Full Title: **Phase-specific premotor inhibition modulates leech rhythmic motor output**

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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 single 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 (analogous to that formed by vertebrate Renshaw cells), shapes the leech crawling motor pattern. The study included a quantitative analysis of putative 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. To ensure smooth movements, diverse regulatory mechanisms operate at different hierarchical levels within the motor system, narrowing down the degrees of freedom of a highly distributed system with multiple units. The overall functional organization and operational logic of motor systems, comprising the components at all levels of the hierarchy and their feedforward and feedback interactions, remain, 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); this includes 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. Note that in the context of this manuscript fictive crawling refers to the rhythm generation but does not include the connectivity that rules intersegmental interactions and the consequent intersegmental lags.

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 functionally analogous to mammalian Renshaw cells (Szczupak, 2014). These spinal cord cells deliver inhibitory signals to the motoneurons in proportion to the motoneuron activity, forming an activity-dependent feedback mechanism that regulates motoneuron output (Alvarez and Fyffe, 2007).

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 Szczupak, 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 could operate as modulators of motoneuron activity. A previous study (Rodriguez et al., 2012) indicated that NS membrane potential oscillates in tune with the *crawling* motor pattern and that this premotor neuron can indeed modulate motor activity. In that study *crawling* was monitored by the recording of a particular motoneuron, the dorsal excitor cell 3 (DE-3). However, the wide connectivity of NS with several excitatory motoneurons that fire during *crawling*, called for a more comprehensive readout of the motor pattern.

The present study aimed to evaluate the influence of the premotor NS neuron across the different stages of *crawling*. For this purpose, we describe the pattern of activity of different neurons during *crawling* which were simultaneously monitored through extracellular nerve recordings while manipulating the NS membrane potential. The study reveals that the premotor neuron mediated a reduction of the firing frequency of motoneurons recruited during a specific phase of *crawling*.

88 **RESULTS**

89 **NS membrane potential oscillations during *crawling***

90 To study the role that premotor NS neurons play in fictive crawling (*crawling)* we first analyzed
91 the correspondence between NS electrophysiological activity and the motoneuron output during
92 this motor pattern. Since motoneuron DE-3 (dorsal excitor cell 3), which innervates the
93 longitudinal dorsal muscle fibers, is the most robust proxy of *crawling* (Puhl and Mesce, 2008),
94 extracellular recordings of this motoneuron in the DP nerve were combined with NS intracellular
95 recordings. As shown previously (Rodriguez et al., 2012), the NS membrane potential showed
96 rhythmic oscillations tuned to DE-3 activity, with hyperpolarizations occurring in conjunction
97 with DE-3 bursts and depolarizations during DE-3 silent phases (Figure 2A). Here, a cross-
98 correlation analysis between NS and DE-3 firing frequency revealed a marked anticorrelation,
99 with a cross-correlation index of -0.61 ± 0.03 (mean ± SEM) at a lag close to 0 sec (0.24 ± 0.10
100 s) (Figure 2B). This result indicates that NS hyperpolarization takes place in phase with DE-3
101 bursts.

102 Within the framework of the recurrent inhibitory circuit, NS is the target of inhibitory
103 signals evoked by excitatory motoneurons via chemically-mediated polysynaptic pathways (Rela
104 and Szczupak, 2003, Figure 1C). Thus, it was hypothesized that the NS hyperpolarizations
105 observed during *crawling* resulted from inputs originating in DE-3 and other motoneurons that
106 fire in phase with it (Rodriguez et al., 2012). If this hypothesis was correct, the onset of DE-3
107 bursts should precede the onset of NS hyperpolarization. Because the cross-correlation was
108 performed on binned DE-3 firing frequency curves, the peak lag reported does not offer a proper
109 estimation of the relative timing between the two signals. To gain a more precise assessment, NS
110 intracellular recordings were segmented into individual cycles and aligned with the first spike of

111 the corresponding DE-3 burst (Figure 2C). As shown in the figure, because of the NS membrane
112 potential oscillations it is not possible to establish a sharp onset for the NS hyperpolarizing
113 response. However, the example illustrates that the onset of the DE-3 bursts occurred after NS
114 hyperpolarization was already initiated. These findings rule out the possibility that the NS
115 hyperpolarization was originated by DE-3 bursting. Instead, they suggest that NS and DE-3
116 received concomitant rhythmic inputs of opposite sign—that is, hyperpolarizing inputs in NS and
117 depolarizing ones in DE-3.

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

130 These results indicate that, in addition to the known recurrent inhibitory circuit between
131 NS and motoneurons, NS neurons receive an inhibitory signal during *crawling*, which is in phase
132 with DE-3 activity. This inhibitory signal seems to have originated from the same source that
133 activates DE-3 motoneurons. In that case, it seems puzzling to find a lack of correlation between

134 NS hyperpolarization amplitude and DE-3 firing frequency. However, this observation might be
135 explained by considering that DE-3 is subjected to two counteracting effects at the same time: an
136 excitatory input from the oscillator unit C and an inhibitory input from NS via the rectifying
137 electrical junctions (Figure 1C).
138
139 **Effect of NS on DE-3 activity during *crawling***
140 In a previous study (Rodriguez et al., 2012) we showed that hyperpolarization of NS during
141 *crawling* reduced the firing frequency of DE-3, suggesting that the premotor neuron preserved
142 the influence that exhibits in basal conditions during the rhythmic motor pattern (Rodriguez et
143 al., 2009). Here we aimed at testing whether the inhibitory signal that NS receives during
144 crawling is indeed transmitted to the motoneurons. For this purpose the premotor neuron was
145 transiently removed from the circuit. In line with the properties of the rectifying electrical
146 synapse between NS and the motoneurons, depolarizing NS neurons is equivalent to
147 disconnecting them from the network (Rela and Szczupak, 2007). The work by Rodriguez et al.
148 (2012) showed that NS depolarization increased DE-3 firing frequency but the study lacked
149 appropriate controls that we carried out here.
150 Depolarizing steps applied in NS neurons in the course of *crawling* episodes (Figure 3A)
151 shifted their membrane potential beyond +100 mV, well above the average membrane potential
152 of the motoneurons. An important control introduced here evaluate potential drifts in *crawling*
153 features over time. For this purpose we compared two series of experiments: one in which NS
154 was subjected to depolarizing steps (depo) and a control (ctrl) in which it was not. We assessed
155 changes in cycle period, DE-3 mean firing frequency (mFF), burst duration and duty cycle for
156 both treatments (depo, ctrl) across different epochs (pre, test, post).

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

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

165 As predicted based on the known circuit (Figure 1C), this series of experiments indicates
166 that inhibitory signals onto NS premotor neurons are transmitted to DE-3 motoneurons, thereby
167 limiting their firing frequency by counteracting their excitatory drive during *crawling*.

168

169 **A more comprehensive view of *crawling***

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

179 during the contraction stage, defined by DE-3 activity, while others fire during the elongation
180 phase.

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

187 Multiple intracellular recordings represent a challenge, and therefore recording the
188 activity of different motoneurons during crawling has been restricted to one (Puhl and Mesce,
189 2008) or two (Rodriguez et al., 2012) cells at a time. While multiple extracellular recordings
190 have been performed previously (Eisenhart et al., 2000), these results (Figure 4) present the first
191 quantitative analysis of multiple units activated throughout the *crawling* cycle in this type of
192 recordings. Nerve roots carry both sensory and motor axons; however, the units recorded during
193 *crawling* in isolated ganglia are more likely to be motoneurons. Although the preparation is
194 detached from peripheral inputs, sensory neurons could still receive inputs from the segmental
195 pattern generator. The recorded nerves conduct axons from mechanosensory pressure (P) and
196 touch (T) neurons (Nicholls and Baylor, 1968). However, P cells show no activity during
197 dopamine-induced *crawling*, and T cells receive inhibitory inputs in phase with DE-3 bursts
198 (Alonso et al. 2020). Axons from stretch receptors also run through the recorded nerves, but
199 these neurons transmit information passively (Blackshaw and Thompson, 1988, Cang and
200 Friesen, 2000). Therefore, the In-Phase units are expected to be motonerons that control the
201 contraction stage by exciting or inhibiting the longitudinal or circular muscles, respectively, and

202 the Anti-Phase units motoneurons that control the elongation stage, by exciting or inhibiting the
203 circular or longitudinal muscles, respectively. It is plausible that In-Phase-Early-Onset are
204 inhibitors of circular muscles, preparing the conditions for a fast contraction stage. Further
205 studies should confirm this hypothesis.
206
207 **Comparison of the motor pattern in single ganglia and in intact animals**
208 The assumption that the crawling motor pattern described on the basis of extracellular recordings
209 in isolated single ganglia, reflects key characteristics of the behavioral pattern, requires
210 qualitative and quantitative validation. To this end intact leeches were monitored during
211 crawling. The analysis was performed by tracking nine dots painted along the dorsal longitudinal
212 midline of the leech, which divided the body into 8 sections (Figure 5A). Because ganglia are
213 located in highly conserved positions relative to the length of the animal (Figure 5B, Kearney et
214 al. 2022), the relative distance from the head can be used to infer which ganglia are included
215 within the 8 sections mentioned above. We focused the analysis on sections delimited by points
216 4-5 and 5-6, that correspond to the range of segmental ganglia included in the *ex vivo* studies
217 (ganglia 8 to 15) (Figure 5A-B). For electrophysiological data the frame of analysis was the
218 cycle of DE-3 rhythmic activity and, equivalently, for the behavioral data the frame of analysis
219 was the crawling step (see Materials and Methods).
220 Figure 5C shows a representative trace of a series of whole length waves, that were
221 divided into discrete steps (see Materials and Methods), and the corresponding measurements in
222 the section delimited by the dots 4 and 5. Within each crawling step, four stages were identified
223 in the studied sections (Figure 5C): the elongation stage (marked by an increase in section
224 length), the contraction stage (marked by a decrease in section length) and two isometric stages

225 flanking the dynamic stages. The plot in Figure 5D shows the section length of different steps by
226 the same animal, normalized by the step duration, and illustrates the variability of the step
227 dynamics.

228 For a quantitative comparison of the *in vivo* and *ex vivo* experiments the following
229 variables were evaluated: period of the rhythmic pattern and duty cycle of the different phases.
230 While the average cycle period (end-to-end) recorded in isolated ganglia was of 15.22 ± 3.21 s (n
231 = 6), the average duration of the leech step was of 7.49 ± 0.50 s (n=6). These values are similar
232 to those reported in previous studies (Puhl and Mesce, 2008, for single ganglia; and Stern-
233 Tomlinson et al., 1986; Baader and Kristan, 1992 and Puhl and Mesce, 2008, for intact animals).
234 Thus, in the intact animal the rhythmic activity was twice as fast as in the isolated ganglion,
235 suggesting that peripheral signals may accelerate the rhythmic behavior, as already described in
236 other animals (Pulver et al., 2015; Fushiki et al., 2016). However, we cannot rule out that
237 activation of the rhythmogenic circuit in isolated ganglia may fail to activate a circuit component
238 responsible for speeding the rhythm.

239 It seems natural to correlate the activity of the Anti-Phase and In-Phase units of *ex vivo*
240 studies with the elongation and contraction stages of *in vivo* behavior, respectively. But the
241 isometric stages displayed *in vivo* at the section level have no obvious counterpart in the
242 electrophysiological recordings of motoneurons in the isolated ganglia. It is important to
243 consider that the rhythmic movement of successive segments along the antero-posterior axis of
244 the animal requires a delay signal that allows the appropriate propagation of the metachronal
245 wave (Puhl and Mesce, 2010; Puhl et al 2012), and this signal is probably absent in the isolated
246 ganglion. In consequence, for the comparison of the duty cycle, the *in vivo* isometric stages were
247 ignored, and only the duration of the dynamic fraction of the step was taken in consideration. In

248 turn, the behavioral duty cycle was calculated as the ratio between the elongation or contraction
249 stage duration over the dynamic step duration. Thus measured, the duty cycle of the elongation
250 stage was 0.65 ± 0.01 and that of the contraction was 0.35 ± 0.01 (Figure 5E). These values are
251 noticeably close to the duty cycle of the Anti-Phase units (0.64 ± 0.07) and of the DE-3
252 motoneurons (0.32 ± 0.03) (Figure 5E) measured in the isolated ganglia.
253 These results show that the dynamic pattern described by multiple motoneurons recorded
254 in isolated ganglia reflects the phase relation observed in the corresponding body section *in vivo*.
255
256 **Effect of NS on the activity of the different types of motoneurons during *crawling***
257 The results shown in Figure 5 support the view that the activity recorded in single isolated
258 ganglia reflects the neuronal control of the rhythmic motor pattern underlying crawling. Based
259 on this premise the recording of multiple units presented above was used to evaluate the
260 influence of NS on units active at different *crawling* stages (Figure 6Ai-ii). Previous work
261 (Rodriguez et al., 2012) and the experiments presented in Figure 3 suggest that NS
262 depolarization releases motoneuron DE-3 from the inhibitory influence of the premotor neuron.
263 The suggested interpretation postulates that NS depolarization, by turning off the rectifying
264 junctions, prevents the transmission of NS inhibitory inputs onto DE-3. Given the widespread
265 connectivity of NS with all the excitatory motoneurons, it is expected that NS manipulation
266 affects the activity of all the units that fire in phase with DE-3 when NS receives inhibitory
267 inputs (Figure 2A). In contrast, units active in anti-phase with DE-3 should not be affected as NS
268 does not receive inhibitory inputs during that stage.
269 To evaluate the effect of NS depolarization, the activity of the motoneurons was
270 characterized using two metrics: maximum bFF (Max bFF) to assess activity level, and relative

271 half width (Rel HW) to evaluate the duty cycle. To enable a comparison with the other units, the
272 data for DE-3 was reanalyzed in this manner.

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

279 The Anti-Phase units also showed a slight but significant decrease in the maximum bFF
280 across time in control conditions, but in this case the effect of NS depolarization was limited to
281 counteracting this decrease (Figure 6B). While in control conditions Anti-Phase motor units
282 exhibited a decrease of around 15% between pre and test epochs, the depo experiments showed
283 no decrement between these two epochs. Notwithstanding, control and depo experiments showed
284 a marked decrease in bFF from test to post (15% and a 20% decrease, respectively). NS
285 depolarization did not affect the maximum bFF of the In-Phase-Early-Onset units (Figure 6B).

286 Regarding the relative HW, the effect of NS depolarization was limited to In-Phase units.
287 Our results show that the relative HW of these units tended to fall across epochs in control
288 conditions and that releasing NS depolarization enhanced this tendency (Figure 6C). In control
289 experiments, the relative HW decreased by about 15% between test and post epochs; and in the
290 depo experiments the decrease was of around 40%.

291 The statistical analyses, as well as the estimated ratios between epochs for each case, are
292 detailed in Supplementary Table 2.

Taken together, the results support the hypothesis presented earlier: the inhibitory signals received by NS in phase with the contraction stage modulated the activity of the motoneurons active in this instance. 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.

298 **DISCUSSION**

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

315 On the other hand, the fact that In-Phase-Early-Onset activity was also unaffected by NS
316 depolarization confirms that these motoneurons constitute a distinct group from the In-Phase
317 units. Furthermore, this suggests that these units might be inhibitory motoneurons that are not
318 connected to the NS via the recurrent inhibitory circuit (Rela and Szczupak, 2003; Wadepuhl,
319 1989).

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

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

336 Previous work showed that leech motoneurons are subjected to a generalized inhibition
337 mediated by GABA (Baca et al., 2008) while the electrically mediated inhibition exerted by NS
338 shown here is phase-specific. The existence of concurrent excitation and inhibition has been
339 revealed in vertebrate motor networks, where it was proposed as a balancing factor (Kishore et
340 al., 2014; Parkis et al., 1999; Petersen et al., 2014). In line with the present results, experiments
341 performed in zebrafish showed that in-phase inhibition originates from a set of premotor neurons
342 that comprise the Renshaw cells (Callahan et al., 2019). Neurophysiological studies in the leech

343 offer the particular advantage of allowing electrical manipulation of individual neurons in adults,
344 where they can be readily recognized by their anatomical and electrophysiological
345 characteristics. In this sense the present study offers clear-cut evidence of the dual signal
346 motoneurons receive in the course of motor control from an identified premotor element.
347 The results further highlight the notion that knowing the connectivity among neurons is
348 not sufficient to deduce its functional operation, as the dynamical interplay among them is highly
349 relevant to understand its physiological role.
350
351 **Structure of *crawling* based on MN activity and behavior**
352 Evaluation of phase specificity required a more comprehensive tracking of motoneuron activity
353 throughout the cycle and we addressed this demand through the analysis of the activity of
354 multiple motoneurons. In the present study we show that in the course of *crawling* DE-3 neurons
355 fire concurrently with neurons that reach the periphery mainly through the PP and the AA roots.
356 Based on the classical description of Ort and collaborators (1974) the spikes in phase with DE-3
357 recorded in the PP root are likely to correspond to VE-4 (ventral-excitor 4), DE-5, DE-7 and VE-
358 8, while those recorded in AA are likely to correspond to DE-107 and VE-108. Intracellular
359 recordings of VE-4, VE-5 showed indeed the coincident firing with DE-3 (Baader, 1997,
360 Eisenhart et al., 2000 and Puhl and Mesce, 2008)
361 To learn how the motoneuron readout compares with the rhythmic body movements that
362 take place in crawling we presented behavioral studies that focused on the sections of the leech
363 body that match the range of ganglia used in the electrophysiological studies. The comparison
364 shows two main differences. The period of the rhythmic animal behavior was half that measured
365 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 known descending brain signals (Puhl et al., 2012) 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 dynamic aspects of the motor behavior at any given section of the animal.

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 $CaCl_2$, 1 $MgSO_4$, 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).

To induce fictive crawling (*crawling*), ganglia were superfused with 75 μM 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). In the present study the minimum and maximum mean period values (end-to-end) in isolated ganglia were 6.32 and 31.24 s, respectively.

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 stages 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

444 could not be split into clusters with well-defined waveforms were discarded. After this
445 procedure, each cluster was considered a single motor unit.
446 To select for rhythmic units, autocorrelations were performed considering the first four
447 cycles of the fictive behavior. Units whose autocorrelogram included at least 2 peaks with a
448 correlation coefficient greater than 0.25 were considered rhythmic.
449
450 **Data Processing**
451 DE-3 spike times were derived from DP nerve recordings using the spike sorting algorithm
452 mentioned above. DE-3 bursts were considered as such if they comprised at least 10 spikes with
453 a mean firing frequency of 8 Hz, where the separation between two subsequent spikes occurred
454 with a maximum separation of 0.5 seconds. Burst duration was calculated as the time difference
455 between the last and first DE-3 spikes in each burst. Cycle period was calculated in two different
456 ways: as the time difference between the last (end-to-end), or the first spikes (start-to-start) of
457 subsequent DE-3 bursts, and is reported accordingly. Duty cycle was calculated as the ratio
458 between burst duration and period. The mean firing frequency (mFF) of each burst was
459 calculated as the number of spikes over the burst duration. The binned firing frequency (bFF) of
460 DE-3, or any other unit, was obtained by binning the number of spikes across the recording using
461 a 0.05 s or a 0.5 s interval, for Figure 2 and Figure 6, respectively. For cross-correlation analysis,
462 the DE-3 bFF was filtered using a Gaussian filter (sigma of 150 ms).
463 For the cross-correlation between NS membrane potential and DE-3 firing frequency, the
464 intracellular recording of NS was resampled to match the data rate of DE-3 bFF. To determine
465 the amplitude of NS hyperpolarization, a baseline membrane potential was established using a
466 Gaussian filter (sigma of 5 s) applied to the entire trace. The amplitude was calculated as the

467 difference between the baseline value at the start of the DE-3 burst and the minimum membrane
468 potential reached by NS during the corresponding burst (see Figure 2A).
469 When NS depolarization was implemented, the cycles including the onset or offset of the
470 current pulse were excluded, and the traces were divided into three epochs: pre, test, and post.
471 Control experiments were treated similarly.
472 When analyzing the activity of the units obtained by spike sorting, the maximum bFF
473 value and relative half width were obtained from curves displaying bFF versus cycle-phase (time
474 normalized to the period of each cycle). The relative half width was calculated as the width at
475 half the maximum bFF value.
476 Data processing was performed using custom written Python scripts.
477
478 **Statistical Analysis**
479 All statistical analyses were performed using R (R Core Team, 2020). The goodness-of-fit for
480 each model was analyzed using the DHARMa package. For every analysis, the structure of
481 random factors was kept maximal whenever possible. The specific random factors considered in
482 each case are detailed in Supplementary Tables 1 and 2.
483 To analyze the correlation between the amplitude of NS hyperpolarization and different
484 *crawling* features (Figure 2D-G), the lmer function from the lmerTest package (Kuznetsova et
485 al., 2017) was used to perform linear mixed models (LMM) with the experiment as a random
486 factor.
487 To analyze the effect of NS depolarization on motoneuron activity, the data was analyzed
488 using mixed-effects models with two fixed factors: epoch (pre, test, post) and treatment (control,
489 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.

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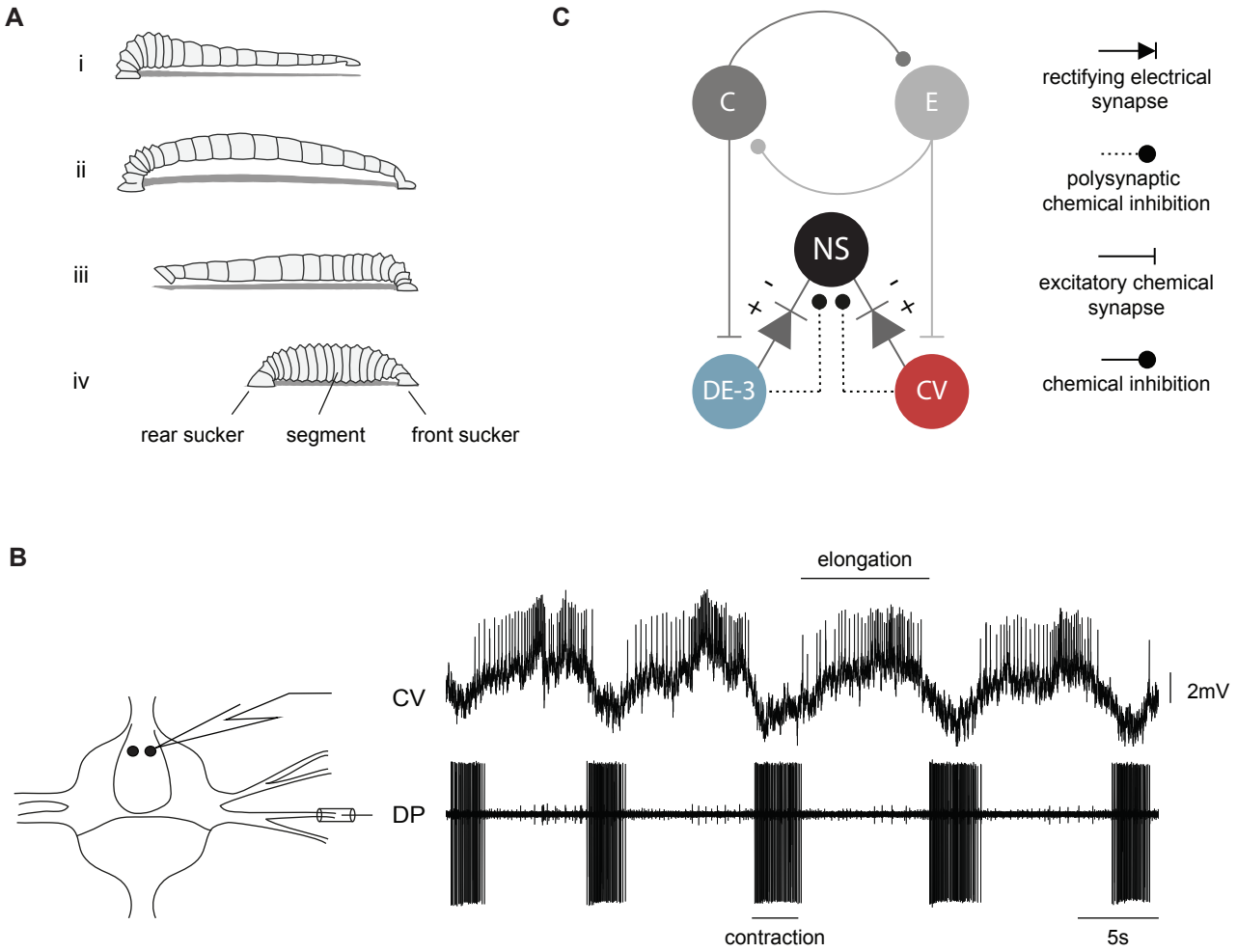


Figure 1. The crawling motor pattern. A. Schematic description of a leech crawling step that results from coordinated waves of elongation (i-ii) and contraction (iii-iv) phases, anchored on front and rear suckers. **B.** Intracellular recording of CV and extracellular recording of DE-3 (in DP nerve) 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.

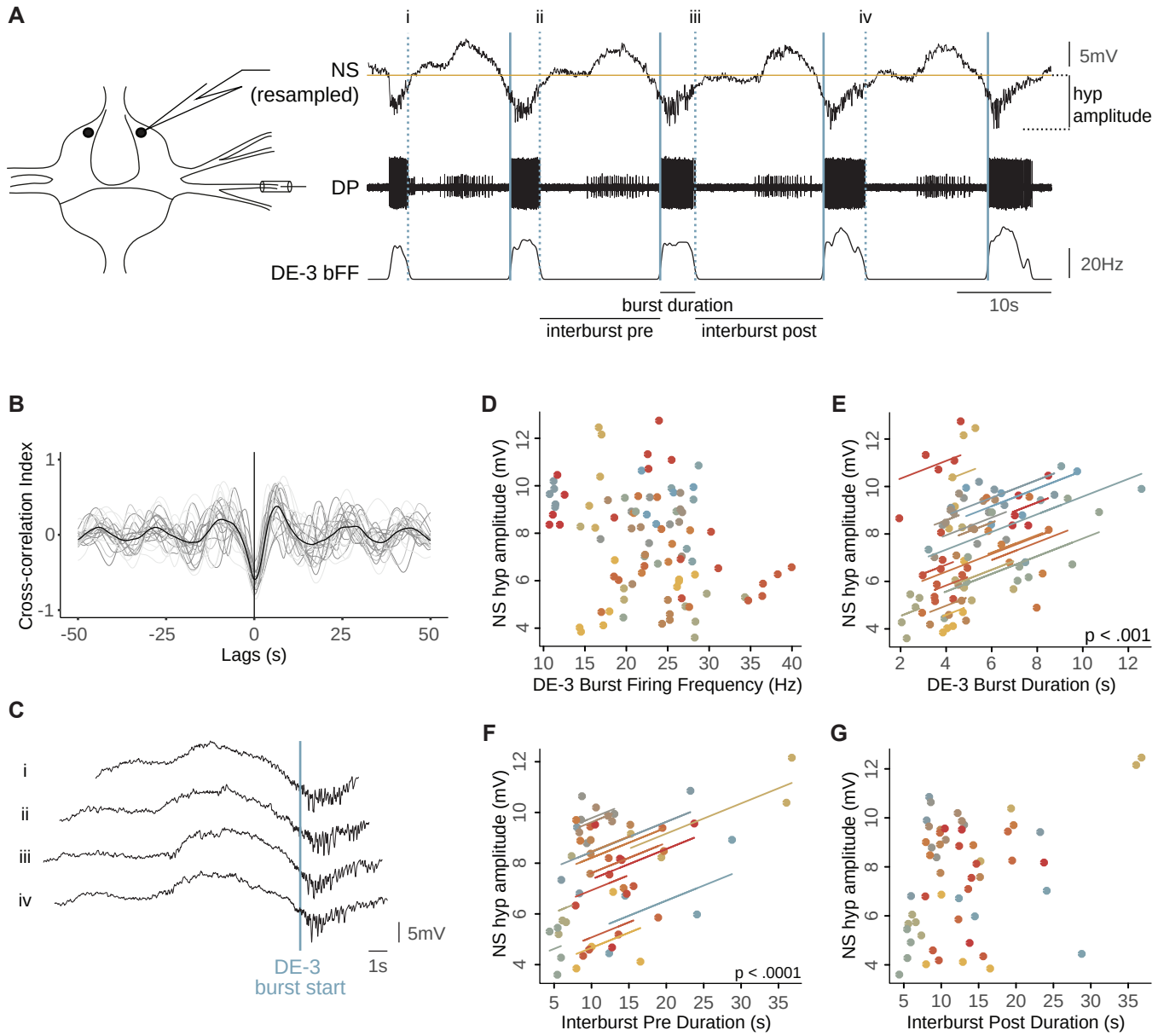


Figure 2. NS activity correlates with the crawling motor pattern. A. NS intracellular recording, DP extracellular recording and DE-3 binned firing frequency (bFF) during a crawling episode. NS recording was resampled to match the data rate of DE-3 bFF. Recording diagram on the left. The blue vertical 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.

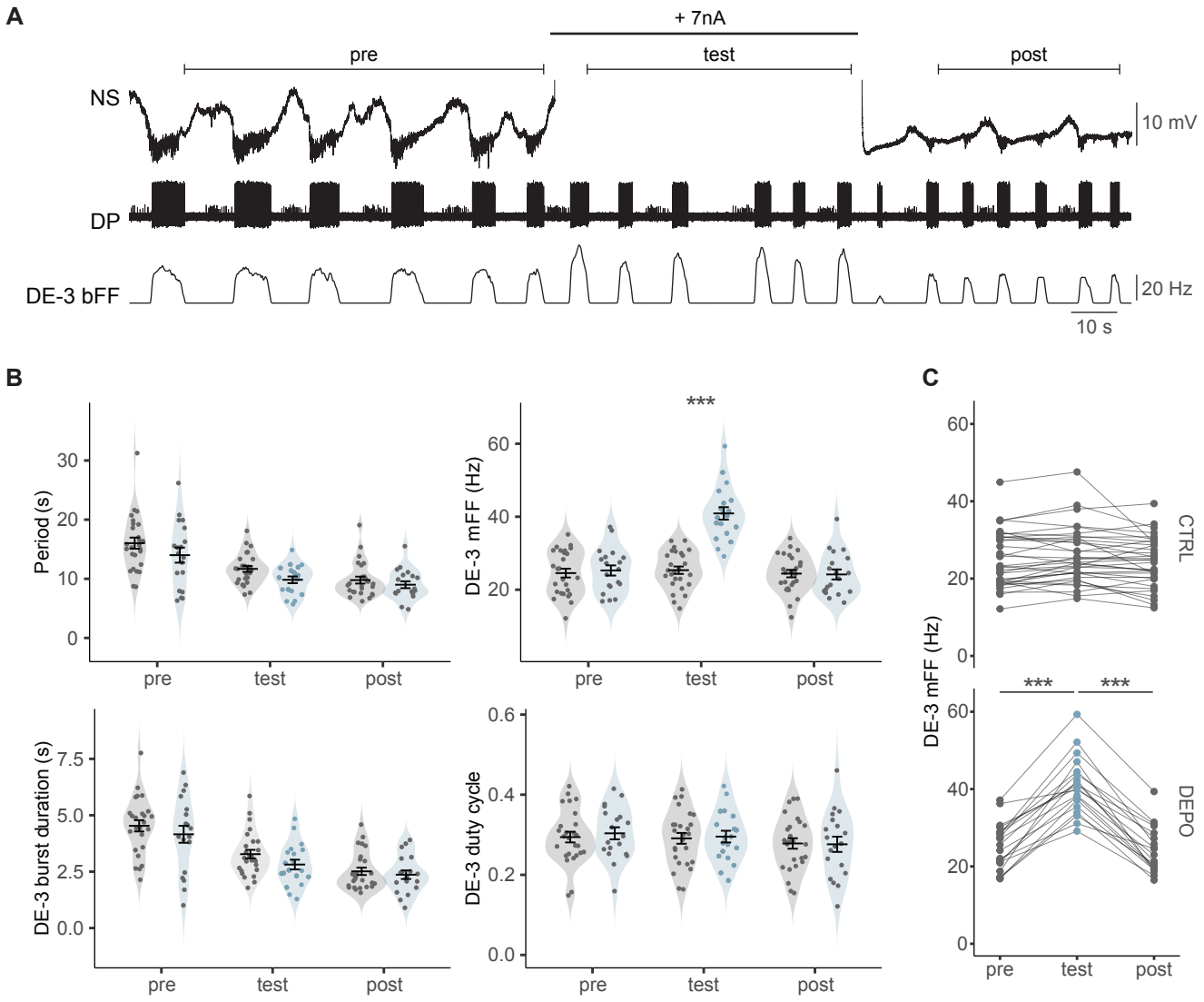


Figure 3. Effect of NS on crawling. A. Intracellular NS and extracellular DP recordings during a dopamine-induced crawling episode. A +7 nA pulse was injected in NS at the time indicated by the thick line. Segments above the recordings mark three epochs: previous (pre), during (test) and after (post) 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 ± 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).

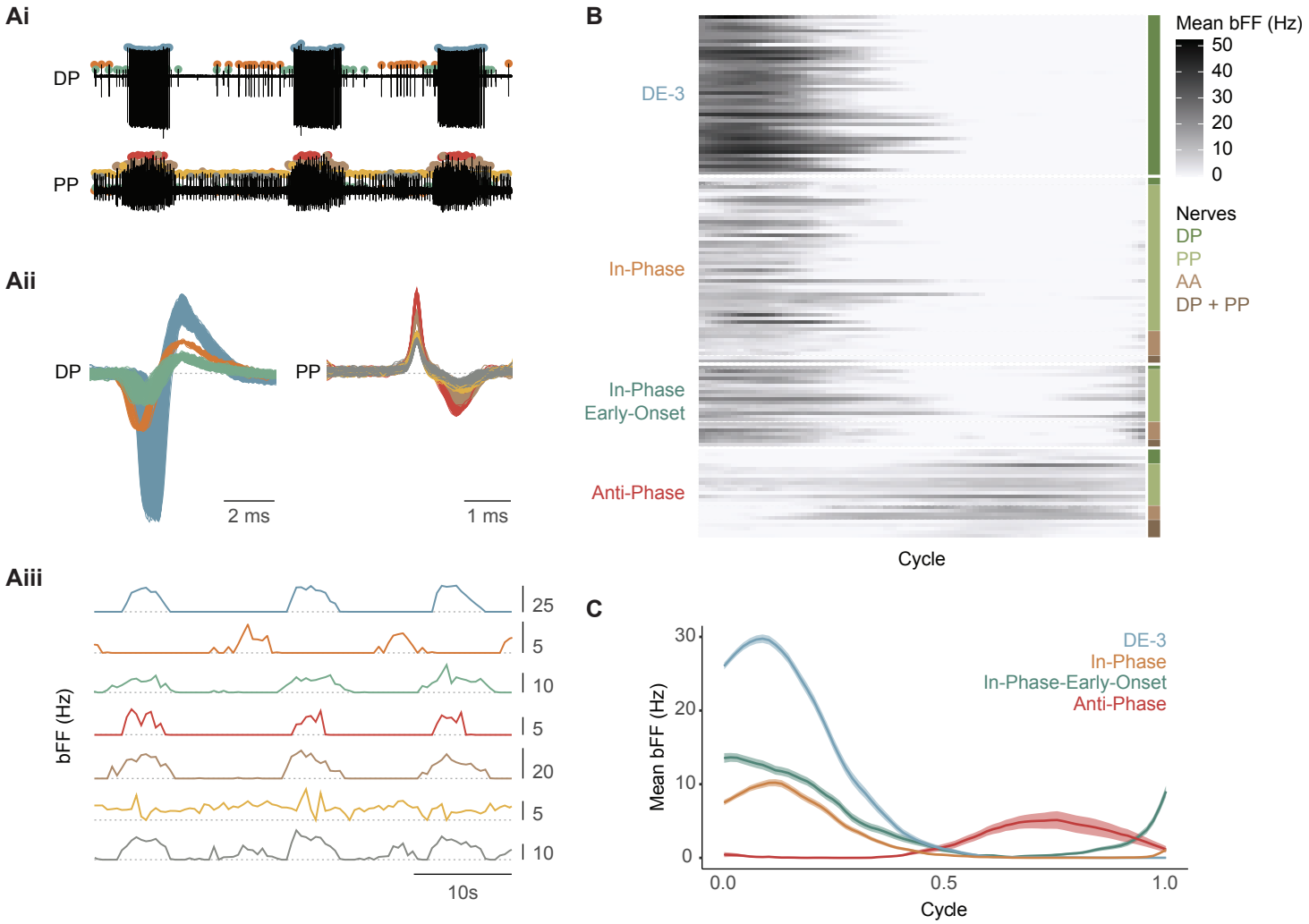


Figure 4. Profile of motoneuron activity during crawling. A.i. Representative example of a spike sorting analysis implemented in paired extracellular recordings of DP and PP nerves during a crawling episode. Each color dot singles out a distinct active unit across the recordings. **A.ii.** Spikes waveforms 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 across 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.

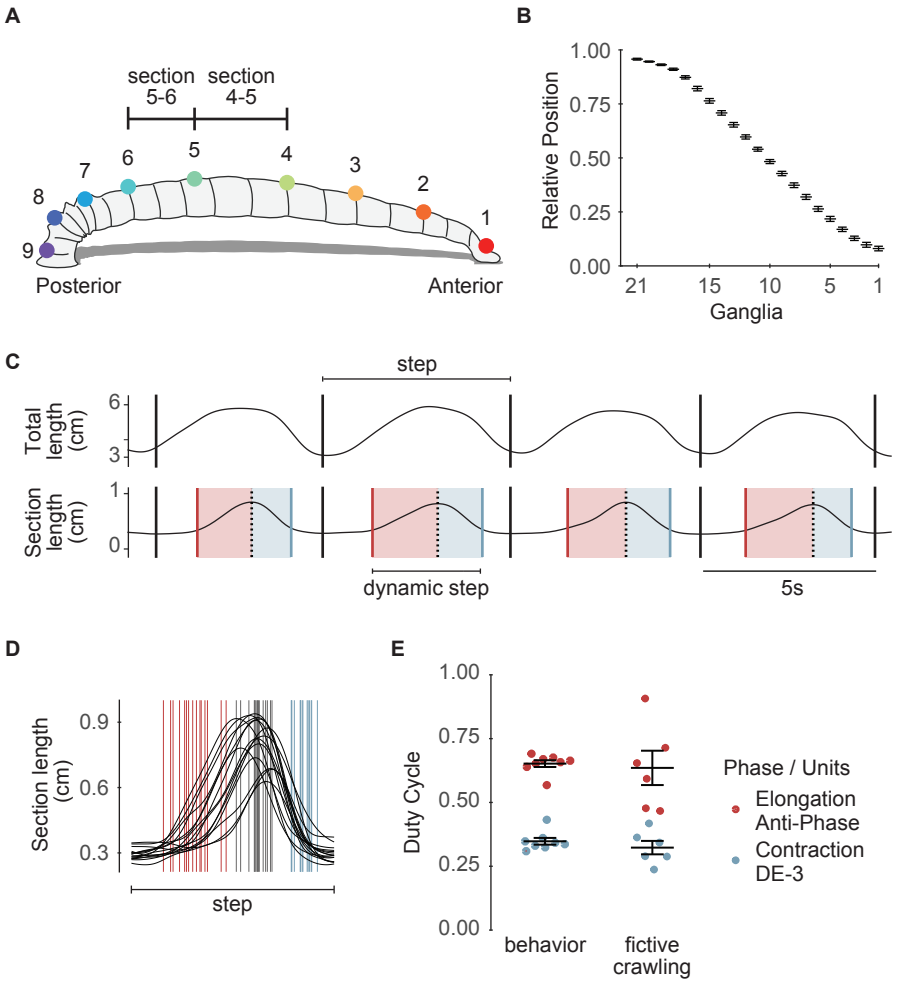
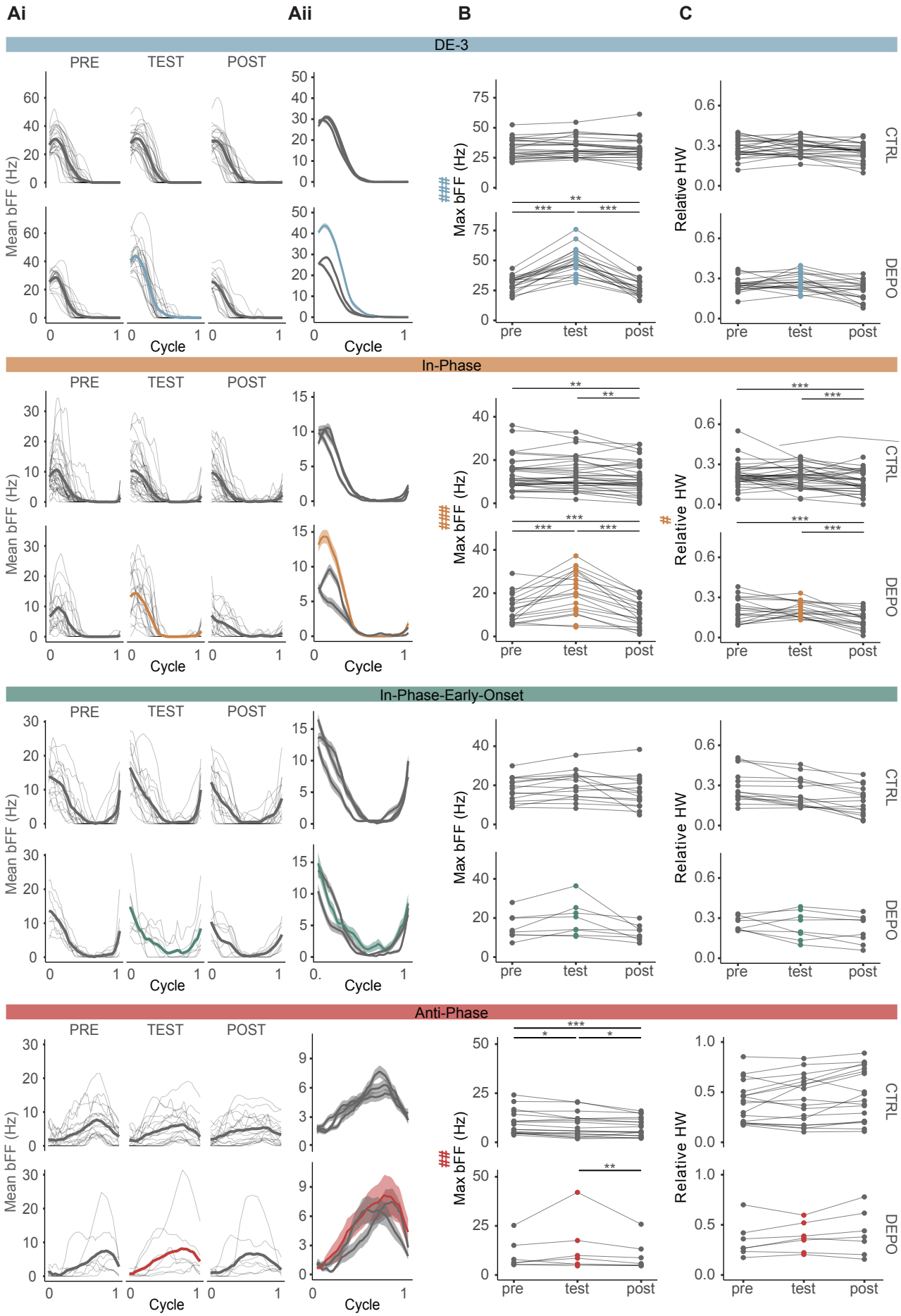


Figure 5. Analysis of leech crawling. A. Schematic representation of a leech with nine points as painted on their dorsal midline. The sections between points 4-5 and 5-6 comprise ganglia 8-15 that were used for the electrophysiological analysis. **B.** Relative position of midbody ganglia 1–21. Mean 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 ± 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 ± SEM across all ganglia (n = 4 cycles for each DE-3 or Anti-Phase motoneuron, 6 ganglia, 6 leeches).


Ai
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Relative HW
CTRL
DEPO
pre
test
post

Figure 6. Effect of NS on the different phases of crawling. A.i. Mean bFF across 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.

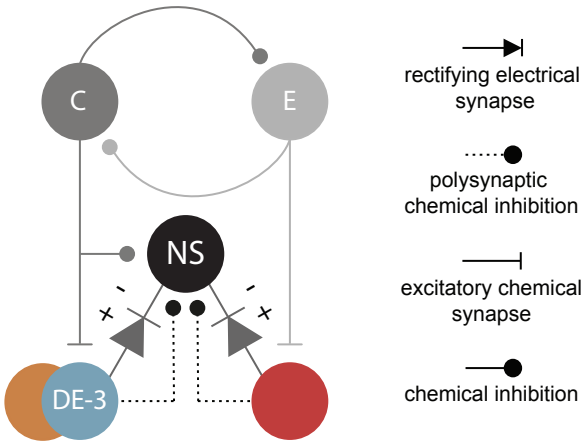

Figure 7. NS in the crawling circuitry. Putative neuronal circuitry as described in Figure 1C, including the connection between the oscillatory circuit and NS. The colors used here match those identifying the units in Figures 4 and 6.