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1 Sodium pump regulation of locomotor control circuits

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17 University of St Andrews) for providing images and data on *Drosophila* third instar
18 larval crawling.

19 Abstract

20 Sodium pumps are ubiquitously expressed membrane proteins that extrude three
21 Na⁺ ions in exchange for two K⁺ ions using ATP as an energy source. Recent studies
22 have illuminated additional, dynamic roles for sodium pumps in regulating the
23 excitability of neuronal networks in an activity-dependent fashion. Here we review
24 their role in a novel form of short-term memory within rhythmic locomotor networks.
25 The data we review derives mainly from recent studies on *Xenopus* tadpoles and
26 neonatal mice. The role and underlying mechanisms of pump action broadly match
27 previously published data from an invertebrate, the *Drosophila* larva. We therefore
28 propose a highly conserved mechanism by which sodium pump activity increases
29 following a bout of locomotion. This results in an ultraslow afterhyperpolarisation
30 (usAHP) of the membrane potential that lasts around 1 minute, but which only occurs
31 in around half the network neurons. This usAHP in turn alters network excitability so
32 that network output is reduced in a locomotor interval-dependent manner. The
33 pumps therefore confer on spinal locomotor networks a temporary memory trace of
34 recent network performance.

35 Introduction

36 Motor systems have evolved to meet the species-specific behavioural requirements
37 upon which animal survival and reproduction depend. To succeed, the underlying
38 motor circuits must be adaptable in the face of the demands placed on individuals by
39 prevailing external and internal conditions. Such circuit adaptations, which may
40 relate to developmental stage and/or hormonal state, are mostly due to changes in
41 the integrative electrical properties of, and synaptic weightings between, component

42 neurons within motor circuits (Harris-Warrick and Marder 1991). Many of these
43 changes are mediated by the opening of ion channels, and the consequent
44 alterations to circuit function can involve both neuromodulation and activity-
45 dependent neuronal plasticity. One disadvantage of this ion channel-based strategy
46 is that the decrease in input resistance that accompanies channel opening could
47 shunt incoming synaptic inputs and decrease the responsiveness of neurons and
48 subsequent network output. This, in turn, could compromise the intended behaviour,
49 and if this involves the escape from a predator, for example, it could be potentially
50 catastrophic for survival. An alternative strategy is for neuronal activity or
51 neuromodulation to affect the function of ion pumps which, since there is no change
52 in input resistance, should not shunt the membrane response and hence preserve
53 the responsiveness of the network to various inputs. Furthermore, changes in the
54 activity of ion pumps can exert effects on the excitability of neurons on a much
55 slower timescale, over many seconds and even minutes, leaving a prolonged
56 memory trace of a neuron's recent activity.

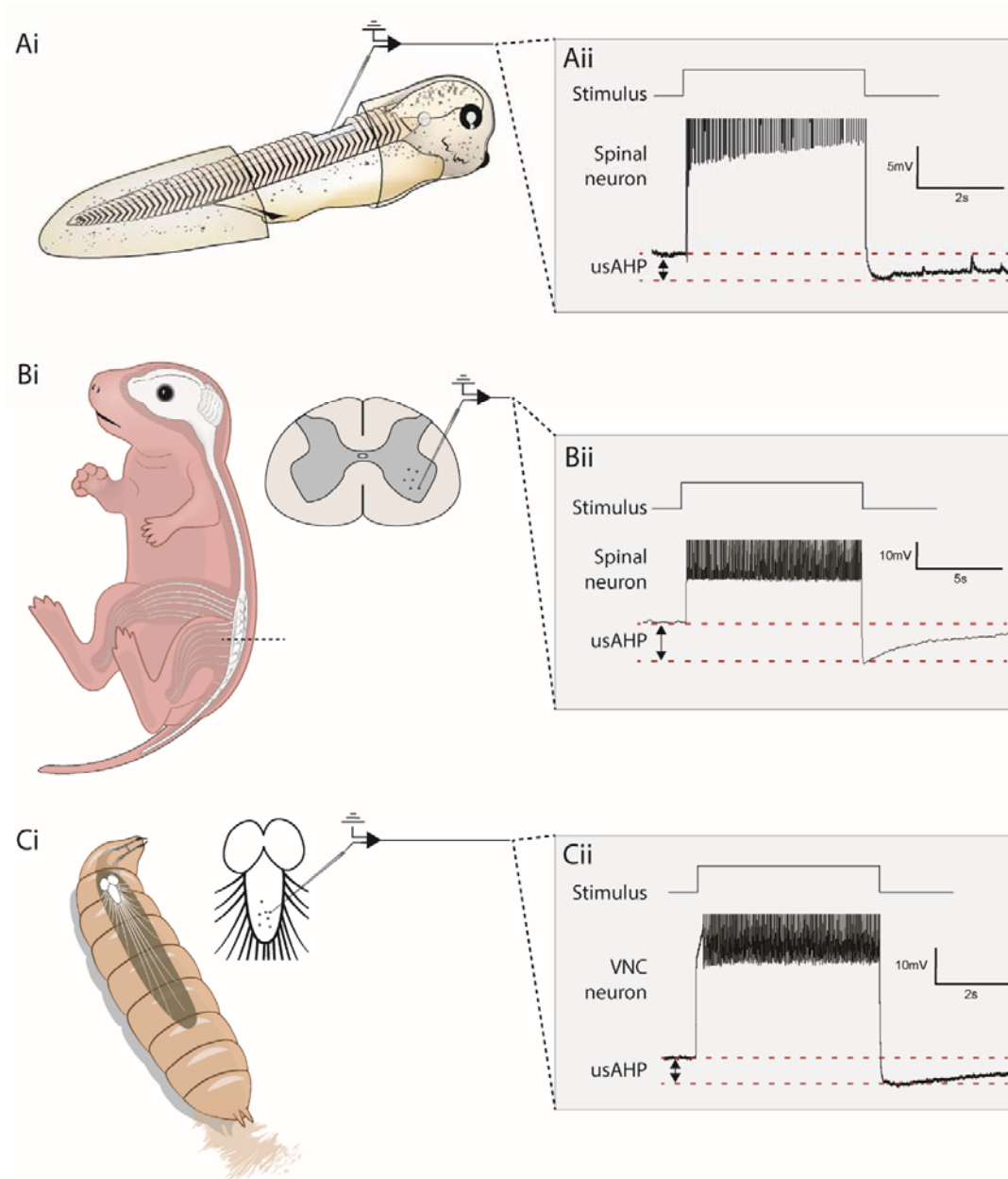
57 The $\text{Na}^+\text{-K}^+$ ATPase (*aka* the Na^+ pump) is one of the most ubiquitously expressed
58 proteins in the animal kingdom, which is most renowned for its role in establishing a
59 gradient of high extracellular Na^+ and high intracellular K^+ ion concentrations across
60 cell membranes. With each Na^+ pump cycle, three Na^+ ions are extruded and two K^+
61 ions flow into the cell, utilizing ATP as an energy source. Because of this charge
62 asymmetry, Na^+ pump activity sets and homeostatically maintains the resting
63 membrane potential upon which neuronal firing relies, and in so doing accounts for
64 more than half of all brain energy consumption (Engl and Attwell 2015).

65 Recently, a novel and dynamic role for the Na^+ pump as an activity-dependent
66 regulator of brain and spinal circuit function has been reported across a wide range
67 of neurons, systems, behaviours and species. Within motor systems, for example,
68 seminal work on crawling in *Drosophila* larvae has demonstrated that high frequency
69 action potential firing of motoneurons causes a pump-mediated hyperpolarization
70 lasting tens of seconds, which in turn influences future locomotory crawling
71 behaviour (Pulver and Griffith 2010). In the present paper, we review and compare
72 similar findings from spinal central pattern generator (CPG) circuits controlling
73 rhythmic locomotion in two phylogenetically disparate vertebrate model systems: the
74 *Xenopus* frog tadpole and the neonatal mouse. As in *Drosophila*, these circuits also
75 possess an intrinsic pump-based mechanism that links future to past network activity.
76 This suggests a highly conserved, pump-mediated dynamic regulation of motor
77 circuit function. In spinal motor circuits, the duration of a bout of locomotion is
78 influenced by previous network activity if two bouts occur within about a minute of
79 each other; a form of short-term motor memory (Picton et al. 2017; Zhang and Sillar
80 2012; Zhang et al. 2015). This motor memory relies on the presence of a pump-
81 mediated ultraslow afterhyperpolarization (usAHP) of up to 10 mV in spinal neurons,
82 which lasts for the same duration of approximately a minute.

83 **Na^+ pump regulation in three locomotor systems**

84 **The ultra-slow afterhyperpolarisation (the usAHP)**

85 In both the tadpole (Figure 1A) and neonatal mouse (Figure 1B), high frequency
86 action potential firing drives the resting membrane potential to a more hyperpolarized
87 level in a subset of motoneurons and interneurons in the spinal cord. A remarkably
88 similar phenomenon has also been reported in *Drosophila* larva motoneurons
89 (Pulver and Griffith 2010; Figure 1C). This hyperpolarization is distinguished from
90 other ion channel-mediated AHPs (e.g. the “fast”, “medium” or “slow” AHP; Storm
91 1987) largely by its duration, with neurons remaining hyperpolarised once activity
92 has stopped for up to one minute. Although the amplitude of a usAHP can vary quite
93 considerably both within and between neuron types, our findings in *Xenopus* and
94 mouse spinal neurons suggest that, on average, the pump AHP involves a
95 hyperpolarization of approximately 5 mV (Figure 1Aii,Bii), remarkably similar to the
96 equivalent event in *Drosophila* larvae (Figure 1Cii).

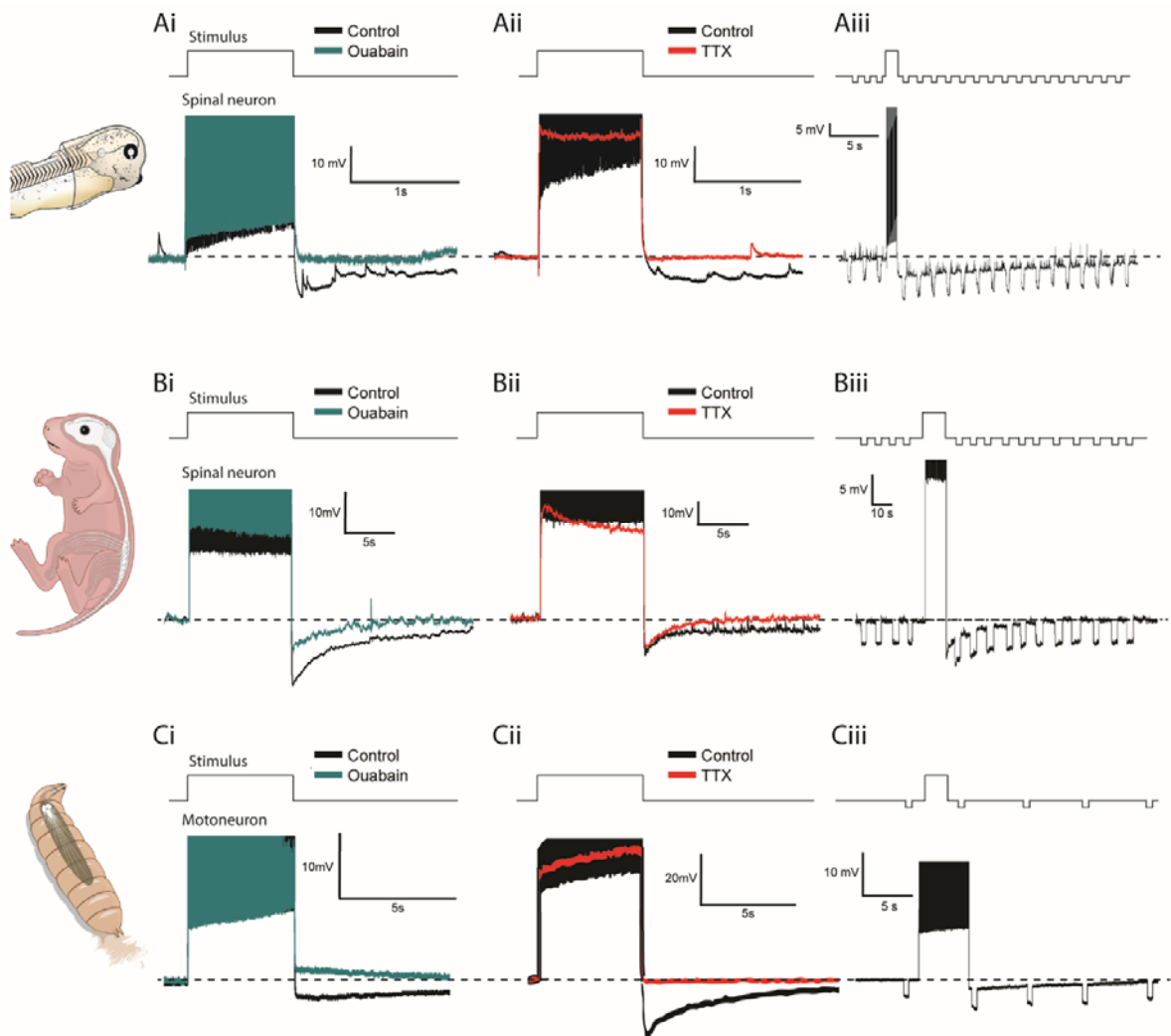


97

98 **Figure 1. The ultraslow afterhyperpolarisation (usAHP) in CPG neurons of three species.** **Ai.** Experimental preparation for
 99 making patch-clamp recordings from an immobilised stage 37/8 *Xenopus* tadpole. **Aii.** Following either swimming, or in this
 100 case a long suprathreshold current pulse, the membrane potential is driven to a more hyperpolarised membrane potential
 101 (the usAHP). **Bi.** Experimental preparation for making patch-clamp recordings from neonatal mice. **Bii.** Following a long
 102 suprathreshold current pulse, a usAHP is observed in spinal motoneurons and interneurons in neonatal mice. **Ci.** Schematic
 103 of a third instar *Drosophila* larva. **Cii.** A usAHP observed in a *Drosophila* motoneuron.

104 Besides its long duration, several other features of the usAHP distinguish it from ion
 105 channel-mediated AHP mechanisms. For example, because it is mediated by the
 106 Na^+ pump, it is selectively blocked by a low concentration of the cardiac glycoside
 107 ouabain (Figure 2Ai,Bi,Ci). The usAHP is also highly dependent on the accumulation
 108 of intracellular sodium that accompanies repetitive action potential firing. Therefore
 109 blocking fast sodium channels with TTX, to prevent action potential generation, also
 110 effectively abolishes the usAHP (Figure 2Aii,Bii,Cii). Thirdly, because the usAHP

111 occurs upon the increased activation of ion pumps, rather than ion channel opening
 112 or closing, there are no detectable changes in conductance, and this can be
 113 observed by measuring a consistent membrane response to small injections of
 114 hyperpolarising current throughout the usAHP (Figure 2Aiii,Biii,Ciii). Perhaps not
 115 surprisingly, there are a number of differences in the features of the usAHP in
 116 tadpoles and mice at the single-cell level. For example, whilst ouabain and TTX
 117 completely abolish the usAHP in tadpoles, a shorter-duration AHP often persists in
 118 many motoneurons and interneurons in mice (Figure 2Bi,ii), presumably due to the
 119 presence of additional, voltage-dependent AHP mechanisms such as the medium
 120 and/or slow AHP, which can persist in the absence of spiking (Rekling et al. 2000).



121
 122 **Figure 2. A cross-species comparison of the basic features of the usAHP.** Ai. The usAHP is abolished by the Na^+ pump
 123 blocker ouabain. Aii. The usAHP is also abolished when fast Na^+ channels are blocked using TTX. Aiii. By measuring the
 124 membrane response to small hyperpolarising current pulse we found no changes in conductance before, during or after the
 125 induction of a usAHP, suggesting the involvement of a Na^+ pump (adapted from Zhang and Sillar 2012). The experimental
 126 manipulations outlined in A have similar results in neonatal mouse CPG neurons (B; adapted from Picton et al, 2017) and
 127 *Drosophila* motoneurons (C; adapted from Pulver and Griffith 2010).

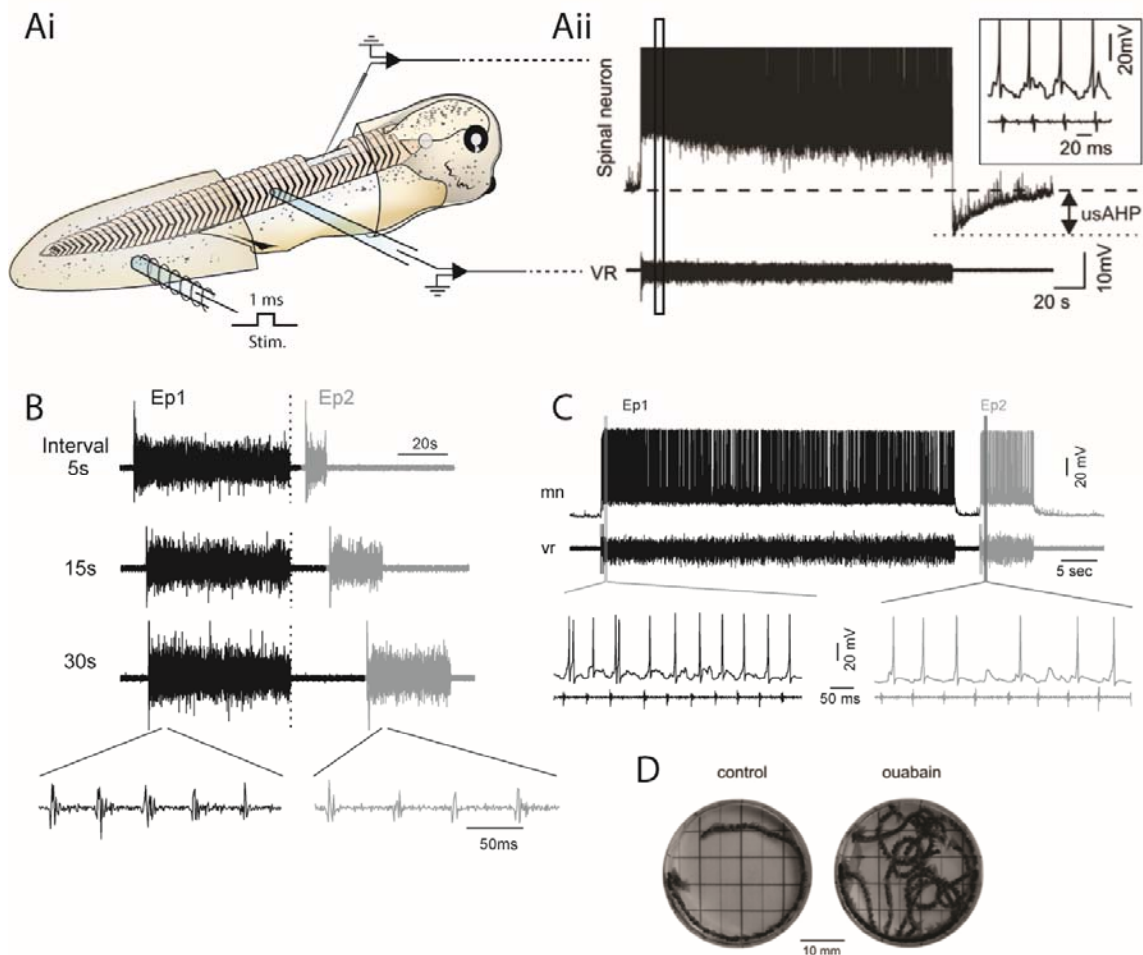
128 **Physiological roles for the Na^+ pump**

129 By its very nature, the usAHP is ideally positioned to function as a spike rate monitor,
130 whose duration and amplitude reflects the integration of spike frequency over time.
131 Furthermore, the usAHP is not only generated in response to artificial current
132 injection protocols used to evoke spikes, but by any stimulus that produces trains of
133 action potentials sufficient to generate a build-up of intracellular sodium (e.g.
134 locomotion, Figure 3Aii). Importantly, because the usAHP recovers over a period of
135 around a minute, it acts as a transient engram of how recently, and how intensely
136 locomotor activity occurred.

137 In *Xenopus* tadpoles, we have explored how this short-term memory of recent
138 activity acts to regulate the interval relationship between evoked episodes of “fictive
139 swimming” (motor output without muscle contraction). When the interval between
140 swim episodes is set to longer than the duration of a usAHP (longer than 1 minute),
141 episodes of evoked swimming in a “well rested” tadpole are statistically identical,
142 both in the duration of a swim episode and all other parameters of swimming (swim
143 frequency, burst durations etc.). However, when this interval is reduced to 30, 15 or
144 5 seconds, the second episode is progressively shorter, slower and weaker, in an
145 interval-dependent manner (Figure 3B; note spike failures in episode 2, Figure 3C).
146 The importance of the Na⁺ pump for this self-regulation of network output becomes
147 clear when the pumps are blocked by ouabain; the animal becomes completely
148 unable to regulate its own locomotor activity, causing it to swim almost indefinitely
149 (Figure 2D).

150 The swim durations and inter-episode intervals involved here may seem short
151 anthropomorphically (tens of seconds), but need to be scaled to be appreciated from
152 a human perspective, and in the broader context of locomotion. If we treat a single
153 tail undulation as equivalent to one human stride, then a typical 2 minute episode of
154 20 Hz swimming (~2400 swim cycles) could be considered broadly equivalent to a 5
155 km sprint for a human (assuming a typical stride length of ~2 metres). This distance
156 could comfortably be covered in around 30 minutes, but imagine resting only for a
157 minute before being stimulated to sprint again while still fatigued; the runner is
158 unlikely to get as far, or locomote at the same speed, as it could from a well-rested
159 start. Whether Na⁺ pumps play a direct role in human fatigue is not yet completely
160 clear, but certainly the evidence for central mechanisms of fatigue is extremely
161 compelling (reviewed in Gandevia 2001). More specifically, there is strong evidence
162 that central fatigue involves an activity-dependent reduction in motoneuron drive
163 (Ranieri and Di Lazzaro 2012; Rossi et al. 2012). Furthermore, it has been shown
164 that human motor axons display an activity-dependent hyperpolarisation following
165 natural activity, which is due to an enhancement of Na⁺ pump activity, and whose
166 duration and amplitude depends on the axonal discharge rate (Kiernan et al. 2004;
167 Vagg et al. 1998). This raises the fascinating possibility that an activity-dependent
168 enhancement of Na⁺ pump activity in spinal neurons may contribute to fatigue during
169 human locomotion. Given the ubiquity of pumps throughout the nervous system they
170 have enormous potential as drug targets, with important implications not only for
171 endurance athletes, but also in the context of diseases associated with fatigue
172 symptoms such as diabetes (Krishnan et al. 2008) and ALS (Ellis et al. 2003), in
173 which sodium pump dysfunction has been implicated.

174 It has long been known that one way to experimentally “fatigue” a neuron is to raise
 175 the levels of intracellular sodium. These experiments were first conducted on the
 176 squid giant axon in the mid 1950’s and, quite unexpectedly, high sodium resulted in
 177 a tonic membrane hyperpolarisation (Hodgkin and Keynes 1956) that turned out to be
 178 mediated by enhanced Na^+ pump activity. In our experiments, we have used a
 179 drug called monensin, a sodium ionophore, to raise the level of intracellular sodium
 180 in spinal CPG neurons. This not only enhances the usAHP by increasing Na^+ pump
 181 activity, but in effect it causes the locomotor network to become chronically fatigued.
 182 Under these conditions, the swim network acts as if it is being activated from an
 183 unrested starting point, resulting in weaker, slower and shorter locomotion.

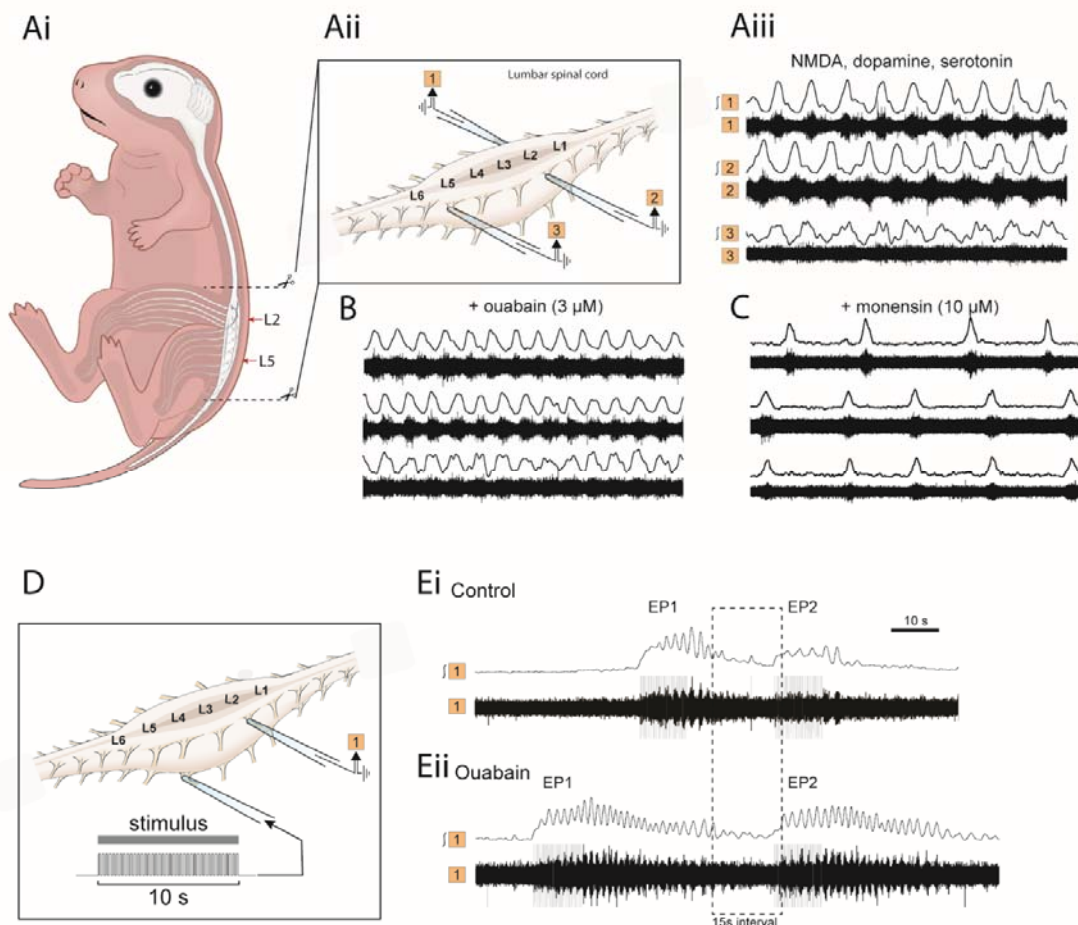


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185 **Figure 3. The usAHP as a short-term memory mechanism in *Xenopus tadpoles*.** Ai. Schematic showing the experimental
 186 set-up. Aii. A brief (1 ms) current pulse to the tail (Stim.) initiates an episode of swimming which is recorded at both the
 187 single cell level (Aii, top) and at the level of overall network output using ventral root recording (Aii, bottom). Note the
 188 prolonged membrane hyperpolarisation (usAHP) in the intracellular trace at the end of the swim episode. Inset shows an
 189 expansion of the recording indicated by the black box showing the intracellular and ventral root traces during swimming. B.
 190 Ventral root recordings showing that an evoked swim episode is shorter and slower when it follows a previous episode after
 191 a 5, 15 or 30 second interval. C. The interval relationship is apparent when activity is evoked within the 1 minute usAHP that
 192 follows swimming, which reduces the spike probability of CPG neurons. D. Real swimming behaviour in a *Xenopus tadpole*
 193 with multiple consecutive video frames overlapped to show swim path in response to touch. When the Na^+ pumps are
 194 blocked using ouabain the tadpole is unable to regulate its activity and swims continuously (adapted from Zhang and Sillar
 195 2012; Zhang et al. 2015).

196 We have also explored the effects of Na⁺ pump manipulation in the lumbar spinal
 197 cord of neonatal mice, using two methods for evoking locomotor activity. Traditionally,
 198 a combination of drugs (dopamine, NMDA, serotonin) is applied to induce a
 199 continuous locomotor rhythm (Figure 4A). Under these conditions, blockade of Na⁺
 200 pumps using ouabain causes the rhythm frequency to increase (Figure 4B).
 201 Conversely, raising the levels of intracellular sodium using monensin, which
 202 indirectly activates the Na⁺ pump, causes the opposite effect (Figure 4C). Whilst this
 203 reveals the importance of the Na⁺ pump for frequency control, it obviously cannot
 204 address the role of Na⁺ pumps in regulating intervals between locomotor episodes.

205 In order to address this question in a similar way to our earlier tadpole experiments,
 206 we switched to using dorsal root sensory stimulation to evoke individual, more
 207 natural bouts of locomotor activity (Figure 4D,E). In much the same way as in
 208 tadpoles, episode 2 is clearly influenced by episode 1 so long as the interval is
 209 shorter than 1 minute (Figure 4Ei). This relationship breaks down in the presence of
 210 ouabain such that episode 2 is now similar to episode 1 in duration, frequency and
 211 amplitude (Figure 4Eii).



212

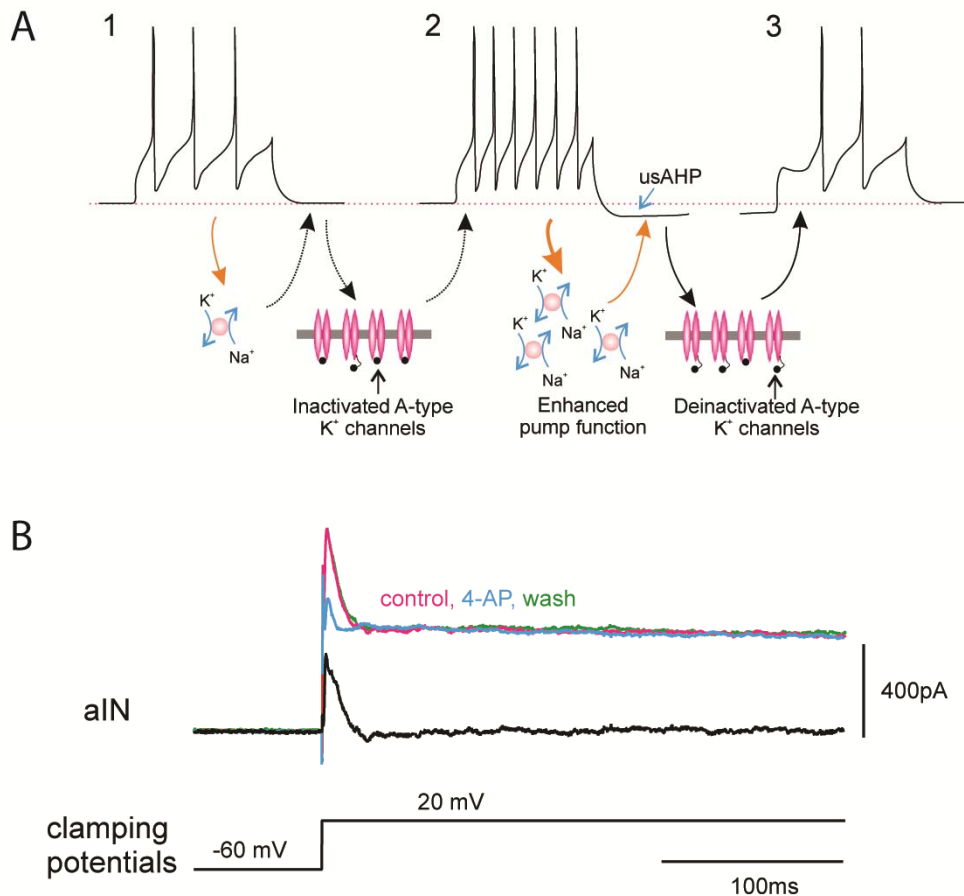
213 **Figure 4. Na⁺ pump manipulation in the neonatal mouse preparation.** Ai. Schematic depicting neonatal mouse spinal cord
 214 preparation. Aii. Glass suction electrodes are attached to the first or second lumbar ventral roots (L1, L2) on the left and
 215 right sides of an isolated spinal cord to record flexor-related activity, and a third electrode is attached to the fifth ventral
 216 root (L5) to record extensor-related activity. Aiii. Raw and rectified/integrated traces showing drug-induced activity on the
 217 left and right L2 roots and the right L5 root. B. Na⁺ pump blockade increases the frequency of locomotor bursting. C.

218 Activation of the Na^+ pump has the opposite effect of slowing locomotor burst frequency. **D.** For sensory stimulation, an
219 electrode was attached to the fourth or fifth dorsal root (L4 or L5) to deliver current pulses to initiate locomotion. **Ei.** When
220 two episodes of locomotor output are evoked with a short interval (15 s), the second episode is both shorter and slower
221 compared to this first episode. **Eii.** Following blockade of the Na^+ pump, not only are episodes longer and faster compared
222 to control, but the interval relationship is abolished (Adapted from Picton et al. 2017).

223 **Mechanism linking usAHP and A-current**

224 The Na^+ pump-mediated usAHP clearly plays an important role in allowing locomotor
225 networks to regulate their output in relation to past activity. However, it is not
226 immediately obvious how the relatively modest membrane hyperpolarisation (~ 5 mV)
227 caused by increased activation of the Na^+ pump can cause dramatic changes in
228 neuronal excitability, especially since there is no obvious change in conductance. A
229 likely possibility is that in different systems, different voltage-dependent currents are
230 affected by the change in membrane potential. Two currents that appear to have
231 important interactions with sodium pump currents in CPG networks are I_h and I_A
232 (Kueh et al. 2016; Pulver and Griffith 2010; Zhang et al. 2015).

233 Pulver and Griffith (2010) showed in *Drosophila* larva motoneurons that the pump-
234 mediated AHP brought the membrane potential into a range that caused the de-
235 inactivation of an A-type potassium current, I_{shal} , which in turn introduced a delay to
236 the first spike when activity resumed. Classically, channels mediating I_A are largely
237 inactivated at the resting membrane potential but are de-inactivated by
238 hyperpolarisation, so that when the neuron is next excited by a depolarising input the
239 rate of depolarisation is slowed by I_A . We found precisely this mechanism at play in
240 tadpole spinal neurons (Zhang et al. 2015). When a usAHP was induced by a high
241 frequency train of action potentials (Figure 5A2), the delay to firing in response to a
242 brief current pulse was longer compared with before the induction of a usAHP
243 (Figure 5A3 vs. 5A1). The presence of a 4-AP-sensitive A-type potassium current
244 was confirmed using voltage clamp recordings (Figure 5B). Whether a similar
245 mechanism involving an A-type potassium current contributes to the role of the
246 usAHP in neonatal mice is yet to be confirmed, but this possibility seems likely.



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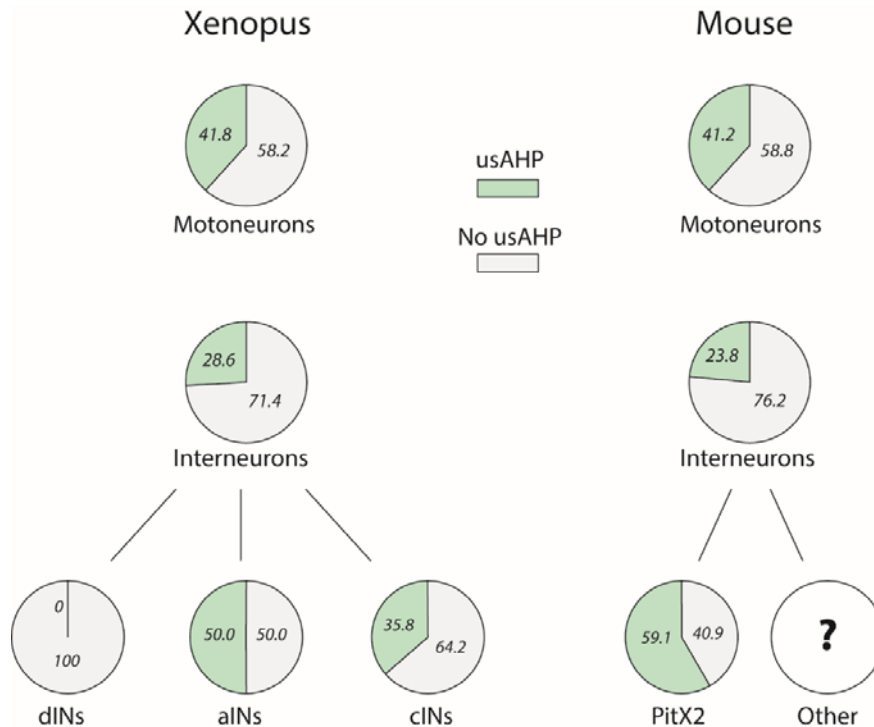
248 **Figure 5. An A-type potassium current links the usAHP to inhibition of firing in *Xenopus* spinal neurons.** **A.** Summary of
 249 the mechanism illustrating how the Na⁺ pump and A-type K⁺ current are involved in the short-term memory of motor
 250 network output. At rest, most A-type K⁺ channels are inactivated. Weak activity (1) does not increase Na⁺ pump current
 251 sufficiently to hyperpolarize the membrane potential so when the membrane potential is subsequently depolarized above
 252 threshold (2) most A-type K⁺ channels cannot be activated, and thus the first spike delay is unaffected. Stronger activity (2)
 253 can potentiate Na⁺ pump function and induce a larger pump current which hyperpolarizes the membrane potential (usAHP).
 254 This hyperpolarization removes the inactivation of A-type K⁺ channels, so that when depolarized above threshold (3), the A-
 255 type current is large enough to impede membrane depolarisation, prolonging first spike delay, and reducing the total
 256 number of spikes to a given depolarising input. **B.** Voltage clamp evidence for a 4-AP-sensitive A current in a spinal
 257 ascending interneuron (aIN). 4-AP preferentially blocks transient K⁺ currents. Red current trace is control, blue is in 4-AP and
 258 green is wash. Black trace is the difference in currents between control and 4-AP. (Adapted from Zhang et al. 2015).

259 **Heterogenous distribution**

260 The functional anatomy of the tadpole spinal network is known in considerable detail
 261 (Roberts et al. 2010), such that the presence or absence of a usAHP can be
 262 ascribed to each class of spinal neuron that participates in locomotory swimming. In
 263 three of the four main CPG classes (motoneurons (MNs), commissural interneurons
 264 (ciNs) and ascending interneurons (aiNs)), we found that approximately half of
 265 neurons display a usAHP; while in the other half of each subtype it is absent (Figure
 266 6, Zhang and Sillar 2012). Furthermore, in one entire class, the excitatory rhythm-
 267 generating descending interneurons (diNs), the usAHP is absent altogether. The fact
 268 that diNs appear to be spared the influence of a usAHP presumably explains why
 269 some residual rhythm-generating capability remains regardless of how short the

270 inter-swim interval is (e.g. Figure 3B, 5s interval). However, the firing of dINs relies
 271 on rebound from mid-cycle inhibition coming from cINs on the contralateral side of
 272 the spinal cord, and therefore the impact of I_A on cIN firing will indirectly compromise
 273 dIN firing, and in turn the maintenance of the swim rhythm. The explanation for a
 274 lack of a pump current in dINs is yet to be determined, but one possibility is that they
 275 do not possess specific sodium pump isoforms responsible for mediating the usAHP
 276 (see discussion). Alternatively, the usAHP may be masked in this cell type by an
 277 equal, but opposite depolarising current, such as a persistent sodium current, or an
 278 I_h current, which may also become activated during intense spiking protocols
 279 (Darbon et al. 2004; Gullledge et al. 2013; Wang et al. 2012). This possibility is
 280 currently under investigation, with preliminary evidence suggesting that this may be
 281 the case.

282 A similar heterogenous usAHP distribution is present in the neonatal mouse CPG
 283 (Figure 6, Picton et al. 2017). For MNs, a very similar proportion to the tadpole
 284 (~40%) display the usAHP. For interneurons, there are many more classes in the
 285 mouse compared to the tadpole (Kiehn 2016), but around a quarter of unidentified
 286 interneurons that were recorded displayed a usAHP. This proportion is similar to that
 287 in tadpole interneurons when cINs, aINs and dINs are pooled. Although the identity
 288 of all the specific interneuron classes displaying a usAHP in neonatal mice is not yet
 289 known, one type of modulatory neurons, the cholinergic pitx2 class, was found to
 290 display a usAHP in around 60% of the population (Picton et al. 2017).



291

292 **Figure 6. Heterogenous distribution of the usAHP among neuron types in *Xenopus* tadpoles (Zhang and Sillar 2012) and**
 293 **neonatal mice (Picton et al. 2017).**

294 **Discussion**

295 **Na⁺ pumps: intrinsic memory through a spike-rate monitor**

296 Networks of neurons require the intrinsic capacity to monitor their own activity,
297 allowing for the initiation of important homeostatic control mechanisms that adjust
298 their output in light of past activity. Changes in neuronal and synaptic function often
299 begin with changes in ionic conductances. The activity of a neuron may be reflected
300 in changes in intracellular calcium concentration, leading to the activation of a range
301 of downstream signalling pathways including protein phosphorylation and ion
302 channel modulation. However, the clearance of calcium itself, mediated primarily by
303 the calcium pump, is often relatively rapid (Benham et al. 1992), and therefore
304 calcium influx is usually not considered to be responsible for electrical changes in the
305 time scale of tens of seconds. Another ion intrinsically linked to neuronal activity is
306 sodium, whose intracellular levels also rise rapidly during spiking before decaying
307 slowly over tens of seconds after activity has ceased (Rose 2002). The Na⁺ pump is
308 the primary means of restoring intracellular sodium concentrations. It is therefore
309 strategically positioned both to homeostatically control changes in intracellular
310 sodium levels resulting from neuronal firing, and to link neuronal activity to intrinsic
311 excitability. It was shown as early as the 1950's that rises in intracellular sodium can
312 cause a prolonged membrane hyperpolarisation (Coombs et al. 1955), and that this
313 effect is mediated by the activation of the Na⁺ pump (Connelly 1959; Ritchie and
314 Straub 1957).

315 This phenomenon has since been reported in a range of neuronal types at every
316 level of the motor pathway. For example, pump-mediated AHPs have been reported
317 in the *sensory neurons* of a range of species including insects (French 1989),
318 lamprey (Parker et al. 1996), leech (Arganda et al. 2007; Baylor and Nicholls 1969;
319 Scuri et al. 2002), crayfish (Nakajima and Takahashi 1966; Sokolove and Cooke
320 1971), frogs (Davidoff and Hackman 1980; Kobayashi et al. 1997), horseshoe crabs
321 (Smith et al. 1968) and rats (Gordon et al. 1990). Similar post-tetanic AHP
322 mechanisms mediated by the sodium pump have also been found in the
323 *interneurons* of numerous species including the leech (Tobin and Calabrese 2005),
324 *Aplysia* (Gage and Hubbard 1968; Pinsker and Kandel 1969) and rats (Darbon et al.
325 2002; 2003; Krey et al. 2010; Tsuzawa et al. 2015). Finally, the *motoneurons* of
326 diverse species have also been shown to display a spike-dependent, pump-
327 mediated hyperpolarisation, including in the motor axons of lizards (Morita et
328 al. 1993), guinea pigs (del Negro et al. 1999), rats (Ballerini et al. 1997; Gage and
329 Hubbard 1966) and humans (Kiernan et al. 2004; Vagg et al. 1998). In several
330 networks, these activity-dependent hyperpolarisations have been shown to perform
331 important roles in shaping the rhythmic output of the network itself; from
332 neurosecretory networks in the snail brain (Nikolić et al. 2008, 2012; Tsai and Chen
333 1995), to rhythmic networks in the rat brain including the suprachiasmatic nucleus
334 (Wang et al. 2004, 2006, 2012) and midbrain dopaminergic neurons (Johnson et al.
335 1992). More recently, sodium pumps have also been found to play an important role
336 in shaping the output of hippocampal neurons (Azarias et al. 2013; Gullledge et al.
337 2013; Gustafsson and Wigström, 1983), striatal neurons (Azarias et al. 2013),
338 cerebellar purkinje fibres (Forrest et al. 2012) and neurons in the auditory pathway
339 (Kim et al. 2007, 2012). Sodium pumps thus play important roles throughout the

340 nervous system and across diverse species, and participate at every level of the
341 motor pathway; from modifying sensory information, to the integration and relay of
342 this information by interneuronal networks, right through to the regulation of the final
343 motor output by motoneurons. However, only recently has the functional importance
344 of the sodium pump as a spike-rate monitor been explored in depth in the spinal
345 CPG networks controlling vertebrate locomotion.

346 Because of the close link between intracellular sodium levels and Na^+ pump activity,
347 pharmacological tools that raise the levels of sodium in a neuron can be useful for
348 studying the effects of increased Na^+ pump activity. Hence monensin, a sodium
349 ionophore that exchanges one sodium ion intracellularly for one proton extracellularly,
350 has been used extensively in studying sodium pumps (e.g. Kueh et al. 2016; Wang
351 et al. 2012; Zhang et al. 2015). Monensin essentially acts as a proxy for intense
352 spiking, imposing on neurons the pharmacological equivalent of a long train of high
353 frequency action potentials. In both *Xenopus* and mouse spinal neurons, monensin
354 increases Na^+ pump activity, hyperpolarising the membrane potential to the level
355 attained by the usAHP. Locomotor activity, again in both species, becomes shorter
356 and slower under monensin as if the network has been intensely active for a long
357 period of time. Thus, monensin appears to chronically fatigue spinal networks by
358 maximally activating the Na^+ pump autoregulation mechanism. Monensin has also
359 recently been used to study the role of Na^+ pumps in the heartbeat network of the
360 leech, where a fascinating interaction between a pump current and a depolarising I_h
361 current was revealed (Kueh et al. 2016). Directly increasing intracellular sodium
362 concentration using a modified intracellular solution could be used in future studies
363 to confirm these findings.

364 **Molecular and cellular basis for activity-dependent pump activation**

365 The pump-based mechanisms that link future to past network activity transcend
366 major phylogenetic boundaries and occur on multiple levels; from the molecular to
367 the cellular and circuit levels.

368 At the molecular level, there is an emerging hypothesis that there exist both tonic
369 and dynamic contributions of the sodium pump to membrane potential, and that
370 these contributions rely partly on the heterogeneity of subunit composition of the
371 pumps. In neurons in general, the α -subunit of the Na^+ pump takes one of two forms
372 with different affinities for intracellular sodium; $\alpha 1$ (high affinity) or $\alpha 3$ (low affinity).
373 Thus, at typical resting intracellular sodium levels, the $\alpha 1$ is maximally active, whilst
374 the $\alpha 3$ remains inactive, or sub-maximally active, allowing it to act as a sensor for
375 activity-dependent rises in sodium (Azarias et al. 2013; Dobretsov and Stimers,
376 2005). The subsequent increase in the activity of $\alpha 3$ -containing sodium pumps is
377 thought to be responsible for generating the transient membrane hyperpolarisation
378 that reduces the excitability of the neuron for tens of seconds. The different isoforms
379 also have differential sensitivity to ouabain, such that low concentrations of ouabain,
380 including those used in our experiments (1-3 μM), selectively block the $\alpha 3$ isoform
381 (Blanco and Mercer 1998; Dobretsov and Stimers 2005). Our pharmacological
382 experiments showing that the usAHP is blocked by these low concentrations of
383 ouabain are therefore in support of the above hypothesis.

384 In mice, both $\alpha 1$ and $\alpha 3$ expression is found throughout the ventral and dorsal horns
385 of the spinal cord, although $\alpha 3$ expression is more widespread (Edwards et al. 2013;
386 Hieber et al. 1991; Watts et al. 1991). However, both $\alpha 1$ and $\alpha 3$ expression appears
387 to be restricted to some neurons and not others. For instance, alpha-motoneurons
388 predominantly express $\alpha 3$, whilst gamma-motoneurons predominantly express $\alpha 1$
389 (Edwards et al. 2013). The functional importance of this difference is not yet clear.
390 Expression of $\alpha 3$ is also found in interneurons, and in our experiments, we
391 specifically focused on $\alpha 3$ expression in one interneuron type, the cholinergic pitx2
392 cells (Zagoraiou et al. 2009). We found $\alpha 3$ expression in around half of this
393 population, which broadly matches the number of pitx2 neurons found to display the
394 usAHP (Picton et al. 2017). This is also similar to previous studies in rats which
395 documented an activity-dependent, pump-mediated hyperpolarisation in around half
396 of cultured spinal interneurons (Darbon et al. 2002, 2003). It will be important in
397 future studies to further characterise $\alpha 3$ expression in other interneuron types. It will
398 also be important to characterise developmental changes in $\alpha 3$ expression. For
399 example, Calyx of Held neurons in young rats have lower expression of $\alpha 3$
400 compared to adults, and this is accompanied by a significantly smaller and shorter
401 duration usAHP (Kim et al. 2007).

402 At the cellular level, we have partially characterised the details of the cascade of
403 events in *Xenopus* tadpoles that link spinal neuron firing to network regulation. This
404 cascade involves the spike-dependent accumulation of sodium ions, which in turn
405 triggers an increase in ion exchange by the Na^+ pump, hyperpolarising the neuron.
406 This hyperpolarisation de-inactivates an A-type potassium channel, and enhanced A-
407 current delays spiking in a subset of spinal motor and interneurons when activity
408 resumes, causing a collapse of swim network activity. Thus, swimming activity
409 evoked within a minute after the end of previous swimming is both shorter in duration
410 and slower in frequency, in a time-dependent manner. In mice, a similar
411 physiological mechanism appears to be at play, but unsurprisingly, additional
412 mechanisms of locomotor bout termination are likely to be involved. For example,
413 unlike tadpoles, blockade of the Na^+ pump does not produce continuous locomotion,
414 but merely extends the duration of evoked locomotor bouts (Picton et al. 2017). It is
415 likely that synaptic depression plays a role in locomotor bout termination, a possibility
416 that has been explored previously in rat spinal neurons in the context of the sodium
417 pumps (Darbon et al. 2002, 2003; Rozzo et al. 2002). We also do not yet know
418 whether A-currents play a role in neonatal mice. As we come to understand more
419 about Na^+ pump currents, we will likely uncover species-specific mechanisms
420 involving a range of other currents, such as the I_h current, which has been shown to
421 have important interactions with pump currents in a number of different brain areas
422 (Gulledge et al. 2013; Kim and von Gersdorff 2012; Rozzo et al. 2002; Trotier and
423 Døving 1996).

424 **Heterogeneity allied to circuit role**

425 The usAHP is a powerful way of reducing network excitability. However, if it were to
426 be homogenously expressed in all CPG neurons then there would be a distinct
427 possibility that the network could render itself completely unresponsive. This, in turn,

428 could be catastrophic because of the requirement to retain a residual capacity to
429 respond to potentially life-threatening stimuli such as an approaching predator. In
430 both tadpole and neonatal mouse spinal locomotor networks there is strong evidence
431 for a heterogenous distribution of the usAHP among spinal CPG network
432 components.

433 There are a number of possible explanations for the heterogenous distribution of the
434 usAHP among neuron subtypes in the spinal cord. One possibility, for which we have
435 preliminary evidence in the mouse (described above) is that the ability of the pump to
436 respond dynamically to intense activity requires the presence of an $\alpha 3$ -containing
437 sodium pump, which is only recruited by high intracellular sodium concentrations
438 achieved following intense neuronal firing. Alternatively, the α subunit may also be
439 subject to direct phosphorylation in some neurons, but not others (Therien and
440 Blostein 2000), which can tune the affinity of the subunit for sodium. A similar
441 mechanism could also involve a set of accessory proteins, known as FXD proteins,
442 which are also subject to phosphorylation (Geering 2006). Thus, it will be important
443 in future studies not only to establish the distribution of $\alpha 1$ and $\alpha 3$ subunit isoforms,
444 but also the expression of FXD proteins in the spinal cord.

445 The importance of the Na^+ pump as an intrinsic locomotor memory mechanism, and
446 its high conservation through evolution, make it a useful target for a range of
447 neuromodulators, and this could also explain differences in usAHP expression. The
448 range of neuromodulators known to impinge on the Na^+ pump is extensive (Therien
449 and Blostein 2000), but dopamine, serotonin and nitric oxide seem particularly
450 important, especially in the spinal cord. Indeed, in mice we showed that the effects of
451 Na^+ pump manipulation were dopamine-dependent, and that dopamine extends the
452 duration of the usAHP (Picton et al. 2017). Whether this involves direct
453 phosphorylation of sodium pumps, or via FXD accessory proteins, or both, is a
454 topic for future experiments.

455 **Phylogenetic conservation**

456 In this paper, we have reviewed the evidence that the activity-dependent increase in
457 Na^+ pump activity, manifest as the usAHP, functions as a simple form of short-term
458 motor memory in animals as diverse as fruitflies, frog tadpoles and neonatal mice.
459 Modern amphibians and mammals diverged from a common ancestor that existed
460 around 360 million years ago. The nervous system underwent dramatic changes to
461 accommodate changes in lifestyle, morphology, and behavioural repertoire, with the
462 number of neurons increasing from around 16 million in adult frogs to around 70
463 million in adult mice. However, many components of the nervous system are known
464 to be highly conserved (Katz 2016; Katz and Harris-Warrick 1999; Keifer and
465 Summers 2016). The basic architecture of many neural circuits appears to have
466 been retained through evolutionary time, with extant species displaying variations on
467 a theme rather than completely new circuit architecture. Thus, we can often identify
468 conserved principles of circuit function and this often appears to be true for the
469 circuits controlling locomotor behaviours, including at the cellular and molecular
470 levels (Goulding and Pfaff 2005). The neuronal Na^+ pump is especially highly
471 conserved between vertebrates in terms of its structure and function, with around 96%

472 cross-species similarity (Dobretsov and Stimers 2005; Takeyasu et al. 1990). This
473 implies that the Na⁺ pump plays an important and conserved neuronal function. Our
474 own mammalian lineage diverged from the common ancestor with mice around 65
475 million years ago (O'Leary et al. 2013), and so it will be interesting in future studies,
476 especially with a rise in the use of human induced pluripotent stem cells (iPSCs), to
477 study whether the sodium pumps embedded in human spinal motoneurons and
478 interneurons also play a similar role in neuronal self-regulation.

479 **Dysfunction of the Na⁺ pump**

480 Na⁺ pumps are receiving increasing attention in mammalian systems not only for
481 their importance for normal network function, but also for their relevance to both the
482 ageing process and a range of debilitating diseases of the nervous system (de Lores
483 Arnaiz and Ordieres 2014; Holm and Lykke-Hartmann 2016). The $\alpha 3$ Na⁺ pump
484 isoform is highly expressed in the human brain and spinal cord (Peng et al. 1992)
485 and several mutations in the gene encoding this subunit (*ATP1A3*) are known to
486 cause at least three neurological disorders: Alternating Hemiplegia of Childhood
487 (AHC, (Heinzen et al. 2012; Rosewich et al. 2012)); Rapid-onset Dystonia
488 Parkinsonism (RDP, De Carvalho Aguiar et al. 2004; Rodacker et al. 2006); and
489 Cerebellar ataxia, Areflexia, Pes cavus, Optic atrophy and Sensorineural hearing
490 loss (CAPOS) syndrome (Demos et al. 2014). Furthermore, a wide range of other
491 disorders are also known to involve changes in the activity of the $\alpha 3$ Na⁺ pump
492 isoform. In recent studies, the $\alpha 3$ isoform has been shown to directly interact with
493 both SOD1 (Martin et al. 2007; Ruegsegger et al. 2016), and α -synuclein
494 (Shrivastava et al. 2015), in ALS and Parkinson's Disease mouse models,
495 respectively. This aggregation leads to reduced $\alpha 3$ activity and a general inability to
496 respond to rises in intracellular sodium (Ellis et al. 2003; Shrivastava et al. 2015).
497 Given that dysfunction of $\alpha 3$ also contributes to epilepsy (Krishnan et al. 2015) and
498 bipolar disorder (Kirshenbaum et al. 2012), it is possible that the inability to respond
499 dynamically and homeostatically to activity-induced rises in intracellular sodium may
500 be a general feature of pump disorders involving the $\alpha 3$ isoform (Azarias et al. 2013;
501 Benarroch 2011).

502 Genetically modified zebrafish and rodent disease models have been used to
503 explore the underlying mechanisms of Na⁺ pump deficiency. *ATP1A3* knockdown
504 zebrafish display abnormal motor activity accompanied by depolarization of spinal
505 sensory neurons (Doganli et al. 2013). Homozygous knock-out mice for $\alpha 1$ are
506 embryonic lethal (James et al. 1999), whilst homozygous $\alpha 3$ knock-out mice die
507 shortly after birth (Moseley et al. 2007). However, a number of $\alpha 3$ knock-in mouse
508 lines have been developed and heterozygote mice all show severe motor deficits
509 (DeAndrade et al. 2011; Hunanyan et al. 2015; Ikeda et al. 2013; Kirshenbaum et al.
510 2011; Moseley et al. 2007; Sugimoto et al. 2014). The hyperactivity phenotype in
511 these mice is especially pronounced, with mutant mice showing almost continuous,
512 high frequency locomotor activity compared to control mice. The $\alpha 3$ -mutation affects
513 Na⁺ pumps throughout the nervous system, including presumably the spinal cord,
514 and therefore this phenotype may relate to the role of the $\alpha 3$ Na⁺ pumps explored in
515 this review. Indeed, this behavioural phenotype would be predicted by the effects

516 covered in this review using low concentrations of ouabain; namely, longer duration
517 bouts of locomotion with a higher frequency of limb movements, and a general
518 inability to regulate locomotion.

519 **Summary**

520 Na^+/K^+ exchange pumps are ubiquitously distributed, abundantly expressed and
521 phylogenetically conserved proteins that are often viewed as molecular automata
522 engaged exclusively in the maintenance of ionic distributions across cell membranes.
523 Here, we have discussed recent data in *Xenopus* tadpoles, neonatal mice and also
524 *Drosophila*, showing that Na^+ pumps respond dynamically to changes in intracellular
525 sodium that accompany intense neuronal firing. This capacity endows networks of
526 the spinal cord with a homeostatic control mechanism to shape motor output in an
527 activity-dependent manner. Moreover, despite the ubiquity of Na^+ pump distribution
528 among network neurons, their ability to respond homeostatically to the changes in
529 intracellular sodium triggered by activity may result from the highly targeted insertion
530 of $\alpha 3$ -containing pumps in selected neurons and neuronal subtypes. The possibility
531 that the balance of $\alpha 1$ to $\alpha 3$ expression is a mutable entity that can change during
532 development, or with circuit use, is an exciting idea that should be pursued in the
533 future.

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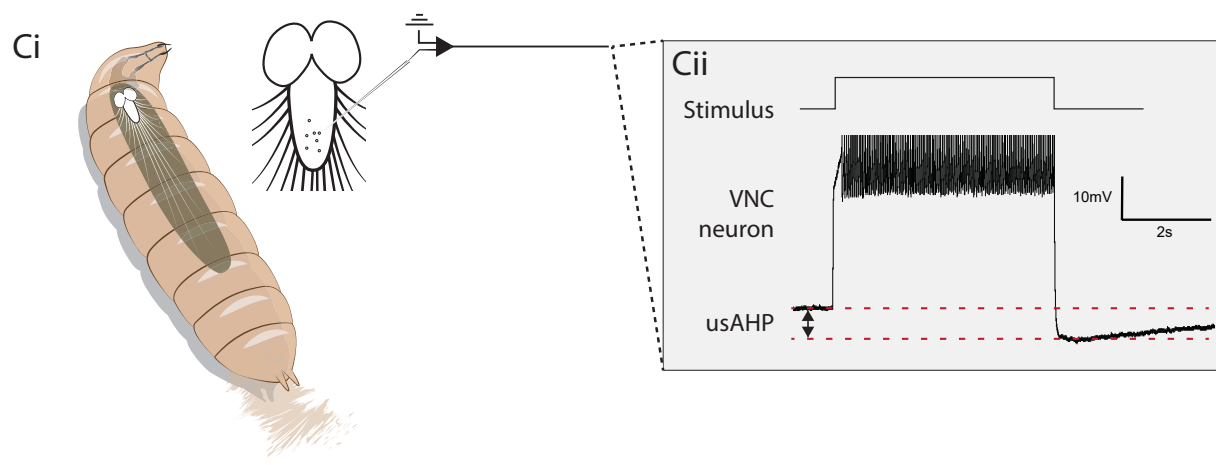
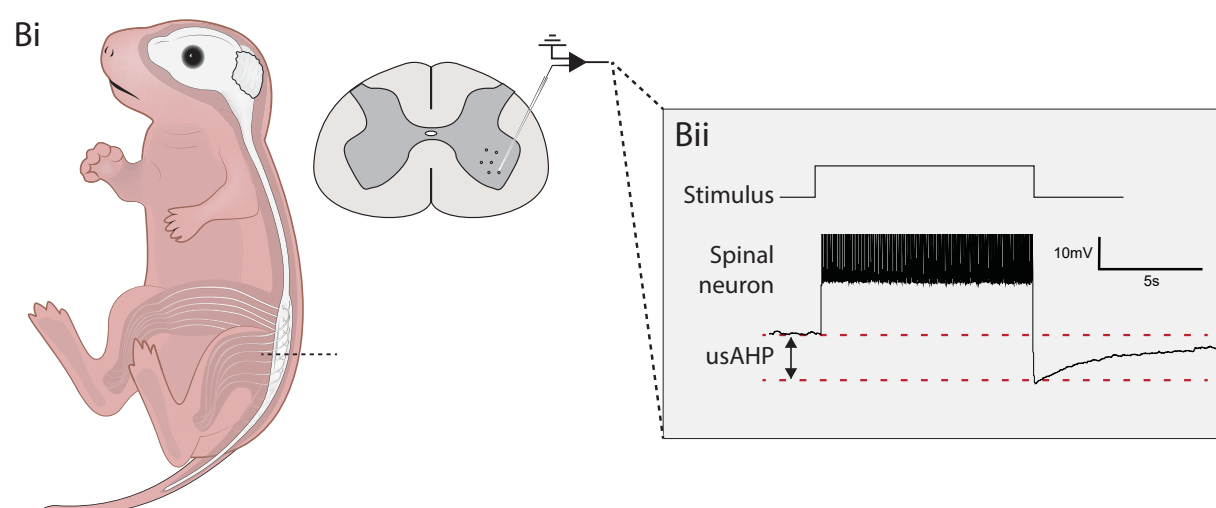
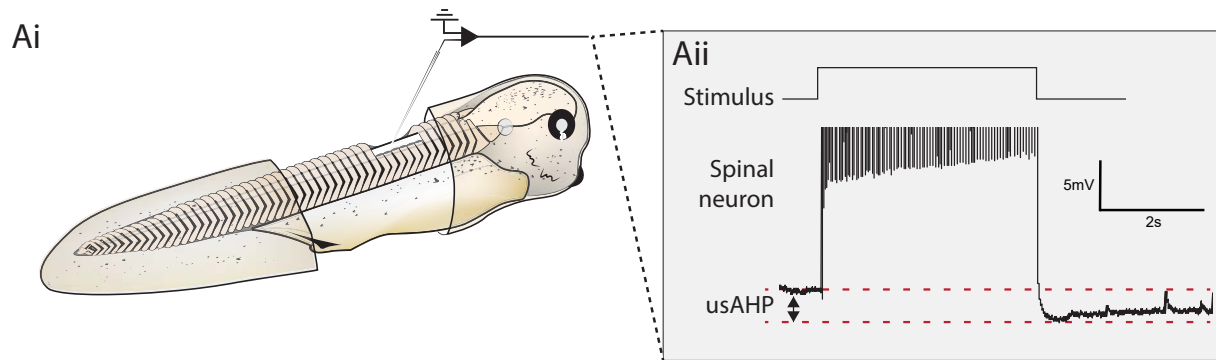
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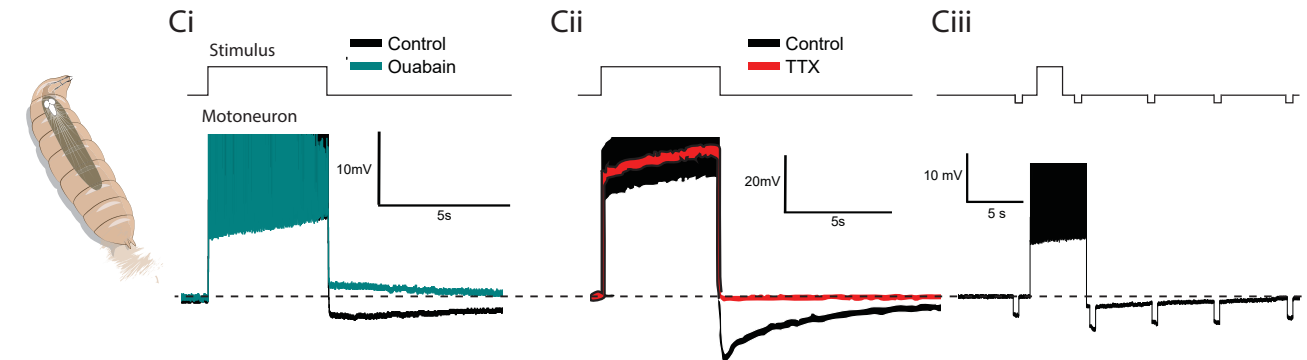
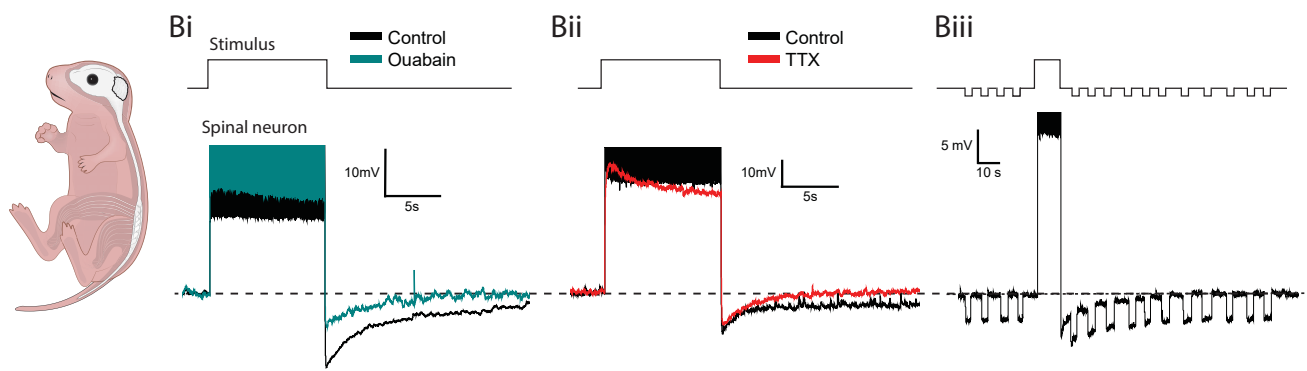
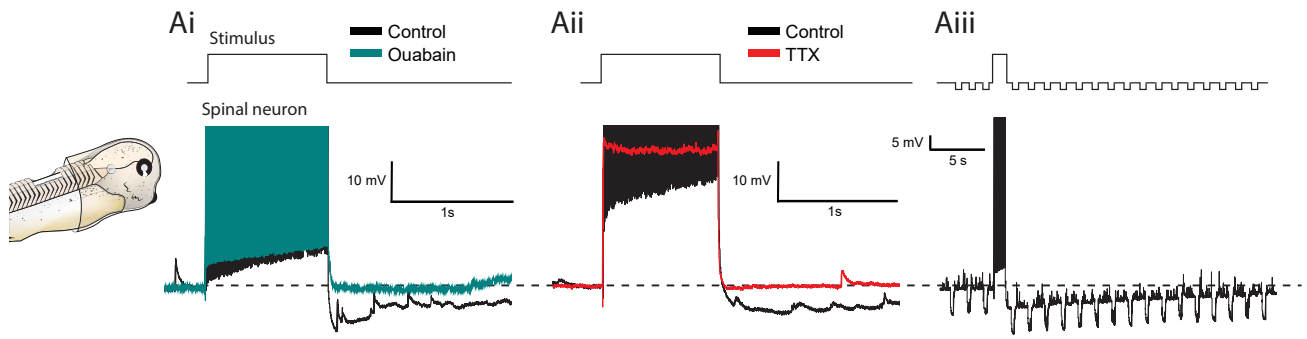
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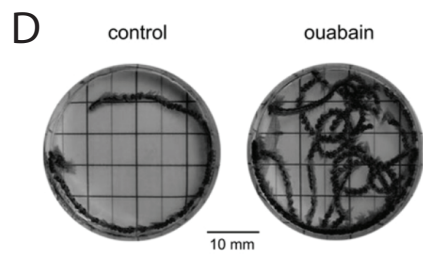
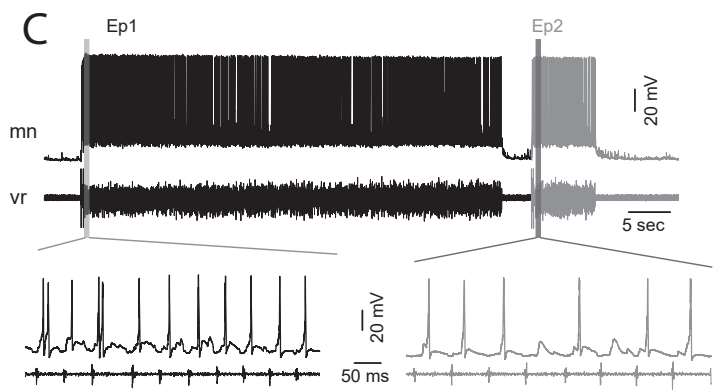
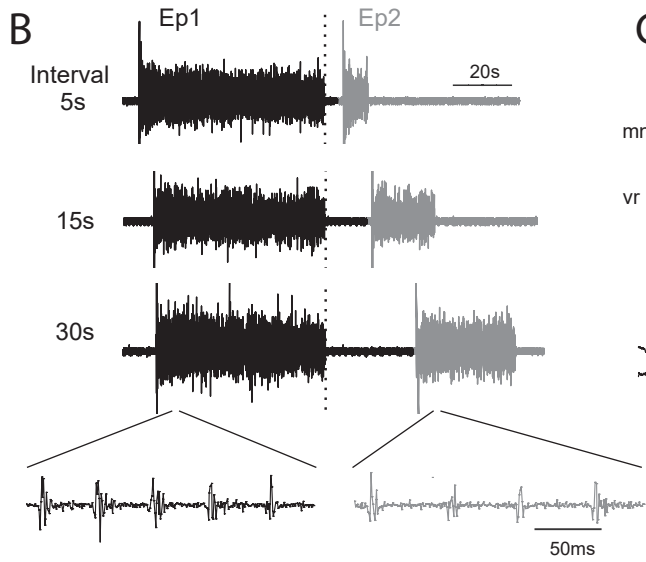
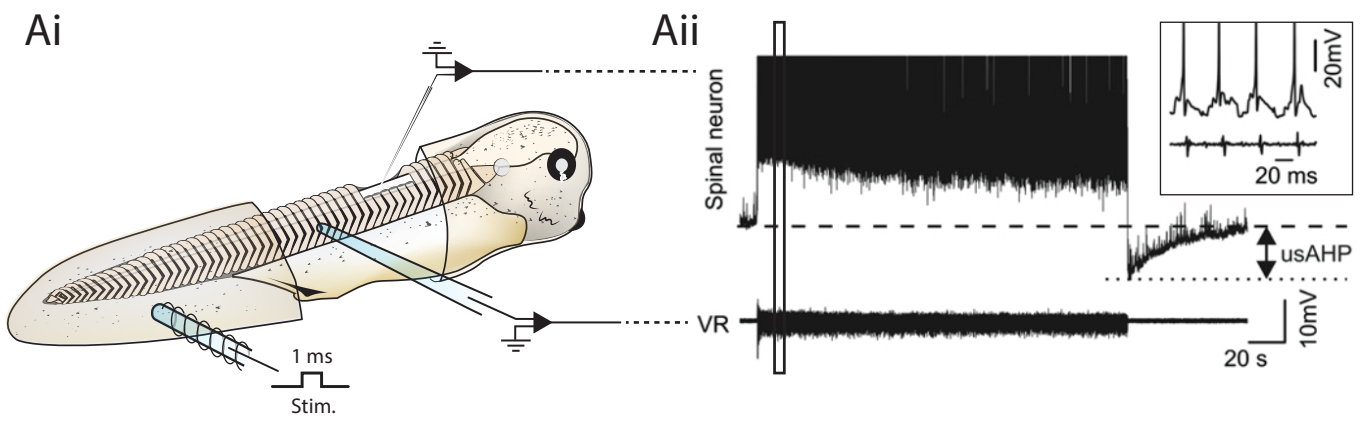
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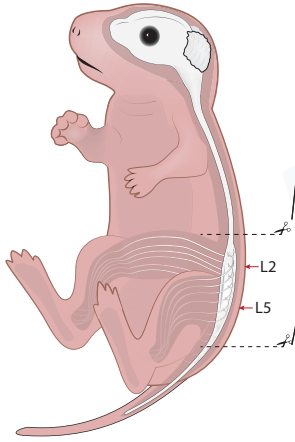
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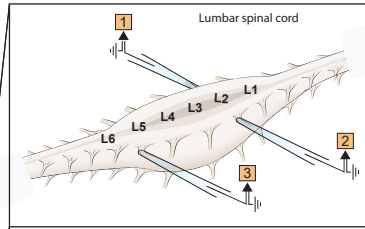




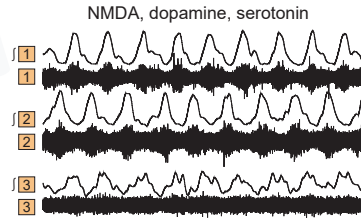
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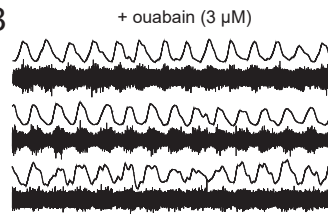
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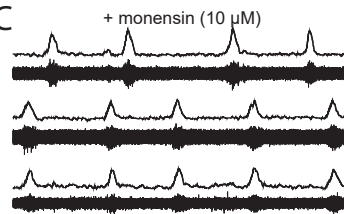
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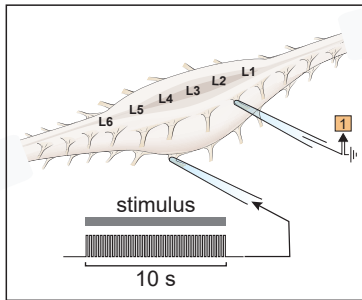
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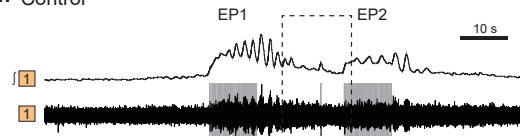
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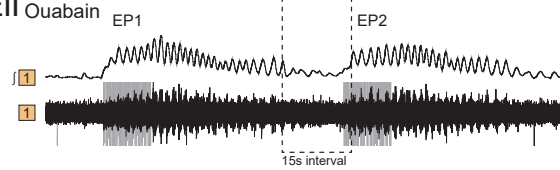
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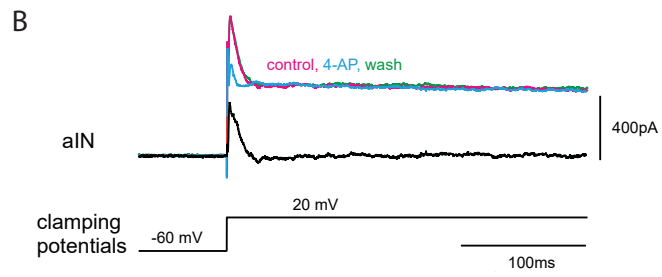
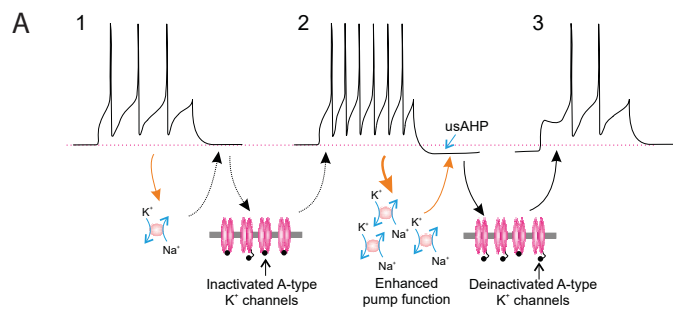


Ei Control

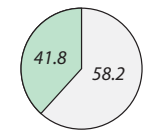


Eii Ouabain





Xenopus



Motoneurons

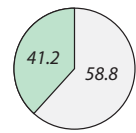
usAHP



No usAHP



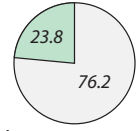
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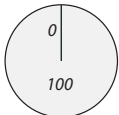
Motoneurons



Interneurons



Interneurons



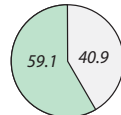
dINs



aINs



cINs



PitX2



Other