Novel and highly distributed classes of hindbrain neuronal activity contribute to sensory processing and motor control in the *Xenopus laevis* tadpole

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Abstract

An animal’s survival depends heavily on the selection and execution of timely and well-coordinated motor responses. The brainstem controls the activity of spinal neural circuits to produce and modify movements. However, important questions remain unanswered about the origin of this descending control and how brainstem neuronal activity integrates sensory inputs and determines motor functions. Here, we record hindbrain extracellular activity in response to trunk skin stimulation, which in turn leads to fictive swimming in the hatchling *Xenopus laevis* tadpole. We identify four distinct classes of single unit activity, distributed along the hindbrain rostro-caudal axis, whose firing patterns correlate to distinct motor states. We observe different firing patterns in response to stimulation that leads to fictive swimming versus the application of a weak stimulus which does not evoke movement. We identify differences in the temporal activation of the four classes of hindbrain activity in relation to the initiation of fictive swimming. We propose a simple network encompassing the novel neuronal populations embedded within the currently known sensory pathway and central pattern generators of the tadpole brainstem. By identifying the contribution of the individual supraspinal neuronal populations we build a better understanding of how the brain controls and modulates movement.

Introduction

In nature animals must select, execute, and adapt their motor behavior to specific aims such as feeding, escaping from a predator, or finding a mate, with the ultimate goal of surviving and reproducing. From nudibranchs (Sakurai & Katz, 2016) to lamprey (Stephenson-Jones, Samuelsson, Ericsson, Robertson, & Grillner, 2011), from cats (Opris et al., 2019) to humans (MacKinnon, 2018), all animals are required to coordinate and timely activate central and peripheral neuronal circuits in order to perform the most advantageous movement. Most of the sensory input animals receive from the environment leads to motor activity. This can be driven by the nature of the stimulus (e.g. withdrawal from the source of harmful heat), or by the instinctive need to inspect the surroundings (e.g. eye movement to follow a moving object).

In adult vertebrates, the combined activation of neural circuits in the forebrain, brainstem and spinal cord generates a fine-tuned and rich repertoire of motor behaviors, including locomotion (Goulding, 2009; Ruder & Arber, 2019). Spinal CPG (central pattern generator) circuits are activated and via integration of ipsilateral activation and contralateral inhibition (Crone et al., 2008; Wallén-Mackenzie et al., 2006) they enable the animal to move efficiently (Capelli, Pivetta, Soledad Esposito, & Arber, 2017; Noga, Kriellaars, Brownstone, & Jordan, 2003).

Different brain regions have been identified as key components for the generation of movement (Grillner & El Manira, 2020) with the basic neural circuit being conserved from lamprey to humans (Grillner & El Manira, 2020). The basal ganglia play a major role in descending control over the MLR (mesencephalic locomotor region) (Crone et al., 2008; Goulding, 2009). This circuit relies on tonic inhibition of the MLR at resting conditions provided by GABAergic neurons from GPi (globus pallidus pars interna) and SNr (substantia nigra pars reticulata) (Sakurai & Katz, 2016). When striatal projections silence these highly active inhibitory neurons, the MLR excitation over reticulospinal neurons (RS) is released (Noga et al., 2003; Wallén-Mackenzie et al., 2006). Recently, key glutamatergic neurons in the MLR have been found to be sufficient to induce locomotion in mice (Roseberry et al., 2016) and in a more caudal region of the mouse brainstem, namely the LPGi (lateral paragigantocellular nucleus), the activation of vGlut2-
expressing neurons has been shown to be sufficient to start locomotion and increase its speed (Capelli et al., 2017).

Despite the advances in the field and although the spinal neural circuits have been extensively studied in higher vertebrates (O. Kiehn, 2006; Ole Kiehn, 2016) unravelling the supraspinal control of movement in such animals (Armstrong, 1988; Dietz, 2010; Shik & Orlovsky, 1976) remains challenging, and the neuron-to-neuron pathways that coordinate motor decision and movement execution have not been fully elucidated yet (Arber, 2012; Bouvier et al., 2015; Caggiano et al., 2018; Ferreira-Pinto, Ruder, Capelli, & Arber, 2018; Parker, 2010).

The spinal neuronal circuits responsible for swimming in the hatchling Xenopus laevis tadpole have been extensively characterized. The reticulospinal descending interneurons (dINs), that drive spinal CPG activity, have been anatomically and functionally described (Li et al., 2001; Li, Soffe, Wolf, & Roberts, 2006; Roberts, Li, & Soffe, 2010). Furthermore, we have recently shown that the tadpole responds to trunk skin stimuli by initiating swimming with a long and variable delay of 50-150ms (Buhl, Soffe, & Roberts, 2015; Koutsikou et al., 2018; Roberts et al., 2019). This delay is characterised by accumulation of excitation on dINs, which resembles the accumulation of excitation proposed for complex brain circuits active during motor decision-making (Carpenter, 1999; Gold & Shadlen, 2007).

In the hatchling tadpole, whole-cell recordings of caudal hindbrain reticulospinal neurons (dINs) showed a slow and variable increase in synaptic excitation following skin stimulation. This in turn allowed us to infer the existence of candidate neurons pre-synaptic to dINs, namely hindbrain extension neurons (hexNs; Koutsikou et al., 2018). It has been proposed that hexNs extend the short-lived sensory signal carried by the sensory and sensory pathway neurons (Rohon-Beard cells and dorsolateral ascending -dla- and dorsolateral commissural -dlc- neurons, respectively (Clarke Jd Fau Hayes & Roberts, 1984; Roberts & Clarke, 1982)), thus allowing the animal to ‘procrastinate’ prior to making the simple motor decision, to swim or not to swim. Ultimately, and only if the stimulus delivered is strong enough, this build-up of excitation in the dINs reaches a firing threshold, which in turn marks the start of swimming (Buhl et al., 2015; Li et al., 2006). The variable and long process of accumulation of excitation recorded in dINs has been mimicked through modelling by an excitatory recurrent network within the brainstem sensory pathways (Ferrario et al., 2021; Koutsikou et al., 2018).

Despite the evidence above indicating that the fundamental elements of descending motor control are present at an early developmental stage, the neurons activating the motor pathways in the Xenopus tadpole are currently undefined. We implemented a top-down approach to uncover the firing characteristics of the neuronal components responsible for the accumulation of excitation in reticulospinal interneurons (dIN’s) prior to swim initiation. We recorded extracellular activity in the hindbrain of the tadpole, and we analyzed single unit firing patterns in response to skin stimulation during distinct motor states, including swim initiation.

We identified novel firing patterns across the tadpole’s hindbrain that indicate an overall increased neuronal activity in the time between the sensory trunk skin stimulation and motor output. We classified four hexN subpopulations based on their firing activity, which differentially contributed to the accumulation of excitation in the hindbrain prior to the start of swimming. These neuronal populations exhibit all the features necessary for the motor decision-making process (to swim or not to swim) taking place in the simple embryonic brain of the Xenopus laevis tadpole. We finally propose a simple hindbrain neural network that includes the newly identified hexN sub-populations and their proposed connections.
within the existing well-defined sensory, sensory-pathway and CPG neuronal circuitry of the tadpole’s CNS.

**Results**

Extracellular hindbrain neuronal activity was recorded from immobilised tadpoles in response to electrical trunk skin stimulation above and below the threshold for swimming (fig. 1A). Single unit hindbrain neuronal activity was studied in four distinct motor states: 1) at rest, 2) following stimulation which did not initiate swimming (stim/no start), 3) following stimulation which led to swim initiation (stim/start), and 4) during sustained swimming (swimming) (fig. 1Aiv). Spike sorting analysis (see Materials and Methods) was used to isolate and subsequently categorise single units. All units were mapped along the rostro-caudal axis of the tadpole’s hindbrain, which was divided into three sectors defined by anatomical landmarks (in rostro-caudal order: midbrain-hindbrain border, otic capsule and obex, respectively) as shown in fig. 1Aiii.
Figure 1

A) Experimental design. Ai. Lateral view of stage 37/38 Xenopus laevis tadpole; anatomical features are indicated on the head (eye and otic capsule) and along the body (spinal cord and trunk muscles). Grey dotted line on the head represents the area where the skin was removed to expose the brain. Aii. Dorsal
view of stage 37/38 X. laevis tadpole with extracellular suction electrodes positioned as per experimental conditions. Grey dotted line marks the area where the skin was removed to make the hindbrain accessible. In different experiments, the brain electrode (hindbrain) was positioned in one of the three hindbrain sectors as depicted in fig. 1Aiv. The two VR (ventral root) electrodes were positioned at the 5th inter-myotomal cleft on both sides of the tadpole’s body (left VR, right VR). The stimulating electrode (stimulus) was attached to the skin on the right side of the body at the level of the anus. Scale bar as in Ai. Aiii. X. laevis tadpole’s hindbrain as it appears when the skin is removed. The hindbrain was visually divided into three sectors along the rostro-caudal axis using well-defined anatomical landmarks, i.e. MHB (yellow arrow), otic capsules (blue arrow) and obex (green arrow): rostral sector (1), otic capsule level (2) and caudal sector (3). Aiv. Examples of recordings for the four different motor states analysed (200 ms): rest, stimulation/no start of swimming, stimulation/start of swimming, sustained swimming. Raw traces for hindbrain extracellular activity (hb, black trace) and right and left VR recording (rVR and lVR, grey traces) are presented. Red arrowheads indicate stimulus delivery, blue arrowhead indicates the start of swimming.

B, C) Command units and swim driver units are activated at the start of swimming. Bi) Average heat map of command units recorded in the hindbrain (25 units in 11 animals, minimum of 4 trials per motor state per units). Coefficients of variation (CV=standard deviation/mean) were calculated for each time bin (5 ms) on the number of spikes fired by single units during the same 5 ms in the four motor states. Bii) Examples of spikes fired by one command unit recorded in the four motor states investigated (rest, stimulation/no start, stimulation/start, swimming) 150 ms are reported for each example. Red lines indicate spikes fired by the unit, and they are presented above the respective extracellular hindbrain recording raw trace (black trace). Fictive swimming is shown by rhythmic right and left VR bursts (grey trace, rVR and lVR). Red arrowheads represent the delivery of the electrical stimulus; blue arrowhead indicates the start of swimming. Ci) Average heat map of swim driver units recorded in the hindbrain (11 units in 8 animals, minimum of 4 trials per motor state per units). Coefficients of variation (CV=standard deviation/mean) were calculated for each time bin (5 ms) on the number of spikes fired by single units during the same 5 ms in the four motor states. Cii) Examples of spikes fired by one swim driver unit recorded in the four motor states investigated (rest, stimulation/no start, stimulation/start, swimming) 150 ms are reported for each example. Green lines indicate spikes fired by the unit, and they are presented above the respective extracellular hindbrain recording raw trace (black trace). Fictive swimming is shown by rhythmic right and left VR bursts (grey trace, rVR and lVR). Red arrowheads represent the delivery of the electrical stimulus; blue arrowhead indicates the start of swimming.

D) Distribution of command and swim driver units. Di. Bar charts representing percentages for non-significant (grey), swim driver (green) and command units (violet) in the entire hindbrain (Di), and in the different hindbrain sectors as depicted in Aiv (Dii, rostral sector; Diii otic capsule level; Div, caudal sector). Percentages were calculated over the total number of units recorded in the hindbrain (Di, 54/90 = 60.0% non significant units; 11/90 = 12.2% swim driver units, 25/90 = 27.8% command units), and over the total number of units recorded in each of the hindbrain sectors (Di-rostral sector, 21/33 = 63.6% non significant units, 2/33 = 6.1% swim driver units, 10/33 = 30.3% command units; Di-otic capsule level, 26/36 = 72.2% non significant units, 3/36 = 8.3% swim driver units, 7/36 = 19.4% command units; Di-caudal sector, 7/21 = 33.3% non significant units; 6/21 = 28.6% swim driver units, 8/21 = 38.1% command units).
Novel hindbrain firing patterns involved in control of swimming and its initiation

We identified two novel firing patterns that were correlated with swim initiation in the tadpole. This was achieved by counting the number of spikes fired by individual units during 150 ms trials in each of the four experimental motor states (fig. 1Aiv); two-way ANOVA was then run to categorise the different units.

25 units out of 90 (recorded in 18 animals) showed significantly higher activity at the start of swimming in response to skin stimulation (‘stimulus X swim interaction’ units, p<0.05, two-way ANOVA; 150 ms were analysed starting from stimulation, fig. 1Bii). We named these units, with firing rate higher only at motor initiation, ‘command units’. Command units were highly active only when the electrical stimulus was strong enough to induce swimming in the tadpole (the experimental condition referred to as ‘stimulation/start’), while they were mainly silent at rest, during sustained swimming, and when the stimulation failed to trigger swimming (fig. 1B). 11 units (out of 90 units in 18 animals) showed increased firing activity following trunk skin stimulation, both at the initiation and during sustained swimming (‘swim effect’ units, p<0.05, two-way ANOVA; fig. 1Ci, Cii). These units, which were more active both at the start of movement, as well as during continuous swimming, were named ‘swim driver units’. Swim driver units were mostly inactive when the animal was not swimming, i.e. at rest and when stimulation did not lead to swimming (fig. 1C). Both command and swim driver units were recorded throughout the hindbrain (fig. 1D), with an overall prevalence for command units over swim driver units (27.8% for command vs 12.2% for swim driver units, fig. 1Di). In the rostral area of the hindbrain (sector 1, fig. 1Aiii), 30.3% of the recorded units were command units (10/33 units), while 6.1% (2/33 units) fell into the swim driver category (fig. 1Dii). At the level of the otic capsule (sector 2, Fig. 1 Aiii), 19.4% of the units were found to be command units (7/36 units) and 8.3% were swim driver units (3/36 units) (fig. 1Diii). The highest percentage of command and swim driver units was recorded in the most caudal area of the hindbrain (sector 3, Fig. 1Aiii), where 38.1% of the units recorded were command units (8/21 units), and 28.6% (6/21 units) were swim driver units. (fig. 1Div). Percentages are calculated over the total number of units recorded within a single hindbrain sector. 54 out of 90 recorded units did not change their firing rate in response to trunk skin stimulation, or during swimming (‘non-significant units’, p>0.05, two-way ANOVA). Such units were recorded throughout the hindbrain: 63.6% in the rostral area (21/33 sector’s units), 72.2% (26/36 sector’s units) at the otic capsule level, 33.3% (7/21 sector’s units) in the caudal area (fig. 1Dii-iv, percentages calculated over the total number of units recorded within each hindbrain sector). These units showed stable, low firing in every motor state tested (rest, stimulation/no start, stimulation/start and swimming, S. Fig. 1A). We concluded that these units were not implicated in the control of swim initiation and therefore they were excluded from further analysis.

CPG neurons are also activated as soon as swimming starts. We recorded 3 CPG units, one in each hindbrain sector, and we fully characterised them in order to rule out the possibility of swim driver units to exhibit CPG-like activity. None of the swim driver units presented in this study showed features of rhythmic activity as observed in the CPG unit showed in S. Fig. 1Bii.

Command units are activated earlier than swim driver units, and both firing patterns are distributed throughout the hindbrain

Following stimulation, command units firing increased to a peak (0.48 ± 0.21 Hz, mean ± SEM) 11 ms before swimming started (fig. 2Ai) and then slowly decreased to become silent during sustained swimming (fig. 2Ai). Swim driver units also increased their activity rate before the start of swimming (fig. 2Ai), but there was no clear peak in their firing activity. Instead, swim driver units firing rates were stable as
swimming became continuous (0.06 ± 0.08 Hz 10 ms before start vs 0.06 ± 0.09 Hz 10 ms after swimming initiation; data expressed as mean ± SEM). Spikes fired by command units were recorded earlier before movement initiation in comparison to the spikes fired by swim driver units (fig. 2Aii). Command units recorded in the central area of the hindbrain (otic capsule level) were responsible for the firing rate peak at swimming initiation, with a peak of 1.28 ± 2.23 Hz recorded 10 ms before the start (fig. 2Bi). Command units detected in the rostral and caudal sectors increased their firing in a less abrupt fashion (fig. 2Bi), contributing nevertheless to the overall augmented activity, which was prolonged after the initiation of swimming (0 to ~150 ms after the start, fig. 2Ai). However, the onset and distribution of the spikes fired at the start of movement by command units in the three hindbrain sectors did not differ (fig. 2Bii). Swim driver units recorded in the three hindbrain areas showed prolonged firing at the initiation of swimming (fig. 2Ci), resulting in a constant activity that persisted during sustained swimming. The distribution of the spikes fired by swim driver units did not differ among the three hindbrain areas (fig. 2Cii). Both command and swim driver units showed a distribution of firing activity throughout the three hindbrain sectors, leading us to consider the two populations to be dispersed throughout the hindbrain.
**Figure 2**

**A)** Command and swim driver units are activated at different latencies.  
**Ai**) Firing rates of command (violet line) and swim driver units (green line) recorded before movement initiation and in the first 500 ms of sustained swimming. Dotted grey line (ms=0) marks the start of fictive swimming. Data are presented as mean (solid lines) ± SEM (shaded area). Wilcoxon matched pairs signed rank test, p<0.0001; command units 0.0925 ± 0.0139, swim driver units 0.0299 ± 0.0031 (mean ± SEM). Command units N=25, swim driver units N=11; minimum 4 trials/unit. ****p<0.0001. Top right insert: close-up view of Ai, showing increased firing rate in command (violet line) and swim driver (green line) units at swimming initiation.  

**Aii**) Scatter plot of spikes fired by command units (violet) and swim driver units (green) before movement initiation and during the first 500 ms of sustained swimming. Dotted grey line (ms=0) marks the start of fictive swimming. Black solid lines indicate median values (median ± SD= 72.69 ± 104.4 ms for command units;
median ± SD = 160.8 ± 144.3 ms for swim driver units). Kolmogorov-Smirnov test, p<0.0001; 1145 spikes from 25 command units, 288 spikes from 11 swim driver units. ****p<0.0001.

B, C) Command units and swim driver units show different firing rates throughout the hindbrain. Bi) Firing rates of command units recorded in the rostral sector (blue), at the otic capsule level (red) and in the caudal sector (green) of the hindbrain. Dotted grey line (ms=0) marks the start of fictive swimming. Data are presented as mean (solid lines) ± SEM (shaded area). Repeated measures one-way ANOVA with Bonferroni correction for multiple comparisons, p=0.0003; rostral vs OC level, mean diff.=0.0725; rostral vs caudal, mean diff.= 0.0376; OC level vs caudal, mean diff.= 0.1101. Rostral sector N=10 units; otic capsule level N=7 units; caudal sector N=8 units; minimum 4 trials per unit. ***p<0.001. Top right insert: close-up view of Bi that shows increased firing rate in rostral (blue line), otic capsule level (red line) and caudal (green line) units at swimming initiation.

Bii) Scatter plot of spikes fired by command units recorded in the rostral sector (blue), at the otic capsule level (red) and in the caudal sector (green) of the hindbrain. Dotted grey line (ms=0) marks the start of fictive swimming. Black solid lines indicate median values (median ± SD = 71.94 ± 93.29 ms for rostral units; median ± SD = 65.97 ± 117.5 ms for otic capsule level units; median ± SD= 78.32 ± 109.5 ms for caudal units). Kruskal-Wallis test, p=0.0618; 558 spikes from 10 rostral units, 284 spikes from 7 otic capsule level units, 303 spikes from 8 caudal units. Ci) Firing rates of swim driver units recorded in the rostral sector (blue), at the otic capsule level (red) and in the caudal sector (green) of the hindbrain. Dotted grey line (ms=0) marks the start of fictive swimming. Data are presented as mean (solid lines) ± SEM (shaded area). Repeated measures one-way ANOVA with Bonferroni correction for multiple comparisons, p=0.0003; rostral vs OC level, mean diff.= 0.0390; rostral vs caudal, mean diff.=-0.0137; OC level vs caudal, mean diff.=-0.0254. Rostral sector N=2 units; otic capsule level N=3 units; caudal sector N=6 units; minimum 4 trials per unit. ****p<0.0001. Top right insert: close-up view of Ci that shows increased firing rate in rostral (blue line), otic capsule level (red line) and caudal (green line) units at swimming initiation.

Cii) Scatter plot of spikes fired by swim driver units recorded in the rostral sector (blue), at the otic capsule level (red) and in the caudal sector (green) of the hindbrain. Dotted grey line (ms=0) marks the start of fictive swimming. Black solid lines indicate median values (median ± SD = 127.8 ± 148.6 ms for rostral units; median ± SD = 190.2 ± 160.7 ms for otic capsule level units; median ± SD = 162.2 ± 134.1 ms for caudal units). Kruskal-Wallis test, p=0.2837; 63 spikes from 2 rostral units, 73 spikes from 3 oc level units, 152 spikes from 6 caudal units.

Subpopulations of command and swim driver units are differentially activated

Based on their distinctive firing patterns, we identified two subgroups among command units. The first group, ‘first level command units’, showed increased firing in the stimulation/start trials (fig. 3Ai and Aii), but was also slightly active when the stimulus delivered was not strong enough to cause swim initiation (fig. 3Bi and Bii). On the contrary, the second group, named ‘second level command units’, was active only when the stimulation led to a motor response (fig. 3Ai, Aii and 3Bi, Bii). In the time prior to swim initiation, spikes fired by first level command units were recorded at shorter latencies compared to those fired by second level command units (fig. 3C, negative area of the graph). After swimming had started, the temporal distribution of spikes fired by first and second level command units did not differ (fig. 3C, positive area of the graph). Similarly, the swim driver population could be divided into two subpopulations. First level swim driver units were active when stimulation was delivered to the animal, irrespective of the motor outcome (fig. 3Di, Dii and 3Ei, Eii). On the other hand, second level swim driver firing was detected only when the electrical stimulus led to swimming (fig. 3Di, Dii and 3Ei, Eii). Contrary to command units, both first and second level swim driver units showed the same temporal distribution of spikes prior to
movement initiation (fig.3F, negative area of the graph), as well as after swimming had become continuous (fig. 3F, positive area of the graph).

**Figure 3. Subpopulations of command and swim drive units are activated differently.**

Aii) Firing rates of first (pink line) and second level (violet line) command units recorded before movement initiation and in the first 500 ms of sustained swimming. Dotted grey line (ms=0) marks the start of fictive swimming. Data are presented as mean (solid lines) ± SEM (shaded area). Wilcoxon matched pairs signed rank test, p=0.0511; first level command units 0.0695 ± 0.0100 ms, second level command units 0.1094 ± 0.0180 ms (mean ± SEM). First level command units N=11, second level command units N=14; minimum 4 trials/unit. Aii) Examples of spikes fired by one first level command unit (top trace, pink lines) and one
second level command units (bottom trace, violet lines), recorded in the stimulation/start motor state. Spikes fired by the units are presented above the respective extracellular hindbrain recording raw trace (hb, black trace). Fictive swimming is shown by rhythmic VR bursts (grey trace, VR). For clarity, only the VR with the first burst, marking swimming initiation, is shown here. Red arrowheads represent the delivery of the electrical stimulus; blue arrowhead indicates the start of swimming. 

**Bii)** Firing rates of first level (pink line) and second level (violet line) command units recorded in the first 500 ms after stimulation. Data are presented as mean (solid lines) ± SEM (shaded area). Wilcoxon matched pairs signed rank test, p<0.0001; first level command units 0.0149 ± 0.0018 ms, second level command units 0.0000 ± 0.0000 (mean ± SEM). First level command units N=11, second level command units N=14; minimum 4 trials/unit. ****p<0.0001.

**C)** Scatter plot of spikes fired by first level command units (pink) and second level command units (violet) before movement initiation and during the first 500 ms of sustained swimming. Dotted grey line (ms=0) marks the start of fictive swimming. Black solid lines indicate median values. Kolmogorov-Smirnov test on data recorded before swim initiation, p=0.0187; first level command units -25.75 ± 50.27 ms; second level command units -17.19 ± 26.90 ms (median ± SD). 60 spikes from 11 first level command units, 120 spikes from 14 second level command units. Kolmogorov-Smirnov test on data recorded after swim initiation, p=0.0923; first level command units 91.09 ± 94.18 ms; second level command units 88.95 ± 104.5 ms (median ± SD). 450 spikes from 11 first level command units, 565 spikes from 14 second level command units. *p<0.05.

**Di)** Firing rates of first level (blue line) and second level (green line) swim driver units recorded before movement initiation and in the first 500 ms of sustained swimming. Dotted grey line (ms=0) marks the start of fictive swimming. Data are presented as mean (solid lines) ± SEM (shaded area). Wilcoxon matched pairs signed rank test, p<0.0394; first level swim driver units 0.0237 ± 0.0035, second level swim driver units 0.0350 ± 0.0042 (mean ± SEM). First level swim driver units N=5, second level swim driver units N=6; minimum 4 trials/unit. *p<0.05.

Discussion

This work presents the first evidence of the distributed and diverse hindbrain neuronal excitability accounting for the long and variable latency to swim initiation in the *X. laevis* tadpole. Threshold and subthreshold trunk skin electrical stimuli evoked distinct hindbrain activity and motor outputs, allowing us to categorise hindbrain units based on their firing patterns and latencies in relation to the initiation of swimming. We identified two groups of the recently proposed hindbrain extension neurons (hexNs; (Koutsikou et al., 2018)), and based on their firing properties, we named them ‘command’ and ‘swim driver’ units.

We showed that both hexN types had the ability to extend the sensory memory based on their variable firing latency and frequency, following stimulation above and below the threshold for swimming (fig. 2A). Their firing patterns were also in agreement with well-established theories on sensory memory and motor decision-making, based on the existence of a variable accumulation of excitation to a threshold for
movement initiation (Brody & Hanks, 2016; Carpenter & Williams, 1995; Gold & Shadlen, 2007; Noorani & Carpenter, 2016).

Furthermore, these units’ firing patterns cannot be ascribed to any of the well-known cell types of the tadpole central nervous system (Roberts et al., 2010). The firing of both types of units differed significantly from the early and mostly single-spike firing of sensory pathway neurons (dla and dlc) (Roberts & Clarke, 1982), as well as the rhythmic and late firing of dINs, key in the initiation and maintenance of locomotor patterns (Koutsikou et al., 2018; Li et al., 2006). Indeed, both types of units fired earlier than the start of locomotion indicated by the first VR burst, and with variable latencies across trials. This agrees with both the latency of synaptic potentials previously recorded on dINs, as well as their long and variable firing (Buhl et al., 2015; Ferrario et al., 2021; Koutsikou et al., 2018; Roberts et al., 2010). This suggests that both command and swim driver units could act pre-synaptically to dINs.

Furthermore, we identified subtypes of both command and swim driver units. Both groups’ subtypes were categorised as ‘first level’ and ‘second level’ units based on their firing patterns in response to subthreshold stimuli; stimulation that did not lead to initiation of fictive swimming. First level units across both groups fired in response to subthreshold stimuli, even if at lower frequency, when compared to the firing after suprathreshold stimulation. This is in full agreement with the presence of synaptic potentials and accumulation of excitation on dINs (Koutsikou et al., 2018), even when the stimulus does not lead to dIN firing and thus to initiation of fictive swimming.

Based on our current findings, we propose a supraspinal mechanism of descending motor control, which includes the newly identified hindbrain units, as depicted in fig. 4. We suggest that command units work as sensory processors in the hindbrain of the tadpole, being postsynaptic to sensory pathway neurons (dlas and dlc)s. Swim driver units act at later stages, providing the necessary overall excitation to dINs in the hindbrain. This is supported by swim driver units’ average spiking latency, which is longer compared to command units’ firing. Initially, the sensory information received by Rohon-Beard cells in the skin is carried to the brain by dlc and dla neurons, it is then weighted and integrated by the proposed sensory processing centre in the hindbrain, comprised by first and second level command units (fig. 4). When the stimulus delivered is strong enough to lead to movement, first and second level command units fire and will excite both subpopulations of swim driver units (stimulation/start; fig. 4A), which in turn provide the cumulative excitation to dINs, allowing them to reach their firing threshold. The firing of dINs will lead to motor neuron excitation and the initiation of the undulatory movement of swimming (Li, Roberts, & Soffe, 2009; Li et al., 2006).

All first level units were also active when the trunk skin stimulus applied was below the threshold for swimming (stimulation/no start, fig. 4B), in contrast to second level units which were inactive in similar conditions. The firing of first level units will still lead to depolarisation of dINs, but only below their firing threshold, thus not allowing swim initiation.

We hypothesise that the second level command population is less likely to fire due to its electrical membrane properties providing the neuronal circuit with the means to discriminate between stimulus intensities. In this scenario, second level command units, as well as first level ones, will receive synaptic input from dlc and dla neurons. However, they will not be activated due to their higher firing threshold. On the contrary, first level command units will be activated at lower stimulus intensities. Once active, second level command units would excite second level swim driver units, which will provide, together with first level swim drivers, strong excitation to dINs (fig. 4A). A different firing likelihood for first and
second level command populations might also explain the slightly delayed firing of second level units before swimming starts, compared to first level command units (fig. 3C).

Although it is not possible to precisely locate the neuronal somata through extracellular recordings, we discovered that swim driver unit firing was preferentially localised in the caudal portion of the hindbrain (fig. 1D), while command units firing was more dispersed along the hindbrain. This anatomical layout might partially reflect the function of the distinct neuronal populations, i.e. swim driver units would be excited, and thus controlled, by command units. This layout across the tadpole hindbrain is in agreement with studies in complex vertebrate brains, where neurons involved in motor decision-making and planning processes have diverse spatial and temporal firing profiles, and they are intermingled across different brain areas (Svoboda & Li, 2018).

In this study we provide the first direct evidence of the spatial and temporal ‘extension’ of sensory information across the tadpole’s hindbrain. We attribute to this hindbrain neuronal activity a major role in the accumulation of excitation on reticulospinal neurons, whose firing, or lack of, will in turn manifest into the tadpole’s binary motor decision to swim or not to swim, respectively. We believe that the identification of the neuron-to-neuron pathways and how individual cells modulate aspects of the tadpole’s swim behavior are the important next steps in unravelling the role of supraspinal brainstem control on motor output.
Figure 4. Proposed neural mechanism for sensory processing and motor descending control in the X. laevis hindbrain.

**A** Scheme of the proposed neural circuit active when a suprathreshold stimulus is delivered to the tadpole’s trunk skin leading to swim initiation (stimulation/start). Solid arrows represent known synaptic connections, solid line boxes indicate known circuits (sensory pathway and swimming). Dotted arrows and boxes represent proposed connections and circuits in the hindbrain (sensory processing, motor planning, descending motor control). A higher firing rate is represented by thicker arrows, compared to the same arrows in **B**. Red star represents stimulation that is strong enough to lead to swim initiation.

**B** Scheme of the proposed neural circuit active when a subthreshold stimulus is delivered to the tadpole’s trunk skin, failing to initiate swimming (stimulation/no start). Solid arrows represent known synaptic connections, solid line boxes indicate known circuits (sensory pathway and swimming). Dotted arrows and boxes represent proposed connections and circuits in the hindbrain (sensory processing, motor planning, descending motor control). A lower firing rate in the various synaptic connections is represented by thinner arrows, compared to the same arrows in **A**. Smaller than in **A**, red star represents a weaker stimulation, which does not lead to swim initiation. Red ‘X’ indicates that the tadpole does not start to swim.
Materials and Methods

Ethics, animal care and preparation

*Xenopus laevis* embryos were supplied by the European *Xenopus* Resource Centre (EXRC; Portsmouth, UK). Animal care and all experimental procedures on *Xenopus laevis* tadpoles were approved by the University of Kent Animal Welfare and Ethical Review Body (AWERB) committee. *Xenopus laevis* tadpoles at developmental stage 37/38 (Nieuwkoop & Faber, 1956) were used and all experiments were conducted at room temperature (19-22°C).

Tadpoles were briefly anesthetized with 0.1% MS-222 (ethyl 3-aminobenzoate methanesulfonate, Sigma-Aldrich), and subsequently immobilized by immersion in a 10 μM α-bungarotoxin (Invitrogen) solution for 50 minutes. Both solutions mentioned above were made in saline (NaCl 115 mM, HEPES 10 mM, NaHCO3 2.4 mM, KCl 3 mM, MgCl2 1mM, CaCl2 2mM) adjusted to pH 7.4. Animals were then mounted onto a rotating Sylgard block submerged in saline, and dissection was carried out as previously described (Buhl et al., 2015; Li et al., 2001). Briefly, the skin covering the brain and trunk muscles on both sides was removed, giving access to the entire hindbrain and to myotomal clefts (Buhl et al., 2015; Li et al., 2001). The trigeminal nerves were severed at both sides of the body to prevent initiation of swimming in response to propagation of skin impulse (James & Soffe, 2011; AM Roberts, 1996; A. S. Roberts, Charles A, 1971).

Electrophysiology

Extracellular ventral root recordings, indicative of fictive swimming, in combination with extracellular recordings of hindbrain neuronal activity, were performed on immobilized *X. laevis* tadpoles at stage 37/38. Two borosilicate glass suction electrodes (tip diameter ~50μm) filled with saline were attached to both sides of the tadpole’s body (fig.1Ai, Aii), approximately at the level of the 5th myotomal cleft (Buhl et al., 2015; Li et al., 2001). A third glass suction electrode (tip opening ~30μm) filled with saline was used to record extracellular hindbrain neuronal activity (fig.1Ai, Aii). The hindbrain recording electrode was randomly positioned in one of the three hindbrain areas depicted in fig. 1Aiii. The electrode’s location was annotated based on its position relative to anatomical landmarks, *i.e.* the midbrain-hindbrain border (MHB), otic capsules and the obex (fig. 1Aiii). Ventral root and hindbrain extracellular activity were amplified, filtered and digitized via a 1041Power (CED, Cambridge, UK) and recorded in Signal 7 (CED, Cambridge, UK). Electrical stimulation was delivered in single square pulses through a glass suction electrode, wrapped in silver wire and filled with saline. This stimulating electrode was attached to the trunk skin at the level of the anus (fig. 1Aii). Both intensity (V) and duration (ms) of the stimulus were set in each experiment as the smallest values required to evoke fictive swimming. All animals initiated fictive swimming after a stimulation within the range of 3.5-4.5 V and 0.25-0.4 ms. Ventral root and hindbrain neuronal activity were recorded in four motor states: 1) at rest, when no stimulus was applied to the tadpole’s skin and ventral root activity was absent; 2) stimulation/no start, when the stimulus delivered was not strong enough to produce fictive swimming; 3) stimulation/start, when the stimulus delivered triggered fictive swimming; 4) swimming, during sustained fictive swimming (fig. 1Aiv).

Data analyses

Spike sorting, based on single spikes’ size and shape, was carried out on all hindbrain extracellular recordings using Spike2 10.00 (CED, Cambridge, UK), and single units were visually evaluated for spike
shape consistency. The number of spikes fired by individual units was counted during 150 ms trials in each of the four motor states (fig. 1Aiv). Randomly chosen 150 ms repetitions throughout the recording were analysed for ‘rest’ and ‘swimming’ states. For the two states where stimulation was applied (‘stimulation/start’ and ‘stimulation/no start’), the time frame analysed was stimulation (t=0) +150 ms. A minimum number of 4 trials were analysed for each of the four motor states in each experiment. Two-way ANOVA with Geisser-Greenhouse correction was run (GraphPad Prism 9) on the number of spikes counted for each unit in the four motor states described above. Stimulation and swimming were the two factors tested in the two-way ANOVA. Depending on the statistical outcome of the two-way ANOVA, units considered for further classification and firing pattern analysis were: 1) units which showed a p value <0.05 for the interaction between stimulation and swimming; 2) units which showed a p value <0.05 for the swimming factor (swim effect). Coefficient of variations (CV=standard deviation/mean) were used to create the heat maps presented in the figures. CV was calculated for each time bin (5 ms) on the number of spikes fired by each unit during the same 5 ms.

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Supplementary Figure 1

A. Neural activity of non significant units is not correlated to swim behaviour. Ai. Average heat map of non significant units recorded in the hindbrain (54 units in 14 animals, minimum of 4 trials per motor state per units). Coefficients of variation (CV=standard deviation/mean) were calculated for each time bin (5 ms) on the number of spikes fired by single units during the same 5 ms in the four motor states. Aii) Examples of spikes fired by one non significant unit recorded in the four motor states investigated (rest, stimulation/no start, stimulation/start, swimming) 200 ms are reported for each example. Orange lines indicate spikes fired by the unit, and they are presented above the respective extracellular hindbrain recording raw trace (black trace). Fictive swimming is shown by rhythmic bursts of the right VR (grey trace, rVR). Red arrowheads represent the delivery of the electrical stimulus; blue arrowhead indicates the start of swimming.

B. Central Patter Generator (CPG) units. Bi. Average heat map of CPG units recorded in the hindbrain (3 units in 3 animals, minimum of 4 trials per motor state per units). Coefficients of variation (CV=standard deviation/mean) were calculated for each time bin (5 ms) on the number of spikes fired by single units during the same 5 ms in the four motor states. Bii) Examples of spikes fired by one non significant unit
recorded in the four motor states investigated (rest, stimulation/no start, stimulation/start, swimming) 200 ms are reported for each example. Green lines indicate spikes fired by the unit, and they are presented above the respective extracellular hindbrain recording raw trace (black trace). Fictive swimming is shown by rhythmic bursts of the right VR (grey trace, rVR). Red arrowheads represent the delivery of the electrical stimulus; blue arrowhead indicates the start of swimming. Bii. Scatter plot of spikes fired by one rostral CPG (blue), one CPG recorded at the otic capsule level (red) and one caudal CPG (green) at the initiation of swimming. Dotted grey line (ms=0) marks the start of fictive swimming. Black solid lines indicate median values. Kruskal-Wallis test, p=0.356; rostral CPG unit 261.9 ± 141.5 ms; CPG unit at the otic capsule level 248.4 ± 143.9 ms; caudal CPG unit 205.9 ± 120.6 ms. Values expressed as median ± SD. 69 spikes from 1 rostral unit, 110 spikes from 1 unit at the otic capsule level, 15 spikes from 1 caudal unit.