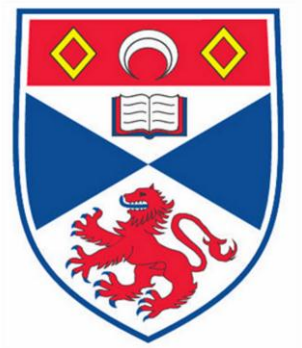
Activity-dependent development of the locomotor network in *Xenopus laevis* larvae

Franziska Bender



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I, Franziska Bender, received assistance in the writing of parts of this thesis in respect of language, grammar and spelling or syntax, which was provided by Steven Currie and Dr. Hong Yan Zhang.

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Abbreviations

5-HT	serotonin
αBT	α-bungarotoxin
ACh	acetylcholine
AChR	acetylcholine-receptor
ADP	adenosine
aIN	ascending interneuron
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
ATP	adenosine tri-phosphate
aVOR	angular vestibuloocular reflex
CA	control animal
cIN	commissural interneuron
CNS	central nervous system
EPSP	excitatory postsynaptic potential
GABA	gamma aminobutyric acid
Glu	glutamate
HA	high-activity group
hdIN	dIN hindbrain population
HFN	neuron with high firing probabilities
IR	Input resistance
LA	low-activity group
LFN	neurons with low firing probabilities
LTP	long-term potentiation
MFN	neuron with medium firing probabilities
mhr	mid-hindbrain GABAergic reticulospinal neuron
mPSP	miniature post-synaptic potential
NA	noradrenaline
NMJ	neuromuscular junction
NT	neurotrophin
NO	nitric oxide
NPP	neurotransmitter phenotype plasticity
IPSP	inhibitory postsynaptic potential
PBS	Phosphate buffered saline
RB	Rohon-Beard
RMP	resting membrane potential
TH	firing threshold
TTX	tetrodotoxin
usAHP	ultraslow afterhyperpolarisation

Abstract

The impact of activity in *Xenopus* embryonic and larval development was studied with regards to the locomotor system and developmental rate. The data suggest that pharmacologically suppressing neuronal and muscular activity decreases, and enhancing swimming activity, by raising tadpoles in a rotating water column, increases the rate of development. Moreover the latter adapted their swimming behaviour in accordance to the treatment: their swimming was characterized by longer episode durations and more frequent turning manoeuvres. Patch clamp recordings revealed that spinal neurons of agitated animals receive more synaptic drive, probably deriving from descending INs, enabling the animals to maintain swimming for long time periods. An increase in the incidence of miniature potentials suggests that synaptic connections are strengthened. Probably in response to the increase in synaptic excitation the intrinsic excitability, and thereby the probability of signal transmission, decreased. I argue that this represents a case of homeostatic plasticity and serves to prevent over-excitation and maintain pattern generation in the network. Stronger bursts might be explained by enhanced signal transmission from motoneurons to muscle cells. The amount of extrasynaptic AChRs at the NMJ appeared reduced. In contrast, when activity was suppressed behavioural, ventral root and patch data were comparable to those of control animals, suggesting that the locomotor system can develop normally up to stage 42 in the absence of electrical activity. However, animals appeared less excitable, indicating that the establishment of sensory systems might indeed require extrinsic inputs.

Introduction

I. The nervous system is plastic

During development neurons are assembled into circuits according to genetic instructions. However the ultimate form and function of the nervous system is influenced by many factors. Spontaneous electrical activity can regulate synaptic, intrinsic and structural properties of neurons. Due to this plasticity organisms have the capacity to adapt behaviour in response to a changing environment.

In 1949 Hebb proposed the idea that memory storage in the nervous system results from activity-dependent changes in synaptic strength. Since then synaptic plasticity has been documented in various model organisms, cell types and synaptic components. Depending on the duration and frequency of stimulation (Castellucci et al., 1986) neurons undergo short-term plasticity changes, expressed as strengthening or weakening of the synapse, or long-term changes, which are associated with the growth of new synaptic connections. The latter require protein synthesis and both pre-synaptic and post-synaptic mechanisms. During intermediate stages spontaneous transmitter release serves as an orthograde signal for recruiting postsynaptic mechanisms for long-term plasticity in *Aplysia* (Jin et al., 2012). Spontaneous miniature post-synaptic potentials (mPSPs) were discovered as early as 1952 (Fatt and Katz) and represent the quanta of transmitter released by a single vesicle. Changes in the frequency of mPSPs are supposed to arise from changes in transmitter release probability, size of vesicle pools, synapse size and/or synapse number.

In 1973 another form of plasticity, termed intrinsic plasticity (or "non-synaptic plasticity") was identified by Bliss and Lomo. In contrast to synaptic plasticity, which involves changes at the synapse between neurons, intrinsic plasticity alters the properties within a single neuron.

Hence it affects the entire neuron's excitatory properties, typically through modulation of ion channel currents causing alterations in spike generation, conductance, firing threshold, depolarisation level etc. Thereby intrinsic plasticity determines whether synaptic inputs finally lead to an AP or not.

Intrinsic plasticity plays an important role in homeostasis. The Hebbian learning rules operate at the two-cell level: enhanced input from the pre-synaptic element causes strengthening of the synaptic connection. The neurons “learn” and subsequent stimuli will produce stronger excitation of the post-synaptic neuron (termed long-term potentiation, LTP). In contrast, when the input decreases the connection weakens (termed long-term depression, LTD). However this form of plasticity has a strong destabilizing effect on network activity, because positive feedback would drive synaptic strengths sooner or later towards their maximum or minimum values (Eliot, 1994, illustrated in fig. 1). In neuronal circuits, like a central pattern generator (CPG) controlling rhythmic movements, each layer is driven by activity in the preceding layer. Consequently synaptic and intrinsic properties must be adjusted to achieve stable activity patterns. Circuits can be set to the correct strength by adjusting the intrinsic excitability according to the strength of incoming signals. When network activity is reduced, the probability and duration of spike firing increased (Desai et al., 1999), and vice-versa when activity is increased (Garcia et al., 1992; Li et al., 1996). Neuronal excitability adapts, for instance, through functional changes in K^+, Ca^{++} and Na^+ channels. In this way firing rates are kept relatively constant and stability is maintained (LeMasson et al., 1993; Miller, 1996).

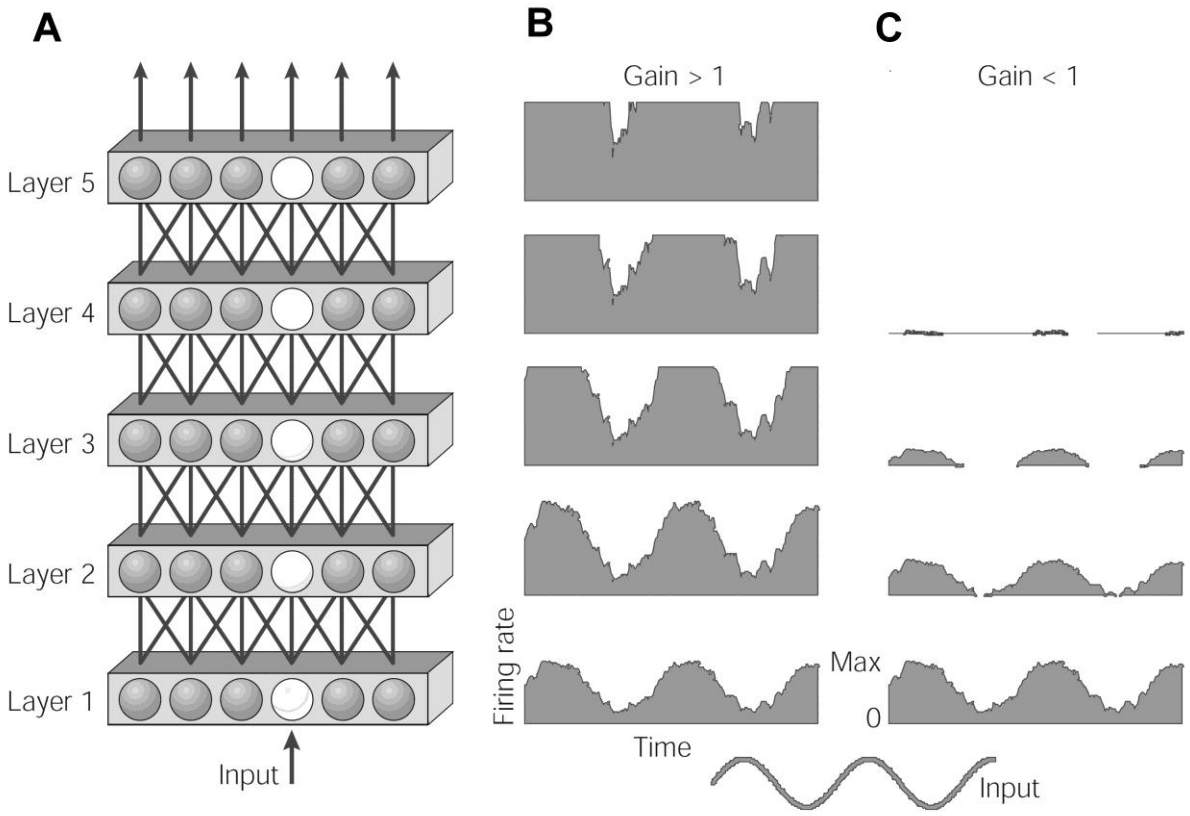

Fig. 1. Neuronal circuits underlie homoestatic plasticity

A. The problem of propagating patterned activity over several layers of a neuronal circuit is illustrated schematically. Applied to the network controlling locomotion in tadpoles the model can be interpreted as follows: activity in RB neurons (layer 1) drives activity in sensory interneurons (layer 2), which excite CPG neurons (layer 3), among these are motoneurons (layer 4) which eventually transmit the signal to the muscle cells (layer 5) which respond with contractions. B. Enhanced signal transmission in one layer would cause signal amplification of several neurons in subsequent layers through the principle of Hebbian plasticity and specificity will be lost because at high activity levels all neurons would fire, regardless of the firing pattern at the first layer. C. In contrast, when transmission is down-regulated activity will die out. The problem is applicable to recurrent circuits. Therefore neurons have to adapt synaptic strength and excitability in a homeostatic manner. **Adapted and modified from Turrigiano and Nelson, 2004.**

Moreover, during the formation and maintenance of functional neuronal circuits structural plasticity is of particular importance. Neurotrophins (NTs) mediate changes in the morphology and survival of neurons and are expressed in an activity-dependent manner. In *Xenopus* nerve-muscle cultures NT-4 is secreted in a activity-dependent way and plays a role in synaptic plasticity, as a modulator of presynaptic transmitter release (Wang and Poo, 1997).

The present study aims to provide more insights into the activity-dependence of development of the neuronal circuit controlling locomotion, the CPG. The CPG has the ability to rapidly mature into a highly specialized network that processes and responds to extrinsic information. Throughout development it has to function stably, in spite of massive synaptic and intrinsic changes. Although the CPG can develop in the absence of motor or sensory input, there is increasing evidence that electrical activity has a wide impact on its assembly and modulation. Peristaltic crawling develops in the complete absence of sensory input in *Drosophila* embryos, however the polarity of movement is deranged (Suster and Bate, 2002). Motor axon pathfinding events are highly dependent on the normal pattern of bursting activity: a decrease in frequency resulted in dorsal–ventral pathfinding errors, while frequency increases led to segmental pathfinding errors in chick (Hanson and Landmesser, 2004, 2006). Mutant mice which lack the choline acetyltransferase enzyme and are therefore deprived of motoneuronal activity showed abnormalities in right-left and flexor-extensor coordination and an elongated cycle period (Myers et al., 2005).

Despite the increasing number of insights in this field it is not yet well understood how activity during early development influences CPG function. The CPG controlling *Xenopus* tadpole swimming provides an excellent model system to study this question due to the simplicity of the spinal cord network, the rapidity of development and extensive foregoing research, providing detailed knowledge of neuroanatomy (Roberts and Clarke, 1982; Roberts, 2000), physiology (Kahn and Roberts, 1982a, b) and development (Sillar et al., 1992a, b; 1995a, b).

Nevertheless research in *Xenopus* so far has mainly been limited to studies on the effect of reduced or increased activity in pre-hatching tadpoles, during the time when the innervation of axial musculature starts (at 1 d after fertilization, stage 22). The tadpole development is introduced below.

However, the results, summarized below, are contradictory. Haverkamp and Oppenheim (1986) reported that chronic immobilization of *Xenopus* embryos, reared from stage 17 to 35 in chloretone or lidocaine, displayed reduced swim speed and swim episode duration, but increased spontaneous swimming in *Xenopus* at stage 35 to 40. However this effect disappeared at later stages (44/45). The authors therefore concluded that the transient decrease in swimming behaviour was induced by a residual drug effect. Moreover the electrical activity recorded from ventral roots and the anatomy of the motoneurons appeared unaltered (Haverkamp, 1983).

More recently, in contrast, it was reported that neurotransmitter phenotype plasticity (NPP) is activity-dependent in the nervous system of *Xenopus* and follows a homeostatic principle (reviewed in Demarque and Spitzer, 2011). When spike activity is increased, by overexpression of Na^+ channels, the number of spinal neurons expressing inhibitory transmitters is increased and vice-versa, when Ca^{++} spikes are suppressed by overexpression of K^+ channels (Borodinsky et al, 2004). The same is true for the neuromuscular junction (NMJ; Borodinsky and Spitzer, 2007).

By implanting agarose beads loaded with Ca^{++} or Na^+ channel blockers into the neural tube at 20 h of development, suppressed activity induced an extrajunctional distribution of nAChRs, as well as an overall increase in NMDA and AMPA type glutamate receptors at 3 days of development. When the beads were loaded with veratridine, a voltage gated Na^+channel activator, the expression of excitatory receptors (nicotinic, NMDA and AMPA receptors) decreased while the level of inhibitory receptors ($GABA_A$ and glycine receptors) increased at the NMJ. In general the number of synaptic puncta in the axial musculature was increased in both conditions, compared to the control.

On the other hand, a different study showed that blockage of the NMJ results in decreased repetitive firing of pre-synaptic motoneurons by an increase in the refractory period in *Xenopus* neuron/myocyte co-cultures through feedback regulation (Nick and Ribera, 2000). Hence, decreasing post-synaptic activity during development down regulates pre-synaptic excitability. NPP has been further described for neuromodulators (Dulcis and Spitzer, 2008; Demarque and Spitzer, 2010). Increased activity enhances expression of inhibitory, dopaminergic neurons, which regulate skin pigmentation, and vice versa. However for serotonin in the raphe, which increases burst duration and intensity (Sillar et al., 1998; Fig.1 C), the opposite is true. The activity-dependent expression of 5-HT was found to be mediated by the transcription factor Lmx1b (Demarque and Spitzer, 2010). In addition, activity has been shown to influence the establishment of *Xenopus* sensory systems. When free-swimming *Xenopus* tadpoles are exposed for 4-5 hours to persistent visual stimulation the excitability of optic tectal neurons increases, due to an enhancement of sodium currents (Aizenman et al., 2003). At the same time the excitatory synaptic drive decreases. Together the changes are thought to improve signal-to-noise by making the visual system less responsive to background activity but more responsive to brief, strong visual stimuli.

II. Locomotion in *Xenopus* embryo and larvae

II.1. The *Xenopus* swim CPG

Swimming in *Xenopus* tadpoles is produced by a CPG which establishes a rhythmic motor activity pattern that alternates across the body and propagates rostrocaudally with a delay between muscle segments (Kahn and Roberts, 1982). Thereby an undulation of trunk and tail is produced. Tonic and phasic excitatory input as well as reciprocal mid-cycle inhibition is mediated by interneurons. Motoneurons contribute positive feedback excitation to themselves and premotor interneurons (Roberts and Perrins, 1995).Sensory and descending signals allow changes in movement frequency, intensity and duration.

Swimming can be initiated by various stimuli, which excite different sensory pathways. Photic stimuli, for instance light dimming, excite pineal ganglion cells (Foster and Roberts, 1982) which transmit the signal to tonic firing diencephalic-mesencephalic interneurons projecting to the hindbrain from where the locomotion network is excited (Jamieson and Roberts, 1999). *Xenopous* tadpoles furthermore respond to tactile stimulation. Two different pathways are known: i) Rohon-Beard (RB) mechanosensory neurons; and ii) the electrically excitable skin whose constituent cells generates long, cardiac-like impulses (Roberts, 1969). At pre-hatching stages embryos respond to noxious stimuli, like pokes, and immature RB neurons are suggested to provide a transitory route for skin impulses to excite the CNS to generate a response (Roberts, 1971; James and Soffe, 2011). Their large growth cones are probably sensitive to signals from the overlying epithelium (Roberts and Tayler, 1983; Roberts and Patton, 1985). During development this skin impulse is lost and sensitivity to light touch (strokes) extends, as the number of mechanosensory free nerve endings increases (Roberts and Hayes, 1977).

These unmyelinated peripheral neurites form a loose network under the skin (Roberts and Hayes, 1977) and are excited in response to tactile or electrical stimuli (Clarke et al., 1984) conducting a single AP to excite the locomotor network. RB neurons are the largest neuronal cell bodies in the spinal cord and hence their conduction velocity is relatively high: in stage 37/8 about 0.3 m/s (Clarke et al., 1984). In more developed tadpoles conduction is more rapid. For instance a conduction velocity range of 0.6-0.8 m/s was reported in stage 45-49 tadpoles (Spitzer, 1976). The somata of RB neurons are located in the dorsal spinal cord (Hughes, 1957). RB neurons are glutamatergic, their synapses are dominated by AMPARs, so they produce large and fast EPSPs which helps them toinitiate swimming with a localized mechanical stimulus that only excites a few RB neurons (Boothby and Roberts, 1995).

RB neurons make strong synapses, enabling them to recruit many sensory pathway interneurons: dorsolateral ascending (dla) and dorsolateral commissural (dlc) interneurons (Sillar and Roberts, 1988; Roberts and Sillar, 1990), which in turn excite the neurons of the CPG (Li et all, 2003). Glutamatergic dla neurons excite the ipsilateral side of the cord and glutamatergic dlc neurons the contralateral side (Clarke and Roberts, 1984; Sillar and Roberts 1988, 1992; Roberts and Sillar, 1990). (Li 2003)

The CPG consists of ascending interneurons (aINs), commissural interneurons (cINs), descending interneurons (dINs) and motoneurons (MNs). CPG neurons are rhythmically active during swimming and are responsible for generating swimming(Roberts et al., 2010; Li., 2011). During swimming, all rhythmic neurons receive phasic inhibition from aINs and cINs and phasic excitation from dINs which also generate a tonic background depolarization (Roberts et al., 2010). Glycinergic aINs gate incoming signals from the sensory pathway during swimming to limit motorneuron firing by inhibiting dlc neurons (Li et al., 2002; Li et al., 2004).

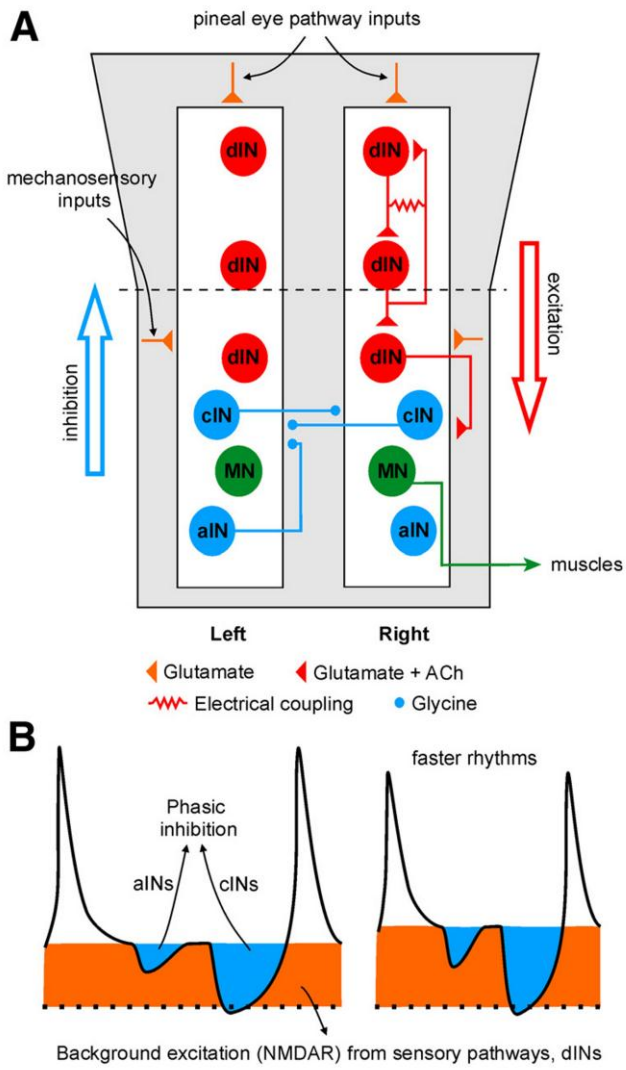

Fig. 2. Swimming activity is maintained by excitatory drive from dINs

A. The CPG in Xenopus tadpoles consists of dINs, cINs, aINs and MNs. Glutamatergic dINs in the caudal hindbrain and rostral spinal cord provide excitatory drive during swimming (red arrow). Rostral dINs provide positive feedback and excite spinal cord dINs which propagate the signal. Phasic inhibition arises from glycinergic cINs and aINs (blue arrow). Cholinergic MNs transmit excitation to muscle cells.. B. During faster rhythms tonic excitation, as well as phasic inhibition is increased in dINs. That is accompanied by an increase in tonic depolarisation and a decrease in spike height. **(adapted from Li and Moult, 2012)**

Glycinergic cINs produce phasic mid-cycle inhibition of the contralateral side (Dale, 1985; Soffe et al., 2001), thereby coupling the rhythm generators on each side of the cord and coordinating the rhythmic alternation (Kahn & Roberts, 1982; Soffe & Roberts, 1982; Soffe et al. 1984). Glutamatergic dINs directly excite all ipsilateral CPG neurons via descending axons (Li et al., 2006; Soffe et al., 2009). They evoke unitary EPSPs consisting of two components: fast EPSPs mediated by AMPARs result in phasic excitation, underlying spikes, and slow EPSPs mediated by NMDARs which sum from cycle by cycle due to their long duration and thereby induce a tonic excitation (Dale and Roberts, 1985). The background tonic excitation is required to maintain swimming activity and the amplitude correlates with rhythm frequency (Soffe and Roberts, 1982; Soffe et al., 1984; Dale and Roberts, 1984; Sillar and Roberts, 1993). Glutamate is coreleased with acetylcholine which activates nicotinic acetylcholine receptors (Li et al., 2004). DINs also feed long-duration glutamate excitation back onto themselves via ascending axons, thereby maintaining strong depolarisation (Li et al., 2004). Furthermore they are electrically coupled and if the coupling is blocked rhythm-generation becomes less reliable (Li et al., 2009). They are rhythmically active and fire once on each cycle of swimming (Li et al., 2004). The firing pattern is maintained in larval stages (HongYan Zhang, personal communication). The dIN hindbrain population (termed hdINs) also have ascending axons projecting to the brain and providing positive feedback excitation to sustain swimming (Li et al., 2006, reviewed by Roberts et al., 2010). At the current level of knowledge swimming is maintained by the following mechanism (Roberts et al., 2008): hdINs fire earliest during swimming and feed back long duration excitation to ipsilateral hdINs which prevents them for firing as they are less excitable when depolarised. The contralateral side is inhibited by excitation and firing of glycinergic cINs.

When inhibition decays the hdINs fire and inhibit the contralateral side (first side), hyperpolarising the neurons (hdINs) and allowing them thereby to fire on rebound. Rebound firing can occur in dINs solely in response to negative current pulses or IPSPs when they are depolarized (Soffe et al., 2009); they are otherwise very difficult to re-excite shortly after firing.

The excitation of MNs results in activation of the trunk muscles and finally induces behavioural output. Neighbouring MNs are electrically coupled, which helps to make those exciting a given segment fire synchronously (Perrins and Roberts, 1995; Wolf et al., 2009; Zhang et al., 2009). MNs also make central nicotinic cholinergic synapses with each other (Perrins and Roberts, 1995) and some other CPG neurons to provide positive feedback (Soffe and Roberts, 1982). By stage 42, larval MNs have differentiated in terms of their morphology into those that supply predominantly dorsal, medial or ventral muscle regions. Moreover they can discharge multiple action potentials in each cycle and display a wider spectrum of firing probabilities during swimming: high-, medium-, or low-probability MNs can be distinguished (Zhang et al., 2011).

II.2. Control of episode duration in *Xenopus* embryo and larvae

Swimming episodes terminate when the tadpole hits an obstacle in its swim path: the rostral cement gland detects physical contact and mediates the activation of the mid-hindbrain GABAergic reticulospinal neurons (mhr, Roberts and Blight, 1975; Boothby and Roberts, 1992; Perrins et al., 2002). Mhr neurons inhibit all spinal neurons and a single mhr neuron can stop the whole swimming network (Li et al., 2003). Normally this pathway is rhythmically inhibited by glycinergic interneurons.

In addition, swimming also stops spontaneously, without activation of the mhr-pathway. So far no ubiquitous "stopping signal" has been found. The duration and strength of locomotor activity is modulated by descending pathways from the brain and the sensory system. The impact of modulatory systems increases during development and so does the variability of swimming motor patterns. Hence in *Xenopus* larvae locomotor output becomes less predictable than in embryos. Furthermore, the idea has recently arisen that the CPG provides an elaborate, intrinsic system which can store information about its own previous activity.

Although a relationship between inter-swim interval and episode duration had already been noted circumstantially much earlier, it was only recently that the mechanism underlying this form of short-term memory in the CPG was understood (Zhang and Sillar, 2012). The model works as follows. During activity Na^+ enters the cell as a result of firing and the resulting increase in intracellular Na^+ concentration which is thought to increase Na^+/K^+ pump activity. The stronger the firing pattern of a neuron during swimming, the higher becomes the intracellular Na^+ concentration, and the more Na^+ is exchanged for K^+ by the pump. This results in a hyperpolarization of the neuron because the pumps extrude 3 Na^+ ions and bring in two K^+ ions so there is a net removal of positive charge It can take one minute before the membrane potential returns to rest . That long hyperpolarization, which accumulates during episodes, is termed ultraslow after-hyperpolarisation (usAHP).

It has been argued that the firing probability of CPG neurons and consequently their recruitment duringduring swimming is reduced during the usAHP (Zhang et al., 2011). Hence the overall network excitability is reduced shortly after swimming termination. The magnitude of this reduction depends on the strength and duration of the preceding episode. This model supports the idea that episode duration depends on the excitability of CPG neurons.

Flexibility of circuit output is generated on different levels and regulated homeostatically, as mentioned above. Firing probability of neurons within a network can increase either in correlation with an increase in neuronal excitability, as shown above, or in correlation with enhanced input. The excitatory drive of the CPG that maintains swimming activity derives from dINs and is glutamatergic (Li et al., 2006; Soffe et al., 2009). Accordingly application of D-serine, which is an agonist at the NMDA receptor glycine site, increases the number of spontaneous episodes, as well as episode duration and variations in the amplitude and frequency of the locomotor rhythm (Issberner and Sillar, 2007). This is accompanied by an increase in rhythm frequency, tonic depolarisation amplitude of spinal neurons during swimming and the occurrence of mEPSPs (E.R. Björnfors, unpublished data). These observations strongly indicate a correlation between NMDA mediated drive and rhythm duration, as well as frequency and variability. Glutamate transmitter and receptor regulation plays an important role in synaptic plasticity (Klein et al., 1982; Malinow and Malenka, 2002). A plastic, activity-dependent change at glutamatergic synapses might therefore alter episode durations on the long-term.

The impact of neuromodulators and neurotransmitters on swimming episode durations has also been described. Increasing levels of the free radical gas nitric oxide (NO), a metamodulator of the *Xenopus* CPG, decreases swim episode duration as well as rhythm frequency (McLean and Sillar, 2000).

Its effects on the duration of swim episodes involve a direct effect on the release of GABA (McLean and Sillar, 2004). Due to the boosting effect of serotonin (5-HT) on rhythm frequency, intracellular Ca^{++} accumulates more rapidly (Demarque and Spitzer, 2010). Activation of Ca^{++}-dependent K^{+}-channels is suggested to cause hyperpolarization of interneurons (Dale and Kuenzi, 1997) which triggers a swimming terminating cascade. A correlation between firing strength and episode duration was also proposed by Dale and Gilday (1996). They argued that ATP released by unidentified CPG neurons during swimming increases the excitability of spinal CPG neurons by inhibiting voltage-gated K^{+} currents while adenosine has the opposing effect as it inhibits Ca^{++} currents. Following release into the extracellular space during swimming ATP is converted to adenosine (by a 5' ectonucleotidase) causing activity to slow down and eventually stop. These data suggest that rundown of motor-pattern generation results from an interplay of many systems at different circuit levels and is not completely understood yet.

II.3. Control of rhythm frequency in *Xenopus* embryo and larvae

As discussed above, swimming in *Xenopus* tadpoles is episodic and exhibits run-down. In response to skin stimulation swimming typically starts at a frequency of around 20 Hz. Over time the frequency of swimming gradually decreases to around 10 Hz, at which point the embryo spontaneously stops swimming (Kahn *et al.*, 1982 ; Kahn and Roberts, 1982; Wall and Dale, 1995). However when the skin receives sensory input during swimming the frequency can increase again, because interneurons which were previously silent can be recruited and start to fire again (Sillar and Roberts, 1992). Accordingly the firing reliability of inhibitory CPG neurons is highest at episode beginning and drops when swimming slows down (Li et al., 2002).

Therefore it was suggested that swimming frequency is dependent on the number of interneurons that are active on each cycle of swimming, because more active interneurons mean more synaptic drive. Furthermore changes in frequency are correlated with the level of tonic depolarisation (Soffe and Roberts, 1982; Soffe et al., 1984; Dale and Roberts, 1984; Sillar and Roberts, 1993). But which pathways drive the CPG? It is already known that stronger excitation within the rhythm-generating networks due to higher levels of glutamate receptor activation leads to increased rhythm frequency in lamprey and mammals (Brodin et al., 1985; Alford and Grillner, 1990; Cazalets et al., 1992; Talpalar and Kiehn, 2010). In *Xenopus* the firing rate of glutamatergic dINs is thought to dictate swimming frequency as reticulospinal dINs fire earliest on each swimming cycle (Soffe et al., 2009) and provide tonic and phasic excitation of CPG neurons. Their background excitation is correlated with their firing frequency (Li et al, 2012). Strong depolarization can promote faster NMDAR-dependent pacemaker firing in dINs (Li et al., 2010). Electrical coupling between dINs may contribute to the background depolarization because the coupling coefficient is highest for low-frequency signals (Li et al., 2009). Furthermore frequency was correlated with higher phasic inhibition (namely higher IPSPs amplitude produced by cINs and aINs) in dINs (Li et al, 2012). Rhythmic inhibition contributes to rebound firing in dINs (Soffe, 2009; Li, 2011). In conclusion, both strong background excitation and phasic inhibition can promote faster tadpole swimming. In contrast, no correlation was found between phasic EPSPs of dINs and frequency. Elevated network excitation affects MN firing and output and eventually results in faster swimming (Roberts and Kahn, 1982).

Rhythm frequency is influenced by modulatory substances. Serotonin has a boosting effect on swimming, increasing the intensity and duration of ventral root bursts but without much change in cycle periods, whereas NA produces the opposite effect of decreasing the swimming frequency (Sillar et al., 1992b; McDearmid et al., 1997).

Furthermore, serotonin reduces the amplitude of mid-cycle IPSPs recorded from motoneurons whereas NA increases them. A decrease in the frequency of mini IPSPs recorded in the presence of tetrodotoxin (TTX) and serotonin suggests a pre-synaptic modulation of the mid-cycle glycinergic inhibition (McDearmid et al., 1997).

III. The early development in *Xenopus* tadpoles

Xenopus embryos progress through five behavioural stages between the end of neurulation (stage 20) and the accomplishment of free swimming (stage 33, Muntz, 1975): I, non-motile, the myotomal muscles begin to differentiate (stage 20-22); II, pre-motile, first APs are initiated (stage 22-24; Baccaglini and Spitzer, 1977); III, early flexure, innervation of the axial musculature starts, reflex responses occur and peripheral nerves are present (stage 24–27). The NMJ contains receptors for acetylcholine (ACh), glutamate (Glu), GABA, and glycine. Later on AChR levels increase and expression of the remaining receptors is suppressed (Borodinsky and Spitzer, 2007). IV, early swimming, first behaviours like struggling and tail swimming can be observed, but the tadpole does not move efficiently through the water (stage 28–33). During the free-swimming phase, in contrast, undulatory movements of the myotomal musculature are fully developed and the embryo is able to swim through the water.

In hatching *Xenopus* embryos (stage 37/38, day 2) the spinal network generating output for swimming is, however, still relatively restricted and inflexible, as motoneurons fire only a single action potential in every cycle of activity (Sillar and Roberts, 1993; Fig.1 A). In contrast in *Xenopus* larvae (stage 42, day 3) motoneurons can discharge a variable number of action potentials per swimming cycle and, moreover, the ventral root discharge can vary in duration and intensity from one cycle to the next (Sillar et al., 1991, see Fig.1 B).

This rise in flexibility of motor patterns is in part due to an increase of innervation by modulatory neurons. In particular serotonergic projections only reach the rostral spinal cord in hatchling embryos, while they also reach more caudal regions in *Xenopus* larvae (van Mier, 1986).

Moreover, the higher the developmental stage, the more sophisticated become the sensory systems and the more reliable do the animals respond to stimulation. The number of lateral line receptors, which enable tadpoles to detect water currents, increases throughout development (Roberts et al., 2009). While stage 32 tadpoles only have as few as three or four neuromasts on each side of the head, during further development more neuromasts appear. Hence, with age, the proportion of animals reacting to currents, by swimming against the direction of flow, or suction, by swimming away, increases (Scharrer, 1932; Shelton, 1971, Simmons et al., 2004). Swimming into water currents is thought to help the tadpole to maintain its position in turbulent water and avoid being swept away by currents. Likewise, during development from embryo to larvae, the number of mechanosensory free nerve endings in the skin increases, making the animal more sensitive to light touch (Roberts and Hayes, 1977; James and Soffe, 2011). The development and differentiation of the vestibular system starts at stage 18/20, and at stage 25 it starts to function. Vestibular receptors provide information about orientation and control posture (Deliagina et al., 2006). However the angular vestibuloocular reflex (aVOR) is not fully functional before stage 47 (Lambert et al., 2008; reviewed in Straka and Simmers, 2011).

The influences of environmental change on tadpole development have indeed been commonly reported: a rise in temperature causes an increase in swim cycle frequency but a decrease in swim bout duration (Sillar and Robertson, 2009), tuned by the NO/cGMP pathway (Robertson and Sillar, 2009). Tadpoles which experience high predator pressure develop longer fins, which is unexpectedly not correlated with enhanced swimming (Doherty et al., 1998) and food deprivation as well as simulation of the "drying pond effect" results in earlier metamorphosis in *Rana temporaria*, induced by a CRH-dependent mechanism (Loman, 1999). This project addresses the question whether activity has an impact on the developmental rate in *Xenopus* embryo and larvae (stage 21 to 42). Furthermore it aims to investigate the influence of early neuronal and muscular activity on the locomotor system in stage 42 *Xenopus* larvae, by means of behavioural analysis, ventral root recordings and whole cell patch clamp recordings from spinal neurons.

Methods

Animals

All experiments were performed on *Xenopus laevis* tadpoles between stage 21 and stage 43. Animals were obtained by hormone assisted (HCG injection; 1,000 U/mL; Sigma) matings of adults selected from an in-house breeding colony. Fertilized ova were collected and reared in enamel trays at 17-23°. All experiments comply with UK Home Office regulations and have been approved by the University of St Andrews Animal Welfare Ethics Committee.

At stage 21 tadpoles were separated into three groups. Control animals (CA) were reared in an enamel tray, unless otherwise stated. In one experimental group activity was suppressed. This "low-activity" (LA) group was reared in a beaker, unless otherwise stated, containing an anaesthetic (0.015% (3-aminobenzoic acid ester (MS-222)) at 1:20 saline in distilled water (pH 7.4). The "high-activity" (HA) group, in contrast, was kept in 50 ml polypropylene centrifuge tubes (Sigma-Aldrich, dimensions: 11,5cm length and 3cm diameter). One tube contained 10-15 tadpoles in approximately 30 ml tap water. A small hole (radius ~ 2mm), cut in the middle of the tube, served to supply oxygen. As the tubes were only filled to one third the water did not leak out. One to four tubes per batch were glued to a rotator (TAAB) with a speed of 4 rpm that caused the tube to rotate end-over end once every 7.5 seconds. The water movement stimulated animals to swim (see figure 3). The rotator was connected to a time clock. Rotation was paused once per hour for 15 minutes duration, as that turned out to decrease the mortality rate. Behavioural and electrophysiological analyses were conducted on stage 42 tadpoles (unless otherwise stated) and experimental animals were allowed a minimum of 30 minutes recovery time before the start of the experiment.

In one experiment tadpoles of all groups were raised in circular plastic tubes. As this rearing condition led to abnormal behaviour (see below) CA and LA tadpoles were usually raised in an enamel tray and a beaker, respectively. To study the developmental rate CA tadpoles were reared as well in a beaker containing 100 ml 1:20 saline in distilled water (pH 7.4) and HA tadpoles were reared as usual in plastic tubes containing then 30 ml 1:20 saline in distilled water.

Development

To compare the rate of development under the three conditions (LA, CA, HA) 50 stage 21 embryos were selected for each group and the egg membranes were removed using forceps to allow access of the anaesthetic. For this experiment animals of all groups were kept in centrifuge tubes (with 3 tubes per group), which were positioned in close proximity. Three times a day the stage of development was noted in every group, respectively, and photographs were taken.

Behaviour

To study swimming behaviour tadpoles were placed individually in an enamel tray containing tap water. Swimming behaviour was recorded with a high speed camera (Casio Exilim, at 30 fps) after tactile stimulation using human hair mounted on a Pasteur pipette. Five such gentle strokes were given to each animal, with intervals between swimming episodes of at least 1 min duration. Videos were analysed using ImageJ wrMTrck Plugin (J. S. Pedersen; Northwestern University). The programme measured automatically the swimming duration, distance travelled as well as the average and maximal horizontal speed for each episode.

Speed was calculated as distance per time for each 1/30s swimming segment (frame by frame). Tadpole positions during swimming were plotted in two dimensions.

Electrophysiology

Tadpoles were briefly anesthetized with 0.1% MS-222 and the trunk skin was then gashed using etched tungsten needles to facilitate immobilization in 12.5 μM α-bungarotoxin saline (20-40 min). The tadpoles were then pinned to a Sylgard block in a bath of saline containing 115 NaCl, 3 KCl, 2 CaCl2, 2.4 NaHCO3, 1 MgCl2 and 10 Hepes (in mM, pH 7.4), unless otherwise stated. Both sides of the trunk skin overlying the myotomal muscles were removed. Extracellular recordings of fictive swimming were made with suction electrodes from ventral roots at intermyotomal clefts. Whole-cell patch recordings, in current clamp mode, from semi-intact immobilized *Xenopus* larvae were performed as described by Li et al., 2002. The whole of the ventral part of the trunk was removed, as the preparation was illuminated from below. The dorsal parts of rostral myotomes were freed from the spinal cord of immobilized animals, and the spinal cord was opened to the neurocoel for patch electrode access. Loose tissue and cells were removed. The animal was moved to the recording chamber and tilted to an angle that allowed the exposed cell bodies to be seen under the microscope. The electrodes were pulled from borosilicate glass capillaries containing a fine filament (Harvard Apparatus Ltd) on a Narishige PP830 pipette puller. Patch pipettes were filled with 0.1% neurobiotin in the intracellular solution (in mM: 100 K-gluconate, 2 MgCl2, 10 EGTA, 10 Hepes, 3 Na2ATP, 0.5 NaGTP adjusted to pH 7.3 with KOH) and had resistances of 10-20 MΩ. They were motor driven and advanced to contact exposed interneuron somata under a 40x water immersion lens. Extracellular signals were amplified using differential AC amplifiers (A-M Systems model 1700), and whole-cell recordings with an Axoclamp 2B amplifier.

Signals were digitized using a CED power 1401 and stored and processed on a PC computer using Axoscope or Spike2 software (v. 3.21, Cambridge Electronic Design, Cambridge, UK).

Fictive swimming was initiated by a 1-ms current pulse delivered by a Digitimer DS2 isolated stimulator using a glass suction electrode placed on the tail skin, or by dimming the light. The stimulus was set to threshold for each animal. Interswim intervals were set to at least one minute. For the purpose of comparing electrical properties of cells the following stimulation protocols were run during patch experiments. Square pulses of hyperpolarizing current of -10 pA were injected through the recording electrode to estimate input resistances.

Incremental steps (10 pA) of depolarazing pulses (200 ms), were applied to measure firing threshold, current/voltage (I/V) relationship and frequency/current (F/I) relationship. Long suprathreshold depolarizing current pulses were applied until cells terminated firing to check for us AHPs (Zhang and Sillar, 2012). In order to distinguish inhibitory from excitatory potentials the membrane potential was depolarized to beyond the reversal potential of IPSPs by positive current injection. Miniature potentials (mPSPs) were counted manually during silent periods between swim episodes recording times of 60 minutes per cell. They were defined as small postsynaptic potentials occurring during interswim periods. They were not evoking action potentials. However mPSPs were not measured in the presence of the action potential blocker TTX. Maximal and average amplitudes of three representative miniature potentials for each cell were measured manually.

Anatomy

Rhodamine staining. To study the localization of acetylcholine-receptors in the myotomal musculature tadpoles were incubated for 30 min in 12.5 μM rhodamine-conjugated α-bungarotoxin. The skin was left intact. After washing in PBS the tissue was cleared and

mounted as described above. All materials were purchased from Sigma-Aldrich, unless otherwise stated.

Analysis

Electrophysiological data were analyzed using Dataview software (version 8.1.0, W. J. Heitler, School of Biology, University of St Andrews, St Andrews, UK). Raw data were analysed with Open Office Calculator. To test significance unpaired t-tests were applied. Differences were considered as significant at $P < 0.05$. Values are expressed as means ± sd. Data were tested for normality of distribution with the one-sample Kolmogorov-Smirnov test. The Pearson-correlation coefficient, followed by an F-test, served as a measure for correlation. Figures were arranged with the image manipulation programme GIMP.

Results

The aim of this project was to investigate the impact of activity during early development, on morphology, behaviour and motor output (part I), as well as on passive and active electrical properties of spinal cord neurons and their activity during fictive swimming (part II), in *Xenopus laevis* larvae. For this purpose, control activity (CA) animals, kept under conventional laboratory conditions, were compared with two different experimental groups: high activity (HA) reared in a rotating tube; and low activity (LA) reared in MS-222.

I. Morphology, behaviour and motor output.

The HA group animals were reared in a centrifuge tube, glued to a rotator (see fig. 3A). When the rotator was switched on, the tube would rotate around its lateral axis at a speed of 4 rpm. Since the tube was filled to one third with water, the rotation produced a constant turnover of the solution every 15 seconds. The function of this apparatus was to simulate turbulent environmental conditions. During the treatment tadpoles were observed to fall into one of two groups. A small proportion became attached by their cement gland to the tube, usually to the top side, and remained there despite the lack of water for half of the time during rotation. These animals were excluded from analysis. The majority of the tadpoles reacted by maintaining swimming throughout the treatment: they followed the water stream when the water was displaced (fig. 3 C-D and F-G) or otherwise swam towards the water surface while spinning around the longitudinal tube axis, perhaps in order not to collide with the tube wall (fig. 3 B, E and H). The more advanced the developmental stage of the tadpoles, the higher the proportion of them that actually swam.

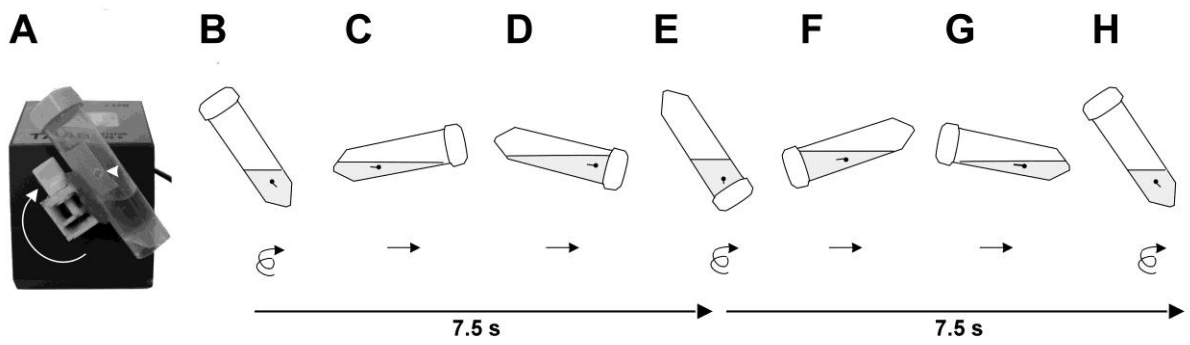

Fig. 3. Experimental setup for activity induction in *Xenopus* tadpoles

A. 50ml centrifuge tubes (2cm diameter), glued to a rotator with a rotational speed of 4rpm (white arrow), were used to increase the activity of tadpoles during development. Tubes were filled to one third with tap water. Therefore the water did not leak through the air hole, which was cut in the middle of the tube (2mm radius; white arrow head). B-H. A single rotation of the tube. Due to the rotation the water was upended 8 times per minute. C-D; F-G. The majority of tadpoles followed the movement of the water (black arrows). B; E; H. When the movement stopped (black arrows) tadpoles usually swam upwards along a corkscrew path. Up to 15 tadpoles were kept in one tube, and 1-4 tubes were run per batch. The rotator was connected to a time switch and the tadpoles were allowed around 10 minutes of recovery per hour.

Clearly the treatment did not permit them to swim either fast or far, as the tube was small in size (length = 11.5 cm; radius = 1.5 cm) and contained only 20-30 mls of water. Occasionally tadpoles would die, despite the water being supplied with oxygen (fig. 3A, arrowhead points at hole). Death of animals could be predicted if they were observed neither to swim nor to attach to the plastic, but instead to float in the water and these animals were removed from the experiment. Whether these animals died as a result of the over excitation or would have died anyway could not be determined. The surviving animals showed high activity during the treatment and were selected for further experiments. In contrast, tadpoles of the second experimental group were deprived of all neuronal activity during development through immobilization with MS-222. It was surprising that despite this treatment the majority of animals not only survived, they also appeared normal (although sometimes paler), and their morphology once they reached stage 42 was similar to the CA and HA groups (see fig. 4).

I.1. HA tadpoles developed faster

I observed that blocking activity delayed the normal progression of development in *Xenopus* tadpoles, while increasing activity had the reverse effect and accelerated their development (fig. 4E). To confirm this observation I compared the development of animals of the three groups from embryonic to larval stages. Animals were separated at stage 21 (fig. 4A), a non-motile stage (according to Muntz, 1964). The egg membranes were removed, or at least opened, using forceps in order to allow access of MS-222. After 15 hours HA animals had reached stage 28 (31/50) or stage 29, LA animals stage 28 (34/50) or stage 29 and CA animals stage 27 (16/50) or stage 28. Seven and a half hours later (fig. 4B) all groups comprised stage 33/34 animals. Some HA animals (11/48), as well as some CA animals (4/50) had already developed to stage 35/6. In contrast the majority of LA animals (45/49) had only developed to stage 32. Thirty nine hours after separation, almost all LA tadpoles (43/48) had developed to stage 37/8, while the CA group also included stage 35/36 (20/50) animals. The HA tadpoles, in contrast, had already become larvae, with 21/47 animals at stage 40 and the remainder (26/47) at stage 39. Three hours later the CA group (41/50 at stage 37/8) had caught up with the LA group (46/48 at stage 37/8), while the HA tadpoles had reached stage 40 (26/47) or stage 41. Within the next 6 hours the CA tadpoles (46/49 at stage 39) overtook the LA tadpoles (45/48 at stage 38), while HA tadpoles had still developed the farthest (45/47 at stage 41). All animals of the latter group had reached the larval stage about half a day later (fig. 4C). Around that time I observed LA animals at stage 40 (41/45) and CA animals at stage 40 (26/49) to 41. The latter required another 10 to 12 hours to reach the larval stage, while LA tadpoles needed at least another 17 hours to develop to stage 42 (fig. 4D). The developmental rate in the three conditions was tracked in the same way on a second occasion with similar results. Furthermore, on every occasion when tadpoles were prepared for experiments ($n > 50$), similar developmental differences to those described above were noticed.

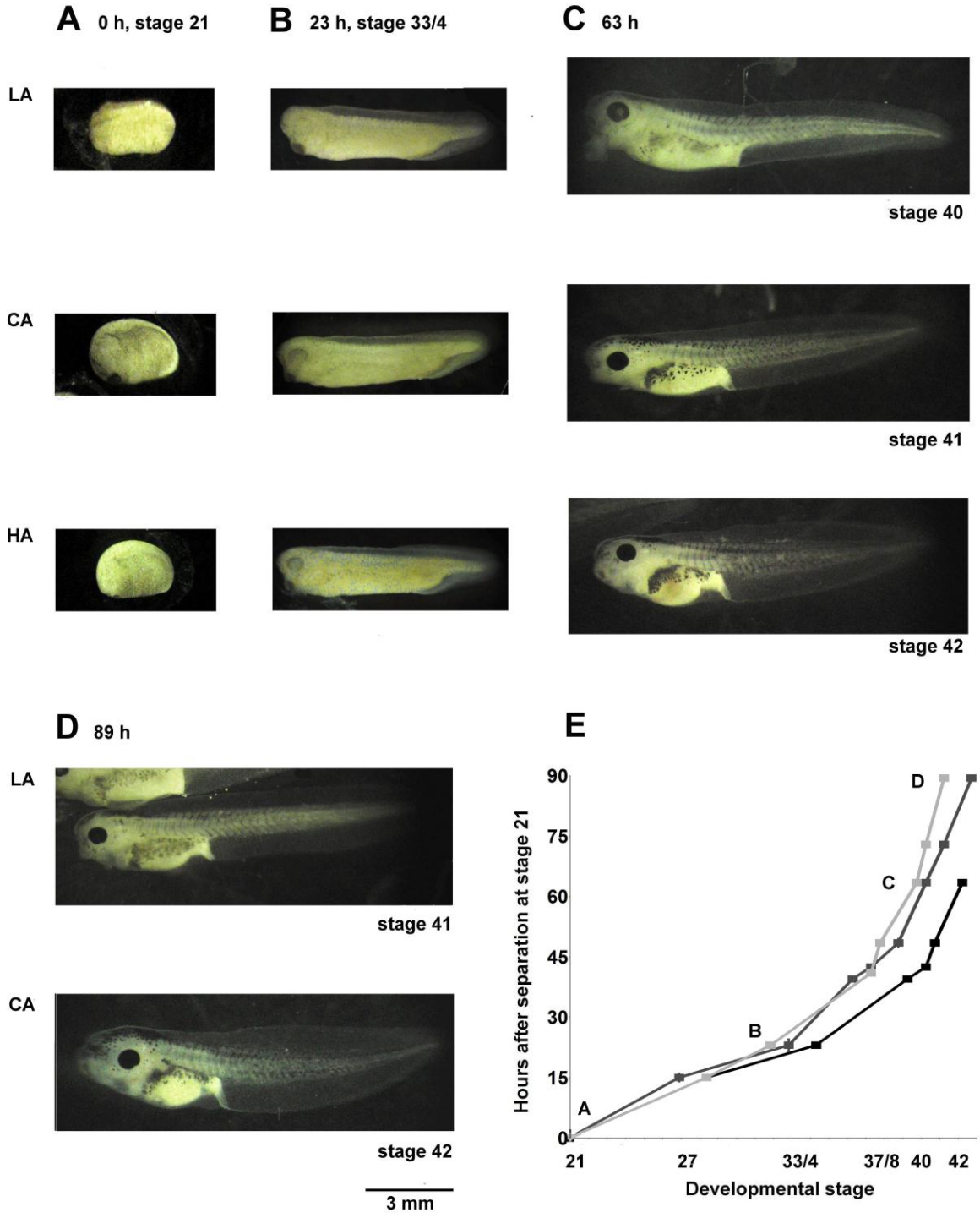

Fig. 4. The effect of activity on the rate of tadpole development

A. Tadpoles were selected at stage 21, before the beginning of movement. In one experimental group activity was increased (HA, black squares in E), while in the other group activity was suppressed (LA, light grey squares in E). Control animals (CA, grey squares in E) developed under standard laboratory conditions. B. After 23 hours tadpoles had reached stage 33/34 in all groups. C. However, 31 hours after separation tadpoles had reached different stages of development, depending on their activity. HA tadpoles had developed to stage 42 about 63 hours, and control tadpoles about 89 hours after separation. D. At that time point the majority of LA tadpoles were retarded in their development. E. Graph showing a positive correlation between the rate of activity and the speed of development, with HA tadpoles developing most rapidly. Note that the x-axis represents the developmental stage, which is not directly correlated with time.

All of the experiments described below were conducted in stage 42 animals (unless otherwise stated), but when considering these data it should be borne in mind that the age of the animals differed between the three groups with HA animals being about a day younger than the others in real time.

I.2. Free swimming episode durations were higher in HA tadpoles

The different experimental conditions also led to interesting changes in locomotor behaviour after the animals were removed from either the rotator (HA) or the MS-222 (LA). The primary, and most striking, observation concerned the propensity to swim. After being released from the rotator and transferred to still water, tadpoles would carry on swimming incessantly, as if they were still in the rotating water column. The other two groups, for the most part, lay dormant. I concluded that this behaviour was due to a decrease in likelihood to terminate swimming once initiated, rather than due to an increase in the probability of occurrence of spontaneous swimming episodes. This was because the incessant swimming eventually stopped and, after 10-20 minutes of recovery, the number of spontaneous episodes within a 30 minute period in HA tadpoles (3 spontaneous episodes counted in 4 tadpoles observed) was similar to that in CA tadpoles (2 episodes/4 tadpoles). No data of LA tadpoles have been collected here. In contrast, video analysis revealed that, once initiated, swimming in HA animals lasted on average 25.9±26s ($n_{episodes}$=42, $n_{tadpoles}$=9, see figure 5B), while the duration of free swimming episodes in CA animals was far shorter (4.3±5.9 s with $n_{episodes}$=56 and $n_{tadpoles}$=12). Swimming episodes were evoked by poking the animals with a human hair. Individual HA tadpoles swam for extremely long periods, with the longest duration recorded being over 20 minutes. This type of swimming was exceptional and therefore these data have been valued as outliers in order not to increase the standard deviation dramatically.

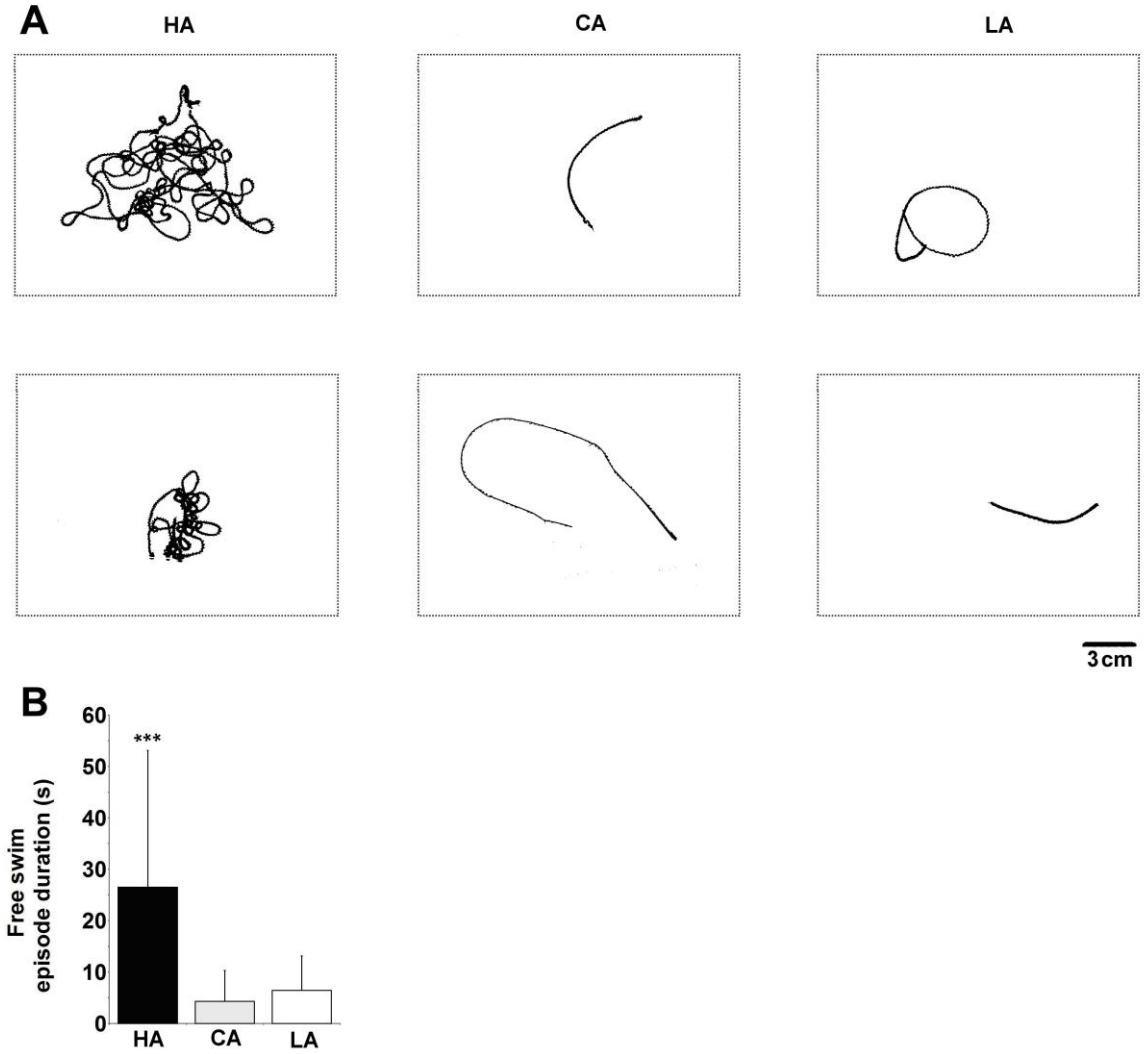

Fig 5. Xenopus larvae maintained swimming for longer time periods when they had experienced high activity during development

To study the effect of activity on free swimming durations, videos of tadpoles were taken after termination of the treatment. The swimming paths of representative episodes from HA, CA and LA tadpoles are illustrated (A). The swimming paths of the latter were similar, but HA animals swam significantly longer (B). **** P<0.001.*

Nevertheless the increased episode duration was significantly different from control or immobilized animals ($p<0.001$). Animals deprived of activity in MS-222 during embryonic and early larval development swam apparently normally shortly after removal from the MS-222. This was surprising given that their locomotor system had never been activated and yet had developed normally. With an average duration of 6.4±6.7 s ($n_{episodes}=35$, $n_{tadpoles}=7$) swim episodes were not significantly different from control animals ($p>0.05$). Figure 5A illustrates the tracked swimming paths of 2 episodes, respectively, in one HA tadpole (Ai), one CA tadpole (Aii) and one LA tadpole (Aiii) and shows that the HA tadpoles swim longer and take a more circuitous path than either CA or LA animals.

I.3. Fictive swimming episode durations were increased in experimental tadpoles

These observations show that activity has a significant effect on both the rate of tadpole development and also on the duration and direction of swimming behaviour and suggest that there may be associated changes to the underlying motor control, CPG, networks of the spinal cord and brainstem. To gain initial insights into the effects of activity upon CPG output the rhythmic swimming motor patterns of the experimental animals were recorded extracellularly in α-bungarotoxin-immobilized tadpoles. Spontaneous and evoked episodes were analysed. Animals were allowed at least 1 minute rest between episodes.

I expected that my measurements would broadly match the behavioural results described above. In fact, average fictive swimming episodes lasted 175±155 s ($n_{episodes}=132$, $n_{tadpoles}=28$) in HA animals, hence about 6 times longer ($p<0.001$) than in CA animals (44±58 s; $n_{episodes}=146$, $n_{tadpoles}=30$; see figure 6A). In LA animals fictive swimming was also significantly increased ($p<0.001$) compared to CA animals (80±89 s; $n_{episodes}=67$, $n_{tadpoles}=9$) but still shorter ($p<0.001$) than in HA animals. Spontaneous activity was compared in two HA, two CA and three LA animals from the same batch and under the same experimental conditions.

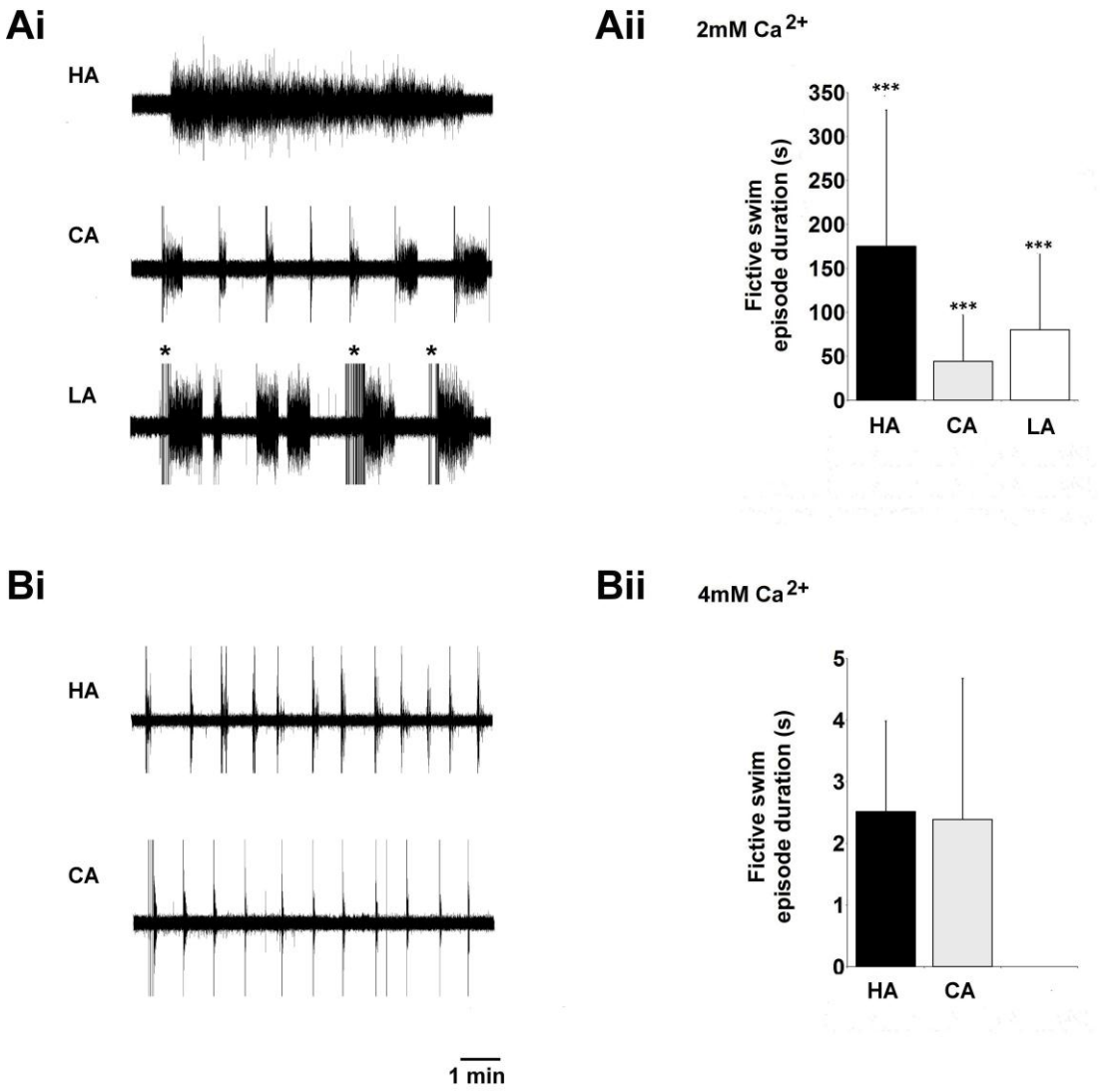

Fig. 6. Experimental tadpoles show increased fictive swim episode durations

Ai. Three representative ventral root recordings demonstrate that average episodes in HA animals were about 4 times, and in LA animals about 2 times longer than in CA animals. Aii. Histogram showing mean episode durations. The differences were significant and similar to the results from behavioural experiments. B. High calcium concentrations (4 mM) reduced episode durations and removed the effect of activity during development. Note also that LA animals required stronger stimuli to elicit swimming (asterisked; described in part II). **** P<0.001.*

The number of spontaneous episodes, counted within 20 minutes of recording time, did not appear different in HA (11 and 3, respectively) and CA animals (11 and 5, respectively), but was reduced in LA animals (only one spontaneous episode occurred in 3 animals measured). However, as episodes in HA animals were longer the relative time of activity was increased in the HA animals (84% and 35%), compared to the CA animals (6% and 9%) and MS-222 animals (2% in 1 animal, 0% in the other two).

In general, fictive swim episodes were longer than free swim episodes and the alterations between groups were more pronounced, when recordings were conducted in saline containing 2 mM calcium. High Ca^{++}-concentrations (4 mM) reduced episode durations significantly ($p<0.001$) to 2.5±1.5 s ($n_{episodes}=36$, $n_{tadpoles}=5$) in HA animals and to 2.4±2.3 s ($n_{episodes}=26$, $n_{tadpoles}=5$) in CA animals (fig. 6B). Thus the effect of activity during development was removed when the Ca^{++}-concentration was high ($p>0.05$). When the calcium concentration was increased from 2 mM to 4mM during the course of an experiment episode durations decreased dramatically (not illustrated). The effect was reversible; after washout initial episode durations were observed. Different Ca^{++}-concentrations have been applied as the actual Ca^{++}-concentration of the extracellular environment in *Xenopus* larvae has not been determined yet.

I.4. Horizontal swim speed was decreased in HA tadpoles

Besides changes in swim duration certain aspects of the swimming capabilities of HA animals appeared altered. First of all they appeared to cover comparatively short distances considering the long swimming durations. Recordings of evoked swimming episodes in response to gentle strokes were analysed with ImageJ wrMTrck Plugin. Distances covered from frame to frame (hence per 1/30 second) were measured in the horizontal plane and average and maximal speed was calculated for each episode.

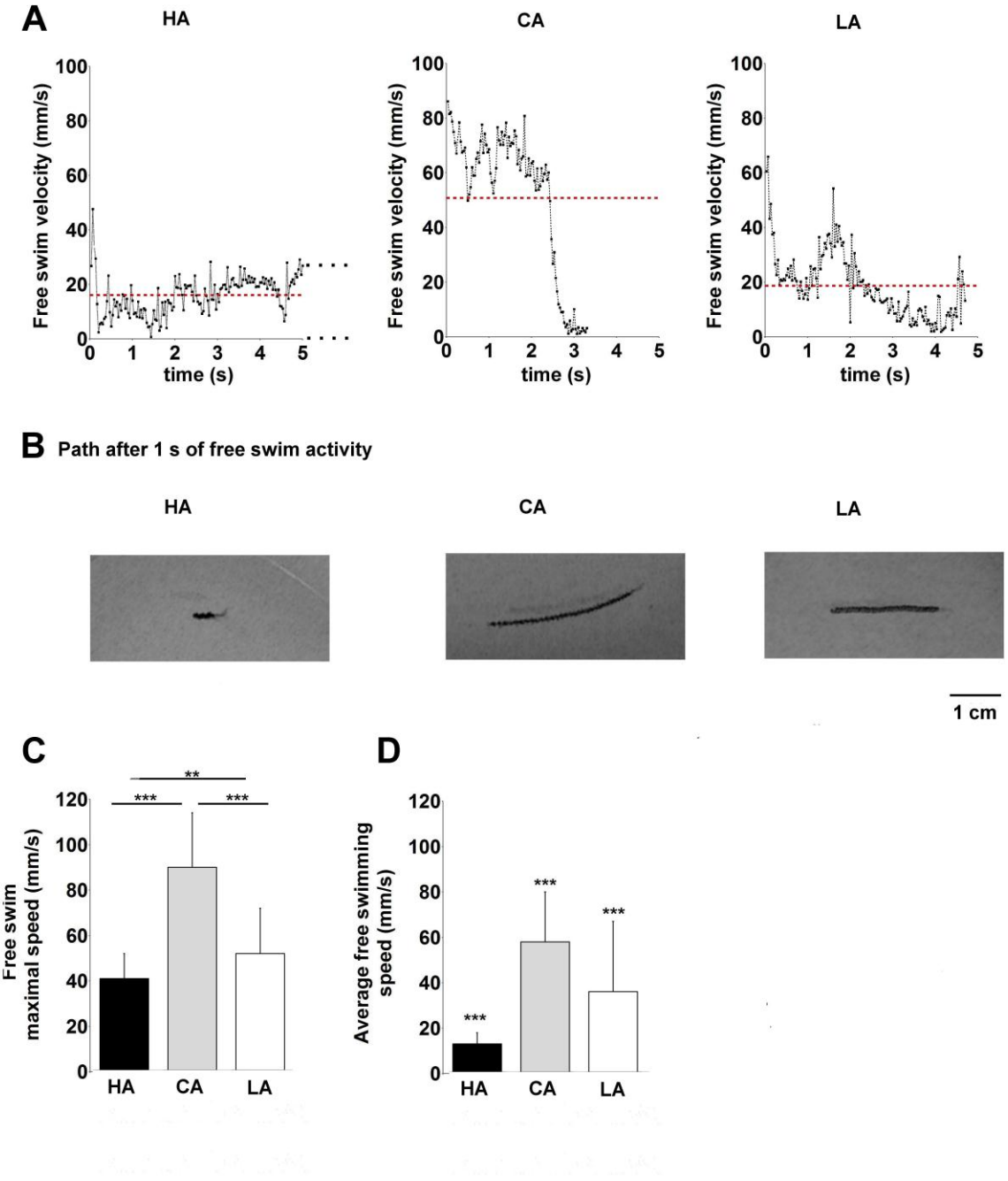

Fig. 7. Control animals covered distances most rapidly

A. The changes in horizontal swimming speed within one representative episode of an agitated (HA), a control (CA) and an immobilized (LA) tadpole are illustrated. HA episodes are long, therefore only the start of the episode is presented (indicated by dotted line). The average speed (red, dotted line) as well as the maximal speed was highest in CA and lowest in HA tadpoles. Note that episodes usually started with a very high speed, which then reduced. B. The distances covered after one second of swimming activity are illustrated in one representative episode. The CA tadpole covered more distance per time than the LA tadpole. The swimming path of the HA tadpole was shortest. C, D. When data were pooled differences between the groups for maximum and average speed were significant.. *** P<0.01, *** P<0.001.*

In control tadpoles horizontal speed describes quite precisely the actual swimming speed as they swim horizontally without any apparent inclination of the body. That is because they have a density close to that of water (Campeny and Casinos, 1989) and the body is ballasted by the dense yolk sac. LA animals oriented themselves in the water like control animals. However, in HA tadpoles the swimming trajectory became more upwards (angle not measured), with a strong vertical (v_x) component, reducing the horizontal (v_y) vector length. Moreover, they were often observed to swim on the spot with the body oscillating but without obvious forwards propulsion. Hence it has to be born in mind that the actual speed (v) is higher in HA tadpoles, than the horizontal component measured. The magnitude of the horizontal speed in response to tactile stimulation will be considered as a measure of swim speed. Both control and experimental animals responded to tactile stimulation frequently with a "C-start" like escape behaviour.

Xenopus larvae could reach extremely high maximal horizontal speed when developed under normal conditions. Within one second they easily traversed the enamel tray (around 10cm width) once. The highest horizontal speed measured was 140 mm/s. The average horizontal speed in 12 control animals ($n_{episodes}$=37) was 58±22 mm/s and the average maximal horizontal speed was 90±23 mm/s (see fig. 7, C-D). LA animals ($n_{episodes}$=26) could not accelerate their speed as much ($p<0.001$). Here the fastest tadpole, out of seven, reached a velocity of 93 mm/s; the average horizontal speed was 31±19 mm/s and the average maximal speed was 53±18 mm/s. In HA animals ($n_{episodes}$=25, $n_{tadpoles}$=9) the average horizontal speed was reduced dramatically to 13±6 mm/s ($p<0.001$). The highest horizontal speed measured was 66 mm/s, hence less than half of that measured in CA animals ($p<0.001$) and the average maximal horizontal speed was 41±11 mm/s, much reduced compared to CA ($p<0.001$) and LA animals ($p<0.01$).

Consequently HA tadpoles did not cover distances as rapidly as CA or LA tadpoles (illustrated in fig. 7B). Whether that was due to a reduction in tail beat frequency will be discussed in the following.

I.5. Initial swim frequencies were highest in HA tadpoles

The frequency of ventral root bursts, reflecting the cycle frequency of CPG output, was compared in the three groups. Ventral root burst swimming frequency in stage 37/38 *Xenopus* tadpoles usually ranges between 10 and 20 Hz (Kahn et al., 1982; Kahn and Roberts, 1982) although this range increases somewhat by stage 42 (Sillar and Simmers, 1992). Tadpoles initiate swimming with high ventral root burst frequencies and the swimming frequency then declines during the course of an episode. This was true for free swimming (see fig. 7A-B), as well as for fictive swimming (see fig. 8D). Ventral root bursts during the first 30 cycles of an episode in HA tadpoles occurred at a frequency of 22.7±2.8 Hz ($n_{episodes}$=33, $n_{tadpoles}$=13). These initial ventral root burst frequencies were significantly higher ($p<0.001$) than in both LA (19±3 Hz, $n_{episodes}$=23, $n_{tadpoles}$=6) and CA (18.8±2.2 Hz, $n_{episodes}$=69, $n_{tadpoles}$=14) tadpoles (see fig. 8A and D). I considered whether there might be a correlation between episode duration and ventral root burst firing frequency, but in stage 42 tadpoles frequencies vary considerably between episodes. However, I detected a significant correlation (F-test, $p<0.001$) between both the initial (r_1=0.86, r_2=0.8) and also the final ventral root burst frequency (r_1=0.77, r_2=0.86) and episode duration in two stage 37/8 control animals (Pearson-correlation, fig. 8F, $n_{episodes}$=13 and 14), but data from stage 37/8 are required to verify this observation.

The differences in initial ventral root burst frequencies did not have an impact on the average bursting frequencies, which turned out to be very similar in all groups with 17.3±2.6 Hz ($n_{episodes}$=12, $n_{tadpoles}$=8) in HA, 17.3±1.6 Hz ($n_{episodes}$=26, $n_{tadpoles}$=4) in CA and 16.8±2.26 Hz ($n_{episodes}$=22, $n_{tadpoles}$= 5) in LA tadpoles ($p>0.05$; fig. 8B).

Figure 8E illustrates that the ventral root burst frequencies were distributed similarly in all groups within one episode (data points have been sorted in descending order).

Increasing activity during development did not have an impact on ventral root burst frequencies at the end of episodes (defined as the mean of the last 30 cycles). Episodes terminated in these animals at 15.4±2.4 Hz ($n_{episodes}$=33, $n_{tadpoles}$=13), compared to 16±2.1 Hz ($n_{episodes}$=50, $n_{tadpoles}$=14) in animals whose development was normal (fig. 8C).

However, the analysis revealed that the MS222 immobilized LA group ceased swimming with the lowest ventral root burst frequencies (14.1±2.1 Hz, $n_{episodes}$=20, $n_{tadpoles}$= 6)($p<0.05$).

Taken together HA tadpoles showed the broadest range of frequencies ($p<0.001$), with average differences between initial and ceasing frequencies of 7.3±0.9 Hz per episode ($n_{episodes}$=33, $n_{tadpoles}$=13) compared to 4.3±2.2 Hz ($n_{episodes}$=19, $n_{tadpoles}$=6) in immobilized and 3.3±1.7 Hz in control animals ($n_{episodes}$=38, $n_{tadpoles}$=14).

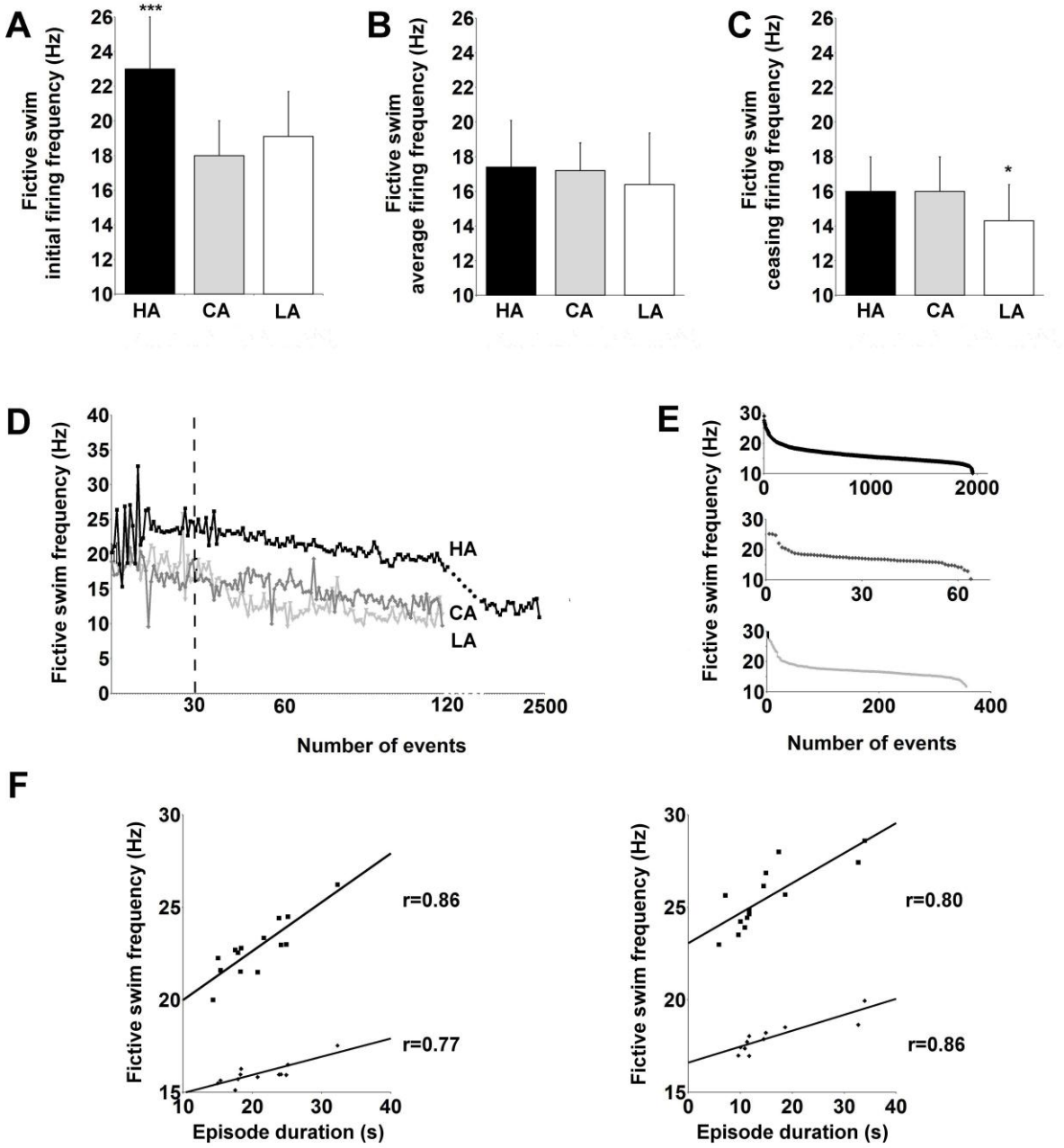

Fig. 8. HA tadpoles showed the highest instantaneous burst frequencies

A-C. Pooled data of VR burst frequency analysis are represented in histograms. A. The initial 30 bursts of an episode (see dashed line in D) occurred with the highest frequency in HA animals. B. Average frequencies, however, did not differ. C. In LA animals the last 30 bursts of an episode were of significantly low frequency. D. The distribution of frequency over time in a representative episode of an HA (black line), CA (dark grey line) and LA (light grey line) and summarizes the finding described above. Note that the episode in HA was much longer (illustrated by dotted line). E. Frequencies of one episode, respectively, were sorted in descending order to illustrate that mean frequencies are similar, in spite of differences in episode durations as well as instantaneous and terminal frequencies. F. In stage 37/38 tadpoles a significant correlation between episode duration and initial (upper line) as well as terminal (lower line) frequency was found in the two animals measured.
*P<0.05, *** P<0.001.

I.6. HA tadpoles changed their swimming direction frequently

The video analysis revealed that HA experimental animals required much more time than control animals to cover the same distances. However this was not correlated with a reduced ventral root burst frequency, but instead with a higher initial ventral root burst frequency. Why do experimental animals then appear to swim slower? Either something has changed in their mode of swimming and/or the way in which swimming is analysed is influencing the results (as mentioned above). Usually hatchling *Xenopus* tadpoles swim with a relatively straight trajectory. They spend 99 % of their time motionless (Jamieson and Roberts, 2000) and only move when stimulated, whereupon they would escape in the opposite direction (Boothby and Roberts, 1995). As soon as they come into contact with an obstacle swimming terminates and they become attached to the object by a thread of mucus secreted by their cement gland (Roberts and Blight, 1975). Consequently their swimming paths appeared comparatively straight (fig. 9B, CA). The same was true for tadpoles with suppressed activity (fig. 9B, LA). Only very rarely did their swimming path cross itself. The difference for animals reared in the rotating tube (HA), is evident in figure 9B. The animals would repeatedly change their swimming direction twisting and turning to both the left and right sides in a seemingly random fashion. Only very rarely would they swim in a straight line. Consequently their swim paths involved many intersections, on average 4.4±2.9 per 10 cm distance travelled ($n_{episodes}$=33, $n_{tadpoles}$=7; fig. 9A). In contrast the swim paths of the CA or LA group only crossed on average 1.54±0.77 ($n_{episodes}$=54 $n_{tadpoles}$=13) or 1.16±0.89 ($n_{episodes}$=31, $n_{tadpoles}$=6) times per 10 cm distance. In ventral root recordings made from α-bungarotoxin immobilized animals changes in the ”direction” of fictive swimming are evident as variations in burst intensity between the two sides (Sillar et al., 1995; Zhang et al., 2011). This phenomenon was indeed very pronounced in agitated HA animals, but was also observed to a lesser extent in the other groups as well (fig.9 D). In addition, the right-left alternation appeared normal.

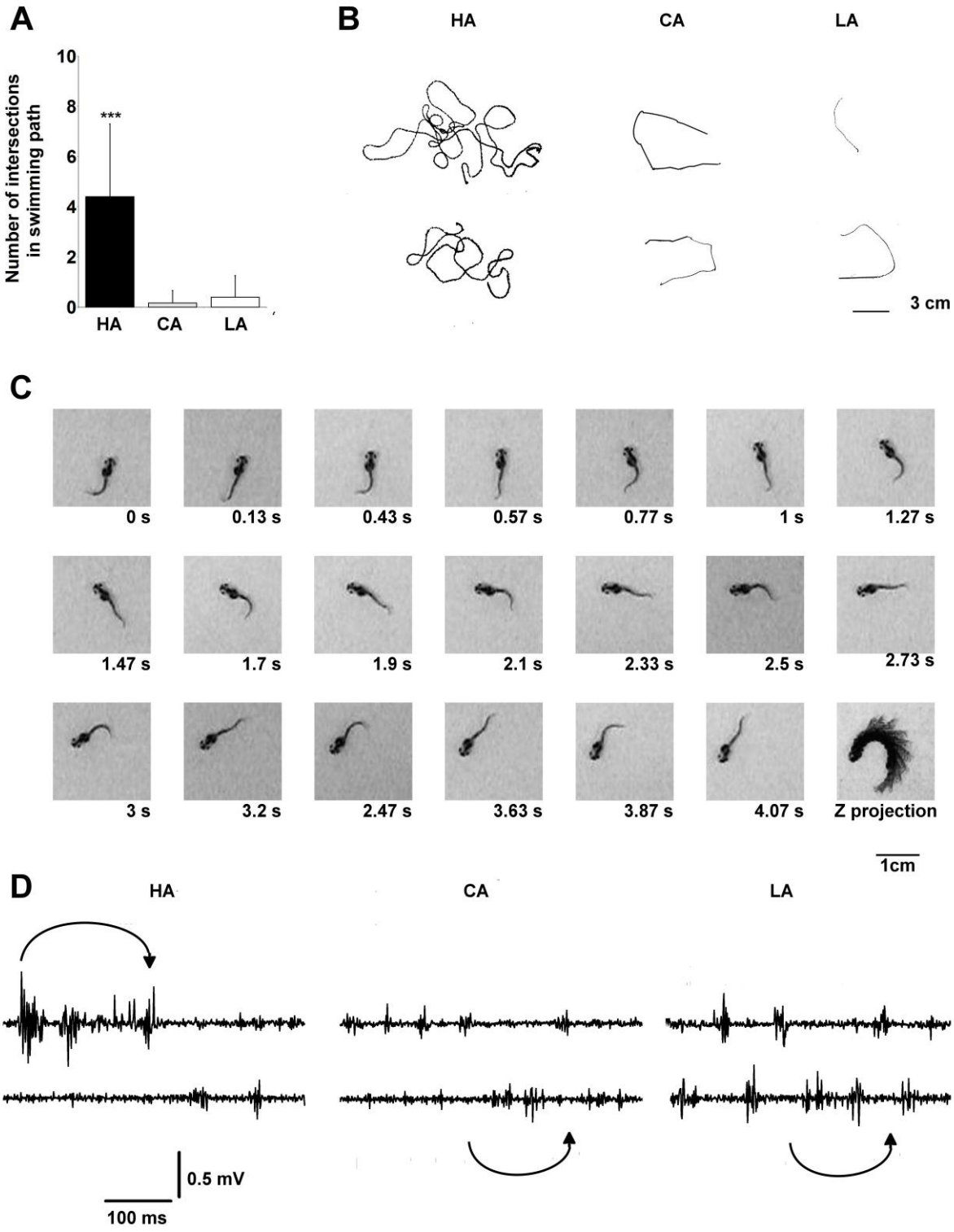

Fig. 9. HA animals changed direction more often during swimming

A. In HA animals the number of intersections of the swimming path per 10 cm distance covered was higher than in CA or LA animals. B. That was because they would twist and turn to both sides during swimming. C. During turns HA animals performed high amplitude waves to one side, to allow for sharp twists . The HA animal presented here rotates by 180 degrees within 4 seconds. D. Fictive turning manoeuvres are evident in VR recordings as variations in burst intensity between the two recording sides (indicated by arrow). **** P<0.001*

I.7. Turning manoeuvres decreased horizontal swim speed

A reduction of horizontal swimming speed due a dramatic increase in the amount of turning manoeuvres seems likely. Data described here derive from a separate set of experiments in which all animals were reared in circular plastic tubes. Hence CA and LA tadpoles presented here lay on the bottom of the curved tube wall during differentiation and growth. Therefore the notochord eventually became crooked to the left or to the right hand side. Due to this deformation the tadpole would constantly turn to the left or the right side, respectively, during swimming and their swimming paths would describe traces of the outlines of the tube in which they developed (fig. 10A; the dimension of the base of the tube is illustrated as a circle in the lower right corner). In HA tadpoles that phenomenon was prevented due to almost permanent rotation of the tubes.

Evoked swimming episodes were analysed. In CA and LA animals horizontal swimming speed was reduced when performing a lot of turns during episodes (see fig. 10). When the number of intersections per distance approached the value measured in agitated HA tadpoles (fig. 10B), the average horizontal speed was reduced in CA animals ($n_{episodes}$=6) and LA animals ($n_{episodes}$=6) to around 30 mm/s (fig. 10C) and the maximum horizontal speed lowered in the CA animal (but rose in the LA animal) to about 90 mm/s (fig. 10D). This was not accompanied by more vertical swimming and the horizontal swim speed in HA tadpoles was still significantly ($p<0.001$) lower.

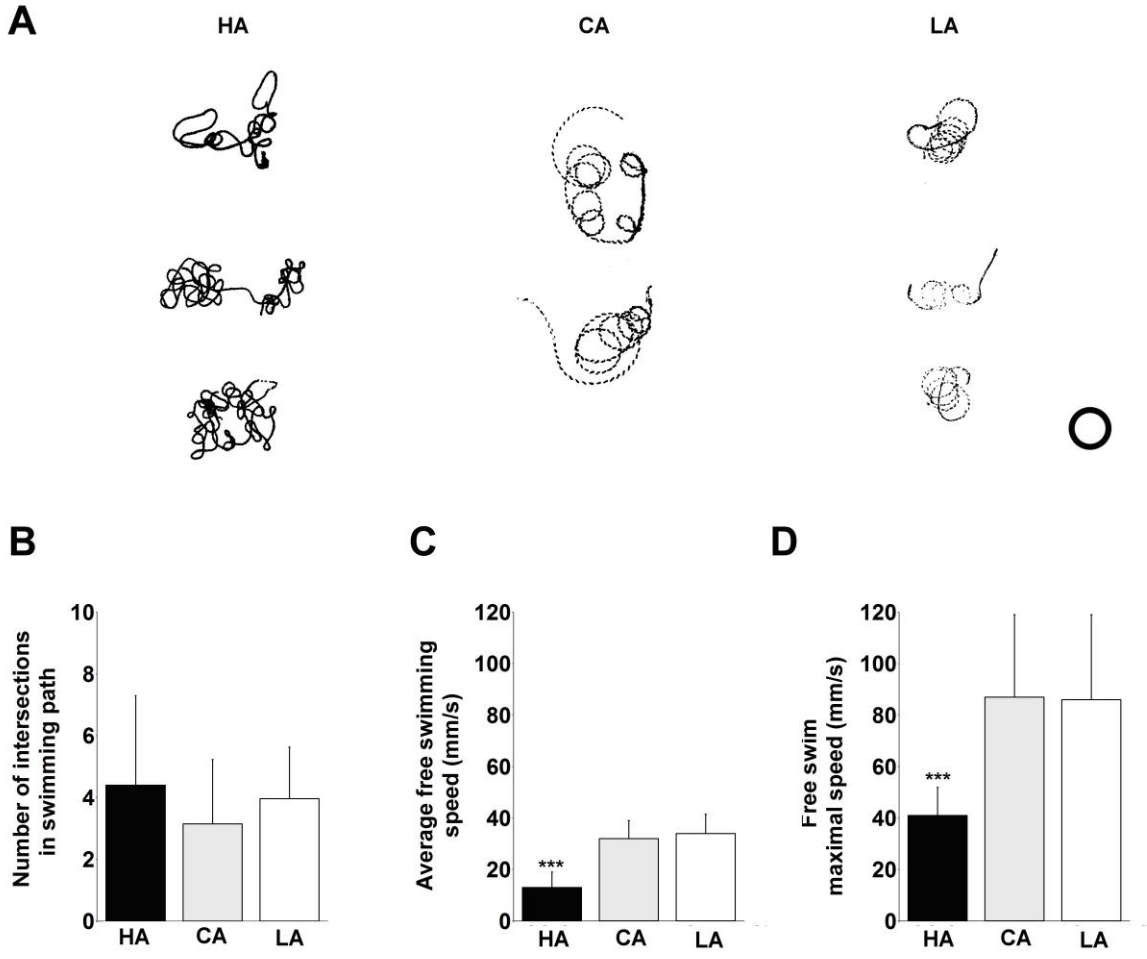

Fig. 10. Swimming speed was reduced in CA and HA animals as well, when they turned a lot during free swimming

A. HA tadpoles changed their swimming direction frequently during free swimming. When CA and LA tadpoles were raised in a curved plastic tube their notocord became crooked and therefore they swam in circles which matched the dimensions of the tube (circle illustrates tube base dimensions). In contrast to HA animals, they would always turn either to the left or to the right side, respectively. B. In these animals the number of intersections per 10 cm distance covered approached the value of HA animals. C-D, Accordingly the average and maximal swimming speed was reduced (compare with fig.7, C and D). However, agitated tadpoles in comparison still showed the lowest horizontal velocity. **** P<0.001.*

I.8. HA tadpoles showed excessive bending during swimming

As described above, HA tadpoles perform a lot of sharp turns during swimming. In fig. 9C the trajectory of an HA tadpole is illustrated which rotates by 180 degree within 4 seconds. Such behaviour requires strong body waves of high amplitude, as the water exerts a viscous torque on the surface of the tadpole that opposes the rotation. Moreover, HA animals could be observed to swim on the spot while, nevertheless, remaining at the water surface. Figure 11 A illustrates that HA animals indeed showed a higher bending magnitude during free swimming.

During fictive swimming neither the burst durations nor the burst amplitudes appeared to have increased (see 11B). However there was high degree of variability between recordings and a more exhaustive analysis with more data is required.

To investigate whether the distribution of AChRs at the neuromuscular junction might vary between groups receptors were stained with α-BT-rhodamine (see fig. 11 C). In fact in HA animals the staining appeared more concentrated than in CA animals, and in LA animals more blurry. A higher density of AChRs could be an indicator of more precise and efficient signal transmission from motoneurons to muscle cells. The experiment was conducted two times and three animals were stained per group per experiment, respectively. More data have to be collected and quantified to confirm this funding.

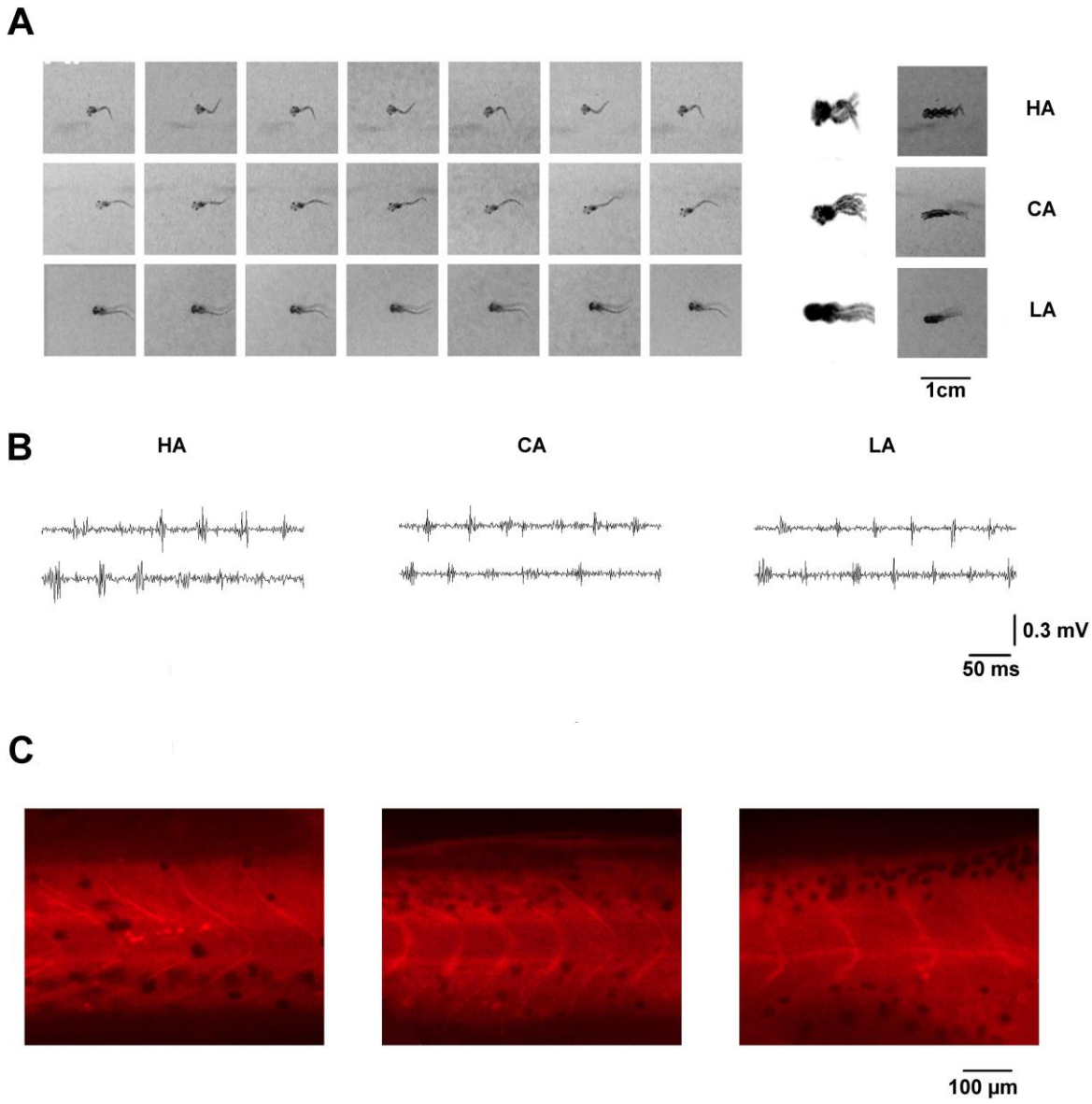

Fig. 11. Rotator-treatment caused more excessive bending during swimming.

A. Images of seven alternating tail twitches of representative animals of the three groups are presented. One can recognize that in the agitated tadpole sequence (HA) the tail is curved more strongly during swimming. When comparing the overlays of these frames the alteration is more obvious. On the right, one can find the distance covered after the short swim sequence. B. Potentially, stronger bending might involve stronger bursting of ventral roots. The burst duration did not appear altered however. Whether the amplitude of VR bursts is higher in agitated tadpoles cannot be established at present. C. AChRs stained with α-BT-rhodamine. The staining of receptors at the neuromuscular junction appeared more concentrated in HA, and more blurry in LA tadpoles.

II. Influence of activity on properties of spinal cord neurons and their activity during fictive swimming.

The behavioural and ventral root recordings presented in the preceding section have revealed alterations in experimental tadpoles concerning episode duration, swimming speed, burst frequency and swimming trajectory. Therefore I expected to find differences in the electrical properties and/or synaptic connections of spinal cord neurons controlling locomotion and their activity during swimming. Most experiments have been conducted on the agitated animals (HA; n=62 patched neurons), as the behavioural alterations were more pronounced than in the MS-222 group (LA; n=16 patched neurons), when compared with control animals (CA; n=44 patched neurons).

Recorded neurons were classified according to their firing activity during fictive swimming and their firing threshold (TH), as described for motoneurons by Zhang et al., 2011. Representative neurons, recorded in HA animals, are illustrated in figure 12. Neurons with a high firing probability during swimming (HFNs) showed a very low current threshold for firing (10-60 pA in CA). In neurons with a medium firing probability (MFNs) action potential (AP) firing was less reliable during swimming. The firing threshold was slightly increased compared to HFNs (10-90 pA in CA). Motoneurons and CPG neurons with higher current threshold are recruited only during periods of stronger swimming activity. Usually they would fire repetitively at the beginning of episodes but then the firing rate would decrease, but could increase again when swimming frequency accelerated. Neurons with low firing probabilities (LFNs) showed an even higher firing threshold (100-140 pA in CA). During swimming they depolarised, but fired very rarely or not at all. They were only recruited during very intense swimming periods. The firing threshold was the highest in these neurons (160-750 pA in CA).

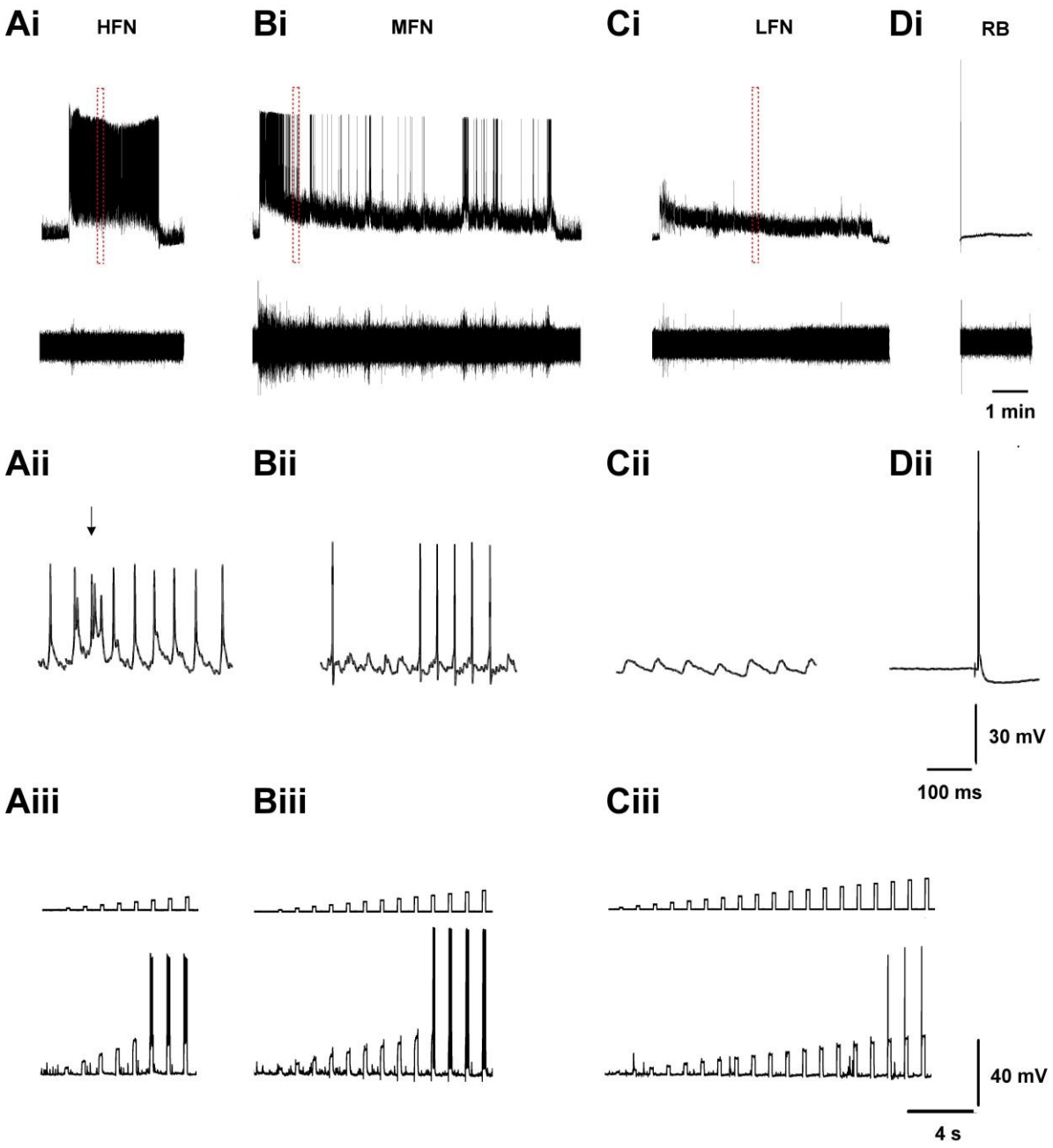

Fig. 12. Firing activity and current threshold of spinal cord neurons in Xenopus tadpoles

Ai-Aiii. HFNs fired throughout each episode and on almost every cycle, as can be seen in the expanded trace, and had the lowest thresholds for firing. Bi-ii. MFNs fired at episode beginning and often during periods of intense swimming. Biii. The current threshold was slightly higher than in HFNs. Ci-Cii. Action potentials were elicited very rarely in LFNs, or not at all. Ciii. As the name implies the firing threshold was highly increased compared to HFNs or MFNs. When swimming speed was accelerated neurons got more depolarised and the probability of AP firing increased (see for instance in Aii, arrow). Di. RB neurons fired once at the beginning of an episode a very large AP. Dii. They did not depolarise during swimming as they don't receive input from the locomotor network. Illustrated data derive from patched neurons of HA animals. Note the long periods of activity of the neurons.

RB sensory neurons can fire a single, long-duration action potential at the start of an episode when their receptive field is stimulated. That was observed in two RB neurons and can be explained by the stimulation electrode being positioned over the peripheral neurites of the respective RB neuron.

II.1. Neurons in agitated tadpoles received more synaptic input

During locomotor activity MNs and CPG-neurons (aINs, cINs and dINs) depolarise due to the synaptic excitation provided by the locomotor network. The higher the swimming frequency, the stronger the depolarisation and consequently the level of firing activity (Roberts and Kahn, 1982; Zhang et al, 2011). Some neurons were observed to increase their firing up to 5 spikes per cycle during periods of high excitation (fig. 13 C). Other neurons, for instance dINs, did not change their firing pattern of one spike per cycle throughout swimming activity, but their depolarisation amplitude changed with rhythm frequency, peaking near the starts of episodes when frequency was at its highest (see fig. 13 A, one dIN measured).

The depolarization amplitude was defined as the change in membrane potential within the first second at the beginning of an episode, measured at mid-cycle (see fig. 14-16, red dotted line in excerpts indicates depolarisation level). Generally the depolarization was higher in LT neurons than in MT neurons (data described below). My data suggest that after increased activity during development neurons received a greater tonic excitatory input during swimming (fig. 13 and 14). The average depolarisation amplitude was 18±5 mV ($n_{neurons}$=26) in HA neurons, compared to 11.8±5.4 ($n_{neurons}$=26) in CA and 11±2.2 mV ($n_{neurons}$=27) in LA neurons ($p<0.001$).

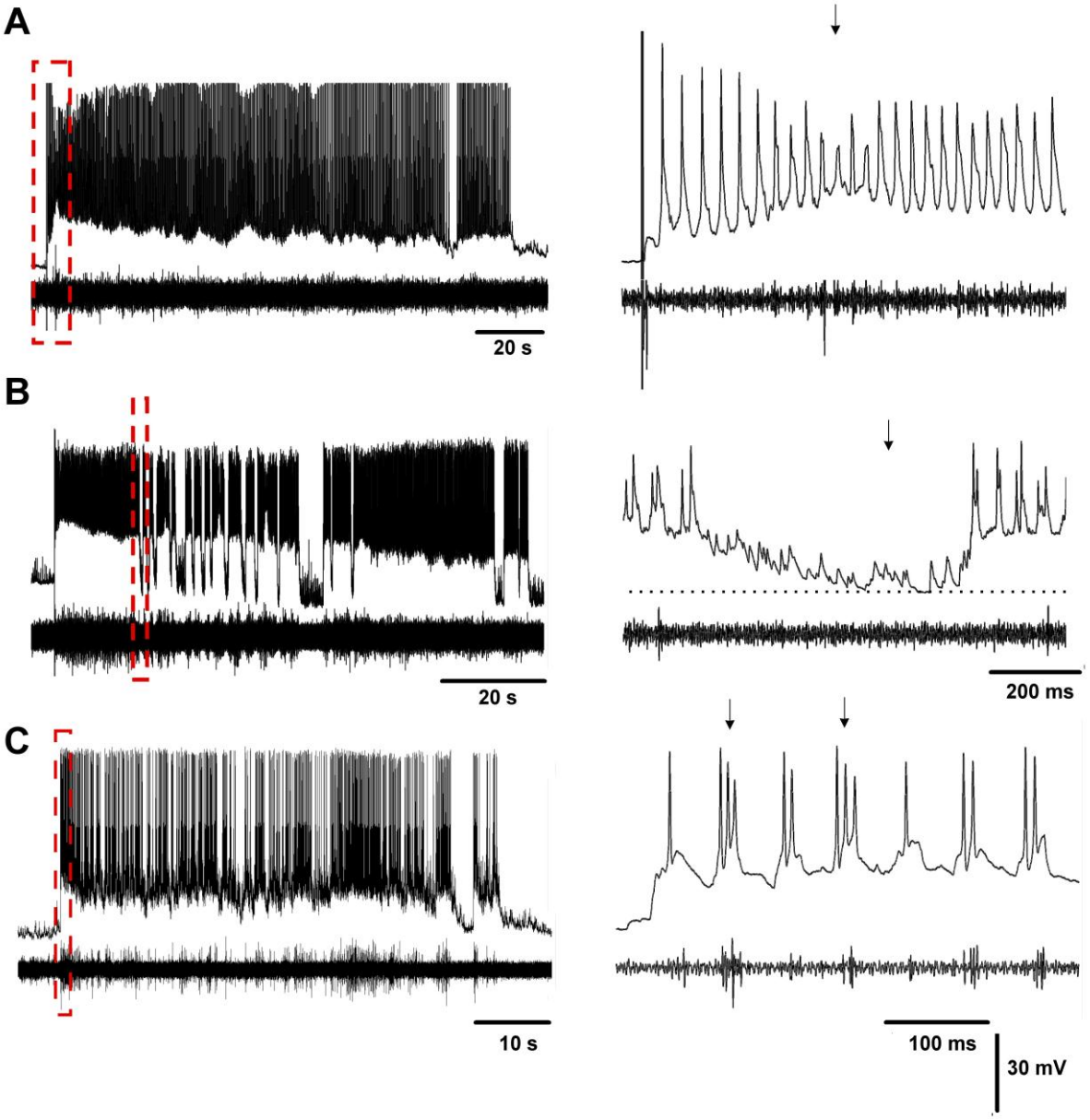

Fig. 13. During high frequency swimming neurons are more depolarised

Each episode of swimming generally began with a large tonic depolarization and terminates after a gradual repolarization. Within an episode phases of increased depolarization coincide with an increased in swim frequency. A. DINs fire one AP per cycle. Increase in frequency is correlated with increased depolarisation and decreased spike height (arrow). B. In an HFN the membrane potential hyperpolarizes back towards the resting potential during phases of low activity (see arrow, dotted line displays RP). C. In addition to a rise in depolarisation amplitude CPG neurons (except for dINs) also increase the number of spikes fired per cycle (arrow).

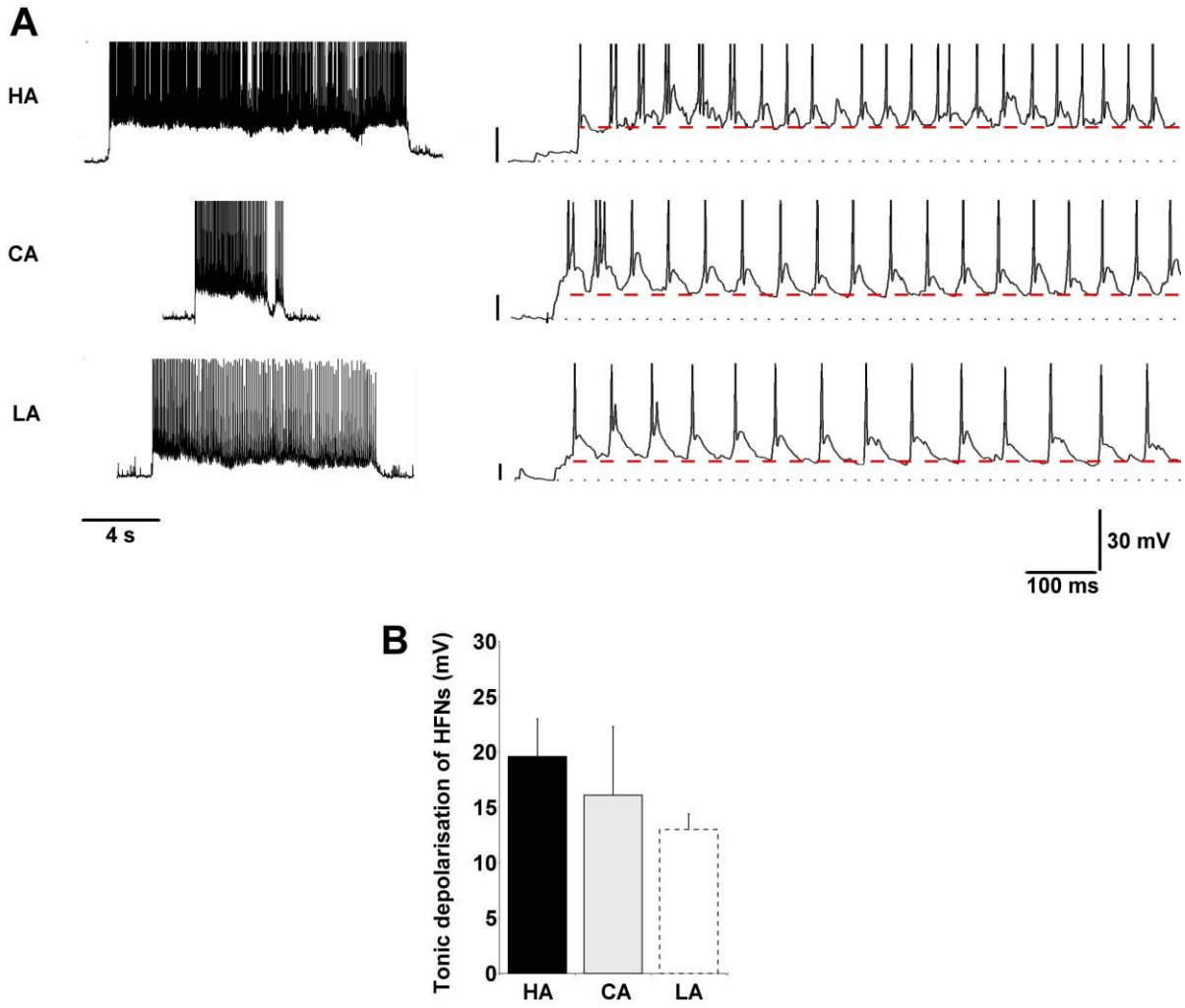

Fig. 14. HFNs of HA animals depolarised the most during fictive swimming.

A Representative traces of HFNs from HA, CA and LA tadpoles are presented. HFNs fire on almost every cycle one or more APs, depending on cell type and degree of activity (see excerpts). Depolarisation amplitude was measured within the first second of the episode (illustrated in excerpts). Here and in subsequent figures the dashed red lines indicate the depolarisation level, defined at the level of mid-cycle inhibition, the vertical lines to the left of the excerpts illustrate the depolarisation amplitude and the RMP between episodes is shown dotted. The action potential peaks have been chopped. Note that the initial firing frequencies are higher in HA tadpoles (described in part I). High-frequency swimming is known to be correlated to strong background excitation. B. Pooled data of HFNs revealed highest depolarisation amplitudes in HA tadpoles and lowest in LA tadpoles. However the differences were not significant. Data derive from 1-2 episode analysed per cell.

In HA animals HFNs were depolarised by 19.6±3.5 mV, thereby raising the membrane potential (MP) to -33±2.9 mV ($n_{neurons}$=9), while in HFNs of CA animals the MP had risen by 16.2±6.2 mV to -30±5.8 mV ($n_{neurons}$=7; $p>0.05$; fig. 14). In LA animals only two HFNs were measured (increase by 13±1.4 mV to -32.5±3.5 mV). The strongest change was found in MFNs (see fig. 15). In HA tadpoles the tonic depolarisation of MFNs was almost as pronounced as in HFNs. The average amplitude was 18.9±6.2 mV, depolarising the MP to -37±7mV ($n_{neurons}$=15), compared to only 10±4.2 (-42.1±6 mV, $n_{neurons}$=15) in CA ($p<0.001$) and 10.3±2.7mV (-48±10.4 mV, $n_{neurons}$=6) in LA tadpoles. Accordingly the spike height during swimming was slightly decreased in MFNs of HA (58±21 mV, $n_{neurons}$=12), compared to CA tadpoles (64±14.5 mV, $n_{neurons}$=10, $p>0.05$). Spikes occurring during the first second of activity were measured. The increased tonic depolarization in MFNs was also accompanied by a slight increase in firing; the probability of AP firing within the first 10 cycles of an episode was 62±42% in HA MFNs ($n_{neurons}$=6) and 44±41% ($n_{neurons}$=10) in CA MFNs. LFNs depolarised on average by 16.8±3.7 mV to -41.8±2.5 mV ($n_{neurons}$=3) in HA tadpoles and by 12.2±2.5 mV to -47.5±4.8 mV ($n_{neurons}$=3) in CA tadpoles (see fig. 16). Hence the same tendency was observed as described above.

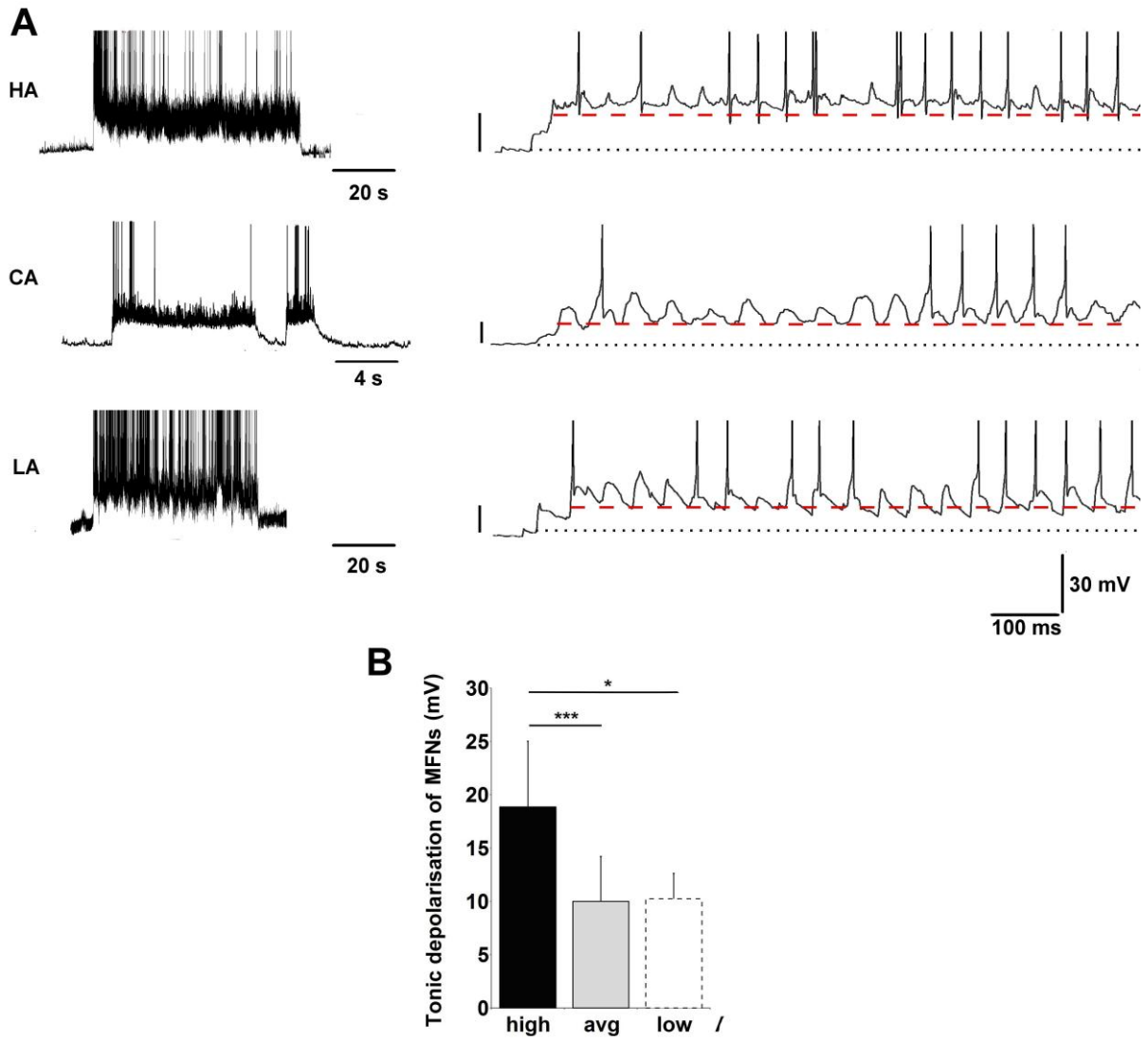

Fig. 15. The increase in tonic depolarisation amplitude in HA animals was most pronounced in MFNs

A. The increase in depolarization amplitude appears to be accompanied by increased initial firing frequencies and is larger in HA neurons. B. The difference in tonic depolarization amplitude in MFNs of HA tadpoles was significant. *$P<0.05$, ***$P<0.001$.

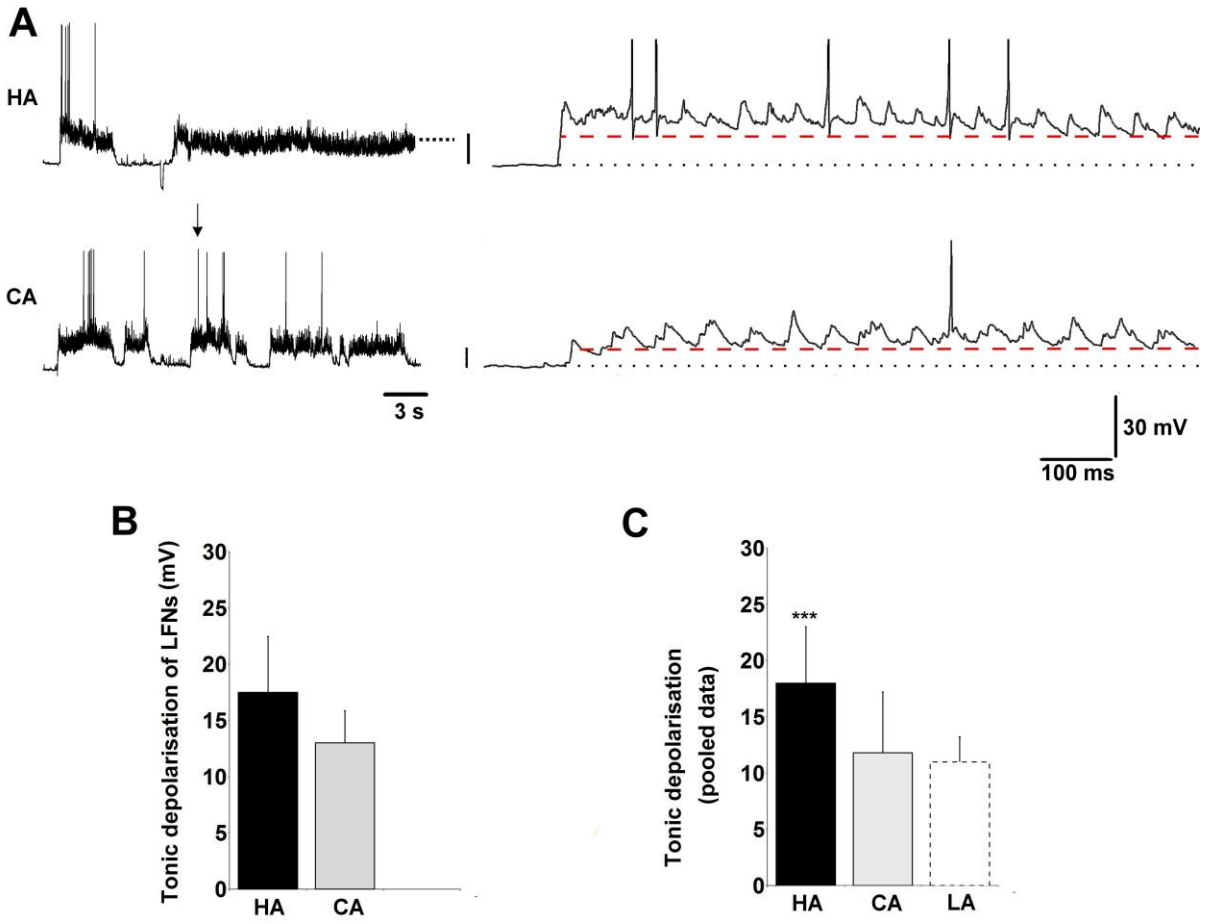

Fig. 16. LFNs of HA animals depolarised more during fictive swimming.

A. LFNs also appeared to depolarise more at the beginning of swimming in agitated HA tadpoles . Note the different time scale indicating that HA episodes were much longer than CA episodes. B. However differences were not significant. C. Pooling the data of HFNs, MFNs and LFNs for each group revealed significantly stronger excitation of HA neurons. ****P<0.001.*

Table 1. Mean +/- SD of tonic depolarisation amplitude (mV) measured just before episode termination

HFNs			n	MFNs			n	LFNs			n
HA	7.8±7.1	-44±5.7	10	HA	6.7±4.4	-48±4.8	15	HA	8.7±4.9	-51±3.9	3
CA	8.2±5	-39±6.5	11	CA	8.6±5.4	-47±6.8	15	CA	3±3.6	-55.7±3	3
LA	8±5.7	-38±7.8	2	LA	4.2±2.6	-54±16	6	LA	-	-	-

When firing stopped the depolarisation fell back to the resting potential. The average depolarization amplitude at the end of episodes did not differ between groups: 7.5±5 mV ($n_{neurons}$=25) in agitated, 6.7±5.4 mV ($n_{neurons}$=21) in control and 5.14±3.6 mV ($n_{neurons}$=7) in immobilized tadpoles (see table 1).

It could be hypothesized that the increase in tonic drive to HA neurons during swimming results from stronger glutamatergic connections allowing larger NMDA receptor-mediated components to summate; as a result more frequent spontaneous synaptic events in the silent periods between swim episodes might be expected. Indeed miniature postsynaptic potentials (mPSPs), counted during one minute periods in the intervals between swim episodes occurred at a higher frequency (2.86±2.4 Hz; $n_{neurons}$=32) in HA neurons than in CA (1.46±1.08 Hz; $p<0.05$; $n_{neurons}$=22) or LA neurons (1.73±1.17 Hz; $n_{neurons}$=7; see fig. 17).

The amplitudes of the three largest mPSPs and three average mPSPs, occurring within one period of one minute recording time, were measured for each neuron. Means from neurons of the same experimental group were pooled. Maximal amplitudes were measured at 10.4±5.1 mV ($n_{neurons}$=21) for HA neurons, 8.9±3.9 mV ($n_{neurons}$=16) for CA neurons and 11.1±4.2 mV ($n_{neurons}$=6) for LA neurons (fig.17 B). Average mPSPs amplitudes were 3.95±1.6 mV ($n_{neurons}$=21) in HA neurons, 3.53±1 mV ($n_{neurons}$=16) in CA and 4±0.8 mV ($n_{neurons}$=6) in LA neurons. As no significant differences were found between the three groups ($p>0.05$), amplitudes were not further analysed.

In all groups mIPSPs as well as mEPSPs were detected, when the membrane potential was depolarized for 30 seconds above the reversal potential of IPSPs by positive current injection through the recording pipette (fig.18). If the increased depolarization described above were to be explained by reduced inhibition then the mIPSP rate should be decreased in HA neurons. The probability of mIPSP occurrence, however, did not differ between HA and CA neurons ($p>0.05$): on average 39±8 % of miniature potentials were inhibitory in HA neurons (n=5) and 31±19 % in CA neurons (n=7; fig.18 B). The incidence of synaptic inputs was higher after the end of an episode and the majority of them were inhibitory (fig. 18 C and D). As shown by Reith and Sillar (1999), stage 42 swim episodes often end coincident with a barrage of GABAergic IPSPs.

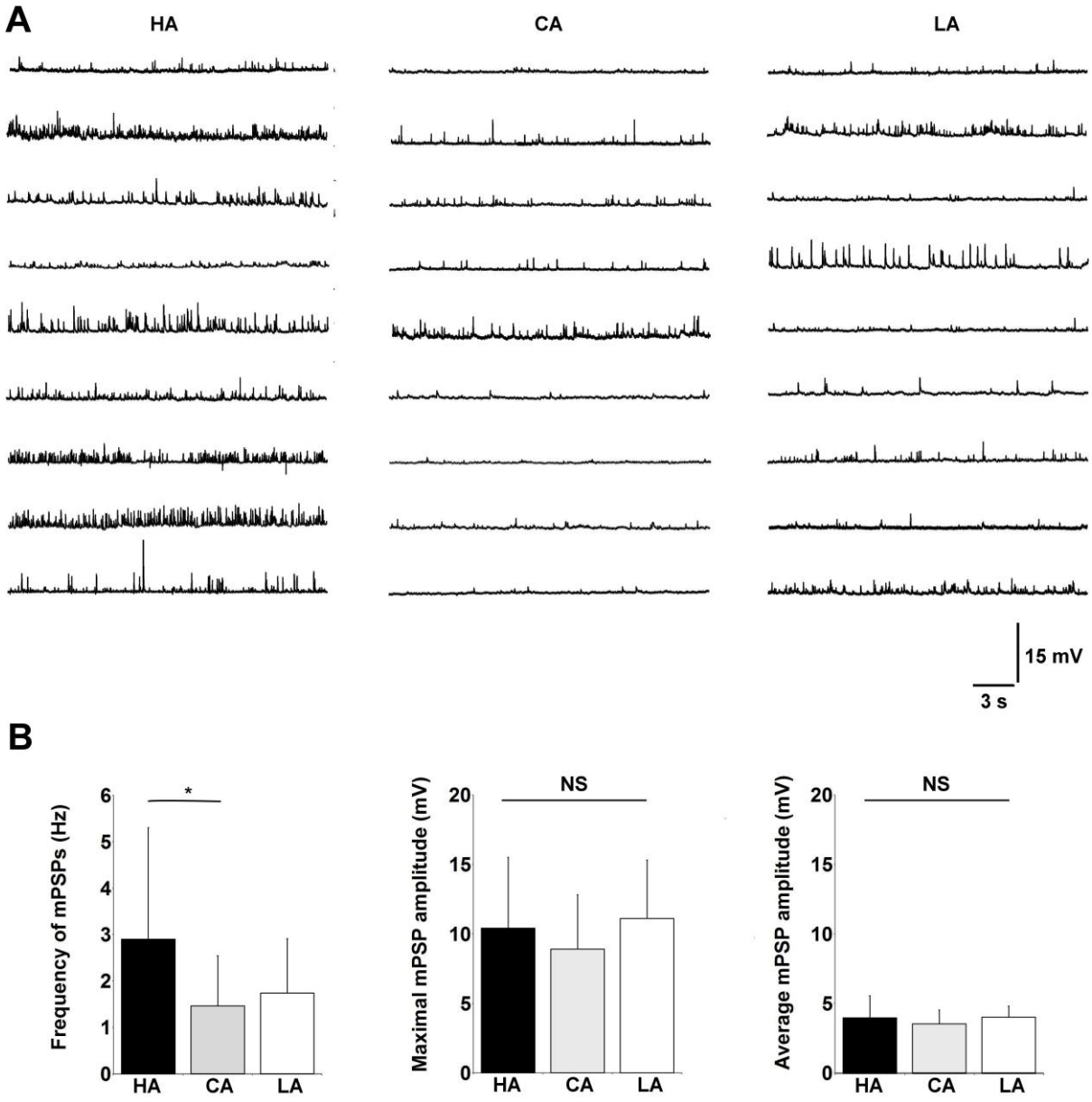

Fig. 17. Frequency of spontaneous vesicle release is higher in HA animals.

A. Nine representative traces from quiescent period of recordings from neurons of agitated animals (HA), control (CA) and MS-222 animals (LA). B. Miniature potentials occurred at a significantly higher frequency in HA neurons than in the other groups. For LA neurons mPSP frequency did not differ significantly from CA neurons. The amplitudes of mPSPs was not significantly different between the three experimental groups. Three highest and three average amplitudes, respectively, occurring within one minute of recording time, were measured in each neuron. * $P < 0.05$.

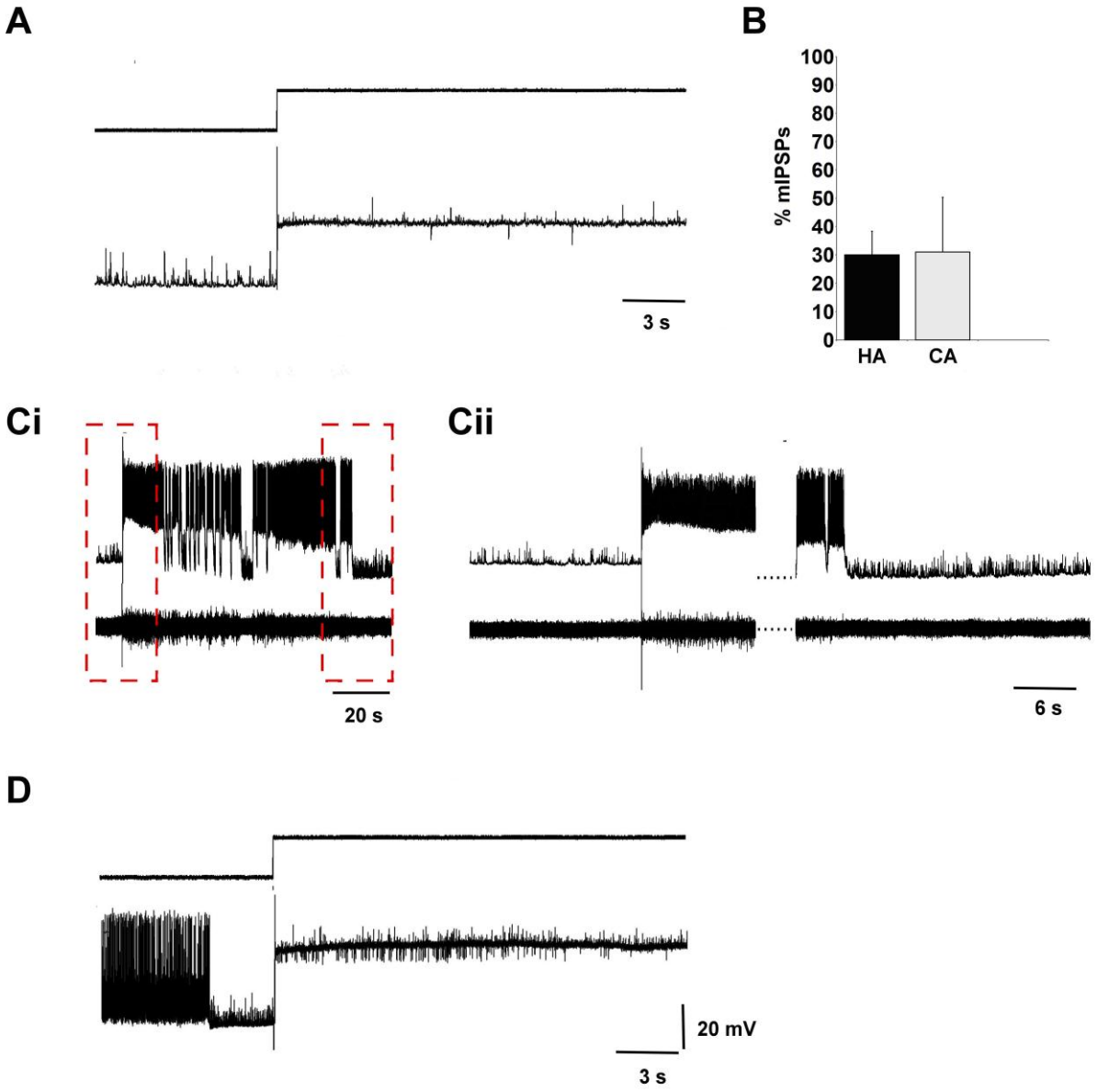

Fig. 18. The proportion of mIPSPs versus mEPSPs did not appear altered

A. Membrane potentials were depolarised beyond the reversal potential of IPSPs, by injection of positive current, to distinguish inhibitory from excitatory mPSPs. Trace displayed here derives from an HA neuron. Note that the neuron receives a mixture of mEPSPs and mIPSPs. B. The number of inhibitory and excitatory spontaneous potential was measured during 30 seconds of recording time per neuron. In both HA and CA neurons the percentage of mIPSPs was about 30 %, hence not different. C. The frequency of mPSPs is higher after, then before episodes. D. These potentials are mainly inhibitory.

Table 2. Comparison of RMP (mV) in rhythmically active CPG neurons and RBs

HFNs		n	MFNs		n	LFNs		n	RBs		n
HA	-54.7±4.2	10	HA	-55.7±6.6	13	HA	-61±3.9	5	HA	-70.4±9.5	6
CA	-54±5	9	CA	-57±4.7	13	CA	-60±6	4	CA	-70±6.4	4
LA	-53	1	LA	-60.3±9.4	6	LA	-	-	LA	-69.5±0.7	2

The changes in the activity of neurons as a result of the different experimental treatment during development was not, however, accompanied by changes in the resting membrane potential (RMP) and no significant differences were found in this parameter between the three groups ($p>0.05$, see table 2). Generally in neurons which were more active during swimming the RMP was more depolarised than in neurons with a high firing threshold. When data of the three groups were pooled the difference in RMP between HFNs (-54±4.3 mV, $n_{neurons}=20$) and LFNs (-60±4.7 mV, $n_{neurons}=8$) became significant ($p<0.01$). MFNs had RMPs between those of HFNs and LFNs (-57±6.4 mV, $n_{neurons}=32$; $p>0.05$). The RMP in RB neurons (-70.3±8 mV, $n_{neurons}=10$) was significantly more negative than in HFNs ($p<0.001$), MFNs ($p<0.001$) and LFNs ($p<0.01$).

II.2. HA tadpole neurons were less excitable

My results above suggest that neurons of HA animals are more depolarised during swimming (see fig. 14-16) due to enhanced synaptic excitation. Indeed, HA neurons required larger depolarizing currents to reach spike threshold (figure 19 A-B). A series of rectangular positive current pulses (200msec) of increasing amplitudes (in 10 pA steps) was applied via the recording patch pipette. The neurons displayed spike frequency adaptation; during application of a supra-threshold current pulse neurons usually started to fire with a high frequency which then decreased and was lowest at the end of the pulse. The higher the intensity of the current pulse the higher was the initial firing frequency. The TH of HFNs significantly ($p<0.05$) increased from 26±17 pA in CA animals ($n_{neurons}=10$) to 53±31 pA in HA animals ($n_{neurons}=9$). Likewise in MFNs the average TH was with 40±26 pA lower in CA animals ($n_{neurons}=14$) than in HA animals (63.4±34.5 pA; $n_{neurons}=12$; $p>0.05$). The average TH in MFNs of LA animals was close to the value measured in CA animals (36.7±24.2 pA; $n_{neurons}=6$). Insufficient data on LFNs were collected to draw conclusions yet, but higher currents were required to induce firing in HA neurons (168±71 pA; $n_{neurons}=5$) than in CA neurons (127±23 pA; $n_{neurons}=3$).

Neurons of HA animals started firing at higher current injections with an increased instantaneous frequency (measured between the first two spikes in the train at current threshold; fig. 19 C). In HFNs of HA animals ($n_{neurons}=7$) the initial firing frequency was 32.5±17 Hz, compared to 21.8±11 Hz in CA animals ($n_{neurons}=7$). The same was observed in MFNs: 32.7±14 Hz in HA ($n_{neurons}=9$) versus 22±9 Hz in CA animals ($n_{neurons}=10$). When these data were pooled the differences were significant ($p<0.05$).

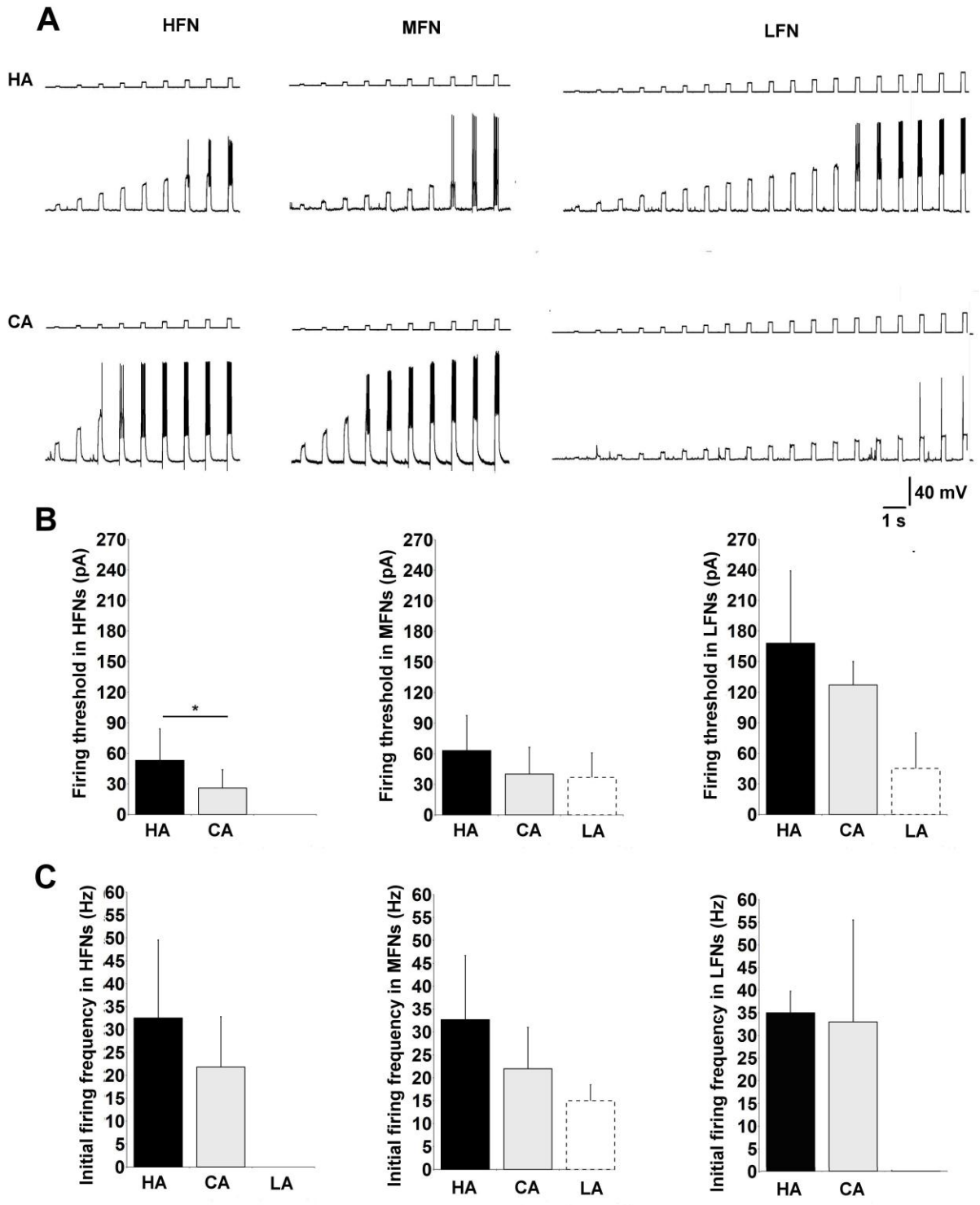

Fig. 19. HA neurons showed a higher firing threshold than CA neurons

A. Examples of firing thresholds in neurons of agitated tadpoles (HA) and control tadpoles (CA) illustrated by steps of increasing current (upper traces) and the neuron's response (lower traces): low firing threshold neurons (HFNs) in this case 70pA versus 30pA, medium threshold neurons (MFNs, 80pA and 40pA, respectively) and high threshold neurons (LFNs, 160pA and 140pA). B. Differences were significant in HFNs. C. Higher thresholds were accompanied by increased instantaneous firing frequencies. These differences were observed in all neuron types. When data of HFNs and MFNs were pooled the alteration became significant. In LA neurons not enough data were collected.*$P<0.05$.

Data from LA animals ($n_{neurons}$=4) were again quite similar to CA animal data. The average initial firing frequency of MFNs and HFNs was 20.5±11.6 Hz. No differences were found in LFNs: 35±4.2 Hz in HA ($n_{neurons}$=2) and 32.5±22.5 Hz in CA neurons ($n_{neurons}$=3).

Analysis of the relationship between current injection and voltage change (V/C) revealed that CA neurons at same current injection levels reached higher depolarisation levels than HA neurons (see fig. 20). That might explain the higher firing TH in HA neurons. Differences in V/C-relationship were found in neurons with a low threshold (10-40 pA, fig.20 A, $n_{CA\text{-}neurons}$=13, $n_{HA\text{-}neurons}$=13), as well as in neurons with a medium threshold (50-90 pA, fig.20 B, $n_{CA\text{-}neurons}$=7, $n_{HA\text{-}neurons}$=14). However the number of experiments was too small to determine if any changes were significant. The average depolarisation of the membrane potential in response to increasing current pulses was similar in both groups of LFNs (fig.20 C,$n_{CA\text{-}neurons}$=7, $n_{HA\text{-}neurons}$=14). RB neurons of HA tadpoles, in contrast, depolarised slightly more than CA RB neurons at same degrees of current injections up to about 100 pA (fig. 20 D, $n_{CA\text{-}neurons}$=3, $n_{HA\text{-}neurons}$=4).

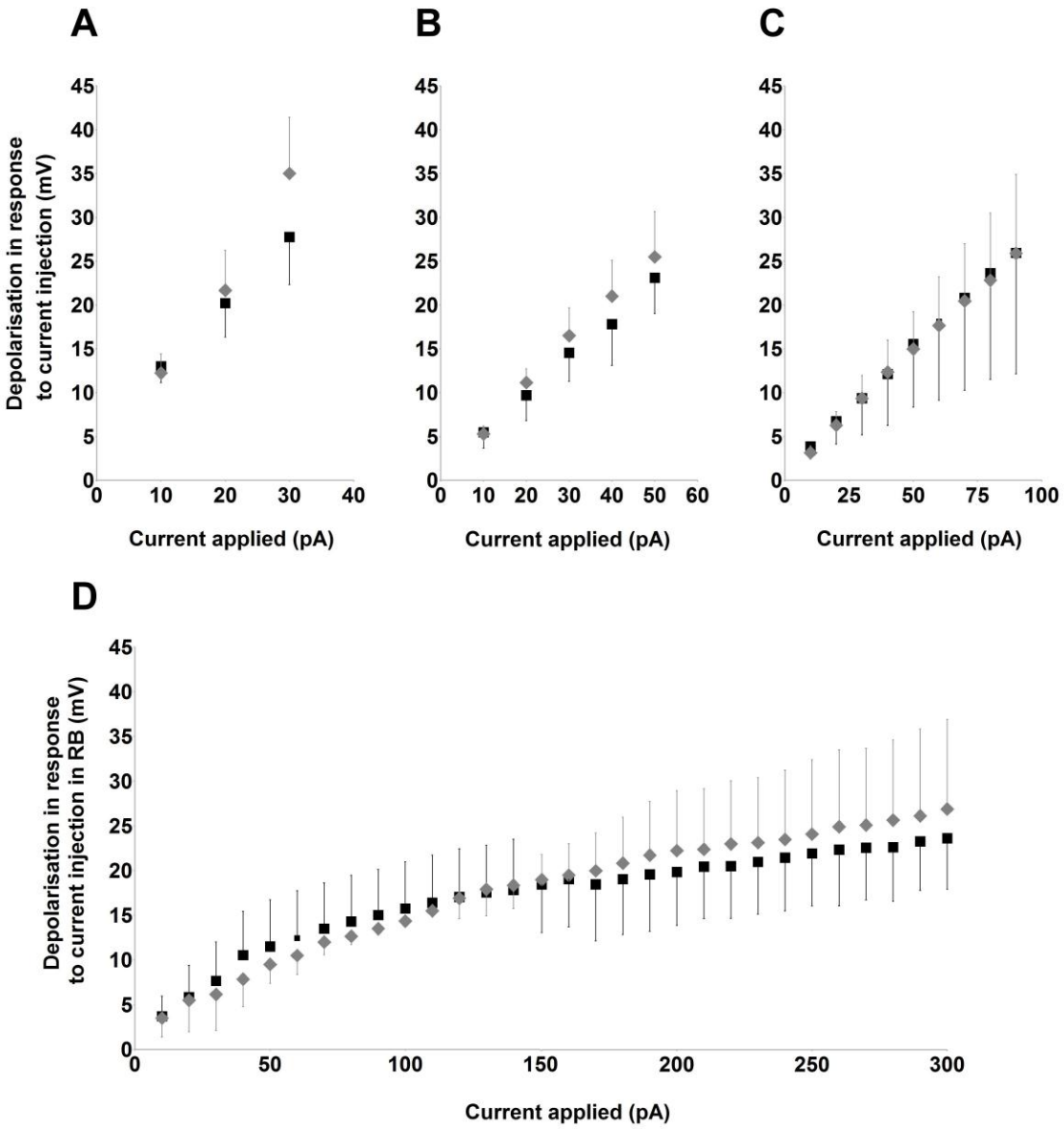

Fig. 20. Relationship between applied current and resulting voltage change (V/I)

The graphs illustrate the relationship between injected current and the resulting change in membrane potential in HA (black squares) and CA neurons (grey diamonds). Depolarisation amplitude was correlated with firing threshold (TH). A-B. In neurons with a firing threshold up to 30 pAand between 30 and 90 CA neurons increased depolarisation amplitude with increasing current steps further than HA neurons. C. No difference was observed in LFNs. D. RB neurons of HA tadpoles, in contrast, depolarised slightly more than RB neurons of control tadpoles in response to current injection up to 100 pA. Differences were not significant, probably due to a low n-number.

The average instantaneous firing frequency was higher in CA neurons than in HA neurons at the same level current injection (see figure 21). When a current of 100 pA was injected CA neurons fired on average at a frequency of 117±41 Hz (n=30) compared to 93±39 Hz in HA neurons (n=27; fig. 21, excerpts I). This difference was reversed when high currents, above 280 pA, where applied. At a current injection of 420 pA CA neurons fired on average with 228±38 Hz (n=8), but HA neurons with 278±33 Hz (n=14; fig. 21, excerpts II). Accordingly the highest frequency measured in control animals (345 Hz) was lower than in HA neurons (384 Hz). Both neurons were HFNs. Moreover at high frequencies HA neurons could maintain spike height better throughout the pulse while CA spikes reduced in amplitude much quicker, when data were pooled (see fig. 21, II). Spike height was decreased by 10 mV (at the end of the current pulse) with an injection of 185±146 pA and at a frequency of 141±70 Hz in CA neurons (n=25), but at 252.4±123 pA (or 189±58 Hz) in HA neurons (n=31; $p<0.05$). On average MFNs of HA neurons ($n_{neurons}=9$) could sustain spike amplitude up to a current pulse amplitude of 266±135 pA and CA neurons ($n_{neurons}=10$) up to injection of 210±143 pA. HFNs reduced spike height (by an amount of 10 mV) quicker: at 221±156 pA in HA neurons ($n_{neurons}=8$) and 139±100 pA in CA neurons ($n_{neurons}=11$).

These data indicate that HA neurons fire normally at lower frequencies than CA neurons, when receiving identical synaptic input. However, when the input is very high HA neurons can maintain firing better than CA neurons, suggesting a change in Na^+ channel inactivation properties, as a decrease in spike amplitude is most likely explained by Na^+ channel inactivation.

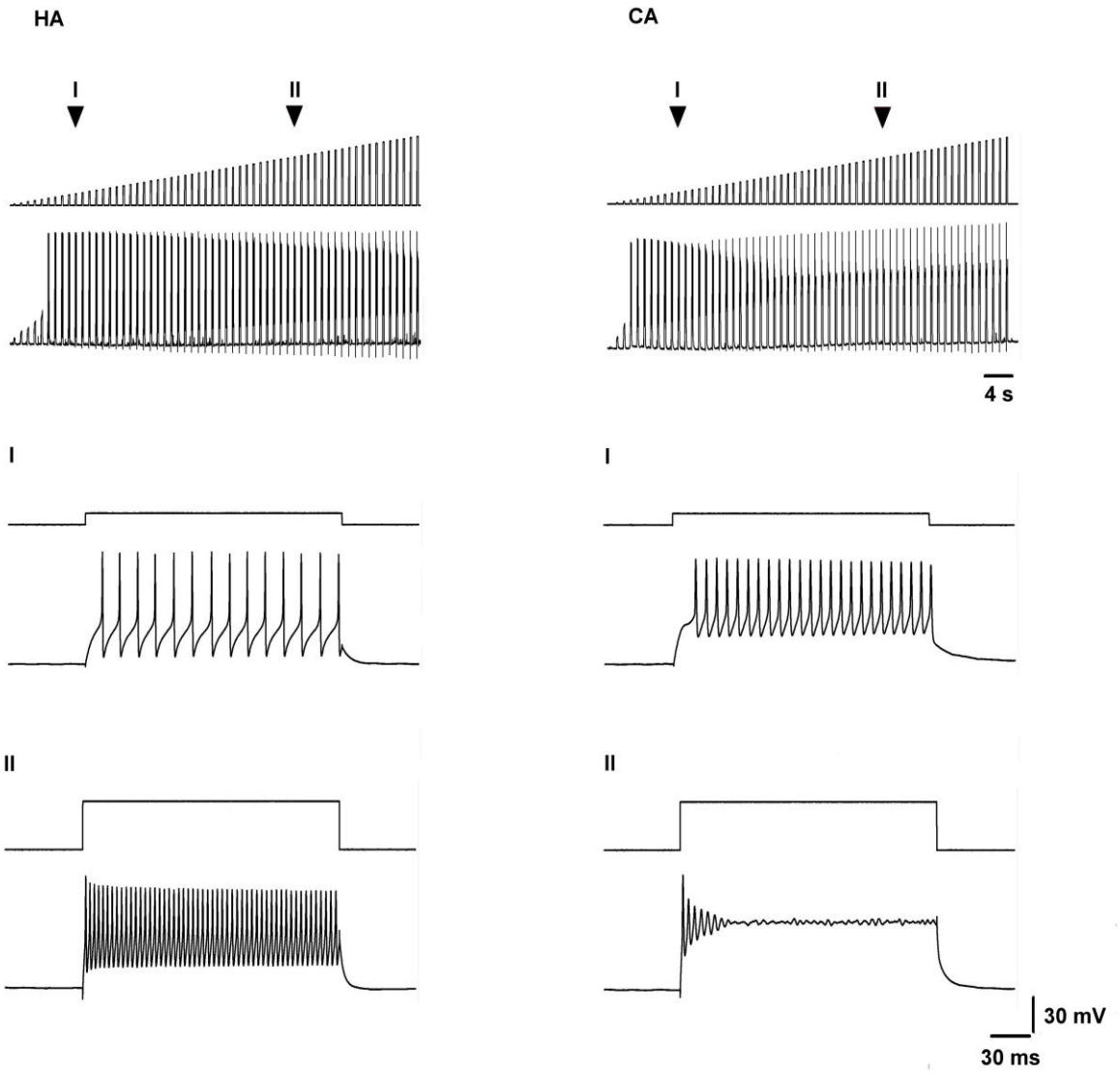

Fig. 21. CA neurons were more excitable but HA neurons could sustain firing better at high current levels

Responses of representative neurons (lower traces) of agitated tadpoles (HA) and control neurons (CA) to a series of current pulses injected via the recording patch pipette (upper traces). At 100 pA (excerpts I) control neurons showed higher instantaneous firing frequencies than HA neurons. When the current was increased (here 420 pA) HA neurons fired usually with higher frequencies, while CA neurons decrease spike amplitude and frequency could stop before the end of the 200ms current pulse.

To gain further insights the relationship between the highest instantaneous frequency and the current intensity was plotted (see figure 22). The first section of the graph approached more a linear relationship for HA (R^2=0.98) than CA neurons (R^2=0.95). To make analysis comparable both were fitted by logarithmic functions (R^2=0.95, $n_{CA\text{-}neurons}$=6-32, $n_{HA\text{-}neurons}$=4-39, see appendix for respective n-numbers, fig. 22B). The y-intercept of the F/I relationship was lower in HA neurons (-261 versus -192 in CA neurons). Hence the plot was shifted to the right, consistent with the higher firing threshold. Moreover the logarithmic coefficient was increased (81 in HA neurons versus 71 in CA neurons), implying a reduction of the slope of the rising phase. Consequently HA neurons increased their firing rate later and more slowly than CA neurons. However at frequencies above 280 pA HA neurons could still increase firing rate further, while CA neurons appeared to reach a plateau ($n_{CA\text{-}neurons}$=22-7, $n_{HA\text{-}neurons}$=24-7, fig. 22C). Graphs were fitted by linear regression lines (R^2=0.91 in HA neurons, R^2=0.61 in CA neurons). The gradient was much steeper in HA neurons (0.35) than in CA neurons (0.08). I concluded from these data that excitability was reduced in HA neurons, as they required more current injection for activity and increased firing intensity slower than CA neurons.

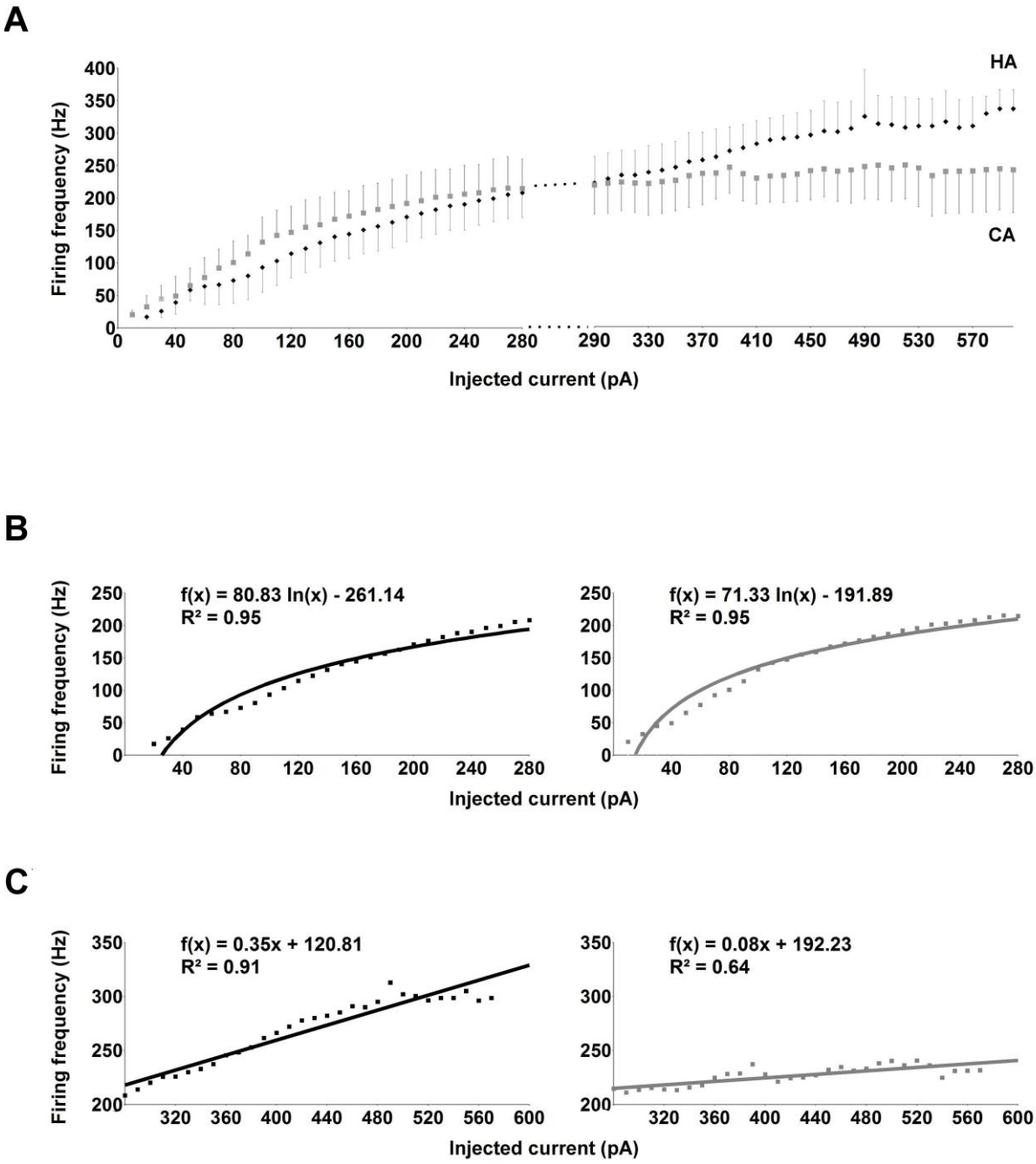

Fig. 22. Relationship between sustained firing rates and applied current (F/I)

A. The first graph displays the average firing rate as a function of injected current in 39 HA and 32 CA neurons. In the beginning CA neurons increased frequency more quickly, but then reached a plateau, while HA neurons were able of increasing frequency further. The 2 graphs crossed at 280 pA current. B-C. For theoretical purposes F(I) was assumed to have first a logarithmic (B) and then (after intersection) a linear form (C). The logarithmic function showed a decreased y-intercept and an increased coefficient for HA neurons. The linear functions revealed an increase of slope in F/I relationship of HA neurons between currents of 280pA and 600pA.

II.3. The input resistance at rest was lower in HA neurons

Increased activity during development was accompanied by a decrease in input resistance (see figure 23). In HFNs of HA animals ($n_{neurons}$=10) input resistance (IR) was 630±382 mΩ at rest and in HFNs of control ($n_{neurons}$=10) 2060±1337 mΩ ($p<0.05$, fig.21 A,B). Only two HFNs were measured in LA animals (400 mΩ). The average IR in MFNs was again lower in HA neurons (659±278 mΩ; $n_{neurons}$=13) than in CA neurons (1413±1143 mΩ, $n_{neurons}$=14) and LA neurons (1583±943 mΩ, $n_{neurons}$=6, fig.23 A). The same tendency was found in RB neurons (see figure 24): in HA animals IR was measured at 267±293 mΩ ($n_{neurons}$=4), but at 452±270 mΩ ($n_{neurons}$=3) in control and 750±212 mΩ ($n_{neurons}$=2) in previously immobilized animals. In LFNs the measured IR in HA neurons ($n_{neurons}$=5) was with 388±161 mΩ close to the control level (400 mΩ, $n_{neurons}$=4, fig. 23 A). The IR is the reverse of conductance. Hence increased activity during development (HA group) resulted in much higher conductances in HFNs, MFNs and RB neurons. IR was not measured during activity.

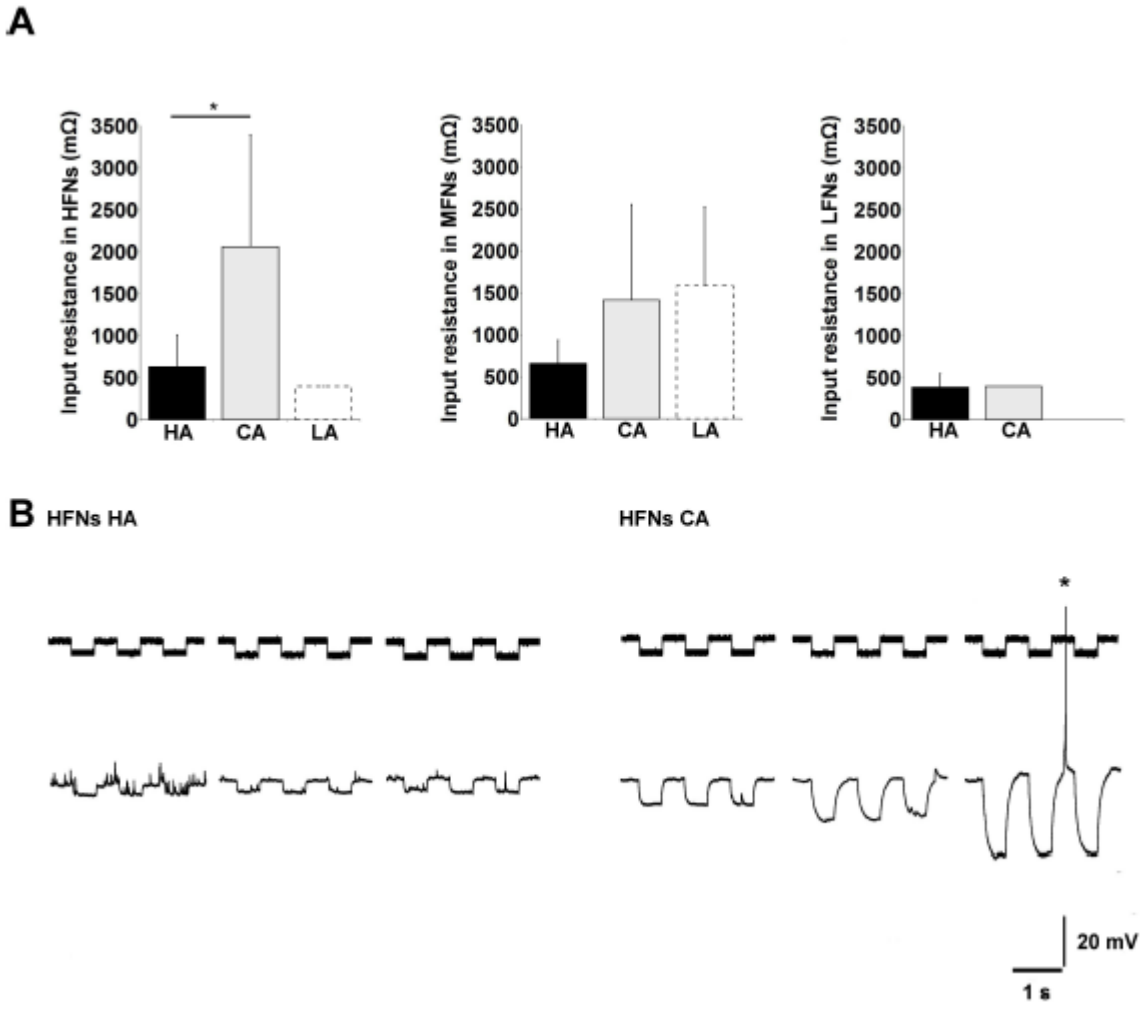

Fig. 23. The IR in HA neurons was reduced, especially in HFNs

A. In HFNs the input resistance of HA neurons was significantly lower than in CA neurons. The same was true for MFNs and LFNs, but differences in these neurons were not significant. B. Negative current pulses of -10 pA (upper traces) and the responses (lower traces) are displayed of three representative HFNs of agitated (HA) and control (CA) animals, respectively. Note that the change in membrane potential was bigger in HFNs of CA animals than in HFNs of HA animals in response to current injection. Note also the increased occurrence of miniature potentials in neurons of experimental animals (as described above). One CA neuron is firing on rebound (indicated by asterisk) *$P<0.05$.

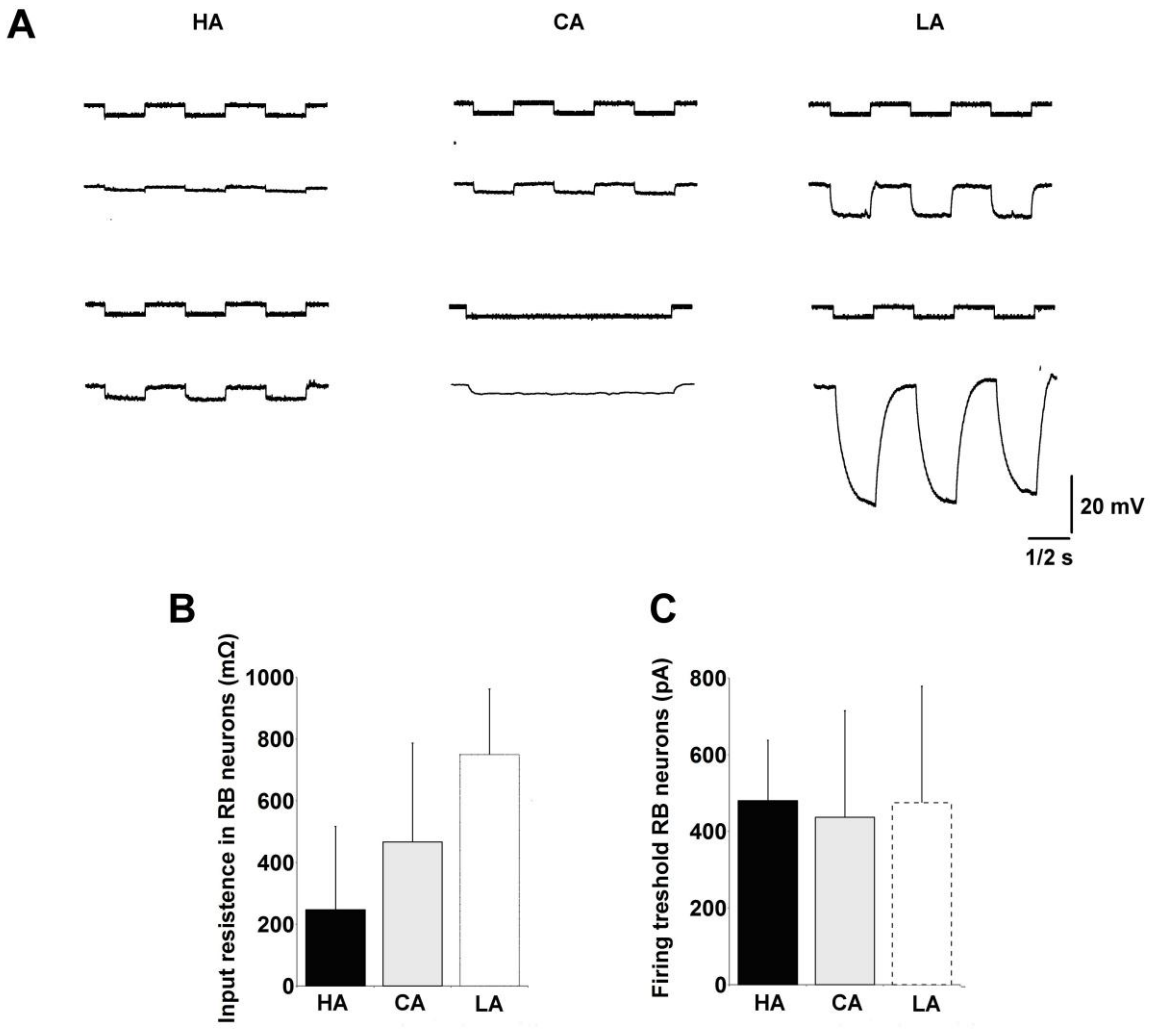

Fig. 24. Membrane conductance and firing threshold in RB neurons

A. The responses of RB neurons (lower traces) to negative current injections (upper traces) in the three groups are illustrated. B. The input resistance was highest in LA neurons. Differences were not significant due to small amount of data. C. The firing threshold was slightly increased in LA and HA neurons, however the n-number is too small to draw firm conclusions.

Although not many RB neurons have been measured it was striking that the conductance in these neurons was much reduced in LA tadpoles. Figure 24 A illustrates the difference in input resistance in LA compared to HA and CA neurons. The firing threshold in RB neurons was slightly higher in LA (475±304 pA; $n_{neurons}$=2) and HA neurons (480±158 pA, $n_{neurons}$=5) compared to control (437±278 pA, $n_{neurons}$=3; fig. 24 C).

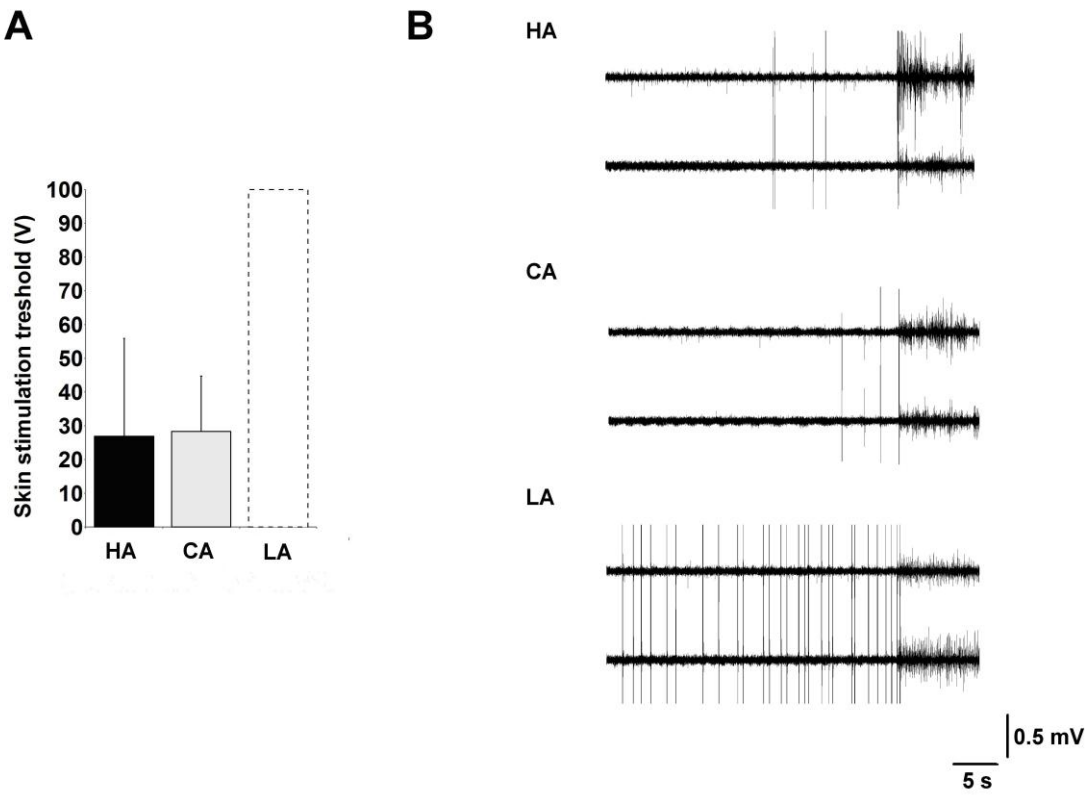

Fig. 25. LA tadpoles required repeated and strong skin stimulation in order to induce swimming

Fictive swimming in CA and HA animals could usually be induced with a single skin stimulus of ~30 V (A) (B, HA and CA). In contrast, tadpoles which had been incubated in MS-222 during development required a repeated tail stimulus at the maximum stimulus output amplitude (100mV). However, once initiated fictive swimming was relatively normal.

An alteration of the electrical properties of RB sensory neurons in the LA group seemed likely because the skin stimulation threshold was much higher compared to both other groups (see figure 25). A very high stimulus (100 V) had to be applied to induce swimming in LA animals. Moreover, the stimulus usually had to be given repeatedly (fig. 25 B). In HA or CA animals swimming could often be induced with a stimulus of 10 V. Only rarely was the threshold found to be 30 V. In free swimming experiments LA animals required repeated skin strokes before they would start to swim (no quantitative data). The stimulus voltage is highly dependent on nonphysiological factors like, for instance, the tip diameter of the electrode or the strength of the batteries of the Digitimer.

Table 3. Action potential properties are not significantly affected

AP peak

HFNs		n	MFNs		n	LFNs		n	RBs		n
HA	27±9	9	HA	28±15.3	12	HA	32±16.3	5	HA	50±8.8	5
CA	36±7.5	9	CA	31±6.6	14	CA	30±10.7	4	CA	45±13.8	3
LA	30	1	LA	29±12.3	6	LA	nm	nm	LA	56±7.8	2

AP width

HFNs		n	MFNs		n	LFNs		n	RBs		n
HA	1.03±0.3	9	HA	1.59±0.5	12	HA	1.26±0.75	5	HA	0.754±0.32	5
CA	1.29±0.45	10	CA	1.14±0.3	14	CA	0.64±0.09	4	CA	0.95±0.07	3
LA	0.96	1	LA	0.96±0.23	6	LA	nm	nm	LA	0.79±0.4	2

**nm: not measured*

Nevertheless within and between experiments the threshold level was relatively constant, but clearly much higher in LA tadpoles than in CA and HA tadpoles. In all animals it was possible to induce swimming by light dimming.

II.4. The form of APs remained mainly unaltered

For each neuron the height and duration at half amplitude of two spikes was measured at threshold in response to depolarising current steps. Spikes had amplitudes of up to 94 mV in HFNs, 91 mV in MFNs and 100mV in LFNs. In RB neurons APs were by far the largest with an amplitude of up to 120 mV ($p<0.01$). The average spike amplitudes were not significantly different in the three groups ($p>0.05$; see fig. 26), although in HA neurons spike height was slightly decreased in HFNs, MFNs and RB neurons.

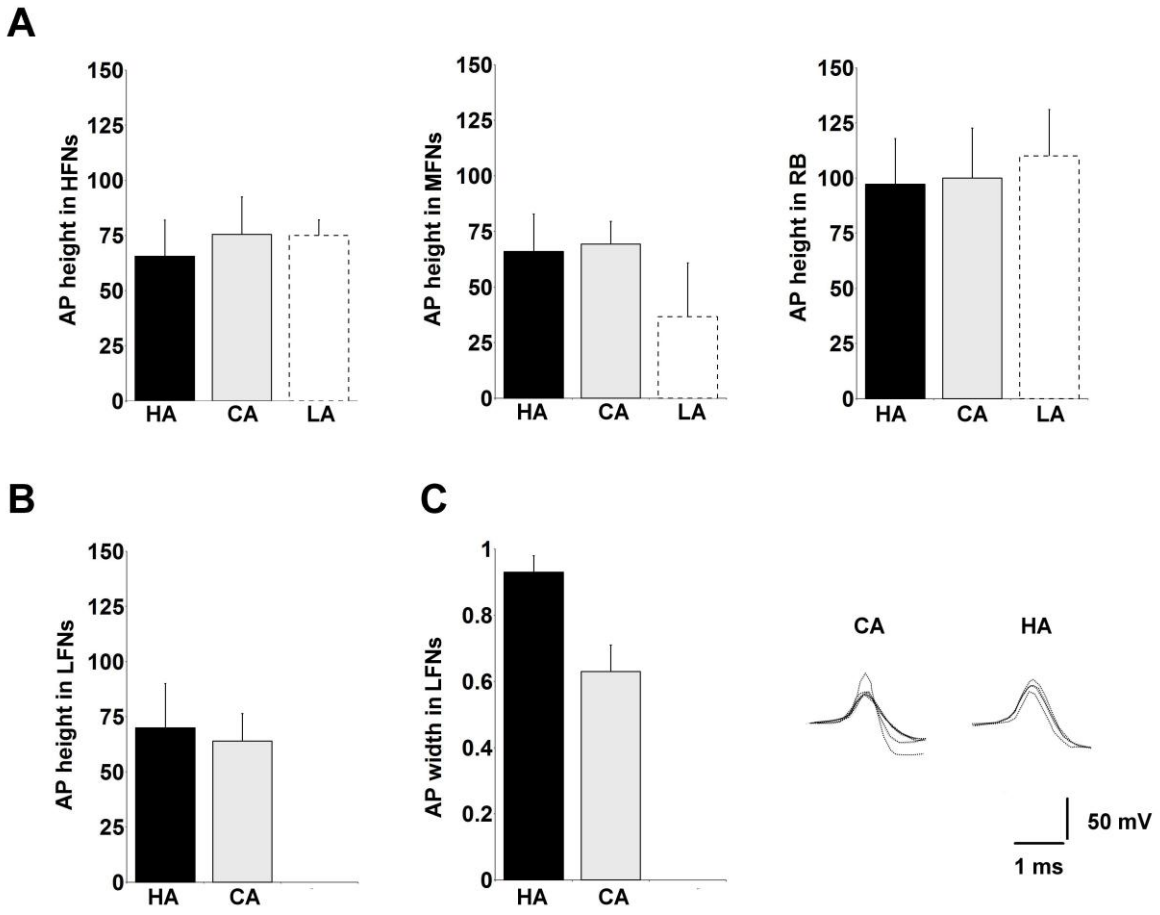

Fig. 26. Spike height and width was slightly increased in HA neurons.

A-B. Spike height was around 60-75 mV in HFNs, MFNs and LFNs and about 90-110 mV in Rohon-Beard (RB) neurons. APs were slightly larger in the control group, however, differences were minor and not significant ($p>0.05$). Only in LFNs AP height was increased in HA neurons. C. That was accompanied by a significant ($p<0.001$) increase in AP width.

In contrast LFNs of HA neurons were larger than those of CA neuron, accompanied by a significant ($p<0.01$) increase of AP width in LFNs of the HA group (1.26±0.7 ms; $n_{neurons}=4$) compared to LFNs of the control group (0.63±0.08 ms; $n_{neurons}=4$). In the other neuron types the AP duration was similar between groups. AP widths ranged from 0.58 to 1.92 ms in HFNs, from 0.48 to 2.21 ms in MFNs, from 0.55 to 2.6 ms in LFNs and from 0.45 to 1.2 ms in RB neurons (table 3).

UsAHPs occurred after high frequency firing in neurons of all groups. The usAHP derives from an increase in Na^+/K^+ pump activity and accumulates during swimming episodes (Zhang and Sillar, 2012). The usAHP amplitude depends on the number of APs fired during swimming or current injection. Following continuous firing induced by supra-threshold current injections a third (9/27) CA neurons showed usAHPs, with amplitudes ranging from 1 to 10 mV (5.9±3.5 mV). In HA neurons post-current injection usAHPs occurred in a similar proportion (30%; 4/13 neurons with amplitudes of 3-12.5 mV (6.5±5.2 mV). Furthermore 3/22 CA neurons displayed a postswim usAHP (2±1 mV) and 6/18 HA neurons (2.9±1.2 mV). Hence usAHP probability or amplitude was not apparently altered in HA neurons.

Discussion

The goal of this study was to determine whether locomotor activity during development influences the morphology and behaviour of *Xenopus* larvae. Two different approaches have been taken in the experimental groups: i) activity has been suppressed pharmacologically (LA), by application of a sodium channel blocking local anaesthetic, MS-222; and ii), activity has been increased by raising tadpoles in a rotating water column (HA). The latter has the advantage that there can be no unknown drug side-effects and that natural activity patterns occur. The treatments began at pre-motile stages of the animals in order to maximize possible effects; later in development neuronal circuits may become more robust, less variable and hence less manipulable.

Control animals were reared under normal laboratory conditions at room temperature and consequently the probability of them swimming was very low (Jamieson and Roberts, 2000). The control situation mimics best the normal environmental conditions, as the natural habitats of *Xenopus* tadpoles are ponds. However, this study also showed that *Xenopus* tadpoles can survive under turbulent environmental conditions during a time when their locomotor apparatus first starts to function. Moreover, their swimming behaviour adapts to the prevailing conditions. Sensory and locomotor networks are changed in a way that the final larval motor output is dramatically different in the different tadpole groups. One has to remember that cellular, network and behavioural changes will likely depend on the way activity was induced and that it is possible that that the results would have been different, maybe even opposing, if a different protocol had been applied, for example if tadpoles were poked frequently.

Surprisingly small alterations were observed when animals were deprived of activity, indicating that development up to the larval stage (stage 42) does not necessarily require neuronal activity to construct a fully functional and near-normal locomotor system. It has been reported previously that functional, although altered, CPGs can develop in spite of disruption of motor activity (Milner and Landmesser, 1999) or sensory input (Suster and Bate, 2002). However, early embryonic movements and neuronal activity are assumed to help in fine-tuning the connectivity patterns within the network.

I. The influence of activity on development in *Xenopus* larvae

Although siblings, and raised together under identical laboratory conditions, some tadpoles of a given batch can always be observed to develop faster than others. That might of course be because of differences in the time point of fertilization. However, even when animals were collected at the same stage of development (stage 21), some of those were farther in development than others later on. That might not solely be due to genetic factors. The results of this study suggest that the rate of development is linked to the level of activity in *Xenopus* tadpoles: when swimming activity is increased development accelerates, and the opposite was the case when neuronal activity was suppressed. The further development proceeded the more frequent became episodes of spontaneous swimming and the greater became the differences in rate of development. During free-swimming stages (stage 32-42), quicker development of HA tadpoles became most pronounced, supporting the assumption that development was indeed accelerated by increased swimming activity and not by other factors. Accelerated development might be advantageous: the higher the stages of development are reached the more mature motor and sensory systems will be and the better equipped the animals will be to handle the turbulent environmental conditions.

Immobilized animals initially developed at the same rate as control animals and it was not until later embryonic stages (37/38) that it became apparent that development was delayed. The more advanced the developmental stage, the more retarded was the development in LA animals up until larval stage 42.

Acceleration or deceleration of tadpole development has been reported frequently. Temperature, for instance, has a big impact as tadpoles are ectothermic. In warmer water their body temperature increases, their metabolism accelerates and they develop faster. This could also be linked to the syndrome in which reduced water levels signal a drying pond and the organismal response is to accelerate through metamorphosis and hence prevent desiccation (Loman, 1999). As activity influences metabolism it is also likely to have an impact on development. The consumption of the yolk sac is is a measure of metabolic rate. This decreases in size earlier in HA tadpoles, due to the accelerated development.

It cannot be excluded, however, that factors other than activity influenced the developmental rate in the experimental animals. Although the animal density was virtually identical, it is possible that HA tadpoles were somehow more aware of the presence of their conspecifics. For example, the higher the motility the more likely becomes tactile contact between animals. An influence of conspecific density on development has for instance been reported in *Rana* (Wilbur, 1972 and 1976). In addition, the environmental conditions could have influenced the ageing of the tadpoles. Generally alterations in the rates of growth and development depend upon the activity of endocrine systems. For instance it has been commonly reported that metamorphosis is accelerated by environmental changes which induce an increase in the level of stress hormones. Release of corticotropin-releasing hormone (CRH) induces elevation of the plasma concentration of circulating corticosteroids. The metabolic rate increases and stored energy is mobilized (“fight or flight” response).

Respiration and heart rate increases and energy is redirected to locomotory structures (Beckerman et al., 2007; Slos and Stoks, 2008; Sapolsky 2002). Interestingly glucocorticoids are further known to influence development of organ systems and even to affect learning and memory processes. It has not been studied so far whether the stress hormone system is already functional in *Xenopus* embryos and larvae. However glucocorticoid receptor gene expression, for instance, is regulated from developmental stage 32 on (Gao et al., 1994). It could bring further insights to compare the heart rate between the three groups.

On the neuronal level it is well-established that proliferation, differentiation and migration depend on activity, both in the embryonic and in the mature nervous system (reviewed in Mennerick and Zorumski, 2000; Zito and Svoboda, 2002; Goda and Davis, 2003). Activity stimulates outgrowth of neuronal process, refinement and growth of synapses and neuronal survival. Growth factors are expressed in an activity-dependent way in *Xenopus* (Wang and Poo, 1997). HA stage 42 larvae are about a day younger in age compared with equivalent stage LA larvae. Nevertheless the output generated by the locomotor system in both groups was similar to that typically seen in control larvae. This indicates that the neuronal circuits controlling locomotion developed quicker when motor activity was increased. Moreover it might be suggested that electrical signals deriving from the nervous system stimulate other tissues, for example muscle cells, to accelerate growth and development. Besides, it should be borne in mind that not only the motor system of HA animals was more active, but also their sensory and visual systems.

II. The influence of activity on locomotor output in *Xenopus* larvae

When developing under normal conditions, in still water, tadpoles are rarely observed to swim spontaneously. If they receive tactile or photic stimuli they can escape by swimming away until they contact an obstacle and become attached to it by their cement gland. HA animals were raised in turbulent water. Several aspects of locomotor output were altered in parallel including swimming episode duration, bending amplitude, horizontal swimming speed, alignment in the water and the degree of turning manoeuvres. Why these behavioural changes turn out to be an suitable adaptation to the conditions will be discussed below. In contrast the chance of survival of HA tadpoles in their natural habitat could be reduced if their increased movements attract the attention of predators and if they did not react with a typical flight response when perceiving their presence. It is interesting that these basic and highly conserved mechanisms are not genetically determined but can adapt to the respective environmental requirements. The adaptation of locomotion was maintained long after the treatment was terminated. Thus I assume that motor and sensory systems were undergoing long-term plasticity changes during development.

On the rotator HA tadpoles were swimming against the water currents, which they perhaps detected with their lateral line organs (Scharrer, 1932; Gorner, 1963). The number of receptors increases from embryonic to larval stages (Roberts et al., 2009). The higher the developmental stage the tadpoles had reached, the higher was the proportion of tadpoles actually swimming on the rotator. They usually did not show sensitization towards the stimuli, presumably because with every turnover the resulting water currents were different. If quiescent animals bounced against the tube wall such stimuli could generate cardiac-like skin impulses which excite the CNS and evoke swimming in embryos (Roberts, 1969; James and Soffe, 2011).

During development this skin-impulse is gradually lost, but tactile stimulation then excites the RB neurite network underneath the skin which transfer the signal to descending sensory interneurons and eventually excite the CPG. Hence, as HA tadpoles constantly received sensory stimuli they maintained swimming throughout the treatment.

A reduction in swimming efficiency could be advantageous for HA tadpoles as they were kept in small tubes; if they were to swim very fast they would have bounced more often against the wall, or they would have to decelerate and accelerate in speed within a very short time period. I suppose that changes in swimming mode resulted in altered kinematics of the animals in the water. However an in-depth analysis would go beyond the scope of this thesis. Here I attempt to find explanations for the reduction of horizontal speed. Maybe the higher bending amplitude increased water turbulence and therefore the drag on the animal. Additionally the tilted orientation of the animal in the water might increase the angle of attack of water streams and counter thrust. Besides it is possible that turning increased the drag, as the water flow opposes the movement direction of the animal.

As mentioned above the bending amplitude was increased in HA tadpoles. This could, in principle, be caused by longer burst durations which can be detected in VR recordings. However, motor bursts did not appear different between groups. Hence the systems affected might not have been measured or blocked in this preparation: a possible candidate is the NMJ. In VR recordings the output of motoneurons is measured, but not the incoming signal at the muscle. Moreover cholinergic signal transmission was blocked by application of α-BT. An indicator for efficiency of signal transmission from motoneurons to muscle cells is the number and distribution of AChRs. Embryonic *Xenopus* muscle cells initially express receptors not only for ACh, but also for glutamate, GABA and glycine. However, AChR expression prevails as maturation progresses (Hall and Sanes, 1993).

Initially these receptors are present at a moderate level throughout the myotube surface with occasional high-density patches. Sensitivity to ACh is a prerequisite for the establishment of nerve-muscle synapses (Katz and Miledi, 1964) and receptor patches are assumed to be potential sites of innervation (Sytkowski, 1973).

Later in development AChRs are highly concentrated at the postsynaptic membrane, and virtually absent extrasynaptically. This clustering involves redistribution of AChR proteins and localized synaptic synthesis of AChRs. The local synthesis results from enhanced transcription of AChR genes by subsynaptic nuclei and by repression of extrasynaptic nuclei (Merlie and Sanes, 1985; Schaeffer et al., 2001). This process is controlled by muscle cell activity (Schuetze and Role, 1987).

AChRs were visualized in larvae muscle cells by staining with α-BT-rhodamine. High density rows of AChRs on the opposite side of the active zones of the motoneurons were stained most intensively in all groups. These rows, which correspond to intermyotomal clefts, appeared most distinct in HA larvae. That might indicate that AChRs are more concentrated at the postsynaptic side in larvae whose muscle activity was dramatically increased throughout development. In agreement with this finding it was observed that HA tadpoles required longer incubation times in α-BT for paralysis, however no quantitative data were collected. It might also be insightful to compare the muscle size between the three groups. In LA larvae, in contrast, staining looked more diffuse. That might indicate a higher percentage of extrasynaptically located AChRs. It has been shown previously that degradation of extrasynaptic AChRs depends on activity, as they disappear when embryonic skeletal muscle activity begins (Diamond and Miledi, 1962; Dennis and Ort, 1977). Accordingly, during normal development, the density of extrajunctional AChRs is highest at stage 36 and then declines to less than 3-fold over the next 10 days of development (Chow and Cohen, 1983).

Moreover their elimination can be delayed when neuromuscular transmission is blocked pharmacologically (Gordon and Vrbova, 1975; Burden, 1977). On the other hand extrajunctional AChRs were eliminated by direct electrical stimulation of denervated adult skeletal muscle (Lomo and Westgaard, 1975).

AChRs were certainly activated more often in HA animals which would be expected to lead to stabilization of receptors, while other receptors are inactivated or removed (Changeux et al., 1976; Rotzler et al., 1991). A better matching of pre- and postsynaptic activity favours more reliable and precise signal transmission and could explain the enhanced bending in HA tadpoles. Although the overall morphology was very similar to control animals it might be that certain systems in HA animals, for example the NMJ, already harboured features which are usually acquired later in development. Moreover it is possible that different transmitter matching has taken place in the three groups. The transmitter phenotype of the NMJ is altered when neuronal activity is perturbed (Borodinsky and Spitzer, 2007): when activity was suppressed extrajunctional expression of AChRs increased, at the expense of junctional AChR expression. That might be the case in LA tadpoles. However, enhanced activity caused a decrease in AChRs and an increase in the level of inhibitory transmitters. Plasticity was induced by pre-synaptic signals through enhancement of neuronal calcium spike activity in spinal neurons (Borodinsky and Spitzer, 2007). In HA tadpoles muscle activity increased, but an increase of spinal neuron activity need not automatically follow (discussed below). Maybe refinement of transmitter matching was induced by a feedback loop from the muscle cells. However more data have to be collected and quantified to confirm this hypothesis.

Activity also plays a role in regulating electrical coupling of muscle cells during development. Electrical coupling is widespread until the embryos begin sustained and frequent swimming at about stage 36. With increasing locomotor activity electrical coupling of muscle cells becomes reduced. Single muscle cells, however, remain coupled as late as stage 45 (Armstrong et al., 1983). Usually uncoupling of muscle cells is accompanied with cell fusion. However, in *Xenopus* myotomes the muscle cells remain mononucleate until stage 45 (Muntz, 1975). If embryos are treated with tricaine or α-BT before the onset of uncoupling, muscle cells remain coupled, indicating that cholinergic activation is required for gap junction elimination. Correspondingly, muscle activity probably stimulates the elimination of gap junctions between developing muscle cells (Armstrong et al., 1983).

Electrical coupling is proposed to coordinate reflex responses before innervation is established. Perhaps due to high activity in HA tadpoles, muscle cells are uncoupled too early and orchestration of muscle differentiation during development is prevented. That might lead to abnormal muscle activity later on, like aimless turning in the absence of environmental cues.

III. The influence of activity on sensory systems in *Xenopus* larvae

As mentioned above the number of turning manoeuvres per swimming episode was dramatically increased in HA tadpoles. In addition it was observed that when CA or HA tadpoles were kept in circular dishes (radius ~ 3 cm) they would perform more loops later on in development. The amplitude and duration of muscle activity on one side, relative to the other, increases with increasing turn angle. Movement-related sensory feedback is probably not required, as turning manoeuvres can be detected in VR recordings when muscle activity is blocked. Which factor/s caused circular swimming in HA tadpoles can only be speculated upon at present.

Turning could be produced by asymmetric descending inputs from the brainstem to the spinal oscillator network. The brain receives and integrates sensory, visual and vestibular input. Accordingly tactile stimulation during swimming results in turning manoeuvres. However, HA tadpoles did not receive tactile input during swimming. Maybe due to increased head and body waves in HA tadpoles, without visual field-stabilizing eye movements (Lambert et al., 2008), they changed swimming direction more often, as they always attempt to swim in gaze-direction. However the eyes become functional quite late in development: phototransduction begins in the *Xenopus* retina at stage 39. The stimulus threshold is initially high and then falls rapidly from stage 39-43 (Witkovsky et al., 1976). It might be possible that the eyes respond earlier to photic stimulation when they receive more input. Later in development visual experience refines for instance the projection of the retina (Cline and Constantine-Paton 1989) and the circuitry in the tectum in *Xenopus* (Pratt et al., 2008). Moreover it is possible that the vestibular system is disturbed in HA tadpoles. Spatial reception requires sensing of environmental cues, which are then integrated and interpreted by the CNS.

Changes of the environment can result in failure to integrate spatial information properly and degrade the animals' ability to move and function properly. For instance an abrupt reduction in gravity results in forward, outside loops in *Xenopus* (Wassersug, 1992). Hence spatial perception or interpretation might be disrupted in HA tadpoles. Maybe they can't perceive differences between right-left and/or below-above, as they developed in a rotating water column.

HA tadpoles appeared to be very sensitive to tactile stimuli. The opposite was the case in LA tadpoles. Here repeated stimuli had to be given to excite both free- and fictive swimming. That could imply that the sensory system is modified under the different experimental conditions. In LA tadpoles the input resistance of RB neurons was increased, indicating a decrease in conductance and therefore a decrease in cell size and/or number of conducting channels. The opposite was the case in RB neurons of HA tadpoles. Hence RB neurons might conduct signals more rapidly in animals which received a lot of prior stimulation compared with animals whose activity was suppressed throughout development. Furthermore, it is conceivable that the maturation of RB neurons is retarded. A delay in outgrowth of neurites might explain the decreased sensibility to light strokes in behavioural experiments. However, the animals should then still be sensitive to noxious pokes, which excite the large growth cones of immature RB neurons (Roberts, 1971; James and Soffe, 2011). The notion that deprivation of sensory input causes disorders in development has been reported in a number of earlier studies. For instance, when *Xenopus* were dark-reared from embryonic stages on the intertectal system was disrupted later in life (Keating et al., 1991).

IV. The influence of activity on spinal neurons in *Xenopus* larvae

In HA tadpoles the output of the locomotor network was of longer duration and higher initial firing frequency. The spinal neurons of HA tadpoles received increased synaptic input; during swimming episodes tonic excitation was increased and during silent periods the frequency of miniature potentials was higher. On the other hand HA neurons were less excitable. The decrease in IR and the increase in firing threshold suggest that increased activity induced changes in cell properties.

IV.1. HA spinal neurons received increased synaptic input

Swimming normally terminates when tadpoles contact an obstacle, but it can also stop spontaneously. Therefore is has been proposed that swimming durations are partly regulated by an intrinsic signalling mechanism. The excitatory drive maintaining swimming activity derives mainly from dINs (Li et al., 2006). DINs generate a tonic depolarisation of CPG neurons by inducing long duration NMDAR-mediated EPSPs which sum from cycle to cycle (Dale and Roberts, 1985). Depolarisation is maintained partly because they are electrically coupled and, moreover, excite each other in a positive feedback network (Li et al., 2004 and 2009).

Application of D-serine, a ligand at the NMDAR glycine binding site, shows similar results to those observed in HA tadpoles: increased episode durations, depolarisation amplitude and spontaneous transmitter release (Issberner and Sillar, 2007; E.R. Björnfors, unpublished data). Moreover, burst amplitudes and frequencies were shown to become more variable, which might increase the likelihood of tuning manoeuvres during free swimming.

The higher depolarization amplitude in HA spinal neurons could indicate an increased input from and/or response to dINs. In turn this could be explained by an increase of the number of dINs, increased input to dINs or a strengthening of their synaptic connections. An increase of firing probability in dINs, however, is not likely, because they fire reliably one spike per cycle and this property persists into stage 42 (H.Y. Zhang, unpublished data). Increased tonic excitation of dINs, along with an increase in phasic inhibition, leads to faster rebound firing and therefore higher cycle frequencies (Li and Moult, 2012), as observed in HA neurons. A drop in frequency, in contrast, can be explained by fewer neurons firing (Sillar and Roberts, 1993). Maybe when starting swimming with a high initial firing frequency long swimming episodes become more likely. However, if this were to be the case the phenomenon is certainly overshadowed by other mechanisms regulating episode durations. Accordingly no correlation was found in stage 42 larvae between episode duration and initial rhythm frequency.

I propose that higher frequencies and prolonged episodes in HA tadpoles might be mediated by increased tonic excitation from dINs and increased phasic inhibition, deriving from aINs and cINs. Maybe CPG neurons receive stronger input due to plastic changes at the synaptic level. Indeed miniature potentials occurred more frequently in HA neurons, than in CA or LA neurons. Spontaneous transmitter release is proposed to play an important role in synaptic plasticity and is often accompanied by growth of new synapses (see, for instance, Kim et al, 2003). The phenomenon can be caused by changes at the pre- or postsynaptic side, or both: for instance by an expansion of vesicle pools, a presynaptic increase of docked vesicles and/or a higher density of postsynaptic receptors. As the mPSP amplitude did not appear different between groups the latter can probably be excluded. I therefore suggest that plastic changes have taken place presynaptically, rather than at the postsynaptically.

On the other hand, spontaneous vesicle release was found to occur at an early stage of long-term facilitation and is supposed to act as an orthograde signal for recruiting the postsynaptic mechanism for intermediate-term facilitation in *Aplysia* (Jin et al., 2012a,b). Hence it might be suggested that HA larvae are found in an intermediate stage of synaptic plasticity and additional postsynaptic changes are about to follow. Moreover the percentage of excitatory mPSPs was not different from the value measured in control neurons, indicating that the increase in spontaneous release was not due to enhanced synaptic input from solely one class of interneuron, for instance dINs, but rather that the entire circuit was more strongly connected in the HA group.

As shown by Zhang and Sillar (2012) episode duration is also influenced by previous network activity: during activity Na^+ enters the neuron and increases the Na^+/K^+ pump activity. Exchange of Na^+ for K^+ results in hyperpolarization of the neuron which can last up to one minute and is therefore called ultraslow hyperpolarization (usAHP). The amplitude and duration of the usAHP depends on preceding neuronal activity, therefore the usAHP encodes a "short-term memory" of network performance.

Hyperpolarized neurons are less likely to be recruited during locomotion. Consequently the excitability of the network is reduced for a period after episode termination when the enhanced pump function hyperpolarizes a proportion of network neurons. However, the long swimming durations in HA tadpoles cannot be explained by a reduction of Na^+/K^+ pump activity. Even when control animals were inactive for long time periods they never showed episode durations as long as those observed in HA tadpoles. Moreover a reduction of excitability after swimming termination was observed in HA tadpoles the same as in control tadpoles. Patch experiments revealed that HA neurons also showed usAHPs, following swimming as well as in response to current injection.

Moreover the amplitude of the usAHPs was increased in HA neurons, probably due to enhanced and prolonged activity during swimming.

Besides, the membrane potential of control and HA neurons was not significantly different. Maybe the usAHP, as a conserved mechanism for short-term memory, is relatively immutable.

A parallel mechanism for regulating swimming episode durations was proposed in 1996 by Dale and Gilday. They demonstrated that rundown of swimming is regulated by the conversion of ATP (released in the CNS) to adenosine, which reduces calcium currents, depresses neuron excitability and leads to a slowing of swim frequency and episode termination. Maybe that mechanism is somehow reduced in HA tadpoles by decreased neuronal excitability.

IV.2. HA spinal neurons were less excitable

Developing neural circuits evolve rapidly into highly specialized networks that process and relay information. This is achieved by means of synaptic and intrinsic plasticity. Intrinsic properties are determined by the number and distribution of ion channels and ultimately control whether synaptic input will lead to an action potential. Stimulation of a neuron often leads to increased IR and a reduced firing threshold hence higher excitability. In neurons of HA tadpoles, however, the opposite was the case. On average HA neurons showed smaller changes in membrane voltage compared with CA neurons when receiving identical input suggesting that the density of ion channels in the membrane of HA cells could be higher. The lower IR in HA neurons could also indicate that cells were larger, and therefore contained more channels.

Perhaps the increased activity stimulated the release of growth factors in HA neurons. Neurotrophin, for instance, is released in an activity-dependent way in *Xenopus* nerve-muscle cultures (Wang and Poo, 1997).

At first glance it might be surprising that increased activity and increased episode duration was accompanied by lower excitability of spinal neurons. However, it has often been reported that increased activity leads to a reduction in receptor density (Ehlers, 2003). This process, called homeostasis (input-output stability) allows neurons to adjust their intrinsic excitability in response to the amount and strength of incoming signals to maintain relatively invariant response properties (Miller, 1996). Neurons of various types are known to use a series of homeostatic mechanisms to maintain their synaptic and intrinsic properties within a stable functional range or to maintain a specific firing pattern (LeMasson et al., 1993; Turrigiano et al., 1994). Homeostatic regulation of intrinsic properties plays a central role in the functional development of neural circuits. Decreasing intrinsic excitability in response to up-regulation of voltage-gated Na^+ currents, as an active homeostatic process, was reported in *Xenopus* tectal cells (Pratt and Aizenman, 2007). At high frequencies HA neurons were found to maintain AP amplitude better than CA neurons which could have been the result of either an increase of the number of Na^+ channels or reduced Na^+ channel inactivation. The decrease in input resistance of HA neurons could also indicate a larger population of K^+ channels mediating faster repolarisation of cells and consequently leading to more rapid de-inactivation of Na^+ channels.

IV.3. The influence of activity on modulatory systems

Episodes can terminate either spontaneously or when the tadpole encounters an object. It might be argued that, due to the circuitous trajectory of the HA tadpoles, they were less likely to reach the sidewall of the chamber and therefore swimming termination was less likely. However this was not the reason. During VR recordings in immobilized tadpoles, when not receiving sensory signals, differences in swimming episode durations between groups were even greater. During free swimming HA tadpoles were often observed to swim into obstacles but still continued to swim. In fact it was hardly possible to stop swimming by contacting their cement gland, for instance with a hair. Apparently HA tadpoles received so much excitatory drive that the stopping response was unable to overcome it. On the other hand it is possible that the GABAergic inhibition from brainstem IR was reduced in HA tadpoles or that the normal developmental degradation of the cement gland occurred prematurely in HA tadpoles.

Episode duration and swimming frequency is modulated by serotonergic neurons. An increase in the incidence of serotonergic neurons has a boosting effect on motoneurons and causes increased swimming frequencies, as observed in HA tadpoles. However that effect is accompanied by accumulation of intracellular Ca^{++} which activates Ca^{++} sensitive K^{+}-channels and thereby triggers a swimming terminating cascade (Dale and Kuenzi, 1997; Wedderburn and Sillar, 1994; Demarque and Spitzer, 2010). Hence it seems unlikely that the number of serotonergic neurons is increased in HA tadpoles.

V. Summary

When exposing tadpoles to frequent stimulation during development they respond with prolonged swimming activity. Their nervous system adapts to this condition and thereafter long duration swimming is maintained, even after turbulent conditions are stopped. I suggest that CPG neurons receive increased synaptic excitation from dINs. More synaptic input from dINs, for example due to an increased dIN population or enhanced transmitter release, would result in stronger excitation of cINs and aINs and motoneurons. However, their excitability is reduced, perhaps due to a compensatory homeostatic process; as synaptic drive increases, intrinsic excitability begins to decrease. Hence enhanced excitatory presynaptic inputs do not implicitly lead to more action potentials and as a result neurons will be protected from over-excitation, causing for instance depletion of vesicle pools, saturation and desensitization of receptors, excessive calcium entry and ultimately cell death. Whether or not the excitability of dINs was decreased in HA tadpoles cannot be determined at present, as only one dIN was recorded. All in all, due to persistent input, activity is maintained, allowing for long swimming, but not all cells are induced to fire more on each cycle, so that over-excitation of the system is prevented.

A reduction in the intrinsic excitability of motoneurons could reduce the strength of synaptic output and stimulate an upregulation of postsynaptic AChRs at the NMJ. On the other hand, the receptors at the NMJ constantly receive input, due to almost permanent swimming activity, which might facilitate the stabilization of receptors at the synapse and degradation of extrajunctional receptors. These incoming signals, however, might not be sufficiently strong to initiate a down regulation cascade of receptor density at the muscle cell, which would be the case when cells were overexcited.

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Appendix

Appendix 1. Instantaneous firing frequency (Hz) in response to current injection (10-400 pA) in CA neurons

	Injected current (pA)																																								
	10	**20**	**30**	**40**	**50**	**60**	**70**	**80**	**90**	**100**	**110**	**120**	**130**	**140**	**150**	**160**	**170**	**180**	**190**	**200**	**210**	**220**	**230**	**240**	**250**	**260**	**270**	**280**	**290**	**300**	**310**	**320**	**330**	**340**	**350**	**360**	**370**	**380**	**390**	**400**
Neuron																																								
1	18	36	50	61	77	83	103	115	116	130	133	137	145	152	159	167	161	169	169	175																				
2				24	50	73	95	109	120	136	153	167	161	174	143	186	198	195	207	217	217	226	223	238																
3			18	29	49	78	104	122	149	172	185	204	200	213	217	217	217	227	233	227	238	244	238	250	250	244	256	250	256	256	263	263	263	270	270	270	270	278	278	278
4		9	40	45	70	89	104	125	139	146	153	169	180	195	198	208	213	222	225	232	238	238	240	245	253	253	259	260	260	261	261	266	272	274	274	278	282	283		
5		10	18	29	49	77	104	123	149	172	183	200	200	216	217	218	204	233	228	235	240	238	246	243	245	251	247	251	254	259	256	260	262	256	260	265	270	274	272	272
6		34	64	85	104	124	138	145	153	158	164	169	172	182	184	187	191	191	188	198	198	207	205	209	209	212	210													
7										20	37	47	59	75	81	87	109	119	128	136	143	156	158	164	166	171	176	195	203	201	198	210	210	215	222	220	227	236	235	238
8					44	97	111	123	139	156	167	169	179	192	200	213	208	217	222	227	238																				
9						16	42	83	105	145	156	175	196	200	217	227	233	244	250	263	263	270	270	270	286	286	294	303	294													
10	23	43	62	81	100	119	128	147	154	161	172	175	189	200	204	204	200	208	208	213	217	217	217	222	227	233	233	238	222	233	244	233	238	244	244	238	238	238	256	270
11			21	50	58	83	93	109	119	122	130	133	137	141	139	154	154	154	164	167	175	175	182	189	192	196	204	222	227	222	222	233	233	233	233	233	238	238	244	250
12	20	31	45	51	63	70	79	81	88	93	94	101	110	109	112	119	119	123	130	125	132	135	139	139	137	139	143	141	145	145	149	147	149	147	156	156				
13		60	83	104	114	130	137	141	145	149	152	154	164	169	169	172	179	175	189	185	185	192	204	196	204	200	192	204	217	200	208	217	200	200	192	200	208	213	204	
14		16	34	47	69	84	95	111	114	122	122	130	133	141	139	141	156	154	152	156	152																			
15				43	52	68	74	87	94	103	110	119	122	128	135	143	154	152	159	159	167	172	179	179	185	185	192	196	196	200	204	208	208	208	213	213	217	217	217	227
16					13	36	65	90	110	123	128	145	152	164	164	172	175	185	192	192	200	204	213	213	208	217	217	222	227	238	244	233	238									
17						47	55	58	78	85	89	102	115	123	125	133	143	149	156	161	169	167	175	182	189	189	196	196	196	213	200	208	217	213	222	227	233	227	227	233
18						35	62	85	97	97	109	116	120	133	135	141	147	156	154	167	172	175	185	185	185	196	192	192	204	204	208	208	217	213	222	222	217	233	222	238		
19																																								
20		19	34	46	63	75	88	94	106	115	125	135	143	152	156	164	169	182	185	189	189	200	204	208	213	217	222	217	222	227	233	233	233	250	250	250	256	256	263	263
21	11	32	56	81	96	115	132	10	154	169	182	189	196	204	213	222	233	227	238	250	256	263	256	256	263	263	278	278												
22			37	57	65	74	80	104	109	114	130	135	145	152	149	161	167																							
23					25	35	49	56	68	81	84	94	93	97	109	115	116	109	118	123	125	128	120	123	128	122	128	135	137	128	130	133	128	125	122	135	116	127	179	189
24					44	65	79	78	85	101	83	120	81	74	90	109	122	114	143	145	139	139																		
25														58	88	106	125	111	13	135	140.8	144	153	163	169	178	181.8	172	178.8	192	185.1	188	204	227.2	227.2	238	244	243.9	250	250.0
26					16	28	67	86	77	114	123	127	134	147	145	164	161																								
27														19	30	43	58	74	81	105	119	139	142	152	156	185	200	208	217	227	233	238	243	263	256	270				
28			14	21	27	27	34	40	44	51	58	63	59	74	74	91	88	93	100	98	109	116	126	126	136	143	145	155	161	170	178	181	178	181	196	188	104	204	222	217
29					62	70	95	103	123	137	143	159	164	169	179	189	192	196	196	200	208	217	222	222	227	222	238														
30	20	41	59	69	85	99	111	120	130	135	143	149	154	159	167	169	175	182	182	189	192	192	196	200	200	213														
31			22	26	35	44	42	51	61	62	68	65	78	83	83	89	94	98	101	104	106	108	112	118	119	120	122	128	130	132	132	137	141	139	141	143	147	147	147	152
32	31	60	74	99	111	128	135	152	159	169	175	185	189	196	208	213	222	222	227	227	238	238	244	244	244	250	256	250	256	256	263	256								
33									15	41	51	80	76	95	103	116	120	115	130	137	149	152	161	161	172	179	175	192	185	192	196	208	208	204	222	213	213	222	233	238
34	10	20	30	40	50	60	70	80	90	100	110	120	130	140	150	160	170	180	190	200	210	220	230	240	250	260	270	280	290	300	310	320	330	340	350	360	370	380	390	400

Appendix 2. Instantaneous firing frequency (Hz) in response to current injection (410-800pA) in CA neurons

Injected current (pA)

Neuron	410	420	430	440	450	460	470	480	490	500	510	520	530	540	550	560	570	580	590	600
1																				
2																				
3	278																			
4																				
5																				
6																				
7	250	246	251	253	254	265	267	264	274	279	279	280	285							
8																				
9																				
10																				
11	244	244	244	250	244	250	256	256	250	263	263	256	256	270						
12																				
13																				
14																				
15	227	227	227	227	227	238	238	238	244	244	250	244	250	256	256	263	263	263	270	270
16																				
17	233	238	244	244	244	250	250													
18																				
19																				
20	256	256	263	263	278	278	278	278	286	286	294	286	294	303	294	303	303	303	303	303
21																				
22																				
23	179	182	185	182	182	189	182	185	182	189	189	182	189	182	182	189	192	185	189	185
24																				
25	263.1	270	270.2	270	277.7	285	294	295	303	394	294	303.0	312.5	322	303	303	322	312	322	322
26																				
27																				
28	217	227	232	227	222	232	232	232	238	250	243	250	243	250	243	250	256	256	263	270
29																				
30																				
31	156	154	156	156	161	156	172	167	164	169	167	169	169	169	167	169	167	175	172	182
32																				
33	233	244	238	238	244	250	244	250	256	250	256	256	270	270	278	270	278	278	286	286
34	410	420	430	440	450	460	470	480	490	500	510	520	530	540	550	560	570	580	590	600

Neuron	610	620	630	640	650	660	670	680	690	700	710	720	730	740	750	760	770	780	790	800
1																				
2																				
3																				
4																				
5																				
6																				
7																				
8																				
9																				
10																				
11																				
12																				
13																				
14																				
15	256	256	256	256	263	270	270	270	270	270	270	270	270	278	278	278	294	286	294	294
16																				
17																				
18																				
19																				
20	313	313	313	323	313	313	323	323	333	323	333	333	333	333	333	345	345	333	345	
21																				
22																				
23	189	196	192	196	196	192	189	192	196	192	192	189	189	196	204	192				
24																				
25	322	322	322																	
26																				
27																				
28	256	277	270	285	228	228	285	285	285	285	285	285	285	294	294	294	312	294	303	
29																				
30																				
31	175	182	179	179	182	182	182	182	182	189										
32																				
33	294	294	303	313	313	313	313	323	323	345	333	345								
34	610	620	630	640	650	660	670	680	690	700	710	720	730	740	750	760	770	780		

Appendix 3. Instantaneous firing frequency (Hz) in response to current injection (10-400pA) in HA neurons

Neuron	Injected current (pA)																																						
	10	20	30	40	50	60	70	80	90	100	110	120	130	140	150	160	170	180	190	200	210	220	230	240	250	260	270	280	290	300	310	320	330	340	350	360	370	380	390	400
1								13	23	46	74	108	127	127	144	144	154	166	166	180	196	196	196	196																	
2																																								
3																127	130	135	147	154	156	172	167	175	179	182	185	189	200	208	208	213	217	222	227					
4							28		33	46	56	67	75	88	95	103	107	114	125	131	139	140	151	146	161	159	169	182	173	186	193	196	204	201	203					
5			13	27	54	73	99	116	125	144	151	159	175	180	189	195	198	204	206	216	214	224	225	228	231	236	243	241	242	244	251	249	252	249	258	260	257	258	263	261
6																																								
7			18	43	60	78	92	102	111	126	131	135	143	150	150	159	160																							
8							27	34	54	56	61	78	81	85	98	89	109	106	112	118	120	127	133	130	140	146	153	156	157											
9			34	70	93	110	130	141	154	164	172	185	185	189	204	213	213	217	227	233	233	244	256																	
10						52	48	63	74	86	91	94	112	113	117	129	132	139	142	149	156	157	168	168	179	180	183	189	195	192	191	204	195	203						
11						20	47	38	86	97	99	119	125	132	134	139	148	150	157	160	164	173	172	178	186	186	189	194	206	203	210	211	205	214	211	222	229			
12		14	19	36	56	90	81	88	102	133	128	164	139	156	159	169	175	192	192	200	208	208	213	217	227	233	233	238	250	256	263									
13						21	31	45	65	75	88	104	111	128	137	147	164	172	182	189	200	204	213	227	227	238	244	244	250	263	270	278	278	286	278	294	294	294	303	303
14			41	68	79	101	109	122	137	141	145	152	164	167	169	175	172																							
15					61	75	90	101	115	127	139	139	154	167	167	172	182	179	192	192	200	204	204	208	217	217	222	222	233	227	238	238	238	244	250	250	250	256	256	256
16				31	54	57	72	86	92	103	118	123	133	135	147	152	159	164	172	175	185	185	189	196	200	208	213	213	217	222	222	227	227	238	238	238	250	250	250	250
17				10	23	38	48	60	72	81	93	99	111	125	132	141	149	159	164	172	172	182	192	189	204	208	213	213	222	222	233	233	238	244	250	250	256	263	263	270
18		15	25	44	59	79	67	82	105	127	129		141	161	178	181	204	217	227	238	243	238	256	278	270	263	278	294	294	303	294	294	313	312	303	333	323	323	323	323
19											35	44	50	60	68	70	83	92	100	109	116	125	135	141	156	156	167	164	169	175	213	185	185	189	192	200	204	208	217	
20		22	33	47	54	64	70	79	85	78	97	95	103	106	114	108	120	120	132	130	133	137	137	141	143	147	147	149	156	159	161	161	159	159	169	169	167	172		
21								39	48	65	93	127	120	128	141	149	169	175	196	213	196	200	213	227	233	238	250	250	263	263	270	270	278	286	286	294	303	303	303	303
22									32	38	54	68	76	85	98	106	115	125	137	147	151	164	172	175	181	188	196	204	212	217	222	227	232	238	238	244	250	250	256	263
23							46	67	58	80	85	106	108	120	131	135	140	156	156	166	166	169	178	181	200	196	200	204	212	212	217	217	222	222	232	232	238	243	243	250
24				21	50	66	81	103	115	130	141	143	156	167	179	185	196	196	196	208	208	222	227	227	238	238	238	244	244	250	256	256	263	263	263	270	270	270	278	278
25								56	71	93	108	125	139	145	156	164	172	179	185	189	200	204	208	217	222	222	233	244	250	250	256	250	263	256	278	278	278	278	286	286
26							21	22	26	25	31	40	41	44	53	62	64	73	73	85	93	90	100	104	105	123	125	128	129	135	137	147	149	156	153	158	169	169	178	178
27			24	36	51	70	91	110	123	149	156	167	185	192	204	212																								
28													55	90	103	103	121	128	149	152	163	172	178	175	196	200	185	196	212	222	217	232	232	243	243	243	250			
29						16	31	39	46	62	75	86	90	103	107	111	117	125	131	147																				
30								42	43	56		88	100	115	135	135	142	153	154	164	185	193	192	200	208	204	212	204	200	204	212	222	232	238	256	243	256	256	263	270
31									57	68	113	131	138	156	163	175	181	188	196	200	204	212	212	212	222	217	232	217	238	243	238	250	256	250	250	263	256	277	277	277
32					65	80	92	102	116	125	128	145	145	156	163	145	175	172	178.5	178	170	185	196	204	185	200	200	208	208	208	208	213	222	222	212	222	217	227	227	227

Appendix 4. Instantaneous firing frequency (Hz) in response to current injection (410-800pA) in CA neurons

Injected current (pA)

Neuron	410	420	430	440	450	460	470	480	490	500	510	520	530	540	550	560	570	580	590	600
1																				
2																				
3																				
4																				
5	266	269	270	268																
6																				
7																				
8																				
9																				
10																				
11																				
12																				
13	313	303	313	313	323	323	323	323	333	333	333	345	345	345	357	345	345	345	357	357
14																				
15	263	263	270	270	263															
16	256	263	270	263																
17	278	278	278	286	286	286	294	294	294	294	294	303	303	303	313	303	313	313	313	313
18	333	333	344	344	344	357	357	344	457	344	357	357.1	357.1	357.1	370.3	357.1	370.3	370.3	370.3	370.3
19																				
20																				
21	313	313	313																	
22	263	270	270	277	278	286	286	286	303	295	303	312.5	312.5	303.0	312.5	322.5	312.5	312.5	312.5	322.5
23	250	256	250	256	256	263	256	263	270	277	277	285	285	285	285	294	294	294	303	303
24	278	278	286	286	294															
25	286	294	294	313	303	313														
26	196	192	196	196	204	196	208	217	212	217	227	227	238	238	238	243	243			
27																				
28																				
29																				
30	277	294	285	294	303	303	303	322	322	344										
31	285	285	285	303	285	294	294	312	312	312	312	322	322	322	333					
32	227																			
33																				

Neuron	610	620	630	640	650	660	670	680	690	700	710	720	730	740	750	760	770	780	790	400
1																				
2																				
3																				
4																				
5																				
6																				
7																				
8																				
9																				
10																				
11																				
12																				
13	357																			
14																				
15																				
16																				
17	323	313	323	323	323	323	323	323	323	333	323	333	333	345	333	345	345	345	345	
18	370.3	357.1	384.6	384.6	370.3	370.3	384.6154													
19																				
20																				
21																				
22	322.5	322.5	333.3	322.5	322.5	322.5	333.3	333.3	344.8	344.8276										
23	312	323	312	312	322	322	322	333	323	333	344									
24																				
25																				
26																				
27																				
28																				
29																				
30																				
31																				
32																				
33																				

Appendix 5. Instantaneous firing frequency (Hz) in response to current injection (10-630pA) in LA neurons

Neuron	Injected current (pA)																																							
	10	20	30	40	50	60	70	80	90	100	110	120	130	140	150	160	170	180	190	200	210	220	230	240	250	260	270	280	290	300	310	320	330	340	350	360	370	380	390	400
1	17	44	68	103	99	119	128	138	148	175	184	189	198	214	221	233	244	249	260	263	270	273	272																	
2								28	37	45	74	96	121	132	147	161	172	185	198	200	200	203	212	218	228	232	232	242	248	252	250	254	258	270	263	271	270	284	281	282
3									37	45	74	96	120	132	145	161	172	185	200	200	204	213	222	233	233	233	244	250	250	256	256	263	270	270	278	278	286	294	294	294
4		37	44	65	86	104	122	135	152	159	172	182	192	200	208	217	222	227	233	238	244	244	250	250	256	263	263	270	278	270	270	278	278	278	286	286	294	286	294	303
5									23	52	78	93	103	116	122	139	152	156	164	164	169	182	189	189	192	204	200	208	217	217	222	227	233	238	244	238				
6					11	41	70	74	79	90	112	115	127	135	132	135	143	145	152	156	127	132	135	147	147	156	159	156	161	169	169	182	179	175	182	185	196	196		
7									34	53	82	71	91	96	111	106	119	130	133	139	141	139	154	149	154	159	167	169	167	175	161	167	179	179	182	189	192	185		
8								33	32	125	159	182	204	217	222	238	244	250	250	263	270	270	278	286	294	303	303	313	303	313	323	323	323	333	333	333	357	333	345	357
9				17	55	39	72	83	105	116	114	147	114	111	132	132	159	135	149	145	161	141	182	161	169	182	189	182	192	192	185	182	196	200	185	204	182	200	200	208

Neuron	Injected current (pA)																						
	410	420	430	440	450	460	470	480	490	500	510	520	530	540	550	560	570	580	590	600	610	620	630
1																							
2	287	233	298																				
3	303	303	303	313	313	313	313																
4	294	303	303	303	303	313	313	313	313	313	323	313											
5																							
6																							
7																							
8	345	357	357	357	370	385	370	400	400	400	400	417											
9	213	208	200	213	213	208	217	213	222	217	213	222	204	227	222	222	233	204	233	227	217	233	