Dynamics and Reproducibility of a Moderately Complex Sensory-Motor Response in the Medicinal Leech

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Submitted 19 December 2003; accepted in final form 26 April 2004

Garcia-Perez, Elizabeth, Davide Zoccolan, Giulietta Pinato, and Vincent Torre. Dynamics and reproducibility of a moderately complex sensory-motor response in the medicinal leech. J Neurophysiol 92: 1783–1795, 2004. First published April 28, 2004; 10.1152/jn.01240.2003. Local bending, a motor response caused by mechanical stimulation of the leech skin, has been shown to be remarkably reproducible, in its initial phase, despite the highly variable firing of motoneurons sustaining it. In this work, the reproducibility of local bending was further analyzed by monitoring it over a longer period of time and by using more intact preparations, in which muscle activation in an entire body segment was studied. Our experiments showed that local bending is a moderately complex motor response, composed of a sequence of four different phases, which were consistently identified in all leeches. During each phase, longitudinal and circular muscles in specific areas of the body segment acted synergistically, being co-activated or co-inhibited depending on their position relative to the stimulation site. Onset and duration of the first phase were reproducible across different trials and different animals as a result of the massive co-activation of excitatory motoneurons sustaining it. The other phases were produced by the inhibition of excitatory and activation of inhibitory motoneurons, and also by the intrinsic relaxation dynamics of leech muscles. As a consequence, their duration and relative timing was variable across different preparations, whereas their order of appearance was conserved. These results suggest that, during local bending, the leech neuromuscular system 1) operates a reduction of its available degrees of freedom, by simultaneously recruiting groups of otherwise antagonistic muscles and large populations of motoneurons; and 2) ensures reliability and effectiveness of this escape reflex, by guaranteeing the reproducibility of its crucial initial phase.

**INTRODUCTION**

Understanding of the nervous system from the perspective of systems neuroscience requires the identification of those features of the neural activity that are reproducible from trial to trial (Bialek and Rieke 1992; Gerstner et al. 1997; Lestienne 2001; Shadlen and Newsome 1998; Stevens and Zador 1998). A prerequisite for this investigation is the analysis of the reproducibility of a repeated behavior. In a previous analysis of the leech local bending reflex (LB), it was shown that this simple sensorimotor response, initiated by a light mechanical stimulation of the skin (Kristan 1982; Lockery and Kristan 1990a), was more reproducible than the firing of individual motoneurons sustaining it (Zoccolan et al. 2002). Reproducibility of the LB, however, was established only for the peak amplitude of its initial phase, lasting ~1 s, and in a reduced preparation. Moreover, the variability of its onset and duration across different animals was not studied in a systematic way.

Therefore it was interesting to extend this analysis to later phases of the LB by monitoring it over a longer period of time (~30 s) and by using more intact preparations, in such a way to characterize this behavior in its integrity and complexity.

LB in the leech is also a suitable model to investigate basic properties of motor control such as the neuromuscular basis of coordinated motor behavior, where different classes of muscle fibers are recruited in a flexible way and at specific phases to produce a range of motor actions, from escape reflexes to locomotor patterns. One current view of spinal motor systems suggests that such a variety of motor behaviors in vertebrates is the result of the flexible combination of a small number of behavioral units (for review, see Tresch et al. 2002) controlling groups of functionally related muscles and often referred to as synergies (Bizzi et al. 2000; d’Avella and Bizzi 1998; Tresch et al. 1999). In this view, grouping together synergistic muscles allows the nervous system of vertebrates to cope with the many degrees of freedom related to the control of limbs, muscles, motor units, spinal motor circuits, etc. (Tresch et al. 2002). This process can be more directly assessed in the neuromuscular systems of invertebrates because of the lower number of available degrees of freedom. For instance, in a previous study, it has been shown that the first phase of the LB in the leech is produced by the linear sum of the patterns of body wall deformation induced by the activation of two distinct classes of otherwise antagonistic muscles: longitudinal and circular muscles (Zoccolan and Torre 2002). Therefore one of the goals of this study was to assess if a similar co-activation of longitudinal and circular muscle fibers could be observed during other phases of the motor response, thus supporting the hypothesis that such muscles act synergistically during the whole behavior.

In this work, a quantitative analysis of the pattern of body wall deformation produced by local bending allowed this motor behavior to be decomposed into a sequence of four distinct phases. The increasing variability of the motor response across all phases was tracked, and the dynamics of muscular activation and inhibition was correlated to the biomechanical properties of leech muscles and to the firing pattern of the motoneurons co-activated during the reflex. In addition, our experiments investigated the simultaneous recruitment of circular and longitudinal muscles across the different phases of the reflex. Overall, these results provide a better understanding of the motor organization of the local bending and suggest that a similar flexible combination of basic behavioral units can...
underlie the construction of motion in invertebrates and higher animals.

METHODS

Animals and preparations

Medicinal leeches (*Hirudo medicinalis*) were obtained from Ricarimpex (Eysines, France) and kept at 5°C in tap water dechlorinated by aeration for 24 h. Different types of body wall preparations were used, and their pictures are shown on the right of Fig. 1. On Fig. 1, left, a sketch of the leech (in which the body of the animal is represented as a “tube”) shows how the leech body was dissected to obtain these preparations. The procedure is in accordance with the regulations of the Italian Animal Welfare Act and was approved by the local authority veterinary service.

The simplest preparation (Fig. 1A) consisted of a semi-section of the leech body wall, about three segments in length, isolated from the rest of the body. One boundary was formed by the dorsal midline (DM), and the other boundary was between the lateral edge (LE) and the ventral midline (VM) of the animal. The body wall was flattened and fixed with pins to the bottom of the recording chamber but was allowed to deform during muscle contraction. The middle segment was kept innervated by its ganglion, which was cleaned and exposed (Fig. 1A, bottom right) to allow intracellular recordings from motoneurons and mechanosensory neurons. This preparation, which will be referred to as the half body wall preparation, has been extensively used to study local motor behavior of the leech (Kristan 1982; Mason and Kristan 1982; Norris and Calabrese 1987; Zoccolan and Torre 2002; Zoccolan et al. 2002). However, since it does not allow monitoring the leech motor responses contralateral to the stimulated site, we performed most of the experiments using more intact preparations. Two of them, which will be referred to as whole body wall preparations, are shown in Fig. 1, B and C. These preparations were obtained by cutting the leech body longitudinally along the ventral midline (Fig. 1B, left, dashed line) or along the dorsal midline (Fig. 1C, left, dashed line). As in the other preparation, about three segments in length were isolated, flattened, and fixed with pins. The middle segment was kept innervated by its ganglion. In the case of the dorsal cut (Fig. 1C), the body wall could be stretched and fully elongated along the annuli direction in such a way as to obtain a complete view of the whole body segment. In the case of the ventral cut (Fig. 1B), only a dorsal view of the body segment from one lateral edge to the other could be obtained. This is because the body wall could not be completely stretched along the annuli without breaking the nerve roots emerging from the ganglion.

In most of the experiments performed on the whole body wall preparations, the ganglion was not exposed (Fig. 1B, right). However, in some preparations (e.g., Fig. 1C, right), the leech body was dissected, according to the suggestion of Prof. William Kristan, by opening a little hole in the ventral side of the body wall, just around the ganglion. This allowed performing intracellular recordings from mechanosensory neurons in the ganglion using a sharp microelectrode (Fig. 1C, right, white asterisk).

An isolated leech segmental ganglion with exposed nerve roots was used to monitor the pattern of motoneuron activation during LB. Five suction pipettes were used to perform parallel extracellular recordings from the anterior anterior (AA), anterior medial (MA), and posterior posterior (PP) roots and from the two bifurcations (DP:B1 and DP:B2) of the dorsal posterior root (Arisi et al. 2001; Pinato et al. 2000; Stent et al. 1978). Spikes recorded from these roots were classified according to dimension and shape and were identified by impaling each motoneuron as previously described (Arisi et al. 2001; Pinato et al. 2000). In this way, it was possible to characterize the firing activity of a large fraction of all leech motoneurons: the excitatory motoneurons of the longitudinal muscles (cells 3, 4, 5, 6, 8, 107, 108, and L), the excitor of the flatterner muscles (cell 109), the excitor of the oblique muscles (cell 110), and two inhibitory motoneurons of longitudinal muscles (cells 101 and 102) (Arisi et al. 2001; Ort et al. 1974; Stent et al. 1978). The only excitor motoneuron of circular muscles that was identifiable in the extracellular recordings was the ventral circular excitor, usually named CV, which, to avoid confusion with the coefficient of variation (CV), will be referred to as CIV.

All preparations used in this study were dissected from the central region of the leech body (between the 8th and the 14th segment). They were kept in a Sylgard-coated dish at room temperature (20–24°C) and bathed in Ringer solution containing (in mM) 115 NaCl, 1.8 CaCl₂, 4 KCl, 12 glucose, and 10 Tris maleate buffered to pH 7.4 (Muller et al. 1981).

Depending on the preparation used, LB was initiated either by intracellular stimulation of one or two mechanosensory pressure (P) cells or by mechanical or electrical stimulation of the skin (see *Mechanical and electrical stimulation of the leech skin*). When the reproducibility of LB was assessed by repeated stimulation of the...
preparation, stimuli were delivered with a period of 3–5 min to avoid possible adaptation mechanisms in the reflex pathway.

**Intracellular recordings**

Electrical activity of motoneurons and mechanosensory neurons was monitored by intracellular recordings with sharp electrodes (resistance, 30 MΩ, filled with 4 M potassium acetate) using Axoclamp-2b amplifiers (Axon Instruments, Foster City, CA). Intracellular and extracellular voltage recordings were digitized at 10 kHz, stored on a personal computer, and analyzed with Clampex 8 (Axon Instruments).

**Imaging and behavior analysis**

Skin deformations were quantified by computing the optical flow from image sequences of the contracting leech skin (Zoccolan and Torre 2002; Zoccolan et al. 2001, 2002). Images were acquired at 5 or 8.3 Hz by a standard CCD camera mounted on a dissecting microscope and were digitized and stored on a personal computer using a frame grabber DT3155 (Data Translation) with the acquisition software Axon Imaging Workbench 2.2 (Axon Instruments). The method for computing the optical flow is based on finding the best correlation between patches of successive images and is fully described in a previous work (Zoccolan et al. 2001). The same technique was used to follow the displacement of a specific point of the leech skin. Once the optical flow is computed, it can be further analyzed by decomposing it in its elementary deformations. This is done in two steps. First, the optical flow in a given area is approximated by a linear vector field. The optimal criterions for choosing this area are widely discussed in Zoccolan and Torre (2002). Moreover, the results of the linear analysis have been found to be very robust against variation in the size of the linearization area (Zoccolan 2002). Second, from this linear approximation, the elementary deformations are obtained (Zoccolan et al. 2001). They provide a compact and quantitative characterization of the pattern of skin deformation produced by the contraction of leech muscles (Zoccolan and Torre 2002).

Based on the shape and the structure (i.e., the elementary deformations) of the optical flow, different phases of LB were identified. Their timing was precisely quantified by tracking the displacement of selected points on the surface of the body wall preparation. For this kind of analysis, six to nine points to track were selected on the leech body wall, in such a way as to subsample the whole optical flow. Namely, the image plane was partitioned into six to nine rectangular windows of identical size. One point was randomly sampled inside each window from a two-dimensional (2D) Gaussian distribution centered on the center of the window and with σ equal to one-half the width of the shortest window side. This sampling procedure avoided selecting unreliable points near the edges of the preparation. Finally, the displacement of each tracked point was plotted versus time, and the phase identification was based on those points whose displacement was largest, smoothest, and with clear maxima, minima, inflection points, and plateaus. If no such points were found, the sampling procedure was repeated until at least two points (one ipsilateral and one contralateral) could be found that provided enough details about the time course of the different phases of the LB. Details on the method used to identify onset and offset of each phase are provided in RESULTS.

**Mechanical and electrical stimulation of the leech skin**

In some experiments, a brief (200–500 ms) mechanical stimulus (~20 mN) was delivered to the body wall preparation to initiate LB. The stimulus consisted of a poke with a nylon filament (two filaments are visible in Fig. 1B, right, marked by asterisks) driven by a solenoid (347–652 RS components), as previously described (Lewis and Kristan 1998; Pinato and Torre 2000). In the experiments where it was necessary to obtain intracellular recordings from motoneurons, the motor response was initiated by an electrical pulse of 0.5–1 mA delivered to the skin through a glass suction pipette (Kristan 1982; Mason and Kristan 1982).

**Terminology**

In the experiments in which the body wall preparation was mechanically stimulated, the terms “ipsilateral” and “contralateral” refer to the stimulation site. The same terms when applied to sensory neurons and motoneurons are defined with reference to the field of innervation. In the experiments in which the body wall preparation was electrically stimulated and intracellular recordings were performed on motoneurons, the terms “ipsilateral” and “contralateral” refer to motoneurons whose field of innervation is, respectively, on the same side or on the opposite side of the stimulus site.

**RESULTS**

**Pattern of body wall deformation during LB**

The pattern of skin deformation during LB was analyzed in the whole body wall preparation (ventral cut) shown in Fig. 1B, in which a mechanical stimulus was delivered near one lateral edge of the preparation (note the position of the nylon filaments in Fig. 1B, right). Figure 2 shows the result of two of these experiments (one on the left, the other on the right), in which a mechanical stimulus of ~20 mN, lasting 500 ms, was applied to the top lateral edge of the preparation (touch location is indicated by the circle in Fig. 2, A and D). The gray background in each panel of the figure shows the annular margins and part of the texture of the preparation. The skin deformation induced by the stimulation was quantitatively characterized by computing the optical flow (Zoccolan et al. 2001), as described in METHODS. Image sequences of the deforming leech body wall were acquired for a duration varying between 15 and 30 s after mechanical stimulation. As a consequence, muscle contractions or relaxations occurring after 30 s were described only by visual inspection.

Three phases were clearly distinguishable in the motor response analyzed in the left column of Fig. 2. The first phase (Fig. 2A) was a contraction around the stimulus site. The shape of the optical flow, with the arrows pointing to the stimulus site along both the longitudinal and the transverse direction, indicates that longitudinal and circular muscles ipsilateral to the stimulus site were activated. The expansion of the contralateral body wall dominated the second phase (Fig. 2B) of the motor response. The shape of the optical flow, with the arrows pointing outward along both the longitudinal and the transverse direction, is consistent with the relaxation of longitudinal and circular contralateral muscles. The third phase (Fig. 2C) was a similar strong relaxation of ipsilateral longitudinal and circular muscles that did previously contract during the first phase.

The right column of Fig. 2 describes the motor response induced by a similar mechanical stimulation in a different preparation. This appears—at first sight—rather different from that shown in the left column. However, the same first two phases were clearly identifiable in both the motor responses. In fact, the contraction of ipsilateral muscles around the stimulus site (Fig. 2D) was followed by the relaxation of contralateral body walls (Fig. 2, E and F). In this case, however, the duration of the ipsilateral contraction was longer (Fig. 2, D and E, lasting >5 s), allowing the superposition of the ipsilateral
contraction and contralateral relaxation (Fig. 2E, lasting \(\sim 2.4\) s). The ipsilateral contraction was almost finished in the last 10 s of the deformation (Fig. 2F), while the contralateral relaxation was still large.

The optical flows shown in Fig. 2 are typical examples of body wall deformations in an isolated semi-intact leech body segment performing the LB in response to a mechanical stimulus delivered at the skin surface. Similar experiments were performed on a total of 14 different whole body wall preparations obtained by ventral cut (5/14), dorsal cut (6/14), and dorsal cut with hole (3/14). The skin of these preparations was stimulated on different locations (left and right dorsal, lateral, ventral side), for a total of 26 different stimulations. Visual inspection of the obtained optical flows showed that, in all these experiments, the skin deformation was qualitatively similar to that shown in Fig. 2. Further quantitative analysis confirmed this conclusion.

![Image](image_url)

**Fig. 2.** Optical flows describing the body wall deformations occurring in 2 different whole body wall preparations (ventral cut) during LB. A–C: patterns of body wall deformation induced in the 1st preparation (the same shown in Fig. 1B) by a mechanical stimulus (20 mN, 500 ms) delivered near its lateral edge (touch location is marked by a circle in A). Each panel shows a specific phase of the motor response evoked by the stimulus. Phase duration is indicated by the text between columns, D and E: patterns of body wall deformation induced in a different preparation by similar mechanical stimulus (20 mN, 500 ms; touch location is marked by a circle in D). Similar phases, but with different timing, were identified. Note that the magnification in the left column is \(3 \times\) to enhance the visualization of the optical flows. Numbers in A point out the location of 2 selected points whose displacement is shown Fig. 3. Gray background in each panel is a sketch of the preparation used in each experiment.

**Determination of onset and offset for the different phases of LB**

The dynamics of the motor response analyzed in Fig. 2, A–C, was also studied by tracking the displacement of selected points on the surface of the body wall preparation (Fig. 2A, points 1 and 2), as shown in Fig. 3. For this kind of analysis, six to nine points to track were randomly selected on the leech body wall (see METHODS) until at least two points (one ipsilateral or contralateral), on which the final identification of the different phases of the LB could be based, were found.

The left column of Fig. 3 shows the trajectory of point 1 (ipsilateral to the stimulus site) on the image plane (Fig. 3A), as well as the time course of its X displacement (Fig. 3C) and its time derivative (Fig. 3E). The right column (Fig. 3, B, D, and F) shows the same analysis for point 2 (contralateral to the stimulus site), while Fig. 3G summarizes the duration and timing of the identified phases.

Timing and duration of these different phases were precisely quantified by identifying plateaus, minima and maxima in the time course of the X (and Y) displacement of points 1 and 2. Indeed, for each trial, the onset and offset of the different phases of LB can be determined by computing the derivatives of the time evolution of the coordinates X and Y. These derivatives \(dX/dt\) and \(dY/dt\) were computed by the numerical convolution with the derivative of a Gaussian filter (Oppenheim and Schafer 1989). The onset of the first phase (Fig. 3, A, C, and G, red line) coincides with the time at which \(dX/dt\) for point 1 becomes larger than zero and its offset coincides with the time at which \(dX/dt\) becomes again almost equal to zero. Similarly, the duration of the second phase (Fig. 3, B, D, and G, green line) can be detected by looking at time interval in which the derivative \(dX/dt\) of the second point is different from zero (Fig. 3F). The onset of the third phase (Fig. 3, A, C, and G, blue line) coincides with the end of the plateau in \(X_1(t)\) (Fig. 3C) and can be detected by looking at the time in which \(dX_1/dt\) becomes again different from zero (Fig. 3E). Note how this method allows the detection of overlapping phases of the motor response occurring in different areas of the body segment (overlapping bars in Fig. 3G).

In addition, the trajectory of ipsilateral and contralateral points on the image plane (Fig. 3, A and B) provides much more information about the spatial and temporal kinematics than force or displacement transducers can do. For instance, it is clear from Fig. 3 that the pathway along which a point on the leech skin moves during the relaxation phase (Fig. 3A, blue line) is not the same as that along which it moved during the contraction phase (Fig. 3A, red line). In fact, the surface of the leech body wall moves along a sort of loop during LB, and this loop is not yet closed in Fig. 3, A and B, because an additional phase is lacking: the re-contraction of all ipsilateral and contralateral muscles until they reach their resting state. This hysteretic pattern of deformation of leech body wall during LB was further investigated by measuring the motor response over a longer time.

**Simultaneous relaxation of circular and longitudinal muscles during the late phases of LB**

In our previous analysis of LB, we decomposed the optical flows obtained from imaging the half body wall preparations...
into their elementary deformations: expansion (E), rotation (ω), horizontal (S₁), and oblique (S₂) shear (Zoccolan and Torre 2002). Such analysis revealed that longitudinal muscle contractions are almost pure negative horizontal shears (S₁ < 0), whereas circular muscle contractions are the sum of positive horizontal shear (S₁ > 0) and negative expansion (E < 0). As a consequence, during simultaneous activation of longitudinal and circular muscles, the horizontal shears cancel each other, and the main component of the resulting deformation is a large negative expansion. Based on this analysis, we found that the first phase of the LB (i.e., the ipsilateral contraction) is sustained by the coactivation of longitudinal and circular muscles, because, in all tested preparations, its main elementary deformation was a large negative E (Zoccolan and Torre 2002). We use the term compression for such a characteristic pattern of muscle activation. Since relaxation of longitudinal and circular muscles is characterized by the same elementary deformations but with reverse sign (i.e., S₁ > 0 for longitudinal and S₁ < 0 + E > 0 for circular relaxation (see Zoccolan and Torre 2002), in this study, we investigated if simultaneous relaxation of circular and longitudinal muscles could be consistently observed in the late phases of the LB.

The analysis based on computing the elementary deformations of the optical flow was first applied to the body wall deformations recorded in the preparations obtained by dorsal cut. In these preparations, the stationary (singular) point of the occurring deformation was usually visible, and the linear approximation of the optical flow could reliably be computed in a region around it. Figure 4A shows an example of the pattern of deformation obtained in the contralateral side of such a preparation during the second phase of LB. A positive expansion E was the largest of the resulting elementary deformations (Fig. 4B; note the little shear S₁), thus providing evidence of the simultaneous relaxation of both longitudinal and circular muscles. We refer to such characteristic pattern of muscle activation as expansion.

In general, the elementary deformations could not be directly computed for the experiments performed on the preparations obtained by ventral cut. In fact, for such preparations, the stationary point of the occurring deformation was usually not in the imaged piece of skin. Therefore a preprocessing of the optical flow was necessary to verify that the observed deformation was a radial expansion (consistent with simultaneous longitudinal and circular relaxation) and to estimate the position of the singular point, i.e., the center of the deformation. Such processing is based on assessing how close to a pure radial expansion is the measured optical flow. This is done by finding the position of the singular point that minimizes the mean angular error between the direction of the arrows of the measured optical flow and that of a perfectly radial vector field (pure E > 0). Figure 4C shows how such analysis was applied to quantify how close to a radial expansion was the optical flow previously shown in Fig. 2B. Here the black arrows represent the direction of the measured optical flow and the green arrows, the direction of the pure radial field centered in the position marked by the X. Note that all the arrows in both fields were normalized to 1, since only their direction and not their length were compared. The goodness of fit to the radial field was quantified by computing the distribution of the angular difference (or angular error) between the directions of each pair of corresponding arrows in the two fields (see Fig. 4D). Figure 4E reports the mean value of this angular error for the defor-
The mean was computed over the largest area of the optical flow of Fig. 4C (in red). Figure 4G shows a population analysis performed on all tested preparations, during the second and third phase of LB (diamonds and circles, respectively; 14 experiments). The positive expansion $E$ was consistently the main elementary deformation ($\gg |S_1|$), thus providing a strong evidence of the simultaneous relaxation of circular and longitudinal muscles during these late phases of LB.

**Hysteretic behavior of leech body wall and reproducibility of LB**

The hysteretic pattern of deformation of the leech body wall during LB was further analyzed by measuring the motor response over a long time. Figure 5 shows the results of one of these experiments, in which a preparation was monitored for 30 s after mechanical stimulation ($\sim 20$ mN for 500 ms) delivered at the dorsal-lateral quadrant (touch location is indicated by the orange circle in Fig. 5A). The initial phases of the resulting deformation were similar to those previously described. An ipsilateral compression (Fig. 5A) was followed by an expansion of contralateral (Fig. 5B) and ipsilateral (Fig. 5C) body wall. In addition to these three phases, a fourth phase could be identified (Fig. 5D). At $\sim 25$ s from the beginning of the motor response, longitudinal ipsilateral muscles started to re-contract (bars on the top of Fig. 5, E or H, provide an approximate measure of the relative timing of the different phases). This is also shown by the trajectories (Fig. 5, G and J) of two selected points (Fig. 5A, points 1 and 2) on the preparation surface. Here, the magenta piece of trajectory (4th phase) helps to close the loop initiated by the previous phases. The loop is still not closed after 30 s (at the end of the recording interval) because leech muscles are extremely slow and require several minutes to come back to their resting state (Arisi et al. 2001; Mason and Kristan 1982; Zoccolan and Torre 2002). Because of limitations of the image acquisition system, the preparation could not be monitored for a long time, but, when the preparation was newly imaged after 3 min from the beginning of the motor response, the position of points 1 and 2 (Fig. 5, G and J, circles) was almost coincident with the initial one. This confirmed that the body wall had finally returned to the resting state (the dashed line in Fig. 5G and J shows an hypothetical trajectory).

In this same experiment, the reproducibility of the body wall displacement during LB was studied by tracking the displacement of points 1 and 2 across 14 different repetitions of the same mechanical stimulation (Fig. 5, E and H, superimposed colored traces). The time evolution of the resulting mean displacement (solid line) and its CV (dotted line) are shown in Fig. 5, F and I. The CV of the mean displacement of both points reached a minimum ($\sim 0.1$) at the peak of the motor response (i.e., at the end of the 1st phase for point 1 and around the middle of the 2nd for point 2). Then, the CV gradually increased with time during the motor response as expected by observing that the displacements across individual trials started to diverge significantly after the peak (Fig. 5, E and H). This trend of the CV was confirmed by similar experiments per-
formed on other two preparations, in which different skin spots were stimulated.

The variability of duration and timing of the kinematics of the LB is shown in Fig. 6. The ipsilateral contraction (in red), the contralateral relaxation (in green), the ipsilateral relaxation (in blue), and the late re-contraction of the all muscles (in magenta) were detected in 14 different whole body wall preparations by looking at the derivative of the displacement of points 1 and 2 in A during LB. Plots show 14 superimposed traces resulting from 14 identical mechanical stimulations performed in the same preparation. F and I: mean displacement over the 14 trials (solid line) and its CV (dashed line) as a function of time during LB. G and J: trajectories of the 2 tracked points in I of the 14 trials, with colors indicating the approximate timing of the 4 phases of LB. These phases are the same shown in A–C and in the bars on the top of E and H. Circles in G and J show the position of the tracked point at the beginning of the next trial. Dashed line is a hypothetical trajectory linking this position to the end of the recorded trajectory. Note that colors in G and J, as well as bars in E and H, underestimate the actual duration of the phases and are meant to provide just an approximate measure of their timing. In fact, these phases were drawn as sequential by setting the onset of each phase as coincident with the actual offset of the previous one, while they were actually overlapping (similarly to that shown in Fig. 3G). This has been done to make the figure simpler and more understandable (timing and duration of LB phases are precisely quantified in Fig. 6).

Pattern of motoneuron activity during LB

The firing pattern of leech motoneurons during LB was studied in the isolated leech ganglion, in which LB was induced by intracellular stimulation of individual P cells. Extracellular suction pipettes were used to record the electrical activity of a large fraction of the motoneurons (12) innervating the muscles located in one-half of the body segment, during stimulation of both ipsilateral and contralateral dorsal and ventral P cells.

Figure 7 reports the results of one of these experiments, in which all the P cells in a ganglion were impaled and induced to fire two spikes. Each column shows the average firing rates (AFRs) of 12 identified motoneurons computed in time bins of 200 ms from n extracellular recordings. The names of the identified motoneurons are written to the right of the last column.

FIG. 5. Dynamics and reproducibility of LB. A–D: 4 phases of LB evoked, in a whole body wall preparation (ventral cut), by a mechanical stimulus (20 mN, 500 ms) delivered to the location marked by the orange circle in A. Note that the 4th phase (D) is a late re-contraction of the longitudinal muscles previously relaxed during the 2nd (B) and 3rd (C) phases. E and H: time course of the displacement of points 1 and 2 in A during LB. Plots show 14 superimposed traces resulting from 14 identical mechanical stimulations performed in the same preparation. F and I: mean displacement over the 14 trials (solid line) and its CV (dashed line) as a function of time during LB. G and J: trajectories of the 2 tracked points in I of the 14 trials, with colors indicating the approximate timing of the 4 phases of LB. These phases are the same shown in A–C and in the bars on the top of E and H. Circles in G and J show the position of the tracked point at the beginning of the next trial. Dashed line is a hypothetical trajectory linking this position to the end of the recorded trajectory. Note that colors in G and J, as well as bars in E and H, underestimate the actual duration of the phases and are meant to provide just an approximate measure of their timing. In fact, these phases were drawn as sequential by setting the onset of each phase as coincident with the actual offset of the previous one, while they were actually overlapping (similarly to that shown in Fig. 3G). This has been done to make the figure simpler and more understandable (timing and duration of LB phases are precisely quantified in Fig. 6).
Before P cell activation (the timing of P cell firing is indicated by the arrow in the bottom left of Fig. 7), all the motoneurons fired at a spontaneous rate of \( \sim 2-10 \) Hz. When the LB was initiated by the firing of the ipsilateral dorsal (P_d) or ventral (P_v) P cell, almost all the excitatory motoneurons of longitudinal muscles were activated at a frequency higher than the spontaneous rate. The firing rate of the dorsal excitors (DEs) transiently increased to \( 20-40 \) Hz (Fig. 7A) after 30–60 ms (mean latency to the 1st spike) from the onset of the P_d firing. Similar behavior was observed for the ventral excitors (VEs) following P_v firing (Fig. 7C). Most of the VEs were also excited after P_d firing (Fig. 7A) but at a much lower rate (5–15 Hz) than DEs. Similarly, the DEs were also excited by P_v firing, but less than VEs (Fig. 7A).

The firing pattern of leech motoneurons was different when contralateral dorsal (P_d) and ventral (P_v) P cells were stimulated. Following P_d firing (Fig. 7B), some of the excitors of longitudinal muscles (cells 3, 4, 5, and 107) were inhibited, with their firing rate decaying from the resting value to 0–1 Hz. Other motoneurons, such as cells 110 and CiV (previously excited by P_v firing), were also inhibited. The remaining longitudinal excitors (cells 6 and 8) were only weakly excited. However, note the sustained and long-lasting excitation of cell 102 (an inhibitor of dorsal longitudinal muscles). The activation of cell P_v (Fig. 7D) caused a similar inhibition in two longitudinal excitors (cells 4 and 5) and in some other excitatory motoneurons (cells 109, 110, and CiV). Also, in this case, the remaining longitudinal excitors (cells 3, 6, 8, and 107) were only weakly excited.

The inhibition of the excitatory motoneurons following contralateral P cell firing was delayed if compared with the excitation of these same motoneurons produced by ipsilateral P cell firing. This is better shown in Fig. 8, which compares the AFRs of four selected motoneurons (data taken from Fig. 7, A and B) following stimulation of the P_d (gray bars) and P_v (white bars). The black arrow in Fig. 8D indicates the onset of the P cell firing. The gray arrows in Fig. 8, A–D, point out the time bin in which the motoneuron-firing rate started to increase above the resting value (excitation onset). Similarly, the white arrows point out the time bin in which the motoneuron-firing rate started to decrease below the resting value (inhibition onset). A comparison between the location of the gray and white arrows shows that the inhibition induced by the P_d firing started one (Fig. 8, A and B) or two (Fig. 8, C and D) time bins later than the excitation produced by the P_v firing. This is equivalent to a delay of \( \sim 200-400 \) ms between P_d firing and inhibition of the excitatory motoneurons.

In summary, the pattern of motoneuron activation that emerges from the experiment presented in Fig. 7 is the following: a spike train fired by a P cell produces an excitation of the ipsilateral motoneurons and a delayed inhibition of the contralateral cells. These results were confirmed by a similar experiment repeated on a different preparation (data not shown). In short, P_d and P_v firing excited almost all longitudinal excitatory motoneurons (cells 3, 4, 5, 6, 107, and 108 and 4, 5, 6, 8, 107, and 108). On the other hand, P_d and P_v firing inhibited several longitudinal excitatory motoneurons (cells 3, 5, and 107 and 3, 5, 6, and 108).
Time course of the inhibition of excitatory motoneurons

To allow a quantitative comparison between the time course of contralateral relaxation and that of the inhibition of contralateral excitatory motoneurons, intracellular recordings were performed from contralateral motoneurons during electrical stimulation of the leech skin. Contralateral motoneurons were impaled, and their resting firing rate was set to the spontaneous value (~2–10 Hz) observed in the extracellular recordings by passing hyperpolarizing current into their cell bodies.

Figure 9 shows the result of four of these experiments in which the behavior of four different contralateral excitatory motoneurons was investigated. The onset of the electrical stimulation applied to the leech body wall (1 mA lasting for 200 or 400 ms) is indicated by the arrows. The effect of the electrical stimulus was the hyperpolarization of the recorded motoneuron and a strong decrease of its firing rate. The motoneuron firing rate started decaying after ~200–400 ms from the stimulus onset and remained below its spontaneous value for 3–10 s. This variable duration of the inhibition of contralateral motoneurons is consistent with the large variability observed for the duration of the second phase of the LB. Some degree of variability was also observed in the effect of the electrical stimulation on contralateral motoneurons. In 12 of 14 performed experiments, the effect of the skin stimulation was the opposite: the contralateral excitatory motoneuron was excited, with its firing rate transiently increasing ≥10 Hz for 2 s.

Excitation of inhibitory longitudinal and circular motoneurons

The behavioral analysis of the LB revealed that the body wall contralateral to the stimulus site relaxed following mechanical stimulation. Extracellular (Fig. 7) and intracellular (Fig. 9) recordings confirmed the inhibition of most of the longitudinal excitors and activation of at least one inhibitory motoneuron (cell 102) during LB. Therefore it was important to assess the kinematic pattern of body wall deformation produced by the excitation of the inhibitory motoneurons and by the inhibition of the excitatory motoneurons.

Figure 10A shows the optical flow associated with the body wall deformation evoked by the stimulation of cell 2, a ventral longitudinal inhibitor. As expected, a relaxation of longitudinal muscles was observed. A similar experiment was hard to repeat on the inhibitory motoneurons of circular muscles because no motoneurons have been fully proven to be circular inhibitors. Since Baader (1997) identified cell 166 as a putative circular inhibitor, we tried to impale and stimulate that neuron, and the resulting pattern of deformation (Fig. 10B) was consistent with the relaxation of dorso-lateral circular muscles. The inhibition of excitatory motoneurons had a similar effect. When a hyper-
polarizing current step was passed into a longitudinal excitor (Fig. 10D, cell 5), the resulting body wall deformation (Fig. 10C) was a relaxation along the longitudinal direction. Similar results were obtained when circular excitors were hyperpolarized (data not shown).

Comparison between the timing of muscle contraction and relaxation

As summarized in Fig. 6, the relaxation of contralateral muscles followed the contraction of ipsilateral muscles by about 2 s. This is consistent with the slow dynamics of muscle relaxation produced by inhibitory motoneurons (Mason and Kristan 1982; Norris and Calabrese 1987). In addition, since the inhibition of contralateral excitatory motoneurons followed the excitation of ipsilateral excitatory motoneurons by only 200–400 ms, it was important to assess the time course of muscle relaxation produced by inhibition of excitatory motoneurons when they were firing at their baseline resting rate (2–10 Hz). For instance, when an intracellular hyperpolarizing current pulse lasting 3 s reduced the firing rate of motoneuron 5 from 10 to ~4 Hz (Fig. 10D, bottom), the longitudinal dorsal relaxation shown in Fig. 10C was observed. This relaxation (its time course is shown in Fig. 10D, top) started after ~2 s from the onset of the current step. Note that, in this example, the
body wall did not recover to its initial state, because the firing rate of cell 5 after the inhibition was higher (30 Hz) than the initial rate (10 Hz), probably because of the injury discharge produced by the movement of the preparation. When the same cell in a different preparation was stimulated by passing a 5-s depolarizing current step (Fig. 10F) into its soma, the resulting muscle contraction (Fig. 10E) was developed within 0.5 s from the onset of the depolarization (time course of the contraction shown in Fig. 10F, top).

Similar experiments were repeated in motoneurons 3, 7, 8, 107, and 108. All these experiments showed that the contraction of the longitudinal muscles follows the excitation of the excitatory motoneurons with a delay of <500 ms. On the other hand, the relaxation of longitudinal muscles follows the inhibition of longitudinal excitatory motoneurons with a longer delay of about 2 s. This large difference between the latencies of muscle relaxation and muscle contraction fully accounts for the observed difference in the timing of the ipsilateral contraction and the contralateral relaxation during LB.

**Discussion**

Our experiments indicate that LB is composed by four distinct phases, always occurring in the same sequence, during which longitudinal and circular muscles in different regions of the body segment are simultaneously, or almost simultaneously, co-activated or co-inhibited. This behavioral characterization has been obtained using different kinds of body wall preparations (Fig. 1), which are planar projections of a single leech body segment. Their main advantage is that muscle deformations can be reliably quantified as planar displacement fields in the simplest functional unit of the leech body, i.e., an isolated segment innervated by its ganglion. However, several limitations are imposed by the use of such preparations. In fact, in intact leeches, muscle contractions work against the hydrostatic skeleton provided by the fluid-filled body tube (Wilson et al. 1996). Therefore we tested by visual inspection the effect of mechanical stimuli delivered to intact leeches. These observations were consistent with previous qualitatively descriptions of this behavior (Kristan 1982; Lockery and Kristan 1990a) and confirmed that the simultaneous recruitment of longitudinal and circular muscles at specific phases during LB could be observed in intact leeches. In addition, they suggest that the optical flow analysis of more intact body wall preparations composed by several innervated segments could be useful to elucidate the intersegmental coordination during LB and its interaction with associated reflexes, such as local body shortening (Wittenberg and Kristan 1992a,b). Finally, in this study, we did not take into account the possible role of stretch receptors in shaping the LB reflex. These receptors, which innervate longitudinal muscles, hyperpolarize in response to stretch of the body wall (Blackshaw and Thompson 1988), thus providing a peripheral sensory feedback during leech motor behavior. For instance, it has been shown that phasic sensory input provided by stretch receptors contribute to the intersegmental phase relationship during swimming (Cang and Friesen 2000) through synaptic interactions with most of the oscillatory swimming interneurons (Cang et al. 2001). Therefore we cannot exclude a similar involvement of stretch receptors in establishing, for instance, the temporal relationship between the different phases of the LB. We believe that combining simultaneous intracellular recordings from stretch receptors (Cang et al. 2001) and LB interneurons (Lockery and Kristan 1990b) with optical flow analysis of body wall deformations occurring in multiple body segments could potentially lead to a complete understanding of the neuromuscular events underlying LB.

**Variability of LB**

In this study, we found that the CV of the mean body wall displacement during LB (see Fig. 5) was initially <0.1 during the first seconds following mechanical stimulation of the skin. After 10 s, the CV invariably increased and reached a value between 0.3 and 0.5. This means that, after a highly reproducible initial phase (the ipsilateral compression), the reliability of LB is slowly degraded.

Note that the value of the CV of the peak displacement reported in Fig. 5 is less than one-half the value reported in our previous analysis (Zoccolan and Torre 2002). The reason for this difference is that, in our previous study, we induced LB by evoking in a P cell the minimal number of spikes (usually 2) necessary to observe a motor response, thus avoiding high-frequency bursts in the excited motoneurons that could prevent the spike identification. In this study, we induced a sustained LB by delivering a mechanical stimulus of ~20 mN lasting for 500 ms, which is able to evoke >10 spikes in a P cell (Lewis and Kristan 1998; Pinato and Torre 2000; Zoccolan and Torre 2002). Therefore the resulting motor response (Fig. 5, F and I) is bigger and longer-lasting than in the case of minimal P cell activation. Since the CV depends on the inverse of the mean displacement, its value must be expected to be remarkably lower in the case of prolonged and sustained skin (and P cell) deformation (Fig. 5).

While the variability of LB amplitude among different trials in the same preparation was initially small, there was a high variability in the timing and duration of the phases of the motor response (with the exception of the 1st) across different preparations and across different trials in the same preparation (Fig. 6).

This variability can be rationalized in the following way. LB is a stereotyped escape behavior, since similar phases were consistently found in all tested animals. The first phase, the ipsilateral compression, is the most crucial since it represents the withdrawal from potential danger. Its effectiveness and reliability are high (CVs are ~0.1 and ~0.17 for its peak amplitude and its duration across trials, respectively) and are guaranteed by the recruitment of a large population of excitatory motoneurons. This population activity overcomes any potential dependence on the specific state of the preparation at the time of the stimulation and therefore ensures also the reproducibility of this first phase across different animals. Later phases are less important for the effectiveness of the escape reflex. The second phase, for example, helps the animal withdraw from the stimulus by relaxing the contralateral side, but it is less crucial than the first. Starting from the second phase, the inhibition of muscles, the inhibition of excitatory motoneurons, and the dynamics of muscle relaxation play major roles in shaping the motor response. As a consequence of the slow (Fig. 10D) (Mason and Kristan 1982; Norris and Calabrese 1987) and variable dynamics of muscle relaxation, the reliability of the LB across trials starts to decrease. For the
same reason, the behavior during later phases might become more sensitive to differences in the overall state of the tested preparations (i.e., differences in the stiffness and basal tension of the muscles or in the level of some modulatory factors), causing the variability across individuals observed in the duration and timing of these later phases. These conclusions are supported by previous works in which the level of serotonin (5HT) the transmitter released by the Retzius cells) has been shown to have a long-lasting modulatory effect on the rate of the relaxation following contractions induced by longitudinal motoneurons, but not on the rate and peak amplitude of the contraction itself (Mason and Kristan 1982).

Pattern of motoneuron activation during LB

Parallel extracellular recordings performed on the isolated ganglion showed that activation of ipsilateral P cells produced the excitation of most of the excitatory motoneurons (DEs and VEs) of longitudinal muscles (Fig. 7, A and C). These findings are consistent with previous reports (Kristan 1982; Lockery and Kristan 1990a), because they confirm the major role of DEs and VEs in mediating, respectively, the dorsal and the ventral LB. On the other hand, these results show that many VEs and DEs can also be excited (but at low rate), rather than be inhibited during the execution of respectively dorsal and ventral LB in a rather variable way from preparation to preparation. This simultaneous activation of VEs and DEs seems to be more consistent with the execution of the lateral bending, which is usually elicited by simultaneous activation of both ipsilateral dorsal and ventral P cells (Kristan et al. 1995; Lockery and Kristan 1990a,b).

The pattern of motoneuron activation following stimulation of contralateral P cells revealed that many DEs and VEs contralateral to the stimulus site were inhibited during LB. This is consistent with previous reports (Lockery and Kristan 1990a) and with the relaxation of contralateral longitudinal muscles observed during the second phase of LB (Fig. 2). In addition, one dorsal inhibitor (cell 102) was strongly activated by contralateral P cell firing, and it is reasonable to assume that other inhibitory motoneurons, which could not be detected from the extracellular recordings, contributed to sustain the relaxation of the contralateral body wall. Similar speculation applies to most of the circular motoneurons, whose signal could not be detected in the extracellular recordings.

Overall, since in the leech ganglion about 24 different excitatory motoneurons have been described (Kristan 1982; Mason and Kristan 1982; Norris and Calabrese 1987; Ort et al. 1974) and we found that ≥11 of them are involved in LB, these results support the conclusion that LB is a collective event, sustained by a population activity at the level of excitatory motoneurons. From a computational point of view, it is interesting to point out how this population activity at the onset of the motor response is effective in guarantying its reproducibility (see also Zoccolan et al. 2002), while the later inhibition of this same population of motoneurons, and presumably, the activation of the population of inhibitory motoneurons, is not able to guarantee the same reliability.

Muscle coordination during leech LB

A major conclusion of this study is the finding that longitudinal and circular muscles were consistently co-activated or co-inhibited during the first three phases of LB. These results suggest that the leech neuromuscular system is capable of controlling a set of functionally distinct but behaviorally related muscles as a single group (or unit) during most of the behavioral cycle and across the entire body segment. Moreover, neuronal recordings showed that a large population of excitatory motoneurons innervating different muscle fibers belonging to the same class (namely, the class of longitudinal muscles) was recruited during the first phase of LB. Taken together, these findings suggest that the leech neuromuscular system is able to perform a remarkable reduction of its degrees of freedom during LB.

We believe that the simultaneous control of otherwise antagonistic muscles by large populations of motoneurons is reminiscent of the concepts of synergies and behavioral modules discussed in the spinal systems literature. Here, growing experimental evidence suggests that a flexible combination of basic spinal modules imposing specific patterns of muscle activation allow the spinal cord to reduce the degrees of freedom required for controlling multi-jointed limbs (Bizzi et al. 2000; d’Avella and Bizzi 1998; Tresch et al. 2002). A similar functional role for the simultaneous recruitment of circular and longitudinal muscles during leech LB cannot be proposed, because it is unlikely that, in invertebrates, simple reflexes such as LB can be produced in a number of different ways (i.e., muscles combinations). However, we believe that the neuronal circuit responsible for the pattern of neuromuscular activation observed during LB can share relevant basic features with the behavioral units proposed for the modular organization of spinal motor systems. The simpler version of such a circuit would be a set of mechanosensory neurons (namely, the P cells) monosynaptically connected to most of circular and longitudinal muscles. However, previous studies on the interneuronal control of the LB have shown the presence of at least one layer of interneurons driving longitudinal motoneurons (Lockery and Kristan 1990b). Based on our results, we speculate that such interneuronal network could act as a behavioral unit simultaneously controlling a large number of longitudinal and circular muscles across all the different phases of the reflex. Therefore we believe that dissecting out the operational properties of the LB interneuronal circuit with a combination of multiple intracellular recordings (Lockery and Kristan 1990b; Wittenberg and Kristan 1992b), optical imaging (Taylor et al. 2003), and quantitative assessment of the behavior (Zoccolan and Torre 2002; Zoccolan et al. 2002) could provide useful insights on the computational mechanisms allowing spinal interneuronal networks to group synergistic muscles into behavioral units.

Acknowledgments

We thank Prof. Bill Kristan for valuable scientific suggestions and help in the design of some experiments described in this manuscript. We also thank Dr. Matthew Tresch for useful discussions about muscle synergies, behavioral units, and the use of these concepts in the context of invertebrate neuromuscular systems.

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