Coapplication of noisy patterned electrical stimuli and NMDA plus serotonin facilitates fictive locomotion in the rat spinal cord

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Dose F, Taccola G. Coapplication of noisy patterned electrical stimuli and NMDA plus serotonin facilitates fictive locomotion in the rat spinal cord. J Neurophysiol 108: 2977–2990, 2012. First published September 5, 2012; doi:10.1152/jn.00554.2012.—A new stimulating protocol [fictive locomotion-induced stimulation (FLstim)], consisting of intrinsically variable weak waveforms applied to a single dorsal root, is very effective (though not optimal as it eventually wanes away) in activating the locomotor program of the isolated rat spinal cord. The present study explored whether combination of FLstim with low doses of pharmacological agents that raise network excitability might further improve the functional outcome, using this in vitro model. FLstim was applied together with N-methyl-D-aspartate (NMDA) + serotonin, while fictive locomotion (FL) was electrophysiologically recorded from lumbar ventral roots. Superimposing FLstim on FL evoked by these neurochemicals persistently accelerated locomotor-like oscillations can be recorded from lumbar (L) 2 and L5 ventral roots (VRs), alternating between the left and right sides of the cord, due to rhythmic activation of flexor and extensor motor pools, respectively (Kiehn and Kjaerulff 1996). This phenomenon is ascribed to the existence of a neuronal spinal network, named central pattern generator (CPG) that, following diverse stimuli (Cazalets et al. 1992; Lev-Tov et al. 2000), automatically triggers the locomotor program that can be recorded extracellularly from VRs and intracellularly from motoneurons and premotoneurons (Kiehn 2006).

Although experimental studies have indicated the possibility to activate the CPG using trains of square pulses applied to dorsal afferents (Marchetti et al. 2001a) or to the cauda equina (Blivis et al. 2007), to date it is impossible to evoke stable locomotion in humans by electrical stimulation of sensory afferents (Selinov et al. 2009). Epidural electrical stimulation associated with intense neurorehabilitation has recently produced encouraging clinical benefits in reactivating, albeit transiently, standing in persons with incomplete traumatic lesions of the spinal cord (Harkema et al. 2011). The lack of effectiveness of electrical stimulation can be attributed to the filtering effect of CPG interneurons receiving synaptic inputs from the spinal rhythm generator to gate the flow of sensory information in the spinal cord (Sillar 1991). Interestingly, a better outcome is observed when stimuli are applied close to the spinal cord region, which putatively contains the CPG (Kiehn and Butt 2003), using epidural (Lavrov et al. 2008a, 2008b) or intraspinal (Gaunt et al. 2006) electrodes.

In the attempt to better activate spinal locomotor circuits, studies on spinalized animals have proposed the association of electrical stimulation of the lumbo-sacral spinal cord to the systemic administration of substances that can activate CPG neurons (Musienko et al. 2011). Using just pharmacological agents to activate the CPG in humans requires, however, doses that are associated to strong collateral effects (Hollenberg 1988; Moreau et al. 1989). From this perspective, it would be more practical to refine the parameters of electrical stimulation, rather than to titrate the concentrations of pharmacological drugs, which possess complex pharmacokinetics and pharmacodynamics. Thus the design of new stimulating protocols, in parallel with a better understanding of the pharmacology of locomotor spinal circuits, might open up a broader repertoire of neurorehabilitative treatments to specifically restore motor functions following a severe spinal cord injury (Musienko et al. 2012).

An innovative protocol of electrical stimulation, named FLstim (fictive locomotion-induced stimulation; Taccola 2011), has recently been shown to evoke locomotor-like activity in vitro. FLstim is obtained by sampling, from one VR, noisy waveforms that previously appeared during fictive locomotion [FL; in the presence of N-methyl-D-aspartate (NMDA) plus serotonin] and delivering the recorded trace to one lumbar dorsal root (DR) or to the cauda equina of the in vitro spinal cord. The clear advantage of FLstim relies on the stimulation strength that is much lower than the minimum one required to induce a reflex response. Compared with the classic protocols of electrical stimulation, which use trains of stereotyped rectangular impulses (Marchetti et al. 2001a), FLstim, regardless of the main frequency of waveforms composing it, induces locomotor-like oscillations of longer duration and with a
greater number of cycles (Taccola 2011). This makes of FLstim a novel tool for the electrical activation of the locomotor CPG.

The present study aims at optimizing the effect of the new FLstim protocol, through the joint application of pharmacological drugs, capable of activating the neurons of the locomotor CPG. Moreover, we have assessed whether a low-amplitude FLstim could facilitate subthreshold concentrations of neurochemicals to trigger the emergence of a locomotor rhythm.

**METHODS**

Electrophysiological recordings. In accordance with the guidelines of the National Institutes of Health and the Italian Act Decreto Legislativo 27/1/92 n. 116 (implementing the European Community directives n. 86/609 and 93/88) and under the authorization of the Italian Ministry of Health, experiments were performed on spinal cord preparations after isolation from neonatal rats as previously reported (Taccola 2011). Although, in the first days after birth, maturation of locomotor networks occurs (Jamon and Clarac 1998; Clarac et al. 2004), for this study we used animals in the range of P0–P4, since, at this stage, the alternating pattern is characterized by stable timing (Kiehn and Kjaerulf 1996; Juvín et al. 2007) and, when electrically stimulating dorsal afferents, comparable effectiveness in activating the CPG is reported (Kiehn et al. 1992; Marchetti et al. 2001a). Since data from P0 and P4 did not differ in terms of FLstim effects, results were pooled together.

All efforts were made to reduce the number of animals and their suffering. Each spinal cord (sectioned from the mid-thoracic region to the cauda equina) was mounted in a small recording chamber (maintained at constant room temperature, 22° C), which was 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 MgCl2·7H2O, 2 CaCl2, 1 NaH2PO4, 25 NaHCO3, and 11 glucose, pH 7.4. AC-coupled recordings (range 0.1–10,000 Hz) from lumbar VRs were obtained by using tight-fitting suction electrodes (Taccola 2011).

In a few experiments, DC-coupled records were also acquired. As a routine, recordings were taken from L2 VRs, which primarily consist of axons driving extensor motoneurons of the same limbs (Kiehn and Kjaerulf 1996). The alternation of discharges between flexor and extensor motor pools and between left (l) and right (r) sides of the cord represents the hallmark of FL (Juvín et al. 2007).

Parameters of spinal network activities. FL rhythm (Cazalets et al. 1992) was induced by the continuous bath-application of NMDA (Tocris, Bristol, UK) plus serotonin [5-hydroxytryptamine (5-HT); Sigma, Milan, Italy].

In accordance with previous studies, the concentration of NMDA was selected in each experiment to produce a stable FL with typical periodicity (2–4 s; Bracci et al. 1998; Beato and Nistri 1999; Bertrand and Cazalets 1999; Pearselein et al. 2005; Juvín et al. 2007). Usually, the concentration of NMDA was 3–6 µM with the expected acceleration of FL period for the larger dose (Kudo and Yamada 1987; Smith and Feldman 1988; Atsuta et al. 1991; Cazalets et al. 1992). This approach allowed us to select the most appropriate period of FL to be tested in experiments with FLstim application as the goal was to explore how these electrical stimuli (of varying frequency) could modulate either slow or fast ongoing patterns. The same approach was used for the concentration of 5-HT (3–10 µM) that is typically added to the NMDA solution to stabilize the rhythm (Pearselein et al. 2005). In this study, we considered as subthreshold a pharmacological stimulation based on half of the lowest concentration of NMDA + 5-HT capable of evoking a stable FL rhythm.

For each preparation, a VR recording was randomly chosen and, from it, at least 20 cycles of FL activity were analyzed for periodicity (defined as the time between the onset of two cycles of oscillatory activity) and amplitude (calculated as the voltage difference expressed in µV between the baseline at the beginning of each cycle and its peak). Variations in period and amplitude occurring during electrical stimulation are indicated as a percentage of prestimulus control conditions.

FL activity was also assessed based on its regularity, expressed by the coefficient of period variation (CV; displayed as standard deviation [SD] mean⁻¹). Correlation among signals arising from pairs of VRs was expressed by the cross-correlation function (CCF), obtained with Clampfit 10.1 software (Molecular Devices). While a CCF greater than +0.5 indicates that two roots are synchronous, a CCF less than −0.5 shows full alternation (Ryckebsch and Laurent 1994; Taccola et al. 2008).

**Designing the FLstim.** FLstim was always elicited by applying a reconstructed series of electrical pulses to a single DR as previously detailed (Taccola 2011). In our experiments, this type of stimulation was ineffective to evoke FL when applied to a VR, either L1 or from L4 to L7 of both sides (n = 7). The method of constructing the protocol for FLstim started with AC-coupled recordings of FL induced by NMDA (4–6 µM) and 5-HT (10 µM) acquired (range 0.1 Hz–10 000 Hz; sampling rate = 500 Hz) from VRs. Epochs (60 s or 30 s) of FL were promptly processed for off-line analysis (Clamp 10.1 software; Molecular Devices) and randomly selected for use. Sampled traces were imported into an Origin 7.5 spreadsheet (Origin-Lab), where the x-axis comprised each sampling time for every epoch duration and the y-axis was used for the corresponding current amplitude. Through Origin software the amplitude of FLstim was optimized to evoke a FL in each preparation. The optimal amplitude of FLstim was in the range of 0.2–0.6 times the threshold, while larger intensities only induced a synchronous rhythm on all four VRs, which was time locked with the stimulating pattern (Taccola 2011).

The two columns of values were then exported (as an ASCII text file) to a multichannel stimulation device STG 4004 (Multi Channel Systems).

The stimulating protocol resulting from this procedure was termed FLstim and was applied to one DR through a bipolar suction electrode, after the neurochemicals were washed off, to allow the preparation to return to baseline conditions.

In accordance with the previously described method (Taccola 2011), the experimental protocol used to generate FL with distinct electrical stimuli applied to a single DR is illustrated in Fig. 1A. In detail, AC-coupled recordings were first obtained from a stable FL rhythm induced by the coapplication of NMDA (5 µM) and 5-HT (10 µM; mean period of oscillations was 2.54 ± 0.36 s; CV = 0.14) and stored. After extensive washout (30 min) and return to control (Ctrl) conditions, a 60-s epoch from VR1L2 (shaded box) was digitized and converted into the electrical stimulation protocol FLstim (see above), delivered to the DRL5 of the same preparation. FLstim, delivered at an amplitude of 0.53 times the threshold (Th, defined as the minimum intensity required to induce a reflex response using a single square pulse of duration = 0.1 ms), evoked a sustained episode of locomotor-like response elicited by FLstim (see above), delivered to the DRL5 of the same preparation. FLstim, delivered at an amplitude of 0.53 times the threshold (Th, defined as the minimum intensity required to induce a reflex response using a single square pulse of duration = 0.1 ms), evoked a sustained episode of locomotion elicited by FLstim applied to a naïve preparation. This approach has demonstrated (see Taccola 2011) that the locomotor-like response elicited by FLstim in the two experimental protocols was comparable in terms of number oscillations (15 ± 4 vs. 20 ± 5 cycles per 60 s epoch).
19 ± 3, \( P = 0.018 \), Mann-Whitney rank sum test; \( n = 7 \)); mean cycle period (2.44 ± 0.35 s vs. 2.73 ± 0.60 s; \( P = 0.262 \), Mann-Whitney rank sum test; \( n = 7 \)); and regularity of oscillations (0.18 ± 0.03 vs. 0.21 ± 0.08; \( P = 0.262 \), Mann-Whitney rank sum test; \( n = 7 \)).

The protocol of FLstim is, therefore, different from the standard train of square pulses applied to a single DR as exemplified in Fig. 1C (Etlin et al. 2010; Dunbar et al. 2010; Marchetti et al. 2001a). In the latter case, the stimulus intensity was chosen to be slightly above \( \text{Th} \) as this is typically sufficient to induce FL (Etlin et al. 2010; Dunbar et al. 2010; Marchetti et al. 2001a). Figure 1C indicates that, on the same preparation, the response induced by a rectangular pulse train (DRtrain) at 2 Hz (amplitude = 1.5 \( \text{Th} \) ) elicited a shorter episode of alternating oscillations, that lasted for 36.81 s, with only 13 cycles whose period and regularity (2.93 ± 0.54 s and \( CV = 0.19 \), respectively) were not different from those seen in response to FLstim.

Previous studies have demonstrated that even stronger DR square stimuli did not improve the length of FL (Atsuta et al. 1990; Delvolvé et al. 2001; Marchetti et al. 2001a).

**Statistical analysis.** Data are expressed as means (±SD), while \( n \) indicates the number of spinal cord preparations. After distinguishing between parametric or nonparametric data using a normality test, all parametric values were analyzed with 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, while, for multiple comparisons, ANOVA on Ranks was first applied, followed by a post hoc test (Dunnett’s method). Statistical analysis was performed using SigmaStat 3.5 software (Systat Software). Results were considered significant when \( P < 0.05 \).

**RESULTS**

**Continuous delivery of FLstim induced reproducible bouts of locomotor-like oscillations.** To assess the reproducibility of the locomotor-like response elicited by FLstim, in 10 experiments, FLstim (average amplitude 0.39 ± 0.14 \( \text{Th} \) ) were repetitively delivered to the same preparations at 5-min interval for a total of 175 min. As indicated in Fig. 2, A and B, in response to stimulation with FLstim, stable episodes of FL were observed even after 175 min, preserving a similar number of cycles (93.6 ± 7.5% of Ctrl; \( P = 0.630 \), Kruskal-Wallis one way ANOVA on Ranks on raw data; \( n = 10 \)), thus demonstrating that FLstim could be used as a long-term routine protocol for activating the spinal CPG in a reproducible manner. On a set of preparations (\( n = 7 \)), FLstim that successfully evoked locomotor-like patterns when applied to a single DR, was then delivered to a lumbar VR and failed to produce any sustained epoch of FL.

Unlike standard trains of stimuli, FLstim could modulate pharmacologically induced locomotor cycles. Figure 3A shows an example of FL induced by NMDA (5 \( \mu \text{M} \)) + 5-HT (10 \( \mu \text{M} \)) with oscillation period of 3.97 ± 0.27 s (\( CV = 0.07 \)), during which the concurrent application of FLstim (applied at 5 min intervals for a total of 175 min) increased rhythm frequency (mean period 2.78 ± 0.18 s) and cycle amplitude (averaged value 116.74% of Ctrl) with similar regularity (mean period \( CV = 0.07 \)). This pattern was typically stable because the rhythm period at the start and after 110 min of uninterrupted FL was 2.94 ± 1.02 and 3.83 ± 0.49 s, respectively (paired \( t \)-test on raw data, \( P = 0.117; n = 4 \)). Similarly, cycle amplitude remained unmodified (0.29 ± 0.15 and 0.25 ± 0.12 mV, respectively; paired \( t \)-test on raw data, \( P = 0.217; n = 4 \)).

When delivering FLstim in the presence of neurochemicals, the double alternation pattern (typical of locomotor-like oscillations as clearly shown on faster time-base in Fig. 3B) was accelerated (Fig. 3A). However, in the 60-s epoch following termination of each FLstim, despite the continuous application of neurochemicals, the rhythm was transiently (30.94 ± 8.85 s) reduced in amplitude (averaged value 91.34% of Ctrl). Correspondingly, the rhythm was slowed down (mean period = 3.46 ± 0.28 s) towards preFLstim values, while regularity remained similar (mean period \( CV = 0.08 \)).

Histograms of Fig. 3C show that, on average taken from four VRs of either side of four spinal cords, FLstim delivery
accelerated FL rhythm ($P = 0.005$, one-way ANOVA followed by Dunnett’s method; $n = 4$), which returned to control when stimulation ceased, with unvaried regularity throughout all the different experimental phases (mean period CV in Ctrl = 0.17 ± 0.06, mean period CV during FLstim = 0.12 ± 0.04, and mean period CV after FLstim = 0.15 ± 0.05; $P = 0.630$, Kruskal-Wallis one-way ANOVA on ranks; $n = 4$). The average amplitude of oscillations regularly increased during each stimulation episode and returned to control after the end of stimulation, as indicated in Fig. 3D ($P = 0.029$, Mann-Whitney rank sum test; $n = 4$). Lack of FL fatigue was also confirmed by the observation that (in the continuous presence of NMDA and 5-HT) the period of FL oscillations evoked by the first FLstim was similar to the value recorded at 110 min (3.41 ± 1.30 s vs. 3.01 ± 0.52 s, respectively; paired $t$-test on raw data, $P = 0.775; n = 4$). Likewise, cycle amplitude (0.28 ± 0.19 vs. 0.30 ± 0.14 mV; paired $t$-test on raw data, $P = 0.537; n = 4$) remained constant.

FL induced by NMDA plus 5-HT was modulated by FLstim even when records had been sampled in DC mode (data not shown). Out of 10 preparations, delivery of FLstim cycles sampled in DC-mode (Taccola 2011) with an average period of 4.09 ± 0.84 s evoked an increase in frequency and amplitude of locomotor oscillations induced by neurochemicals (period = 3.41 ± 0.92 s; amplitude = 132.89 ± 19.78% compared with Ctrl) similar to the one reported in response to stimulation with AC FLstim. At the end of DC FLstim, it was again observed a temporary reduction in period (4.13 ± 1.06 s; $P = 0.038$ vs. stimulation without FLstim, Mann-Whitney rank sum test) and amplitude (94.12 ± 9.70% compared with Ctrl; $P < 0.001$ vs. stimulation with FLstim, Mann-Whitney rank sum test on raw data).

Table 1 summarizes the average value of FL oscillation period by FLstim (intensity of 0.4 Th) in standard solution, by NMDA (5 μM) + 5-HT (10 μM), and by the combination of electrical and chemical stimulation ($n = 17$). The period of oscillations induced by NMDA and 5-HT alone was significantly greater than the one obtained from the alternating oscillations evoked in standard solution by FLstim or the one calculated from alternating cycles during the combined stimulation (respectively $P < 0.001$ and $P = 0.004$, one-way ANOVA followed by Tukey’s test).

Comparing the effect of FLstim or DRtrain on chemically induced FL. In another set of experiments, similar to the ones shown in Fig. 4A, during a stable pharmacologically evoked FL (period = 4.37 ± 0.31 s), DRtrain (frequency = 2 Hz, amplitude = 1.5 Th), or FLstim (intensity of stimulation = 0.6 Th) were alternatively delivered to compare their differential effects. Figure 4A (and Fig. 4B, inset) shows that the DRtrain did not interfere with average FL cycle period (3.91 ± 0.21 s) induced by NMDA and 5-HT. Thus, even if a similar train of weak DR pulses could evoke per se FL, it could not modify the ongoing pattern elicited by neurochemicals. This observation accords with previous data demonstrating that electrical stimuli of an intensity at least double than the one used in the present experiments are necessary to modulate the FL (Kiehn et al. 1992; Taccola et al. 2010).

On the other hand, FLstim of rather weak intensity sped up average FL cycles calculated for the whole stimulation epoch (period decreased to 2.93 ± 0.34 s).
We also investigated if, during the continuous application of FLi stim, the coincidence of a stimulus with the peak or trough of the FL cycle could reset the periodicity of the subsequent oscillation: this is exemplified in Fig. 4C in which FLi stim pulses are shown in correspondence to FL cycles (indicated by circles) from three VRs. Thus, for an average of 6 preparations, the cycle period was $3.22 \pm 0.11006$ s when a FLi stim pulse coincided with either a trough or a peak ($P = 0.56$, Wilcoxon signed rank test). These data show that, on average, FL oscillations were accelerated by FLi stim but were not reset during persistent stimulation.

Histograms in Fig. 4D summarize the mean values collected from five spinal cords, demonstrating that the application of FLi stim (intensity of stimulation $= 0.51 \pm 0.12$ Th) during chemically induced FL (unlike DRtrains) could significantly improve periodicity and amplitude of FL ($P = 0.008$, Mann-Whitney rank sum test).

Comparing the effect of FLi stim or DRtrain in the presence of baclofen. The GABA_B receptor agonist baclofen is a potent inhibitor of excitatory synaptic transmission in the spinal cord by reducing release of glutamate from primary afferents and depressing network excitability (Nistri 1975; Curtis et al. 1981; Bertrand and Cazalets 1998, 1999). Thus we wondered whether baclofen might differentially affect FL evoked by FLi stim or square pulse trains applied to activate afferent fibers impinging upon the locomotor CPG. In fact, it has been demonstrated that baclofen slows down the chemically induced FL in the rat spinal cord (Bertrand and Cazalets 1998, 1999).

As depicted in Fig. 5, A–D, left, and E, baclofen (0.2–1 $\mu$M) dose dependently and reversibly disrupted FL induced by FLi stim (0.2 Th intensity) as cycle period increased with

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**Table 1. Summary of effects produced by neurochemicals, FLi stim, or their combination**

<table>
<thead>
<tr>
<th>Period (means ± SD)</th>
<th>n</th>
<th>Statistics</th>
</tr>
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<tbody>
<tr>
<td>A: NMDA (5 $\mu$M) + 5-HT (10 $\mu$M)</td>
<td>4.01 ± 0.81 s</td>
<td>17</td>
</tr>
<tr>
<td>B: FLi stim 0.4 Th</td>
<td>2.62 ± 0.93 s</td>
<td>17</td>
</tr>
<tr>
<td>C: cumulative effect</td>
<td>2.98 ± 0.90 s</td>
<td>17</td>
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<tr>
<td></td>
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<td>17</td>
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NMDA, N-methyl-D-aspartate; 5-HT, 5-hydroxytryptamine; FLi stim, fictive locomotion-induced stimulation; Th, threshold. All 3 experimental conditions were tested on the same preparation. Statistic was performed by applying one-way ANOVA followed by all pairwise multiple comparison procedures (Tukey’s test) for group pairs as indicated by A–C.
augmenting concentrations of baclofen (Fig. 5D), while the CV was unvaried (0.22 ± 0.07; n = 8). At the concentration of 5 μM (not shown), the locomotor-like response induced by FLstim was replaced by synchronous discharges that reproduced the stimulating pattern.

The locomotor-like response induced by a square pulse DRtrain (Fig. 5, A–D, right) was also strongly (yet reversibly) inhibited with loss of alternating VR cycles at 1 μM baclofen (Fig. 5D, right). Figure 5F summarizes the mean period of oscillations for DRtrains applied at 1.5 Th intensity (n = 8).

Figure 5, B–D, shows that augmenting concentrations of baclofen delayed rhythm onset in a dose-dependent manner and that this effect was stronger during the DRtrain-triggered FL. In particular, for 0.2 μM baclofen, the initial pause was $7.14 \pm 1.46$ s with DRtrain and $3.85 \pm 0.76$ s with FLstim. For 0.5 μM baclofen, the pause was $12.38 \pm 0.13$ s with DRtrain and $6.36 \pm 1.59$ s with FLstim.
Stereotypic acceleration of FL. To verify whether the accelerated average periodicity of FL oscillations observed during the combined pharmacological and FLstim stimulation was related to the main periodicity of stimulating waveforms, experiments were performed in which FLstims of different frequencies were repeatedly delivered to a stable FL, induced by application of NMDA (5 μM) + 5-HT (10 μM). FLstims were obtained from a separate group of experiments by sampling FL traces in the presence of 10 μM of 5-HT plus increasing concentrations of NMDA (from 4 μM to 6 μM), thus providing stimulating protocols of different periodicity (5.10 ± 0.33 s for slow rate FLstim; 4.14 ± 0.33 s for medium rate FLstim; 3.33 ± 0.39 s for fast rate FLstim).

Figure 6A shows an example in which, during FL induced by NMDA + 5-HT (period = 3.98 ± 0.30 s), all three different FLstims decreased cycle period to a similar degree. In fact, in five preparations, the mean FL period induced by neurochemicals alone (4.27 ± 0.75 s) became 3.48 ± 0.79 s for slow rate FLstim, 3.42 ± 0.75 s for medium rate FLstim, and 3.69 ± 1.10 s for fast rate FLstim (P = 0.946, Kruskal-Wallis one way ANOVA on Ranks on raw data; n = 5). Thus period acceleration evoked by FLstim was apparently independent from stimulation periodicity.

Did the FL cycle amplitude depend upon the frequency of stimulation? It is known that neuronal output increases in response to stimulation with sinusoidal inputs close to the oscillation frequency of the networks (Leung and Yu 1998; van Brederode and Berger 2008; Haas et al. 2010). We wanted to clarify whether a resonance phenomenon might have occurred to account for the enhancement of cycle amplitude observed during the joint application of FLstim plus NMDA + 5-HT.

Figure 7A depicts a FL induced by 3 μM NMDA + 10 μM 5-HT (period = 6.22 ± 0.63 s; period CV = 0.10) upon which fast rate FLstim was superimposed. For the whole duration of application (60 s), cycle period dropped to 4.06 ± 0.43 s (CV = 0.11), while, compared with prestimulus values, cycle amplitude increased to 170%. On the same preparation, after wash-
out, concentration of NMDA was raised to 6 μM (keeping the 5-HT concentration at 10 μM) to induce a faster FL (period = 1.38 ± 0.19 s; CV 0.14), while cycle amplitude was reduced to a 61%.

In the graph of Fig. 7C, the percentage of locomotor cycle amplitude during delivery of FLstim (compared with the prestimulus control phase) is plotted against the phase shift between locomotor output and stimulating input, calculated as the difference between the period of FL oscillations before stimulus and the main period of stimulating oscillations. The linear regression analysis obtained from the values collected from 21 spinal cords (y = 8.93x + 116.51; Pearson’s r = 0.57; n = 92) showed that there exists a linear relationship, with a biphasic trend, between the increasing positive values of phase displacement of the two waveforms and the variations in cycle amplitude.

In fact, for positive phase shift values between the two waveforms (Fig. 7, A and C), which correspond to the situation in which the frequency of the afferent stimulating pattern is higher than the frequency of the rhythmic motor output recorded from VRs before FLstim, a strengthened amplitude of locomotor-like cycles appears. Vice versa, for FLstim slower than the frequency of prestimulus FL cycles (Fig. 7, B and C), a reduction in amplitude of oscillations was observed.

**FLstim and neurochemicals cooperate to bring locomotor CPG to threshold.** We explored whether combined subthreshold stimuli of different nature, namely low concentrations of neurochemicals and weak FLstim, could contribute to bring the CPG to threshold for triggering the locomotor program.

For this purpose, we first delivered a FLstim of strength 0.2 Th to elicit a long lasting locomotor episode (duration = 55.10 s) composed of 32 cycles of 1.78 ± 0.38 s period (data not shown). Thereafter, as shown in Fig. 8A, the same FLstim was halved (0.1 Th) and evoked only sporadic events instead of stable alternating oscillations. Then, we applied NMDA + 5-HT at subthreshold concentrations (respectively, 2.5 and 5 μM), which induced noisier baseline activity unable to trigger FL (Fig. 8B, left). When the weak FLstim was superimposed (but not so when using weak 2 Hz DRtrains; data not shown), FL emerged with period = 3.25 ± 0.09 s and CV period = 0.14 ± 0.06 (Fig. 8B, right, and traces on faster time scale in Fig. 8C). Correlograms (Fig. 8D) confirmed the double alternation among pairs of homolateral (upper; CCF = 0.755) and homosegmental (lower; CCF = −0.814) VRs.

This observation was replicated on nine spinal cords. In all tested cases, in the presence of subthreshold concentrations of neurochemicals, weak FLstim (0.31 ± 0.18 Th), evoked locomotor-like oscillations (3.24 ± 1.05 s period) comparable to those detected in the same preparation in the presence of 5 μM NMDA and 10 μM 5-HT (2.80 ± 0.75 s; P = 0.299, paired t-test). In four of these preparations, low intensity (Th...
DR trains were also delivered, which, unlike FLstim, did not facilitate emergence of FL.

Low intensity FLstim delivered in the presence of higher extracellular potassium. We wondered whether increasing network excitability, by elevating extracellular potassium without reaching threshold for FL, could synergize with weak FLstim to evoke a locomotor pattern. In control conditions, weak FLstim (0.08 times Th) induced irregular discharges with no alternating oscillations (Fig. 9A), as indicated by the cross-correlation values reported in Fig. 9A2 for a pair of L2 and L5 homolateral (CCF = -0.110) and homosegmental (CCF = -0.092) VRs. Subthreshold concentrations of NMDA (2 μM) and 5-HT (3 μM), together with the same weak FLstim, elicited a stable FL with 16 ± 1 oscillations (period and CV were 3.49 ± 0.26 s and 0.07, respectively; Fig. 9B), alternating among pairs of L2 and L5 homolateral (CCF = -0.654) and homosegmental (CCF = -0.655) VRs (Fig. 9B2).

While an increased K+ concentration (6 mM) evoked only sporadic discharges (not shown), superimposed weak FLstim elicited a short FL episode with 23 ± 8 cycles, of period and regularity of 1.85 ± 0.04 s and 0.02, respectively (Fig. 9C). These oscillations showed a modest alternating trend among pairs of L2 and L5 homolateral (CCF = -0.408) and homosegmental (CCF = -0.420) VRs, as indicated by the cross-correlogram in Fig. 9C2. When the concentration of potassium was raised stepwise up to 11 mM, no alternating oscillations appeared in the presence of FLstim of subthreshold intensity (n = 4).

DISCUSSION

The main outcome of this study is the demonstration that a new stimulation protocol, which uses a noisy waveform sampled from the motor output during FL (as opposed to a classic train of rectangular pulses), could potentiate the pharmacological stimulation of the CPG to facilitate FL. FLstim regulated the oscillation amplitude in a biphasic manner, as the slowest noisy waveforms decreased the amplitude of VR cycles, while the higher frequency ones enhanced the oscillation size. FLstim in association with subthreshold concentrations of NMDA and serotonin facilitated the emergence and duration of a locomotor rhythm. A similar effect was not observed in case of a generalized increase in spinal cord neuronal excitability, suggesting that, to optimally activate spinal locomotor circuits, it is necessary to combine a selective pharmacological stimulation of CPG elements with an electrical stimulation using a noisy waveform that corresponds to the locomotor pattern.

Delivery of FLstim during FL rhythm modulates the properties of locomotor cycles. The fast periodicity of FL discharges has been traditionally ascribed to the variable number of neurons activated through the gradual recruitment of usually silent premotoneurons (Sillar and Roberts 1993; Grillner 2003). More recently, it has been proposed that the frequency
of rhythmic oscillations is related to a selective switch from an interneuronal population activated at low speed to another group of interneurons that respond only to higher speeds and that seem to be anatomically and genetically distinct from the first ones (Crone et al. 2009; Fetcho and McLean 2010). During locomotor performance, recruitment of V2a interneurons, essential for the alternation between right and left limbs, has been suggested to be dependent on the frequency of the rhythmic synaptic drive from the CPG. At high frequencies, a neuronal subpopulation (normally silent at rest) is selectively added to maintain coordination between the left and right sides during accelerations (Zhong et al. 2011).

Notwithstanding the fact that the detailed topography of neurons recruited during stimulation with FLstim remains to be explored, the increase in cycle amplitude due to stimulation with higher frequencies may correspond to the involvement of a larger number of motoneurons, as a previous study has reported a relation between number of motoneurons and cycle amplitude (Mazzone et al. 2010). On the other hand, the larger cycle amplitude due to stimulation with high frequency waveforms might also be determined by the recruitment of an additional pool of motoneurons within the same segment. In fact, homologous motoneurons possessing distinct intrinsic membrane properties and synaptic drive can contribute to stronger oscillations (Gabriel et al. 2011). We can also suppose that the involvement of a wider population of premotoneurons, selectively recruited by FLstim, could better synchronize motoneurons, with the result of increasing the amplitude of each cycle. It is noteworthy that pharmacological block of spinal inhibition elicits synchronous discharges recorded from all VRs and, at the same time, determines an increase in amplitude of single rhythmic events (Beato and Nistri 1999).

FLstim compared with standard protocols of electrical stimulation. In the present study, by comparing the effect of classic stimulation and FLstim, both of them sequentially applied to the same DR, it emerged as clearly shown in Fig. 4 that only this latter one could modify the properties of alternated cycles. This fact suggests that the mechanisms that render FLstim capable of generating locomotor-like oscillations may be different from those induced by stereotyped rectangular stimuli. One possibility comes from the high intrinsic signal variability contained in FLstim, which corresponds to the high sampling frequency of a FL trace (500 Hz) and may determine a profile of transmitter release to CPG elements that is different from the one induced by a traditional train of 2-Hz impulses. FL epochs evoked by either electrical stimulation protocols inevitably decayed, although they lasted longer with FLstim. By associating FLstim with subthreshold concentrations of neurochemicals, it was possible to significantly prolong the episode of locomotor oscillations. Conversely, in the presence of the same subthreshold concentration of neurochemicals, a classical DRtrain was far less effective in evoking FL cycles. These results suggest that the causes of termination of FL induced by FLstim in control conditions could be compensated, at least in part, by the pharmacological activation of the CPG.

Previous studies have reported that repeated VR stimuli can trigger FL in the mouse spinal cord (O’Donovan et al. 2010).
When FLstim was applied to a single VR, no FL was observed. This result suggests that the antidromic pathways activated by FLstim could not readily access the locomotor CPG that needs to be stimulated by stronger electrical pulses (Mentis et al. 2005) than the ones used in the present investigation. This notion accords with the demonstration that inhibitory Renshaw cells activate different postsynaptic receptors on motoneurons, depending on the stimulation frequency (McCrea et al. 1980; Lamotte d’Incamps and Ascher 2008). As one aim of the current report was to identify patterns of stimulation with minimal deleterious effects and with sustained efficiency on the locomotor CPG, it seemed desirable to carry further experiments with weak intensity FLstim applied to a single DR.

Baclofen differently affects alternating rhythms evoked by FLstim or DRtrain. Baclofen, by acting on pre- and postsynaptic metabotropic GABA_B receptors, depresses synaptic transmission (Nistri 1975; Curtis et al. 1981) and neuronal network activity (Brockhaus and Ballanyi 1998; Brown et al. 2007). In this study we demonstrated that increasing concentrations of baclofen augmented, in a dose-dependent manner, the period of electrically-induced locomotor cycles and reduced the number of oscillations up to a complete suppression for higher concentrations of this drug. An analogous depressant effect of baclofen on the locomotor patterns evoked through direct activation of CPG by neurochemicals has been reported (Bertrand and Cazalets 1998, 1999). The effect of baclofen has been ascribed to the activation of GABA_B receptors that slow down the timing of the motor pattern (due to their direct action on the network) and also inhibit presynaptically the CPG output, with a minor direct effect on motoneurons (Cazalets et al. 1998). In our experiments, the electrically induced locomotor rhythm appeared sensitive to even lower concentrations of baclofen than the ones reported to suppress the chemical FL (Bertrand and Cazalets 1998, 1999). In fact, when the CPG was activated through electrical stimulation of a DR, the well-known reduction of glutamate release from primary afferents (Nistri 1975; Curtis et al. 1981) was likely additive to the effects of baclofen listed above.

The present data showed a differential sensitivity of FL to baclofen depending on the protocol of electrical stimulation. In fact, although significantly slower, the locomotor-like oscillations elicited by FLstim with lower intensity than the ones reported to suppress the chemical FL (Bertrand and Cazalets 1998, 1999). In fact, when the CPG was activated through electrical stimulation of a DR, the well-known reduction of glutamate release from primary afferents (Nistri 1975; Curtis et al. 1981) was likely additive to the effects of baclofen listed above.

Conjoint pharmacological and electrical stimulation facilitates CPG activity. The contrasting ability of FLstim applied to a single DR to modulate FL evoked by NMDA and 5-HT, whereas standard square pulses delivered to the same DR could not do so, clearly indicated that FLstim was the protocol of choice to influence the activity of the locomotor CPG. Thus it
was important to consider how pharmacological agents and FLstim could summate to facilitate locomotor-like patterns. Previous studies have demonstrated that catecholamines prolong the locomotor response induced by tonic epidural electrical stimulation in the spinalized rat (Musienko et al. 2011). This in vivo effect is proposed (Musienko et al. 2012) to arise from the combination of the direct electrical stimulation of spinal circuits, to replace the lost excitatory drive, with the pharmacological agents that would mimic the modulatory action of monoaminergic systems on spinal networks (Conway et al. 1988). In the present experiments, FLstim, in combination with agonists of the glutamatergic and serotoninergic systems, cooperated in facilitating FL rhythm, further validating the close analogy between in vivo observations and in vitro data. In fact, FLstim increased the frequency of stable locomotor oscillations induced by NMDA + 5-HT, a result reminiscent of the better functional outcome of the association of pharmacological and electrical stimuli in the spinal rat (Ichiyama et al. 2008).

The role of neuromodulators may consist in the activation of extrasynaptic serotoninergic receptors (Smeets and González 2000) that positively modulate synaptic inputs (triggered by electrical stimulation) to crucial CPG elements (Hinckley et al. 2010). In support of this notion, it has been shown that epidural spinal cord stimulation recruits the serotoninergic system (Song et al. 2009, 2011) that plays a pivotal role in modulating the activity of locomotor networks (Zhong et al. 2006; Dunbar et al. 2010).

The present data provide the demonstration that stimulation with combined subthreshold electrical and pharmacological stimuli cooperated in activating the in vitro locomotor pattern. Widespread activation of spinal neurons with a high potassium solution did not produce a comparable effect. Although discrete increases in extracellular potassium are likely to trigger FL episodes induced by dorsal afferent stimulation (Marchetti et al. 2001b), it is difficult to activate the CPG by increasing extracellular potassium, as the effective concentration window is very narrow (Bracci et al. 1998). Thus a broad increase in spinal cord excitability evoked by high potassium was not per se sufficient to synergize the effect of a weak FLstim.

The neurochemicals employed in this experimental study to activate the CPG evoke severe central and systemic collateral effects (Hollenberg 1988; Moreau et al. 1989). Nevertheless, the discovery of new drug combinations that can selectively act on the CPG (Guertin et al. 2010), along with the development of innovative systems for a more localized drug delivery (Kang et al. 2009) or with new substrates for the controlled release of neuroactive chemicals directly at the level of the spinal subarachnoid space (Cobacho et al. 2009), may help targeting pharmacological agents to modulate the activity of spinal circuits. Hence, the present study suggests that conjoint electrical stimulation using weak noisy waves with even subthreshold concentrations of neurochemicals may actually be a desirable process to pharmacologically manipulate CPG excitability to restore functional benefits to persons with a spinal cord injury.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: F.D. and G.T. performed experiments; F.D. and G.T. analyzed data; F.D. and G.T. interpreted results of experiments; F.D. and G.T. approved final version of manuscript; G.T. conception and design of research; G.T. prepared figures; G.T. drafted manuscript; G.T. edited and revised manuscript.

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