The modulation of two motor behaviours by persistent sodium currents

in *Xenopus laevis* tadpoles

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Author contributions: E.S. and W.C.L. conception and design of research. E.S., H.J. and W.C.L. performed experiments, analysed data, and prepared figures. E.S. and W.C.L. wrote the manuscript and all authors approved the final version.
Abstract

Persistent sodium currents (I_{NaP}) are common in neuronal circuitries and they have been implicated in several diseases like amyotrophic lateral sclerosis (ALS) and epilepsy. However, the role of I_{NaP} in the regulation of specific behaviours is still poorly understood. Here we have characterized I_{NaP} and investigated its role in the swimming and struggling behaviour of *Xenopus* tadpoles. I_{NaP} was identified in three groups of neurons, namely sensory Rohon-Beard neurons (RB neurons), descending interneurons (dINs), and non-dINs (neurons rhythmically active in swimming). All groups of neurons expressed I_{NaP} but the currents differed in decay time constants, amplitudes, and the membrane potential where I_{NaP} peaked. Low concentrations (1 µM) of the I_{NaP} blocker, riluzole, blocked I_{NaP} for ~ 30 % and decreased the excitability of the three neuron groups without affecting spike amplitudes or cellular input resistances. Riluzole reduced the number of rebound spikes in dINs and depressed repetitive firing in RB neurons and non-dINs. At the behaviour level, riluzole at 1 µM shortened fictive swimming episodes. It also reduced the number of action potentials neurons fire on each struggling cycle. The results show that I_{NaP} may play important modulatory roles in motor behaviours.

Key words: *Xenopus* tadpole, motor behaviour, spinal cord, sodium currents, riluzole

New and noteworthy: In this paper we have characterized persistent sodium currents in three groups of spinal neurons and their role in shaping spiking activity in the *Xenopus* tadpole. We then attempted to evaluate the role of persistent sodium currents in regulating tadpole swimming and struggling motor outputs by using low concentrations of the persistent sodium current antagonist riluzole.
Introduction

Transient sodium currents (I_{Nat}) and persistent sodium currents (I_{NaP}) are generated by the same sodium channels, depending on their opening states. I_{Nat} accounts for the fast depolarisation during action potentials and I_{NaP} have been shown to modulate spiking patterns (Bean 2007; Crill 1996; Theiss et al. 2007a). Ten voltage-gated sodium channels have been identified in mammals and six have been located in the spinal cord and/or dorsal root ganglion where they have been implicated in pain transmission and spasticity after spinal cord injury (Brocard et al. 2016; Catterall et al. 2005; Dib-Hajj et al. 2013; Waxman and Zamponi 2014). The basic organization of the spinal locomotor circuitry and the ion channels expressed show a high degree of similarities in lower vertebrates and in mammals (Grillner and El Manira 2015; Grillner and Jessell 2009; Kiehn 2016; Roberts et al. 2012) and I_{NaP} has been identified in many spinal neurons (Benedetti et al. 2016; Hu et al. 2002; Miles et al. 2005; Tazerart et al. 2008; Theiss et al. 2007a; Tong and McDearmid 2012; Zhong et al. 2007). Low concentration of riluzole blocks the I_{NaP} (Urbani and Belluzzi 2000) and is used to treat amyotrophic lateral sclerosis (ALS) (Bellingham 2011; Benedetti et al. 2016; Devlin et al. 2015; Jenkins et al. 2014; Quinlan et al. 2011), which is associated with hyperactivity of spinal motor neurons and upper motor neurons in the primary motor cortex (Caballero-Hernandez et al. 2016). It is necessary to understand how I_{NaP} modulates motor behaviours in a trackable spinal circuit and assess the actions of riluzole (Benedetti et al. 2016).

Xenopus tadpole spinal and hindbrain circuits controlling swimming and struggling have been mapped using paired whole-cell recordings (Berkowitz et al. 2010; Roberts et al. 2010). Tadpole swimming central pattern generator comprises of descending interneurons (dINs), commissural interneurons (cINs), ascending interneurons (aINs) and motoneurons. Among them, dINs provide the phasic excitation to drive other types of rhythmic neurons while their
own firing is sustained by rebound firing following mid-cycle inhibition or NMDAR-dependent pacemaker properties (Li et al., 2006, Soffe et al., 2009, Li et al., 2010). When the tadpole skin is stimulated repetitively, two types of interneurons are recruited (excitatory commissural interneurons/e-clNs and repetitive firing descending interneurons/dINrs) but the dIN activity is suppressed (Li et al 2007; Li 2015). Tadpole neurons involved in swimming and struggling display different types of firing properties in response to depolarizing current pulses (Li et al. 2007a; Li et al. 2007b; Sautois et al. 2007; Winlove and Roberts 2012). dINs fire a single spike at the onset of depolarizing step currents while other rhythmic neurons show repetitive firing, often with a delay caused by A-type potassium currents (li 2015). The properties of the INaT have been characterized in dissociated tadpole spinal neurons and sensory neurons and sensory interneurons \textit{in situ} (Dale 1995; Winlove and Roberts 2012).

We analyse sodium currents in the neurons involved in tadpoles swimming and struggling \textit{in situ}. We report INaP in the Rohon-Beard neurons (primary sensory neurons, RB), the excitatory descending interneurons (dINs), and other rhythmic neurons in tadpole swimming and struggling (non-dINs) (Roberts et al. 2008). We have used the INaP antagonist, riluzole, at 1 µM to investigate its role in tadpole swimming and struggling.

\textbf{Methods}

All experiment procedures were approved by the local Animal Welfare Ethics committee and comply with UK Home Office regulations. Human chorionic gonadotropin injections were carried out to induce mating between pairs of adult \textit{Xenopus}. Tadpoles at stage 37/38 (Nieuwkoop and Faber 1956) were anaesthetised using 0.1% MS222 (3-aminobenzoic acid ester, Sigma, UK), then immobilised using 12.5 µM α-bungarotoxin (Tocris, UK) and mounted onto a sylgard stage for dissections (Moult et al. 2013a). The saline contained in mM: 127 NaCl, 3 KCl, 2 CaCl2, 2.4 NaHCO3, 1 MgCl2, 10 HEPES, with pH adjusted to 7.4.
Fine dissections were carried out to expose muscle clefts for recording motor nerve (m.n.) activities using a glass suction electrode and neuronal somata in the caudal hindbrain and rostral spinal cord for whole-cell recordings (between the 5th rhombomere segments and the 7th post-otic muscle segment). Intracellular signals were amplified with an Axon Multiclamp 700B, digitised with a Power 1401 mkII and sampled with Signal (version 5, CED, Cambridge).

**Neuron identification and grouping**

The sensory RB neurons were initially visually identified by their large round somata and location on the dorsal edge of the spinal cord. Further RB identification is by their wide action potential and typical firing pattern (Winlove and Roberts 2012) in whole-cell recordings. We group neurons rhythmically active during fictive swimming as dINs and non-dINs (motoneurons, commissural interneurons, ascending interneurons, repetitive firing descending interneurons), which have similar firing properties to current injections (Li et al. 2007a; Sautois et al. 2007). dINs and non-dINs were identified by their responses to dimming the light, which triggers swimming activity. It is possible to distinguish dINs and non-dINs by recording extracellular action potentials with a loose patch electrode. The dINs have monophasic action potentials and the non-dINs have biphasic action potentials (Soffe et al. 2009). Since sensory interneurons are not active during swimming (Li et al. 2007a; Li et al. 2004; Sillar and Roberts 1988), our screening method using loose patch recordings should have systematically excluded them.
**Current clamp recordings**

Whole-cell recording pipettes were filled with a solution containing in mM: 100 K-gluconate, 2 MgCl₂, 10 EGTA, 10 Hepes, 3 Na₂ATP and 0.5 NaGTP with 0.1% neurobiotin (Vector Labs, Burlingame, CA, pH adjusted to 7.4). The inclusion of neurobiotin allowed the revealing of neuronal anatomy after whole-cell recordings in some recordings (Li and Moult 2012). Current clamp recordings of spiking properties were performed in bridge mode and stimulations were in all cases done from membrane potential set at -60 mV by injecting slow DC currents using Multiclamp 700B controller. Microperfusion of riluzole (Tocris, UK) was done by positioning a glass pipette with a tip opening of ~10 µm more than 30 µm upstream to the recorded soma (Li and Moult 2012). A gentle pressure was applied inside the pipette by compressing a connected 50 ml syringe for 100 µl (~ 200 Pa) to eject riluzole. The pipette was moved > 200 µm away from the preparation combined with gentle suction (~ -100 Pa) to stop gravity-driven leakage when not in use.

**Voltage clamp recordings of sodium currents**

After identification of neurons the preparation was bath perfused with a solution containing in mM: 35 NaCl, 40 NMDG, 3 KCl, 10 CaCl₂, 2.4 NaHCO₃, 1 MgCl₂, 10 HEPES, 40 TEA, 1 4-AP, 0.15 CdCl₂, and pH adjusted to 7.4. The neurons were patched with an electrode filled with a pipette solution where 100 mM K-gluconate was replaced with 100 mM caesium-methanesulfonate to block potassium channels from the inside. Liquid junction potential was 5.7 mV, calculated using the Clampex 10.2 junction potential formula. This was corrected during all recordings. Leak currents were subtracted during experiments and serial resistance compensation were done for 70 %. Serial resistances accepted for voltage-clamp recordings were between 10 and 20 MΩ.
Decay time constants were measured by doing a double exponential curve fitting of the recovery phase of sodium currents using the curve fitting function in Signal (Fig 1C). The peak amplitude of the $I_{\text{NaP}}$ was measured by extrapolating a single exponential curve fit to the slow persistent component, where $I_{\text{NaT}}$ was expected to have closed (Fig 1F). The surface area was calculated from the capacitance of the whole cell configuration and on the assumption that neurons had a specific capacitance of 1 µF/cm² (Winlove and Roberts 2012). The current densities were then calculated by dividing $I_{\text{Na}}$ (pA) with the calculated cell surface (µm²). Statistical significance was examined using Kruskal-Wallis or Friedman test with Dunn’s post-hoc test when the datum distribution was not normal or sample size was small. When the data were normally distributed, ANOVA or student t-tests were carried out. Means were given with standard errors.

**Results**

$I_{\text{NaP}}$ and $I_{\text{NaT}}$ in tadpole spinal neurons

Previous studies have not been able to show the presence of any $I_{\text{NaP}}$ in tadpole spinal neurons (Dale 1995; Winlove and Roberts 2012). To reinvestigate this, we applied a voltage ramp (from -80 mV to 20 mV over 200 ms) to RB neurons. The slowly rising depolarization will inactivate the $I_{\text{NaT}}$ and activate the $I_{\text{NaP}}$. The ramp induced an inward current that started to open at about -40 mV and had a peak of -133.38 ± 42.12 pA at around -10 mV ($n = 8$, Fig 1A and 1B). This shows that the RB neurons possess $I_{\text{NaP}}$.

In order to quantify $I_{\text{NaP}}$ properties relative to $I_{\text{NaT}}$, we used voltage steps rather than ramps in RB neurons, dINs and non-dINs. Voltage steps ranged from -80 mV to 30 mV with 10 mV increments. The recovery phase of sodium currents was best fitted with a two-exponential curve, suggesting the presence of a fast-decaying ($I_{\text{NaT}}$) and slowly decaying component ($I_{\text{NaP}}$) (Fig 1C). In order to estimate the size of both currents, we carried out a single
exponential fitting of the latter phase of the currents and extrapolated the size of $I_{NaP}$ when the
combined currents peaked (Fig 1C and 1F). $I_{NaT}$ was calculated by subtracting the $I_{NaP}$
component from the combined peak currents (Fig 1C and D). The peak $I_{NaT}$ in RB neurons
was $-3103.38 \pm 586.25$ pA (Fig 1D, $n = 8$) at steps to $-10$ mV. The $I_{NaT}$ in dINs was $-1516 \pm
296.5$ pA at steps to $10$ mV ($n = 7$) and in non-dINs was $-979.8 \pm 185.86$ pA at steps to $10$
mV ($n = 6$, Fig 1D). The peak decay constant for $I_{NaT}$ was to steps to $-20$ mV in RB neurons
($0.89 \pm 0.21$ ms, $n = 8$) and at steps to $-10$ mV in dINs ($1.00 \pm 0.17$ ms, $n = 7$) and non-dINs
($1.08 \pm 0.20$ ms, $n = 7$), which is shown in Fig 1E. There was no difference in the peak decay
time constants for the $I_{NaT}$ between neuron types ($p > 0.05$).

The ratio between the $I_{NaP}$ and $I_{NaT}$ amplitudes did not differ between RB neurons ($0.19 \pm
0.025$, $n = 8$) and dINs ($0.21 \pm 0.042$, $n = 7$, $p > 0.05$), or in non-dINs ($0.33 \pm 0.076$, $n = 6$, $p$
$> 0.05$, Fig 1G). The current density for the $I_{NaT}$ was higher in 8 RB ($2.02 \pm 0.35$ pA/µm²)
neurons than in 7 dINs ($1.13 \pm 0.17$ pA/µm², $p > 0.05$) and 6 non-dINs ($0.66 \pm 0.058$
pA/µm², $p < 0.01$, Fig 1H). There was no difference in the current density of $I_{NaP}$ among the
three neuron groups (RB neurons ($0.37 \pm 0.086$ pA/µm²); dINs ($0.21 \pm 0.03$ pA/µm²); non-
dINs ($0.25 \pm 0.084$ pA/µm², $p > 0.05$, Fig 1H). These results show that all the three neuron
groups express both fast inactivating $I_{NaT}$ and a slowly inactivating $I_{NaP}$.

**Properties of $I_{NaP}$**

We further analysed the properties of $I_{NaP}$ in these neurons based on the voltage step
experiments. The $I_{NaP}$ in RB neurons started to activate at steps to $-40$ mV and maximum
current was achieved at steps to $-10$ mV ($552.00 \pm 91.17$ pA, Fig 2A and D, $n = 8$). Steps to
more depolarized voltages reduced the amplitude and steps to $30$ mV generated a current of $-170.12 \pm 31.52$ pA (Fig 2A and D, $n = 8$). The decay time constant was also voltage-
dependent and had its maximum of $17 \pm 4.7$ ms at steps to $-10$ mV (Fig 2E, $n = 8$).
Also in the dINs the $I_{NaP}$ first activated at steps to -40 mV. The maximum $I_{NaP}$ was generated at steps to -10 mV (-311.43 ± 49.90 pA, Fig 2B and D, $n = 7$). Steps to more depolarized levels generated smaller $I_{NaP}$ (Fig 2B and D, $n = 7$). The decay constant for the $I_{NaP}$ in dINs had its peak at steps to -10 mV with a decay constant of 9.9 ± 1.5 ms (Fig 2E, $n = 7$).

In the non-dINs the $I_{NaP}$ also first activated at steps to -40 mV. However, the $I_{NaP}$ in the non-dINs had its peak $I_{NaP}$ at steps to 0 mV (-323.83 ± 94.88 pA, Fig 2C and D, $n = 6$). The amplitude of $I_{NaP}$ decreased at more positive steps (Fig 2C and D, $n = 6$). The decay constant of 4.27 ± 0.80 ms at steps to 0 mV in non-dINs ($n = 6$) was faster than that in 8 RB neurons and 7 dINs (Fig 2E, both $p < 0.05$, Student’s unpaired $t$-test). No difference was found between the decay time constants between RB neurons and dINs ($p > 0.05$).

Finally, we tested the action of riluzole (1, 10, and 20 µM) on $I_{NaP}$ in spinal neurons. All three concentrations of riluzole significantly reduced the amplitude of $I_{NaP}$ (Fig 2G). 1 µM reduced the $I_{NaP}$ to 71.7 ± 15.0 % of control (Fig 2F, $n = 8$, 2 dINs, 2 non-dINs and 4 RB neurons; $p < 0.01$), 10 µM reduced $I_{NaP}$ to 44.1 ± 12.7 % of control ($n = 6$ RB neurons, $p < 0.05$) and 20 µM riluzole reduced $I_{NaP}$ to 25.7 ± 6.0 % of control ($n = 6$ RB neurons, $p < 0.01$). The effect of riluzole recovered completely or partially after washout. We also tested the action of 1, 10, and 20 µM riluzole on the $I_{NaT}$. Riluzole at 1 µM reduced $I_{NaT}$ significantly to 74.7 ± 15.9 % of control (Fig 2H and 2I, $n = 7$, 2 dINs, 2 non-dINs and 3 RB neurons, $p < 0.01$). Higher doses of riluzole (10 and 20 µM) significantly reduced the amplitude of $I_{NaT}$ (Fig 2I). At 10 µM $I_{NaT}$ was reduced to 46.8 ± 12.2 % of control ($n = 6$ RB neurons, $p < 0.05$) and at 20 µM riluzole reduced $I_{NaT}$ to 28.8 ± 8.2 % of control ($n = 6$ RB neurons, $p < 0.05$).

These results show that all three groups of neuron express $I_{NaP}$ but the properties of the current vary and that 1 µM riluzole can reduce the amplitude of $I_{NaP}$ and $I_{NaT}$. $I_{NaP}$ in non-dINs peaks at steps to 0 mV, while the current in RB neurons and dINs peaks at steps to -10
mV. There are also differences in the decay time constants, where non-dINs decay significantly faster than the dINs and non-dINs.

The effect of 1µM riluzole on neuronal firing properties

Riluzole is known to affect many other aspects of neuronal function, especially at much higher concentrations than 1 µM (Bellingham 2011; Urbani and Belluzzi 2000). Having demonstrated that riluzole at 1µM could weaken I_{NaP}, we next tested 1µM riluzole in current clamp mode so that we could monitor its effects on neuronal firing in the three groups of neuron. Riluzole at 1 µM did not alter the resting membrane potential, which was -62.15 mV in control, -61.32 in riluzole, and -60.77 mV after washout \((n = 11, p > 0.05)\).

The dINs typically fire single action potentials in response to a depolarizing current pulse. However, hyperpolarizing pulses on top of depolarisation to mimic inhibitory synaptic inputs generate reliable rebound spikes in dINs (Li et al. 2006) (Fig. 3A). Microperfusion of 1 µM riluzole reduced the number of rebound spikes from 10 in control to 1.33 ± 0.76 (from 10 hyperpolarising pulses, Fig 3A1 and 3A2, \(p < 0.001\)). The rebound firing recovered to 7 ± 1.32 spikes after washout (Fig 3A1 and 3A2, \(n = 6, p < 0.01\)). We then tested the effect of riluzole on RB neurons with slow repetitive firing to current injections and found that the number of spikes was reduced from 4.33 ± 0.67 to 1.33 ± 0.33 (Fig 3B1 and 3B2, \(n = 3, p > 0.05\)). The effect recovers partly to 3.00 ± 0.00 spikes (Fig 3B1 and 3B2, \(n = 3, p > 0.05\)).

Finally, we tested the effect of 1 µM riluzole on repetitive spiking in non-dINs and the number of spikes evoked by a 500 ms depolarizing current pulse was significantly reduced from 39.22 ± 6.82 to 12.56 ± 5.55 (Fig 3C1 and 3C2, \(n = 9, p < 0.001\)). This recovered to 28.00 ± 6.76 after washout.

We also monitored the effect of 1 µM riluzole on action potential amplitude, spike threshold and input resistance in the three neuron groups. Firing thresholds were tested by injecting a
10 ms depolarising ramp current with its peak stepped and defined as the highest depolarization before the neuron started to fire (Li 2015). In dINs riluzole significantly increased the spike threshold from $-33.35 \pm 4.98$ mV to $-26.25 \pm 5.67$ mV (Fig 3A3 and D, $n = 4; p < 0.001$) with recovery after washout of riluzole. Riluzole did not affect the spike amplitude or input resistance in dINs (Fig 3A3, E, and F, $n = 6$ for both the spike amplitude and the input resistance, $p > 0.05$). In the RB neurons 1 µM riluzole also increased the spike threshold from $-22.96 \pm 2.73$ mV to $-17.55 \pm 3.72$ mV (Fig 3B3 and 3D, $n = 4$, $p < 0.001$). The effect recovered to $-20.68 \pm 2.84$ after washout (Fig 3B3 and 3D). Riluzole did not change the spike amplitude or input resistance in RB neurons (Fig 3B3, 3E, and 3F, $n = 9$, $p > 0.05$). Finally, in the non-dINs riluzole at 1 µM changed the spike threshold from $-29.02 \pm 7.03$ mV to $-25.34 \pm 7.03$ mV (Fig 3C3 and D, $n = 5$, $p < 0.001$). The effect recovered to $-33.00 \pm 7.38$ mV (Fig 3C3 and D, $n = 5$). Riluzole had no effect on either the spike amplitude or input resistance in non-dINs (Fig 3C3, E, and F, spike amplitude $n = 9$, input resistance $n = 7$, $p > 0.05$).

These results show that 1 µM riluzole affects firing properties in all three groups of neurons without changing the spike amplitude or input resistance. The reduction of repetitive and rebound firing in the three groups of neuron may be due to increased firing thresholds following the partial blockade of $I_{\text{NaP}}$ by riluzole.

Riluzole modulates swimming and struggling

Tadpoles at stage 37/38 generate two different rhythmic motor outputs, swimming and struggling. While swimming is self-sustaining after the initiation by sensory stimulation, struggling normally requires continuous activation of the mechanosensory pathway (Roberts et al. 2010). We next investigated the roles of $I_{\text{NaP}}$ in these two motor patterns using 1 µM
riluzole, which was shown not to have significant effect on synaptic transmission at the spinal level (Zhong et al. 2007).

Swimming episodes in the hatching *Xenopus* tadpole can be induced by dimming the light which activates the pineal eye on top of its forebrain (Jamieson and Roberts 2000). The lengths of swimming episodes induced by dimming the light were 20.4 ± 1.1 s in control (Fig. 4A and 4B, n = 5). Riluzole at 1 µM was locally perfused onto the hindbrain and this reduced the duration of swimming episodes to 13.6 ± 2.1 s (Fig. 4A, 4B, n = 5, p < 0.05). The swim episode duration recovered after wash to 19.4 ± 1.6 s (Fig 4A, 4B). This result shows the I_{NAP} can modulate swimming episode length could result from increased neuronal firing thresholds, or reduced neuronal excitability, in the presence of riluzole (Fig. 3D). We next analysed neuronal firing reliability during swimming, defined as percentage of cycles with neuronal spiking. However, we did not identify any change in firing reliability in 1 µM riluzole (Fig 4D, n = 7 non-dINs, Related samples Friedman’s Two-Way Analysis of variance by ranks, p = 0.82).

The other motor output from the tadpole spinal/hindbrain circuit is struggling, during which neurons fire multiple spikes on each struggling cycle (Li et al. 2007b). In 7 tadpoles, repetitive skin stimulation of the rostral trunk skin was used to induce fictive struggling (Li et al. 2007b). Since we have shown that riluzole application could reduce repetitive firing of non-dINs at rest, we analysed if riluzole could weaken non-dIN firing during struggling. The struggling rhythms became irregular in two tadpoles when 1 µM riluzole was bath-applied. In the other 5 tadpoles, fictive struggling persisted throughout riluzole application (Fig 4C). There was a decrease in the number of spikes per struggling cycle (n = 5 non-dINs, p < 0.05, paired *t*-test, Fig. 4E). During struggling with higher frequencies, there can be fewer spikes.
on each cycle because burst duration is shorter. However, there was no change in struggling frequencies in the presence of riluzole (Fig 4F), suggesting the reduction of neuronal spiking during struggling may be a direct effect of 1 µM riluzole on the recorded neuron.

Discussion

INaP in spinal/hindbrain circuits

INaP has been widely identified in spinal circuits controlling locomotion (Dai and Jordan 2011; Heckman et al. 2008; Tazerart et al. 2007; Theiss et al. 2007b; Zhong et al. 2007; Ziskind-Conhaim et al. 2008) and also preBötzinger complex neurons critical for generating breathing rhythms (Del Negro et al. 2002; Del Negro et al. 2005; Koizumi and Smith 2008; Pace et al. 2007). We have identified INaP in the sensory RB neurons and all groups of neurons involved in rhythmic swimming and struggling activity. The majority of INaP, with other persistent inward currents, may be located on dendrites (Hounsgaard and Kiehn 1993; Lee and Heckman 2000; 1996) whereas others have reported the generation of INaP in the proximal axon (Astman et al. 2006; Osorio et al. 2010). This may explain why clear INaP was not recorded in the isolated *Xenopus* tadpole spinal neurons (Dale 1995), where neurons lose axons and most dendrites during the dissociation process. This study also shows that the activation of INaP will require stepping the membrane potential above -40 mV, peaking at around 0 mV. The absence of INaP in the RB neurons in a previous study may be a result of using depolarising voltage steps below -40 mV (Winlove and Roberts 2012).

Role of INaP in regulating spiking activity

In this study we show that three groups of neuron in the tadpole display INaP but the properties of the current differ slightly between the neuron types. The INaP in all neuron groups started to be activated at voltage steps to -40 mV, which is below the threshold for the
action potential in all three neuron types (Fig 1A, 1B, 2E and 3D) (Li 2015). This suggests that depolarization from synaptic currents or current injections may just need to depolarize the membrane potential to the level to activate $I_{NaP}$. The activation of $I_{NaP}$ then will depolarize the membrane potential further to trigger spiking. Therefore, $I_{NaP}$ can play a role in setting the spike threshold, defined as the highest depolarization before spiking in this study. Indeed, increased $I_{NaP}$ current density is correlated with more negative spike threshold (Bellingham 2013; Kuo et al. 2006). This was supported by the observation that riluzole reduced the amplitude of $I_{NaP}$ and significantly decreased the excitability of all three groups of neuron. One difference between the $I_{NaP}$ in the three neuron groups was $I_{NaP}$ peaked at voltage steps to -10 mV in RB neurons and dINs, while the peak in non-dINs was at steps to 0 mV. The decay time constant also differed where RB neurons had the slowest decay and non-dIN $I_{NaP}$ decayed the fastest. We don’t know how much these differences can be explained by the space-clamping issues which depend on the anatomy of neurons and the distribution of ion channels. One explanation can be that the different groups of neurons express different type of sodium channel isoforms. Non-dINs in the tadpole spinal cord and hindbrain typically show repetitive firing to depolarising current injections and they have higher $I_{NaP}/I_{NaT}$ ratio than the dINs and RB neurons (Fig. 3C1, C2) and fastest $I_{NaP}$ decay time constant. These properties of $I_{NaP}$ may help to shape their repetitive firing, as suggested in the p11-19 rat ventral horn interneurons in the lumbar cord region (Theiss et al. 2007a). The potential of $I_{NaP}$ in setting neuronal firing thresholds has also been reported in the commissural interneurons and motoneurons of neonatal mouse spinal cord (Zhong et al. 2007).

Role of $I_{NaP}$ in regulating tadpole swimming and struggling

$I_{NaP}$ is not thought to be essential in the generation of respiratory-related rhythm because riluzole microinjection in the mouse preBötzinger complex did not perturb respiratory
frequency in a slice preparation (Pace et al. 2007), although $I_{\text{NaP}}$ plays a role in the pacemaker bursting of some neurons therein (Del Negro et al. 2002; Koizumi and Smith 2008). $I_{\text{NaP}}$ also contributes to the induced oscillations in some rodent spinal interneurons (Bouhadfane et al. 2013; Tazerart et al. 2008; Ziskind-Conhaim et al. 2008). $I_{\text{NaP}}$ may help to stabilise the fictive locomotion rhythms in neonatal rats (Tazerart et al. 2007) and is suggested to have an essential role in the generation of neonatal mouse locomotion pattern (Zhong et al. 2007) or in generating the rat masticatory movements (Brocard et al. 2006).

Does $I_{\text{NaP}}$ play a role in the generation of tadpole swimming rhythms? The generation of swimming rhythms relies on rebound/pacemaker firing in dINs (Li 2011). In this report we have shown that riluzole shortened swimming episodes. It is known that tonic depolarisation in dINs during swimming is needed in dIN rebound firing (Li and Moult 2012). $I_{\text{NaP}}$ has been suggested to amplify excitatory synaptic inputs and prolong membrane depolarisation and firing (Lee and Heckman 1996). The activation of $I_{\text{NaP}}$ can enhance NMDAR-mediated depolarisation in dINs during normal swimming and contribute to the maintenance of swimming rhythms. dINs in tadpoles also show large membrane oscillations when their NMDA receptors are activated (Li et al. 2010). The activation threshold for $I_{\text{NaP}}$ is within the oscillation voltage range suggesting $I_{\text{NaP}}$ should play a role in the NMDAR-dependent oscillations although further direct experiments on $I_{\text{NaP}}$ and NMDAR interactions are needed.

Furthermore, the activation of $I_{\text{NaP}}$ in dINs at membrane potentials below their firing threshold may facilitate their rebound firing following inhibition. Indeed, our results show riluzole has decreased dIN rebound firing reliability (Fig. 3A1, A2). However, the firing reliability of rhythmic neurons during swimming is not affected by 1 µM riluzole. dIN firing as a cell group during swimming is very robust due to the extensive electrical coupling among them (Li et al. 2009). Reliable dIN firing can in turn sustain non-dIN firing on each swimming
cycle (Soffe et al. 2009). Since the brainstem dINs play critical roles in the maintenance of tadpole swimming, the shortening of swimming may be a result of reduced activity in a small number of dINs, which can alter swimming activity (Moult et al. 2013b) but missed in our recordings. Another confounding factor is that whole-cell recordings can alter the cytoplasmic ionic composition and lead to rundown of certain ion channel currents, which may cause mismatching changes in neuronal spiking in whole-cell recordings and network activities like swimming. We also cannot exclude the possibility that swimming is shortened due to the presence of some subtle, unobserved effects from riluzole application.

Tadpole struggling rhythms involves high frequency repetitive firing of spinal and hindbrain neurons (non-dINs) whereas dINs appear to play a minor role because their firing is weak (Li 2015; Li et al. 2007a). I_{NaP} has been shown previously to promote repetitive firing in rat ventral horn neurons (Theiss et al. 2007a). Riluzole has similar effect on non-dIN repetitive firing evoked by either current injection (Fig. 3C) or during struggling (Fig. 4C, E). Therefore, the depression of non-dIN repetitive firing by riluzole should affect the generation of struggling rhythms. Meanwhile, RB neurons need to be activated repetitively to evoke struggling rhythms (during repetitive skin stimulation) and they can be another target for riluzole. Riluzole reduced the number of spikes CPG neurons fire on each struggling cycle. This may result indirectly from weakened RB outputs to the CPG circuit and directly from suppression of CPG excitability by riluzole. However, the frequency of struggling rhythms was not affected by riluzole at 1 µM (Fig. 4F), although we still do not fully understand what controls struggling frequencies. The lack of effect can be due to the limited block of I_{NaP} by riluzole at 1 µM. Increasing riluzole concentration will affect chemical synaptic transmission (Bellingham 2011; Mohammadi et al. 2001), calcium (Huang et al. 1997), potassium (Cao et al. 2002) and transient sodium channels (Kuo et al. 2006) and make the results very difficult.
to interpret. Therefore, we do not know if swimming or struggling rhythms could persist 
through a full blockade of $I_{\text{Na}p}$ unless a more specific way to block $I_{\text{Na}p}$ becomes available.

In this study, we have revealed the wide expression of $I_{\text{Na}p}$ in all groups of tadpole spinal 
negrons. Although we have reported some effects of 1 µM riluzole on tadpole swimming and 
struggling behaviour, it is difficult to evaluate how $I_{\text{Na}p}$ shapes the swimming and struggling 
outputs due to the lack of specificity of riluzole blockade of $I_{\text{Na}p}$ and its indiscriminate 
targeting of all neuronal types with $I_{\text{Na}p}$ expression. To genetically modify $I_{\text{Na}p}$ currents 
targeting specific type of neurons in the motor rhythm generation circuits may provide more 
definitive insights into the $I_{\text{Na}p}$ modulation of motor outputs (Brocard et al. 2016).

Acknowledgements: We thank Drs Steve Soffe, Alan Roberts for helpful discussions.

Grants: The work was financial supported by BBSRC (BB/L00111X/1).

Disclosures: The author declare no competing financial interests.
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Figure legends

**Figure 1.** Measurements of $I_{\text{NaT}}$ and $I_{\text{NaP}}$ in spinal neurons. **A.** A voltage ramp in a RB neuron reveals $I_{\text{NaP}}$. **B.** The I-V curve of $I_{\text{NaP}}$ in RB neurons measured from the ramp currents ($n = 8$). **C.** Sodium currents in a RB neuron evoked by a voltage step to -10 mV. The red curve shows the double exponential fitting of the decay of the currents. **D.** I-V curves of $I_{\text{NaT}}$ in RB neurons, dINs, and non-dINs. **E.** The decay time constants of the $I_{\text{NaT}}$ at steps to different voltages in the three neuron groups. **F.** Single exponential fitting (red curve) of the slowly decaying currents. Dotted line indicates estimation of $I_{\text{NaP}}$ at the peak of combined sodium currents. **G.** Ratios of $I_{\text{NaP}}/I_{\text{NaT}}$ in RB neurons, dINs, and non-dINs. **H.** Current densities for $I_{\text{NaT}}$ and $I_{\text{NaP}}$ in RB neurons, dINs, and non-dINs. **p <0.01 in H.**

**Figure 2.** Properties of $I_{\text{NaP}}$ in the three neuron groups and the effect of riluzole. **A.** Sodium currents in a RB neuron (A), a dIN (B) and a non-dIN (C) in response to voltage step to -40 mV (black traces), -10 mV or 0 mV (red traces showing the maximal $I_{\text{NaP}}$) and to 30 mV (blue traces). **D.** Averaged I-V curves for the $I_{\text{NaP}}$ in RB neurons, dINs, and non-dINs. **E.** Decay time constants of $I_{\text{NaP}}$ at steps to different membrane potentials in RB neurons, dINs,
and non-dINs. **F.** The effect of 1 µM riluzole on \( I_{NaP} \) in a RB. **G.** The blocking effect of 1, 10 and 20 µM riluzole on \( I_{NaP} \). **H.** The effect of 1 µM riluzole on \( I_{NaT} \) in a RB neuron. **I.** A graph showing the effect of 1, 10 and 20 µM on \( I_{NaT} \). * indicates \( p < 0.05 \) and ** \( p < 0.01 \).

**Figure 3.** The effects of riluzole on firing properties of the three different neuron groups. **A1.** The effect of 1 µM riluzole on the rebound firing in a dIN. **A2.** A Bar chart summarising the reduction of rebound spikes in dINs (\( n = 6 \)). **A3.** Sub-threshold depolarization and firing at threshold level in a dIN in control (black), 1 µM riluzole (red) and wash (blue). **B1.** The effect of riluzole on a RB neuron with slow, repetitive firing. **B2.** Bar chart showing the reduction of spikes by riluzole (\( n = 3 \)). **B3.** Sub-threshold depolarization and firing at threshold level in a RB neuron in control (black), 1 µM riluzole (red) and wash (blue). **C1.** The effect of riluzole on the repetitive spiking in a non-dIN. **C2.** The reduction of number of spikes by riluzole (\( n = 9 \)). **C3.** Sub-threshold depolarization and firing at threshold in a non-dIN in control (black), 1 µM riluzole (red) and wash (blue). **D.** The effect of riluzole on the spike thresholds in RB neurons (\( n = 6 \)), dINs (\( n = 4 \)), and in non-dINs (\( n = 5 \)). * indicates \( p < 0.05 \), ** \( p < 0.01 \) and *** \( p < 0.001 \). **E.** The lack of effects of riluzole on spike amplitudes in RB neurons, dINs, and non-dINs. **F.** The lack of effects of riluzole on input resistances in RB neurons, dINs, and non-dINs.

**Figure 4.** The effects of 1 µM riluzole on fictive swimming and struggling. **A1.** Motor nerve (m.n.) recordings during swimming evoked by light dimming in control, 1 µM riluzole, and after washout. **B.** The reduction of the swimming episode duration by 1 µM riluzole (\( n = 5 \)). **C.** The effects of riluzole on the number of spikes on each struggling cycle. Dashed grey lines indicate periods of repetitive skin stimulation at the rostral trunk (40 pulses at 30Hz), used to
evoke fictive struggling. **D.** Riluzole does not affect the firing reliability of neurons in swimming (% of cycles with spiking). **E.** Riluzole reduces the number of spikes per struggling cycle (*, p < 0.05). **F.** Struggling frequencies are not affected by riluzole.