

1 **Developmental changes in spinal neuronal properties, motor network configuration and neuromodulation**
2 **at free-swimming stages of *Xenopus* frog tadpoles.**

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9 **Running Head:** Development of tadpole spinal locomotor network

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22 KTS and SPC designed the experiments. SPC performed all experiments and data analysis. KTS and SPC
23 drafted the manuscript.

24

25 ABSTRACT

26 We describe a novel preparation of the isolated brainstem and spinal cord from pro-metamorphic tadpole
27 stages of the South African clawed frog (*Xenopus laevis*) that permits whole cell patch-clamp recordings from
28 neurons in the ventral spinal cord. Previous research on earlier stages of the same species has provided one
29 of the most detailed understandings of the design and operation of a CPG circuit. Here we have addressed
30 how development sculpts complexity from this more basic circuit. The preparation generates bouts of fictive
31 swimming activity either spontaneously or in response to electrical stimulation of the optic tectum, allowing
32 an investigation into how the neuronal properties, activity patterns and neuromodulation of locomotor
33 rhythm generation change during development. We describe an increased repertoire of cellular responses
34 compared to younger larval stages and investigate the cellular level effects of nitregeric neuromodulation as
35 well as the development of a sodium pump-mediated ultra-slow afterhyperpolarisation (usAHP) in these
36 free-swimming larval animals.

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38 **Keywords:** development, locomotion, nitric oxide, neuromodulation, *Xenopus*

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40 **NEW & NOTEWORTHY** A novel *in vitro* brainstem/spinal cord preparation is described that enables whole
41 cell patch-clamp recordings from spinal neurons in pro-metamorphic *Xenopus* tadpoles. Compared to the
42 well-characterised earlier stages of development, spinal neurons display a wider range of firing properties
43 during swimming and have developed novel cellular properties. This preparation now makes it feasible to
44 investigate in detail spinal CPG maturation during the dramatic switch between undulatory and limb-based
45 locomotion strategies during amphibian metamorphosis.

46

47 INTRODUCTION

48 The hatching stage tadpole of the frog, *Xenopus laevis*, (stage 37/38) has one of the most completely
49 described motor control systems of any vertebrate. This has largely been due to the development of a
50 preparation enabling patch-clamp recordings from pairs of synaptically coupled spinal neurons, which has
51 allowed the spinal network for locomotion to be understood in cellular and synaptic detail (Roberts et al.
52 2010). Furthermore, research on the early larval stages - just a day or so later in development, but still before
53 continuous free swimming begins - has provided several insights into how neural networks are modified to
54 enable complex behaviour to emerge as ontogeny progresses (Sillar et al. 1991, 1992, Zhang et al. 2009,
55 2011). Here we describe a novel preparation that forms the foundation for the next steps in the study of
56 motor control in *Xenopus* by enabling whole cell patch-clamp recordings from *in vitro* brainstem/spinal cords
57 isolated from free-swimming pro-metamorphic (stages 50–58 Nieuwkoop and Faber 1956) tadpoles. At
58 these stages, the tadpoles swim almost continuously, sculling with the caudal portion of their tail in order to
59 retain a head-down hover, facilitating their lifestyle as obligate filter feeders (Hoff and Wassersug, 1986). At
60 earlier stages of *Xenopus* development, swimming occurs in prolonged bouts following sensory stimulation
61 and all neurons within the spinal network fire rhythmically throughout the swim cycle to maintain the
62 coordinated muscle contraction underlying forward propulsion (Roberts et al. 2010). The aim of this study
63 was to explore changes in the properties and modulation of spinal neurons that might account for the
64 development of more flexible swimming behaviour and the switch from a primarily sessile existence to one
65 where swimming occurs almost constantly (Currie et al. 2016). For instance, an important intrinsic property
66 recently described in spinal neurons from earlier stages is the activity-dependent ultra-slow
67 afterhyperpolarisation (usAHP; Zhang and Sillar 2012; Zhang et al. 2015), which results from increased
68 activation of the sodium pump. The usAHP is thought to act as a form of internal memory for previous
69 cellular activity. At early stages of development, the usAHP is detectable in about 50% of spinal locomotor
70 neurons but whether this is a transient feature during early development or one that persists later in
71 development is not known. Moreover, the effects of nitric oxide (NO), which is known to potently modulate
72 fictive locomotion at these later stages (Currie et al. 2016), have not been investigated at the cellular level.
73 Since the effects of endogenous NO at these stages are location-specific, complex and different from earlier
74 stages, having switched from an inhibitory to an excitatory influence (Currie et al. 2016), NO's direct effect
75 on spinal neurons is an important next step in understanding the role of nitrergic neuromodulation in this
76 developing system.

77 METHODS

78 *Animals and husbandry.*

79 Experiments were performed on free-swimming, pro-metamorphic stages (50–58) of the South African
80 clawed frog, *Xenopus laevis* (Nieuwkoop and Faber 1956). Animals were obtained by human chorionic
81 gonadotrophin hormone-assisted (1,000 U/ml; Sigma) matings of adults selected from an in-house breeding
82 colony. Fertilized ova were collected and reared in enamel trays until the first free-feeding stages, before
83 being transferred to standard glass aquarium tanks. All procedures complied with the UK Animals (Scientific
84 Procedures) Act 1986 and the European Community Council directive of 24 November 1986 (86/609/EEC)
85 and were approved by the University of St. Andrews Animal Welfare Ethics Committee.

86 *Dissection for whole cell recording.*

87 In order to make single cell patch-clamp recordings in the ventral spinal cord of pro-metamorphic *Xenopus*
88 tadpoles, the dissection employed during extracellular experiments (Combes et al. 2004; Currie et al. 2016)
89 was modified. Briefly, after removal and destruction of the brain under MS222 anaesthesia, the remaining
90 nervous system was cut free from the rest of the body. The caudal extent of the spinal cord was cut to
91 completely isolate the brainstem and spinal cord and this tissue was then pinned down securely with
92 sharpened tungsten wire pins in a recording chamber with a rotatable Sylgard platform. Using a finely
93 etched tungsten needle, the spinal cord was then opened along the medio-lateral midline as far as the
94 neurocoel from approximately the 12th -15th post-otic ventral root. At the caudal extent of this first cut, a
95 second cut was made perpendicularly, approximately as deep as the dorso-ventral midline of the spinal cord,
96 and all the way to the lateral extent of the cord. The free end of the spinal cord was then carefully peeled
97 back towards the rostral end of the animal, removing most or all of the dorsal horn. Once at the level of the
98 12th post-otic ventral root the dorsal portion of spinal cord was cut away using microscissors (see Fig. 1B).
99 This modified preparation gave direct access to the ventral spinal cord, where the spinal circuits involved in
100 motor control are presumed to lie. Moreover, exposing the ventral cord between the 12th and 15th ventral
101 roots gave access to neurons assumed to be primarily involved in axial swimming patterns since they are
102 located caudal to the ventral roots innervating the developing hindlimbs.

103 *Electrophysiology.*

104 The dissection and subsequent electrophysiological recordings were performed in 'HEPES' saline
105 (composition in mM: 115 NaCl, 3 KCl, 2 CaCl₂, 2.4 NaHCO₃, 1 MgCl₂, 10 Hepes, adjusted with 4 M NaOH to
106 pH 7.4). Whole-cell patch-clamp recordings in current clamp mode were made using microelectrodes pulled
107 on a Sutter P97 pipette puller from borosilicate glass capillaries (Harvard Apparatus Ltd.). All recordings were
108 made from neurons at relatively ventral locations in the spinal cord, between the 11th and 16th post-otic

109 ventral root. Patch pipettes were filled with 0.1% neurobiotin in the intracellular solution (composition in
110 mM: 100 K-gluconate, 2MgCl₂, 10 EGTA, 10 Hepes, 3 Na₂ATP, 0.5 NaGTP adjusted to pH 7.3 with KOH) and
111 had resistances of approximately 8MΩ. Extracellular ventral root recordings were made ipsilaterally to the
112 exposed area for patch recording and generally caudally between the 16th and 19th post-otic ventral roots.
113 Recordings in whole-cell mode were amplified with an Axoclamp 2B (Axon Instruments) amplifier and
114 digitized using a CED power1401. All signals were displayed and saved on a PC using Spike2 software and all
115 subsequent analysis performed in Dataview software (v 8.62, courtesy of W. J. Heitler, School of Biology,
116 University of St Andrews, St Andrews, UK).

117 *Neuron labelling.*

118 Neurobiotin (0.1%) in the intracellular solution was used to label neurons for anatomical identification.
119 Following electrophysiological recordings, the brainstem-spinal cord tissue was fixed in 2% glutaraldehyde in
120 0.1 M phosphate buffer (pH 7.2) overnight in the refrigerator (4°C). After they were rinsed with 0.1 M PBS
121 (120 mM NaCl in 0.1 M phosphate buffer, pH 7.2), the animals were (1) washed in two changes of 1% Triton
122 X-100 in PBS for 15 min with agitation, (2) incubated in a 1:300 dilution of extravidin peroxidase conjugate
123 (Sigma Aldrich, UK) in PBS containing 0.5% Triton X-100 for 2–3 hr with agitation, (3) washed again in at least
124 four changes of PBS, (4) presoaked in 0.08% diaminobenzidine in PBS (DAB solution) for 5 min, (5) moved to a
125 second container with 0.075% hydrogen peroxide in DAB solution for 5 min, and (6) washed in running tap
126 water. The nervous system was then dehydrated, cleared in methyl benzoate and xylene, and mounted
127 whole, between two coverslips using Depex. Neuronal anatomy was observed using a Zeiss Axio Imager Ax10
128 at x20 and x40 magnification and measurements were made using Zen Imaging Pro (v10; Zeiss) software.

129 *Pharmacological manipulations.*

130 Saline during electrophysiological experiments was gravity fed from one of two stock chambers. This allowed
131 switching between control and drug conditions via a three-way tap. Saline flowed to waste and was not
132 recirculated. Drugs used were the NO donors S-nitroso-N-acetylpenicillamine (SNAP – 200μM) and
133 Diethylamine NONOate (DEA-NO – 50 – 200μM). No obvious differences in drug effect were seen over this
134 concentration range of DEA-NO (c.f. Currie et al, 2016). Drugs were dissolved in distilled H₂O (18MΩ),
135 aliquoted and then frozen, before being made up to final concentration in standard HEPES saline - the
136 dilution of saline with H₂O vehicle was <0.5%.

137 *Statistical analysis.*

138 Mean data was analysed using either a paired t-test; repeated measures ANOVA with Bonferroni correction;
139 or in the case of normalised data, a Wilcoxon signed rank test and significance reported at either < 0.01 or <
140 0.05. Error bars represent the standard deviation of the mean. For the cumulative probability plots of PSP

141 frequency and amplitude, the Kolmogorov-Smirnov test (K-S test) was employed at a significance level of
142 0.05. The bin sizes were standardised throughout at 0.1Hz for PSP frequency and 0.05mV for PSP amplitude.
143 Statistical analyses were performed in SPSS (version 21; IBM), graphs were produced from custom written
144 MATLAB scripts (The MathWorks, Inc.) and figures arranged in Adobe Illustrator CC (Adobe Systems, Inc.).

145 RESULTS

146 A major aim of this study was to explore developmental changes in neuronal, circuit and neuromodulation
147 properties that accompany the transition to more flexible and continuous tadpole swimming behaviour
148 compared to embryonic and early larval stage. In order to do this we have developed a new preparation that
149 allows for whole cell patch-clamp recordings of neurons within the spinal cord at free-swimming pro-
150 metamorphic stages (50-58; Nieuwkoop and Faber 1956) of *Xenopus* tadpoles (Fig. 1A,B). At these stages of
151 development, bouts of fictive swimming readily occur spontaneously in *in vitro* preparations of the spinal
152 cord and brainstem (Combes et al. 2004; see Fig. 1Ci), but similar bouts can also be evoked by brief electrical
153 stimulation of the optic tectum (Fig. 1Cii). In both cases, the activity of individual rhythmically active neurons
154 can be recorded and assessed simultaneously using this new preparation.

155 Neuronal activity during swimming

156 The data reported in this study derive from 104 neurons (9 identified anatomically as motoneurons (MNs) -
157 see below) recorded in 83 preparations. During spontaneous episodes of swimming, rhythmically active
158 spinal neurons generally fired action potentials in phase with the bursts of activity recorded from ipsilateral
159 spinal ventral roots (Fig. 1C; 50/83 (60.2%) neurons where activity in a ventral root was present for
160 comparison). In a typical neuron, the onset of ventral root activity coincided with or was just preceded by a
161 depolarisation of the membrane potential, which then oscillated in phase with the rhythmic network activity
162 recorded in the ventral root. Preceding the onset of rhythmic activity, 12/83 (14.5%) neurons initially fired
163 tonically and the ventral root recording displayed a corresponding period of tonic discharge (see Fig. 1Ci). As
164 the ventral root began to burst rhythmically these cells also switched into a rhythmic pattern of firing, with
165 volleys of action potentials interspersed with periods of subthreshold activity (Fig. 1Ci middle inset; Cii, inset).
166 Over the course of an episode, the activity often waxed and waned and the neuron was often de-recruited
167 but continued to receive rhythmic synaptic drive in time with the ventral root bursts (see inset in Fig. 1Cii).
168 Rhythmic activity was often followed by tonic ventral root discharge and neuronal firing (Fig. 1Ci) or faded
169 away gradually with sporadic spiking activity (Fig.1Cii). While the majority of recorded neurons fired
170 transiently and only during periods of network activity, a subset (7/104; 6.7%) discharged tonically at rest (Fig.
171 2). However, these neurons were also apparently linked to locomotor output since during network activity
172 their firing was often altered. The changes in firing pattern could be quite subtle, as in the modulation of
173 tonic firing frequency seen in figure 2A, or they could be more dramatic, switching in and out of a rhythmic
174 firing pattern phase-related to the locomotor cycle, as in figure 2B.

175 Basic firing properties

176 The average resting membrane potential of all recorded neurons was -57.8 ± 6.67 mV ($n = 104$ from $N = 83$
177 animals). From rest, the injection of a short (2ms) current pulse was capable of eliciting an action potential
178 with a mean threshold for activation of 487.34 ± 390.89 pA. All but one recorded neuron (see Fig. 3Ci) was
179 capable of firing repetitively during current injection and fired at higher frequency as the amplitude of the
180 current pulse was increased (Fig. 3A). The frequency-current plots in Fig. 3Aiii illustrate representative
181 examples of both a lower threshold (open black circles; also see Fig. 3Ai) and a higher threshold neuron
182 (filled black circles; also see Fig. 3Aii). Both of these neurons were later identified as MNs (see below for
183 details).

184 A novel property found in a small proportion of unidentified neurons in pro-metamorphic tadpoles, was an
185 apparently intrinsic rhythmogenic capacity (Fig. 3Bi). Thus in 2/104 neurons (2%), depolarising current
186 injection resulted in an oscillation of the membrane potential, with superimposed bursts of action potentials
187 interspersed with periods where the membrane potential was re-polarised below spike threshold. The
188 intrinsic membrane oscillation was relatively slow, in the range of 0.5-1Hz, in comparison to the membrane
189 oscillation associated with swimming, which are typically 4-6Hz. The firing pattern was similar to the intrinsic
190 bursting seen in low threshold zebrafish MNs that are recruited during the slowest swimming speeds (Gabriel
191 et al. 2011; Menelaou and McLean 2012), but in *Xenopus* this has never been documented in publications
192 based on many thousands of recordings at the embryonic and early larval stages of tadpole development.
193 The neurons had relatively low rheobases of 110pA and 310pA, respectively, and both fired rhythmically
194 during swimming (Fig. 3Bii).

195 As at early larval stage 42 (Sillar et al. 1991), neurons fired variably during episodes of fictive swimming and
196 could fire multiple spikes during each motor burst (see inset in Fig. 1Cii). The exception to this was a single
197 neuron that had a relatively depolarised resting membrane potential of -50mV and during swimming fired
198 one broad action potential per cycle (Fig. 3Ci-ii – for comparison Fig 3Cii also shows the MN from Fig. 3Aii).
199 Moreover, injection of supra-threshold current was unable to elicit repetitive firing, even at 400% of the
200 rheobase (Fig. 3Ciii). This neuron therefore displayed physiological characteristics reminiscent of an
201 embryonic descending interneuron (dIN; Li et al. 2006; see discussion).

202 Recording from motorneurons

203 Following patch clamp recordings, neurobiotin originating from within the patch solution allowed post-hoc
204 analysis of the anatomy of a subset of individual spinal neurons (see e.g. Fig. 4). Definitive anatomical
205 identification of spinal neurons was not possible in the majority of cases due in part to the quality of the fills,
206 but also due to the lack of conformity of successful stainings with the well-characterised spinal neurons from
207 earlier in *Xenopus* development. Nevertheless, 9/101 (8.9%) filled neurons were confirmed as MNs based on
208 their axonal projections that exited the spinal cord via a ventral root (Fig. 4Ai & iii). The MNs had a medially

209 located soma and generally had dendrites that projected laterally into the marginal zone (Fig. 4Aiv). The
210 primary axon exited the soma and initially ran close to the midline of the spinal cord (Fig. 4Aiv). The axon
211 projected ipsilaterally and caudally over several spinal segments (Fig. 4Ai-iii) before turning and exiting via a
212 ventral root (Fig. 4Ai & iii). All identified MNs projected caudally from the soma and exited via a ventral root
213 between 4 and 9 spinal segments away (mean = 6.33 +/- 1.58 segments). Occasionally physiological
214 characterisation of MNs was also possible (Fig. 4B). In 3/104 recorded neurons, including 1 in addition to the
215 9 anatomically identified MNs, each action potential following supra-threshold current injection was
216 matched 1:1 by an impulse in the ventral root trace (Fig. 4Bi). Further confirmation of MN identity was
217 provided by stimulating the ventral root in these preparations, which elicited antidromic spikes in the
218 recorded neuron (Fig. 4Bii). The spikes occurred reliably following stimulation and at a very short latency
219 (<2ms) confirming their antidromic nature. The identified MNs had a mean resting membrane potential of -
220 59 +/- 5.55mV and a mean rheobase of 408.57 +/- 190.39pA following a 2ms current pulse.

221 Post spike afterhyperpolarisations

222 The output of most neural circuits changes dramatically during development to accommodate maturation of
223 the behaviours they regulate. This is due partly to alterations in the synaptic connections between and
224 electrical properties of the constituent neurons. With regard to the latter, post-spike hyperpolarizations are a
225 defining feature of the responses of many neurons to excitatory inputs. In *Xenopus* tadpoles, action
226 potentials in spinal neurons recorded at embryonic stage 37/38 are characterised by a fast (f)AHP after which
227 the membrane potential typically returns to rest within a few milliseconds (Sautois et al. 2007). A fAHP
228 persists at pro-metamorphic stages and was found in all recorded neurons (Fig. 5Ai). In addition, a subset of
229 neurons at pro-metamorphic stages (15/104; 14%) displayed a pronounced slow (s)AHP following an action
230 potential evoked from rest, which typically lasted 150-200ms (Fig. 5Ai; mean 161.29 +/- 59.99ms (n = 15)).
231 This sAHP resembled similar responses documented in other species, which are mediated by apamin-
232 sensitive Ca²⁺-dependent K⁺ channels (for review see Sah and Faber 2002). However, the sAHP reported here
233 seems to be masked during rhythmic bursting, where only fAHPs were obvious (Fig. 5Aii).

234 At earlier stages of *Xenopus* development (Zhang and Sillar 2012), intense firing activity in spinal neurons
235 triggers an even longer duration AHP (approximately 60s), termed the ultra-slow (us)AHP. At pro-
236 metamorphic stages a usAHP was evident in response to the injection of supra-threshold current pulses (Fig.
237 5Bi) and also following the termination of episodes of rhythmic swimming (Fig. 5Bii). Using the same stimulus
238 paradigm as Zhang and Sillar (2012) – a train of increasing supra-threshold current pulses (Fig. 6Ai) – a direct
239 comparison of the responses of spinal neurons at stages 37/38-42 with those at stages 50-58 was possible.
240 The usAHP was detectable in a far higher percentage of recorded neurons (81/93(87%), including 8/9 (89%)
241 identified MNs) at pro-metamorphic stages (50-58) as compared with stages 37/38-42 (87/202 (43%),

242 including 39/67 (58%) identified MNs; Fig. 6Aii and see Zhang and Sillar 2012). The amplitude of the usAHP,
243 measured as the change in membrane potential between rest and the peak slow hyperpolarisation following
244 current injection, was similar between the two stages of development. On average, a hyperpolarisation of
245 4.84 +/- 2.64mV occurred at stages 37/38-42 (N=20; Zhang and Sillar 2012) while during pro-metamorphosis
246 the hyperpolarisation was 4.66 +/- 2.6mV (N=25; ns; Fig. 6Aii). The duration of the usAHP, measured as the
247 time between the end of the stimulus train and the point where the membrane potential returned to rest,
248 was significantly shorter at pro-metamorphic stages. On average, the usAHP duration measured 19.33 +/-
249 24.50s in neurons from pro-metamorphic stages (N=25; stages 50-58), while at embryonic and early larval
250 stages the usAHP duration was 50.78 +/- 34.21s (N=20; p<0.01; Fig. 6Aii and see Zhang and Sillar 2012).

251 The usAHP described in stage 37/38-42 tadpole spinal neurons is thought to be mediated solely via an
252 increase in the activity of the Na⁺ / K⁺ pump and, as such, the membrane hyperpolarisation is not associated
253 with a detectable change in membrane input resistance (IR; Zhang and Sillar 2012). During pro-
254 metamorphosis the IR of spinal neurons was reduced, but only during the first few seconds of a usAHP (Fig.
255 6B). At 500ms and 2000ms after the end of the stimulus the IR was reduced significantly to 89.45 +/- 8.61
256 and 94.30 +/- 7.35% of control, respectively (N=9; Fig. 6Bii). The change in IR cannot account for the
257 complete recovery of the membrane potential to rest, however, since it returned to control levels (100%) in
258 just 6.67 +/- 7.35s, which was significantly shorter than the duration of the usAHP to the same stimulus that
259 measured 18.58 +/- 15.33s (N=9; p<0.05, t-test; Fig. 6Biii). This suggests the usAHP comprises both a long
260 duration Na⁺ pump-based event lasting the duration of the usAHP and a superimposed event, likely caused
261 by the opening of a membrane ion channel that is active in the early stages of the usAHP.

262 One candidate current to mediate such a response is the hyperpolarisation activated, I_h current. Although
263 not reported in embryonic spinal neurons (stage 37/38), there is evidence that I_h currents emerge by larval
264 stage 42 (Picton (2017) and pro-metamorphic *Xenopus* spinal neurons show strong evidence of possessing I_h
265 channels (Fig. 6C). In 42/104 (40%) neurons recorded in the present study, hyperpolarization of the
266 membrane potential caused a characteristic depolarizing sag potential, while termination of hyperpolarising
267 pulses caused a depolarizing overshoot of the membrane potential (Fig. 6Ci). On some occasions, this post-
268 inhibitory rebound was large enough to cause firing in the neuron (Fig. 6Cii). I_h has not previously been
269 reported in *Xenopus* embryo spinal neurons and is thus another example of a change in the cellular
270 properties that occur during larval development.

271 Based on evidence from earlier stages of *Xenopus* development (Zhang and Sillar 2012; Zhang et al. 2015),
272 we predicted that neurons will be less likely to fire if excited within the period when the usAHP is active. To
273 test this hypothesis the relative excitability of neurons prior to and immediately after the onset of the usAHP
274 was investigated. Neurons with a detectable usAHP showed a reduction in excitability during the membrane

275 hyperpolarisation (Fig. 6D). The latency to first spike following supra-threshold current injection significantly
276 increased by 35% from 13.87 +/- 7.55ms at rest to 17.70 +/- 8.58ms, during the trough of the
277 hyperpolarisation (Fig. 6Diii; N=17; p<0.01). Furthermore, the instantaneous spike frequency of a second
278 spike to the current pulse was significantly reduced to 96% of that in control; 126.17 +/- 47.42Hz at rest to
279 121.73 +/- 48.88Hz during the usAHP (Fig. 6Diii; N=6; p<0.05). In many cases, this led to the same stimulus
280 eliciting less spikes during the usAHP than before (Fig. 6Dii).

281 Modulation by nitric oxide

282 The behavioural repertoire expressed by an individual depends upon its developmental, ecological and
283 arousal states at any given moment in time and neuromodulation plays an important, determinant role in
284 sculpting behaviour to prevailing conditions. NO is known to be a potent inhibitory modulator of locomotion
285 in embryonic and early larval stages of *Xenopus* development where it has been shown to enhance both
286 GABAergic and glycinergic inhibition within the spinal cord (McLean and Sillar 2002, 2004). In contrast, at
287 later pro-metamorphic stages, the effects of NO on the occurrence of spontaneous locomotor activity are
288 excitatory (Currie et al., 2016). Moreover, the excitatory effects are thought to be mediated primarily via the
289 brainstem in these older animals. Together these findings highlight the need to investigate the effects, if any,
290 of NO on spinal neurons during fictive locomotion in pro-metamorphic tadpoles.

291 As well as increasing the occurrence of spontaneous locomotor activity (Currie et al., 2016), bath application
292 of the NO donors SNAP (200µM; N=4) and DEA-NO (50-200µM; N=7) caused a depolarisation of spinal
293 neurons (Fig. 7Ai, Bi). The membrane potential depolarised significantly by 8.34 +/- 7.99% relative to control
294 during NO donor application and subsequently reduced to 6.88 +/- 7.56% of control upon washout (Fig. 7Bi;
295 N=11, p<0.01). On average, the depolarisation was 4.03 +/- 3.21mV relative to control. The same drug
296 application caused a reversible decrease in IR to 94.76 +/- 7.50% of control (Fig. 7Bii; N=9; 5 in DEA-NO & 4 in
297 SNAP, p<0.05), and upon washout, the IR returned to control levels to 99.16 +/- 7.23%.

298 During quiescent periods, frequent depolarising postsynaptic potentials (PSPs) were observed and NO donors
299 were found to significantly increase their frequency (Fig. 8A, Ci). The mean frequency of PSPs increased from
300 1.40 +/- 0.85Hz in control to 2.66 +/- 1.68Hz during NO donor application (Fig. 8Ci; N=10, 4 in SNAP and 6 in
301 DEA-NO; p<0.05). During washout, the PSP frequency returned towards control levels to 1.43 +/- 0.85Hz. The
302 cumulative probability of PSP inter-event interval shifted to the left, indicating a significant increase, in 5/10
303 recorded neurons (see example in Fig. 8Bi; p<0.05). The mean amplitude of PSPs was not significantly altered
304 during NO donor application (Fig. 8A, Cii), however, the cumulative probability of PSP amplitude shifted to
305 the right in 5/10 recorded neurons, indicating that the proportion of large amplitude PSPs was increased
306 significantly compared with control in these cases (Fig. 8Bii; p<0.05).

307 As well as these more general effects, bath application of DEA-NO (50-200 μ M) also specifically reduced or, in
308 some neurons, completely abolished the usAHP following depolarising current injection (Fig. 9A). Overall, the
309 usAHP amplitude reduced significantly to 33.21 \pm 13.40% of the value in control; on average the amplitude
310 was -3.42 \pm 1.54mV in control and -1.04 \pm 0.42mV during DEA-NO application (Fig. 9Ai, ii, iv; N=6, p<0.01).
311 Upon washout, the usAHP amplitude recovered partially to -1.25 \pm 0.24mV or 43.08 \pm 20.13% of control
312 (Fig. 9Aiii-iv). The effects of DEA-NO on the usAHP appear to be independent of the depolarisation during NO
313 donor application since these effects were evident when the membrane potential was the same or even
314 hyperpolarised vs control (e.g. Fig. 9Aii). This result is particularly interesting since it suggests that the Na⁺/K⁺-
315 pump is modulable in *Xenopus* spinal neurons and therefore must be considered when investigating the
316 neuromodulation of spinal CPG networks.

317 DISCUSSION

318 Cellular correlates of spontaneous swimming activity

319 We have described a new preparation that enables patch clamp recordings from spinal neurons of pro-
320 metamorphic tadpoles during spontaneous bouts of fictive swimming. In contrast to earlier stages of
321 development, rhythmically active neurons display a range of firing patterns during swimming activity and in
322 response to depolarizing current pulses. One firing pattern seen in a proportion of neurons that may relate to
323 recruitment and de-recruitment during swimming is illustrated in figure 1C. These neurons fired tonically
324 when the ventral root was discharging low amplitude tonic activity, and then switched into a rhythmic firing
325 pattern when the ventral root was bursting. 3/12 neurons displaying this activity pattern were identified
326 morphologically as MNs (see Fig. 4) and therefore this pattern of firing may underlie the low-amplitude tonic
327 activity recorded in ventral root recordings. Moreover, this pattern of activity would be suitable to provide a
328 basal tone of muscle activation just prior to rhythmic contraction during locomotion. A bilateral stiffening of
329 the muscles immediately rostral to those engaged in propulsive locomotion could be important to generate
330 thrust, without causing unwanted lateral movement of the more rostral regions of the body. Other common
331 features of these neurons were a short period of tonic ventral root discharge and neuronal firing following
332 the end of an episode of rhythmic activity (Fig. 1Ci) or activity diminishing gradually with sporadic spiking
333 activity (Fig.1Cii). This suggests a switch from the relatively abrupt termination of episodes in earlier stage of
334 *Xenopus* development, often coincident with a barrage of GABAergic potentials (Reith and Sillar 1999). The
335 ability to fire in a graded fashion may be a general feature of more mature locomotor networks. In the
336 embryonic tadpole, neurons have two basic states: quiescent and rhythmically active. This may be sufficient
337 for a lifestyle in which movement is solely a means of escape but would be of little use to an animal that
338 needs to move constantly and dynamically, as in free-swimming *Xenopus* larvae. Instead, the larvae require a
339 greater ability to change the direction and speed of locomotion as well as to selectively recruit different parts
340 of the tail. At the cellular level, this flexibility must necessarily involve differential activation of neurons but
341 might also involve different firing patterns such as those described here. Understanding how the firing
342 patterns of neurons in the spinal network map onto an episode of spontaneous swimming will be an
343 important future step in understanding how such a well-coordinated behaviour is controlled.

344 Another novel finding in pro-metamorphic tadpoles is that a proportion of spinal neurons fire tonically from
345 rest (Fig. 2). No such neurons have been reported at earlier stages (37/38-42) of development. The spiking of
346 this type of neuron is modulated during spontaneous motor activity (Fig.2A), and can switch between periods
347 of tonic activity and rhythmic bursting (Fig. 2B). These neurons appear to represent a sub-population of
348 spinal neurons that are continuously active, which is presumably as a result of a different set of intrinsic
349 properties compared with other more typical CPG neurons. If a proportion of MNs were tonically active, this

350 type of activity may contribute to a resting tone in the axial muscle of these animals that is maintained in
351 parts of the tail that are not contributing to on-going locomotion. This type of postural control may be
352 important in *Xenopus* swimming to facilitate their stereotypical 'hovering' during feeding, especially since
353 there will be reduced trimming forces due to the lack of significant forward propulsion during this behaviour
354 (Hoff and Wassersug 1986; Webb 2002). However, the fact that ventral roots are not continually active (i.e.
355 there is no evidence for continuous low amplitude activity) may be an argument against these neurons being
356 MNs. These tonically active neurons appear to represent a newly reported phenotype of ventral spinal
357 neuron in *Xenopus* tadpoles that may be integral components of the neural circuitry required to generate
358 spontaneous motor output. However, it cannot be completely ruled out that removing the dorsal half of the
359 spinal cord during the dissection could contribute to this firing profile.

360 A small subset of neurons (2/104) in the pro-metamorphic spinal cord displayed intrinsic bursting in response
361 to depolarising current injection (Fig. 3B). The membrane oscillations underlying these bursts were slow
362 relative to fictive swim frequency, in the order of 0.5-1Hz. This is in contrast to zebrafish, where a subset of
363 low threshold MNs display very similar, but much faster intrinsic bursting to depolarising current injection,
364 which is thought to contribute to their propensity to be recruited at the lowest swimming speeds (Gabriel et
365 al. 2011; Menelaou and McLean 2012). In the lamprey (Wallén and Grillner 1987) and neonatal rat (Hochman
366 et al. 1994), similar but conditional bi-stability of the membrane potential is expressed in the presence of
367 NMDA. Despite their relatively slow cycle period, the oscillations are proposed to contribute to the rising
368 phase of locomotor cycles in the lamprey, since their frequency is modulated by current injection, mimicking
369 the effects of intrinsic membrane currents during locomotion (Wallén and Grillner 1987). In early larval
370 stages of *Xenopus*, a similar slow oscillation of spinal neuron membrane potential is found to be dependent
371 on both NMDA and 5-HT (Scrymgeour-Wedderburn et al. 1997) and mediates a slow modulation of
372 swimming activity over several consecutive cycles (Sillar and Reith 1998). As in the lamprey and zebrafish, the
373 intrinsically bursting neurons described here could contribute to the fast oscillations of the membrane
374 potential during swimming, reducing the reliance on fast synaptic inhibition for burst termination, as is the
375 case in earlier stages of *Xenopus* development (Soffe et al. 1984; Dale 1985; Soffe 1987). Another possibility
376 is that the intrinsic oscillations may contribute to a slower modulation of swimming that alters the intensity
377 of motor output, and in these animals could be involved in the 'waxing and waning' of activity along the
378 rostral-caudal axis of the body. This would be analogous to the NMDA-dependent modulation seen at earlier
379 stages of development, controlling the relative intensity of motor bursts over the course of multiple cycles
380 (Sillar and Reith 1998).

381 The development of the AHPs and their role in spontaneous network activity

382 In addition to fAHPs, which are found at early embryonic stages of development (Sautois et al. 2007), 14% of
383 recorded pro-metamorphic spinal neurons display a pronounced slow sAHP, which typically lasts 150-200ms
384 (Fig. 5Ai). A very similar sAHP, primarily mediated by apamin-sensitive Ca^{2+} -dependent K^+ channels is found in
385 spinal neurons within the lamprey (Wallen et al. 1989; Hill et al. 1992; el Manira et al. 1994). This additional
386 property of pro-metamorphic spinal neurons may offer additional opportunities for neuromodulation since
387 the sAHP is a target for, for example, 5-HT in lamprey spinal neurons (for reviews see Grillner et al. 2001) and
388 acetylcholine in mammalian MNs (Miles et al. 2007).

389 Nearly half of recorded spinal neurons (43%) at embryonic and early larval stages of *Xenopus* development
390 display a usAHP that is dependent on increased Na^+ / K^+ -pump activity following intense periods of activity
391 (Zhang and Sillar 2012). A similar phenomenon has also been described in MNs regulating crawling behaviour
392 in *Drosophila* larvae (Pulver and Griffith 2010) and spinal CPG neurons in mammalian locomotor networks
393 (Picton et al. 2017). The usAHP is proposed to act as a simple mechanism for short-term memory of cellular
394 activity that dynamically sets the excitability of neurons based on previous activity and that regulates the
395 duration of locomotor bouts in light of previous network output. As we show here, the basic phenomenon of
396 the usAHP persists into later, pro-metamorphic stages of *Xenopus* development (Fig. 5B, 6). However, there
397 are several key differences. Although we cannot be sure we are comparing like with like in terms of cell type,
398 since the majority of neurons recorded in the present study were not identified anatomically, the usAHP
399 occurs in a far higher proportion of rhythmically active spinal neurons (Fig. 6Aii; 87% as compared with 43%).
400 In MNs, the one cell type reliably identified in the present study, the usAHP occurred in 89% of recordings,
401 compared to only 58% of MNs at stages 37/38-42 (Zhang and Sillar 2012), suggesting they are representative
402 of the overall trend. Possible explanations for this more widespread expression of the usAHP are the
403 differential expression of Na^+ / K^+ -pump subunits (Azarias et al. 2013) or their accessory proteins (Cornelius
404 and Mahmmoud 2003), or that Na^+ / K^+ -pump activity is regulated via second messenger pathways (reviewed
405 in Therien and Blostein 2000) and influenced by one of the many neuromodulators known to act on the
406 *Xenopus* spinal CPG (Sillar et al. 2014). NO donors, for instance, were found to reduce the usAHP in these
407 older animals (Fig. 9) and this may contribute to the overall excitatory effect of NO at these stages of
408 development (Fig. 7, 8 and see Currie et al. 2016). At earlier stages of development, NO has net inhibitory
409 effects on *Xenopus* locomotion (McLean and Sillar 2002, 2004) and this may be partially due to fewer
410 network neurons being susceptible to NO's inhibitory effects on the usAHP (Zhang, Picton & Sillar,
411 unpublished observation). A role for NO modulating the Na^+ / K^+ -pump has been suggested in the rat
412 midbrain, where it was shown to enhance NMDA-induced oscillations, mimicking the effects of increasing
413 pump activity (Johnson et al. 1992; Cox and Johnson 1998). In the vasculature, NO donors have been shown
414 to activate the pump (Gupta et al. 1996), however, these results are confounded by reports in both the
415 kidney (Meffert et al. 1994) and cerebral cortex (Sato et al. 1995) where NO donors have been shown to

416 inhibit pump activity. In fact, there is evidence that NO-mediated cell death may be partly via S-nitrosation of
417 the sodium pump, which is one of many metabolic membrane proteins targeted by NO (Jaffrey et al. 2001).

418 As well as occurring in a higher percentage of recorded neurons in free-swimming *Xenopus* larvae, the usAHP
419 is also shorter in duration (Fig. 6Aii) and associated with an initial reduction in IR (Fig. 6B) during pro-
420 metamorphosis. The reduction in IR is only associated with the first few seconds of the usAHP and recovers
421 significantly earlier (approximately 5s) than the duration of the usAHP (approximately 20s; Fig. 6B). Since the
422 change in input resistance is almost certainly associated with the opening of an ion channel, and the usAHP
423 causes a hyperpolarisation, one plausible candidate is I_h. We found evidence for the development of I_h in
424 pro-metamorphic *Xenopus* spinal neurons, since 42/104 (40%) displayed a prominent sag response and PIR
425 following membrane hyperpolarisation (Fig. 6C). It is possible that the usAHP interacts with I_h, which is now
426 prevalent in spinal neurons, speeding up the recovery of the membrane potential. A similar interaction has
427 recently been reported in leech CPG neurons (Kueh et al, 2016). Another possible explanation for the
428 reduction in IR during the initial part of the usAHP is the opening of *Shal*-type I_A channels. Like I_h channels,
429 these are also activated by hyperpolarisation and have been suggested to contribute to the usAHP-like
430 responses in *Drosophila* larvae (Pulver and Griffith 2010).

431 Nitroergic modulation of spinal neurons

432 Given NO's excitatory effect on the occurrence of spontaneous locomotor activity during pro-metamorphosis
433 (Currie et al. 2016), it is perhaps not surprising that NO donors were found to depolarise spinal neurons (Fig.
434 7) and increase PSP frequency (Fig. 8). However, evidence published previously strongly suggests that NO
435 mediates its excitatory effects on spontaneous activity via the brainstem, and has little effect directly on the
436 spinal network itself (Currie et al. 2016). Furthermore, at embryonic and early larval stages, NO also
437 depolarises spinal neurons despite having a potent inhibitory effect on motor output (McLean and Sillar 2002,
438 2004). One possible explanation to reconcile these apparent anomalies is that pre-synaptic facilitation of
439 depolarizing PSPs reflects the potentiation of input synapses coming from brainstem neurons involved in the
440 descending activation of the swim CPG.

441 The increase in PSPs after NO donor application is not surprising given that NO is known to facilitate synaptic
442 transmission in neurons throughout the nervous system (for review see Garthwaite and Boulton 1995). More
443 specifically, in earlier stages of *Xenopus* development, NO is known to increase both GABAergic IPSPs onto
444 MNs, prematurely terminating swim episodes, and NA release onto glycinergic neurons, slowing swim
445 frequency (McLean and Sillar 2002, 2004; Fig. 2.4). NO's highly diffusible nature allows it to act on multiple
446 neurons and synapses simultaneously, and as such any increase in NO concentration would be expected to
447 facilitate local synaptic transmission. An important next step in these experiments is to deduce the origin of
448 the PSPs.

449 In summary, we describe a novel preparation that enables patch clamp recordings from spinal neurons at
450 later stages in the development of a well-characterised motor control system for swimming in the *Xenopus*
451 tadpole. This preparation will now make it feasible to investigate in detail the maturation of a spinal CPG that
452 first appeared during embryonic development and to explore the role of attendant modulatory pathways
453 following the switch to a free-swimming life style, as well as how the new circuitry controlling the limbs
454 becomes incorporated.

455

456

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461 DISCLOSURES

462 The authors declare no competing interests

463

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565

566 FIGURE LEGENDS

567 **Figure 1 - Whole cell patch clamp recordings during fictive locomotor activity.** A: cartoon of a stage 54/55
568 pro-metamorphic *Xenopus laevis* tadpole. Drawing kindly provided by Laurence D. Picton, with permission.
569 Approximate location of patch clamp recordings is indicated. B: schematic of isolated brainstem-spinal cord
570 preparation for whole cell recording. The expanded section illustrates the site of recording with overlying
571 dorsal spinal cord removed. Ci: both whole cell patch clamp and ventral root recordings of a spontaneous
572 episode of fictive locomotion from a stage 55 tadpole. The lower panel shows the firing pattern on an
573 expanded time scale to highlight the transitions between tonic and rhythmic firing during the episode. Cii: As
574 above but for an episode evoked by brief electrical stimulation of the optic tectum. Right hand panel shows
575 the waxing and waning of activity during the episode, including the recruitment and de-recruitment of the
576 spinal neuron – expanded from grey box in left hand panel. * denotes stimulus artefact.

577 **Figure 2 - Neurons with a tonic firing pattern.** A: ventral root and whole cell patch clamp recordings from a
578 stage 56 tadpole. This neuron modulates its firing rate during spontaneous episodes of motor activity (lower
579 panels). At the beginning of the episode when the ventral root activity is highest the neuron fires at 9Hz,
580 while just prior to the episode finishing this has dropped to only 5.5Hz. B: as above although this cell has an
581 even more dramatic increase in firing rate at the beginning of the evoked episode of motor activity. During
582 the episode both neuron and ventral root burst rhythmically (see lower panels).

583 **Figure 3 - Firing properties of spinal neurons.** Ai: example of typical low threshold neuron response to
584 current injection at 1.1x and 2x rheobase. Aii: same as Ai but for higher threshold neuron. Aiii: scatter plot of
585 current pulse vs firing frequency for the two neurons depicted in Ai (open circles) and Aii (filled circles). Bi:
586 neuron displaying intrinsic oscillations following current injection at its firing threshold and at 2x rheobase.
587 Bii: the same neuron during an episode of evoked swimming. Inset is part of trace within the grey box on an
588 expanded time scale. *** denote multiple stimulation artefacts. Ci: example of a presumptive dIN firing a
589 single spike during an episode of swimming. Cii: a spike from the same neuron evoked via current injection
590 (black trace) with a similar evoked spike from the neuron in Fig 3Aii superimposed (grey trace). Ciii response
591 of this neuron to current injection at its firing threshold and at 4x rheobase.

592 **Figure 4 - Recording from motoneurons.** A: neurobiotin fills of two motoneurons (MNs) from pro-
593 metamorphic tadpoles. MNs were primarily identified based on their long descending axon (arrow in Aii,iv)
594 that spanned several spinal segments (numbered in Ai) and exited the spinal cord via ventral root (* in Ai & iii)
595 – soma position in Ai is marked with arrow. The arrowhead in Aiv highlights dendritic arborisations around
596 the soma and the midline is traced with a dashed line in all panels. Unlabelled scale bars represent 50µm. Bi:
597 ventral root and whole cell recording of a different MN following current injection in a stage 56 tadpole. Each
598 spike in the neuron is represented 1:1 in the ventral root trace indicating the neuron projects to that root. Bii:

599 an antidromic spike (lower panel) from the same neuron following brief electrical stimulation of the recorded
600 ventral root (upper panel - * denotes stimulation artefact).

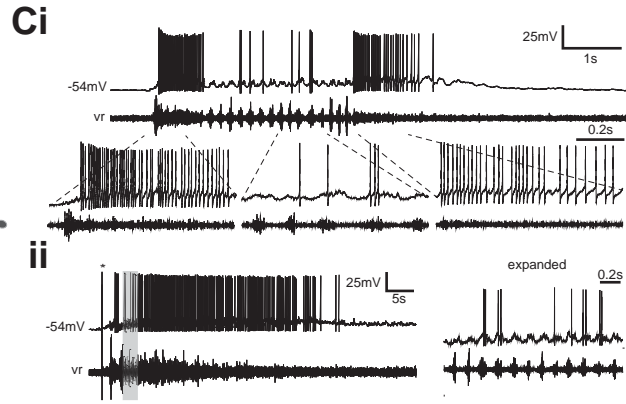
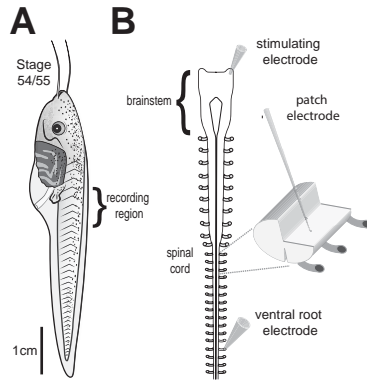
601 **Figure 5 - Afterhyperpolarisations in spinal neurons.** Ai: whole cell recording at rest with 2ms
602 suprathreshold current pulse. Following an action potential both fast (f) afterhyperpolarisation (AFP) and
603 slow (s) AHP are visible (see insert on expanded time scale). Aii: the same neuron during motor activity
604 highlighting the lack of any sAHPs despite the clear presence of fAHPs. Bi: whole cell recording at rest with 2s
605 suprathreshold current pulse. Following the train of action potentials there is an ultra-slow (us) AHP lasting
606 several seconds (see inserts for expansion of the first few seconds). Bii: the same neuron and a ventral root
607 recording during spontaneous motor activity. Following membrane repolarisation at the termination of
608 swimming there is an usAHP lasting several seconds (see insert for expansion of first few seconds).

609 **Figure 6 - The development of the usAHP.** Ai: whole cell recording during 10pA current steps from -50pA to
610 260pA (protocol was stopped after 20th suprathreshold current step). The protocol (originally used in Zhang
611 & Sillar, 2012) drives an usAHP in the neuron and basic parameters can be measured. Aii: direct comparison
612 of usAHP between stages 37/38-42 (N = 25; Zhang & Sillar, 2012) and pro-metamorphic stages 50-58 (N = 20).
613 Bi: whole cell recording with hyperpolarising steps both prior to and after driving an usAHP (NB: action
614 potentials are truncated; see expansion for details of protocol). Bii: graph of input resistance (IR) at
615 successive hyperpolarising steps following the usAHP relative to IR at rest. Biii: graph of mean time taken for
616 IR to return to baseline levels and mean duration of the usAHP to the same protocol (N = 9) Ci: whole cell
617 recording showing a typical response to successive hyperpolarising steps from rest. Membrane sag and post
618 inhibitory rebound (PIR) are highlighted. Cii: whole cell recording from the same cell showing how PIR often
619 leads to rebound action potentials following membrane repolarisation. Di: whole cell recording during
620 successive suprathreshold current steps highlighting the loss of neuronal excitability during the usAHP (NB:
621 action potentials are truncated). Dii: comparison of neuronal response to short suprathreshold current
622 injection before (black trace) and after (grey trace) driving an usAHP. Diii: mean data for the same protocol
623 showing the latency to the first spike (N = 17) and instantaneous firing frequency of the second spike (N = 6)
624 after the usAHP relative to before it. * denotes $p = < 0.05$; *** denotes $p = < 0.01$ from a paired t-test (Aii, Biii)
625 or a Wilcoxon signed rank test (Bii, Diii).

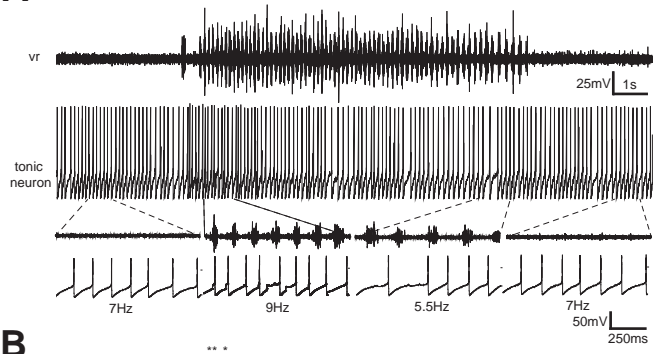
626 **Figure 7 - Nirtergic modulation of pro-metamorphic spinal neurons.** A: ventral root and whole cell
627 recordings during bath application of 200 μ m DEA-NO. A reversible increase in resting membrane potential
628 (RMP) is visible in the whole cell record (Ai, bottom panel). Aii: on expanded time scales examples of
629 spontaneous episodes motor activity are shown. B: graphs of mean RMP (Bi) and input resistance (IR; Bii)
630 relative to control during NO donor application (200 μ m SNAP, N = 4; 200 μ m DEA-NO, N = 7) and during
631 washout. * denotes $p = < 0.05$; *** denotes $p = < 0.01$ from a Wilcoxon signed rank test.

632 **Figure 8 - Nitric oxide increases PSPs in spinal neurons.** A: a whole cell recording from 3 quiescent periods
633 during control, DEA-NO application and washout showing depolarising post-synaptic potentials (PSPs). B:
634 cumulative frequency plots for PSP inter-event interval (Bi) and amplitude (Bii) for the cell recorded in A. C:
635 average data for PSP frequency (Ci) and amplitude (Cii) during control, NO donor application (200µm SNAP, N
636 = 4; 200µm DEA-NO, N = 6) and washout. * denotes $p = < 0.05$ from a repeated measures ANOVA with
637 Bonferroni correction .

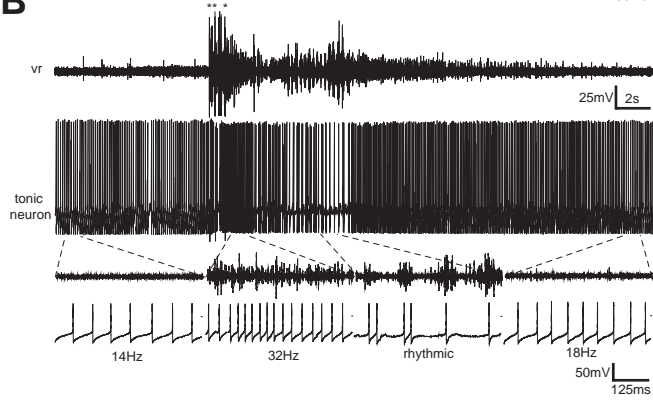
638 **Figure 9 - Nitric oxide blocks the usAHP in pro-metamorphic neurons.** Ai-iii: whole cell recording of response
639 to suprathreshold current steps before (Ai), during (Aii) and after (Aiii) bath application of 200µm DEA-NO
640 (action potentials are truncated). Div: average data of usAHP amplitude relative to control during 200µm
641 DEA-NO application and during washout (N = 6). *** denotes $p = < 0.01$ from a Wilcoxon signed rank test.

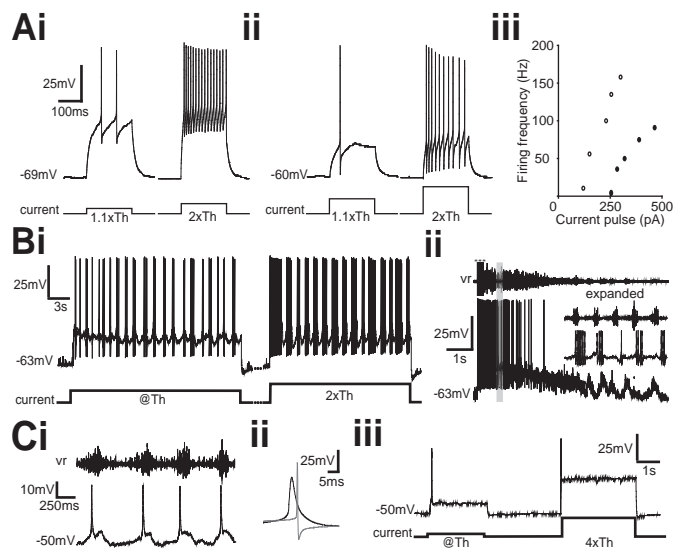


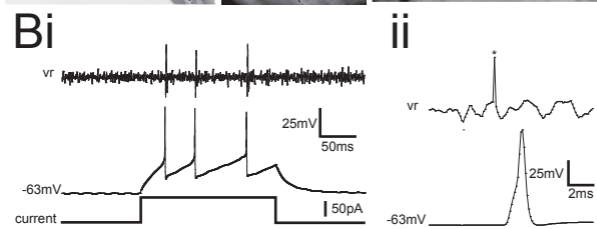
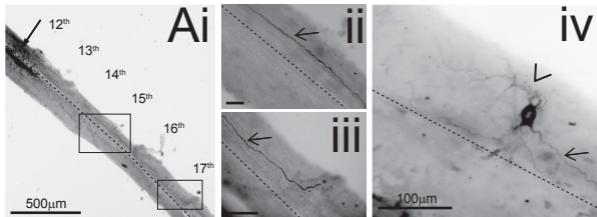
A

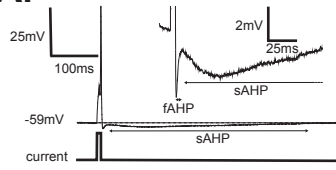
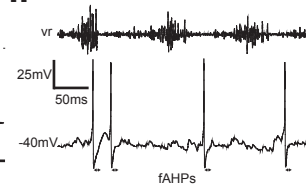
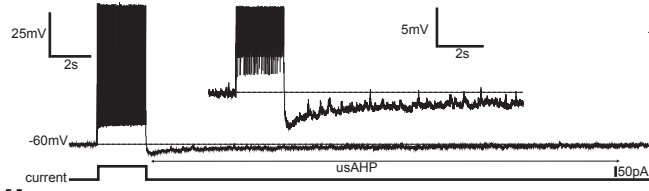


B







Ai**ii****Bi****ii**