Fast and slow locomotor burst generation in the hemi-spinal cord of the lamprey

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Short Title: FAST AND SLOW LOCOMOTOR RHYTHMS IN THE LAMPREY HEMICORD

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ABSTRACT

A fundamental question in vertebrate locomotion is whether distinct spinal networks exist that are capable of generating rhythmic output for each group of muscle synergists. In many vertebrates including the lamprey, it has been claimed that burst activity depends on reciprocal inhibition between antagonists. This question was addressed in the isolated lamprey spinal cord, in which the left and right sides of each myotome display rhythmic alternating activity. We sectioned the spinal cord along the midline, and tested whether rhythmic motor activity could be induced in the hemicord with bath applied D-glutamate or NMDA as in the intact spinal cord, or by brief trains of electrical stimuli. Fast rhythmic bursting (2-12 Hz), coordinated across ventral roots, was observed with all three methods.

Furthermore, to diminish gradually the crossed glycinergic inhibition, a progressive surgical lesioning of axons crossing the midline was implemented. This resulted in a gradual increase in burst frequency, linking firmly the fast hemicord rhythm (6.6±1.7 Hz) to fictive swimming in the intact cord (2.4±0.7 Hz). Ipsilateral glycinergic inhibition was not required for the hemicord burst pattern generation, suggesting that an interaction between excitatory glutamatergic neurons suffices to produce the unilateral burst pattern. In NMDA, burst activity at a much lower rate (0.1-0.4 Hz) was also encountered, which required the voltage-dependent properties of NMDA receptors, in contrast to the fast rhythm.

Swimming is thus produced by pairs of unilateral burst generating networks, with reciprocal inhibitory connections that not only ensure left/right alternation but also downregulate frequency.
INTRODUCTION

The rhythmic movements of the body and limbs that propel vertebrates through space, be it swimming, walking or flying, are mainly generated by specialized circuits confined to the spinal cord. These networks, called central pattern generators (CPGs) for locomotion, normally integrate sensory feedback about body position and the environment together with supra-spinal motor commands (see Grillner 1985). This allows, for example, a squirrel to run along the branches of a tree with unfailing precision. Nonetheless, spinal CPGs are capable of producing stereotyped locomotor output also in complete isolation. This general control structure has emerged from experiments on a wide variety of animals including lampreys, tadpoles, turtles, chicks, rats, cats (see Grillner 1981; Kiehn et al. 1997; Stein and Smith 1997) and recently also humans (Dimitrijevic et al. 1998).

Locomotion is produced by a complex motor pattern with rhythmic and alternating contractions of antagonistic muscles: in the lamprey and many aquatic vertebrates between left and right sides of the body, and in terrestrial and airborne species also between flexors and extensors at each joint of the leg or wing. The coordination between different muscle groups is to some degree flexible (see Stein and Smith 1997), and can even be recombined (compare forward and backward locomotion). This ability of the spinal networks to flexibly reshape the motor pattern has led to the proposal of a modular organization of the CPGs for locomotion (Grillner 1981). Each group of muscle synergists at a joint would be controlled by a dedicated module, the unit burst generator (UBG), autonomously capable of rhythmic output. UBGs would then be dynamically interconnected by reciprocal inhibition or excitation depending on whether alternation, co-activation or more complex patterns are required. In the lamprey (Buchanan 1999)
and other vertebrates, it has instead been suggested that locomotor burst generation depends crucially on reciprocal inhibition between antagonistic centers.

The different flexor and extensor motor nuclei in the tetrapod spinal cord are not sufficiently separated to allow an experimental functional isolation of potential unit burst generators (see Kiehn and Kjaerulff 1998). The situation is somewhat more favorable in the mudpuppy in which the main groups of flexors and extensors in the forelimb area can be separated into two centers (Cheng et al. 1998). Other evidence supporting UBGs mainly comes from experiments in which rhythmic ventral root activity was observed in the presence of antagonists of inhibitory neurotransmission (neonatal rat: Cowley and Schmidt 1995; Kremer and Lev-Tov 1997), or in which unilateral bursting occurred in one muscle group without concomitant activity in the antagonists (cat: Grillner and Zangger 1979; turtle: Stein et al. 1995). In the frog embryo, rhythmic ventral root discharge was observed after longitudinal hemisection of the spinal cord, in the fast pattern of ‘single spike alternation’ of tadpole swimming (Soffe 1989).

In the lamprey it was shown that the spinal cord maintains its rhythm-generating capacity during a blockade of glycinergic transmission with strychnine, with either a fast or a very slow bursting being reported (Cohen and Harris-Warrick 1984; Alford and Williams 1989; Hagevik and McClellan 1994; Aoki et al. 2001). While this supports the existence of unilateral rhythmic networks not requiring reciprocal inhibition for their operation, it leaves open the question of whether these two rhythms are related to the ‘normal’ operation of the CPG for swimming. Moreover, direct tests of the UBG hypothesis, with a longitudinal midline section separating the two sides, lead Buchanan
(1999) to the opposite conclusion that hemicords are unable to generate a rhythmic burst pattern.

We have further investigated this and are now able to show that the lamprey hemicord can express two clear patterns of locomotor rhythmicity, a fast and a slow rhythm. We demonstrate that the fast rhythm, evoked under a broad spectrum of activating protocols, is directly linked to the operation of the network in the intact cord during swimming. Crossed connections are thus not required for the generation of the swimming rhythm. Furthermore the unilateral motor pattern is independent of ipsilateral glycinergic inhibition and presumably due to a burst generating network consisting of interacting glutamatergic interneurons.

**METHODS**

A total of 55 adult lampreys (*Lampetra fluviatilis*) were anesthetized by immersion in tricaine methane sulfonate (MS-222; Sigma, St. Louis, MO; 200 mg/l) and decapitated caudal to the gills. The spinal cord preparations (normally between 8 and 13 segments) were dissected from the region between the gills and the first dorsal fin, together with the dorsal half of the notochord as a mechanical support. After pinning down these preparations in a Sylgard-lined chamber, the meninges were peeled off from the dorsal surface of the cord. During the dissection and thereafter, preparations were perfused with cooled physiological solution (5-10 °C). The Ringer’s solution (containing in mM: 138 NaCl, 2.1 KCl, 1.8 CaCl₂, 1.2 MgCl₂, 4 glucose, 2 HEPES, 0.5 L-glutamine) was bubbled with O₂ and adjusted to pH 7.4 with NaOH.
**Electrophysiology**

Glass pipette suction electrodes were gently placed on the ventral roots to record efferent locomotor activity. Signals were sent to a differential AC amplifier (Model 1700, A-M Systems, Everett, WA) and band-pass filtered between 100 and 500 Hz. The output from the amplifier was acquired at a sampling rate of 2 kHz per channel on a PC equipped with an A/D converter (Digidata 1320A), and running Clampex 8 software (both from Axon Instruments, Foster City, CA). In some experiments the hemi-spinal cord was stimulated by a single train of 1 to 30 pulses (2 ms pulse duration, 30 Hz frequency) delivered to either end of the spinal cord by a large suction electrode applied on the dorsal surface. This was sufficient to elicit a long-lasting bout of motor activity.

**Sagittal sections**

With the aid of a stereoscopic microscope and trans-illumination oriented at an optimal angle of incidence, relevant features of the spinal cord could be viewed and used as a guide during the lesions. The midline appeared as a distinct dark line on a lighter background or as a bright line on a gray background depending on the lighting. The ventro-medial column and cell layer were also readily identified. Hemi-spinal cords were obtained either by sectioning sagittally along the midline \((n = 53)\) or by following a slightly lateral path, lying halfway between the midline and the contralateral cell layer \((n = 34)\). The two types of preparation were equal with regard to the fast locomotor burst activity (2-10 Hz), whereas there was a tendency for those with the lateral section to display an earlier recovery of the NMDA induced slow burst rate (0.1-0.4 Hz) after the lesion. Mid-sagittal and para-sagittal preparations are dealt with together below. Sectioning was performed either with the tip of a fine hypodermic needle (outside...
diameter 0.4 mm; 20 of 55 animals), or using a Micro Feather ophthalmic scalpel (Feather Safety Razor, Osaka, Japan) with a tip angle of 15°, mounted on a micromanipulator. Both instruments were lowered into the spinal cord along the line of sectioning (midline or lateral cut). In later experiments the scalpel was employed due to its bilateral symmetry, which permitted both left and right hemicords to be used.

**Pharmacology**

Efferent locomotor activity was induced both in intact and hemi-spinal cords by perfusing either 0.5-1 mM D-glutamate (Sigma, St. Louis, MO) or 75-150 µM n-methyl-d-aspartic acid (NMDA; Tocris, Bristol, UK). Both agonists have routinely been used to elicit fictive swimming in the lamprey spinal cord (Cohen and Wallén 1980; Grillner et al. 1981). To study the contribution of the voltage-dependence of NMDA receptors to rhythm generation, Magnesium-free Ringer’s was used. This was obtained by replacing MgCl$_2$ with NaCl. In the absence of Mg$^{2+}$, lower concentrations of D-glutamate and NMDA were used to elicit locomotor activity (Brodin and Grillner 1986). Glycinergic inhibition from ipsilaterally projecting interneurons was blocked by perfusing 1 µM strychnine hemisulfate salt (Sigma, St. Louis, MO) for 1 hour (Buchanan and Grillner 1988; McPherson et al. 1994).

**Data analysis**

Ventral root recordings, as a rule 3 minutes in length (except when activating the hemicords electrically), were analyzed using custom scripts run within Axograph 4.6 software (Axon Instruments, Foster City, CA) on a Macintosh G4 computer (Apple Computer, Cupertino, CA). The main parameters characterizing rhythmic activity were extracted from the auto-correlogram (burst frequency and rhythmic quality) and the
cross-correlogram (coordination between ipsi- or contralateral hemisegments) of ventral root recordings. Before calculating these functions, the raw recordings were digitally high-pass filtered (smooth cutoff at 40 Hz) to remove any DC offset introduced after the hardware filter. Then, for each recording, the amplitude of baseline noise was automatically estimated and a multiple of this value was selected as a threshold below which all samples were set to zero. This procedure sets baseline noise to zero and is useful in preventing that fluctuations in noise amplitude, which may occur during a long experiment, influence the estimation of rhythmic quality. Finally, recordings were rectified and correlograms calculated (normalized between 0 and 1). Figure 1 shows three examples of ventral root recording of decreasing rhythmic quality, and their corresponding auto-correlograms. Cycle period (the inverse of burst frequency) was taken as the delay of the second peak in the auto-correlogram (Fig. 1). Rhythmic quality was described using a numerical value, referred to as the coefficient of rhythmicity ($C_r$), which ranges between 0 and 1. This was defined as $C_r = (\alpha - \beta)/(|\alpha| + |\beta|)$ where $\alpha$ and $\beta$ are the height of the second peak and the first trough in the auto-correlogram, respectively (Fig. 1). The higher the coefficient, the more rhythmic the activity at the ventral root, as can be seen by comparing the three samples shown in figure 1, with their corresponding $C_r$. For a discussion of similar techniques see Buchanan (1999). Only records having a $C_r$ equal or above 0.01 were considered rhythmic. In all these cases the raw recordings were inspected by eye, and rhythmicity at the frequency predicted by the auto-correlogram was confirmed.
Statistics

Data is expressed as mean ± S.D. and Student’s paired t-test was used for statistical comparisons. Unless stated otherwise, the value of rhythmic quality associated to each preparation is the highest observed in the course of the experiment, calculated on 3 minutes of continuous recording (see above). The reported duration of the locomotor bouts following electrical stimulation is also the longest obtained from each individual hemicord.

RESULTS

Much of our current knowledge of the factors that contribute to the generation of swimming in the lamprey, has come from experiments in which fictive locomotion was induced in the isolated spinal cord (see Grillner et al. 2000). Most studies have used either bath-applied D-glutamate or NMDA to evoke fictive locomotion (Cohen and Wallén 1980; Grillner et al. 1981; Wallén and Williams 1984), the difference being that D-glutamate activates both AMPA and NMDA receptors to a similar degree (Zhang et al. 1996). Both agonists were therefore tested on the hemi-spinal cord preparations.

The hemi-spinal cords express a fast rhythm in D-glutamate

Fictive swimming was induced in 9 intact spinal cord preparations by perfusion of 0.5 to 1 mM D-glutamate. The quality of rhythmic activity in the intact cords, as expressed by the coefficient of rhythmicity, was always high (average \( C_r = 0.85 \pm 0.06 \); range = 0.75 to 0.94; see Methods). Hemisectioning was performed when the rhythm had reached a steady state, to have a stable control value. In a further 8 spinal cord pieces, D-glutamate
(0.5 to 1 mM) was perfused only after hemisection. Altogether 28 hemicords were studied.

Ventral root activity could be evoked by D-glutamate immediately after the lesion, except in a few cases in which the hemicord remained inactive up to 1 hour. Rostral and caudal ventral roots were rhythmically bursting (Fig. 2 A, lower records) and coordinated with each other (Fig. 2 A lower records). All hemicord preparations ($n = 28$, from 17 animals) expressed fast rhythmic bursting in D-glutamate but the $C_r$ was lower than in the intact spinal cord (average $C_r = 0.27 \pm 0.13$; range = 0.08 to 0.56). This burst activity was readily visible in the raw recordings and was confirmed by the auto-correlation analysis (Fig. 2 A). In all 5 preparations where it was tested, bursting was coordinated along the hemi-spinal cord with a small lag (-3% to +2% of the cycle period, per segment) observed in the cross-correlogram (Fig. 2 A, inset).

Figure 2 B shows the burst rates observed before and after the hemisection in different preparations. We subdivided the frequency axis in consecutive intervals. For each interval, the number of intact and lesioned preparations is represented. The histograms (Fig. 2 B) show that in the intact spinal cords the burst frequency ranged between 1 and 3 Hz (gray bars), while in the hemicords it was faster, and ranged between 3 to 10 Hz (white bars). In the preparations in which the frequency was measured before and after the lesion, a significant increase was observed ($n = 15$; $p < 0.0001$), the frequency after hemisection being on average $270 \pm 81 \%$ of control.

To explore if the frequency could still be modulated in the hemicord preparations, we doubled the concentration of D-glutamate from 0.125 up to 2 mM in steps (Fig. 2 C). Within the range 0.25 to 2 mM, a doubling of D-glutamate concentration always induced
an increase in burst frequency (\(n = 6\); average increase 34 ± 18\%). Little or no activity was present at 0.125 mM and in some cases even at somewhat higher concentrations, while at 2 mM and above the hemicords tended to become quiet after a short time.

In order to determine the minimum substrate necessary to generate this unilateral rhythm, two hemicords (10 segments long) were transected into pieces of different length. Rhythmic activity was still expressed by a 2.5 hemisegment-long piece (\(C_r = 0.26\)) which was the shortest tested, and a 3 hemisegment one displayed synchronized bursting across two ventral roots (Fig. 2 D). The burst frequency of these short hemicords did not differ from that expressed by the original hemicord preparations before the transections (Fig. 2 D).

The hemi-spinal cords express a fast and a slow rhythm in NMDA

A similar approach to the experiments described above was used with NMDA. In part of the intact spinal cord pieces (32 out of 43) 75 to 150 \(\mu\)M NMDA was perfused prior to hemisection, thus inducing fictive swimming (average \(C_r = 0.75 ± 0.19\); range = 0.37 to 0.99). In the hemicords, two types of rhythm were observed, a fast and a slow rhythm. The fast rhythm (2-10 Hz) was observed in one third of the preparations in which it was investigated (14 hemicords from 11 animals, out of 42 hemicords). Figure 3 A shows the fast burst pattern and the coordination between two adjacent hemisegments. This can be seen more clearly by comparing rectified/integrated recordings, which display the intensity of ventral root activity over time (Fig. 3 A, lower records). The quality of the fast hemicord rhythm with NMDA was reduced as compared to D-glutamate (average \(C_r = 0.03 ± 0.015\); range = 0.01 to 0.05) and distinct bursts could be present only in sections
of the recordings (Fig. 3 A). The fast rhythm, however, was always detected or confirmed by the auto-correlogram \((n = 14; \text{Fig. 3 A}_2)\). Cross-correlation analysis shows that this fast rhythm is expressed approximately synchronously in two adjacent ventral roots (Fig. 3 A_3). The fast rhythm was generally detected within the first hours after hemisection and often diminished or vanished as the slow rhythm (see below) appeared and gained in strength.

A slow rhythm (0.1-0.4 Hz) was expressed in 40 hemicords (from 25 animals) out of 47 investigated (Fig. 3 B). We observed a high variability from animal to animal. In some cases it appeared within one hour (average 1st hour \(C_r\) for all preparations = 0.09 ± 0.13; range = 0.00 to 0.56), while in others only several hours after hemisection, gaining progressively in quality during the course of 1-2 days. Irrespective of the time of onset after hemisection, the slow rhythm generally attained a good quality (average \(C_r = 0.28 \pm 0.19\); range = 0.01 to 0.93). Bursting was almost synchronous across nearby ventral roots (Fig. 3 B). The slow rhythm was observed in both the left and right hemicord obtained from the same piece of cord \((n = 4)\).

Among the 14 hemicords which expressed the fast rhythm, 12 also expressed the slow rhythm during the experiment. Of these, 10 expressed both rhythms simultaneously.

The histogram in figure 3 C summarizes the burst frequencies observed before and after the hemisection, during perfusion with NMDA. While fictive swimming in the intact spinal cord was in the range from 0.7 to 3 Hz \((\text{gray bars})\), the fast rhythm in the hemicords occurred between 2 and 10 Hz \((\text{white bars to the right})\), and the slow rhythm between 0.1 and 0.4 Hz \((\text{white bars to the left})\). It is clear from figure 3 C, that the fast and the slow rhythm are separated by a gap in which rhythmicity was never observed. In
preparations where burst frequency was measured both before and after hemisection, the fast rhythm was significantly faster than in control fictive swimming ($n = 14; p < 0.001; 298 \pm 151 \%$ of control), while the slow rhythm was significantly slower ($n = 30; p < 0.0001; 15 \pm 7 \%$ of control).

In 3 hemicords we varied the concentration of NMDA in the range 38 to 300 µM (Fig. 3 D). We could follow the slow rhythm in all three preparations, but the fast rhythm was present only in one of them (Fig. 3 D, top dataset). In both rhythms, a doubling of the NMDA concentration evoked an increase in burst frequency (average increase $27 \pm 9 \%$ and $42 \pm 40 \%$, fast and slow rhythm respectively).

The slow rhythm often observed in NMDA was not detected in any of the experiments with D-glutamate (see above). To consolidate this negative finding, we evoked the slow rhythm in four hemicords using NMDA (average $C_r = 0.27 \pm 0.20; \text{range} = 0.10 \text{ to } 0.50$), and then replaced NMDA with 0.5 to 2 mM D-glutamate. None of these preparations re-expressed the slow rhythm in D-glutamate (not shown).

**The hemi-spinal cords express a fast rhythm after electrical stimulation**

In order to observe the output of the unilateral locomotor networks under a broader range of conditions, we attempted to activate them by brief electrical stimulation of the hemicord, in the absence of exogenous D-glutamate or NMDA. In the intact spinal cord, electrical stimulation of the cut end produces, as a rule, a short episode of ventral root activity outlasting the stimulus by only a few seconds. The duration of such episodes appears to be limited by the intervening action of crossed inhibition (Fagerstedt et al. 2000). Since one main consequence of splitting the cord along the midline is the removal
of inhibition from the contralateral side, a prolonged response to electrical stimulation appeared likely in the hemicord.

After dissection, preparations were perfused with standard Ringer’s solution and hemisected along the midline. Stimulation electrodes delivered a single pulse or a brief train of pulses (1-30) at one end of each hemicord. Figure 4 A shows the activity in two hemicords obtained from the same spinal cord piece, and stimulated with the same parameters. Both display a similar bout of activity composed of clear bursts, as can be seen in the lower records with higher time resolution. In all hemicords tested (n = 8, from 5 animals) a prolonged bout of rhythmic burst activity in the ventral roots was evoked (average bout duration 135 ± 54 s). Activity within the bout was organized in distinct bursts in a frequency range from 12 to 3 Hz (Fig. 4 A and B), with the lower frequencies occurring towards the end of the bout. The fast rhythm was of a good quality (average \(C_r = 0.31 ± 0.13\); range = 0.18 to 0.58) and could be evoked immediately after the lesion, and up to three days later. The ability of the lamprey hemicord to respond to electrical stimulation with a long bout of rhythmic burst activity is similar to that of the intact and hemi-spinal cord of the embryo of *Xenopus laevis* (Soffe 1989).

We also tested the rhythm-generating capability of single spinal hemisegments, which were obtained by transecting the hemicord midway between consecutive ventral roots. A stimulation electrode was placed at one end of a hemisegment and a recording electrode placed on the ventral root. Single hemisegments responded to electrical stimulation with shorter bouts of activity (24 ± 15 s) compared to the longer hemicords. In 14 hemisegments out of 29 investigated this activity was rhythmic (average \(C_r = 0.10 ± 0.13\); range = 0.18 to 0.58).
Progressive midline lesions in D-glutamate: the fast hemicord rhythm is linked to fictive swimming

While the intact cords express rhythmic and alternating locomotor activity in the range 0.7-3 Hz (D-glutamate or NMDA), the hemicords may display two very distinct outputs: a fast rhythm with a frequency of 2 to 12 Hz (D-glutamate, NMDA, electrical stimulation) and a slow rhythm between 0.1 and 0.4 Hz (NMDA). Since rhythm generation in these two, non-overlapping, time frames is likely to involve different neuronal mechanisms, the question arises as to which of the two rhythms is directly related to the operation of the network during fictive swimming. We approached this question by studying the transition between intact and hemisected spinal cord by progressively reducing the number of axons crossing the midline, while monitoring the ventral root output. This was done by producing intermittent micro-lesions along the midline, such that the cuts were intercalated with an unlesioned midline (Fig. 5 A1). Using a tiny scalpel mounted on a micromanipulator, it was possible to perform cuts of the same length and at the same interval (~ 3 per segment) along the entire piece of cord. This density of micro-lesions should be sufficiently high to avoid creating a chain of autonomously functional intact and hemisected spinal cord sections, since most crossed axons extend more than one segment rostrally or caudally (see Fig. 3 in Ohta et al. 1991). Once a stable motor output was observed at a certain percentage midline section (100 × cut length / total length) the lesions could be further extended to a new percentage value,
progressing for example from 15% to 55%, 75% and 100% midline section (Fig. 5 A₁).
The ratio of lesioned length over total length was estimated by eye under the dissection microscope.

Figure 5 A₁ shows that the D-glutamate-induced burst frequency at 55% section is
intermediate between those in control (0%) and after complete hemisection (100%). With
every increase in midline section the burst frequency increased in both ipsi- and
contralateral ventral roots (Fig. 5 A₁ and A₂). This was true for all preparations (n = 4),
which are represented in graphs of burst frequency versus percentage midline section
(Fig. 5 A₂, B, C, D). The quality of burst activity (Cᵣ) is given by the size of the filled
circles, as indicated in figure 5 D. The left-right alternation of fictive swimming
remained at 75% of sectioned midline, but was absent at 90% when the two sides became uncoupled. The motor output expressed in the intermediate preparations forms a
continuum between fictive swimming in the controls (0% section) and the fast rhythm
obtained after full hemisection (100%) both in terms of frequency and of rhythmic
quality (Cᵣ). This continuity of motor behavior during progressive hemisection suggests
that the fast rhythm represents the output of the CPG for swimming, in the absence of
crossed inhibitory connections.

Progressive midline lesions in NMDA: the fast and the slow hemicord
rhythm and their relation to fictive swimming

In NMDA the hemicords may express both fast and slow rhythms, and it was therefore
important to perform the intermittent cuts experiments also during these conditions.
Fictive swimming in the intact cord displays as a rule only one burst pattern (Fig. 6 A₁,
0% section), while most intermediate preparations displayed two rhythms simultaneously, such that bursts at a low frequency also contained high frequency bursts (Fig. 6 A₁, 40% section). Both the fast and slow rhythms alternated bilaterally, as can be seen in the raw recordings at low and high time resolution, as well as in the cross-correlogram (Fig. 6 A₁, 40% section). Upon completion of the progressive lesions, the hemicords displayed the slow rhythm alone (Fig. 6 A₁, 100% section), as they typically do when the slow rhythm is strong (see above). It is evident that the fast rhythm in the intermediate preparations \((n = 4)\) derives from fictive swimming in control, as its frequency increases progressively with the increase in percentage midline lesion (Fig. 6 A₂, B, C). The pattern of continuity between fictive swimming and the fast rhythm that was established in d-Glutamate, is thus confirmed in NMDA. The slow rhythm, on the other hand, appears in the intermediate preparations already at a low frequency and remains at this rate until the cut is complete (Fig. 6 A₂, B, C, D). In one case, the slow rhythm was already detectable in the intact cord superimposed on fictive swimming, and it could be followed through several consecutive cuts up to the slow hemicord rhythm (Fig. 6 D), while maintaining approximately the same frequency. Taken together these data confirm that the fast rhythm, generated by the lamprey hemi-spinal cord under an array of different activating conditions, is directly linked to fictive swimming.

**The role of the voltage-dependent properties of NMDA receptors**

The results presented thus far, suggest that the two types of rhythm expressed in the hemicord are not just separated by an order of magnitude in burst frequency, but are likely to be mediated by different network configurations (see Discussion). The slow
rhythm was only observed in NMDA. NMDA receptors (NMDARs) display a voltage
dependence due to the pore-blocking action of magnesium ions (Nowak et al. 1984; Dale
and Grillner 1986). To test if this voltage dependence is important for the generation of
rhythmcity in the hemicord, Mg$^{2+}$ was removed from the physiological solution, as
previously studied in the intact spinal cord (Brodin and Grillner 1986).

In four hemicords the slow NMDA-induced rhythm (75 or 150 µM; 0.2-0.3 Hz;
average $C_r = 0.09 \pm 0.05$; range = 0.05 to 0.17) was evoked and then NMDA, as well as
Mg$^{2+}$ ions, were washed out. The NMDA concentration was subsequently increased in
steps starting from 10 µM, which was the lower threshold for activation (NMDA is more
effective in Mg$^{2+}$-free solution; Brodin and Grillner 1986). At 40 µM and above, ventral
root activity progressively vanished, as observed previously in the intact spinal cord
when using Mg$^{2+}$-free Ringer’s (Brodin and Grillner 1986). Within this effective range of
NMDA concentrations a slow rhythm was never detected (Fig. 7 A), not even with the
aid of autocorrelation analysis (not shown). On the other hand a fast rhythm was now
present in all preparations ($n = 4$; 4-5 Hz; average $C_r = 0.09 \pm 0.04$; range = 0.04 to 0.14;
Fig. 7 A, *two bottom traces*).

In another two hemicords D-glutamate was perfused to induce the fast rhythm (1 mM;
6-7 Hz; average $C_r = 0.27$). Both D-glutamate and Mg$^{2+}$ ions were then washed out for at
least 1 hour, at which point no activity was present at the ventral roots. D-glutamate was
then reperfused in Mg$^{2+}$-free solution, first at 125 µM and subsequently at 250 µM. The
fast rhythm was expressed in the absence of Mg$^{2+}$ (Fig. 7 B), in both preparations tested
(5-6 Hz; average $C_r = 0.27$). A consistent change in quality with Mg$^{2+}$-free compared to
control was thus not manifest.
In summary the slow rhythm, but not the fast, is crucially dependent on the voltage sensitivity of NMDAR-gating. Moreover, when the slow rhythm was abolished in 0 Mg\textsuperscript{2+}, the fast motor pattern emerged.

**The fast and the slow hemicord rhythms do not require ipsilateral glycinergic inhibition**

Another important question is whether inhibitory neurons participate in shaping patterned activity in the ipsilateral networks of the hemi-spinal cord, for example by promoting burst termination on the ipsilateral side. In the lamprey, two types of glycinergic neurons which project ipsilaterally have been identified: the lateral interneuron (LIN; Rovainen 1974; McPherson et al. 1994) and the small ipsilateral inhibitory interneuron (SiIN; Buchanan and Grillner 1988).

To test this possibility we blocked glycinergic synaptic transmission by applying strychnine (1 µM) both during the fast rhythm in D-glutamate \((n = 6, \text{from 3 animals})\) and during the slow rhythm in NMDA \((n = 6, \text{from 5 animals})\). The effects were investigated after at least 1 hour of drug perfusion. Both rhythms remained in strychnine (Fig. 8), indicating that glycinergic inhibition is not essential for rhythm generation in the hemicord. In the case of the fast rhythm (Fig. 8 A) the frequency was not significantly affected (average frequency change = –6 ± 14 %), while there was a consistent decrease in rhythmic quality in all preparations (average \(C_r\) change = –44 ± 18 %). The frequency of the slow rhythm (Fig. 8 B) was unaffected in 3 out of 6 preparations tested, while it was somewhat decreased in the remaining three (average frequency change = -27 ± 28 %). Remarkably, all three cases in which the frequency decreased and one where it did
not change, were associated with a substantial improvement in quality (Fig. 8 B; average C, change = 153 ± 220 %).

**DISCUSSION**

From the Results follows that rhythmic bursting can be generated by a completely hemisected spinal cord, and thus without contralateral inhibition. The hemicord can express two distinct motor patterns, fast and slow bursting, so defined in relation to the frequency of fictive swimming before hemisection. The fast rhythm (2-12 Hz) could be induced both pharmacologically and by electrical stimulation, in preparations as small as one single hemisegment. The slow rhythm (0.1-0.4 Hz) appeared only with NMDA, and required the NMDAR voltage-dependence. Ipsilateral glycinergic inhibition is not essential for the generation of either rhythm. Of the two motor patterns expressed by the hemicord, only the fast is firmly linked to fictive swimming. This was demonstrated by progressively reducing the amount of crossed connections, and showing that ventral root bursting accelerates, forming a continuum between fictive swimming before hemisection and the fast rhythm in the hemicord. The slow rhythm may thus either represent a non-locomotor-related motor behavior or slow swimming dependent presumably on different network mechanisms.

Past attempts to demonstrate rhythmic output in the lamprey hemicord have been negative or unclear. Ventral root bursting was reported to be replaced by tonic/irregular activity after hemisection of the isolated spinal cord (Grillner et al. 1983; Buchanan 1999) or after photoablation of commissural neurons (Buchanan and McPherson 1995).
These different findings made Buchanan (1999, 2001) conclude that crossed inhibitory connections are required for locomotor rhythm generation. Signs of a fast rhythm were, however, reported in a brainstem-hemicord preparation during reticular stimulation, and after electrical stimulation of the hemicord in strychnine (Grillner et al. 1986).

The reasons for the failure to detect unilateral bursting in previous studies may be due to several factors. A fast rhythm was occasionally observed in NMDA by Buchanan (1999), but considered unrelated to swimming due to its higher frequency. Moreover, in D-glutamate the conditions are more favourable than in NMDA, used by Buchanan. The slow NMDA rhythm may have escaped attention since it often requires a long time to develop. The present demonstration of fast bursting initiated by electrical stimulation is complementary, since it does not depend on a pharmacological activation.

**The hemicord locomotor networks do not require glycinergic inhibition**

An important consideration is the role of inhibition in the generation of hemicord rhythmicity. The question, if small ipsilateral glycinergic interneurons (Buchanan and Grillner 1988) are involved in the burst generation, had been unresolved, although simulations indicate that they are not needed (Hellgren et al. 1992). Our results with strychnine provide direct evidence that glycinergic inhibition is not required for burst generation to occur and neither for burst frequency regulation of either the fast or the slow rhythm.
Mechanisms of hemicord burst generation

Why did the fast rhythm not attain as high quality (C_r) in NMDA, as with D-glutamate and electrical stimulation? One possibility is that the plateau properties endowed onto spinal neurons by specific NMDAR activation (Wallén and Grillner 1987), while important at low burst frequencies (Brodin and Grillner 1986), might instead be an obstacle at high frequencies. This interpretation is supported by the fact that in NMDA, the highest quality fast rhythm (C_r) was observed in Mg^{2+}-free solution. In D-glutamate the activation of both AMPA and NMDA receptors will result in a lesser contribution of the voltage-dependent NMDA-properties (Zhang et al. 1996).

The excitatory glutamatergic interneurons (EINs) excite each other (Parker and Grillner 2000) and activate motoneurons (Buchanan and Grillner 1987), in both cases via monosynaptic connections. Rhythm generation in the hemicord can thus be accounted for by an interaction between ipsilateral EINs, and does not require glycinergic inhibition. A fast rhythmic motor pattern, as observed in the hemicord, could be produced by a population of EINs that, when active, become synchronized through their mutual excitation. Accordingly, the interval between consecutive spikes or bursts of spikes, mainly dictated by the slow afterhyperpolarization (sAHP), would synchronize across EINs. During these periods of quiescence in the interneurons, there will be no action potentials in the motoneurons, thus separating consecutive bursts at the ventral roots. The sAHP is mainly due to activation of Ca^{2+}-dependent K^+ channels (K_{Ca}) but also to a smaller component probably representing Na^+-activated K^+ channels (Cangiano et al. 2002; Wallén et al. 2002). K_{Ca} activation has previously been shown to contribute to burst termination in the intact spinal cord (El Manira et al. 1994). This comparatively
simple neuronal organization can account for the “fast” burst generation in the hemicord both during D-glutamate and after electrical stimulation, and in the intact cord, as demonstrated in simulations (Hellgren et al. 1992; Kotasleski et al. 1999).

In NMDA the fast rhythm is less frequent and often replaced with a slower rhythm (0.1-0.4 Hz), with a reciprocal relationship between the two. Clear analogies exist between the slow rhythm and the NMDA-induced membrane potential oscillations (with a depolarizing plateau) evoked in tetrodotoxin in spinal neurons (Sigvardt et al. 1985; Wallén and Grillner 1985, 1987). The latter have similar frequencies, disappear if NMDA is substituted with D-glutamate (Zhang et al. 1996), and require the voltage-dependent block of NMDARs by Mg$^{2+}$ ions. If a proportion of the EINs would display plateau potentials, they would also mutually excite each other, and therefore be active together. The termination of the plateau potentials is due to activation of K$_{Ca}$ channels caused by the Ca$^{2+}$ entry during the plateau. As the EINs terminate firing one after the other, the remaining cells will lose the input from surrounding EINs. This mechanism could thus account for the slow burst generation.

Finally, in the transition from a fast to a slow NMDA-induced rhythm the two can be expressed simultaneously. This can be explained if part of the EINs display plateau potentials and generate the slow rhythm, while other EINs become synchronized and tick-on in the fast rhythm. The action potentials generated by plateauing EINs could still fire in synchrony with the remaining “fast” EINs. A simple arrangement like this can account for the two superimposed rhythms. Slow rhythms superimposed on fictive swimming have also been observed in the intact lamprey spinal cord (Aoki et al. 2001), and in the tadpole (Reith and Sillar 1998).
**Glycinergic blockade during fictive swimming in the intact spinal cord mimics hemisection**

A partial glycinergic blockade by strychnine leads to an increased locomotor frequency (Grillner and Wallén 1980; Cohen and Harris-Warrick 1984; McPherson et al. 1994). This is in agreement with the frequency changes observed here with progressive midline lesions. With a full blockade of glycinergic inhibition, a tonic activity has been reported (Grillner and Wallén 1980; McPherson et al. 1994), or a fast (1-5 Hz) or very slow synchronous bilateral bursting (Cohen and Harris-Warrick 1984; Alford and Williams 1989; Hagevik and McClellan 1994). The fast bilateral bursting can be explained by a crossed excitatory interaction, and is in the hemicord fast rhythm range.

The contralateral phasic inhibition in the intact spinal cord is thus responsible for generating not only the left-right alternation, but also for slowing down the burst frequency. Between each burst there will be a period of inhibition that delays the occurrence of the subsequent burst and thereby prolongs cycle duration.

**The organization of the spinal locomotor networks in other vertebrates**

The spinal network for swimming in the frog embryo (*Xenopus laevis*) shares many features with that of the lamprey. The frog embryo swims with alternating left-right contractions of the body which, however, are generated by single motoneuronal action potentials in each swim cycle. Unilateral activity can be evoked in the xenopus hemicord having a frequency higher than that of fictive swimming before hemisection (Soffe
In analogy, low doses of strychnine increase the locomotor rate in the intact cord (Dale 1995).

In mammals and other tetrapods the evidence for a UBG organization is more indirect. Unilateral bursting may occur in flexors without concomitant extensor activity, and activity may occur in proximal muscles without rhythmic bursting in distal muscles (Grillner and Zangger 1979; Grillner 1985; Stein et al. 1995; Kiehn and Kjaerulff 1998). In the rat embryo synchronous bursting occurs initially in all muscles of the two hind-limbs, somewhat later left and right limbs start to alternate, and finally also flexors and extensors within a limb (Nishimaru and Kudo 2000). In the newborn rat, a blockade of reciprocal inhibition during fictive stepping, increases the locomotor rate, as in the lamprey and tadpole, and a full blockade can induce slow bursts synchronized across all muscle groups (Cowley and Schmidt 1995; Kremer and Lev-Tov 1997). In the mudpuppy, forelimb flexor and extensor activity could be surgically separated (Cheng et al. 1998). These data taken together suggest that there are different rhythm-generating centers for each group of muscles, and that reciprocal inhibition is not required for rhythmogenesis.

In conclusion, the lamprey locomotor CPG contains left and right rhythm-generating networks distributed along the spinal cord. These unilateral networks internally rely solely on excitation, but are strongly down-regulated in frequency by reciprocal inhibition. These results, taken together with those obtained in other vertebrates, provide support for the notion of a neural organization of the vertebrate motor system with a
series of UBGs that can be recombined in a flexible way to produce different motor patterns.
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FIGURE LEGENDS

Figure 1. The Coefficient of Rhythmicity (C_r). Three examples of ventral root recordings of decreasing rhythmic quality are shown (1st, 3rd and 5th trace on the left), such that burst timing becomes more irregular and activity more tonic. Below each raw recording a rectified/integrated version is shown, which gives a more faithful measure of the amount of spike activity (thin dashed lines represent the level of background noise). The auto-correlograms on the right are derived from the three recordings, and display oscillations the period of which is equal to the inverse of the burst frequency at the ventral roots. The amplitude of these oscillations is instead related to the rhythmic quality: the more rhythmic the ventral root activity, the more pronounced the oscillations. The coefficient C_r is calculated on selected features of the auto-correlogram (formula on the right) and takes values in the range 0 to 1 (see Methods). The C_r of each recording is indicated to the left. These values give a good description of the rhythmic quality of a recording. Note that for the third auto-correlogram an enlargement is also shown (inset) that clearly illustrates its rhythmic nature.

Figure 2. The hemi-spinal cords express a fast rhythm in D-glutamate. A1: Simultaneous recordings from two ipsilateral ventral roots of a hemicord preparation, during perfusion of 1 mM D-glutamate (top pair of traces). Enlargement of a smaller section (bottom pair of traces) shows that motoneuronal activity is organized in distinct bursts of spikes. A2: The auto-correlogram of either ventral root recording contains a series of peaks repeating every 0.18 s, indicating rhythmic activity at the fundamental frequency of 5.6 Hz. A3: The cross-correlogram of the two ventral root recordings has the main peak centered
approximately at a delay of 0 seconds. Bursts are thus synchronous (see also panel A1, bottom pair of traces) except for a small lag (inset). B: Histograms summarizing all the burst frequencies observed before (gray bars) and after hemisection (white bars), in all preparations. Note that each preparation may contribute to more than one frequency bin. C: Different concentrations of D-glutamate were tested in each of 6 preparations. The frequency of rhythmic activity in the hemicords increases with higher levels of the agonist. D1: A 10-hemisegments long preparation was transected to obtain a 3-hemisegments short piece. Rhythmic bursting in D-glutamate was present both in the original (lower trace) and in the reduced preparation (top pair of traces), at similar frequencies. D2: The cross-correlogram of the activity at two adjacent ventral roots of the short hemicord shows that bursts are coordinated even in such a short piece.

Figure 3. The hemi-spinal cords express a fast and a slow rhythm in NMDA. A1: Simultaneous recordings from two adjacent ventral roots of a hemicord preparation, during perfusion of 75 µM NMDA (top pair of traces). The fast rhythm expressed by the hemicord becomes more evident after rectifying-integrating ventral root activity (bottom pair of traces), which gives an indication of spike intensity. A2: Auto-correlation analysis confirms the presence of a rhythm at 3.4 Hz. A3: The position of the main peak in the cross-correlogram of the two recordings shows that bursting occurs synchronously at the two ventral roots (see also panel A1, bottom pair of traces). B: Simultaneous recordings from three ipsilateral ventral roots of a hemicord preparation expressing a slow 0.18 Hz rhythm (150 µM NMDA). C: Histograms summarizing all the burst frequencies observed before (gray bars) and after hemisection (white bars), in all preparations. Two hemicord
rhythms can be clearly resolved, separated by a gap of frequencies. Note that each preparation may contribute to more than one frequency bin. D: Different concentrations of NMDA were tested in each of 3 preparations, of which one expressed the fast rhythm (filled circles) and all expressed the slow rhythm (open circles). Burst frequency increases with higher levels of the agonist.

Figure 4. The hemi-spinal cords express a fast rhythm following electrical stimulation. A: Two hemicords obtained by hemisecting a single piece of spinal cord are stimulated by a brief train of pulses delivered through suction electrodes. A prolonged episode of motor activity is evoked at the ventral roots, outlasting the stimulus by several tens of seconds (top pair of traces). Progressive enlargement of smaller sections (bottom three traces) shows that motoneuronal activity is organized in distinct bursts of spikes at 9-10 Hz. The locomotor frequency decreases progressively during each bout, in parallel with the diminishing spike intensity (not shown). B: Histogram summarizing all the burst frequencies observed after hemisection in all preparations. Note that each preparation generally contributes to more than one frequency bin, and the lower values observed (3-4 Hz) correspond to the activity towards the end of a bout. C: Single hemisegments express the fast rhythm following electrical stimulation.

Figure 5. Progressive midline lesions in D-glutamate demonstrate that the fast hemicord rhythm is linked to fictive swimming. A: Fictive swimming is induced by 0.75 mM D-glutamate in an intact piece of spinal cord. Motor bursts alternate bilaterally as in the freely moving lamprey (0%, top pair of traces). Intermittent cuts are performed uniformly along the entire midline, thereby reducing crossed connections by 15%. Ventral root
bursting becomes faster (15%, not shown). Intermittent cuts are further extended, thus reducing crossed connections by 55%. Bursting at the ventral roots is still alternating bilaterally, but at an even higher frequency (55%, middle pair of traces). This procedure is repeated two more times (75%, not shown), until complete hemisection is achieved with two hemicords expressing the fast rhythm independently of each other (100%, bottom pair of traces). A₂: This graph summarizes the experiment just described. For each of the five stages of cutting, average burst frequency is plotted as a function of percentage midline section. Arrows indicate which data points correspond to the raw recordings displayed in A₁. The size of the filled circles shows the average quality of bursting (Q₀) according to the scale given in panel D (inset). B/C/D: The results of progressive midline lesions in another three preparations. Every reduction in crossed connections evokes an increase in burst frequency.

Figure 6. Progressive midline lesions in NMDA demonstrate that the fast hemicord rhythm is linked to fictive swimming, while the slow rhythm is a separate motor pattern. A₁: Fictive swimming is induced by 75 µM NMDA in an intact piece of spinal cord. Motor bursts alternate bilaterally as in the freely moving lamprey (0%, top pair of traces). Intermittent cuts are performed uniformly along the entire midline, thereby reducing crossed connections by 40%. Two simultaneous rhythms, a fast and a slow, appear at the ventral roots (40%, 2nd pair of traces from the top and enlargement). The bursts in each rhythm alternate bilaterally (40%, traces and corresponding cross-correlogram). Intermittent cuts are further extended and both rhythms persist (85%, not shown). When complete hemisection is achieved, the two resulting hemicords express the slow rhythm
independently of each other (100%, bottom pair of traces) while the fast rhythm is not
detectable. A_2: Summary graph where for each of the four stages of cutting, average burst
frequency is plotted as a function of percentage midline section. Arrows indicate which
data points correspond to the raw recordings displayed in A_1. Filled circle size shows the
average C_r according to the scale given in panel D (inset). B/C/D: The results of
progressive midline lesions in another three preparations. Note that fictive swimming
accelerates when crossed connections are reduced, forming a continuum with the fast
rhythm. The slow rhythm maintains approximately the same frequency throughout
cutting and in one case it is already present in the intact cord (panel D, 0% lower data
point).

Figure 7. The slow rhythm, but not the fast, relies on the voltage-dependence of NMDA
receptors. A: The slow rhythm is induced in a hemicord with 150 µM NMDA (top trace).
NMDA and Mg^{2+} ions are washed out first, and NMDA subsequently reperfused at 20
µM. The hemicord does not express the slow rhythm in the absence of Mg^{2+} (2nd trace
from top) but does express the fast rhythm (enlargements). B: The fast rhythm is induced
in a hemicord with 1 mM D-glutamate (top trace). D-glutamate and Mg^{2+} ions are
washed out first, and D-glutamate subsequently reperfused at 0.25 mM. The hemicord is
capable of expressing the fast rhythm also in the absence of Mg^{2+} (bottom trace).

Figure 8. Both the fast and the slow hemicord rhythms do not depend on glycinergic
inhibition. A: The fast rhythm is induced with 1 mM D-glutamate (top trace). After 1
hour of strychnine perfusion (1 µM) the fast rhythm is still present (bottom trace). In 6
hemicord preparations tested, strychnine did not appreciably modify the burst frequency of the fast rhythm (top graph). B: The slow rhythm is induced with 150 µM NMDA (top trace). After 1 hour of strychnine perfusion (1 µM) the slow rhythm is still present (bottom trace). In all 6 preparations tested, the slow rhythm persisted in strychnine. A decrease in burst frequency was observed in only some of the preparations (bottom graph). Note that the graphs display both the individual results from each preparation, as well as the mean ± S.E. of all experiments.
\[ Cr = \frac{a-b}{a+b} \]

Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
A (NMDA - slow rhythm)
Control (150 μM NMDA)

0 Mg²⁺ (20 μM NMDA)

B (D-glutamate - fast rhythm)
Control (1 mM D-glu)

0 Mg²⁺ (0.25 mM D-glu)

Figure 7
A (D-glutamate - fast rhythm)
Control

1 μM Strychnine

B (NMDA - slow rhythm)
Control

1 μM Strychnine

Figure 8