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Sensory initiation of a coordinated motor response: synaptic excitation underlying simple decision-making

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Key points

- Deciding whether or how to initiate a motor response to a stimulus can be surprisingly slow and the underlying processes are not well understood.
- Neuronal circuitry that allows frog tadpoles to swim in response to touch is well characterised and includes excitatory reticulospinal neurons that drive swim circuit neurons.
- Build-up of excitation to reticulospinal neurons is the key decision-making step for swimming. Asymmetry in this build-up between the two sides allows bilateral initiation while avoiding inappropriate co-activation of motor antagonists.
- Following stronger stimuli, reticulospinal neurons are excited through a trigeminal nucleus pathway and swimming starts first on the stimulated side. If this pathway fails or is lesioned, swimming starts later on the unstimulated side.
- The mechanisms underlying initiation of a simple tadpole motor response may share similarities with more complex decisions in other animals including humans.
Abstract

Animals take time to make coordinated motor responses to a stimulus. How can sensory input initiate organized movements, activating all necessary elements while avoiding inappropriate co-excitation of antagonistic muscles? In vertebrates the process usually results in the activation of reticulospinal pathways. Young *Xenopus* tadpoles can respond to head-skin touch by swimming which may start on either side. We ask how motor networks in the brain are organized, and whether asymmetries in touch sensory pathways avoid co-activation of antagonists while producing coordinated movements. We record from key reticulospinal neurons in the network controlling swimming. When the head skin is stimulated unilaterally, excitation builds up slowly and asymmetrically in these neurons so those on both sides do not fire synchronously. This build-up of excitation to threshold is the key decision-making step and determines whether swimming will start and on which side. In response to stronger stimuli the stimulated side tends to ‘win’ because excitation from a shorter, trigeminal nucleus pathway becomes reliable and can initiate swimming earlier on the stimulated side. When this pathway fails or is lesioned, swimming starts later and on the unstimulated side. Stochasticity in the trigeminal nucleus pathway allows unpredictable turning behaviour to weaker stimuli conferring potential survival benefits. We locate the longer, commissural sensory pathway carrying excitation to the unstimulated side and record from its neurons. They fire to head-skin stimuli but excite reticulospinal neurons indirectly. We propose that asymmetries in the sensory pathways exciting brainstem reticulospinal neurons ensure alternating and coordinated swimming activity from the start.
Abbreviations

dIN, descending interneuron; tIN, trigeminal descending interneuron; rdlc, rostral
dorsolateral commissural neuron; tSt, trigeminal sensory touch receptor; vr, ventral
root
**Introduction**

There has been extensive study on how humans and other animals initiate directed movements like eye saccades, reaching and locomotion in response to sensory stimulation (Gold and Shadlen, 2007; Dubuc et al., 2008; Grillner et al., 2008; Jordan et al., 2008; Goulding, 2009; Romo and de Lafuente, 2013). An emerging consensus is that the decision process to initiate coordinated movements is complex and much slower than simpler reflex responses or the ballistic escape responses of invertebrates or fish (crayfish: Olson and Krasne, 1981; fish: Korn and Faber, 2005).

Sensory information is thought to travel to higher brain centres. Commands are then sent to motor centres in the brainstem to activate eye or limb movements, or locomotion. This decision process is interactive and takes time (often >100 ms) involving the simultaneous activity of large numbers of neurons in different brain regions (Romo and de Lafuente, 2013). In vertebrates, a critical step is the activation of reticulospinal neurons in the brainstem which then activate motoneurons to produce movements (Dubuc et al., 2008; Jordan et al., 2008; Baker, 2011).

Unfortunately, the detailed neuronal pathways activating reticulospinal neurons during a decision are still unclear and major questions remain. For example, what happens during the decision process to ensure that reticulospinal neurons act together to initiate movement? How is the direction of the first movement determined? How is activation of antagonistic muscles ensured while co-activation is avoided?

To understand the decision to initiate coordinated movement at the neuronal level we need a simpler model for investigation. Larval zebrafish have provided valuable insights into neurons controlling swimming (Kimura et al., 2013) but our choice is the hatchling *Xenopus* tadpole which will swim in response to touch on one side of the
head (Boothby and Roberts, 1995). If the touch is strong enough, the tadpole flexes
to either side and then swims off. In immobilized tadpoles we can use skin
stimulation to initiate fictive swimming recorded in ventral roots and define the
neurons and pathways controlling locomotion. Recent studies have shown that a
small population of electrically coupled reticulospinal neurons drive swimming on a
cycle-by-cycle basis. These descending interneurons (dINs) have been characterized
anatomically and physiologically. They form a longitudinal column extending from the
brainstem into the spinal cord and fire once on each cycle of swimming to drive
similar firing in all other neurons active during swimming, including motoneurons
(Soffe et al., 2009; Roberts et al., 2010; Li, 2011; Moult et al., 2013). We also have
detailed information on the head skin sensory system. On each side, 50-80 trigeminal
sensory touch (tSt) receptors innervate the head skin with “free” nerve endings
(Roberts, 1980; Hayes and Roberts, 1983). Each one fires once to touch and
projects a single axon through the hindbrain. Their excitation converges onto a
hindbrain nucleus of approximately 20 trigeminal nucleus neurons (tINs; Buhl et al.,
2012). The tINs directly excite reticulospinal dINs and if this excitation following head
skin stimulation is sufficient, the whole electrically coupled dIN population is recruited
and swimming starts. Modelling of the dIN population response to sensory input has
shown that electrical coupling between dINs is critical to this all-or-none pattern of
recruitment when swimming starts (Hull et al., 2015). Our work has defined how
stimulation of trigeminal head skin afferents can activate a trigeminal nucleus and the
reticulospinal dINs to initiate swimming on the stimulated side of the body. However,
a significant problem remains. The initiation of swimming requires both sides to be
excited following a stimulus to one side so, as in the spinal cord (Li et al., 2003),
there must be sensory pathway neurons with commissural projections to carry
excitation to the unstimulated side.

When animals decide to make even a simple movement in response to a sensory stimulus the activation of different antagonistic muscles must be coordinated. This is true for eye movements, reaching movements, or the initiation of locomotion. In fish and tadpoles there are basically only two segmented blocks of trunk swimming muscles, one on each side, so coordination should be simpler. When skin touch initiates swimming, motoneuron firing is driven by reticulospinal neurons, but how are these neurons excited on both sides of the body while avoiding synchronous co-activation? Once established, swimming contractions alternate on opposite sides; but when reticulospinal neurons on both sides are first excited, their firing must be coordinated so co-activation, and therefore synchronous contraction of muscles on both sides, is avoided. This is not trivial: synchronous firing is a stable state in simple rhythmic model networks coupled by reciprocal inhibition (Wang and Rinzel, 1992) and can occur transiently in models of the tadpole swimming network (Roberts et al., 2014). Curiously, it has occasionally been seen in whole-cell recordings of tadpole reticulospinal neurons following skin stimulation (Li et al., 2014). Since synchronous bilateral motor activity appears to make no behavioural sense for the tadpole, what prevents it during initiation?

To resolve questions about the role of reticulospinal neurons in the initiation of coordinated swimming we examine *Xenopus* tadpole behavioural responses to head skin stimulation. We then use immobilised tadpoles firstly to record ventral root responses to head-skin stimuli, and then to make whole-cell recordings from hindbrain reticulospinal neurons controlling swimming. Video and ventral root
recordings show that when the head is touched on one side the first flexion of swimming can be on either side, but is earlier if swimming starts on the stimulated side than if it starts on the unstimulated side. Using paired whole-cell recordings from the reticulospinal neurons driving swimming we then demonstrate that the sensory pathways to the two sides differ and lead to an organised but asymmetrical build-up of excitation. As a result, alternating firing of the reticulospinal neuron populations on the two sides is established right from the start and synchronous activity is avoided. Lesions are used to locate the commissural pathway neurons activated by sensory stimulation and we then record their responses. Whole-cell recordings and lesions show that sidedness is determined by the success or failure of the stimulated-side pathway. To threshold stimuli, this pathway can fail so the tadpole responds unpredictably. However, to stronger stimuli when this pathway is reliable, the tadpole responds quickly and flexes to the stimulated side first. We conclude that asymmetry between skin sensory pathways on the two sides allows the bilateral initiation underlying a decision to swim while preventing unwanted co-activation of antagonists.
Methods

Animals

Procedures for obtaining developmental stage 37/38 (Nieuwkoop and Faber, 1956) hatchling *Xenopus laevis* (Daudin) tadpoles comply with UK Home Office regulations. All unregulated experiments on the tadpoles have been approved following local ethical committee review. All chemicals were obtained from Sigma (Poole, UK). Experiments were performed at 18-22 °C.

Behaviour

The response of the tadpoles to a touch stimulus (hair, 10 µm tip) applied to the head were analysed using high-speed video recording (Casio Exilim EX-F1, Tokyo, Japan) with 300 frames per second. For this, the animals were placed in a small petri dish (8 cm diameter) base-filled on one side with a layer of Sylgard in an upright (dorso-ventral) position within a groove cut into the edge of the Sylgard so the head and cement gland were unrestrained. An array of LED lights provided even illumination from below. The videos were cut and analysed using the video editing software Adobe ImageReady (Adobe Systems Inc., San Jose, CA, USA) and image processing software ImageJ (NIH, Bethesda, MD, USA).

Electrophysiology

Following brief anaesthesia (in 0.1% MS-222 3-aminobenzoic acid ester) tadpoles were immobilized using 10 µM α-bungarotoxin in saline (115 mM NaCl, 3 mM KCl, 2 mM CaCl₂, 2.4 mM NaHCO₃, 1 mM MgCl₂, 10 mM HEPES adjusted to pH 7.4 with NaOH) for 20-30 min. For experiments involving only ventral root recordings of fictive swimming, the animals were then pinned to a rotatable Sylgard-coated platform in a
small (~2 ml) recording chamber. Skin was removed from either side of the trunk to allow fire polished glass suction electrodes, with tip openings of 40-60 µm and filled with saline, to be placed at clefts between myotomes where motoneuron axons run. Ventral root signals were amplified by a differential amplifier (gain: 1000, SOBS, University of Bristol, UK) and filtered (low: 30 Hz, high: 1 kHz). Fictive swimming was initiated by stimulation of the head skin with either a touch stimulus or with a brief (0.1 ms) current pulse delivered using a glass suction electrode, again with a tip opening of 40-60 µm filled with saline. This excites the peripheral processes of sensory neurons which enter the brain via the trigeminal nerve (Roberts, 1980). Lesions were performed by hand under a Leica M205C stereo microscope (Leica, Wetzlar, Germany), using mounted pins made from 50 µm diameter tungsten wire, etched to a fine point. The position of lesions was judged by eye relative to clear landmarks: otic capsule, hindbrain segments (rhombomeres) and myotome segments. In order to remove the tIN population unilaterally, superficial internal tissue was removed on one side of the hindbrain in rhombomeres 2 to 4 from the midline to edge enclosing the whole extent of the tIN population (see Buhl et al., 2012). Care was taken not to damage the underlying marginal zone including the axons of trigeminal sensory neurons. The extent of the lesion was judged by prior extensive experience of tIN recordings and effects on swim initiation.

For experiments involving whole-cell recording, immobilised animals were pinned to a rotatable Sylgard-coated platform, and the roof of the hindbrain and parts of the inner surface removed to reveal neuron somata. The animals were then repinned in a small recording chamber (~1 ml) where neuron somata could be seen by using a 40x
water immersion lens on an upright Nikon (Tokyo, Japan) E600FN microscope. Once again, fictive swimming was initiated by stimulation of the head skin with a brief (0.1 ms) current pulse and recorded from the ventral roots using saline-filled suction electrodes applied to intermyotome clefts. Whole-cell current clamp recordings were made with an Axoclamp 2B (Axon Instruments Inc., Foster City, CA, USA) in bridge mode, filtered (at 30 kHz) and digitized (sampling rate: 10 or 20 kHz, ADC resolution: 16 bit) using a CED Power1401mkII interface (Cambridge Electronic Design Ltd, Cambridge, UK) and the standard software Signal 3 and 4 (CED). Patch pipettes were filled with 0.1% neurobiotin in intracellular solution (100 mM K-gluconate, 2 mM MgCl2, 10 mM EGTA, 10 mM HEPES, 3 mM NaATP, 0.5 mM NaGTP adjusted to pH 7.3 with KOH) and had resistances of 5-15 MΩ. The liquid junction potential of this solution was measured as +11 mV, but for better comparison with previous data, the values have not been corrected for this.

**Electroporation**

To identify and record from neurons activating the dINs, animals were dissected as before and parts of the hindbrain removed to access dINs. A glass electrode (2-3 MΩ) was filled with the fluorescent marker AlexaFluor488 (dextran, 10000 MW; Invitrogen) and placed at the presumed position of the dIN dendrites. To label neurons projecting onto these dendrites, a positive current pulse train (10 ms, 50 Hz, 20 V) was applied for 15-30 min and the electrode position occasionally altered during the electroporation process. After 2-3 h, the labelled neurons could be seen under a fluorescence microscope and were then recorded together with a dIN.

**Anatomy**
Anatomical identity of the recorded neurons was routinely checked after the experiments using a standard avidin-biotin technique using diaminobenzidine as chromogen. After experiments the animals were left in the saline for 20-25 min allowing neurobiotin to diffuse throughout the whole cell. The animals were fixed in 2% glutaraldehyde for at least 2 h, washed in phosphate buffered saline (PBS, 120 mM NaCl in 0.1 M phosphate buffer) and again in PBS containing 1% TritonX before labelling with ExtrAvidin (1:200) for 3 h. The animals were then washed again in 0.1 M PBS, the neurons stained in 0.8% DAB in phosphate buffer, and then again in buffer containing DAB and 0.03% H₂O₂ for 5 min each. After washing in tap water, the brain and spinal cord were exposed and the specimen dehydrated in an ascending alcohol series and cleared in methyl benzoate. Specimens were mounted ventral side down between coverslips with DePeX on a reversible aluminium microscope slide. Once mounted, the hindbrain lay opened along the ventral midline like an open book. Neurons were observed using an x100 oil immersion lens and traced using a drawing tube. Photographs of the brain were obtained using an x20 objective lens and a CCD camera (DeltaPix DP 200, Maalov, Denmark) and arranged in Adobe Photoshop (Adobe Systems Inc., San Jose, CA, USA). Measurements were made from the scale drawings and corrected for shrinkage during processing (x1.28). The position of the recorded cell bodies was measured during recording using a micrometre connected to the microscope. It is possible that cell bodies could move and change shape slightly during recording and processing.

Statistics/Data analysis

Data analysis was performed using routines purpose-written for Minitab (Minitab Ltd., Coventry, UK). The latencies of postsynaptic potentials (EPSPs) or the first action
potential were measured as the time between the stimulus and the onset of the PSP and averaged for each cell. Additionally for EPSPs the amplitude, the 10-90% rise time, the time to peak, the slope and the width at half-peak-amplitude were measured. The recorded traces, photographs and drawings were imported in and the figures finally arranged using Corel Draw (Corel Corp., Ottawa, Canada). Statistical tests used are stated in the Results and were performed using Minitab or GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA). The outcome of tests was regarded as significant where \( P < 0.05 \). If not stated otherwise, measurements are expressed as mean ± standard deviation (SD) or median and interquartile range (IQR).
Results

Head touch initiates swimming behaviour

When a tadpole, lying on the bottom of a dish with its cement gland detached from
the substrate, is touched anywhere on the head using a fine hair (10 µm tip), high-
speed videos show that it flexes its trunk and then starts swimming, bending
alternately to right and left at 10 - 20 Hz (Fig. 1A, see Roberts, 1990). Following
gentle manually controlled touch the initial flexion is strong and can be towards
(36.7%) or away from the stimulated side (N = 30 tadpoles). The animal then swims
away in an unpredictable direction (Fig. 1B, see Boothby and Roberts, 1995).

Responses to head-skin stimulation can also be studied in immobilized tadpoles by
recording motor nerve activity in the ventral root with suction electrodes applied
between swim muscles in the trunk (Fig. 1C). A touch or stroke with a fine hair or a
0.1 ms current pulse applied via a suction electrode to the head skin can evoke
fictive swimming (Fig. 1C, D). As with the behaviour, the first motor response to
stimulating the head skin can be on either side of the animal (stimulated side: 49%, N
= 10 tadpoles and 95 initiations). Following just suprathreshold electrical skin
stimulation, reaction times measured to the start of fictive swimming were
significantly shorter on the stimulated side (range 15 - 87 ms, median 25 ms, N = 50
episodes) than on the unstimulated side (range 20 – 71 ms, median 35 ms, N = 41
episodes; GLM on 1/latency: p = 0.01; Fig. 1E). Stimulation of the head skin
therefore leads to swimming activity that can initiate on either side but there is an
asymmetry because swimming generally starts earlier on the stimulated side than on
the unstimulated side.
Recruitment of swim reticulospinal neurons on both sides

To study the pathways responsible for asymmetries in the initiation of the swimming behaviour we recorded from key neurons in the swim network. Reticulospinal and spinal descending interneurons (dINs) are electrically coupled, excite each other, fire a single action potential on each swim cycle, fire first on each cycle to excite all other swim neuron types on the same side, show post inhibitory rebound firing to brief negative current pulses while they are held depolarized, and show pacemaker firing during NMDAR activation (see Roberts et al., 2010; Li, 2011; Moult et al., 2013). We have recently defined a two-synapse pathway from head-skin trigeminal touch afferents via trigeminal nucleus interneurons (tINs) to ipsilateral dINs (Buhl et al., 2012). This study also showed that dIN firing precedes ventral root activity when head-skin stimulation initiates swimming starting on the stimulated side. Since swimming can start on the stimulated or unstimulated side, we therefore expect that dINs on both sides will receive excitation.

To look for asymmetries between the responses of dINs to head-skin stimulation on different sides of the body we made whole-cell recordings from pairs of reticulospinal dINs on opposite sides (Fig. 2; N = 6 pairs). These data were supplemented by recordings from single dINs on each side (N = 50). All dINs were identified by their responses to injected current, activity during swimming and anatomy following neurobiotin injection (Li et al., 2006). In response to a weak electrical stimulus to the head skin, dINs on both sides received excitation. To stronger stimuli, this excitation summed and dIN firing occurred. In 95.4% of cases dIN firing preceded the first ventral root burst of swimming (219 swim initiations in 6 pairs of dINs; Fig. 2C, D) consistent with the conclusion that recruitment of the dIN population initiates
swimming (Buhl et al., 2012). The initial excitation on the stimulated side rose rapidly
to a peak which could lead to firing while excitation on the unstimulated side was
smaller but rose steadily to evoke later firing (Figs. 2C, D, 3A, B; see next section for
analysis). It was surprising that when the dIN on one side fired there was often no
obvious sign of reciprocal inhibition in the dIN on the other side.

The patterns of dIN spike firing times on the two sides were very different with firing
alternating on the two sides (measures are from 230 responses in 6 dIN pairs). On
the unstimulated side (Fig. 3C; black bars), the first dIN spikes had a similar and
broadly unimodal distribution regardless of whether ventral root firing on this side
started first (median delay of 39 ms; inter quartile range 29 - 44 ms) or second
(median delay of 33 ms; IQR 29 - 42 ms). These spike times were not significantly
different (Mann-Whitney test: p = 0.22). On the stimulated side, however (Fig. 3C;
red bars), spike delays had two broad peaks. The first peak corresponds to the cases
when ventral root firing started first on the stimulated side (median delay of 12 ms;
IQR 10 - 17 ms), and spikes occur on the early peak of excitation (Fig. 3A; red
traces). If ventral root firing started first on the unstimulated side (at ~30 - 45 ms)
then there is a later peak of spikes on the stimulated side (Fig. 3B; red traces) with a
median delay of 57 ms (IQR 52 - 62 ms). The timing of these later spikes is the same
as the timing of the second burst of spikes (i.e. next cycle) on the stimulated side
when this side started first (55 ms; IQR 49 - 63 ms; Mann-Whitney test: p = 0.67).

The longer delays to dIN spikes on the unstimulated side correspond with the longer
delays to the start of ventral root activity at the onset of swimming described above
(cf. Fig. 1E; black bars). Repeated stimulation with a stimulus intensity just above the
threshold to evoke swimming showed that the first dIN to fire can be on either side
(46% stimulated side; N = 117 stimulations in 10 dINs). Swimming starts on the side
where the recorded dIN fires first.

Measurements of the firing times of reticulospinal dINs after head-skin stimuli show
that they fall into distinct timing windows which ensure that neurons on the stimulated
and unstimulated sides do not fire at the same time but in alternation from the start of
swimming. This dIN firing appears to result from asymmetrical synaptic drive which
ensures that dINs on the two sides fire after ‘preferred’ delays. While the delay on the
unstimulated side seems fixed (Fig. 3; black bars), the stimulated side fires either
earlier or later than the unstimulated side (Fig. 3; red bars). To seek an explanation
for these differences, we examined the synaptic excitation to dINs on each side
following head-skin stimulation.

Synaptic excitation underlying reticulospinal neuron recruitment
To investigate the excitatory synaptic drive to the reticulospinal dINs on each side we
measured their responses to skin stimulus levels sub-threshold for swim initiation in
paired and single recordings. The compound EPSPs seen in dINs differed on the two
sides (Fig. 4) and were analysed for 10 EPSPs in 10 dINs for each side. The delays
to EPSPs on the stimulated side were slightly shorter (7.0 ± 1.7 ms) than those on
the unstimulated side (9.6 ± 1.8 ms; Mann-Whitney test: p < 0.0001). However, the
main difference was that EPSPs on the stimulated side were faster rising than
contralateral EPSPs (10-90% amplitude in 2.2 ± 0.6 ms, slope: 3.1 ± 1.2 mV/ms
compared to 16.7 ± 6.9 ms and 0.5 ± 0.5 mV/ms; both t-test: p < 0.0001; Fig. 4D).
EPSPs on the stimulated side, which we assume are mediated by direct synapses
from trigeminal nucleus tINs (Buhl et al., 2012), fell away after an early peak. EPSPs
on the unstimulated side, mediated by a presently unidentified pathway which we assume must fire repetitively, kept increasing. The sensory-evoked EPSPs in dIN reticulospinal neurons on each side of the body are therefore asymmetric and this could explain the differences in first-spike timings (Figs 2C, D and 3).

Effects of lesions to reticulospinal neuron sensory input pathway

Behavioural experiments show that swimming can start on either side following stimulation of the head-skin on one side (Fig. 1), and our recordings show that, in addition to slowly rising excitation, the reticulospinal dINs on the stimulated side receive short-latency, fast-rising excitation from trigeminal interneurons (tINs; Buhl et al., 2012). In response to stronger stimuli, these tINs fire more and are more likely to activate dINs. Our hypothesis is that if the tIN excitation causes early, same-side dIN spiking, swimming starts on the stimulated side, but if tIN excitation does not cause early spiking, swimming starts on the unstimulated side. To test whether the ipsilaterally projecting tIN population could influence the side on which swimming starts, we surgically removed the region of hindbrain containing the defined cluster of tIN somata on one side while leaving trigeminal central axon projections intact (Fig. 5A). Ventral root recordings from operated animals (N = 5, 10 initiations each) showed that, following lesioning, swimming could still be initiated in a well-organized pattern from the start without any synchronous ventral root activity on both sides (not illustrated).

In response to strong stimuli (twice threshold to initiate swimming), control animal swimming started reliably on the stimulated side (100%; Fig. 5B) and removal of the tIN population led to significantly fewer starts on the stimulated side (40%; Mann-
Whitney test: p < 0.0001). As a control, swimming was initiated by dimming the light (cf. Roberts, 1978) and responsiveness and sidedness was similar before (56% right side) and after tIN removal (58% right side; Mann-Whitney test: p = 0.84). The delay to the first ventral root burst on the stimulated side of 23 ms (median, IQR 19 - 40 ms) was significantly shorter than on the unstimulated side (57 ms, IQR 48 - 95 ms, Mann-Whitney test: p < 0.0001; Fig. 5C). Removal of the tINs increased this delay only on the stimulated side to 80 ms (IQR 52 - 99 ms, Mann-Whitney test: p < 0.0001) while leaving the unstimulated, intact, control side unaffected (treated: 57 ms, IQR 42 - 90 ms, Mann-Whitney test: p = 0.86).

Taken together, these results suggest that the population of the tINs, forming the trigeminal nucleus, is responsible for the short-latency ventral root response on the stimulated side. The source of the slower rising excitation following head-skin stimulation and seen in dINs on each side of the body will be evaluated in the discussion. Even after tIN lesioning, no synchronous left-right motor activity was seen at the start of swimming in any recordings. This means that, in response to a stimulus to the head-skin, the populations of dINs on each side receive asymmetrical input that is independent of, and in addition to, the asymmetric input from tINs. We propose that this asymmetrical input to dINs leads to reliable alternating motor activity right from the start of swimming.

Lesion experiments locate commissural sensory pathway in hindbrain

To try to explain the basis for asymmetry in the sensory excitation to reticulospinal dINs following head-skin stimulation, the next step was to determine the pathway crossing to dINs on the unstimulated side. Trigeminal afferents, tINs and dINs all
exclusively project ipsilaterally. We therefore used lesions with electrical stimulation of the head-skin on the right side to localize areas where commissural axons could carry excitation to the unstimulated (left) side and allow swimming (Fig. 6A; N = 16 tadpoles). Unilateral transection of the right-side hindbrain anywhere in rhombomeres 5 and 6 (Fig. 6A pink) blocked the initiation of swimming (N = 5; Fig. 6B, C), although swimming could still be evoked by stimulating the trunk skin (Fig. 6D). These lesions suggested that there was no sufficient commissural pathway in the rostral part of the hindbrain. However, midline lesions which cut most commissural connections between the two sides of the hindbrain and rostral spinal cord but left some intact caudally in rhombomeres 7 to 8, did not block the initiation of swimming (N = 11; Fig. 6E, F). These experiments suggested that sensory excitation from the trigeminal afferent neurons innervating the head-skin crosses to the other side in the caudal hindbrain at the level of rhombomeres 7 and 8. It should be noted that while axons crossing at this level are sufficient to allow initiation of swimming, suitable crossing axons are not restricted to this region: midline cuts that included rhombomeres 7 and 8 (N = 12) did not block initiation unless extended rostrally and/or caudally. As our aim was to localise a region for further investigation, we did not attempt to define the extent of additional crossing.

**Head-skin stimuli recruit commissural sensory pathway neurons**

In the spinal cord a dorsolaterally positioned longitudinal column of sensory projection neurons with commissural axons are strongly and monosynaptically excited by trunk skin sensory Rohon-Beard neurons and fire at high frequency during above-threshold current injection. These dorsolateral commissural neurons (dlcs) project to the opposite side where they make glutamatergic synaptic connections.
(Clarke and Roberts, 1984; Roberts and Sillar, 1990; Li et al., 2003). If the most rostral members of the dlc population lying in the hindbrain were excited by the descending axons of trigeminal afferents they could carry excitation from head-skin touch to the opposite side. We therefore searched for such neurons by making whole-cell recordings with neurobiotin filling from dorsolaterally located somata in the caudal hindbrain. To find these neurons for recording, we back-filled their crossing axons by electroporating the fluorescent dye AlexaFluor488 using an electrode in the marginal zone on the opposite side.

We named the neurons in the hindbrain responding at short latency to trunk and head-skin stimulation and with evidence of commissural axon projections rostral dorsolateral commissural neurons (rdlcs). These neurons (N = 14) lay in a region 560-880 µm from the mid-hindbrain border. Like dlcs in the spinal cord, they were excited to fire once at short latency (7.7 ± 1.9 ms) by ipsilateral trunk skin stimulation (Fig. 7; Table 1). However, the rdlcs were also excited to fire, and at even shorter latency (6.5 ± 1.7 ms), by head-skin stimuli. If the stimulus threshold to initiate swimming is 100%, the threshold for rdlc EPSPs was 95 ± 3% and for rdlc spiking 97 ± 3%. The large EPSPs in rdlcs following head-skin stimulation were similar to those evoked by trunk stimulation (Table 1; analysed below spike threshold). Basic cellular properties were measured for 14 rdlcs (Table 1). When depolarizing current was injected, rdlcs fired a single action potential at threshold but at higher current levels they fired repetitively (Fig. 7E). Neurobiotin fillings showed that they have dorso-laterally located, uni- or multipolar somata (13-16 µm diameter) with several short dendrites, some of which are on the initial part of the axon (Fig. 7F). Their single axon runs ventrally to cross the midline at a similar longitudinal level as the soma and
on the contralateral side, it bifurcates to ascend into the hindbrain and descend into the spinal cord.

The anatomical and physiological properties of rdlcs and their responses to trunk-skin stimulation correspond to those of spinal dlcs (Clarke and Roberts, 1984; Roberts and Sillar, 1990; Li et al., 2003). The critical additional feature is that rdlcs are also excited following head-skin stimulation (Fig. 7C, D). This excitation is at slightly shorter latencies than that from trunk-skin stimulation where conduction times from a more distant stimulus location would be greater. The EPSP latencies in rdlcs following head-skin stimulation were 4.9 ± 1.3 ms which is similar to latency ranges for EPSPs in spinal cord sensory interneurons following trunk-skin stimulation (dlc: 5.0 to 7.0 ms and dla: 3.5 to 6.7 ms), where their connections from the Rohon-Beard sensory neurons were shown by paired recording to be monosynaptic (Roberts and Sillar, 1990: Li et al., 2003, 2004). It is also within the range of the 4.4 ± 0.5 ms latencies measured in tINs lying more rostral, and therefore closer to the stimulus and which were also presumed to be monosynaptic (Table 1; Buhl et al., 2012). This suggests that rdlc EPSPs result from direct synapses made by the descending axons of primary trigeminal sensory neurons innervating the head skin.

The connections of rdlcs onto dINs were investigated by making paired recordings from them with the excitatory dINs on the opposite, unstimulated side. In 11 paired recordings, neurobiotin filling showed that the rdlc axon crossed ventrally to lie in close proximity to the recorded dIN dendrites. To stimuli which evoked swimming, the rdlcs fired only once and EPSPs were recorded in dINs. However, these EPSPs had long and variable latencies (Fig. 7F, G) and their shapes suggested that they were
the compound sum of presynaptic spikes occurring at different delays. The delays
from rdlc spikes to the onset of dIN EPSPs ranged from 2.9 to 7.1 ms (mean with SD:
4.5 ± 0.9 ms, N = 27 initiations in three pairs; Fig. 7G). These delays are much longer
than seen in other monosynaptic, tadpole synapses (cf. Li et al., 2003; Buhl et al.,
2012). Furthermore, when an rdlc spiked in response to current injection, no PSPs
were seen in the recorded dIN. These observations suggested that additional
neurons lie interposed in the pathway between rdlcs and dINs on the opposite side,
and that this contributes to the delay in the response on the unstimulated side. While
we cannot rule out the possible contribution of other neurons, our results suggest the
rdlcs as strong candidates for bringing the excitation to the contralateral side
following head-skin stimulation, acting via some currently unidentified interposed
neurons.
Discussion

Defining complete, neuron by neuron pathways, from sensory stimulation to the
initiation of coordinated movements is difficult (Vinay et al., 1995; Sparks, 2002;
Viana Di Prisco et al., 2005) even in invertebrates (Esch et al., 2002). To trace the
origins of the vertebrate networks initiating locomotion we chose a very simple case:
swimming of hatchling *Xenopus* tadpoles initiated by touching one side of the head.
Video recordings showed that tadpoles first make a strong flexion whose direction,
like the subsequent swimming path, is variable and unpredictable (Fig. 1A, B;
Boothby and Roberts, 1995). Similar fictive responses, recorded in immobilised
tadpoles (Fig. 1C-E), show the underlying motor activity. We previously defined a
sensory pathway for trigeminal sensory touch (tSt) receptors to initiate swimming on
the stimulated side following head touch (Buhl et al., 2012). In the present study we
investigated how tSt firing on one side also leads to dIN excitation on the
unstimulated side, necessary for bilateral initiation. Whole cell recordings show that
tSts directly excite rostral dorsolateral commissural neurons (rdlcs) to fire once.
These neurons are the most rostral members of a population of spinal sensory
pathway neurons excited by trunk skin afferents and carrying excitation to the
opposite side (Clarke and Roberts, 1984; Roberts and Sillar, 1990; Li et al., 2003).
Perhaps surprisingly, paired recordings from rdlcs and dINs on opposite sides failed
to show direct connections and also suggested that the delays between rdlc firing
and dIN EPSPs following skin stimulation were actually too long for direct
connections. We conclude that instead of directly exciting dINs, rdlcs do so indirectly
via some unidentified neuron type. This step introduces a delay within the initiation
process and also the potential for flexibility.
We will now consider the main questions we have addressed: What is required for the tadpole to decide to initiate locomotion? How does it organize alternation when swimming starts while avoiding synchronous activation of antagonist muscles? We will also ask what makes swimming direction unpredictable. Like other vertebrates (lamprey: Viana Di Prisco et al., 2005; cat: Aoki and Mori, 1981; rat: Vinay et al., 1995), tadpole locomotion can be initiated by head-skin stimulation. The initiation decision requires a series of thresholds to be crossed, leading ultimately to coordinated firing in the bilateral populations of reticulospinal dINs.

**Decision-making steps for initiation of locomotion**

The sensitivity of the touch-sensory nerve endings will determine the first threshold when one sensory neuron fires once. Increasing stimulus strength will recruit more sensory neurons, each firing a single spike (Buhl et al., 2012), but evidence on receptive field overlap suggests that the maximum number will be less than 10 (Roberts, 1980). Sensory neuron spikes then bring sensory pathway neurons with ipsilateral (tlNs) and commissural (rdlcs) axon projections to threshold. This step in the pathway can amplify the excitation from a few sensory neurons by recruitment of more sensory pathway neurons. In the case of tlNs, there is further amplification because they can fire multiply (Buhl et al., 2012). The limited number of sensory pathway neurons may also help to restrict the maximum possible excitation if large numbers of head-skin sensory neurons are excited simultaneously. Finally, the sensory pathway neurons excite the reticulospinal dIN neurons, the critical neurons in the generation of swimming, either directly (tlNs) or indirectly (rdlcs) via unknown neurons.
Our evidence suggests that the decision to swim takes place in the populations of reticulospinal dIN neurons which synaptically drive the other swim neurons, including motoneurons, to fire on each cycle (Roberts et al., 2010; Li, 2011). Our new evidence indicates that, following skin stimulation, excitation builds up in dINs until their firing threshold is crossed on one side. Because they are electrically coupled to each other, recruitment of firing in a few dINs leads to recruitment of the whole dIN population on this side and, as a result, initiation of swimming. As this happens, excitation will also be building up on the other side. Our whole-cell recordings of responses in single or pairs of dINs to head skin stimulation show that their first firing virtually always precedes the start of swimming recorded in the ventral roots (95.4%: 209 of 219 trials). This confirms the results from our previous study of dINs on the stimulated side where dIN firing also preceded ventral root swimming (98%: 170 of 173 trials; Buhl et al., 2012) supporting our view that dIN population firing is the necessary precursor to swimming and the key decision-making step in initiation. Modelling of the hindbrain dIN population has shown that without electrical coupling recruitment is gradual and often incomplete, whereas with electrical coupling recruitment follows a step function: where dINs are recruited all-or-none (Hull et al., 2015). This is exactly what is needed for a tadpole to decide to swim.

Xenopus reticulospinal dINs are probable homologues of Chx10-expressing V2a neurons in larval zebrafish (Kimura et al., 2013), which have similar distribution, anatomy and functions during swimming. The optogenetic activation of V2a neurons in the hindbrain using channelrhodopsin evokes swimming, whereas their inactivation using archaerhodopsin3 or halorhodopsin reliably stops on-going swimming. Taken together with our evidence, this points to these hindbrain neurons being the place
where a simple threshold mechanism together with electrical coupling is the basis for
the decision to swim. Since both sides are depolarized by the time dINs on one side
fire, inhibition from the side starting first could allow rebound firing on the opposite
side and help ensure that the response initiated is bilateral (Moult et al., 2013).

Asymmetric initiation of motor activity

When animals make even simple motor responses they need to be coordinated from
the start and in mammals this may be one reason why reaction times are long and
variable, even for simple eye movements (90 to 400 ms; Gold and Shadlen, 2007). In
the tadpole, reaction times to the start of swimming following skin stimulation are also
long and variable (15 to 87 ms) compared to reflexes (~10 ms; Li et al., 2003).

Perhaps our most striking observation is the very organized firing patterns of
reticulospinal dIN neurons on each side of the body which ensures alternation of
body contractions from the start (Figs 2 and 3). This firing pattern appears to depend
primarily on an asymmetric underlying excitatory drive from the head-skin sensory
pathways to each side of the body. Since reticulospinal dINs are known to excite
reciprocal inhibitory commissural neurons (Li et al., 2006), we would expect to see
IPSPs on one side when dINs are recruited on the other side. Such IPSPs were not
apparent (Figs 2 and 3). This may be because inhibitory cINs are not yet firing or that
the dIN membrane potential is very close to the IPSP reversal potential (Sautois et
al., 2007). Whether there is some additional contribution from reciprocal inhibition
remains to be examined.

How is sensory excitation organized to produce asymmetrical input to the motor
system? The clearest evidence is that trigeminal nucleus neurons (tINs) directly
excite ipsilateral dINs via glutamatergic synapses to produce short-latency EPSPs. These EPSPs can sum to threshold and lead to recruitment of dIN firing at short latencies of ~15 ms (Fig. 2C; Buhl et al., 2012). Swimming then starts on the stimulated side. Regardless of whether the dINs on the stimulated side fire, the next dIN population to be recruited is on the unstimulated side. The excitatory input here is variable and ‘bumpy’ (Fig. 4) but builds up to threshold so firing occurs at ~35 ms. Finally, after the unstimulated side has fired, excitation on the stimulated side builds up in a very variable fashion and dINs fire at ~60 ms. The asymmetrical pattern of sensory pathway excitation to the reticulospinal dINs therefore coordinates a rather variable but strictly alternating response to stimulation.

Our present picture of the pathways exciting reticulospinal neurons following skin stimulation is summarised in figure 8. At present we can account for the early excitation by trigeminal nucleus neurons on the stimulated side but the neurons producing the more slowly increasing excitation on each side remain unknown. These neurons must fire repetitively following excitation from the head sensory pathway to provide long-lasting AMPAR and NMDAR activation of dINs through EPSPs which could sum to bring dINs to their firing threshold after the observed variable delays. Their sensory excitation on the stimulated side could be direct from trigeminal sensory axons or via trigeminal nucleus neurons. On the unstimulated side, we expect these neurons to be excited by commissural sensory pathway neurons (rdlcs) and to be excited more strongly than on the stimulated side. This would produce the required asymmetry of dIN activation. It is interesting that in other motor systems a group of “trigger” neurons is often present. These are excited by sensory input and activate rhythm-generating neurons which are the analogues of
reticulospinal dINs (cf. Brodfuehrer and Friesen, 1986).

Unpredictable direction of response

The decision to respond appropriately to sensory stimulation is central to an animal’s survival. Swimming away from danger is one of the few defence mechanisms for hatchling *Xenopus* tadpoles. This allows them to avoid predators such as adult *Xenopus*, water scorpions, and the larvae of beetles, damselflies and dragonflies (Lawler, 1989). To avoid danger, a widespread strategy among animals is to move away in an unpredictable direction (Domenici et al., 2011). The ballistic escape responses of invertebrates or fish are fast but predictable (crayfish: Olson and Krasne, 1981; fish: Korn and Faber, 2005), and this can be exploited by predators like water snakes which learn which way prey will turn (Catania, 2009). We have found in the tadpole that for stimuli close to threshold, the side contracting first is unpredictable. In addition, regardless of the side of the first bend, the direction of subsequent swimming is very variable (Boothby and Roberts, 1995; and see Fig. 1B). When stronger stimuli are given to the head skin, the latency to dIN firing is shorter and they reliably fire first on the stimulated side. The response becomes a fast but predictable flexion to the stimulated side and our evidence from lesioning suggests that this response depends on tIN excitation to dINs (Fig. 5). Stronger stimuli recruit more tINs to fire more impulses (Buhl et al., 2012). Our hypothesis is that the unpredictable response to weaker stimuli depends on the unreliable firing of the sensory pathway tIN neurons. Weaker stimuli will recruit fewer tINs to fire fewer impulses and make ipsilateral dIN firing, and therefore swimming direction, unreliable. Furthermore, the tINs have another property which could enhance unpredictability. Paired recordings show that their synapses onto dINs fail in 50% of trials (Buhl et al., 2012). In contrast, the commissural pathway exciting dINs on the
unstimulated side leads to a slower and variable build-up of excitation but this leads
to reliable firing within a defined time window. The result is that swimming starts later
on the unstimulated side when excitation within the stimulated side fails to recruit
dINs.

Conclusion
The task faced by the tadpole as it initiates swimming is in essence the same as for
many other directed motor responses. The decision to swim involves key neurons, in
this case excitatory reticulospinal neurons, crossing firing threshold as a result of
incoming signals, in this case from head skin sensory pathways. Even in the very
simple tadpole motor system, reaction times for the initiation of swimming are long
when compared to reflexes. This appears to be a common feature of other decision-
making processes (Gold and Shadlen, 2007). Successful implementation of the
decision again involves dealing with a common requirement in motor systems:
antagonists must both be activated, but synchronous co-activation must be avoided.
In the tadpole, we have shown how this is built in to the decision-making process by
an asymmetry in the pattern of excitation to the reticulospinal neurons. A further
consequence of this asymmetry is that, while for stronger stimuli the response is
largely stereotyped, the direction of response following weaker stimuli is
unpredictable. In the tadpole, this unpredictability may aid survival; in other systems,
mechanisms may be needed to control or prevent it.
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Additional information

Competing interests: The authors declare no conflict of interest.

Author contribution: The experiments were performed in the neurobiology lab at the University of Bristol. All three authors contributed to the conception, design and carrying out of experiments, analysis of data and writing the manuscript. All authors approved the final version of the manuscript.

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Figure and table legends

Figure 1. Swimming in response to head-skin stimulation.

A, The tadpole and video frames at 150 fps of a tadpole viewed from above responding to touch on the left side of the head with a fine hair (arrow) by flexing to the left (frames 4 - 10) and then swimming off (frames 14 - 18). B, Tracings from similar videos after head stimulation on the left side (asterisk) plot head position to show flexion to left or right and swimming in a range of directions (arrows). C, D, In the immobilized tadpole the diagram shows the brain, spinal cord and innervation of head skin by a sensory neuron in the trigeminal ganglion (tg). The positions of suction electrodes to record ventral root activity from swimming muscles (Rr, Rc, Lr, Lc) and to stimulate the right head skin are indicated. Fictive swimming can start on the stimulated side after touch (arrow, C) or current pulse (asterisk, D) stimulation to the head skin. E, Histogram of latencies to the start of swimming in immobilized tadpoles following skin stimulation on the same (red bars) or opposite (black bars) side of the head (bin size: 5 ms).

Figure 2. Excitation and alternating firing of reticulospinal dlNs on both sides after head-skin stimulation.

A, Diagram of the positions of recording and stimulating electrodes. B, Photograph showing a recorded pair of dlNs on either side of the hindbrain (dorsal view, rostral to the left; red rectangle in A) with soma, dendrites and both ascending and descending axons. The descending axon of the left side dlN was damaged during the dissection (at *). C, D, Each panel shows three overlain responses from the right ventral root and the pair of dlNs shown in B. In each case swimming starts following a left head-skin stimulus (at arrowhead) and grey bars show the phase when spikes occur on the
unstimulated side. In C, the first dIN spike is at short latency on the left, stimulated side (red). In D, there is a short-latency EPSP in the left dINs but the first spike is later on the unstimulated right side (black) and is followed by the first spike on the stimulated side.

**Figure 3.** Alternating pattern of first and second reticulospinal dIN spike times on each side of the body following head-skin stimulation.

A, B, Both panels show responses from a different pair of dINs to the one shown in Figure 2. In each case activity starts following a head-skin stimulus (at arrowhead) and grey bars show the phase when spikes occur on the unstimulated side. In A, the first dIN spike is at short latency on the stimulated side (red) while in B, the first spike is later on the unstimulated side (black) and each is followed by the first spike on the other side. C, Plots show spike occurrences on each side at different times after a stimulus to one side of the head (red and pink bars are stimulated side; black and grey are unstimulated side). Measures are from 230 responses in 6 dIN pairs, bin size is 5 ms.

**Figure 4.** Asymmetry in EPSP responses to low-level skin stimulation in dINs on stimulated (red) and unstimulated (black) sides.

A-C, Example EPSPs in response to low level stimulation show differences between the 2 sides in recordings from 3 different dIN pairs. D, Averages of 10 responses from records in C (solid lines) show that the stimulated side EPSPs are earlier and rise faster. Dotted lines either side of curves are SDs.

**Figure 5.** Effects of removing the tIN population on one side on swimming responses
to head-skin stimulation.

A, Diagram showing the stimulation and recording setup and area where tINs were removed (blue). B, Side of first ventral root response (mean, SD) to electrical stimulation and light dimming, in control (black) and treated animals with tINs removed on the stimulated side (blue). Responses to light were similar before and after treatment. C, Delays to first ventral root response (median, IQR) on the stimulated and unstimulated side following head-skin stimulation. Delays increase significantly after removal of the tINs on the treated side only. Asterisks indicate a significant difference of $p < 0.0001$ (Mann-Whitney test).

Figure 6. Lesion experiments showing where trigeminal excitation can cross to the unstimulated side in the caudal hindbrain.

A, Summary of regions of the hindbrain where unilateral transection prevented swimming (pink) or where midline cuts (green) still allowed the initiation of bilateral swimming following a head-skin stimulus applied to the right side (rhombomeres numbered). The trigeminal ganglia on the left side were severed to prevent contralateral sensory access (red line). B, An example of a rostral unilateral transection (pink line). C, Ventral root recordings show this lesion prevented swim initiation to a head-skin stimulus (arrowhead) but not to a trunk-skin stimulus (D). E, An example of a mid-line lesion (green line) which only left a short intact region of caudal hindbrain (arrow). F, This was sufficient to allow initiation of bilateral swimming following a head-skin stimulus to the right side. Ventral root recording positions on right (upper traces) and left (lower traces) sides are indicated in B and E.

Figure 7. Sensory pathway rostral dorsolateral commissural neurons (rdlcs) respond
to head and trunk skin stimulation.

A, Diagram of the preparation viewed dorsally showing the position of the stimulating and recording electrodes. B, Diagram of rdlc neurobiotin fill to show axon extent in hindbrain with enlarged tracing of rdlc with soma, dendrites and axon crossing the midline and ascending and descending on the contralateral side. C, D, Traces show rdlc response to ipsilateral trunk and head-skin stimulation (arrowhead) of increasing strength from black = no response, red = EPSP alone, blue = spike, green = response to strong stimulus. E, Current injected into an rdlc evokes a single spike at threshold and fast multiple firing at higher levels. F, Diagram showing the position of the electrodes for paired recording and a record of responses of rdlc and dIN to right head stimulus which starts swimming as well as low and higher magnification tracings of the recorded cells in box. G, Responses of another rdlc/dIN pair to head-skin stimuli, aligned by rdlc spikes to show EPSPs with variable delays (arrowheads) and shapes. Coupling artefact (*).

Figure 8. Swim initiating network.

A, Diagram of the brain in dorsal view and the head-skin trigeminal pathways initiating swimming on the same or opposite sides. Dots show locations of neuron populations not their real numbers: touch-sensory trigeminal neurons (tSt, yellow), sensory pathway neurons (tIN, pale magenta; rdlc, red), electrically coupled reticulospinal excitatory neurons (dIN, brown). fb – forebrain, hb – hindbrain, mb – midbrain, m – muscles, oc – otic capsule, sc – spinal cord, tg – trigeminal ganglion B, Functional diagram of the network including the central pattern generator (swim circuit) neurons: inhibitory interneurons (cIN, blue; aIN, purple) and motoneurons (mn, green). Solid lines indicate good evidence for monosynaptic connection. Dashed
lines indicate indirect connections (delay). Large circles represent populations of neurons. Small circles (inhibitory) and triangles (excitatory) are synapses and when they contact a box they connect to all neurons in the box.

Table 1. Measurements of rdLc EPSPs and spikes in response to ipsilateral head and trunk skin stimulation and basic properties.

For each parameter the mean, standard deviation and number of measured neurons (in brackets) is given.
### Table 1

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<th>trunk stimulation</th>
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<td>$4.9 \pm 1.3$ ms (20)</td>
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<td>EPSP amplitude (no spike)</td>
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<td>AP width at half peak</td>
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</table>
**A**

![Image of a fish](image)

1 mm

**B**

- flex towards
- flex away

**C**

- stim skin
- stroke skin
- swim muscles
- spinal cord
- hindbrain

**D**

- Lr
- Lc
- Rr
- Rc

**E**

- spike time after stimulus (ms)
- frequency

- stim side start
- unstim side start

*Legend:*

- *: indicates specific events or conditions related to the depicted images and data.
Time for spikes on unstim side

Average

unstim

stim

unstim

stim