

Phase-specific premotor inhibition modulates leech rhythmic motor output

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eLife Assessment

The medicinal leech preparation is an amenable system in which to understand the neural basis of locomotion. Here a previously identified non-spiking neuron was studied in leech and found to alter the mean firing frequency of a crawl-related motoneuron, which fires during the contraction phase of crawling. The findings are **valuable** and the experiments were diligently done and generally **solid**; however, the presentation could improve. The work could be taken to the next level by further experiments, and by providing more overall context for the study.

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Abstract

Understanding how motoneuron activity is finely tuned remains an open question. Leeches are a highly suitable organism for studying motor control due to their well-characterized behaviors and relatively simple nervous system. On solid surfaces leeches display crawling, a rhythmic motor pattern that can be elicited in the isolated nerve cord or even in ganglia isolated from it. This study aimed to learn how this motor output is shaped by concurrent premotor signals. Specifically, we analyzed how electrophysiological manipulation of a premotor nonspiking (NS) neuron, that forms a recurrent inhibitory circuit (homologous to vertebrate Renshaw cells), shapes the leech crawling motor pattern. The study included a quantitative analysis of motor units active throughout the fictive crawling cycle that shows that the rhythmic motor output in isolated ganglia mirrors the phase relationships observed *in vivo*. Taken together, the study reveals that the premotor NS neurons, under the control of the segmental pattern generator, modulated the degree of excitation of motoneurons during crawling in a phase-specific manner.

Introduction

Animal locomotion involves the coordination of multiple muscles under the control of the nervous system. Diverse regulatory mechanisms operate at different hierarchical levels within the motor system, narrowing down the degrees of freedom of a system with multiple units, to ensure smooth movements. The overall functional organization of motor systems remains, to a great extent, an open question in both vertebrates and invertebrates (Kiehn, 2016 [↗](#); Arber and Costa, 2018 [↗](#); Barkan and Zornik, 2019 [↗](#); Grätsch et al., 2019 [↗](#)), including the mechanisms that operate at the level of the nerve cord (Grillner and El Manira, 2020 [↗](#); McLean and Dougherty, 2015 [↗](#); Gal, 2017; Sengupta and Bagnall, 2023 [↗](#); Calabrese and Marder, 2024 [↗](#)). Analysis of this organization across different organisms, developmental stages, and behavioral repertoires has provided fruitful information. Leeches have been highly useful to study motor control due to their robust repertoire of motor behaviors, which are executed by a relatively simple body plan and controlled by a similarly simple nervous system. This system is composed of a chain of 21 midbody ganglia flanked by head and tail brains (Wagenaar, 2015 [↗](#)), where each midbody ganglion contains the sensory and motor neurons that innervate the corresponding segment (Muller et al., 1981 [↗](#)).

On solid surfaces, leeches exhibit a robust rhythmic motor pattern known as crawling (**Figure 1A** [↗](#)). This behavior results from waves of elongation and contraction of the body along its antero-posterior axis, as the animal is anchored on the posterior and anterior suckers, respectively (Stern-Tomlinson et al., 1986 [↗](#), **Figure 1A** [↗](#)). Fictive crawling (*crawling*) can be monitored in the isolated nervous system, where identified interneurons and motoneurons can be recorded intracellularly and extracellularly (Baader, 1997 [↗](#); Baader and Kristan, 1992 [↗](#); Eisenhart et al., 2000 [↗](#)). The pattern is characterized by the alternating activation of the motoneurons, such as CV and DE-3, that innervate circular and longitudinal muscles, respectively (**Figure 1B** [↗](#)). This pattern can be readily evoked by dopamine in the isolated nerve cords (Puhl and Mesce, 2008 [↗](#)), in short chains of ganglia (Kearney et al., 2022 [↗](#)), and in single isolated ganglia (Puhl and Mesce, 2008 [↗](#); Rodriguez et al., 2012 [↗](#)). These studies established that each ganglion contains the network responsible for producing the rhythmic motoneuron activity compatible with crawling.

Studies on motor behaviors in diverse organisms show that, at the final stage of neural processing, motoneuron activity is controlled by multiple signal sources (El Manira, 2023 [↗](#); Zhen and Samuel, 2015 [↗](#); Rotstein et al., 2017 [↗](#)). Analysis of the nature and effects of these signals on the motor pattern is an active field of research. The present work has focused on the role played by nonspiking (NS) premotor neurons on leech crawling. These neurons are present as bilateral pairs in each segmental ganglion and are functional homologs of the Renshaw cells (Szczipak, 2014 [↗](#)). Each pair of NS neurons is at the center of a recurrent inhibitory circuit mediated by chemical and electrical synaptic connections with the motoneurons (**Figure 1C** [↗](#)) (Rela and Szczipak, 2003 [↗](#); Rodriguez et al., 2009 [↗](#)). In the context of this circuit the activity of excitatory motoneurons evokes chemically mediated inhibitory synaptic potentials in NS. Additionally, the NS neurons are electrically coupled to virtually all excitatory motoneurons via rectifying junctions that conduct when the transjunction NS-motoneuron potential is negative. In physiological conditions this coupling favors the transmission of inhibitory signals from NS to motoneurons. Given this recurrent inhibitory circuit, NS premotor neurons can operate as modulators of motoneuron activity. This study aimed to evaluate the role of the known connectivity of the premotor NS neuron (**Figure 1C** [↗](#)) in shaping the crawling motor pattern. The experimental results reveal that NS mediates a reduction of the firing frequency of motoneurons recruited during a specific phase of crawling.

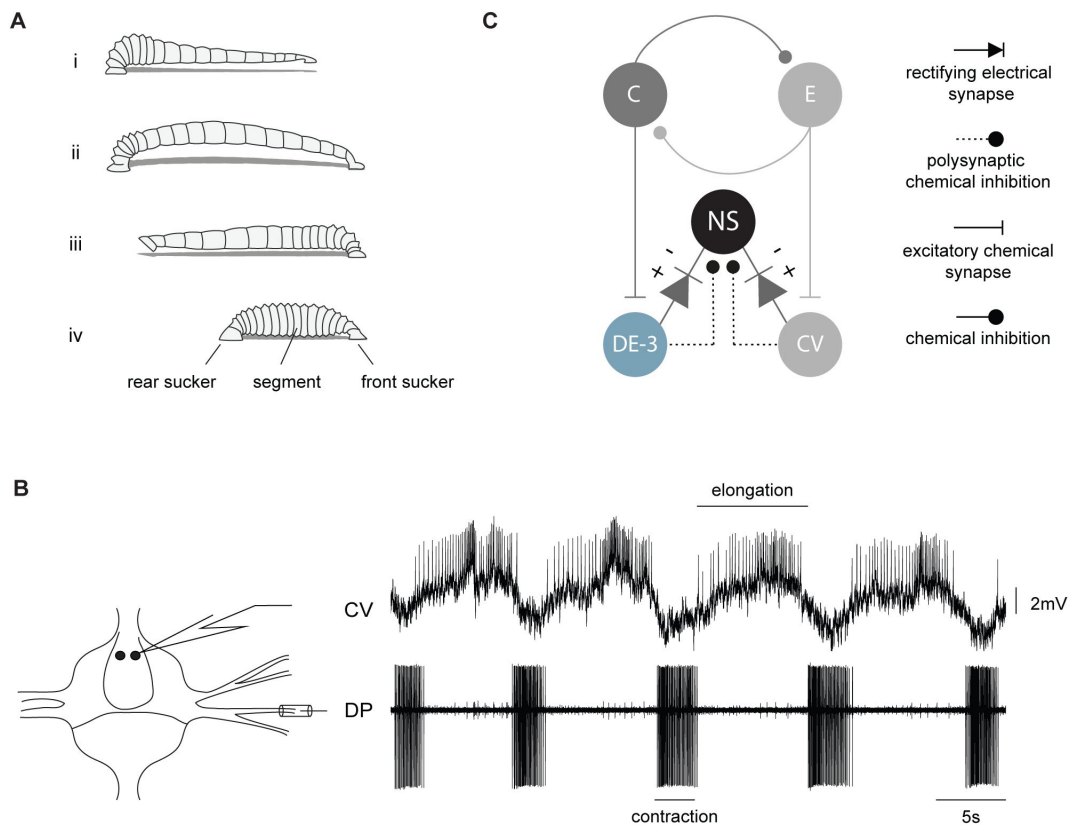


Figure 1.

The crawling motor pattern.

A. Schematic description of a leech crawling step that results from coordinated waves of elongation (i-ii) and contraction (iii-iv) phases, anchored on front and rear suckers. **B.** Intracellular recording of CV and extracellular recording of DE-3 (in DP nerve) motoneurons during a dopamine-induced crawling episode in an isolated midbody ganglion. Recording diagram on the left. **C.** Putative neuronal circuitry underlying crawling and the recurrent inhibitory circuit in a midbody ganglion. Units C and E correspond to contraction and elongation units of the segmental oscillator, respectively. DE-3 and CV are examples of motoneurons involved in each phase. NS neuron is connected to DE-3 and CV (Rela and Szczupak, 2003; Rodriguez et al., 2009) through chemical and electrical synapses; the + and - symbols indicate the polarity at which the rectifying synapse conducts.

Results

NS membrane potential oscillations during *crawling*

To study the role that premotor NS neurons play in fictive crawling (*crawling*), we first analyzed the correspondence between NS electrophysiological activity and the motoneuron output during this motor pattern. Since motoneuron DE-3 (dorsal excitor 3), which innervates the longitudinal dorsal muscle fibers, is the most robust proxy of *crawling* (Puhl and Mesce, 2008 [DOI](#)), extracellular recordings of this motoneuron in the DP nerve were combined with NS intracellular recordings. The NS membrane potential showed rhythmic oscillations tuned to DE-3 activity, with hyperpolarizations occurring in conjunction with DE-3 bursts and depolarizations during DE-3 silent phases (**Figure 2A** [DOI](#)). A cross-correlation analysis between NS and DE-3 revealed a marked anticorrelation, with a cross-correlation index of -0.61 ± 0.03 (mean $\pm$ SEM) at a lag close to 0 sec (0.24 ± 0.10 s) (**Figure 2B** [DOI](#)). This result indicates that NS hyperpolarization takes place in phase with DE-3 bursts.

In the frame of the recurrent inhibitory circuit, NS is the target of inhibitory signals evoked by excitatory motoneurons via chemically-mediated polysynaptic pathways (Rela and Szczupak, 2003 [DOI](#), **Figure 1C** [DOI](#)). Thus, it could be hypothesized that the NS hyperpolarizations observed during *crawling* resulted from inputs originating in DE-3 and other motoneurons that fire in phase with it. If this hypothesis was correct, the onset of DE-3 bursts should precede the onset of NS hyperpolarization. Because the cross-correlation was performed on binned DE-3 firing frequency curves, the peak lag reported does not offer a proper estimation of the relative timing between the two signals. To gain a more precise assessment, NS intracellular recordings were segmented into individual cycles and aligned with the first spike of the corresponding DE-3 burst (**Figure 2C** [DOI](#)). As shown in the figure, because of the NS membrane potential oscillations it is not possible to establish a sharp onset for the NS hyperpolarizing response. However, the example illustrates that the onset of the DE-3 bursts occurred after NS hyperpolarization was already initiated. These findings rule out the possibility that the NS hyperpolarization was originated by DE-3 bursting. Instead, they suggest that NS and DE-3 receive concomitant rhythmic inputs of opposite sign—that is, hyperpolarizing inputs in NS and depolarizing ones in DE-3.

To further analyze the putative concomitant inputs from the pattern generator onto NS and DE-3, we evaluated the correlation between different features of their rhythmic activity (**Figure 2A** [DOI](#)). We found no relationship between the amplitude of NS hyperpolarization and DE-3 mean firing frequency. However, surprisingly, the former exhibited a high correlation with DE-3 burst duration (**Figures 2D-E** [DOI](#)). This result led to examining how the amplitude of NS hyperpolarization related to the time between consecutive DE-3 bursts (interbursts time), preceding (pre) and following (post) the NS hyperpolarization (**Figure 2A** [DOI](#)). The data show a significant correlation of the amplitude of NS hyperpolarization with the pre-interburst duration, but not with the post-interburst duration (**Figure 2F-G** [DOI](#)). Taken in consideration the putative model presented in **Figure 1C** [DOI](#), in which the oscillator units C and E inhibit each other, the longer the activity of unit E, the larger the rebound in unit C and its downstream effects on NS hyperpolarization and on DE-3 burst duration.

These results indicate that, in addition to the known recurrent inhibitory circuit between NS and motoneurons, NS neurons receive an inhibitory signal during *crawling*, which is in phase with DE-3 activity. This inhibitory signal seems to have originated from the same source that activates DE-3 motoneurons. In that case, it seems puzzling to find a lack of correlation between NS hyperpolarization amplitude and DE-3 firing frequency. However, this observation might be

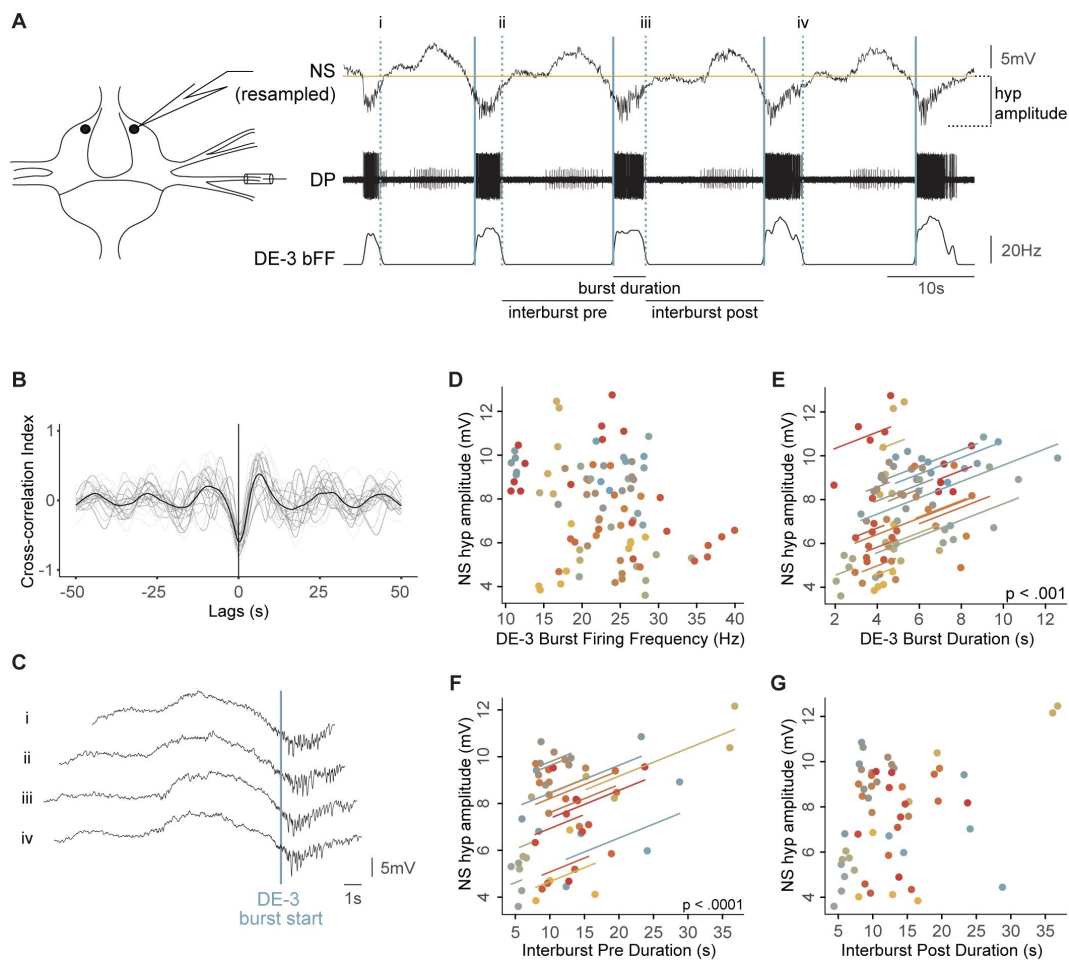


Figure 2.

NS activity correlates with the crawling motor pattern.

A. NS intracellular recording, DP extracellular recording and DE-3 binned firing frequency (bFF) during a crawling episode. NS recording was resampled to match the data rate of DE-3 bFF. Recording diagram on the left. The blue vertical line marks the beginning and the blue dotted line the end of each DE-3 burst. Different crawling features and NS hyperpolarization amplitude (NS hyp amplitude) are indicated. **B.** Cross-correlograms of the DE-3 bFF curves and NS resampled traces. Each gray trace corresponds to an individual experiment. The black curve corresponds to the mean cross-correlogram. ($n = 23$ crawling episodes containing 5 cycles, obtained in 23 ganglia from 18 leeches). **C.** Successive cycles of the NS recording shown in panel A, segmented at the times indicated by the dotted lines and aligned by the times indicated by solid blue lines indicated in A. **D.** NS hyperpolarization amplitude as a function of DE-3 Burst FF. **E.** Like in D, as a function of DE-3 burst duration. **F.** Like in D, as a function of interburst lag preceding DE-3 bursts. **G.** Like in D, as a function of the interburst lag after DE-3 bursts. For D-G, $n = 19$ crawling episodes, with 5 cycles each. In D to G, the correlation was tested using linear mixed-effects models, contemplating different experiments as a random variable. In D and G, $p > 0.05$, and in E and F, $p < 0.05$. The lines correspond to regressions obtained from the linear mixed-effects model. The coefficients were 0.374 ± 0.089 mv/s for E and 0.122 ± 0.034 mv/s for F.

explained by considering that DE-3 is subjected to two counteracting effects at the same phase: an excitatory input from the oscillator unit C and an inhibitory input from NS via the rectifying electrical junctions (**Figure 1C** [↗](#)).

Effect of NS on DE-3 activity during *crawling*

To test whether the inhibitory signal in NS is transmitted to the motoneurons, the premotor neuron was transiently removed from the circuit. In line with the properties of the rectifying electrical synapse between NS and the motoneurons, depolarizing NS neurons is equivalent to disconnecting them from the network (Rela and Szczupak, 2007 [↗](#)).

Depolarizing steps applied in NS neurons in the course of *crawling* episodes (**Figure 3A** [↗](#)) shifted their membrane potential beyond +100 mV, well above the average membrane potential of the motoneurons. To account for potential drifts in *crawling* features over time, we compared two series of experiments: one in which NS was subjected to depolarizing steps (depo) and a control (ctrl) in which it was not. We assessed changes in cycle period, DE-3 mean firing frequency (mFF), burst duration and duty cycle for both treatments (depo, ctrl) across different epochs (pre, test, post).

Transient NS depolarizations had no effect on the *crawling* period (measured in end-to-end cycles, see Materials and Methods), DE-3 burst duration and duty cycle (**Figure 3B** [↗](#)), but produced a 60% increase in DE-3 mFF (**Figure 3B-C** [↗](#)).

In the control series the analysis of *crawling* features over time shows that cycle period and DE-3 burst duration decreased over epochs, while the duty cycle remained constant. Similar results were obtained when cycles were segmented from start-to-start (data not shown). Thus, throughout the dopamine perfusion the motor pattern exhibited an acceleration but the duty cycle remained stable.

This series of experiments proves that, as predicted from the known circuit (**Figure 1C** [↗](#)), inhibitory signals onto NS premotor neurons counteracted the excitatory drive onto DE-3 motoneurons during *crawling*, limiting its firing frequency.

A more comprehensive view of *crawling*

As indicated above, the activity of motoneuron DE-3 has been widely used as a proxy of *crawling*. However, focusing on a single motoneuron neglects possible effects of NS on other phases of the motor pattern. To evaluate whether the effect of the premotor NS neuron extends to other motoneurons active during *crawling*, we performed extracellular recordings of DP, AA and PP root nerves. Single units were identified using a spike sorting algorithm (**Figure 4Ai** [↗](#)-Aii) that enabled a systematic analysis of their activity. The firing pattern of detected units (**Figure 4Aiii** [↗](#)) was expressed as binned firing frequency (bFF). Because DE-3 is used as a reference marker of the cycle, segmentation of the traces was performed using the start-to-start approach (see Material and Methods). This rhythmic activity shows that some units fire during the contraction phase, defined by DE-3 activity, while others fire during the elongation phase.

Plotting the bFF of the different rhythmic units as a function of phase within a *crawling* cycle (start-to-start) allowed for the manual classification of the unit. Based on their activity profile relative to DE-3 three types were identified: In-Phase, Anti-Phase, or In-Phase-Early-Onset (**Figure 4B** [↗](#)). The In-phase units fire simultaneously with DE-3, the Anti-Phase units fire in between DE-3 bursts, and the In-Phase-Early-Onset are active in phase with DE-3 but start firing before DE-3 burst onset (**Figure 4C** [↗](#)).

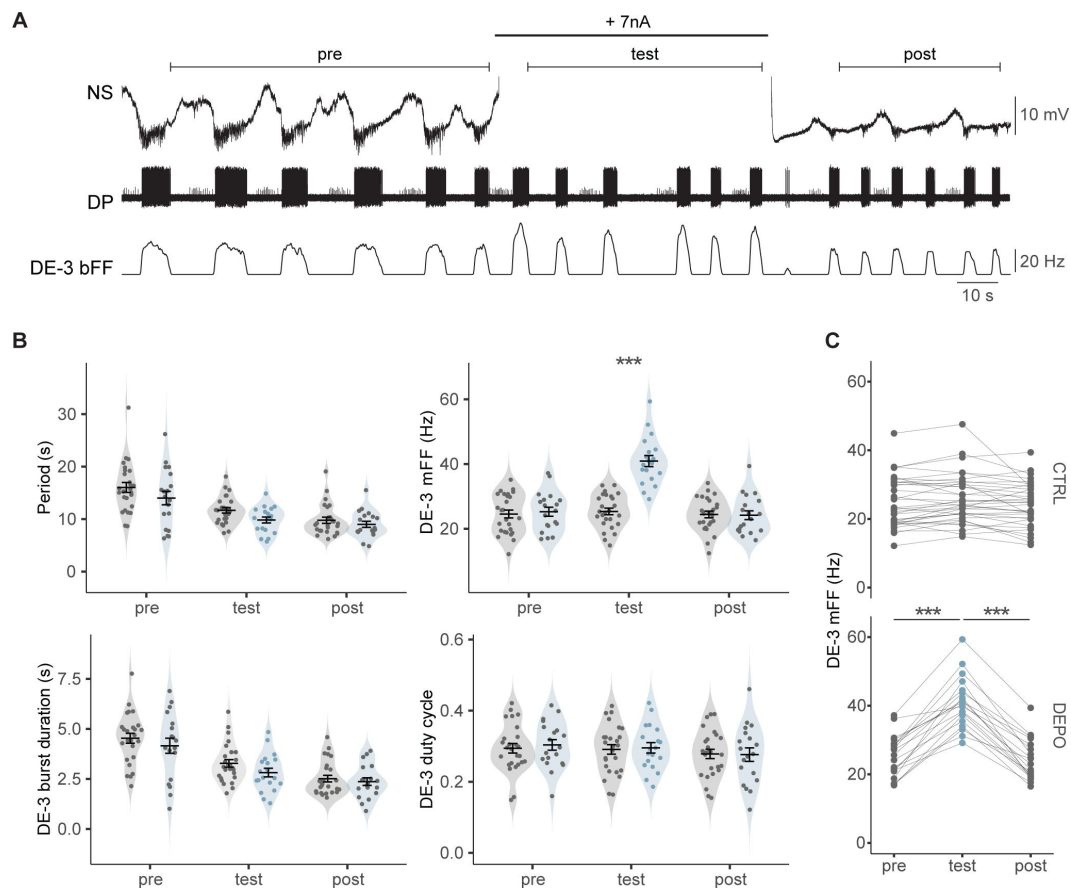


Figure 3.

Effect of NS on crawling.

A. Intracellular NS and extracellular DP recordings during a dopamine-induced crawling episode. A +7 nA pulse was injected in NS at the time indicated by the thick line. Segments above the recordings mark three epochs: previous (pre), during (test) and after (post) the pulse. **B.** Crawling period (end-to-end), DE-3 mFF, burst duration and duty cycle for cycles in the three epochs marked in A, for control experiments where no current was injected in NS (gray violins, $n = 120$ cycles from 26 episodes in 26 ganglia from 16 leeches) and for experiments where NS was depolarized (light blue violins, $n = 92$ cycles from 19 episodes in 19 ganglia from 15 leeches); each point corresponds to the mean value during the epoch for each individual experiment. Bars provide the mean $\pm$ SEM. *** $p < 0.001$ (interaction between epoch and treatment factors). **C.** Mean DE-3 mFF for the three epochs from each individual experiment for control (CTRL) and depolarizing (DEPO) tests. Lines link dots that correspond to each individual experiment. *** $p < 0.001$ (pairwise simple contrasts, Δ pre-test = -15.591 ± 1.037 Hz and Δ test-post = 16.648 ± 0.919 Hz).

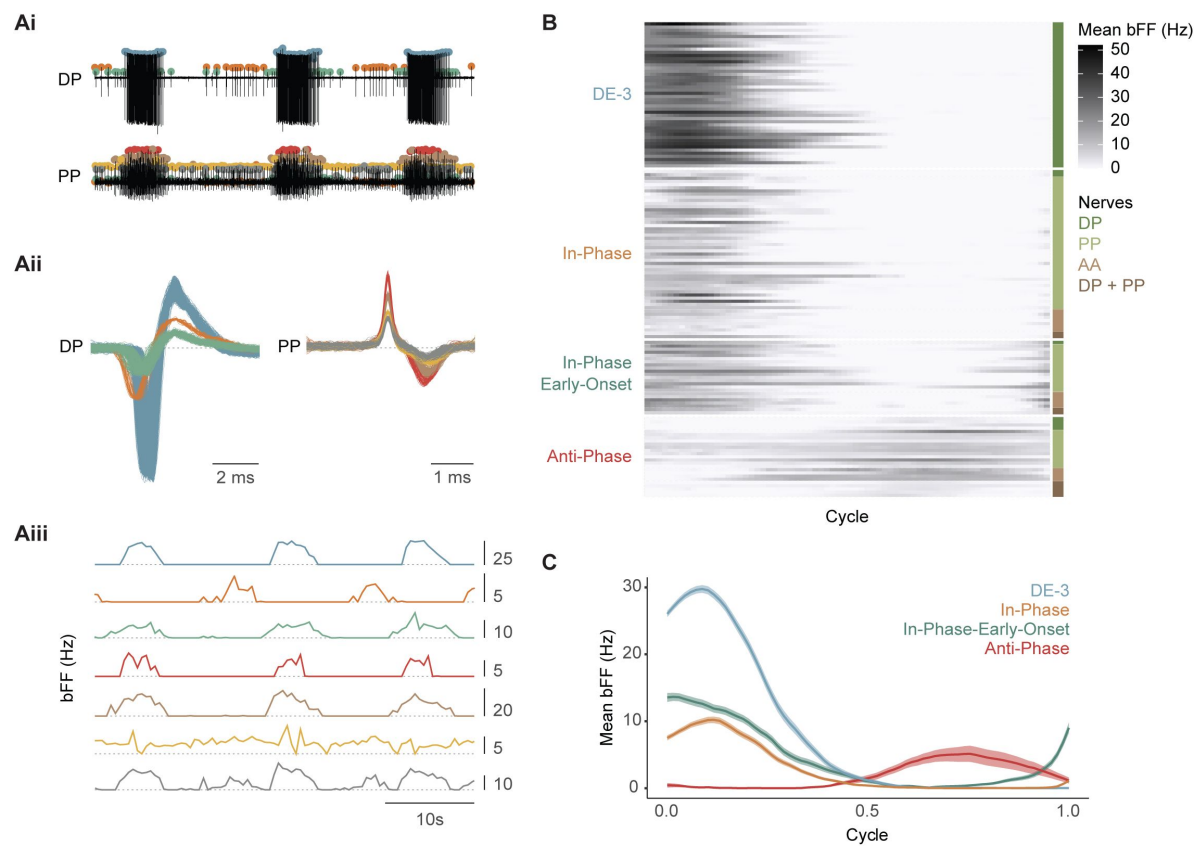


Figure 4.

Profile of motoneuron activity during crawling.

A.i. Representative example of a spike sorting analysis implemented in paired extracellular recordings of DP and PP nerves during a crawling episode. Each color dot singles out a distinct active unit along the recordings. **A.ii.** Spikes waveforms corresponding to spikes detected in the DP and PP nerves in Ai. **A.iii.** bFF calculated for each unit identified in A. The horizontal dotted lines indicate bFF = 0 Hz. Scales on the right are in Hz. **B.** Raster plot showing mean bFF along a cycle, set from the first spike of a DE-3 burst to the first spike in the next burst, obtained from 147 rhythmic units identified in DP, PP and AA nerves (n = 46 ganglia in 24 animals). The values are the mean of 4 crawling cycles for each unit. The units were classified into four groups, indicated on the left. The nerves from which the motoneuron activity was recorded are indicated on the right. The gray scale on the right indicates the bFF value in Hz. **C.** Mean bFF for all the units included in each group. The shaded area represents the standard error of the mean.

While multiple extracellular recordings have been performed previously (Eisenhart et al., 2000 [↗](#)), these results present the first quantitative analysis of motor units activated throughout the *crawling* cycle. The In-Phase units are expected to control the contraction stage by exciting or inhibiting the longitudinal or circular muscles, respectively, and the Anti-Phase units to control the elongation stage by exciting or inhibiting the circular or longitudinal muscles, respectively. It is plausible that In-Phase-Early-Onset are inhibitors of circular muscles, preparing the conditions for a fast contraction phase. Further studies should confirm this hypothesis.

Comparison of the motor pattern in single ganglia and in intact animals

The assumption that the above description of the crawling motor pattern *ex vivo*, in isolated single ganglia, represents the neural correlate of the actual behavior requires qualitative and quantitative validation. To this end intact leeches were monitored during crawling. The analysis was performed by tracking nine dots painted along the dorsal longitudinal midline of the leech, which divided the body into 8 sections (**Figure 5A** [↗](#)). Because ganglia are located in highly conserved positions relative to the length of the animal (**Figure 5B** [↗](#), Kearney et al. 2022 [↗](#)), the relative distance from the head can be used to infer which ganglia are included within the 8 sections mentioned above. We focused the analysis on sections delimited by points 4-5 and 5-6, that correspond to the range of segmental ganglia included in the *ex vivo* studies (ganglia 8 to 15) (**Figure 5A-B** [↗](#)). For electrophysiological data the frame of analysis was the cycle of DE-3 rhythmic activity and, equivalently, for the behavioral data the frame of analysis was the crawling step (see Materials and Methods).

Figure 5C [↗](#) shows a representative trace of a series of whole length waves, that were divided into discrete steps (see Materials and Methods), and the corresponding measurements in the section delimited by the dots 4 and 5. Within each crawling step, four stages were identified in the studied sections (**Figure 5C** [↗](#)): the elongation stage (marked by an increase in section length), the contraction stage (marked by a decrease in section length) and two isometric stages flanking the dynamic stages. The plot in **Figure 5D** [↗](#) shows the section length of different steps by the same animal, normalized by the step duration, and illustrates the variability of the step dynamics.

For a quantitative comparison of the *in vivo* and *ex vivo* experiments the following variables were evaluated: period of the rhythmic pattern and duty cycle of the different phases. While the average cycle period (end-to-end) recorded in isolated ganglia was of 15.22 ± 3.21 s ($n = 6$), the average duration of the leech step was of 7.49 ± 0.50 s ($n=6$). The latter value is similar to that reported in previous studies (Stern-Tomlinson et al., 1986 [↗](#); Baader and Kristan, 1992 [↗](#)). Thus, in the intact animal the rhythmic activity was twice as fast as in the isolated ganglion, suggesting that peripheral signals may accelerate the rhythmic behavior, as already described in other animals (Pulver et al., 2015 [↗](#); Fushiki et al., 2016 [↗](#)). However we cannot rule out that activation of the rhythmogenic circuit in isolated ganglia may fail to activate a circuit component responsible for speeding the rhythm.

It seems natural to correlate the activity of the Anti-Phase and In-Phase of *ex vivo* studies with the elongation and contraction stages of *in vivo* behavior. But the isometric stages displayed *in vivo* have no obvious counterpart in the electrophysiological recordings. It is important to consider that the rhythmic movement of successive segments along the antero-posterior axis of the animal requires a delay signal that allows the appropriate propagation of the metachronal wave, and this signal is probably absent in the isolated ganglion. In consequence, for the comparison of the duty cycle, the *in vivo* isometric stages were ignored, and only the duration of the dynamic fraction of the step was taken in consideration. In turn, the behavioral duty cycle was calculated as the ratio between the elongation or contraction stage duration over the dynamic step duration. Thus measured, the duty cycle of the elongation stage was 0.65 ± 0.01 and that of the contraction was

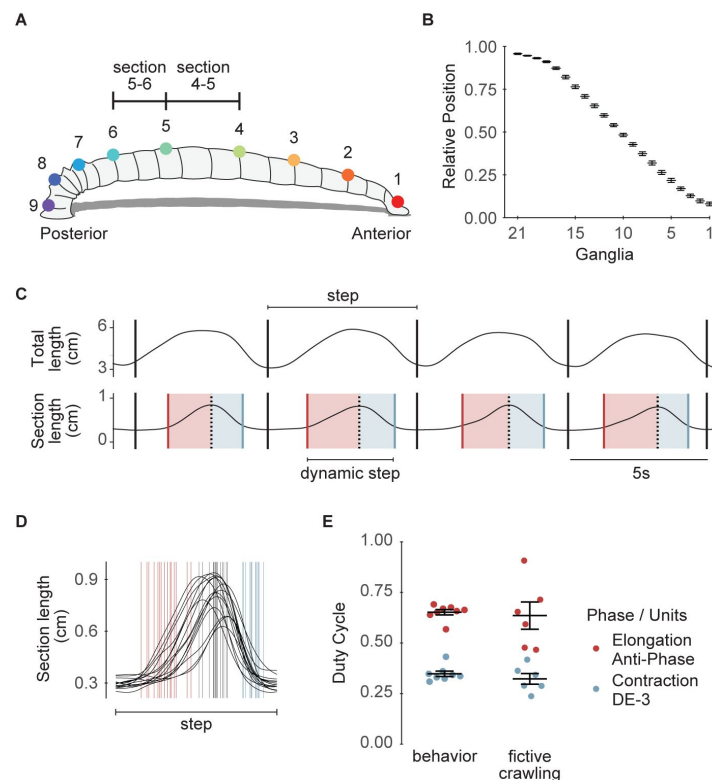


Figure 5.

Analysis of leech crawling.

A. Schematic representation of a leech with nine points as painted on their dorsal midline. The sections between points 4-5 and 5-6 comprise ganglia 8-15 that were used for the electrophysiological analysis. **B.** Relative position of midbody ganglia 1–21. Mean and standard error of the mean are shown ($n = 3$) (Kearney et al. 2022). **C.** Representative example of the changes in total length and section 4-5 of a leech during four steps as the animal was crawling. The limits of each step are indicated by black vertical lines (see Materials and Methods); red and blue vertical lines mark the start of elongation and the end of contraction, respectively; black dashed vertical lines indicate the maximal section length. Light red and blue shades indicate elongation and contraction phases, respectively. **D.** Superimposed length curves of a section across random multiple steps ($n = 15$ of 76 total steps for this animal) of one leech. **E.** Duty Cycle of the elongation and contraction phases measured in crawling behavior or in fictive crawling. Behavioral variables were calculated as the duration of each phase relative to the sum of elongation and contraction phases (dynamic step in C), excluding isometric phases. Points represent the mean for each leech, while bars and lines indicate the mean $\pm$ SEM across all leeches ($n = 249$ steps from 8 leeches). For the Duty Cycle of DE-3 and Anti-Phase units, points correspond to the mean for each ganglion, with bars and lines showing the mean $\pm$ SEM across all ganglia ($n = 4$ cycles for each DE-3 or Anti-Phase motoneuron, 6 ganglia, 6 leeches).

0.35 ± 0.01 (**Figure 5E** [↗](#)). These values are noticeably close to the duty cycle of the Anti-Phase units (0.64 ± 0.07) and of the DE-3 motoneurons (0.32 ± 0.03) (**Figure 5E** [↗](#)) measured in the isolated ganglia.

These results show that the dynamic pattern described by motoneurons recorded in isolated ganglia reflects the phase relation observed in the corresponding body section *in vivo*.

Effect of NS on the activity of the different types of motoneurons during *crawling*

The results shown in **Figure 5** [↗](#) support the view that the activity recorded in single ganglia reflects the neuronal control of the rhythmic pattern underlying crawling. Based on this premise the recording of multiple units presented above was used to evaluate the influence of NS on units active at different *crawling* stages (**Figure 6Ai** [↗](#)-ii). Does NS depolarization affect the activity of other units In-Phase with DE-3? How are Anti-Phase units affected by this experimental maneuver? The activity of the motoneurons was characterized using two metrics: maximum bFF (Max bFF) to assess activity level, and relative half width (Rel HW) to evaluate the duty cycle. To enable a comparison with the other units, the data for DE-3 was reanalyzed in this manner.

NS depolarization produced an increase in DE-3 Max bFF of around 60% (as shown above), while this variable remained stable throughout control experiments (**Figure 6B** [↗](#)). Different from DE-3, In-Phase units showed a marked decrease in the maximal bFF along time in control conditions, but similar to DE-3, this variable underwent an increase of around 50% upon NS depolarization (**Figure 6B** [↗](#)). Given the temporal drop detected in the control series this increase is probably an underestimation of the NS effect on the In-Phase firing frequency.

The Anti-Phase units also showed a marked drop in the maximum bFF along time in control conditions but in this case the effect of NS depolarization was limited to counteracting this decrease (**Figure 6B** [↗](#)). NS depolarization did not affect the maximum bFF of the In-Phase-Early-Onset units (**Figure 6B** [↗](#)).

Regarding the relative HW, the effect of NS depolarization was limited to preventing the decrease of this variable over time only for In-Phase units. It is possible that by increasing the firing frequency of the In-Phase units, the NS depolarization kept the firing span of these units at a more stable level (**Figure 6C** [↗](#)).

The statistical analyses performed for these results are detailed in **Supplementary Table 2** [↗](#).

In summary, the rhythmic NS hyperpolarizations selectively limited the activity of motoneurons that are active in phase with its inhibitory inputs, specifically those controlling the contraction phase. This selective effect highlights the role of NS in modulating specific phases of the motor pattern without disrupting the overall coordination of elongation and contraction phases. It is worth noting that the fact that the firing frequency of Anti-Phase MNs was not affected by NS depolarization indicates that this experimental manipulation did not cause a generalized change in the activity of MNs but achieved the desired goal of temporarily removing the rhythmic NS hyperpolarization. On the other hand, the fact that In-Phase-Early-Onset activity was also unaffected by NS depolarization confirms that these motoneurons constitute a distinct group from the In-Phase units. Furthermore, this suggests that these units might be inhibitory motoneurons that are not connected to the NS via the recurrent inhibitory circuit (Rela and Szczupak, 2003 [↗](#); Wadeuhl, 1989 [↗](#)).

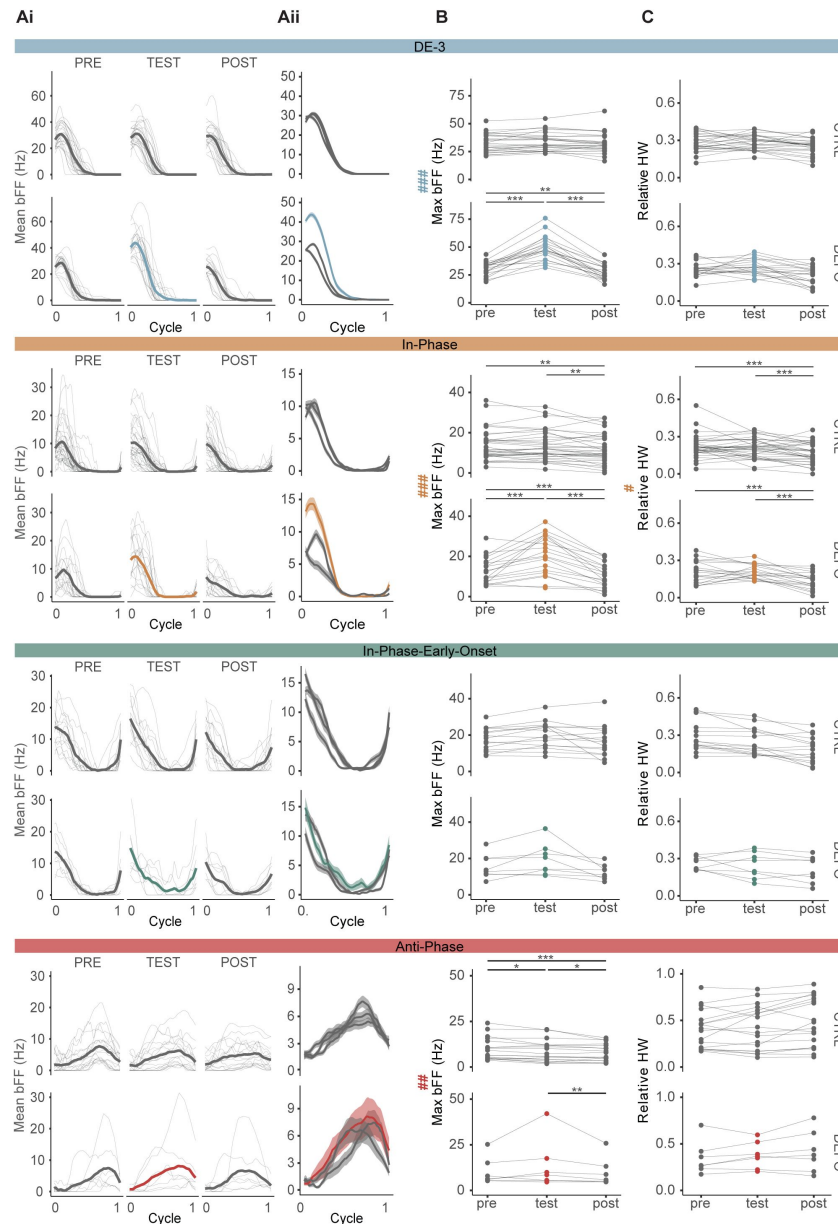


Figure 6.

Effect of NS on the different phases of crawling.

A.i. Mean bFF along cycles in pre, test, and post epochs for each unit within the four groups described in [Figure 4](#). For each unit type the upper line of graphs presents control experiments, where no current was injected in NS, and the bottom line, depolarization experiments, where 5-7 nA current steps were injected in NS during the test epoch. Thin lines show the mean bFF (including 4 - 6 cycles) for each experiment, and thick lines show the overall mean. **A.ii.** Superimposition of the overall bFF mean of the three epochs. The shaded area presents the standard error of the mean. **B & C.** Maximum bFF (Max bFF) and Relative Half Width (Relative HW) measured in each individual unit. A-C are plotted for each of the four groups of units indicated. Lines connect the mean values across each epoch within the same experiment. Mixed-effects models with two fixed factors were performed; on the left of the B and C panel, # indicates a significant interaction between treatment and epoch factors; * indicates significant differences for pairwise simple contrasts between epochs, within each treatment. *, **, and *** (or #) indicate p < 0.05, p < 0.01 and p < 0.001, respectively. [Supplementary Table 2](#) describes the statistical models performed. For DE-3, n = 26 control and 20 depolarization ganglia, from 46 ganglia; for In-Phase, n = 38 units for control and 21 units for depolarization, from 30 ganglia; for Anti-Phase, n = 18 units for control and 7 units for depolarization experiments, from 22 ganglia; and for In-Phase-Early-Onset, n = 15 units for control and 8 units for depolarization experiments, from 23 ganglia.

Discussion

The present study shows that phase specific inhibitory signals, delivered by the functional homolog of Renshaw cells in the leech (Szczupak, 2014), modulate the degree of excitation of the motoneurons responsible for the contraction phase of *crawling*. This effect was phase specific, as motoneurons active in the rest of the cycle were not affected. NS is a premotor neuron that operates at the center of a recurrent inhibitory circuit (Rela and Szczupak, 2003; Rodriguez et al., 2009) and thus the initial hypothesis suggested that the effect on *crawling* was mediated entirely by this circuit. However, the results show that the inhibitory signal did not originate at the level of the motoneurons themselves but, most probably, at the crawling oscillator. The marked temporal correlation between the neurons active during the contraction phase and NS hyperpolarizations suggests that the excitatory signals onto the motoneurons and the hyperpolarizing signals onto NS were driven by the same upstream element (e.g. the element in the oscillator that controls the contraction phase). This result further feeds the homology between vertebrate Renshaw cells and leech NS neurons in that the formers are not only targeted by motoneuron inputs but by rhythmogenic premotor elements as well (Nishimaru et al., 2006). Nonetheless, the results do not rule out that the existing recurrent inhibitory circuit contributes to the hyperpolarization of NS.

In summary, we propose that the crawling oscillator releases a dual signal onto the motoneurons that control the contraction phase: a direct excitatory input that elicits the rhythmic bursts and a concurrent inhibitory input via NS (**Figure 7**). Coherent with this interpretation, the activity level of the Anti-Phase units was not influenced by these inhibitory signals.

The corroboration of a dual counteracting set of actions on motoneuron output calls for a functional interpretation. In a characterization of the electrophysiological properties of leech motoneurons it was shown that DE-3 can reach a firing frequency that exceeds 100 Hz (Perez-Etchegoyen et al., 2011) while the maximal muscle tension that this motoneuron can evoke is achieved at around 25 Hz (Mason and Kristan, 1982). Additionally, it is relevant to consider that the population of motoneurons that activate longitudinal muscles is linked by ohmic coupling that can result in mutual excitation (Fan et al., 2005; Ort et al., 1974; Ashaber et al., 2021). As a result, when all the activators of longitudinal muscles are excited during the contraction phase, the excitatory spread can lead to runaway motoneuron activity (Hennequin et al., 2017). An inhibitory signal imposed on NS can spread to all the motoneurons simultaneously (Rela and Szczupak, 2003; Rodriguez et al., 2009; Wadepuhl, 1989) and thus limit the activity of the whole motoneuron population.

Previous work showed that leech motoneurons are subjected to a generalized inhibition mediated by GABA (Baca et al., 2008) while the electrically mediated inhibition exerted by NS shown here is phase-specific. The existence of concurrent excitation and inhibition has been revealed in vertebrate motor networks, where it was proposed as a balancing factor (Kishore et al., 2014; Parkis et al., 1999; Petersen et al., 2014). In line with the present results, experiments performed in zebrafish showed that in-phase inhibition originates from a set of premotor neurons that comprise the Renshaw cells (Callahan et al., 2019). Neurophysiological studies in the leech offer the particular advantage of allowing electrical manipulation of individual neurons in wild-type adults, where they can be readily recognized by their anatomical and electrophysiological characteristics. In this sense the present study offers clear-cut evidence of the dual signal motoneurons receive in the course of motor control from an identified premotor element.

The results further highlight the notion that knowing the connectivity among neurons is not sufficient to deduce its functional operation, as the dynamical interplay among them is highly relevant to understand its physiological role.

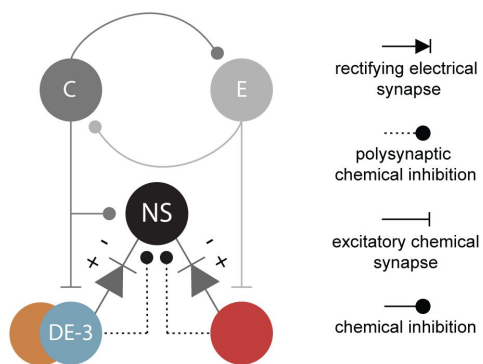


Figure 7.

NS in the crawling circuitry.

Putative neuronal circuitry as described in **Figure 1C**, including the connection between the oscillatory circuit and NS.

Structure of *crawling* based on MN activity and behavior

Evaluation of phase specificity required a more comprehensive tracking of motoneuron activity throughout the cycle and we addressed this demand through the analysis of the activity of multiple motoneurons. In the present study we show that in the course of *crawling* DE-3 neurons fire concurrently with neurons that reach the periphery mainly through the PP and the AA roots. Based on the classical description of Ort and collaborators (1974) the spikes in phase with DE-3 recorded in the PP root are likely to correspond to VE-4 (ventral-excitor 4), DE-5, DE-7 and VE-8, while those recorded in AA are likely to correspond to DE-107 and VE-108.

To learn how the motoneuron readout compares with the rhythmic body movements that take place in crawling we presented behavioral studies that focused on the sections of the leech body that match the range of ganglia used in the electrophysiological studies. The comparison shows two main differences. The period of the rhythmic animal behavior was half that measured in the fictive behavior at the isolated ganglia. In addition, at the behavioral level the actual elongation-contraction gestures were flanked by isometric stages, while in dopamine-induced *crawling* studied in isolated ganglia the rhythmic activity of the motoneurons appears as the alternation between units In-Phase with DE-3 and Anti-Phase of DE-3, with no clear references for isometric phases. The absence of descending brain signals and/or peripheral signals are assumed as important factors in determining the cycle period and the sequence at which the different behavioral stages take place. However, it is noteworthy that taking the isometric stages aside, the sequence of events, and the proportion of the active cycle dedicated to elongation and contraction were remarkably similar in both experimental settings. This suggests that the network activated in the isolated ganglion is the one underlying the motor behavior.

Materials and methods

Biological Preparation

Leeches (*Hirudo* sp.), weighing 1–3 g, were sourced from commercial suppliers (Niagara Leeches, Cheyenne, WY, United States and Biopharm, UK) and kept at 20°C in artificial pond water. These animals are hermaphrodites.

Studies were conducted in isolated single ganglia obtained from segments 8th to 13th. The ganglia were dissected with one dorsal posterior (DP), posterior (PP) and anterior (AA) nerve roots attached. The tissue was bathed in normal saline (in mM: 115 NaCl, 4 KCl, 1.8 CaCl₂, 1 MgSO₄, 10 Hepes, 10 glucose; pH 7.4) at room temperature (20–25°C) and pinned to Sylgard (Dow Corning) in a recording chamber. The sheath covering the ganglion was dissected away, exposing the neuronal cell bodies to the external solution.

Electrophysiological Recordings

Intracellular somatic recordings were conducted using microelectrodes pulled from borosilicate capillary tubing (A-M Systems, Inc., Carlsborg, WA, United States), filled with 3 M potassium acetate (resistance 20–40 MΩ). The electrodes were connected to an Axoclamp 2B amplifier (Axon Instruments; Union City, CA, United States) operating in bridge mode. Extracellular activity from DP, PP, and AA nerves was recorded using suction electrodes connected to a differential a.c. amplifier (Neuroprobe 1700, A-M Systems, Inc., Carlsborg, WA, United States). Intra- and extracellular recordings were digitized through an analog-digital converter (Digidata 1440, Axon Instruments, Union City, CA, United States) and acquired with Clampex 9.2 (Axon Instruments, Union City, CA, United States) at a sampling rate of 20 kHz. Premotor NS neurons were identified by their soma location and electrophysiological properties (Rela et al., 2009 [DOI](#)).

To induce fictive crawling (*crawling*), ganglia were superfused with 75 μ M dopamine hydrochloride (Sigma-Aldrich, St. Louis, MO, United States), freshly prepared at the beginning of each experimental day (Puhl and Mesce, 2008). Only one episode was evoked per ganglion. The rhythmic motor pattern was monitored via extracellular recording of the DP nerve, where the largest spike corresponds to the DE-3 motoneuron, one of the motoneurons innervating the longitudinal muscles (Ort et al., 1974).

When testing the effect of NS neurons, depolarizing pulses of +5 nA to +7 nA were injected into its soma through the intracellular electrode. Between four and five *crawling* cycles before and after a depolarizing pulse were left unaffected to obtain control pre and post rhythmic epochs.

Behavioral Experiments

Leeches were anesthetized by placing individuals at -20°C for 5 to 10 minutes. Then the skin of the animal was dried, and nine points of water-based paint were applied along its dorsal longitudinal midline (Figure 5A). When the animals recovered they were allowed to crawl onto a graph paper sheet covered with a transparent plastic surface. Crawling was filmed at 60 fps with a digital camera (Sony ILCE-A6400) supported above the arena. The position of each point painted on the leech skin was tracked using DeepLabCut version 2.3.5 (He et al., 2016; Insafutdinov et al., 2016; Mathis et al., 2018; Nath et al., 2019). To this end, 338 frames randomly selected from 66 videos of 8 leeches were used for manually labeling the painted dots, and used for training a ResNet_50-based neural network for automating the detection.

To characterize crawling, steps were defined by the frames in which the algorithmic sum of the following values was at a minimum: 1) the whole length of the leech, 2) the velocity of the most anterior dot, and 3) the velocity of the most posterior dot (Figure 5C). Steps where DeepLabCut failed to detect any of the nine points on the animal were discarded. After dividing the animal displacement into steps, we performed a quantitative analysis of the changes in the length of the animal sections (defined between each pair of points) over time. Specifically, the sections delimited by the painted points 4-5 and 5-6 were chosen since they include the range of ganglia considered in the electrophysiological studies (8-15). The length traces of each leech were filtered using a Gaussian filter (sigma of 0.25 s). For each step, the duration of the elongation and contraction phases within a section was calculated by using the maximum length as the boundary between these phases. The onset of elongation and the end of contraction were determined by detecting knee points, as described by Satopaa et al. (2011, Figure 5C).

Spike Sorting

Extracellular recordings were spike sorted using a custom-written algorithm which relies on a template matching-based method based on (Pouzat et al., 2002), incorporating a t-SNE model (Pedregosa et al., 2011; Van der Maaten and Hinton, 2008). Because spike sorting is sensitive to misclassification, the resulting output was manually curated to address whether a single cluster of waveforms was self-consistent with a single neuron (Hill et al., 2011). During manual curation, all clusters of putative spike events were evaluated. Clusters containing two well defined groups of waveforms were split and pairs of clusters with similar waveforms and temporally coordinated activity were merged. Clusters that had inconsistent waveforms and could not be split into clusters with well-defined waveforms were discarded. After this procedure, each cluster was considered a single motor unit.

To select for rhythmic units, autocorrelations were performed considering the first four cycles of the fictive behavior. Units whose autocorrelogram included at least 2 peaks with a correlation coefficient greater than 0.25 were considered rhythmic.

Data Processing

DE-3 spike times were derived from DP nerve recordings using the spike sorting algorithm mentioned above. DE-3 bursts were considered as such if they comprised at least 10 spikes with a mean firing frequency of 8 Hz, where the separation between two subsequent spikes occurred with a maximum separation of 0.5 seconds. Burst duration was calculated as the time difference between the last and first DE-3 spikes in each burst. Cycle period was calculated in two different ways: as the time difference between the last (end-to-end), or the first spikes (start-to-start) of subsequent DE-3 bursts, and is reported accordingly. Duty cycle was calculated as the ratio between burst duration and period. The mean firing frequency (mFF) of each burst was calculated as the number of spikes over the burst duration. The binned firing frequency (bFF) of DE-3, or any other unit, was obtained by binning the number of spikes along the recording using a 0.05 s or a 0.5 s interval, for **Figure 2** and **Figure 6**, respectively. For cross-correlation analysis, the DE-3 bFF was filtered using a Gaussian filter (sigma of 150 ms).

For the cross-correlation between NS membrane potential and DE-3 firing frequency, the intracellular recording of NS was resampled to match the data rate of DE-3 bFF. To determine the amplitude of NS hyperpolarization, a baseline membrane potential was established using a Gaussian filter (sigma of 5 s) applied to the entire trace. The amplitude was calculated as the difference between the baseline value at the start of the DE-3 burst and the minimum membrane potential reached by NS during the corresponding burst (see **Figure 2A**).

When NS depolarization was implemented, the cycles including the onset or offset of the current pulse were excluded, and the traces were divided into three epochs: pre, test, and post. Control experiments were treated similarly.

When analyzing the activity of the units obtained by spike sorting, the maximum bFF value and relative half width were obtained from curves displaying bFF versus cycle-phase (time normalized to the period of each cycle). The relative half width was calculated as the width at half the maximum bFF value.

Data processing was performed using custom written Python scripts.

Statistical Analysis

All statistical analyses were performed using R (R Core Team, 2020). For every analysis, the structure of random factors was kept maximal whenever possible. The specific random factors considered in each case are detailed in **Supplementary Tables 1** and **2**.

To analyze the correlation between the amplitude of NS hyperpolarization and different *crawling* features (**Figure 2D-G**), the `lmer` function from the `lmerTest` package (Kuznetsova et al., 2017) was used to perform linear mixed models (LMM) with the experiment as a random factor.

To analyze the effect of NS depolarization on motoneuron activity, the data was analyzed using mixed-effects models with two fixed factors: epoch (pre, test, post) and treatment (control, depolarization). All models included motor units as a random factor. Each ganglion was also included as a random factor. The effect of NS depolarization was analyzed with the `glmmTMB` function from the `glmmTMB` package (Brooks et al., 2017) to perform a generalized linear mixed model (GLMM) or with the `lmer` function to perform a LMM. For the GLMM, we used a student-t distribution (`t_family`) with the identity link or Gamma family with a log link as indicated in **Supplementary Tables 1** and **2**. The significance of a factor was determined with a likelihood-ratio test for the GLMM or an F-test for the LMM. This was done by comparing the

model including the factor with a model dropping that factor. Pairwise simple contrasts were performed within the treatment factor and between the epoch factor using the *emmeans* package and the Tukey correction were applied to obtain the p values.

To analyze a possible temporal drift on *crawling* features we considered the data of control experiments and used mixed-effects models with epoch (pre, test, post) as a fixed factor and units as a random factor. **Supplementary Tables 1** [↗](#) and **2** [↗](#) specifies the models used for each analysis.

Supplementary tables

Effect of NS depolarization on <i>crawling</i> features. (n units = 26 ctrl, 19 depo / n leeches = 16 ctrl, 15 depo).					
Variable	Model, random effects	Significance of factor interactions	Pairwise simple contrasts		
			Comparison	Ratio	p value
DE-3 FF	LMM intercept - unit slope - epoch	<.0001	ctrl depo / pre post / pre post / depo	1.04 1 0.96	0.6345 0.9815 0.4654
			depo depo / pre post / pre post / depo	1.62 0.96 0.59	<.0001 0.3909 <.0001
DE-3 burst duration	GLMM - Gamma family, log link intercept - unit slope - epoch	0.3077	-	-	-
period	GLMM - Gamma family, log link intercept - units	0.2107	-	-	-
DE-3 duty cycle	LMM intercept - unit slope - epoch	0.8309	-	-	-
Effect of temporal drift on <i>crawling</i> features. (n units = 26 ctrl, 19 depo / n leeches = 16 ctrl, 15 depo)					
Variable	Model, random effects	Significance of factor	Fixed effects		
			Epoch	Estimate	p value
DE-3 burst duration	GLMM - Gamma family, log link intercept - unit	<.0001	pre (intercept) depo post	4.40 3.16 2.44	<.0001 <.0001 <.0001
period	GLMM - Gamma family, log link intercept - unit	<.0001	pre (intercept) depo post	15.45 11.45 9.47	<.0001 <.0001 <.0001
DE-3 duty cycle	LMM intercept - unit	0.1623	-	-	-

Supplementary Table 1.

Statistical Models Performed for the Effect Analysis of NS depolarization on *crawling* features and DE-3 activity during the fictive behavior.

Variable	Model, random effects	Significance of factor interactions	Pairwise simple contrasts		
			Comparison	Ratio	p value
Effect of NS depolarization on motoneuron activity during <i>crawling</i> .					
DE-3 (n units = 26 ctrl, 20 depo / n cycles = 110 ctrl, 99 depo / n leeches = 16 ctrl, 16 depo)					
Max FF	LMM intercept - unit slope - treatment and epoch	<.0001	ctrl depo / pre post / pre post / depo depo depo / pre post / pre post / depo	1.04 1 0.96 1.59 0.89 0.56	0.5512 0.9995 0.5406 <.0001 0.0066 <.0001
Rel HW	LMM intercept - unit slope - treatment and epoch	0.1596	-	-	-
In-Phase (n units = 34 ctrl, 19 depo / n cycles = 149 ctrl, 93 depo / n experiments = 17 ctrl, 11 depo / n leeches = 13 ctrl, 8 depo)					
Max FF	GLMM - t_family, identity link intercept - experiment/unit	<.0001	ctrl depo / pre post / pre post / depo depo depo / pre post / pre post / depo	1.01 0.92 0.91 1.52 0.86 0.56	0.9628 0.0046 0.0014 <.0001 0.0005 <.0001
Rel HW	GLMM - t_family, identity link intercept - experiment/unit	0.01492	ctrl depo / pre post / pre post / depo depo depo / pre post / pre post / depo	0.93 0.78 0.84 0.99 0.60 0.61	0.1788 <.0001 0.0007 0.9840 <.0001 <.0001
Anti-Phase (n units = 18 ctrl, 7 depo / n cycles = 78 ctrl, 34 depo / n experiments = 15 ctrl, 7 depo / n leeches = 12 ctrl, 7 depo)					
Max FF	GLMM - t_family, identity link intercept - experiment/unit	0.00148	ctrl depo / pre post / pre post / depo depo depo / pre post / pre post / depo	0.91 0.82 0.90 1.10 0.94 0.85	0.0104 <.0001 0.0180 0.0505 0.4132 0.0025
Rel HW	GLMM - t_family, identity link intercept - experiment/unit	0.9791	-	-	-
In-Phase-Early-Onset (n units = 15 ctrl, 8 depo / n cycles = 66 ctrl, 45 depo / n experiments = 12 ctrl, 5 depo / n leeches = 11 ctrl, 5 depo)					
Max FF	LMM intercept - experiment/unit	0.0891	-	-	-
Rel HW	GLMM - t_family, identity link intercept - experiment/unit	0.173	-	-	-

Supplementary Table 2.

Statistical Models Performed for the Effect Analysis of NS depolarization on different groups of units activity during *crawling*.

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Reviewer #1 (Public review):

Summary:

The Szczupak lab published a very interesting paper in 2012 (Rodriquez et al. J Neurophysiol 107:1917-1924) on the effects of the segmentally-distributed non-spiking (NS) cell on crawl-related motoneurons. As far as I can tell, the working model presented in 2012, for how the non-spiking (NS) cell impacts the crawling motor pattern, is the same functional model presented in this new paper. Unfortunately, the Discussion does not address any of the findings in the previous paper or cite them in the context of NS alterations of fictive crawling. Aside from different-looking figures and some new analyses, the results and conclusions are the same.

Strengths:

The figures are well illustrated.

Weaknesses:

The paper is a mix of what appears to be two different studies and abruptly switches gears to examine how closely the crawl patterning is in the intact animal as compared to the fictive crawl patterning in the intact animal. Unfortunately, previous studies in other labs are not cited even though identical results have been obtained and similar conclusions were made. Thus, the novelty of the results is missing for those who are familiar with the leech preparation. The lack of appropriate citations and discussion of previous studies also deprives the scientific community of fully comprehending the impact of the data presented and the science it was built upon.

(1) Results, Lines 167-170: "While multiple extracellular recordings have been performed previously (Eisenhart et al., 2000), these results present the first quantitative analysis of motor units activated throughout the crawling cycle. The In-Phase units are expected to control the contraction stage by exciting or inhibiting the longitudinal or circular muscles, respectively, and the Anti-Phase units to control the elongation stage by exciting or inhibiting the circular or longitudinal muscles, respectively."

The first line above is misleading. The study by Puhl and Mesce (2008, J. Neurosci, 28:4192-420) contains a comprehensive analysis of the motoneurons active during fictive crawling with the aim of characterizing their roles and phase relationships and solidifying the idea that the oscillator for crawling resides in a single ganglion. Intracellular recordings from a number of key crawl-related motoneurons were made in combination with extracellular recordings of motoneuron DE-3, a key monitor of crawling. In their paper, it was shown that motoneurons AE, VE-4, DI-1, VI-2, and CV were all correlated with crawl activity, and fired repeatedly either in phase or out-of-phase with DE-3. They were shown to be either excitatory or inhibitory.

At a minimum, the above paper should be cited. The submitted paper would be strengthened if some of these previously identified motoneurons were again recorded with intracellular electrodes and concomitant NS cell stimulation. The power of the leech preparation is that cells can be identified as individuals with dual somatic (intracellular) and axonal recordings (extracellular). The shortfall of this aspect of the study (Figure 5) is that the extracellular units have not been identified here. In fact, these units might not even be motoneurons. They could represent activity from the centrally located sensory neurons, dopamine-modulated afferent

neurons or peripherally projecting modulatory neurons. Essentially, they may not have much to do with the crawl motor pattern at all.

(2) Results Lines 206-210: "with the elongation and contraction stages of in vivo behavior. However the isometric stages displayed in vivo have no obvious counterpart in the electrophysiological recordings. It is important to consider that the rhythmic movement of successive segments along the antero-posterior axis of the animal requires a delay signal that allows the appropriate propagation of the metachronal wave, and this signal is probably absent in the isolated ganglion."

The so-called isometric stages, indeed, have an electrophysiological counterpart due in part to the overlapping activities across segments. This submitted paper would be considerably strengthened if it referred to the body of work that has examined how the individual crawl oscillators operate in a fully intact nerve cord, excised from the body but with all the ganglia (and cephalic ganglion) attached. Puhl and Mesce 2010 (J. Neurosci 30: 2373-2383) and Puhl et al. 2012 (J. Neurosci, 32:17646 -17657) have shown that "appropriate propagation of the metachronal wave" requires the brain, especially cell R3b-1. They also show that the long-distance projecting cell R3b-1 synapses with the CV motoneuron, providing rhythmic excitatory input to it.

For this and other reasons, the paper would be much more informative and exciting if the impacts of the NS cell were studied in a fully intact nerve cord. Those studies have never been done, and it would be exciting to see how and if the effects of NS cell manipulation deviated from those in the single ganglion.

(3) Discussion Lines 322-324. "The absence of descending brain signals and/or peripheral signals are assumed as important factors in determining the cycle period and the sequence at which the different behavioral stages take place."

The authors could strengthen their paper by including a more complete picture of what is known about the control of crawling. For example, Puhl et al. 2012 (J Neurosci, 32:17646-17657) demonstrated that the descending brain neuron R3b-1 plays a major role in establishing the crawl-cycle frequency. With increased R3b-1 cell stimulation, DE-3 periods substantially shortened throughout the entire nerve cord. Thus, the importance of descending brain inputs should not be merely assumed; empirical evidence exists.

(4) Discussion Lines 325-327: "the sequence of events, and the proportion of the active cycle dedicated to elongation and contraction were remarkably similar in both experimental settings. This suggests that the network activated in the isolated ganglion is the one underlying the motor behavior."

The results and conclusions drawn in the current manuscript mirror those previously reported by Puhl and Mesce (2008, J. Neurosci, 28:4192- 420) who first demonstrated that the essential pattern-generating elements for leech crawling were contained in each of the segmental ganglia comprising the nerve cord. Furthermore, the authors showed that the duty cycle of DE-3, in a single ganglion treated with dopamine, was statistically indistinguishable from the DE-3 duty cycle measured in an intact nerve cord showing spontaneous fictive crawling, in an intact nerve cord induced to crawl via dopamine, and in the intact behaving animal. What was statistically significant, however, was that the DE-3 burst period was greatly reduced in the intact animal (i.e., a higher crawl frequency), which was replicated in the submitted paper.

In my opinion, the novelty of the results reported in the submitted manuscript is diminished in the light of previously published studies. At a minimum, the previous studies should be cited, and the authors should provide additional rationale for conducting their studies. They need to explain in the discussion how their approach provided additional insights into what has already been reported.

Reviewer #2 (Public review):

The paper is well-written overall. The findings are clearly presented, and the data seems solid overall. I do have, however, a few major and some minor comments representing some concerns. My major comments are below.

(1) This may seem somewhat semantic, yet, it has implications on the way the data is presented and moreover on the conclusions drawn - a single ganglion cannot show fictive crawling. It can demonstrate rhythmic patterns of activity that may serve in the (fictive) crawling motor pattern. The latter is a result of the intrinsic within single-ganglion connectivity AND the inter-ganglia connections and interactions (coupling) among the sequential ganglia. It may be affected by both short-range and long-range connections (e.g., descending inputs) along the ganglia chain.

(2) The point above is even more critical where the authors set to compare the motor pattern in single ganglia with the intact animals. It would have made much more sense to add a description of the motor pattern of a chain of interconnected ganglia. The latter would be expected to better resemble the intact animal. Furthermore, this project would have benefitted from a three-way comparison (isolated ganglion-interconnected ganglia-intact animal).

(3) Two previous studies by the same group are repeatedly mentioned (Rela and Szczupak, 2003; Rodriguez et al., 2009) and serve as a basis for the current work. The aim of one of these previous studies was to assess the role of the NS neurons in regulating the function of motor networks. The other (Rodriguez et al., 2009) reported on a neuron (the NS) that can regulate the crawling motor pattern. LL 71-74 of the current report presents the aim of this study as evaluating the role of the known connectivity of the premotor NS neuron in shaping the crawling motor pattern. The authors should make it very clear what indeed served as background knowledge, what exactly was known about the circuitry beforehand, and what is different and new in the current study.

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