Segmental oscillators in axial motor circuits of the salamander:
distribution and bursting mechanisms.

Dimitri Ryczko 1, Vanessa Charrier 1, Auke Ijspeert 2, Jean-Marie Cabelguen 1

(1) Pathophysiology of Spinal Networks, Neurocentre Magendie, INSERM U 862, Bordeaux University, 146 rue Léo Saignat, F-33077 Bordeaux Cedex, France.
(2) Swiss Federal Institute of Technology, Lausanne (EPFL), CH-1015, Lausanne, Switzerland.

Running head: Rhythmogenic capacity of spinal segments.

Number of text pages: 49.
Number of figures: 8. (6 out of 8 are color figures).
Number of tables: 0.
Number of supplementary figures: 2. (2 out of 2 are color figures).
Number of words: Abstract = 246 / Introduction = 418 / Discussion = 2873.
Number of references: 120.

Contact information:
Jean-Marie CABELGUEN
INSERM U862 - Neurocentre Magendie
146 rue Léo Saignat
F-33077 Bordeaux Cedex, France
Tel: 33 5 57 57 40 52
Fax: 33 5 57 57 40 51
Email: jean-marie.cabelguen@inserm.fr

Copyright © 2010 by the American Physiological Society.
ABSTRACT

The rhythmic and coordinated activation of axial muscles that underlie trunk movements during locomotion are generated by specialized networks in the spinal cord. The operation of these networks has been extensively investigated in limbless, swimming vertebrates. But little is known about the architecture and functioning of the axial locomotor networks in limbed vertebrates. We investigated the rhythm-generating capacity of the axial segmental networks in the salamander (Pleurodeles waltlii). We recorded ventral root activity from hemisegments and segments that were surgically isolated from the mid-trunk cord and chemically activated with bath-applied N-methyl-D-aspartate (NMDA). We provide evidence that the rhythmogenic capacity of the axial network is distributed along the mid-trunk spinal cord, without an excitability gradient. We demonstrate that the burst generation in a hemisegment depends on glutamatergic excitatory interactions. Reciprocal glycinergic inhibition between opposite hemisegments ensures left-right alternation and lowers the rhythm frequency in segments. Our results further suggest that persistent sodium current contributes to the rhythmic regenerating process both in hemisegments and segments. Burst termination in hemisegments is not achieved through the activation of apamine-sensitive Ca$$^{2+}$$-activated K$$^+$$ channels and burst termination in segments relies on crossed glycinergic inhibition. Together, our results indicate that the basic design of the salamander axial network is similar to most of axial networks investigated in other vertebrates, albeit with some significant differences in the cellular mechanism that underlies segmental bursting. This finding supports the view of a phylogenetic conservation of basic building blocks of the axial locomotor network among the vertebrates.

KEYWORDS: spinal cord, axial CPG, segmental oscillators, salamander.
INTRODUCTION

During locomotion in vertebrates, the rhythmic and coordinated activations of axial and limb muscles are generated by spinal networks, referred to as Central Pattern Generators (CPGs), which are activated from the brainstem (Grillner 1981). It is convenient to distinguish the network that controls axial motion (axial locomotor CPG) from those that control limb motion (limb locomotor CPGs) (Ijspeert 2008). The functional organization of the limb locomotor CPGs has been extensively investigated in mammals, and different models have been developed (Kiehn 2006; McCrea et al. 2008). The axial locomotor CPGs have been studied in greater detail in limbless, swimming vertebrates (lamprey, *Xenopus* tadpole). Neurobiological experiments and computer modeling suggest that the axial CPG for swimming consists of a double chain of oscillators (pools of neurons that produce a rhythmic activity) that are connected by reciprocal inhibition to produce left-right alternation (reviewed in Grillner 2006; Roberts et al. 2008).

By contrast, little is known about the intrinsic functioning of the axial locomotor CPGs in limbed vertebrates. Propagated waves of motor activity have been observed during chemically-induced locomotor-like activity in the isolated axial cord of the adult salamander and in the newborn rat (Delvolvé et al. 1999; Falgairolle et al. 2006). Furthermore, in the salamander, chemically-activated isolated portions (2-3 segments) of the axial cord can generate rhythmic ventral root activity that is characterized by left-right alternation (Delvolvé et al. 1999). This finding suggests that the rhythm-generating capability is distributed along the spinal cord in the salamander, as in limbless swimming vertebrates. However, the presence of gradients of oscillator burst frequencies along the spinal cord remains an open question. Moreover, nothing is known regarding the synaptic connectivity and the intrinsic neuronal properties that are involved in the rhythm-generation of spinal segments in the salamander.
The goal of this study was to investigate the rhythm-generating capacity of the segmental networks from the axial cord in the salamander. To address this issue, which is relevant to all limbed vertebrates, we made use of single hemisegments and segments that were surgically isolated from the mid-trunk cord (i.e., between the cervical and the lumbar regions) and chemically activated with N-methyl-D-aspartate (NMDA).

We directly demonstrate that the global architecture of the oscillatory segmental networks in the salamander is similar to that of the swimming segmental networks in the lamprey. Our results further support the hypothesis that certain oscillator circuits (“modules”) have been preserved during evolution, albeit with different bursting mechanisms (Grillner 2006). Part of this work has been previously reported in abstract form (Ryczko et al. 2007).
METHODS

In vitro spinal cord preparations

Experiments were performed on in vitro isolated spinal cords from juvenile (9-12 mo post hatching) salamanders. Animals (*Pleurodeles waltlii*) obtained from Blades Biological Ltd. (United Kingdom) were kept in an aquarium at 17°C and fed twice per week with *Chironomidae larvae*. Snout-vent lengths ranged between 6.0 and 8.2 cm (mean ± Standard Deviation: 7.1 ± 0.5 cm) at the time of the experiments. Surgical procedures, handling, and housing of the animals were in accordance with protocols approved by the INSERM Ethics Committee and conformed to NIH guidelines. Briefly, animals (n = 35) were deeply anesthetized by immersion in a 0.1% aqueous solution of tricaine methanesulfonate (MS-222; Sigma). After evisceration, the preparation was placed into a dissection dish containing ice-cold amphibian Ringer solution (in mM: NaCl, 130; KCl, 2.1; CaCl₂, 2.6; MgCl₂, 0.2; HEPES, 4; glucose, 5; NaHCO₃, 1), saturated with O₂ (pH 7.4). The entire brain was exposed and the part rostral to the mesencephalon was removed and discarded. Thereafter, a dorsal laminectomy was performed to expose the first 20 segments of the spinal cord, and the spinal cord was disconnected from the brainstem by a transection at the level of the obex. The preparation (“whole spinal cord”, Fig. 1A) was then pinned down, dorsal side up, in a Sylgard-lined chamber and superfused (5 ml/min) with cooled (7°C) and oxygenated amphibian Ringer’s, to which was added the irreversible neuromuscular blocking agent α-bungarotoxin (α-BGTX; Sigma; 2 µM). The whole spinal cord was kept under such conditions for approximately 12 hours before the experiment began. On the following day, the temperature of the Ringer’s solution was progressively raised to 17°C before recording procedures began. Since the skin over the limbs, the tail and a part of the head was not removed, poisonous skin secretions (Nowak and Brodie 1978; Heiss et al. 2009) might diffuse into the bath during the 12-hour incubation, and might alter the properties of the spinal
networks. However, the high tolerance of urodeles against their own toxins has been demonstrated (Brodie and Gibson 1969).

Lesion experiments

With the aid of a stereoscopic microscope (Leica, Germany), relevant features of the spinal cord (approximate diameter: 0.75-0.87 mm) could be viewed and were used as a guide during the creation of lesions. The dorsal midline appeared as a distinct dark line on a lighter background, and the ventral roots (VRs) were easily identifiable. The mid-trunk spinal cord (6th to 12th spinal segments, Fig. 1A) was surgically separated into isolated segments or hemisegments (approximate length: 1.0-2.0 mm, Fig. 1B-C). As the 6th to 12th VRs specifically innervate the axial musculature (Francis 1934) and the networks controlling the limb locomotor movements are located in more rostral (2nd to 4th) and more caudal (15th to 18th) spinal segments (Cheng et al. 1998; Székely and Czéh 1976), the mid-trunk spinal cord is specifically involved in the rhythmic capabilities of the axial network. The activities of 41 hemisegments and 36 segments were recorded. Axial segments were surgically isolated from the mid-trunk spinal cord using ophthalmic scissors (FST, France), with transverse sections being performed at the junction between two successive pairs of VRs. Axial hemisegments were obtained by cutting the mid-trunk spinal cord, first sagittally along the midline using a microscalpel (30° model, Sharpoint), then transversely between successive VRs using the ophthalmic scissors. In all experiments, the completeness of the lesions was verified by gently displacing the sectioned pieces of the spinal cord.

Recordings

Before surgical transections, control recordings were performed on the mid-trunk ventral roots (6th to 12th spinal segments) from the whole spinal cord (Fig. 1A). The recording sessions started after stable patterns of efferent activity had emerged (i.e., after a 2- to 12-hour recovery period following the surgical transections). The VR activities were recorded by use
of tight-fitting glass suction electrodes (see Delvolvé et al. 1999). The neurograms were amplified 10000 times, band-pass filtered (10 Hz-1 kHz), sampled (4.8 kHz per channel), and stored on a computer for further analyses with the use of the Cambridge Electronic Device Micro 1401 mk II system.

Pharmacology

Chemicals were bath applied to the preparation at a constant flow rate of 10 ml/min with a peristaltic pump (Gilson, France). Rhythmic VR activities were induced by adding N-methyl-D-aspartate (NMDA, 20 µM) to the bath in combination with D-serine (10 µM) which potentiates the effect of NMDA (Johnson and Ascher 1987; Kleckner and Dingledine 1988), as previously described in the salamander (Cheng et al., 1998; Delvolvé et al. 1999). This NMDA concentration was selected because an initial series of experiments provided evidence that lower concentrations either induced irregular rhythmic patterns or did not trigger rhythmic VR bursts. In comparison, bath application of NMDA at concentrations ≤10 µM with D-serine (10 µM) induces rhythmic VR activities in the in vitro brainstem-spinal cord preparation from Pleurodeles waltlii, while at higher NMDA concentrations (>15 µM) the VRs display a tonic activity (Delvolvé et al. 1999). This difference probably results from a higher excitability of the spinal cord when connected to the brainstem. Accordingly, the cycle durations are much shorter in the brainstem-spinal cord preparation (4.9 ± 1.2 s; Delvolvé et al. 1999) than in the present preparation (9.57 ± 1.54 s; see Results). In some experiments (n = 23 hemisegments, n = 18 segments), apamine (0.5 µM) was applied concomitantly with NMDA (20 µM) and D-serine (10 µM) to induce rhythmic VR activities (see Results).

The involvement of synaptic mechanisms and intrinsic neural properties in rhythm generation was investigated pharmacologically. The non-NMDA glutamate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione disodium salt hydrate (CNQX) (10, 20 and 40 µM) to selectively block AMPA/Kainate receptors as previously described in spinal...
neurons (Alford and Grillner 1990; Tsvyetlynska et al. 2005) including amphibian spinal
neurons (Rioult-Pedotti 1997; Lavrov and Cheng 2004); strychnine (20 µM) to completely
block glycine receptors, as previously reported for amphibian spinal neurons (Jovanovic
et al. 1999; Kalinina et al. 2009); the gap junction blocker carbenoxolone (100 µM) (Rozental
et al. 2001) to block the electrical coupling between spinal neurons (Tresch and Kiehn 2000;
2002) (see discussion); apamine (0.5 µM), a selective blocker of small conductance Ca^{2+}-
activated K^+ channels (SK channels) (Blatz and Magleby 1986) that are responsible for the
Ca^{2+}-activated K^+ current (IK_{Ca}) expressed by amphibian spinal neurons (Wall and Dale 1995;
Chevallier et al. 2006); ZD 7288 (100 µM), a selective and irreversible blocker of cyclic
nucleotide-modulated (HCN) channels (Shin et al. 2001) that underlie the hyperpolarization-
activated cation current (Ih) expressed by salamander motoneurons (Chevallier et al. 2006).
Furthermore, based on previous results on rodent spinal neurons, we used riluzole (5, 50 and
100 µM) to reduce or block the persistent Na^+ current (INa_{(P)}) (Tazerart et al. 2007; Zhong et
al. 2007; Ziskind-Conhaim et al. 2008). Stock solutions of riluzole were made in
dimethylsulfoxide (DMSO) and diluted in the extracellular solution; the final DMSO
concentration in the extracellular solution was 0.5% for 50 µM riluzole. Note that DMSO
concentrations up to 4% have no effect on the NMDA-induced rhythmic motor activities
expressed by in vitro spinal cords from salamander (Lavrov and Cheng 2008). Other
chemicals were dissolved in amphibian Ringer’s solution and the pH was adjusted to pH 7.4
using NaOH. All chemicals were obtained from Sigma (France).

Data analysis and statistics

Episodes of rhythmic motor activity of 600 s in duration were analyzed using custom
scripts run within Spike2 software (version 5) for Windows (CED, UK) (Fig. 1D). Raw
recordings were rectified and filtered with a time constant of 50 ms. Additional channels were
used to monitor lowpass filtered copies of the rectified-filtered traces (time constant: 500 ms).
Presence of a stable rhythm was assessed with autocorrelograms obtained from the rectified and filtered motor activity. The stability of the rhythmic activity was assessed by measuring the Peak to Trough Correlation Coefficient (PTCC) on autocorrelograms by subtracting the minimum negative value of the correlation coefficient of the first negative peak from the maximum value of the first positive peak (Fig. 1E). A perfectly stable rhythm would have a PTCC value of -2 (see Madriaga et al. 2004). Based on the periodicity predicted by the autocorrelogram, the onsets and offsets were marked manually for each cycle with a cursor (resolution < 1ms) during visual inspection of the raw and the rectified-integrated traces (Delvolvé et al. 1997; 1999; Chevallier et al. 2004). A post hoc signal analysis (MATLAB version 7, MathWorks) revealed that the onsets/offsets determined by this method follow a threshold-based detection process: bursts are detected when the rectified-integrated signal exceeds the average noise level of the VR recording by a value estimated at 5% of the average peak to peak value of the rectified-integrated signal. The onsets and offsets were imported into a spreadsheet program for calculating and graphing the motor parameters. The cycle durations were measured as the interval between the onsets of burst activity in successive cycles. The duty cycles (burst proportions) were calculated as the duration of burst activity (onset-to-offset) divided by the cycle period. The left-right coordination pattern for a segment was calculated as the ratio of the delay between the onsets of right and left VR bursts and cycle duration. In some experiments, the amplitude of the burst was evaluated by measuring the maximum value on the rectified-filtered trace between the onset and the offset of the cycle. Unless stated otherwise, all data are expressed as the mean ± Standard Deviation (SD). Differences between samples were analyzed using a statistical software package (SigmaStat 3.0). Non-parametric tests were used for data analyses. For independent samples, the Mann-Whitney test was used, whereas the Wilcoxon test was used for dependent samples. For all motor parameters, statistical changes were taken to be significant at p < 0.05.
RESULTS

The in vitro whole spinal cords (n = 19) that were superfused with a combination of NMDA (20 µM) and D-serine (10 µM) expressed a long-lasting (several hours) rhythmic motor pattern which consisted of left-right alternation and waves of VR activity traveling caudally along the mid-trunk (i.e. 6th to 12th VRs) (Fig. 1A, 1D). This motor activity could be related to the operation of the axial network for swimming (Delvolvé et al. 1999; Chevallier et al. 2008), although the cycle durations (9.57 ± 1.54 s) and the intersegmental phase lags (6.17 ± 3.76 % of cycle duration) were much longer than those previously reported during swimming in intact animals (ranges: 0.23-0.75 s and 1.46-1.86 % of cycle duration, respectively) (Chevallier et al. 2004; Delvolvé et al. 1997). These differences in cycle durations and phase lags between preparations are probably due to differences in spinal cord excitability and/or sensory inputs (Friesen and Cang 2001).

The motor parameters (cycle duration, burst duration, duty cycle, rhythm stability, and left-right coordination) of a given VR activity were compared before (whole spinal cord preparation) and after performing surgical isolation of the corresponding segment or hemisegment (Fig. 1). We also evaluated the motor parameters of isolated hemisegments and segments in various pharmacological conditions.

Isolated axial hemisegments can generate a rhythmic motor activity

A total of 18 out of 41 surgically-isolated axial hemisegments (44 %) could exhibit a rhythmic motor activity in the presence of a combination of NMDA (20 µM) and D-serine (10 µM) in the bath (Fig. 2A1). The mean cycle duration of burst activity generated by the isolated hemisegments was significantly shorter than that of the activity generated by the whole spinal cord (p < 0.001, Wilcoxon test, n = 18) (Fig. 2C1). Moreover, the mean duration of the bursts generated by axial hemisegments was significantly shorter than that of the bursts
Rhythmogenic capacity of spinal segments

generated by the whole spinal cord (p < 0.001, Wilcoxon test, n = 18) (Fig. 2C1). However, despite shorter burst durations, the mean duty cycle of the bursts generated by the axial hemisegments was significantly longer than that of the bursts generated by the whole spinal cord (p < 0.001, Wilcoxon test, n = 18) (Fig. 2C1).

The mean PTCC value of the NMDA-induced bursting activity in axial hemisegments was significantly smaller than the PTCC of the activity that was generated by the whole spinal cord (p < 0.001, Wilcoxon test, n = 18). This finding indicated that the NMDA-induced bursting activity generated by hemisegments had a lower stability than that generated by the whole spinal cord (Fig. 2B1-C1).

Interestingly, the rhythm-generation capability of axial hemisegments was similar along the mid-trunk cord. Indeed, there was no significant correlation between the frequency of the bursts generated by a given axial hemisegment and its location along the mid-trunk cord (R = 0.14, p = 0.57, n = 18; Spearman correlation analysis) (Fig. 3A, left panel). Furthermore, there was no significant correlation between the stability of the rhythmic activity generated by a given hemisegment and its location along the mid-trunk spinal cord (R = 0.30, p = 0.22, n = 18; Spearman correlation analysis) (Fig. 3A, right panel).

**Isolated axial segments can generate a left-right alternating motor pattern**

After adding NMDA (20 µM) and D-serine (10 µM) to the bath, a total of 16 out of 36 isolated axial segments (44 %) could generate a rhythmic, left-right alternating motor pattern (Fig. 2A2).

The mean cycle duration of the bursting activity generated by axial segments was significantly shorter than that of the activity generated by the whole spinal cord (p < 0.01, Wilcoxon test, n = 16) (Fig. 2C2). By contrast, the mean burst duration of the bursts generated by axial segments was not significantly shorter than that of the bursts that were generated by
the whole spinal cord (p > 0.05, Wilcoxon test, n = 16) (Fig. 2C2). Consequently, the mean
duty cycle of the bursts generated by axial segments was significantly longer than that of the
bursts that were generated by the whole spinal cord (p < 0.05, Wilcoxon test, n = 16) (Fig.
2C2).

Isolated axial segments displayed a left-right alternating pattern that was very similar
to that which occurred in the whole spinal cord (p > 0.05, Wilcoxon test, n = 8) (Fig. 2C2).
The mean PTCC value of the bursting activity generated by the axial segments was
significantly smaller than the PTCC of the activity that was generated by the whole spinal
cord (p < 0.001, Wilcoxon test, n = 16) (Fig. 2C2). This finding indicated that the bursting
stability was lower in axial segments than in the whole spinal cord (Fig. 2B2-C2).

There was no correlation between the frequency of the bursts, or the stability of the
rhythmic activity generated by a given axial segment, and its location along the mid-trunk
spinal cord (respectively, R = -0.17, p = 0.53; R = 0.29, p = 0.28, n = 16, Spearman
correlation analysis) (Fig. 3B). This finding suggests that a gradient for oscillator burst
frequencies is not present along the salamander mid-trunk spinal cord.

The left bar chart in Fig. 3C summarizes the normalized mean burst frequencies
observed in axial hemisegments (n = 18) and segments (n = 16) during perfusion of NMDA
(20 µM) and D-serine (10 µM). The normalized mean frequency of the axial segments was
significantly lower than the normalized mean frequency of axial hemisegments (p < 0.001,
Mann-Whitney test). The right bar chart in Fig. 3C shows that the rhythm stability of the
rhythmic activity was not significantly more stable in segments than in hemisegments (p >
0.05, Mann-Whitney test).

Effects of apamine on the rhythmic motor activity expressed by hemisegments and
segments.
A \( \text{Ca}^{2+} \)-activated \( \text{K}^{+} \) current that is sensitive to apamine (\( I_{K_{\text{Ca}}} \)) has been shown to control the frequency of the NMDA-induced fictive locomotion in the lamprey (El Manira et al. 1994). Moreover, we have previously shown that spinal motoneurons of the salamander are endowed with \( K_{\text{Ca}} \) channels (Chevallier et al. 2006). Therefore, we next investigated whether apamine, as a blocker of \( K_{\text{Ca}} \) channels, would influence the rhythm-generating capability of axial hemisegments and segments in the salamander.

Apamine (0.5 \( \mu \text{M} \), 10-20 min) was added to the solution during the generation of ongoing rhythmic activity that was induced by bath application of NMDA (20 \( \mu \text{M} \)) and D-serine (10 \( \mu \text{M} \)). In each hemisegment (\( n = 9 \)) and segment (\( n = 6 \)) that we tested, \( K_{\text{Ca}} \) channel blockade did not disrupt the ongoing NMDA-induced rhythmic activity, but improved the signal-to-noise ratio of the recorded rhythmic activity (Fig. 4A1-A2).

Furthermore, in some experiments, rather than inducing rhythmic bursting, bath application of NMDA (20 \( \mu \text{M} \)) and D-serine (10 \( \mu \text{M} \)) induced tonic discharges in VRs from isolated axial hemisegments (\( n = 23 \), Fig. 4D1) or segments (\( n = 18 \), Fig. 4D2). In such cases, bath application of apamine (0.5 \( \mu \text{M} \)) was able to switch the tonic motor pattern to a rhythmic and left-right alternating one.

In hemisegments (Fig. 4C1), the cycle duration and the burst duration were not significantly altered during apamine application (\( p > 0.05 \), Wilcoxon test, \( n = 9 \)). However, in the presence of apamine, the duty cycle was significantly reduced and there was a significant increase in the stability of the motor rhythm (in both cases \( p < 0.05 \), Wilcoxon test, \( n = 9 \)). In segments (Fig. 4C2), the cycle duration, the burst duration, the duty cycle (\( n = 6 \) in both cases) and the left-right coordination pattern (\( n = 3 \)) were not significantly affected during bath addition of apamine (in all cases \( p > 0.05 \), Wilcoxon test). Interestingly, the stability of the motor rhythm was significantly increased during bath application of apamine (\( p < 0.05 \), Wilcoxon test, \( n = 6 \)) (Fig. 4B2-C2).
Note that apamine alone (i.e., without NMDA and D-serine) was unable to induce rhythmic motor bursting in axial segments (n = 2, not illustrated).

**Ih controls several parameters of rhythmic motor activity expressed by segments and hemisegments.**

The hyperpolarization-activated inward cationic current \( \text{I}_h \) has previously been described in spinal motoneurons from salamanders (Chevallier et al. 2006). Since \( \text{I}_h \) could contribute to the subthreshold depolarizing currents that initiate rhythmic bursting (Pape 1996), we tested whether blocking \( \text{I}_h \) pharmacologically with ZD 7288 (100 µM) would affect the rhythmic motor activity expressed by hemisegments and segments.

We observed that blocking \( \text{I}_h \) with ZD 7288 (100 µM, 20-30 min) did not affect the ability of axial segments (Fig. 5A2) and hemisegments (Fig. 5A1) to express a rhythmic activity when in the presence of NMDA (20 µM) D-serine (10 µM) and apamine (0.5 µM).

The motor parameters of the rhythmic activity generated by segments were affected in a complex way during ZD 7288 application (Fig. 5C2). The cycle duration and the rhythm stability were significantly increased (\( p < 0.05 \), Wilcoxon test, \( n = 5 \)). The burst duration was unaffected (\( p > 0.05 \), Wilcoxon test, \( n = 5 \)), the duty cycle was significantly reduced (\( p < 0.05 \), Wilcoxon test, \( n = 5 \)), and the left-right coordination was not significantly affected (\( p > 0.05 \), Wilcoxon test, \( n = 4 \)).

Almost every motor parameter of hemisegments (\( n = 5 \)) exhibited a similar trend (Fig. 5C1). Indeed, the cycle duration was significantly increased (\( p < 0.05 \), Wilcoxon test) the burst duration was not significantly affected (\( p > 0.05 \), Wilcoxon test), and the duty cycle was significantly decreased (\( p < 0.05 \), Wilcoxon test). Contrasting with segments, the rhythm stability was not significantly affected by bath application of ZD 7288 (\( p > 0.05 \), Wilcoxon test).
**Effects of riluzole on the rhythm-generating capability of hemisegments and segments.**

The persistent sodium current ($I_{Na(P)}$) has been implicated in the function of the locomotor pattern-generating network in the adult lamprey (Hu et al. 2002) and in the neonatal rodent spinal cord (Tazerart et al. 2007; Zhong et al. 2007; Tazerart et al. 2008; Ziskind-Conhaim et al. 2008). To assess the contribution of $I_{Na(P)}$ to the rhythm-generation of axial hemisegments and segments in the salamander, NMDA-induced bursting was examined in the presence of riluzole, a relatively selective pharmacological blocker of $I_{Na(P)}$ in spinal neurons (Tazerart et al. 2007; Zhong et al. 2007).

We observed that bath application of 5 µM riluzole could abolish the rhythmic VR activity expressed by hemisegments after 40-50 min of application in two out of three hemisegments tested (Supplementary Fig. 1). There was a progressive decrease of the burst amplitude (measured each minute) that was very significantly correlated ($p<0.001$, Spearman correlation analysis) with the duration of application of riluzole (40 min), until the bursting activity was eventually vanished after 40-50 min in two out of the three hemisegments tested. There was no significant effect of riluzole on the cycle duration and rhythm stability during the time the motor pattern was getting weaker (in both cases, $p > 0.05$, Spearman correlation analysis, $n = 3$ hemisegments).

Bath application of 50 µM riluzole caused bursting cessation more rapidly (20-40 min) ($n=3$ hemisegments) (Supplementary Fig. 1). As previously reported, it was difficult to wash out the effects of riluzole (typically 2-3 hours at 50 µM) (Tazerart et al. 2007; Zhong et al. 2007).

As with hemisegments, bath applied riluzole (50 µM during 20 min and subsequently 100 µM during 20 min) did not affect significantly the cycle duration and stability of the NMDA-induced bursting of segments (in both cases, $p > 0.05$, Wilcoxon test, $n = 6$), while it
The rhythmic bursting of axial hemisegments and segments requires the activation of AMPA/kainate receptors.

In the lamprey spinal cord, the activation of AMPA/kainate receptors contributes to the rhythmic bursting of the cord (Brodin et al. 1985; Alford and Grillner 1990) and hemicord (Cangiano and Grillner 2005). To test for similarities between the lamprey and salamander axial networks, we investigated the contribution of AMPA/kainate receptors to the rhythm-generating capability of single hemisegments and segments from the salamander mid-trunk cord.

We discovered that blockade of the AMPA/kainate receptors with 40 µM CNQX (Alford and Grillner 1990) abolished the NMDA-induced motor bursting of both hemisegments (n = 5) and segments (n = 4) in a reversible manner (Fig. 6). Furthermore, a dose-response study (10 to 40 µM CNQX) performed on two hemisegments, one of which is illustrated on Fig. 6A1, revealed that the CNQX-suppressant effect increased in a dose-dependent manner. The burst amplitude started to decrease progressively and totally disappeared with the concentration of 40 µM of CNQX both in hemisegments (Fig. 6A1-A2) and in segments (Fig. 6B). We observed that after a few hours, the rhythmic bursting systematically recovered from the vanishing effect of CNQX in every hemisegment (5/5) and segment (2/2) tested for wash out (Fig. 6).

The left-right alternation in axial segments, but not the rhythm generation, involves the activation of glycinergic receptors.
To test the possibility that the glycinergic interneurons previously described in the spinal cord of the salamander (Jovanovic et al. 1999) contribute to the generation of the hemisegment rhythmicity and to the left-right alternating pattern, we blocked the glycinergic synaptic transmission by adding strychnine (20 µM) to the perfusate during 50-60 min.

We observed that both axial hemisegments and segments still displayed a rhythmic motor activity in the presence of a combination of NMDA (20 µM), D-serine (10 µM), and strychnine (20 µM) in the bath (Fig. 7A1-A2). However, the left-right coordination pattern displayed by axial segments switched from alternation to synchrony during the application of strychnine (Fig. 7A2). These results reveal that glycinergic inhibition is not required for the rhythmicity of hemisegments and segments and that the left and right parts of a segment are coupled in antiphase by a reciprocal glycinergic inhibition, in parallel with a reciprocal excitation.

The mean cycle duration, the burst duration, and the duty cycle of the NMDA-induced bursting activity generated by isolated hemisegments were not significantly altered during bath application of 20 µM strychnine (p > 0.05, Wilcoxon test, n=7) (Fig. 7C1). However, there was a significant increase in the rhythm stability in the presence of strychnine (p < 0.05, Wilcoxon test, n=7) (Fig. 7B1-C1).

In axial segments, the left-right alternating pattern under NMDA and D-serine was replaced by a left-right synchronous pattern when 20 µM strychnine was added to the bath (p < 0.05, Wilcoxon test, n = 5) (Fig. 7A2-C2). Furthermore, the mean cycle duration of the NMDA-induced rhythmic activity was significantly shorter when 20 µM strychnine was added to the bath (p < 0.05, Wilcoxon test, n = 8) (Fig. 7C2). Whereas the burst duration was significantly reduced, the duty cycle was not affected (respectively, p < 0.05 and p > 0.05, Wilcoxon test, n = 8) (Fig. 7C2). In contrast to the hemisegments, the rhythm stability of the segments was not significantly affected by the addition of 20 µM strychnine to the bath (p >
Interestingly, there was no significant difference between the cycle durations of hemisegments and segments in the presence of 20 µM strychnine (p > 0.05, Mann-Whitney test, n = 7 hemisegments vs. n = 8 segments).

The bursting in axial hemisegments and segments and the left-right alternation in axial segments do not involve electrical coupling.

As electrical coupling among spinal neurons is known to contribute to the synchronization of their electrical activity (Perrins and Roberts, 1995; Li et al. 2009), we tested the effects of the gap junction blocker carbenoxolone on the rhythm-generating capabilities of isolated hemisegments and segments from the salamander mid-trunk cord.

Bath application of carbenoxolone (100 µM, 30-40min) did not affect the motor parameters of the bursting activity that was generated by either axial hemisegments or segments in presence of NMDA (20 µM) D-serine (10 µM) and apamine (0.5 µM).

Indeed, the cycle duration, the burst duration, the duty cycle, and the rhythm stability of the bursting activity generated by isolated hemisegments were not significantly modified during bath application of 100 µM carbenoxolone (p > 0.05, Wilcoxon test, n = 5) (Supplementary Fig. 2).

Similarly, the cycle duration, the burst duration (n = 8), the duty cycle (n = 8), the left-right coordination pattern (n = 5), and the rhythm stability (n = 8) of the NMDA-induced bursting activity generated by isolated segments were not significantly affected during bath application of 100 µM carbenoxolone (30-40 min) (in all cases, p > 0.05, Wilcoxon test) (Supplementary Fig. 2).
DISCUSSION

The hemisegmental oscillators

Our study provides direct evidence that each hemisegment surgically isolated from the mid-trunk cord of the juvenile salamander has the capacity to generate rhythmic motor activity when pharmacologically activated with NMDA. We further demonstrate that, whereas activation of AMPA/kainate receptors is required for hemisegmental rhythm generation, activation of glycinergic receptors is not essential. This finding suggests that the rhythmic bursting in axial hemisegments is generated by a pool of purely excitatory neurons that operate through AMPA/kainate synapses, as previously reported for burst generation in the lamprey hemicord (Cangiano and Grillner 2003). In the embryonic *Xenopus*, surgically isolated half of the brain and spinal cord can generate a rhythmic motor activity which requires NMDA receptor-mediated excitation, but not glycinergic inhibition (Soffe 1989). Similarly, in the lumbar hemicord of neonatal rats a rhythmic motor activity can be induced by the activation of NMDA and/or AMPA/kainate receptors in the absence of fast synaptic inhibitions (Bracci et al. 1996; Nakayama et al. 2002). Furthermore, application of *N*-methyl D,L-aspartate or NMDA/5-HT combination to isolated lumbar hemisegments of neonatal rats can induce rhythmic motor discharges which are not suppressed during subsequent addition of strychnine to the bath (Cowley and Schmidt 1997; Kremer and Lev-Tov 1997; see also Bonnot and Morin 1998 in the newborn mouse).

There is some evidence that the glutamatergic excitatory neurons which generate rhythmogenesis in the lamprey hemicord form a recurrently interconnected network (Cangiano and Grillner 2005). It is thought that a similar mechanism is present in neonatal rodent hemicords (Bracci et al. 1996). The excitatory cells directly responsible for rhythm-generation have not been yet identified, although candidate neurons have been proposed both in the lamprey (Cangiano and Grillner 2005), in the mouse (Hägglund et al. 2010), and in the
rodent (reviewed in Kiehn et al. 2008). In the mudpuppy, interneurons located in the brachial spinal cord can display rhythmic firing patterns during NMDA-induced forelimb locomotion (Cheng et al. 2002), but their contribution to rhythmogenesis of the forelimb locomotor network is unknown.

Previous studies provided evidence that electrical coupling synchronizes motoneurons within one segment during swimming in *Xenopus* embryos (Perrins and Roberts 1995; Zhang et al. 2009). A similar mechanism could contribute to the synchronization of activity of hemisegmental neurons for bursting. However, in *Xenopus* tadpoles and newborn mice, electrical coupling between some locomotor-related interneurons and/or motoneurones declines as development proceeds (Hinckley and Ziskind-Conhaim 2006; Zhang et al. 2009). This can explain our observation that carbenoxolone had no significant effect on the oscillatory behavior of hemisegments (and segments) in juvenile salamanders. However, the relatively low efficacy of carbenoxolone weakens this conclusion (Li et al. 2009). Therefore, further studies utilizing more effective gap junction blockers are required to address this issue.

**Role of crossed reciprocal connections in burst generation in segments**

Our study also provides evidence that the intrinsic bursting frequency of surgically-isolated hemisegments is higher (around two fold greater) than that of segments. This suggests that the reciprocal connections between rhythmogenic left and right hemisegments, albeit not necessary for rhythmogenesis, downregulate the bursting frequency of segments. A similar conclusion was drawn from the effect of a complete midsagittal lesion in *in vitro* spinal cord preparations from adult lampreys (Cangiano and Grillner 2003) and *Xenopus* embryos (Soffe, 1989). However, in larval lampreys the reciprocal connections between the left and hemi-spinal cords seem critical for the generation of the swimming rhythm elicited by electrical stimulation of the brain (Jackson et al. 2005).
In contrast to the above results, the bursting frequency of surgically-uncoupled two halves of the lumbosacral spinal cord is lower than that of the intact spinal cord in neonatal rodents (rats and mice) (Kudo and Yamada 1987; Bracci et al. 1996; Whelan et al. 2000; see however Kremer and Lev-Tov 1997) and turtles (Samara and Currie 2007). This difference may reflect a species difference and/or a difference in the functional organization of intrasegmental crossed connections between axial and limb segmental networks. However, more experiments are needed to determine the contribution of developmental changes to the left-right coordinating mechanisms in rodents (Nakayama et al. 2002).

In our study, the bursting frequency of segments is increased during bath application of strychnine (20 µM), while that of hemisegment is not affected. This suggests that crossed glycinergic connections are involved in the regulation of the rhythmicity of the motor pattern displayed by segments. Similarly, bath application of strychnine (0.5-30 µM) during expression of the forelimb locomotor activity in the \textit{in vitro} preparation of the brachial spinal cord of the mudpuppy results in increase in bursting frequency (Jovanovic et al. 1999). However a previous study provided evidence that in addition to block completely the glycine-evoked response in frog spinal motoneurons, strychnine at a concentration of 20 µM also reduces the response mediated by GABA\textsubscript{A} receptor activation (Kalinina et al. 2009). Therefore we cannot exclude the possibility that some of the strychnine effects we observed in our experiments were mediated partially via GABA\textsubscript{A} receptors. Selective GABA\textsubscript{A} blockers (e.g. gabazine; Heaulme et al. 1986) should be used to elucidate the contribution of GABA\textsubscript{A} receptors in segmental/hemisegmental rhythmogenesis.

By contrast, several studies indicate that complete blocking of glycinergic receptors with bath application of strychnine can induce variable effects on the motor bursting expressed by isolated spinal cords of the lamprey (Alford and Williams 1989; Cohen and Harris-Warrick 1984; Hagevik and McClellan 1994; Cangiano and Grillner 2003), \textit{Xenopus}
embryo (Soffe 1989; Green and Soffe 1998), adult zebrafish (Gabriel et al. 2008) and newborn rodents (reviewed in Nishimaru and Kakizaki 2009). However, it is known that the functional projection distances of individual commissural interneurons that provide reciprocal (glycinergic) inhibition between the two sides of the spinal cord extent over several segments in lampreys (Buchanan 1982; Ohta et al. 1991; Fagerstedt et al. 2000), *Xenopus* embryos (Soffe et al. 2001) and newborn rats (Kiehn 2006). Therefore, the action of strychnine on isolated spinal cords likely depends on the overall length of preparations (surgically-isolated segment vs. portion of the spinal cord) because of differences in the architecture of the inhibitory networks that mediate left-right alternation. Moreover, there may be differences regarding the effects of glycinergic receptors activation between adult and larval animals.

Mechanisms for oscillation in hemisegments and segments

Several ionic currents have been shown to determine the intrinsic properties of neurons involved in rhythm generation by spinal motor networks (reviewed in Harris-Warrick 2002). Their relative contribution to the bursting activity of hemisegments/segments in the salamander is still unknown. Nevertheless, our study provides some information about the contribution of \( I_{Na(P)} \) and \( Ih \) to hemisegmental/segmental bursting.

Our results show that bath application of riluzole does not affect the frequency of NMDA-induced bursting in axial hemisegments/segments, while it reduces the amplitude of the rhythmic VR discharges. Similar results have been previously reported for fictive locomotor patterns expressed by isolated spinal cords from neonatal rodents (Tazerart et al. 2007; Zhong et al. 2007). The effects of riluzole on the spinal networks for locomotion in rodents have been attributed to blockade of \( I_{Na(P)} \) (Tazerart et al. 2007; Zhong et al. 2007; Ziskind-Conhaim et al. 2008). It is likely that similar mechanisms occurred in axial hemisegmental/segmental networks of the salamander, but this remains to be tested.
Interestingly, some of the neurons that control the hindlimb locomotor rhythm in neonate rats express \( I_{\text{Na}(P)} \)-dependent (riluzole-sensitive) bursting properties (Tazerart et al. 2008). Similarly, the oscillator interneurons that control leech heartbeat express a persistent \( \text{Na}^+ \) current which is thought to play a crucial role both in burst formation and oscillation (Opdyke and Calabrese 1994).

Our results reveal that blockade of \( I_h \) does not affect the ability of axial hemisegments/segments to express rhythmic bursting. They further suggest that \( I_h \) exerts an important control on the rhythmic activity expressed by segments: decreasing \( I_h \) both increases the cycle duration and decreases the duty cycle in segments. Similar results have been reported for the rhythmic burst produced by the heartbeat network of the leech (Olsen and Calabrese 1996; Lu et al. 1999; Hill et al. 2001; Sorensen et al. 2004). Altogether these results are consistent with the view that \( I_h \) acts as a depolarizing leak current that contributes to the triggering of a new burst, rather than playing an essential role in the generation of a rhythm (Kiehn 2000). \( I_h \) contributes to the generation of oscillatory activity in the leech heartbeat network, however (Angstadt and Calabrese 1989).

Mechanisms of burst termination

During our experiments, the duration of the bursts expressed by single hemisegments was not affected by blockade of glycinergic receptors. This finding indicates that burst termination in single hemisegments does not rely on a buildup of recurrent glycinergic inhibition onto the pool of excitatory cells that generate bursting.

Several previous studies have provided insights into other possible mechanisms for burst termination. Apamine-sensitive \( K_{\text{Ca}} \) channels play a crucial role in burst termination during fictive swimming in the lamprey (Hill et al. 1992; El Manira et al. 1994; but see Meer and Buchanan 1992) and \textit{Xenopus} larvae (Sun and Dale 1998; but see Wall and Dale 1995).
Here, a blockade of the apamine-sensitive $K_{Ca}$ channels did not affect the duration of the bursts expressed by single hemisegments. This finding suggests that under our experimental conditions, the $K_{Ca}$ channels that are activated by $Ca^{2+}$ entry through NMDA channels (and likely also $Ca^{2+}$ channels) did not contribute to termination of the bursts that were expressed by single hemisegments. This finding might reflect species differences or differences between the rhythm-generating mechanisms in surgically-isolated hemisegments from those underlying oscillations in the intact network.

The activation of Na$^+$-activated K$^+$ channels ($K_{Na}$) that mediate the $Ca^{2+}$-insensitive component of the sAHP (Cangiano et al. 2002; Wallén et al. 2007) has recently been proposed as an additional mechanism for controlling burst termination, especially during high frequency bursting (Wallén et al. 2007, Hess et al. 2007, Huss et al. 2007). Whether this control mechanism is operational during bursting in axial hemisegments of the salamander is currently unknown.

The present results provide clear evidence that burst duration in single segments is regulated by crossed glycinergic inhibitory neurons. Interestingly, it has been shown that synaptic depression of the reciprocal inhibition can act as a burst termination mechanism in the struggling network of the *Xenopus* tadpole (Li et al. 2007).

**Stability of hemisegmental/segmental bursting**

The variability in the stability of the motor rhythm generated by the isolated lamprey spinal cord has been related to the extent of the multisegmental propriospinal projections (Miller and Sigvardt 2000; Ayali et al. 2007). It is likely that the propriospinal neurons described in the salamander (Davis et al. 1989) played a similar role during the NMDA-induced motor rhythm that was generated by the isolated whole spinal cord. The absence of these propriospinal neurons in segments and hemisegments could explain the lower regularity
of their NMDA-induced bursting activity. Moreover, the contribution of other conductances (e.g., $K_{Na}$) underlying the spike frequency adaptation in spinal neurons cannot be excluded.

It has recently been shown that SK channels can exert negative feedback on excitatory synapses in the amygdala (Faber et al. 2005). More specifically, blockade of SK channels with apamine (0.1 µM) was found to potentiate fast glutamatergic synaptic potentials (Faber et al. 2005). In that study, the authors demonstrated that the inflow of calcium through the NMDA channels activated SK channels, which in turn shunted the resulting excitatory postsynaptic potential. This mechanism might explain our observation that the signal-to-noise ratio increased during the bath application of apamine; thus, a blockade of the $K_{Ca}$ channels increased the efficiency of the glutamatergic synaptic drive. This mechanism might also explain the transformation of non-rhythmic NMDA-perfused hemisegments and segments into a rhythmic state after the additional bath application of apamine.

Interestingly, our experiments in which we applied specific blockers provided evidence that $I_K(Ca)$ and $I_h$ control the stability of bursting in axial segments of the salamander. As these conductances are targeted by neuromodulatory systems (e.g. cholinergic, Chevallier et al. 2006; serotonergic, Kiehn 2000), they might provide some degree of variability in the bursting of motor axial networks, which is essential for an enhanced robustness and a larger working range (Parker and Bevan 2007).

**Organization of the mid-trunk network**

Our results show that each axial segment of the salamander can be considered as a pair of oscillators that are coupled by reciprocal inhibitory (glycinergic) connections, which ensure left-right alternation. The left-right synchronous bursting that we observed in segments during blockade of glycinergic synaptic transmission further suggests that pairs of hemisegments are connected by relatively strong reciprocal inhibitory connections in parallel with reciprocal
excitatory connections. A similar conclusion has been drawn from results obtained in lamprey (Cohen and Harris-Warrick 1984; Hagevik and McClellan 1994), adult zebrafish (Gabriel et al. 2008) and tadpoles (Soffe 1989). Interestingly, previous studies have found parallel inhibitory and excitatory crossed segmental pathways responsible for left-right coordination pattern of the limbs during stepping in the mudpuppy (Jovanovic et al. 1999) and mammals (reviewed in Butt et al. 2002). The balance between crossed excitation and inhibition onto motor networks appears to be essential for increasing their dynamics to produce the appropriate motor pattern in response to either external conditions or internal goals (Berg and Hounsgaard 2009).

Therefore, it is reasonable to assume that the mid-trunk network of the salamander resembles a lamprey-like swimming network (double chain of hemisegmental oscillators; see Grillner 2006), as suggested by our previous in vitro study (Delvolvé et al. 1999) and predicted by our modeling study (Ijspeert et al. 2007) (Fig. 8). A similar organization has been proposed for the swimming network in the \textit{Xenopus laevis} embryo (Kahn and Roberts 1982; Roberts and Tunstall 1990; Tunstall et al. 2002), the swimmeret beating circuit in the crayfish (Paul and Mulloney 1986; Skinner and Mulloney 1998; Jones et al. 2003), the hindlimb scratching network in the turtle (Mortin and Stein 1989), the tail-moving network in the neonatal rat (Gabbay et al. 2002), and the hindlimb stepping network in neonatal rodents, embryonic chick and cat (reviewed in Kiehn and Butt 2003). Moreover, it has recently been suggested that the axial networks that control trunk movements during stepping in the newborn rat (Falgairolle and Cazalets 2007) and in humans (De Sèze et al. 2008) is composed of a double chain of coupled segmental oscillators. Although the activities recorded in the whole spinal cord have been related to the operation of the swimming network (Delvolvé et al. 1999), whether the activities of isolated hemisegments/segments recorded in this study embody the output of the network activated during swimming and/or stepping remains to be
determined. Furthermore we cannot rule out the possibility that hemisegmental/segmental networks in intact and lesioned cords differ functionally. Indeed, NMDA-evoked bursting in surgically isolated hemisegments and segments could result from alterations in the cellular and synaptic properties after surgery (Hoffman and Parker 2010). Whether the axial spinal cord of the salamander contains more than one specialized rhythm generating network per segment, as suggested for locomotor movements in the horizontal and vertical planes in the lamprey (Aoki et al. 2001) is also currently unknown.

Our results provide evidence that a higher bursting frequency for isolated mid-trunk segments than for the whole spinal cord. This could result from coupling between mid-trunk segmental oscillators and/or from coupling of mid-trunk segmental oscillators with limb oscillators, which have lower intrinsic frequencies than the mid-trunk ones (Ijspeert et al. 2007). Ascending and descending pathways have been found within the spinal cord of the salamander (Davis et al. 1989; Munoz et al. 1997), but their contribution to intersegmental coupling remains to be determined.

Our results further reveal that a gradient of oscillator frequency is absent along the mid-trunk cord of salamanders, as previously reported in adult lampreys (Cohen 1987). Similarly, no difference has been found between the frequencies of the hemisegmental oscillators controlling the swimmeret movements in the crayfish (Mulloney 1997). In contrast, the ability of isolated ganglia to generate swim-like oscillations is not uniformly distributed along the ventral nerve cord in the leech (Hocker et al. 2000). Furthermore, a rostrocaudal gradient of oscillator excitability has been found along the spinal cord in \textit{Xenopus laevis} embryos (Roberts and Alford 1986; Tunstall and Roberts 1994) and larval lampreys (Hagevik and McClellan 1994; but see McClellan 1994). This difference might result from developmental changes in the strength of the coupling between hemisegments or the descending drive to the segmental networks (Sillar et al. 1992). This possibility does not
Rhythmogenic capacity of spinal segments

exclude that a gradient of segmental oscillator excitability could also be produced by a
gradient of descending drive from the brain during natural (tadpole) or chemical/electrical
(lamprey) stimulation of the brain (Hagevik and McClellan 1994). Interestingly, a chain of
coupled oscillatory networks distributed over the lumbar spinal cord and with a rostrocaudal
excitability gradient has previously been proposed for a variety of rhythmic activities
displayed by the hindlimbs (reviewed in Kiehn and Butt 2003).

Conclusion

Altogether, these results strongly suggest a phylogenetic conservation of certain basic
principles of organization and function in the axial locomotor network in vertebrates: it is
composed of multiple distributed rhythm-generating core networks (Grillner 2006; Ijspeert
2008). Notwithstanding these similarities in axial network design across the vertebrate
phylum, there are some interspecies differences in the cellular mechanisms that underlie
segmental bursting. These differences are probably a reflection of the increase in the
flexibility of the axial motor network when moving from limbless to limbed animals
(Cabelguen et al. 2010).
ACKNOWLEDGEMENTS

The support from the European Community (LAMPETRA grant: FP7-ICT-2007-1-216100) and ANR (ImNet grant: ANR-07-NEURO-015-01) is gratefully acknowledged. D.R. received a fellowship from the MENRT. DR’s current affiliation: Groupe de Recherche sur le Système Nerveux Central, Département de Physiologie, Université de Montréal, C.P. 6128, succ. Centre-ville, Montréal (Québec), Canada, H3C 3J7.
REFERENCES


116. Wallén P, Robertson B, Cangiano L, Löw P, Bhattacharjee A, Kaczmarek LK, Grillner S. Sodium-dependent potassium channels of a Slack-like subtype contribute...


**FIGURE LEGENDS**

**Figure 1: Spinal cord preparations and motor parameters.**

A: The whole spinal cord preparation. A dorsal laminectomy was performed to expose the first 20 spinal segments, and the spinal cord was disconnected from the brainstem by a transection at the level of the obex (black horizontal arrow). Extracellular recordings were performed from right (r) and left (l) ventral roots (VRs), using glass suction electrodes at the level of mid-trunk spinal cord (6th to 12th spinal segments, grey region). B-C: The isolated axial segment and hemisegment preparations. Hemisegments and segments from the mid-trunk spinal region were surgically isolated. Extracellular recordings were performed from the corresponding VR, using glass suction electrodes. D: Example of rhythmic motor pattern recorded from two rVRs and one lVR, induced by bath application of N-methyl-D-aspartate (NMDA, 20 µM) and D-serine (10 µM) in the whole spinal cord preparation. The pattern consists of left-right alternation and waves of VR activity traveling caudally along the mid-trunk. The measured motor parameters (cycle duration, burst duration and delay) are shown. The left-right coordination pattern for a segment was quantified by calculating: delay/cycle duration. In some experiments, the amplitude of the burst was evaluated by measuring on the rectified-filtered trace the maximum value between the onset and the offset of the cycle. The rectified-integrated trace (∫) is illustrated for each recording. E: Autocorrelogram derived from the rectified-integrated trace from the VR recording display oscillations the period of which is equal to the inverse of the burst frequency at the VR. Autocorrelograms were used to evaluate the stability of the rhythmic activity by measuring the Peak to Through Correlation Coefficient (PTCC), calculated by subtracting the minimum negative value of the correlation coefficient of the first negative peak (AC min) from the maximum value of the first positive peak (AC max). The stability increases with the absolute value of the PTCC (cf. Madriaga et al. 2004).
Figure 2: Single hemisegments and segments of the mid-trunk spinal cord can express a rhythmic motor activity. **A1-A2:** Rhythmic motor activities recorded from the 11th right ventral root (VR) and from the 7th left (l) and right (r) VRs, before (left) and after (right) a surgical isolation of the corresponding hemisegment (A1) /segment (A2). Diagrams of the recording arrangements are presented on the top. The rectified-integrated trace ($\int$) is illustrated for each recording. **B1-B2:** Autocorrelograms derived from the rectified-integrated VR recording illustrated in A1-A2. Note that the stability of the rhythm, measured by the value of the Peak to Trough Correlation Coefficient (PTCC) (up down black arrow) was higher for the whole spinal cord, than for the isolated hemisegment and segment. This corresponds to the general impression obtained from the raw data in A1-A2. **C1-C2:** Bar charts summarizing the evaluated motor parameters of the ventral root activity for 18 single hemisegments (C1, right blue bars), 16 single segments (C2, right blue bars), and the corresponding whole spinal cord before isolation (C1-C2, left grey bars). Data are presented as the means ± SD. Each dot illustrates the value for one hemisegment /segment. *** p < 0.001, ** p < 0.01; * p < 0.05; n.s. not significant, Wilcoxon test.

Figure 3: Absence of gradient in bursting frequency and rhythm stability along the mid-trunk spinal cord. **A:** Bar charts show the bursting frequency (left) and PTCC (right) for several isolated axial hemisegments (H). For each H of the mid-trunk (6th to 12th), the bursting frequency and the PTCC are expressed as a percentage of the corresponding values measured along the whole spinal cord before the H was surgically-isolated (abscissa). The bars are ranked according to the rostrocaudal location of the H (ordinate). The number of experiments is indicated in brackets. Data are presented as the means ± SD. Each dot illustrates the value for one H. **B:** Bar charts show the bursting frequency (left) and PTCC (right) for several isolated axial segments (S). Same representation as in A. **C:** Bar charts show the mean
bursting frequency (left) and the mean PTCC (right) for 18 isolated H and 16 isolated S. Similar representation as in A. In each case, the rhythmic activity was induced by bath application of NMDA (20 μM) and D-serine (10 μM). *** p < 0.001, n.s. not significant, Mann-Whitney test.

Figure 4: Effects of apamine on the rhythmic motor activity expressed by hemisegments and segments. A1-A2: The motor bursting recorded from the isolated 10th hemisegment and the left-right alternating motor pattern recorded from the isolated 12th segment during bath application of NMDA (20 μM) and D-serine (10 μM) (control condition, left) was not disrupted by the addition of apamine (0.5 μM, 10-20 min) to the bath (right). Note the increase in the signal-to-noise ratio. B1-B2: Autocorrelograms derived from the rectified-integrated ventral root (VR) recording illustrated in A1-A2. Note that both for hemisegments and segments, the stability of the rhythm measured by the value of the Peak to Trough Correlation Coefficient (PTCC) (up down black arrow) was higher when apamine was added to the bath. C1-C2: Bar charts summarizing the evaluated motor parameters of ventral root rhythmic activity for 9 isolated hemisegments (C1) and 6 isolated segments (C2) in the control condition (left grey bars) and during application of apamine (0.5 μM, 10-20 min) (right blue bars). Data are presented as the means ± SD. Each dot illustrates the value for one hemisegment/segment. * p < 0.05, n.s. not significant, Wilcoxon test. D1-D2: In some experiments, bath application of NMDA (20 μM) and D-serine (10 μM) induced a tonic motor activity in isolated axial hemisegments (D1) or segments (D2). Addition of apamine (0.5 μM, solid horizontal blue bar) to the bath switched the tonic activity to a robust patterned rhythmic activity.
Figure 5: Effects of ZD 7288 on the rhythmic motor activity expressed by single axial hemisegments and segments. A1-A2: The rhythmic activity was induced by bath application of a combination of NMDA (20 μM), D-serine (10 μM), and apamine (0.5 μM) (control condition, left). Motor bursting were recorded in the control condition from the isolated 10th hemisegment (A1, left) and from the left (l) and right (r) ventral roots (VRs) of the 10th segment (A2, left), and during bath application of ZD 7288 (100 μM, 20-30 min) (A1-A2, right). Note the increase in cycle duration of the motor bursting both in the 10th hemisegment and in the 10th segment. The left-right alternating motor pattern was preserved. B1-B2: Autocorrelograms derived from the rectified-integrated VR recording illustrated in A1-A2. Note that for segments, the value of the PTCC (up down black arrow) was higher when ZD 7288 was added to the bath. C1-C2: Bar charts summarizing the motor parameters of the VR rhythmic activity for 5 isolated hemisegments and 5 isolated segments in the control condition (left grey bars) and during application of ZD 7288 (100 μM, 20-30 min) (right blue bars). Data are presented as the means ± SD. Each dot illustrates the value for one hemisegment/segment. * p<0.05, n.s. not significant, Wilcoxon test.

Figure 6: Effects of CNQX on the rhythmic motor pattern expressed by single axial hemisegments and segments. A1: Effect of gradually increasing concentrations of CNQX on the motor bursting induced by bath application of a combination of NMDA (20 μM), D-serine (10 μM) and apamine (0.5 μM) in the 12th hemisegment. Raw data, rectified-integrated traces of the ventral root (VR) recordings and autocorrelograms are illustrated for each concentration of CNQX (20-30 min). Recovery at 2 h after wash-out of CNQX is shown (right). A2: Temporal evolution of motor parameters under increasing concentrations of CNQX (solid horizontal blue lines) in the same hemisegment as illustrated in A1. For each motor parameter, each dot illustrates the mean ± SD during 1 minute. For each motor
parameter, the mean value in the control condition is illustrated by the horizontal dotted line. Note the disappearance of the rhythm with the 40 μM concentration of CNQX, as illustrated by the raw recording and its autocorrelogram in A1. B: In this experiment, rhythmic activity was recorded from the left (l) and right (r) VRs of the 7th segment when bath-applying NMDA (20 μM) and D-serine (10 μM) (control condition). The motor bursting recorded in the control condition (left) was blocked by the addition of CNQX (40 μM, 20-30 min) to the bath (middle). Recovery at 2 h after wash-out of CNQX is shown (right).

Figure 7: Effects of strychnine on the rhythmic motor pattern expressed by single axial hemisegments and segments. A1-A2: rhythmic activity was induced by bath application of NMDA (20 μM) and D-serine (10 μM) (control condition). The motor bursting recorded from the isolated 8th hemisegment in the control condition (A1, left) was not disrupted by the bath application of strychnine (20 μM, 50-60 min) (A1, right). The motor bursting recorded from the left (l) and right (r) ventral roots (VRs) of the 8th isolated segment in the control condition (A2, left) was also preserved during application of strychnine (20 μM, 50-60 min) to the bath. However, the left-right alternating pattern in the control condition switched to a synchronous pattern during the application of strychnine (A2, right). B1-B2: Autocorrelograms derived from the rectified-integrated VR recording illustrated in A1-A2. Note that for the hemisegment, the value of the PTCC (up down black arrow) was higher when strychnine was added to the bath. C1-C2: Bar charts summarizing the evaluated motor parameters of the ventral root (VR) rhythmic activity for 7 isolated hemisegments and 8 isolated segments in the control condition (left grey bars) and during bath application of strychnine (20 μM, 50-60 min) (right blue bars). Data are presented as the means ± SD. Each dot illustrates the value for one hemisegment or segment. * p < 0.05, n.s. not significant; Wilcoxon test.
Figure 8: Diagram of the proposed model for the axial segmental network. The network is divided into left and right ‘half centers’ (blue circles), which are hemisegments that innervate the ipsilateral myotomes. Each half center comprises a pool of coupled excitatory interneurons (red circles) and a pool of inhibitory interneurons (black circles). The excitatory interneurons project to the ipsilateral motoneurons (Mns), to the ipsilateral inhibitory interneurons, and to the contralateral excitatory interneurons. The inhibitory interneurons project to the contralateral inhibitory and/or to the contralateral excitatory interneurons. The axial segmental networks are distributed along the mid-trunk and are mutually coupled.

Supplementary figure 1: Effects of riluzole on the rhythmic motor pattern expressed by single axial hemisegments. A: Ongoing effect (40th to 50th minute) of a 5 µM concentration of riluzole on the motor bursting induced in the left (l) 11th hemisegment by bath application of a combination of NMDA (20 µM), D-serine (10 µM) and apamine (0.5 µM). Note the disappearance of the rhythm. B: The motor bursting, induced by NMDA (20 µM) and D-serine (10 µM) and apamine (0.5 µM), recorded from the right (r) 12th segment in the control condition (left) was blocked by the addition of riluzole to the bath (50 µM, 20-30 min) (middle). Recovery at 4 h after wash-out of riluzole is shown (right). C: Temporal evolution of motor parameters when bath-applying a 50 µM concentration of riluzole (solid horizontal blue line) in the same hemisegment as illustrated in B. For each motor parameter, each dot illustrates the mean + SD during 1 minute. Note the disappearance of the rhythm within 20-30 min of bath-applied riluzole, as illustrated by the raw ventral root (VR) recording in B.

Supplementary figure 2: Effects of carbnoxolone on the rhythmic motor pattern expressed by single axial hemisegments and segments. A1-A2: rhythmic activity was induced by bath application of NMDA (20 µM) and D-serine (10 µM) and apamine (0.5 µM)
Rhythmogenic capacity of spinal segments

The motor bursting recorded from the isolated right 11th hemisegment in the control condition (A1, left) was not disrupted by the bath application of carbenoxolone (100 μM, 30-40 min) (A1, right). The motor bursting recorded from the left (l) and right (r) ventral roots (VRs) of the 8th isolated segment in the control condition (A2, left) was also preserved during application of carbenoxolone (100 μM, 30-40 min) to the bath. The left-right alternating pattern in the control condition was also preserved during the application of carbenoxolone (A2, right). B1-B2: Autocorrelograms derived from the rectified-integrated VR recording illustrated in A1-A2. Note that both for the hemisegment and the segment, the value of the PTCC (up down black arrow) is not affected when carbenoxolone was added to the bath. C1-C2: Bar charts summarizing the evaluated motor parameters of the VR rhythmic activity for 5 isolated hemisegments and 8 isolated segments in the control condition (left grey bars) and during bath application of carbenoxolone (100 μM, 30-40 min) (right blue bars). Data are presented as the means ± SD. Each dot illustrates the value for one hemisegment or segment. n.s. not significant; Wilcoxon test.
## PTCC Calculation

The PTCC (Phase-Temperature Coefficient of Cycle) is calculated as:

$$PTCC = (AC_{\text{min}} - AC_{\text{max}})$$

where

- $AC_{\text{min}}$ is the autocorrelation coefficient at the minimum lag.
- $AC_{\text{max}}$ is the autocorrelation coefficient at the maximum lag.

### Rhythm Stability

- $0 > PTCC > -2$ indicates good rhythm stability.
- $PTCC < 0$ indicates reduced rhythm stability.

### Cycle, Burst, and Delay

- **Cycle**: The regular oscillation of the signal.
- **Burst**: The high-intensity part of the signal.
- **Delay**: The period of latency between cycle and burst.

### Amplitude Levels

- $5 \mu V, 50 \mu V, 100 \mu V, 200 \mu V, 250 \mu V$

### Figures

**A**: Diagram of a biological specimen with anatomical labels.

**B**: Schematic showing a comparative scale of 1 mm.

**C**: Schematic showing a comparative scale of 0.5 mm.

**D**: Waveforms illustrating cycle, burst, and delay with amplitude levels.

**E**: Graph depicting the autocorrelation coefficient (AC) against lag (s) with PTCC calculation and rhythm stability.
Excitatory interneuron
Inhibitory interneuron