Flexibility of the axial central pattern generator network for locomotion in the salamander

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Submitted 10 November 2014; accepted in final form 23 December 2014

Much of what we know about the dynamics of the locomotor system in tetrapods is derived from studies examining the adaptation of limb movements during terrestrial stepping in various conditions (Pearson 2000; Rossignol et al. 2006). However, several studies suggest that the axial musculature plays important roles in the adaptation of locomotor patterns to changes in either external conditions or internal goals. In cats and dogs, the amplitude and the patterns of activation of back muscles during stepping are shaped by the physical environment (level/up-slope/down-slope) (Wada et al. 2006a, 2006b) and the form of stepping (trotting vs. galloping) (Carlson et al. 1979; English 1980; Ritter et al. 2001; Schilling and Carrier 2010; Tokuriki 1974; Zomlefer et al. 1984). A speed and physical environment dependency in the epaxial muscle function has also been evidenced in lizards (Ritter 1996; Sharpe et al. 2013). In adult salamanders, several studies have provided evidence of a high degree of variability in the axial kinematics and in the activity pattern of the main epaxial muscle (dorsalis trunci) during locomotion, depending on physical environment (aquatic vs. terrestrial) and gait (stepping/swimming; walking/trotting; forward/backward walking) (Ashley-Ross 1994; Ashley-Ross et al. 2009; Ashley-Ross and Bechtle 2004; Ashley-Ross and Lauder 1997; Deban and Schilling 2009; Delvolvé et al. 1997; Edwards 1977; Frolich and Biewener 1992).

Several neural mechanisms could be proposed to account for the adaptation of the axial muscle pattern during ongoing locomotion in limbed vertebrates. These include an intrinsic flexibility in the operating mode of the spinal central pattern generator (axial CPG) producing axial muscle contractions during locomotion (Grillner 1981). Indeed, a few studies suggest that the spinal axial CPG for locomotion, even without descending and sensory inputs, is capable of producing a wide range of rhythmic patterns in axial motor activities. During pharmacologically induced fictive locomotion in high spinal cats, the muscle nerves innervating the lumbar back muscles can display a variety of rhythmic patterns corresponding to the different gaits observed in freely moving animals (Koehler et al. 1984). In vitro isolated spinal cords of salamanders, chemically activated with N-methyl-D-aspartate (NMDA), can produce backward- and forward-propagating waves of motor activity along the spinal cord (Charrier and Cabelguen 2013; Delvolvé et al. 1999; Ryczko et al. 2010a). However, it is difficult to make a clear relationship between the two distinct axial motor patterns observed in vitro and the various locomotor behaviors exhibited by freely moving salamanders.

An essential prerequisite for investigating the intrinsic flexibility of the axial CPG for locomotion in tetrapods is to precisely define the different axial patterns underlying the different forms of locomotion in vivo. This can be accomplished by recording kinematics of the body axis and electromyographic (EMG) activity of the axial musculature in freely moving animals. Among vertebrates, adult salamanders offer a remarkable opportunity to investigate this issue since they are able to exhibit a rich locomotor repertoire. Indeed, swimming and stepping (on land and underwater) are part of the natural behaviors of these animals. In addition to these main forms of locomotion, salamanders are capable of short episodes of backward stepping while maneuvering on land (Ashley-Ross and Lauder 1997). Our preliminary observations further suggested that adult salamanders display rhythmic struggling movements when firmly grasped by the body (Lamarque et al. 2013). A similar approach was used during rhythmic struggling movements since this would give some insight into the flexibility of the axial motor system. Our results show that each of the forms of locomotion and the struggling behavior is characterized by a distinct combination of mid-trunk motor patterns and cycle durations. Using in vitro electrophysiological recordings in isolated spinal cords, we observed that the spinal networks activated with bath-applied N-methyl-D-aspartate could generate these axial motor patterns. In these isolated spinal cord preparations, the limb motor nerve activities were coordinated with each mid-trunk motor pattern. Furthermore, isolated mid-trunk spinal cords and hemicords could generate the mid-trunk motor patterns. This indicates that each side of the cord comprises a network able to generate coordinated axial motor activity. The roles of descending and sensory inputs in the behavior-related changes in axial motor coordination are discussed.
as displayed by *Xenopus* tadpoles (Green and Soffe 1996; Kahn and Roberts 1982), larval zebrafish (Liao and Fetchko 2008; Sankrithi and O’Malley 2010), newt embryos (Soffe et al. 1983), and lampreys (Hsu et al. 2014; Islam et al. 2006; Kasicki and Grillner 1986; Saitoh et al. 2007).

Here we focused on the trunk located between the two girdles (“mid-trunk”), since there is a wealth of kinematic and/or EMG data on its functioning during the two basic locomotor behaviors of salamanders: swimming and forward land stepping (reviewed in Cabelguen et al. 2010; Schilling 2011). Moreover, the part of the axial locomotor CPG controlling the mid-trunk movements can be investigated in vitro (Delvolvé et al. 1999; Ryczko et al. 2010a). Our results show that the different locomotor behaviors in salamanders are associated with different mid-trunk kinematics, mid-trunk muscular patterns, and rhythm cycle durations. They also provide evidence that the isolated mid-trunk spinal cord pharmacologically activated with bath-applied NMDA generates multiple stable motor patterns that could be identified with those observed during the in vivo motor behaviors. Our preliminary results have been previously published in abstract form (Lamarque et al. 2009; Ryczko et al. 2009).

### MATERIALS AND METHODS

#### Ethics Statement

All procedures were in accordance with the guidelines of the INSERM Ethics Committee. The protocol was approved by the Animal Care and Use Committee of Bordeaux (France) (Permit No. A50120148). All surgery was performed under tricaine methanesulfonate anesthesia, and care was taken to minimize the number of animals used and their suffering.

#### Animals

Experiments were performed on 84 salamanders (*Pleurodeles waltli*). Animals were purchased from Blades Biological, kept in an aquarium at 17°C, and fed twice per week with *Chironomus* larvae and veal heart. Among the 84 animals used in the present study, 67 juvenile salamanders with snout-vent length (SVL) ranging from 4.9 to 8.5 cm were used for in vitro experiments, 12 adults (SVL 8.3–10.2 cm) were used for electromyography, and 5 adults (SVL 8.1–8.7 cm) were used for kinematic recordings.

#### Surgical Procedures

Animals were anesthetized by immersion in a 0.1% aqueous solution of tricaine methanesulfonate (MS-222; Sigma).

**Kinematic experiments.** Nine or ten white plastic beads (diameter 2 mm) were glued to the skin along the dorsal midline. The rostrocaudal position of the beads was expressed as a fraction of the SVL. Beads were placed from the tip of the snout (0.00 SVL) down to the rostral part of the tail (1.10 SVL) (see Fig. 2A). In some cases additional beads were glued onto the forelimb elbow joints or hindlimb knee joints or onto more caudal locations on the dorsal midline of the tail, but these were not considered here for kinematic analysis. The animals were allowed to recover from anesthesia for 1–2 h before video recordings. After each experiment, the beads were removed under anesthesia and the animals were put back in their housing aquarium.

**Electromyography experiments.** EMG electrode implantation was performed as previously described (Chevallier et al. 2004; Delvolvé et al. 1997). Briefly, pairs of 70-μm insulated stainless steel wires with bare ends (exposure 0.5 mm) separated by ≈1.0 mm were inserted 3–4 mm relative to the dorsal midline through small skin incisions into two ipsilateral mid-trunk myomeres of the dorsalis trunci muscle (0.56 SVL and 0.80 SVL). The insulated portions of the wires proximal to the tips were glued to the skin with cyanoacrylate adhesive. All electrode wire pairs were then gathered to make a light and flexible cable (length 1 m) that was sutured to the skin of the animal’s back. Sufficient slack was provided along implantation sites and points of attachment on the back so that the salamander could move in an unimpeded fashion. After electrode implantation, animals were allowed to recover from anesthesia for 1–2 h. At the end of the experiments, the electrodes were also removed under anesthesia before the animals were put back in their housing aquarium.

**In vitro experiments.** The animals were transferred to a cold (8–10°C) Ringer solution (in mM: 130 NaCl, 2.1 KCl, 2.6 CaCl₂, 0.2 MgCl₂, 4 HEPES, 5 glucose, and 1 NaHCO₃) saturated with O₂ (pH 7.4). Thereafter, a dorsal laminectomy was performed to expose the first 20 segments of the spinal cord, and the spinal cord was disconnected from the brain stem by a transverse section at the level of the obex (“whole spinal cord preparation”). Ventral roots (VRs) of the mid-trunk (6th to 12th VRs) were dissected from surrounding tissues and cut to allow easy access with suction electrodes. In 11 of the 67 preparations, two ipsilateral muscle nerves, one innervating forelimb protractors (the nervus extensorius caudalis; Francis 1934) and one innervating hindlimb protractors (the fibularis branch of the nervus sciaticus; Francis 1934), were also dissected from surrounding tissues and transversally cut to allow access with suction electrodes. The whole spinal cord preparation was then pinned down, dorsal side up, in a Sylgard-lined recording chamber and continuously superfused (5 ml/min) with cold (7°C) oxygenated Ringer solution containing the irreversible neuromuscular blocking agent α-bungarotoxin (Sigma; 2 μM). After 12 h of recovery, the temperature of the Ringer solution was raised to 17°C before recording procedures were initiated.

After control recordings performed on the whole spinal cord, in 11 of the 67 preparations the mid-trunk cord (6th to 12th spinal segments) was surgically isolated by transecting the cord between the 5th and 6th spinal segments and between the 12th and 13th spinal segments with ophthalmic scissors (Fine Science Tools, Heidelberg, Germany). In 6 of the 67 preparations, the isolated mid-trunk spinal cords were further sectioned sagittally along the midline to obtain mid-trunk hemiscord preparations. The sagittal sectioning was performed with a microscalpel (30° model; Sharpont). Isolated hemiscord preparations and isolated mid-trunk preparations were obtained from different animals.

#### Kinematic Recordings and Analysis

Salamanders were videotaped (15 or 60 frame/s) from above with a digital CCD camera (FOculus FO234SC, New Electronic Technology) and a Pentax 8- to 48-mm zoom lens. Parallax effects were negligible as the camera was placed a long distance (1 m) from the working section relative to the width of the video field (20 × 25 cm). Scaling was performed based on the known distance between two marks located on the track or on the tank. During forward terrestrial stepping, backward terrestrial stepping, and struggling, video recordings were performed on a rough paper surface. Swimming and forward underwater stepping were recorded on the bottom of a plastic tank (25 × 45 cm) filled with 5–6 cm of tap water, so that the surface of the water was above the height (1.5–2.0 cm) of the animal. Animals either moved voluntarily or were encouraged to do so by gentle touching or squeezing of their tail. Backward terrestrial stepping occurred either spontaneously or in response to a water jet that was gently applied onto the nose of the animal. Struggling was induced as long as the pelvic girdle was firmly grasped (i.e., the behavior stops as soon as the tonic grasping stimulation ends). Backward swimming was never observed, either spontaneously or following the tactile stimulation of the anterior part of the body known to induce backward swimming in lampreys (Islam et al. 2006).
The frame data acquired over an IEEE1394 link were processed with a custom program (Mtracking; K. Karakasiliotis, EPFL) running on an IBM-compatible computer to extract the x-y coordinates of the beads. The coordinates were imported into MATLAB (MathWorks) to reconstruct the trajectory of each bead within the video field. A regression analysis was performed to determine the overall moving direction of the animal. For each video frame, the body midline profile was drawn using a linear interpolation of the x-y coordinates of the midline markers. The locomotor speed of the animal was computed from movements of the most anterior bead (0.00 SVL) and converted to SVL per second (SVL/s). Only bouts in which the animal was moving in a straight line and at a constant speed were selected for further analysis. For the fastest locomotor mode observed (swimming), the onsets of EMG bursts were marked as previously described (Charrier and Cabelguen 2013; Delvolvé et al. 1999; Ryczko et al. 2010a). The cycle duration was defined as the time interval between the onsets of two successive motor bursts. The in vitro phase lag was defined as the delay between the onsets of the VR bursts expressed as a percentage of cycle duration divided by the intervening number of segments. In the legends of Figs. 4–7, the mid-trunk intersegmental phase lag values corresponding to the mean vector angle values illustrated on the circular plots are given for convenience. To this end, the mean vector angle value was converted in percentage and divided by the number of intervening segments.

Wavelet transformations and analyses were done with the MATLAB-driven Spinalcord software developed by Mor and Lev-Tov (2007), an increasingly used tool to analyze VR electrophysiological recordings (e.g., in mice: Hägglund et al. 2013; Humphreys and Whelan 2012; Nakanshi and Whelan 2012; in rats: Etlin et al. 2013). Wavelet transformation allows building a representation of the power of the frequency domains of the signal over the time course of the recording. A wavelet is a wave-shaped function with a zero mean, localized in frequency and time. The software allows performance of continuous wavelet transformation of the recorded signal. It consists of decomposing the recorded signal into a set of coefficients of a mother wavelet function (the Morlet wavelet) and of scaled (i.e., stretched in time) and translated versions of the mother wavelet function (for more information, see Grinsted et al. 2004; Mor and Lev-Tov 2007). Continuous wavelet transformation was performed on the rectified-filtered signals obtained from the VRs or the limb nerves. To examine whether two signals (e.g., 2 VR signals, 2 limb nerve signals...) have common power in some frequency domains and consistent phase relationships over time, we used the coherent cross-power wavelet transformation (CXT). The CXT combines the results obtained from the cross-wavelet transform (XWT) and the wavelet transform-coherence (CWT) algorithms (see Mor and Lev-Tov 2007). The XWT is the complex conjugation of the continuous wavelet transform obtained for each of the two recorded signals. The CWT measures how coherent the XWT of the two recorded signals is in time/frequency domains and thus resembles a cross-correlation coefficient (see Grinsted et al. 2004; Mor and Lev Tov 2007). Color plots were used to illustrate in time/frequency domains the significantly coherent cross-power between two recorded signals (CXWT; Mor and Lev-Tov 2007). The x-axis represents the time course of the recording and the y-axis the frequency domains. The frequencies are displayed with a logarithmic scale. The colors illustrate in logarithmic scale the power of each frequency domain over the time course of the recording, with warm colors (red) indicating high power and cold colors (blue) indicating low power. Only significantly coherent cross-power is illustrated. The significance of the coherence was determined with Monte Carlo estimation methods (see Grinsted et al. 2004; Mor and Lev-Tov 2007). The high-power regions of interest located in the close neighborhood of the rhythm frequency were delineated graphically and used for further statistical analysis of the coherence and phase relationship between the two signals. The phase relationships are illustrated as a vector on circular plots (Mor and Lev-Tov 2007). The edge artifacts that might distort the spectrogram are delineated by the cone of influence (opaque white region in the spectrograms) (see Grinsted et al. 2004; Mor and Lev-Tov 2007).
Statistics  

Data are given as means ± SD unless specified otherwise; n is the number of animals unless stated otherwise. Statistical analyses were performed with SigmaPlot 11.0 (Systat). Statistical differences were considered to be significant when P < 0.05. Correlations between variables were evaluated with the Pearson product moment correlation test. To compare two dependent groups, the paired t-test was used when the assumption of normality and equality of variance were respected; otherwise the Wilcoxon signed-rank test was used. To compare two independent groups, the t-test was used when the assumption of normality and equality of variance were respected; otherwise the Mann-Whitney rank sum test was used. To compare more than two dependent groups, we used the parametric one-way repeated-measures analysis of variance (ANOVA) or the nonparametric Friedman repeated-measures ANOVA on ranks, followed by a Student-Newman-Keuls test. To compare more than two independent groups we used the parametric one-way repeated-measures ANOVA or the nonparametric Kruskal-Wallis one-way ANOVA on ranks, followed by a Student-Newman-Keuls test.

To examine the number of peaks in the distribution of the intersegmental phase lags generated in vitro by the whole spinal cord and the mid-trunk spinal cord and mid-trunk hemiscord preparations, a two-step mathematical analysis was carried out with MATLAB as previously done by us (Charrier and Cabelguen 2013). In the first step, we generated from the in vitro data a set of candidate distribution models comprising one, two, three, or four peaks. For this purpose, Gaussian mixture distribution models comprising k peaks (k = 1, 2, 3, or 4) were built using an expectation maximization algorithm (gmdistribution.m in MATLAB). In the second step, we identified which model was the closest to the in vitro data among the set of candidate distribution models. To this end, we used Akaike’s information criterion (aic in MATLAB), a measure of model quality that allows quantification of the information lost (i.e., the magnitude of the difference) when comparing the candidate model and the real data set. The better the candidate model is, the smaller is the information lost, and the smaller is the value of the aic. For instance, for the whole spinal cord data, the Gaussian mixture model with three peaks had the smallest aic (aic = 517.86 for k = 1; aic = 504.44 for k = 2; aic = 497.83 for k = 3; aic = 499.56 for k = 4), indicating that a trimodal (i.e., 3 peaks) distribution was the best model. To illustrate the distributions of the intersegmental phase lags generated by the whole spinal cord and the mid-trunk spinal cord and mid-trunk hemiscord preparations, a probability density estimate was calculated from the in vitro data using a kernel smoothing function estimate (ksdensity in MATLAB).

RESULTS  

Kinematic and EMG activities  

The snapshots in Fig. 1 show representative body profiles of the same individual during four different locomotor modes (i.e., swimming, forward terrestrial stepping, forward underwater stepping, and backward terrestrial stepping) and during struggling. Supplemental Movie S1 illustrates a couple of cycles for each behavior.1 Note the rhythmic lateral bending of the trunk during each motor behavior, the absence of rhythmic movements of limbs during swimming, and the large movements of the tail during forward underwater stepping. As reported by others (Ashley-Ross and Lauder 1997; Deban and Schilling 2009), unlike terrestrial stepping, aquatic stepping was characterized by periods of suspension with no limbs in contact with the substrate. This feature can be attributed to the buoyant support of the water. Average speed was highest (2.61 ± 0.53 SVL/s, n = 4) during swimming and lowest (0.21 ± 0.10 SVL/s, n = 5) during backward terrestrial stepping (Table 1). Forward stepping speeds were in between these extremes, and no significant difference was observed when the animals were stepping forward on land or underwater in this study (Table 1).

We found that each motor behavior was characterized by a typical pattern of axial curvature and muscle activity along the mid-trunk (i.e., the 0.55–0.85 SVL region; Delvovélé et al. 1997) (Fig. 2). The terms “kinematic intersegmental phase lag” and “EMG intersegmental phase lag” refer exclusively to measurements performed at the level of the mid-trunk region.

Swimming. Swimming (Fig. 2C) was characterized by waves of axial body curvature that propagated posteriorly (i.e., rostrocaudal waves) along the mid-trunk with a kinematic intersegmental phase lag of 2.85 ± 0.76% (n = 4), which was larger than during any other motor behavior recorded (P < 0.05 to P < 0.001, Student-Newman-Keuls test) except backward terrestrial stepping (P > 0.05, Student-Newman-Keuls test) (Table 2). Correspondingly, we observed rostrocaudal waves of EMG activity propagating along the mid-trunk with a phase lag of 1.89 ± 0.25% (n = 12) (Table 3). The mean EMG intersegmental phase lag (n = 12) was lower than that of the mean kinematic intersegmental phase lag (n = 4) (P < 0.05, Mann-Whitney rank sum test), indicating that the EMG muscle activity traveled down the body faster than the mechanical wave, as is the case in most anguilliform and carangiform swimmers (dogfish: Grillner 1974; eel: Grillner and Kashin 1976; tench: Blight 1977; lamprey and trout: Williams et al. 1989; for review see D’Aoust et al. 2001) (see Tables 2 and 3). During EMG recordings, the swimming cycle duration (0.36 ± 0.04 s, n = 12) was significantly shorter than during any other motor behavior in all animals (P < 0.05, Student-Newman-Keuls test, n = 12/12) (Table 4). The averaged swimming cycle duration was not different in the animals examined during kinematic (0.32 ± 0.03 s, n = 4) and EMG (0.36 ± 0.04 s, n = 12) recordings (P > 0.05, t-test).

Forward terrestrial stepping. On ground, salamanders generally switched to forward stepping, with standing waves of axial body curvature that displayed a kinematic intersegmental phase lag of 0.53 ± 0.16% (n = 5) along the mid-trunk (Fig. 2D). This phase lag was different from any other behavior tested (P < 0.05 to P < 0.01, Student-Newman-Keuls test), except forward underwater stepping (P > 0.05, Student-Newman-Keuls test) (Table 2). Correspondingly, EMG waves were recorded along the mid-trunk with a phase lag of 0.04 ± 0.09% (n = 12), which was significantly smaller (P < 0.05, Student-Newman-Keuls test) than during swimming in all animal tested (Table 3). Here again the mean EMG intersegmental phase lag (n = 12) was lower than the kinematic intersegmental phase lag (n = 5) (P < 0.001, t-test), indicating that the EMG muscle activity wave was faster than the mechanical wave. During EMG recordings, the cycle duration during forward terrestrial stepping (0.90 ± 0.18 s, n = 12) was significantly longer (P < 0.05, Student-Newman-Keuls test) than during swimming in all animals tested (Table 4). The averaged cycle duration was shorter (P < 0.001, t-test) during EMG (0.90 ± 0.15 s, n = 12) than during kinematic (1.34 ± 0.14, n = 5) recordings because the surface on which the animals were made to step forward on
ground was more slippery during EMG than during kinematic recordings (see MATERIALS AND METHODS; see similar observation in humans in Cappellini et al. 2010). Slippery surfaces are useful for easily recording several locomotor cycles (e.g., Epstein and Graham 1983; Gruhn et al. 2006 in the stick insect), but these are known to modify the motor patterns by altering the mechanical relation between the limb and the ground. In humans, stepping on slippery surfaces increases trunk rotations and inclinations and limb motion speed, decreases the step length, and displaces the body center-of-body mass (Cappellini et al. 2010). In salamanders, larger tail movements and modification of the tail EMG activity occur when stepping forward on a slippery surface (Bicanski et al. 2013). This could also contribute to the differences observed between the kinematic and EMG intersegmental phase lag values reported during this mode in the present study.

**Forward underwater stepping.** The mean value of the kinematic intersegmental phase lag (0.04 ± 1.57%, n = 5) indi-

Fig. 1. Frame sequences showing from top to bottom 1 complete stride for 5 motor behaviors of *Pleurodeles waltlii*. White markers were tracked for kinematic analysis during swimming (A), forward terrestrial stepping (B), forward underwater stepping (C), backward terrestrial stepping (D), and struggling (E). A black dot was added on each frame to illustrate animal progression. Interframe time intervals are indicated for each behavior. Scale bars, 2 cm. Snapshots are from the same individual. Note the head-to-tail wave during swimming (A) and backward terrestrial stepping (D), the standing wave during forward stepping on land (B) or underwater (C), and the tail-to-head wave during struggling (E).
cated the presence of a standing wave in the mid-trunk during forward underwater stepping. The SD of the kinematic intersegmental phase lag during forward stepping was higher underwater (1.99 ± 1.01%, n = 5) than on ground (0.34 ± 0.04%, n = 5) (P < 0.01, Mann-Whitney rank sum test) (compare mid-trunk curvature angle variability in Fig. 2, D and E). This indicated that a variety of mechanical waves, without a clear bias to positive or negative phase lag values, were observed when salamanders used forward stepping underwater (Fig. 2E). The mean kinematic intersegmental phase lag along the mid-trunk was different from that observed during any other motor behavior (P < 0.01, Student-Newman-Keuls test) except forward terrestrial stepping (P > 0.05, Student-Newman-Keuls test) (Table 2). The EMG pattern of the mid-trunk was in between waves traveling caudally and rostrally, with a phase lag of 0.86 ± 0.50% (n = 9), which was smaller than during swimming in 78% of animals tested (P < 0.05 Student-Newman-Keuls test, n = 7/9) but higher than during land forward stepping in 89% of animals tested (P < 0.05 Student-Newman-Keuls test, n = 8/9) (Table 3). In contrast to forward terrestrial stepping, here the kinematic (n = 5) and EMG (n = 9) intersegmental phase lags were not different (P > 0.05, Mann-Whitney rank sum test), suggesting that different musculoskeletal properties (i.e., body stiffness) could be involved depending on the environmental condition and thus modulate the EMG-mechanical delay in the mid-trunk. As for the kinematic intersegmental phase lag, the SD of the mean EMG intersegmental phase lag during aquatic forward stepping (0.74 ± 0.15%, n = 9) was significantly higher than on land (0.43 ± 0.14%, n = 12) (P < 0.001, t-test), indicating a higher variability of the mid-trunk EMG pattern during aquatic stepping. In addition to differences in body stiffness, the periods of suspension without ground contact that occur underwater could also contribute to increase the variability of the mid-trunk motor pattern. During EMG recordings, the underwater forward stepping cycle duration (1.29 ± 0.28 s, n = 10) was significantly longer than during forward land stepping in 8 of 10 animals tested (P < 0.05, Student-Newman-Keuls test; Table 4). The averaged cycle duration during forward underwater stepping was similar in the animals examined during kinematic (1.06 ± 0.16 s, n = 5) and EMG (1.29 ± 0.28 s, n = 10) recordings (P > 0.05, t-test).

Backward terrestrial stepping. Salamanders could spontaneously display backward terrestrial stepping. This behavior did not often occur. In front of an obstacle, animals had a tendency to turn back and then step forward. Interestingly, during this behavior we observed waves of body curvature propagating caudally along the mid-trunk with a phase lag of 3.47 ± 2.12% (n = 4) (Fig. 2F). This indicated that head-to-tail traveling waves of body curvature could occur both in the presence (i.e.,

Table 1. Locomotor speed in salamanders

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<td>Speed, SVL/s n Swim.</td>
<td>2.61 ± 0.53 4</td>
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<td>Forw. ter. step.</td>
<td>0.61 ± 0.05 5</td>
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<td>Forw. und. step.</td>
<td>0.73 ± 0.09 5</td>
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<td>Backw. ter. step.</td>
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Mean ± SD locomotor speed for n animals is indicated in snout-vent length (SVL) per second. Kinematic recordings obtained from 5 animals were pooled and compared. The fastest locomotor mode was swimming, and the slowest was backward terrestrial stepping. The speeds of forward stepping on ground or underwater were in between, with no significant speed difference between them (n.s., P > 0.05). *P < 0.05, ‡P < 0.001, Student-Newman-Keuls test after a 1-way repeated-measures analysis of variance (ANOVA).

Fig. 2. Diversity of axial motor patterns in salamanders. A: axial movements were video-tracked (n = 5 animals). For convenience, the locations of the beads located on the dorsal midline are magnified by large white circles. B: electromyographic (EMG) activities were recorded from 2 ipsilateral mid-trunk muscles (n = 12 animals). SVL, snout-vent length. C–G: axial maximal curvature angles (left) and EMG activity (right) during swimming (C), forward terrestrial stepping (D), forward underwater stepping (E), backward terrestrial stepping (F), and struggling (G). The anatomical locations of the EMG electrodes (B), of the forelimb (FL) and hindlimb (HL) girdles (gray bars in C–G), and of the mid-trunk (blue bars in C–G) are indicated as a SVL fraction, with 0.05 SVL corresponding to 1 axial segment (see MATERIALS AND METHODS).

J Neurophysiol • doi:10.1152/jn.00894.2014 • www.jn.org
during backward land stepping) and in the absence (i.e., during swimming) of rhythmical movements of the limbs. The kinematic intersegmental phase lag during backward land stepping was not different from that observed during swimming ($P > 0.05$, Student-Newman-Keuls test) but was higher than that measured in any other motor behavior ($P < 0.01$ to $P < 0.001$, Student-Newman-Keuls test) (Table 2). The waves of EMG activity propagated down the mid-trunk with a phase lag of $3.93 \pm 1.47\%$ ($n = 5$). This EMG intersegmental phase lag was not different from that observed during swimming in $80\%$ of animals tested ($P > 0.05$, Student-Newman-Keuls test, $n = 4/5$) but was higher than during any other motor behavior in all animals tested (forward land stepping $n = 5/5$, forward underwater stepping $n = 4/4$, struggling $n = 2/2$; $P < 0.05$, Student-Newman-Keuls test) (Table 3). In contrast to swimming, no difference was observed when comparing the kinematic ($n = 4$) and the EMG ($n = 5$) intersegmental phase lags ($P > 0.05$, t-test). This difference with swimming could be related to multiple factors, nonexhaustively including the different environments, the mechanical consequences of limb contacts on ground, or the much slower frequency of movements.

Whereas the EMG intersegmental phase lags were highly similar during swimming and backward land stepping, the cycle duration during backward terrestrial stepping ($2.25 \pm 1.32\, s$, $n = 5$) was much longer than during swimming in all animals tested ($n = 5/5$, $P < 0.05$, Student-Newman-Keuls test) (Table 4). During EMG recordings, the cycle duration was also longer during backward land stepping than during forward land stepping in all animals tested ($n = 5/5$, $P < 0.05$, Student-Newman-Keuls test) but was longer than underwater forward stepping in only $50\%$ of cases ($n = 2/4$, $P < 0.05$, Student-Newman-Keuls test) (Table 4). The closest motor rhythm to backward land stepping was that recorded during struggling in $67\%$ of the cases ($n = 2/3$, $P > 0.05$, Student-Newman-Keuls test) (Table 4). The averaged backward stepping cycle duration was not different in the animals examined during kinematic ($2.98 \pm 2.02\, s$, $n = 4$) and EMG ($2.25 \pm 1.32\, s$, $n = 5$) recordings ($P > 0.05$, Mann-Whitney rank sum test).

**Struggling.** During struggling (Fig. 2G), caudorostral waves of body curvature were observed, with a kinematic intersegmental phase lag of $-2.33 \pm 1.55\%$ ($n = 5$) that was more negative than during any other motor behavior recorded ($P < 0.01$ to $P < 0.001$, Student-Newman-Keuls test) (Table 2). This other slow behavior was associated with a caudorostral EMG intersegmental phase lag along the mid-trunk ($-3.61 \pm 0.87\%$, $n = 9$) that was also lower than during any other motor behavior recorded (swimming $n = 9/9$; forward land stepping $n = 9/9$; forward underwater stepping $n = 7/7$; backward land stepping $n = 2/2$; $P < 0.05$, Student-Newman-Keuls test) (Table 3). No significant difference was observed between the kinematic and EMG intersegmental phase lag values ($P > 0.05$, t-test). Except when compared with backward land stepping, the cycle duration ($2.66 \pm 0.63\, s$, $n = 10$) was longer than in any other motor behavior in all animals tested during EMG recordings ($P < 0.05$, Student-Newman-Keuls test) (Table 4). The averaged cycle duration during struggling was similar in the animals examined during kinematic ($3.13 \pm 0.77\, s$, $n = 5$) and EMG ($2.66 \pm 0.63\, s$, $n = 10$) recordings ($P > 0.05$, t-test).

The plots in Fig. 3 show the mean kinematic and mean EMG intersegmental phase lags along the mid-trunk for all animals, across the different motor behaviors. Note that only $9$ of the $17$ animals used in this study [i.e., EMG ($n = 12$) and kinematic ($n = 5$) experiments pooled] exhibited episodes of several successive backward steps. Only these episodes have been analyzed and included in the plots. These plots show that the mean kinematic intersegmental phase lags per animal ranged

<table>
<thead>
<tr>
<th>Table 2. Behavior-dependent use of axial kinematic waves by salamanders in vivo</th>
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<tr>
<td>-----------------------------------------------</td>
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<tr>
<td>Swim.</td>
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<tr>
<td>Forw. ter. step.</td>
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<tr>
<td>Forw. und. step.</td>
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<tr>
<td>Backw. ter. step.</td>
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<tr>
<td>Strug.</td>
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</table>

Mean ± SD mid-trunk kinematic intersegmental phase lags for $n$ animals are indicated. Kinematic intersegmental phase lags recorded in the mid-trunk of $5$ animals were pooled and compared. Most kinematic intersegmental phase lags were different from one motor behavior to another. Nonsignificant differences were found between the intersegmental phase lag values of head-to-tail traveling waves during swimming and backward terrestrial stepping and between the lag values of standing waves during forward underwater stepping and forward terrestrial stepping (n.s., $P > 0.05$). $^P < 0.05$, $^P < 0.01$, $^P < 0.001$, Student-Newman-Keuls test after a 1-way repeated-measures ANOVA.

<table>
<thead>
<tr>
<th>Table 3. Behavior-dependent use of axial EMG waves by salamanders in vivo</th>
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<td>-----------------------------------------------</td>
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<tr>
<td>Swim.</td>
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<td>Forw. ter. step.</td>
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<td>Forw. und. step.</td>
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<tr>
<td>Backw. ter. step.</td>
</tr>
<tr>
<td>Strug.</td>
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</table>

Mean ± SD mid-trunk electromyographic (EMG) intersegmental phase lags for $n$ animals are indicated. EMG intersegmental phase lags calculated from EMG recordings of the mid-trunk muscles of $12$ animals were compared individual by individual. The percentage of preparations that were significantly different is reported ($P < 0.05$, Student-Newman-Keuls test after a Friedman repeated-measures ANOVA on ranks), with the corresponding number of animals in parentheses. Most phase lags were different from one motor behavior to another. All except $1$ individual showed a similar EMG intersegmental phase lag during backward terrestrial stepping and swimming. During forward underwater stepping the EMG pattern was in between a standing wave and a head-to-tail traveling wave, as the axial phase lag was inferior to that recorded during swimming in $78\%$ of cases and superior to that recorded during forward terrestrial stepping in $89\%$ of cases.

$J\text{Neurophysiol} \cdot \text{doi:10.1152/jn.00894.2014 \cdot www.jn.org}$
between values as positive as 5.20 ± 3.58% of cycle duration for rostrocaudal waves and −4.54 ± 1.34% of cycle duration for caudorostral waves. The plot in Fig. 3B further shows that each motor behavior could confidently be associated with a cluster of EMG intersegmental phase lags, which ranged from 6.40 ± 1.48% to −4.80 ± 1.80% of cycle duration. The clustering of kinematic intersegmental phase lags is somewhat less clear in Fig. 3A.

**Motor Patterns Generated by Isolated Whole Spinal Cords**

*Three stable patterns of intersegmental coordination.* We then investigated whether the diversity of axial motor patterns observed in vivo could be produced by isolated spinal cord networks. In previous studies, rostrocaudal and caudorostral waves of mid-trunk VR activity were observed in salamander spinal cord preparations with the brain stem attached and in isolated spinal cords (Charrier and Cabelguen 2013; Delvolvé et al. 1999; Ryczko et al. 2010a). However, no detailed, extensive documentation of the whole range of patterns that the spinal cord can generate at the mid-trunk level is available. Here we examined in detail the intrinsic variability of the axial motor patterns generated by spinal cord preparations isolated from the supraspinal centers and sensory inputs. We also recorded the forelimb and hindlimb motor output together with the mid-trunk and evaluated the coupling relations between these components.

We have recorded from the mid-trunk VRs in isolated whole spinal cords (n = 67) pharmacologically activated by bath application of a mixture of NMDA (20 μM) and d-serine (10 μM). The recordings revealed a bursting motor pattern characterized by a left-right alternation and a flexible intersegmental coordination. We confirm that the intersegmental coordination could consist of caudally propagated waves (Fig. 4A) or rostrally propagated waves (Fig. 4C) as previously reported (Charrier and Cabelguen 2013; Delvolvé et al. 1999; Ryczko et al. 2010a). In addition, we provide evidence that the spinal cord could also produce standing waves (i.e., with a near-zero phase lag) (Fig. 4B). Wavelet transformations were used to estimate the stability of these patterns over time (Fig. 4, D–F). The stable coherent cross-power (CXWT) between the wavelet transformations of two VR recording data sets, together with the stable phase relationships (Fig. 4, D–F, insets), indicated a stable coupling between VR bursting activities over relatively long data episodes (~5 min in Fig. 4) for each of the three types of mid-trunk motor patterns.

The plot in Fig. 4G illustrates the distribution of the mean intersegmental phase lags obtained from all the in vitro preparations. Note that the positive phase lag values were associated with caudally propagated waves and the negative phase lag values with rostrally propagated waves. As some preparations could produce more than one stable pattern of activity (see below), we analyzed a total of 80 patterns from the 67 in vitro preparations. The mean intersegmental phase lag ranged from −12.61% to +12.44% of cycle duration. A Gaussian mixture model analysis (Charrier and Cabelguen 2013; see MATERIALS AND METHODS) revealed that the intersegmental phase lag showed a trimodal distribution, stratified into three peaks of positive, null, and negative phase lag values (Fig. 4H).

### Table 4. Behavior-dependent cycle durations used by salamanders in vivo

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<tbody>
<tr>
<td>Swim.</td>
<td>0.36 ± 0.03</td>
<td>12</td>
<td>100% (12/12)</td>
<td>100% (12/12)</td>
<td>100% (5/5)</td>
<td>100% (10/10)</td>
</tr>
<tr>
<td>Forw. ter. step.</td>
<td>0.90 ± 0.15</td>
<td>12</td>
<td>100% (12/12)</td>
<td>80% (8/10)</td>
<td>100% (5/5)</td>
<td>100% (10/10)</td>
</tr>
<tr>
<td>Forw. und. step.</td>
<td>1.29 ± 0.28</td>
<td>10</td>
<td>80% (12/12)</td>
<td>100% (5/5)</td>
<td>50% (2/4)</td>
<td>100% (8/8)</td>
</tr>
<tr>
<td>Backw. ter. step.</td>
<td>2.25 ± 1.32</td>
<td>5</td>
<td>100% (5/5)</td>
<td>100% (5/5)</td>
<td>50% (2/4)</td>
<td>33% (1/3)</td>
</tr>
<tr>
<td>Strug.</td>
<td>2.66 ± 0.63</td>
<td>10</td>
<td>100% (10/10)</td>
<td>100% (10/10)</td>
<td>100% (8/8)</td>
<td>33% (1/3)</td>
</tr>
</tbody>
</table>

Mean ± SD cycle duration for n animals is indicated. Cycle durations measured from the axial muscular activity recorded in 12 animals were compared individual by individual during the 5 motor behaviors. The percentage of preparations that were significantly different is reported (P < 0.05, Student-Newman-Keuls test after a Friedman repeated-measures ANOVA on ranks), with the corresponding number of animals in parentheses. Fast rhythms were observed during swimming. Slow rhythms were observed during struggling and backward terrestrial stepping. Cycle durations during forward terrestrial or underwater stepping were in between.

---

*Mid-trunk kinematic phase lags (% of cycle duration / 0.05 SVL)*

A: distribution of mid-trunk kinematic intersegmental phase lags recorded from 5 animals. B: distribution of mid-trunk EMG intersegmental phase lags recorded from 12 animals. Each dot illustrates the intersegmental phase lag (mean ± SD) recorded per behavior in 1 animal. If an animal showed multiple bouts of the same behavior, we pooled the values from these multiple bouts to generate a single mean value (i.e., dot) per behavior per individual. The color code used to differentiate the motor behaviors is similar to that in Fig. 2.

---

**Fig. 3.** Comparison of kinematic and EMG phase lags during 5 motor behaviors in salamanders. A: distribution of mid-trunk kinematic intersegmental phase lags recorded from 5 animals. B: distribution of mid-trunk EMG intersegmental phase lags recorded from 12 animals. Each dot illustrates the intersegmental phase lag (mean ± SD) recorded per behavior in 1 animal. If an animal showed multiple bouts of the same behavior, we pooled the values from these multiple bouts to generate a single mean value (i.e., dot) per behavior per individual. The color code used to differentiate the motor behaviors is similar to that in Fig. 2.

---

**Fig. 4.** A: distribution of mid-trunk kinematic intersegmental phase lags recorded from 5 animals. B: distribution of mid-trunk EMG intersegmental phase lags recorded from 12 animals. Each dot illustrates the intersegmental phase lag (mean ± SD) recorded per behavior in 1 animal. If an animal showed multiple bouts of the same behavior, we pooled the values from these multiple bouts to generate a single mean value (i.e., dot) per behavior per individual. The color code used to differentiate the motor behaviors is similar to that in Fig. 2.
with either slow (backward stepping) or fast (swimming) cycle durations (see Discussion).

Switches between mid-trunk motor patterns. Spontaneous switches in the direction of propagation of motor waves generated by the spinal cord have previously been reported in in vitro brain stem-spinal cord and tail-spinal cord preparations of salamander bathed in an NMDA solution (Branchereau et al. 2000; Charrier and Cabelguen 2013; Delvolvé et al. 1999). However, in these studies no detailed analysis of the switches between the intersegmental coordination patterns generated by the mid-trunk cord has been performed.

In the present study, 12 whole spinal cord preparations of the 67 recorded (16%) exhibited spontaneously alternating head-to-tail and tail-to-head propagated VR activities along the mid-trunk (Fig. 5, A and B). In 2 of 12 preparations the switch was definitive, while in the remaining 10 preparations the direction of wave propagation could reverse once or several times during the ongoing experiment.

These changes in the output of the mid-trunk motor network were clearly detected and reliably quantified by using the wavelet transformations in time/frequency domains of the recording data of two ipsilateral VRs (Fig. 5C). On average, the value of the intersegmental phase lag shifted from 6.00 ± 1.74° to −7.68 ± 3.39° of cycle duration when switching from head-to-tail to tail-to-head waves (P < 0.001, n = 12, Wilcoxon signed-rank test). Concomitantly, the cycle duration very significantly increased from 9.54 ± 2.06 s to 16.37 ± 4.00 s when the direction of propagation of motor waves switched from head-to-tail to tail-to-head (P < 0.001, n = 12, Wilcoxon signed-rank test).

Interestingly, as illustrated by the insets in Fig. 5C, the absolute value of the intersegmental phase lag was identical (P > 0.05, n = 12, paired t-test) after a switch, despite dramatic changes in the longitudinal delay between bursts expressed by successive VRs (2.05 ± 1.30 s vs. 4.04 ± 2.33 s, P < 0.001, n = 12, paired t-test). This suggested that the absolute phase difference between mid-trunk segments was independent of the cycle duration.

Importantly, in the preparation illustrated in Fig. 5, the axial rhythm frequency and intersegmental phase lag after the secp-
ond switch were not different from the values they had before the first switch, as if the spinal cord could “recall” the pattern it was generating before the first switch (compare regions 1, 2, and 3 in Fig. 5C). This indicated that in our experiments each spinal cord tended to express one stable phase lag, apart from when a switch occurred (see also Fig. 4G).

We mostly observed switches between caudally propagated and rostrally propagated waves, and vice versa. Switches
between caudally propagated, rostrally propagated, and standing waves were observed in a single experiment (not illustrated).

In 11 isolated whole spinal cords, we examined the motor patterns displayed by forelimb and hindlimb nerves. Coherence analyses revealed that the rhythmic activities exhibited by forelimb and hindlimb nerves were frequency locked and well coordinated both with each other (“interlimb coordination”) and with the mid-trunk VR activities (“axial-limb coordination”) (Fig. 5, D–F). Interestingly, changes in the interlimb coordination pattern were associated with changes in the direction of propagation of mid-trunk VR activities (Fig. 5F, insets). These findings are in line with the observation of a variety of coordinating patterns of activation of hindlimb and lumbar back muscle nerves during fictive locomotion in the high spinal cat (Koehler et al. 1984).

Together these results showed that the spinal cord can quickly switch between distinct motor patterns that involve the limbs and the mid-trunk, as is the case during in vivo locomotion.

**Coupling strengths.** A coherence analysis was performed to estimate the strength of the coupling between the mid-trunk segments (“mid-trunk coupling”) during the rhythmic VR activities induced by bath application of NMDA and d-serine (see Miller and Sigvardt 2000; Mor and Lev-Tov 2007). The strength of the mid-trunk coupling was quantified as the amplitude of the coherence between signals displayed by two VRs, one rostral and one caudal, in the high power frequency band corresponding to the axial rhythm frequency. A similar analysis was performed to evaluate the strength of the interlimb coupling. In 9 of the 11 preparations examined, the analyses revealed that during caudally propagated axial waves of VR activity, a strong mid-trunk coupling (coherence: 0.93 ± 0.03), and a strong, albeit weaker (F < 0.001, Student-Newman-Keuls test), interlimb coupling (0.80 ± 0.04) exist (Table 5).

Table 5. **Evaluation of coupling strength by coherence measurements**

<table>
<thead>
<tr>
<th>Coupling Strength (coherence)</th>
<th>n</th>
<th>Mid-Trunk vs. Mid-Trunk</th>
<th>Forelimb vs. Mid-Trunk</th>
<th>Hindlimb vs. Mid-Trunk</th>
<th>Forelimb vs. Hindlimb</th>
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<tbody>
<tr>
<td>Mid-trunk vs. mid-trunk</td>
<td>0.93 ± 0.03</td>
<td>9</td>
<td>‡</td>
<td>‡</td>
<td>‡</td>
</tr>
<tr>
<td>Forelimb vs. mid-trunk</td>
<td>0.87 ± 0.05</td>
<td>9</td>
<td>‡</td>
<td>‡</td>
<td>‡</td>
</tr>
<tr>
<td>Hindlimb vs. mid-trunk</td>
<td>0.82 ± 0.04</td>
<td>9</td>
<td>‡</td>
<td>‡</td>
<td>n.s.</td>
</tr>
<tr>
<td>Forelimb vs. hindlimb</td>
<td>0.80 ± 0.04</td>
<td>9</td>
<td>‡</td>
<td>‡</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

Mean ± SD coherence for n preparations is indicated. Coupling strengths were evaluated between limb nerve and mid-trunk VR signals recorded from isolated spinal cords stimulated with NMDA (20 μM) and d-serine (10 μM). Coherence was measured in the time/frequency regions of significantly coherent cross-power in the frequency band of the axial rhythm, as delineated on the cross-wavelet transformations of the recorded signals (see region 1 in Fig. 5, C–F). The couplings were evaluated in preparations displaying a rostrocaudal wave of axial activity (9/11 preparations) with intersegmental phase lag values ranging from ±4.12% to ±9.78% of cycle duration. The significant differences in coherence are reported. n.s., P > 0.05; †P < 0.01, ‡P < 0.001, Student-Newman-Keuls test after a 1-way repeated-measures ANOVA.
Together these results indicated that the isolated mid-trunk network was able to generate the three types of motor waves. The limb and tail networks are thus not necessary to generate these patterns, but they contribute to slow down the mid-trunk motor rhythm and can also influence the phase lag value when they are coupled to the mid-trunk network.

Mid-trunk hemicords. In a next step, we asked whether the cross-connections between the left and right sides of the mid-trunk spinal cord were necessary to generate a longitudinally coordinated motor activity. As mid-trunk hemicords are capable of producing rhythmic motor bursts (Ryczko et al. 2010a), we examined the motor activities generated by isolated mid-trunk hemicords (i.e., a chain of hemisegments) bathed in NMDA (20 μM) and d-serine (10 μM).

The VR recordings in Fig. 7 show that rostrally propagated waves (Fig. 7A), standing waves (Fig. 7B), and caudally propagated waves (Fig. 7C) could be recorded from VRs in isolated mid-trunk hemicords. Significantly coherent cross-power was observed during these three motor patterns over several cycles, as shown by the wavelet transformations.
tained from the VR signals recorded from one hemicord preparation (Fig. 7, D–F). The plot in Fig. 7G shows the averaged data for the eight motor patterns obtained from the six animals used in this study. The intersegmental phase lag values ranged from \(-14.11 \pm 4.76\%\) (rostrally propagated waves) to \(+13.14 \pm 4.74\%\) (caudally propagated waves). The median value of the whole range of recorded phase lags was \(+1.13\%\), indicating that the isolated mid-trunk preparation also had a tendency to produce head-to-tail propagated waves. Similarly to the isolated mid-trunk preparations, the Gaussian mixture model analysis indicated that the mid-trunk hemicord preparations displayed a unimodal distribution of phase lag values (peak mean \(+1.08 \pm 7.37\%\)) (Fig. 7H).

These results indicated that cross-connections between the left and right sides of the mid-trunk spinal cord were not necessary to generate a longitudinally coordinated motor activity. However, the left-right cross-connections seem to be important to increase the stability of the intersegmental phase lag. Compared with the whole spinal preparation, the activity was coordinated between the hemisegments (Fig. 7) but the value of the intersegmental lag could vary much more. Indeed, the mean of the intersegmental lag SD was significantly higher (P < 0.05, t-test) in isolated mid-trunk hemicords (7.53 \pm 3.49\% of cycle duration, n = 8 patterns from 6 preparations) than in isolated mid-trunk spinal cords (4.49 \pm 2.31\% of cycle duration, n = 11 patterns from 11 preparations). Perhaps...
because of these considerable variations, the mean intersegmental phase lag of isolated hemicords was not significantly different from that of whole spinal cords (2.39 ± 3.08% and 6.58 ± 3.77%, respectively) \((P > 0.05, \text{paired } t\text{-test}, n = 5)\). In addition, consistent with the previous report that hemisegments oscillate faster than the whole spinal cord (Ryczko et al. 2010a), the mean cycle duration significantly decreased from 9.25 ± 1.24 s to 5.94 ± 2.18 s when mid-trunk hemicords were dissected out of the spinal cords \((P < 0.05, \text{paired } t\text{-test}, n = 5)\). As illustrated in Fig. 7, D–F, whereas a slow rhythm was systematically associated with caudorostral waves in whole spinal cords, the phase lag in the same hemicord preparation could change without any noticeable difference in rhythm frequency. Consequently no correlation was found between intersegmental phase lag and cycle duration in hemicords \((R = -0.08, P > 0.05, \text{Pearson product moment correlation})\). Finally, the average cycle duration recorded in hemicords \((7.28 ± 2.48 s, n = 8 \text{ patterns from } 6 \text{ preparations})\) was not significantly different \((P > 0.05, t\text{-test})\) from that recorded in isolated mid-trunk spinal cords \((6.01 ± 1.41 s, n = 11 \text{ patterns from } 11 \text{ preparations})\). Since isolated hemisegments oscillate faster than isolated segments (Ryczko et al. 2010a), this suggested that the longitudinal couplings between hemisegments could contribute to slow down the motor rhythm generated by hemicords.

Taken together these results revealed that each side of the mid-trunk spinal cord comprises a neural network capable of producing rostrocaudal, standing, and caudorostral waves of motor activity and that the left-right cross-connections increase the stability the longitudinal phase lag.

In conclusion, our in vitro experiments revealed that the isolated whole spinal cord, the isolated mid-trunk spinal cord, as well the isolated mid-trunk hemicord can produce the variety of axial motor patterns observed in freely moving animals and can rapidly switch between them. However, a comparison between the in vitro and the in vivo axial motor patterns revealed quantitative differences: the motor rhythm was \(\approx 9–10 \text{ times faster and the range of intersegmental phase lag was } \approx 2.5 \text{ times narrower in vivo}\).

**DISCUSSION**

**Flexibility of Mid-Trunk Locomotor Patterns**

Our results indicate that the environment subtly influenced the mid-trunk locomotor pattern during forward stepping. On ground, our data confirm that salamanders exhibit standing kinematic and EMG waves along the mid-trunk (Ashley-Ross 1994; Ashley-Ross et al. 2009; Frolich and Biewener 1992; Karakasiliotis et al. 2013). Underwater, a kinematic standing wave was observed in the mid-trunk (as previously reported by Ashley-Ross et al. 2009; Deban and Schilling 2009), whereas most animals exhibited waves of EMG activity traveling down the mid-trunk. One additional difference is that trunk muscles exhibit an EMG activity of lower amplitude during aquatic stepping than during land stepping, despite similar bending kinematics (Deban and Schilling 2009; see also Fig. 2, D and E). This decrease in muscle activity is attributable to reduced weight-bearing underwater (Deban and Schilling 2009; Masumoto et al. 2005). Overall, these observations emphasize the difficulty of reliably inferring the kinematic phase lag from the EMG phase lag, and conversely. Indeed, axial undulations emerge from complex interactions between the muscle contractions, the biomechanics of the body, and the environment (Chiel and Beer 1997; Knüsel and Ijspeert 2011; Nishikawa et al. 2007).

The observations mentioned above for the mid-trunk support the view that only minimal changes in the basic stepping pattern are necessary to adapt forward stepping to different environments (Ashley-Ross et al. 2009). This concurs with the observation that salamanders exhibit very similar limb kinematics during terrestrial and aquatic stepping, although the footfall patterns are different (Ashley-Ross et al. 2009; Ashley-Ross and Betchel 2004). However, it is worth noting that the kinematic/EMG patterns of the tail during forward land or aquatic stepping are more complex and variable than those exhibited by the mid-trunk (Bicanski et al. 2013; Cabelguen et al. 2014; Delvové et al. 1997; Karakasiliotis et al. 2013). This suggests that the tail plays a more important role than the mid-trunk in adapting forward stepping to the environment in salamanders. Previous studies in several vertebrates have stressed the role of the tail in the adaptation of stepping movements in various environments (see, e.g., Libby et al. 2012; Siegel 1970; Walker et al. 1998).

Here we report in salamanders that the direction of land stepping is accompanied by drastic changes in kinematic and muscular activity patterns in the mid-trunk and in cycle duration values. Terrestrial stepping in the forward direction was coupled with standing waves, whereas in the backward direction it was associated with posteriorly traveling waves. This indicates that the backward pattern in salamanders is more complex than a simple reversal of the forward pattern (Ashley-Ross and Lauder 1997). Our results also confirm that the cycle duration is longer and more variable during backward stepping than during forward stepping in salamanders (Ashley-Ross and Lauder 1997). Similar results were reported when comparing forward and backward swimming in lamprey (Islam et al. 2006). Conversely, the cycle duration is not different or slightly shorter during backward stepping in cats (Buford et al. 1990; Zelenin et al. 2011) and humans (de Sèze et al. 2008; Grasso et al. 1998).

Our results indicate that salamanders can exhibit posteriorly propagated motor waves during both backward stepping and swimming, i.e., when animals are moving in two opposite directions. How rostrocaudal waves of lateral trunk flexion help the limbs to achieve their propulsive role during backward stepping remains to be investigated. Interestingly, the lamprey uses waves of lateral body undulations and EMG activity propagated posteriorly along the body during forward swimming and anteriorly during backward swimming (Islam et al. 2006). By contrast, humans walking either forward or backward on a treadmill use in both cases waves of EMG activity propagated posteriorly along the spinal column (de Sèze et al. 2008). Taken together, these findings suggest that during evolution the main locomotor function of the axial musculature would have been reconfigured from a propulsive role of the body to a stabilizing role of the trunk (Fischer and Witte 2007; Ritter et al. 2001; Schilling 2011; Schilling and Carrier 2010).

Furthermore, the kinematic and EMG intersegmental phase lags were similar between swimming and backward stepping, although the frequencies of the locomotor movements were very different (see also Islam et al. 2006, in the lamprey).
Therefore, our results suggest that the neural mechanism generating the intersegmental coordination is independent of the cycle duration, as previously shown in the lamprey (Matsushima and Grillner 1992; Wallén and Williams 1984).

Our results confirmed our preliminary observation that struggling movements can be exhibited by salamanders (Lamarque et al. 2009). In hatching *Xenopus* tadpoles, struggling would result from a reconfiguration of the swimming network, which involves a combination of shared neurons and a recruitment of specific new neurons (Li et al. 2007). It is not known whether a similar mechanism is present in the salamander. However, since struggling in the salamander involves rhythmic limb movements while swimming does not, a putative reconfiguration of the swimming network would include the addition of the neurons of the oscillatory networks of the limbs.

In conclusion, our in vivo results show in the salamander that each of the forms of locomotion and struggling behavior is characterized by a distinct combination of mid-trunk motor patterns and cycle durations.

**Spinal Mechanisms of Phase Lag Generation in Trunk Networks**

Our in vitro study indicates that the isolated mid-trunk cord is capable of producing a range of rhythmic motor patterns when activated with bath-applied NMDA. The intersegmental phase lags ranged from positive values (i.e., posteriorly propagated waves) to negative values (i.e., anteriorly propagated waves), including all the values that could be observed in intact animals. Thus we propose that the mid-trunk cord contains neuronal networks that can generate the basic mid-trunk patterns that are appropriate to generate any form of rhythmic motor behavior exhibited by intact salamanders. This would be consistent with previous studies showing that the various forms of axial locomotor patterns exhibited by the lamprey (Hsu et al. 2014; Matsushima and Grillner 1992; McClelland and Grillner 1983), dogfish (Grillner 1974), *Xenopus* embryo (Soffe 1991), and cat (Koehler et al. 1984) can be generated by spinal networks alone. This also concurs with the observation in invertebrates that some patterns generated by the isolated stomatogastric CPG can drive the muscular and kinematic patterns observed in vivo (crab: Diehl et al. 2013). Similarly in the leech heartbeat system, the intersegmental phase delays that typically relate to distinct motor patterns are set up and maintained without sensory feedback (Calabrese 1979).

Our study provides evidence that surgically isolated mid-trunk hemicords are able to express the different types of longitudinal coordinations observed during the four locomotor modes and during struggling. This suggests that the capability of generating intersegmental phase lag on each side does not require mutual influences between the two halves of the mid-trunk cord. However, the left-right commissural neurons contributed to the stabilization of the mid-trunk coordination according to our results. Similar conclusions were 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). Our results are also consistent with the observation in salamanders that isolated mid-trunk hemisegments can produce rhythmic output (Ryczko et al. 2010a). The rhythmogenic capability of axial or limb networks on each side of the spinal cord has been observed in several vertebrate species by using a variety of techniques and preparations. These include electrical stimulation of isolated axial hemicords and hemisegments in lampreys (Cangiano et al. 2012; Cangiano and Grillner 2005), chemical stimulation of midsagittally sectioned spinal cords in neonatal rats (Kjaerulf and Kiehn 1996; Kudo and Yamada 1987), as well as optogenetic stimulation of glutamatergic neurons of each side of the spinal cord in mice (Hagglund et al. 2013). Together these results are in favor of the “unit burst generator” theory (Grillner 1981).

The mechanisms responsible for phase lag generation in the axial network remain to be elucidated in salamanders. In the swimmeret of the crayfish, the mechanism for intersegmental coordination has been described in detail at the cellular level. The swimmeret system is a short chain of four segmental oscillators and only produces caudorstral waves, with a constant intersegmental phase lag of 0.25. In each segment, coordinating neurons send ascending and descending signals reflecting the current state of the segmental rhythm to the rostral and caudal neighbors via excitatory synapses whose strength decreases with distance. This information is integrated in the neighboring segment by a “hub” neuron that, in turn, influences via electrical coupling the local rhythm generator, and this controls the timing of the segment output level (Mulloney et al. 2006; Mulloney and Hall 2007; Namba and Mulloney 1999; Smarandache et al. 2009; Smarandache-Wellman et al. 2014; Smarandache-Wellman and Grätsch 2014; for review see Mulloney and Smarandache-Wellman 2012). The intersegmental coupling is asymmetric: the coordinating neurons sending ascending signals use stronger synapses (Smarandache et al. 2009) and generate more spikes per cycle (Mulloney et al. 2006) than those sending descending signals. Whether a similar organization exists in neural systems constituted by a longer chain of segmental oscillators remains to be determined. In contrast, the salamander has around 40 axial segmental oscillators and can display multiple patterns with a large spectrum of intersegmental lags (Charrier and Cabelguen 2013; Ryczko et al. 2010a; present study). To explain intersegmental coordination in longer chains like the lamprey axial system, three different organizations based on different coupling relations were proposed: 1) balanced ascending and descending coupling (“trailing oscillator hypothesis,” Matsushima and Grillner 1992), 2) dominant descending coupling (Hagevik and McClelland 1994), and 3) dominant ascending coupling (Cohen et al. 1992; Kopell et al. 1991; Williams et al. 1990) (for review see Bicsanski et al. 2013). The three mechanisms can be used to generate traveling waves with modeling, but so far mechanisms 1 and 2 are more strongly supported by the available biological data. Our observation in isolated spinal cords that coherence between mid-trunk VR activities is not different between head-to-tail and tail-to-head waves suggests that the coupling strength between the axial segments does not change from one wave type to another.

Some of our results can be interpreted through the prism of the evolution of spinal networks. The spinal network in the salamander was proposed to be constituted of an axial network inherited from limbless vertebrates like the lamprey, extended by phylogenetically more recent limb networks (Grillner and Wallén 1985; Ilspeert et al. 2007; Ryczko et al. 2010a). In the present experiments the distribution of intersegmental phase lag in the whole spinal cord exhibited three preferred peaks, 

J Neurophysiol • doi:10.1152/jn.00894.2014 • www.jn.org
while in the lamprey the phase lag varies from positive to negative values around a single positive peak (Matsushima and Grillner 1992). This difference could result from the presence of limb and/or tail networks in salamanders. In support of this possibility, the isolated mid-trunk spinal cord, as well as the hemiord, only showed a single positive peak in their distribution of phase lag values, just like the lamprey spinal cord. The inherited lamprey network would have been extended by the addition of limb networks and by a “modularization” of the axial network at the tail level, which shows two peaks of phase lag (positive and negative lags, see Charrier and Cabelguen 2013; for review see Cabelguen et al. 2014). Such a modularization of the locomotor CPG may have underlain the enrichment of the locomotor repertoire of tetrapods during the transition from water to land.

The salamander’s axial network may present an even more complex modularization if different premotor and motoneuron groups are recruited as a function of speed as shown in the axial network of the zebrafish (e.g., Ampatzis et al. 2014; McLean et al. 2007, 2008) (see also in mice limb networks; Talpalar et al. 2013). In salamanders, different spinal modules might be used from one motor pattern to another as shown in the zebrafish, where the active spinal cells during swimming and struggling comprise different assemblies of specialized and multifunctional cells (Liao and Fetcho 2008). In the Xenopus embryo, even the rhythmonic mechanisms would change between swimming and struggling (Li et al. 2007). The presence of such modularizations in the salamander is probable when taking into account the similarities of spinal neuron populations in the salamander, Xenopus, and zebrafish axial networks (for review see Ryczko et al. 2010b). Finally, whether different networks control the dorsal and ventral parts of the axial muscles as in lampreys (Aoki et al. 2001; Buchanan 2011) and zebrafish (Bagnall and McLean 2014) remains to be explored.

Coupling Between Limb and Axial Spinal Networks

The isolated mid-trunk spinal cord and hemiord displayed an increased bursting frequency when disconnected from the limb and tail networks. This is in accordance with our previous results showing that the axial oscillators are slowed down by intrinsically slower limb networks (Ijspeert et al. 2007; see also Ryczko et al. 2010a). The disconnection of limb and tail networks did not prevent the mid-trunk from generating the three types of intersegmental coordination. However, it removed the correlation between positive phase lags and fast cycle durations, making the isolated mid-trunk cord resemble a “lamprey-like” spinal cord (Matsushima and Grillner 1992; Wallén and Williams 1984).

Our results demonstrate that mid-trunk traveling waves can coexist with limb movements during rhythmic movements at high frequency (i.e., swimming) and low frequency (i.e., backward land stepping and struggling). Importantly, our in vitro experiments showed that waves of fictive motor activities traveling up or down along the cord were well-coordinated with rhythmic activation of the limb motor nerves. Taken together, these observations are consistent with the hypothesis that the coupling between the limb and axial CPG networks is local (i.e., only involves a coupling of limb oscillators to the nearest axial segments) (Bicanski et al. 2013; Ijspeert et al. 2005; Knüsel et al. 2013). Our previous simulations suggest that this configuration is able to produce traveling waves when the limbs are rhythmically activated, e.g., during backward stepping or struggling (see Ijspeert et al. 2005; Knüsel et al. 2013).

Observations in newborn rats (Falgairolle and Cazalets 2007), dogs (Schilling and Carrier 2010), and humans (de Séze et al. 2008) have revealed posteriorly propagating activation of the axial muscles associated with traveling waves of trunk bending during walking. Waves of motor activity propagated along the spinal cord have also been observed during fictive stepping in the newborn rat (Falgairolle and Cazalets 2007) and fictive scratching in the decerebrate cat (Cuejar et al. 2009). Together these results suggest that the neural mechanism ensuring the coexistence of traveling waves of lateral motion with rhythmic limb movements may have been conserved in vertebrates.

Our coherence measurements in the isolated spinal cord indicate that the coupling strength between the forelimb and hindlimb networks is the weakest whereas the coupling between axial oscillators in the mid-trunk is the strongest. Between these extremes, the forelimb-axial coupling is stronger than the hindlimb-axial coupling. This asymmetry could be linked to the functional difference between forelimbs and hindlimbs. It is unknown whether the coupling between the limb networks is direct or involves synaptic relays in the mid-trunk network, as previously shown by physiological experiments in the neonatal rat (Juvin et al. 2012). Experiments are also needed to decipher whether consistent changes in coupling strength between the limb and axial networks are associated with pattern transitions.

Role of Descending Control

The role of supraspinal structures in the control of the behaviors characterized in the present study is not fully understood. Swimming and forward stepping can be elicited by stimulation of a brain stem region called the mesencephalic locomotor region (MLR). Its level of activation positively controls the frequency of forward stepping and swimming as well as transitions between these modes (Cabelguen et al. 2003). In vertebrates this region projects to reticulospinal cells that in turn activate the locomotor CPG (for review see Ryczko and Dubuc 2013). Struggling could involve spinal/hindbrain neurons that would be recruited by the tonic sensory input evoked when the animal is held, as reported in the Xenopus embryo (Li et al. 2007). The neural center controlling backward locomotion has not been identified in any vertebrate. Reticulospinal cells are expected to be involved, as some of those are active during backward swimming in lampreys (Zelenin 2011). In cats, cells from the motor cortex specifically discharge during backward locomotion (Zelenin et al. 2011).

Whether and how descending commands contribute to the reconfiguration of the spinal motor networks from one motor pattern to another remains to be explored in salamanders. It is conceivable that, in vivo, the descending inputs could “override” the spinal cord programs to obtain a more flexible motor repertoire. This would explain our observation that slow rostrocaudal waves are displayed during backward terrestrial stepping whereas the slowest rhythms generated by the isolated spinal cord are associated with caudorostral waves. Such a role
for descending inputs would be consistent with that of the descending command neurons that reconfigure intersegmental coordination during swimming in the leech (Puhl et al. 2012). Leech crawling behavior relies on both local interoscillator coupling and long-distance descending inputs from the cephalic ganglion, in contrast to swimming, which is more easily induced without the cephalic ganglion (Puhl and Mesce 2010). This suggests that swimming would be an emerging property of the circuit while crawling would require modifications of the preestablished interoscillator couplings by descending signals from the cephalic ganglion (Puhl and Mesce 2010). The descending inputs could influence the axial intersegmental coordination by differentially activating parts of the spinal cord network, as suggested in a detailed modeling study of the lamprey CPG (Kozlov et al. 2009). Our modeling work on the salamander suggests that a wide range of intersegmental phase lags and cycle durations could be obtained independently through changes in the uncoupled frequencies (i.e., activations) of the axial and limb networks (Knüsel et al. 2013).

Role of Sensory Feedback

Our observations confirmed that the bursting frequency is lower during in vitro experiments than during in vivo locomotion and struggling (Delvolvé et al. 1999; Ryczko et al. 2010a). This discrepancy might be attributable to the pharmacological activation of the spinal system used in the present study. But, interestingly, a reduction in bursting frequency was reported in isolated spinal cord preparations from mudpuppy (Wheatley et al. 1992), lamprey (Guan et al. 2001), turtle (Juranek and Currie 2000), neonatal rodents (Cazalets et al. 1992; Cowley and Schmidt 1997; Kudo and Yamada 1987; Whelan et al. 2000), and cat (Grillner and Zangger 1979; Miller and Van der Meché 1976) compared with intact animals. The higher rhythm frequencies observed in intact animals may partly result from an excitatory influence of movement-related afferent feedback (cf. Rossignol et al. 1988).

The movement-related sensory inputs contribute to the intersegmental coordination during swimming in vertebrates and invertebrates (lamprey: Grillner and Wallén 2002; Guan et al. 2001; Tytell and Cohen 2008; leech: Kristian and Calabrese 1976; Pearce and Friesen 1984). It is conceivable that the nature of sensory feedback that is fed onto the CPG would change from one locomotor pattern to another in salamanders. A reversal of the effects of sense organs that signal forces on a leg when switching from forward to backward stepping was reported in the stick insect (Akay et al. 2007). Moreover, our previous simulations emphasized the role of sensory inputs from the limbs and trunk in the expression of either propagated or standing waves of muscle activity in the trunk during locomotion (Harischandra et al. 2011; Ijspeert et al. 2005).

Finally, descending commands and sensory feedback most probably interact to differentially affect the spinal networks from one behavior to the other. In the lamprey, body movements are fed onto the CPG neurons via mechanosensory edge cells (Grillner et al. 1984). Interestingly, the motoneuron responses evoked by rhythmic movements of the spinal cord can dramatically change after brain stem stimulation (Hsu et al. 2013), suggesting that a reconfiguration of the inputs provided by stretch receptors can be achieved by descending commands.

ACKNOWLEDGMENTS

The authors gratefully thank Yoav Mor and Aharon Lev-Tov for having generously provided a copy of the Spinalcore software and for useful discussions on wavelet transformations. The authors thank Örjan Ekeberg for his valuable comments on a previous version of the manuscript.

GRANTS

Support from the European Community (LAMPETRA grant: FP7-ICT-2007-1-216100), the Fondation pour la Recherche Médicale (FRM, DBC 20101021008), and the Swiss National Science Foundation (Project 140714) is acknowledged. D. Ryczko received fellowships from the Ministère de l’Educaton Nationale, de la Recherche et de la Technologie (MENRT) and the FRM.

DISCLOSURES

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

AUTHOR CONTRIBUTIONS


REFERENCES


Cabelguen JM, Charrier V, Mathieu A. Modular functional organisation of the axial locomotor system in salamanders, Zoology (Jena, Germany) 117: 57–63, 2014.


