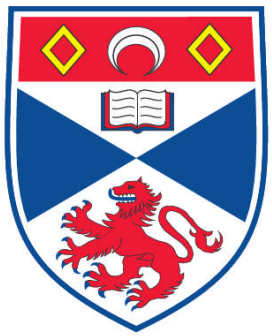
THE DEVELOPMENT OF FREE-SWIMMING IN *XENOPUS LAEVIS* LARVAE

Nicholas William Scott

A Thesis Submitted for the Degree of MPhil
at the
University of St. Andrews



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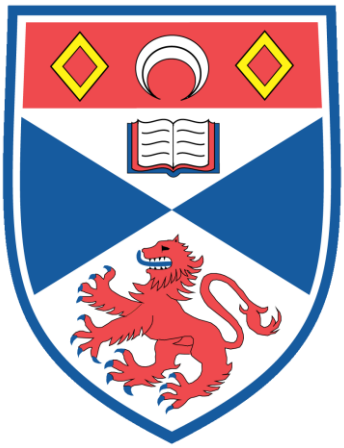

THE DEVELOPMENT OF FREE-SWIMMING IN *XENOPUS LAEVIS* LARVAE

By
Nicholas William Scott B.Sc.

A thesis submitted to the University of St Andrews for the Degree of Master of Philosophy

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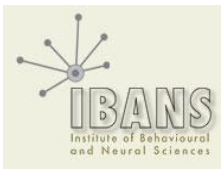

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I, Nicholas William Scott, hereby certify that this thesis, which is approximately 21,000 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

I was admitted as a research student in September, 2010 and as a candidate for the degree of Master of Philosophy in September, 2010; the higher study for which this is a record was carried out in the University of St Andrews between 2010 and 2011.

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II. Supervisor's declaration:

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Acknowledgements

I would firstly like to pay tribute to my supervisor Professor Keith Sillar.

I consider Keith to be a lifelong mentor and friend and this thesis describes more how we got to know each other, rather than how we use to know each other.

Many say that there are only two types of supervisor: the older academic who knows everyone and the young enthusiastic academic who is keen to foster your interests and skills. Keith is an individual who can and does encapsulate both of these two extremes.

I would also like to thank Dr Hong-Yan Zhang, who is a beacon of hope on any dark day, the technical support staff under the excellent management of Isobel and to John Macintyre.

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Life in A26. There are too many excellent memories of my time in this lab to recall here. That could be a thesis in its own right. All I would say is that each day started with coffee and conversation with Stephen. A great many topics were discussed, some scientific, some personal and others simply hilarious and I am so grateful to Stephen to have had the pleasure to share this place with him. My mental photograph of the year was of Keith, Stephen and I standing around A26 listening to one of Keith's sensational jokes.

I would like to thank my friends Stephen, Em, Tobias and Rose for being kind to me during a difficult year.

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Contents

Abstract

In *Xenopus laevis* frog tadpoles, highly self-motive, free-swimming behaviour emerges at the onset of feeding. This is in contrast to the earlier post-hatched larval form, which is capable of escape swimming when stimulated, but normally lies dormant.

This developmental transition in behaviour has been documented here and studied in a semi-intact preparation developed to examine the motor output from larvae at the onset of filter feeding. There is a progressive increase in spontaneous motor activity during this period, where spontaneous fictive swimming occurs in episodes of variable duration but with significantly larger burst durations. This spontaneous activity persists after removal of both the fore- and midbrain, but is absent in spinalised preparations. The spontaneous activity is similar to NMDA (100μM) induced rhythm but shows greater periodic variability in the frequency and occurrence of swimming activity. The activity is not dependent on inhibitory synaptic transmission, but is under the control of central GABAergic restraint, as blocking this inhibition with bicuculine (10μM) increased spontaneous locomotor activity. This is distinct to the role of glycinergic inhibition which influences the character of the ventral root bursts, as strychnine (5μM) caused an initial increase in frequency before bursts were synchronised on

both the left and right sides, but not the presence of spontaneous activity. However, activity is abolished by the persistent sodium current blocker riluzole (5μM) and enhanced by veratridine (90nM) which potentiates the current, which may suggest that it originates in neurons with pacemaker-like properties - possibly within the hindbrain.

Evidence is also provided which shows that the neuromodulatory gas nitric oxide becomes an excitatory modulator of the *Xenopus* swimming network at the onset of a free-swimming existence, switching from having a global inhibitory role on locomotion in early larval life. The nitric oxide donor DEA/NO (200μM) increased spontaneous fictive swimming in the semi-intact preparation. In contrast, the nitric oxide synthase inhibitor L-NAME combined with PTIO, which sequesters nitric oxide, decreased spontaneous fictive swimming.

It is proposed that the emergence of this rhythmic, spontaneous motor activity parallels the increase in swimming at the onset of feeding, suggesting a direct behavioural role for spontaneous network activity in the developing animal.

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Abbreviations

5-HT	5-Hydroxytryptamine (Serotonin)
ATP	Adenosine triphosphate
aINs	Ascending interneurons
cGMP	Cyclic guanosine monophosphate
cINs	Commissural interneurons
CPGs	Central pattern generators
DEA/NO	Diethylammonium-1-(*N*,*N*-diethylamino) diazen-1-ium-1,2-diolate
dINs	Descending interneurons
dla	Dorsolateral ascending interneurons
dlc	Dorsolateral commissural interneurons
dpf	Days post-fertilisation
INaP	Persistent sodium current
Mhr	Mid-hindbrain interneurons
MN	Motoneurons
NMDA	*N*-Methyl-D-aspartic acid
NO	Nitric oxide
RB	Rohon-Beard neurons
RS	Reticulospinal neurons
sGC	Soluble guanylate cyclase
VR	Ventral root

" causa latet, vis est notissima fontis."
"The cause is hidden; but the enfeebling power of the fountain is well known."

Ovid (43 BC - 17 AD) Metamorphoses, 4. 287.[1]

Chapter I
General Introduction

Vertebrate locomotion relies on circuits of spinal interneurons, to generate and coordinate the rhythmic activation of motor neurons, translating nervous action into outwardly expressed behaviour (Grillner 2003; Goulding 2009). These central pattern generators (CPGs) have their own intrinsic activity but normally require descending drive from supra-spinal loci to initiate rhythmic motor output (Brown 1912; Grillner and Zangger 1975; Grillner and Wallen 1985). Developmentally, such networks are assembled while the animal is still an embryo (Marder and Rehm 2005) and are required to function after birth, or hatching from the ovum, in order to allow the organism to seek out food and avoid predation. Critically, the motor control system must also mature with age and produce an output appropriate to the animal's changing biomechanical, hormonal and dietary demands. For example, during development in the lamprey, a filter-feeding larva is transformed into a parasitic adult scavenger (Youson and Sower 2001). Great demands, therefore, are placed on the CPG throughout development and understanding how networks can function within this changing landscape is of much interest.

The development of *in vitro* spinal preparations, where the spinal cord is isolated from sensory and descending inputs, have allowed many of the cellular components and

[1] The quotation refers to the story of Salmacis and Hermaphroditus, in which the nymph Salmacis is infatuated but rejected by Hermaphroditus, son of Hermes and Aphrodite. Salmacis prays for their union in the waters in which Hermaphroditus bathes. The gods answer and the fountain is cursed so that they are united into one impotent being, hence the origin of the term 'hermaphrodite' and that all men who bathe there will now suffer the same fate. The quotation is usually translated as "The cause is hidden, but the result is well-known".

connections of the CPG to be deciphered (Wallen and Williams 1984; Kwan et al. 2009; Zagoraiou et al. 2009). However, how these networks are activated *in vivo* remains poorly understood, as such endogenous or spontaneous activity has proven difficult to study experimentally. Indeed, as Sherrington (1947) writes:

"… reflex action represents certain advantages for physiological description. It can be studied free from complication by that type of 'nerve' activity which is called autochthonous (or 'spontaneous') and generates intrinsically arising rhythmic movements … [however] studied in that self-contained animal group, the Vertebrates, behaviour seems to become less and less reflex as the animal individual becomes more and more completely individuated."

Therefore, the advantage of studying intrinsic activity, rather than evoked activity, is that the range of behaviours available to the animal - which becomes more elaborate with age - can be brought under investigation. The development of free-swimming in larvae of the amphibian *Xenopus laevis* can provide an experimental approach to help elucidate this critical aspect of animal behaviour – how animals execute control and drive their own locomotion.

The remit of this investigation, laid out in the chapters ahead, is to describe the development of free-swimming behaviour and to detail the spontaneous motor activity found in the larval preparations. It is proposed that the emergence of this rhythmic, spontaneous motor activity parallels the developmental increase in swimming at the onset of feeding.

1.1 *Xenopus laevis* larval locomotion

Larvae of the African-clawed toad, *Xenopus laevis*, are a simple and exquisite vertebrate model system which moves through two major changes in locomotory strategy during development (Li et al. 2001). Initially, in the early larval phase,

immediately after hatching from the embryo (stage 37/38; Nieuwkoop and Faber 1956)[2] the tadpole utilises tail-based swimming; there is a characteristic left-right alternation between axial muscle contractions propagating in a rostral-caudal direction (Kahn and Roberts 1982). This swimming pattern matures in the course of early larval development (Sillar et al. 1991) but is then replaced by hind-limb based locomotion, as the animal undergoes metamorphosis, where the tail is absorbed as the hind-limbs grow and become motile, forming the young frog (Combes et al. 2004; Beyeler et al. 2008; Sillar et al. 2008).

1.1.1 A CPG for Swimming

The axial swimming CPG at stage 37/38 (see Figure 1) has been extensively researched and is one of the most described vertebrate neural networks (Roberts et al. 2010). Using immobilised preparations, motor activity in the form of ventral-root discharges can be recorded directly from the inter-myotomal clefts, wherein lie the motor neuron axons. This extracellular measure of 'fictive' swimming has been combined with intracellular recordings – relating the activity of individual neurons to characteristics of the motor output; such as burst duration, cycle period and the duration of swim episodes (Li et al. 2007). These episodes of swimming are usually initiated in preparations by using a stimulating electrode placed on the tail to deliver a short (around 2 msec) electrical shock, although a pineal-dependent light dimming response may also be used (Jamieson and Roberts 2000). The subsequent bout of evoked swimming activity shares many characteristics with the evoked swimming observed behaviourally when the tail skin is touched. The stimulation of the tail activates the Rohon-Beard (RB) mechanoreceptors which in turn excite second-order dorsolateral interneurons (dlc and dla) providing

[2] *Xenopus laevis* tadpoles are staged throughout according to Nieuwkoop and Faber (1956).

excitatory synaptic input onto the CPG (Sillar and Roberts 1988). The skin is also electrically excitable and will respond to noxious stimuli, but does so in immature larvae, stage 31 and earlier, by activating the RB neurons (Roberts 1969; James and Soffe 2011). This brief activation of the animals' sensory system is therefore, translated into prolonged swimming activity.

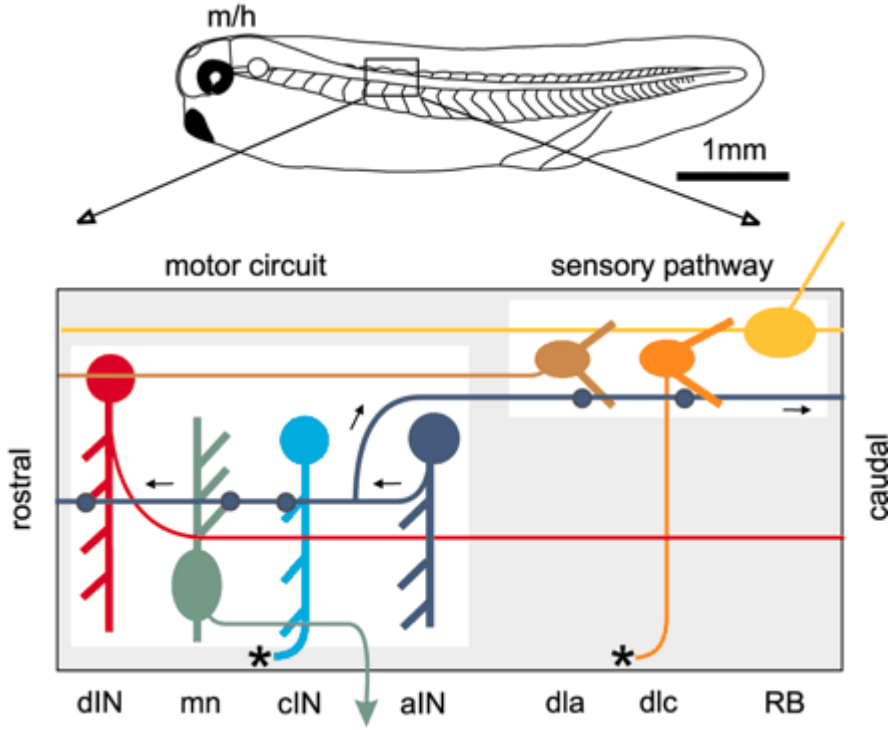

Figure 1. The *Xenopus* tadpole and its spinal neurons. A *Xenopus* tadpole at the time of hatching (stage 37/38), with its head to the left, the border between midbrain and hindbrain (m/h), and the spinal cord lying under segmented swimming muscles. Below, diagram of a length of spinal cord viewed from the side to show the neurons. Sensory pathway: dlc (orange) and dla (brown) are excited by skin mechanosensory RB neurons that have no dendrites. Motor circuit: glycinergic aINs (dark blue) have ascending and descending axons that can inhibit other swimming CPG components: mn (grey), excitatory dINs (red), glycinergic reciprocal inhibitory (cINs; blue). aINs also can form inhibitory synapses with the sensory dla and dlc INs. For each neuron class, the circle/oval is the soma; oblique lines indicate the dorso-ventral extent of the dendrites; and the thin line shows the axon projection (asterisk indicates that the axon crosses ventrally to the opposite side). Each class of neuron actually forms a longitudinal column of 50-150 neurons on each side of the cord. Note Kolmer-Agduhr cells are not included in diagram. (Adapted from Li et al. 2004)

Descending interneurons (dINs) in the hindbrain and rostral spinal cord have been found to play a critical role in generating excitation within this swimming network, driving motor neurons and other spinal neurons as well as feeding-back excitation onto

each other (Li et al. 2006). Using an elegant combination of single-cell physiology, anatomy, lesions and pharmacology, Li and colleagues (2004) have shown that dINs fire action potentials before all spinal neurons and do so with a co-release of the excitatory neurotransmitters, glutamate and acetylcholine. Therefore, they provide the drive and the ability to sustain motor activity after the momentary stimulus has passed.

The propagation of the muscle contractions from the rostral (head end) of the animal to the caudal tail, also suggests that dINs in the hind-brain and rostral spinal cord fire before their more caudal counterparts and this activity spreads down, with left-right alternation. This alternation is strongly dependent on the glycinergic inhibitory commissural interneurons (cINs), which inhibit neurons on the opposite side of the cord, including the motor neurons (MNs) (Soffe et al. 1984), and hence provides further support for Brown's Half-Centre model of spinal motor function (Brown 1912). At this stage of development, there is also electrical coupling between MNs, which helps to provide synchronous firing of MNs innervating the same muscle cleft (Zhang et al. 2009). The Kolmer-Agduhr cells were not shown in Figure 1 and their role in the nervous system generally, and in the generation of swimming specifically, remains uncertain – despite the fact that they are GABAergic with long descending axons (Dale et al. 1987). Recent work published in the larval zebrafish 2-4 days post fertilisation (dpf), suggests that they are involved in providing excitation for swimming although they are GABAergic, the chloride equilibrium is set such that it promotes chloride outflow and therefore depolarisation, and are critical for the frequency of swimming (Wyart et al. 2009).

Episodes of swimming at stage 37/38 will terminate for a combination of reasons. Behaviourally, the tadpoles will stop when their cement gland is physically disturbed upon swimming into something. This has been demonstrated under experimental conditions, where activation of cement gland afferents excites descending GABAergic

mid-hind brain reticulospinal (mhr) neurons, inhibiting MNs and the CPG (Boothby and Roberts 1995). Furthermore, Boothby and Roberts also suggest that this pathway provides a tonic background inhibitory control of the network, thereby contributing to the behavioural quiescence at this stage. Additionally, it has been shown that a build-up of adenosine due to the activity-dependent breakdown of ATP, released in the spinal cord during swimming, can cause inhibition and bring episodes to their end (Dale and Gilday 1996). There has also been recent evidence which links the duration of episodes with past activity in the swimming network, through the generation of an ultra-slow afterhyperpolarisation (usAHP) following activity-dependent up-regulation of the Na^+/K^+ ATPase (Zhang and Sillar 2012). Finally, it has to be noted that the spontaneous end of a swimming episode, could well in part be due to a run down in excitability.

At this stage of development the swimming repertoire is rather limited, with relatively stereotyped ventral root bursts (Sillar et al. 1992). However, around a day later at stage 42 (Nieuwkoop and Faber 1956) the swimming pattern becomes more variable, with MNs - which had previously been confined to firing only a single spike per cycle - now able to fire multiple times (Sillar et al. 1992). A great deal of the networks' maturation is due to neuromodulation, which alters the properties and inter-connections of neurons, rather than the fast acting changes in receptor currents normally attributable to 'classical' neurotransmission (Harris-Warrick and Marder 1991; Dickinson 2006).

1.1.2 Development and neuromodulation

Serotonin (5-HT) has been shown to play a key role in the developing tadpole locomotor network, where descending serotonergic fibres from the raphe nucleus in the brain innervate progressively more of the spinal cord from stage 37/38 causing an increase in burst durations, depression of mid-cycle inhibition and modulation of

NMDA receptor-mediated responses (Wedderburn and Sillar 1994; Sillar et al. 1998). Indeed, effects of 5-HT are also found in the larval zebrafish where quiescent periods of activity are decreased (Brustein et al. 2003; Brustein et al. 2005), in the neonatal mammalian spinal preparation (Liu et al. 2009) and in the isolated lamprey spinal cord (Bass and Baker 1997) for assisting in rhythm generation.

Noradrenaline, on the other hand, has an inhibitory role on swimming output early in development in *Xenopus*, acting through the α2-adrenoreceptor to enhance mid-cycle glycinergic inhibition, thereby slowing the swimming frequency (McDearmid et al. 1997; Fischer et al. 2001). It is clear therefore that descending projections from the brain play an important role in not only exciting spinal motor networks, but are also critical to their ontogeny as well. The interplay between different neuromodulators has also been effectively shown in this motor network, particularly in reference to nitric oxide (NO).

NO is a diatomic labile gas that is able to freely diffuse through cell membranes. It is either produced within nitrergic cells from nitric oxide synthase (NOS) or can be released from molecular stores. NO subsequently activates soluble guanylate cyclase (sGC) increasing cyclic GMP or causes S-nitrosylation of target proteins in effector cells (Hess et al. 2005). In the early larval phase of *Xenopus*, NO has been shown to be produced by three distinct populations of neuron in the brainstem and acts to inhibit the locomotor network, acting both directly by enhancing GABAergic transmission - shortening episodes - and in series by enhancing the action of noradrenaline which slows the swimming rhythm by enhancing glycinergic inhibition (McLean and Sillar 2003; McLean and Sillar 2004). This complicated modulatory action of NO becomes even more intriguing later in development, as NO appears to switch its effect on swimming output, when motor activity is sampled from metamorphic larvae (Personal Communication, D. Combes).

1.1.3 **Metamorphosis**

Metamorphosis in anuran amphibians involves a striking post-embryonic transformation of larval tadpoles into juvenile froglets, characterised by a change in morphology with the loss of the tail to the growth of hind-limbs, from gills to lungs and from filter-feeding to a predatory existence (Shi 2000). The motor control system must not only change along with these demands, it must also remain functional throughout this development process. This is in contrast to metamorphosis in, for example, the holometabolous insects, who sequester themselves away from the external environment in a cocoon, as they dismantle one body form and transition from a larva to a pupa and then to the adult form. They therefore do not have the same demands on avoiding predation and seeking out prey (Consoulas et al 2000).

Combes and colleagues (2004) developed a series of *in vitro* preparations where the brain-stem and spinal cord are isolated from representative stages both before the onset of metamorphosis, during and after the transition. At pre-metamorphic stages (50-54), recordings from exposed ventral roots show many similar characteristics of the fictive swimming recorded at younger stages of development, such as a characteristic left/right alternation and inter-segmental rostro-caudal delay, whereas at stages 60/61, just before metamorphic climax (stage 65) the larval hind-limbs have developed and are fully functional and work together with the tail to generate thrust (compare Figure 2 and Figure 3). In both preparations, as well as those sampled in-between, fictive motor activity is generated spontaneously by the preparations, although NMDA (2-25μM) can also be bath applied to trigger motor output (Combes et al. 2004).

The action of neuromodulators changes at these older stages of larval development, where NA had previously provided an inhibitory effect on locomotion by slowing

swimming frequency, at later metamorphic stages it has the opposite effect on axial tail rhythm – increasing its frequency (Rauscent et al. 2006). Combes and colleagues (unpublished observations) have also shown that NO similarly changes in its effect on motor output, switching from a global inhibitory role to being an excitatory modulator of tadpole swimming.

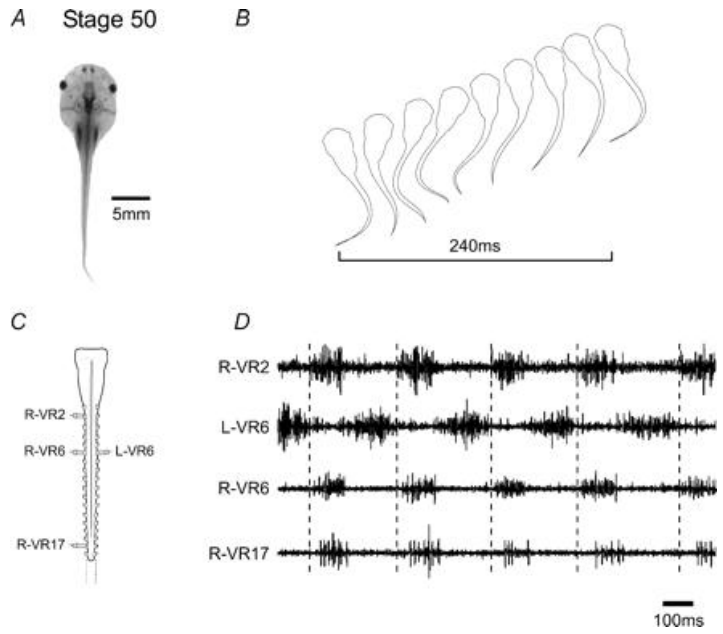

Figure 2. Stage 50 tadpole (*A*), schematic of undulatory swimming movements and forward propulsion generated by alternate bilateral contractions of axial myotomes with a characteristic rostro-caudal delay along the body (*B*). When the brainstem and spinal cord are isolated *in vitro* (*C*), extracellular recordings from selected spinal ventral roots (*D*; recorded roots indicated in *C*) reveal spontaneous bursting in axial motorneurons which, as with swimming *in vivo*, alternates across the cord and propagates rostro-caudally (dotted lines indicate delay between VR2 and VR17). Note that the horizontal bar in *B* indicates a complete cycle. (Adapted from Combes et al. 2004)

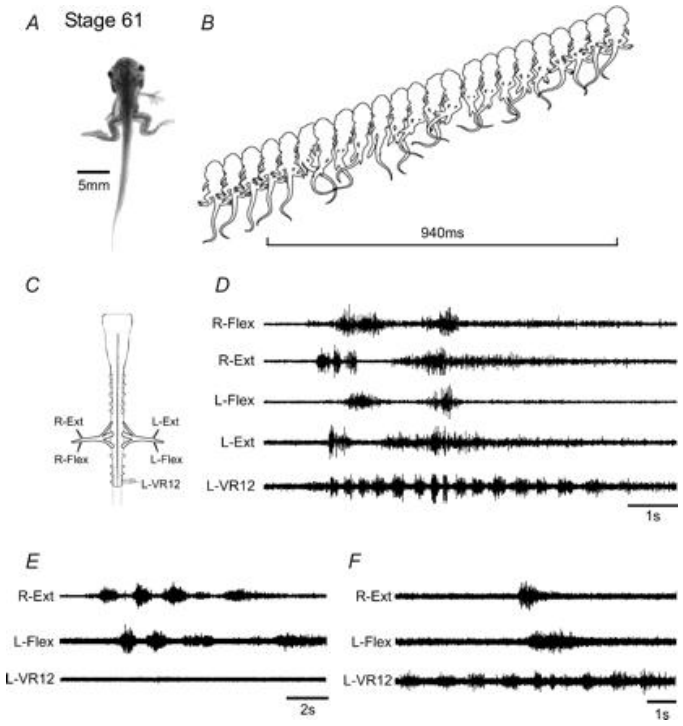

Figure 3. Stage 61 tadpole (*A*) the hind-limbs are now fully functional and a combination of rhythmic bilateral limb kicks and tail undulations are used to propel the animal (*B*). The isolated cord (*C*) can generate spontaneous motor patterns (*D*) appropriate for both locomotor modes: higher frequency bursting in tail spinal segments and slower, bilaterally synchronous bursting in lumbar hind-limb motorneurons corresponding to fictive kicking. As *in vivo*, the two motor programmes may be co-ordinately active (as in *D*) or can operate independently, with bursting expressed solely in limb motorneurons (*E*), or more frequently, activity occurring only in axial motorneurons (*F*). Note that the horizontal bar in *B* indicates a limb-kick cycle. (Adapted from Combes et al. 2004)

In addition, the distribution of NOS positive neurons in the tadpole changes between early larval development and metamorphosis (see Figure 4). Nitrergic neurons are restricted to the brainstem up until the limb buds start to form at stage 48, where NOS positive cells progressively appear throughout the spinal cord as well during metamorphosis (Ramanathan et al. 2006).

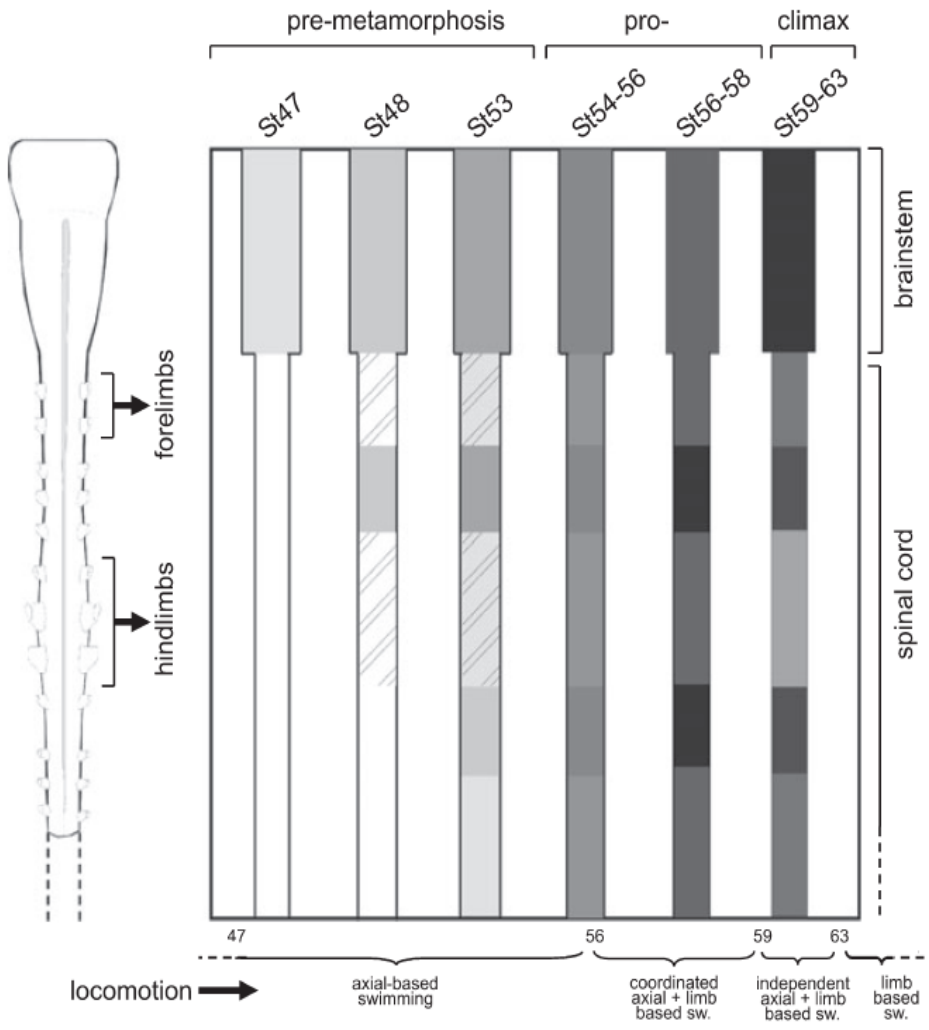

Figure 4. Changing distribution of nitrergic neurons during metamorphosis. Schematic summarises the approximate changing distribution, sequence and intensity (as a function of shading) of NADPH-d reactivity during metamorphosis in *Xenopus laevis*. NOS-positive spinal neurons are first observed at pre-metamorphic stage 48, coinciding with limb bud emergence and by stage 51 two longitudinal clusters are clearly evident (shaded) – which increase in NADPH-d reactivity (darker shading at stage 53). These clusters are in spinal areas flanking the emerging limb motor pools (hatched). During pro-metamorphosis, when both axial- and limb-based swimming begins to occur, neurons in previously unstained spinal areas begin to show weak NADPH-d reactivity, which then also increases as metamorphosis progresses. By pro-metamorphic stage 57, intense neuronal staining is seen throughout the length of the spinal cord, and this persists until the metamorphic climax when a decline in staining in the lumbar region is observed. (Adapted from Ramanathan et al. 2006)

Therefore, it is clear that over the course of development new demands are placed on the motor network in *Xenopus* and with this changing circumstance come differences not only in the complement of networks but also in the actions of neuromodulators on these

networks. Although metamorphosis is the most dramatic transformation for the developing tadpole, there is another critical change during ontogeny - when the animal joins the water column as a filter-feeder.

1.1.4 Free-Swimming

At the moment of hatching from the embryonic envelop (ca. stage 37/38), the animal is capable of generating evoked episodes of swimming, but usually lies dormant or hangs from the cement gland, as Alan Roberts (2010) notes, "Like many other newly hatched tadpoles [*Xenopus*] spends 99% of its first day doing nothing …". Although maturation, often through greater modulation, of the network is important throughout the next day, as the tadpole gets larger and the swimming CPG becomes more flexible – the overall amount of spontaneous, or endogenous, swimming activity remains at low levels during these first few days of larval life (see Results, Figure 10).

At these early larval stages (stages 37/38 to 42), the tadpole still possesses a yolk sac, which becomes smaller as the animal grows, so locomotion is generally restricted to tactile disturbances (Boothby and Roberts 1995) or changes in illumination (Foster and Roberts 1982; Jamieson and Roberts 2000). However, as the animal continues to develop the yolk eventually runs out, the alimentary canal develops (see Figure 5) and the mouth opens, with the first food appearing in the intestine by stage 46 (Nieuwkoop and Faber 1956). The animal therefore requires to free-feed, first emerging at stage 45, and adopts a suspension filter feeding strategy (Nieuwkoop and Faber 1956), which coincides with the emergence of this free-swimming and more active lifestyle for the animal. Accompanying this new dietary demand, is an increase in length and head size (shown in Figure 6) and therefore the animal has to generate more spontaneous activity to allow it to move through the three-dimensional water column, buccal pumping water over the internal

gills to breath and filtering out microorganisms to eat (Lambert et al. 2008). The larval zebrafish also goes through a similar transition from 3 days post-fertilisation (dpf) to 5 dpf - where the animal starts feeding (see Figure 7) (Khan and Roberts 1982; Brustein et al. 2003; Thirumalai and Cline 2008). The zebrafish, however, adopts a hunting strategy reflected in their beat and glide swimming, distinct from the suspension filter feeding observed in *Xenopus* and more like the *Hymenochirus* tadpoles which are macrophagous carnivores (Stehouwer and Farel 1980).

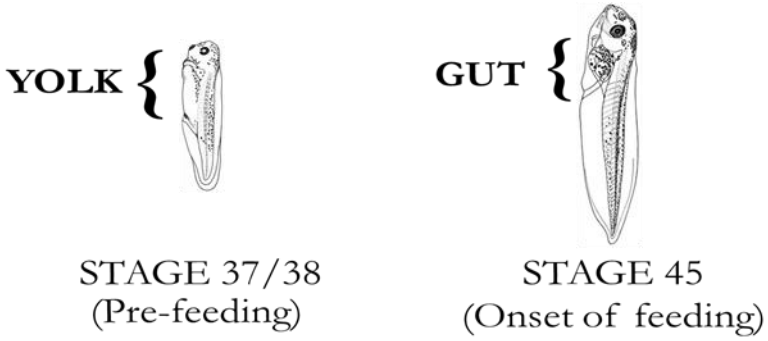

Figure 5. Onset of feeding in *Xenopus*. Drawings of *Xenopus laevis* tadpoles at the time of hatching (stage 37/38), with yolk sac, and at the onset of feeding (stage 45) with the gut and anus shown with no yolk sac present (Drawings from Nieuwkoop and Faber 1956).

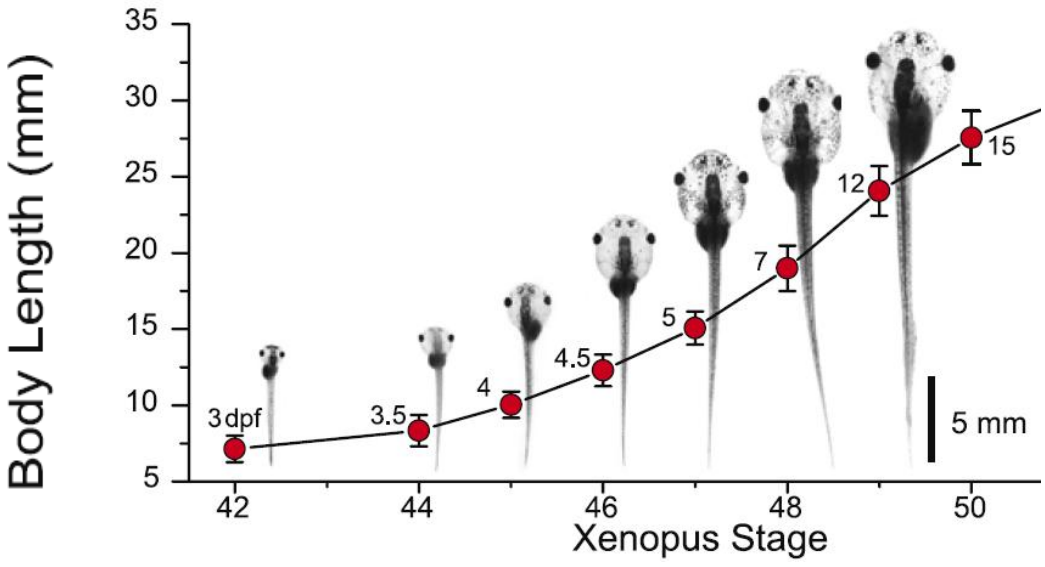

Figure 6. Changes in *Xenopus* larval length at the onset of feeding. Superimposed on the graph is a photomontage of larval *Xenopus* from stages 42– 49 showing the significant change in length and head size with age. The peak growth in larval size occurs between stages 46 and 49. Adjacent to each marker on the graph are the ages of the larvae in days postfertilisation. (Adapted from Lambert et al. 2008)

The developing zebrafish has a very stereotyped developmental series of motor behaviours, starting with spontaneous coiling at 17 hours post-fertilisation, which is dependent on local spinal cord circuits and differs from the myogenic contractions in the dog-fish embryo which are independent of neural control (Harris and Whiting 1954). After the development of burst swimming in the hatched larva, the growing animal becomes capable of generating a more sophisticated beat and glide swim – essential for the fish's predatory requirement for feeding. Invading serotenergic modulation plays an important part in this change, reminiscent of the previously mentioned role of descending serotonergic fibres in the development of a more complex swimming repertoire in *Xenopus* (Sillar et al. 1994) (see section 1.1.2).

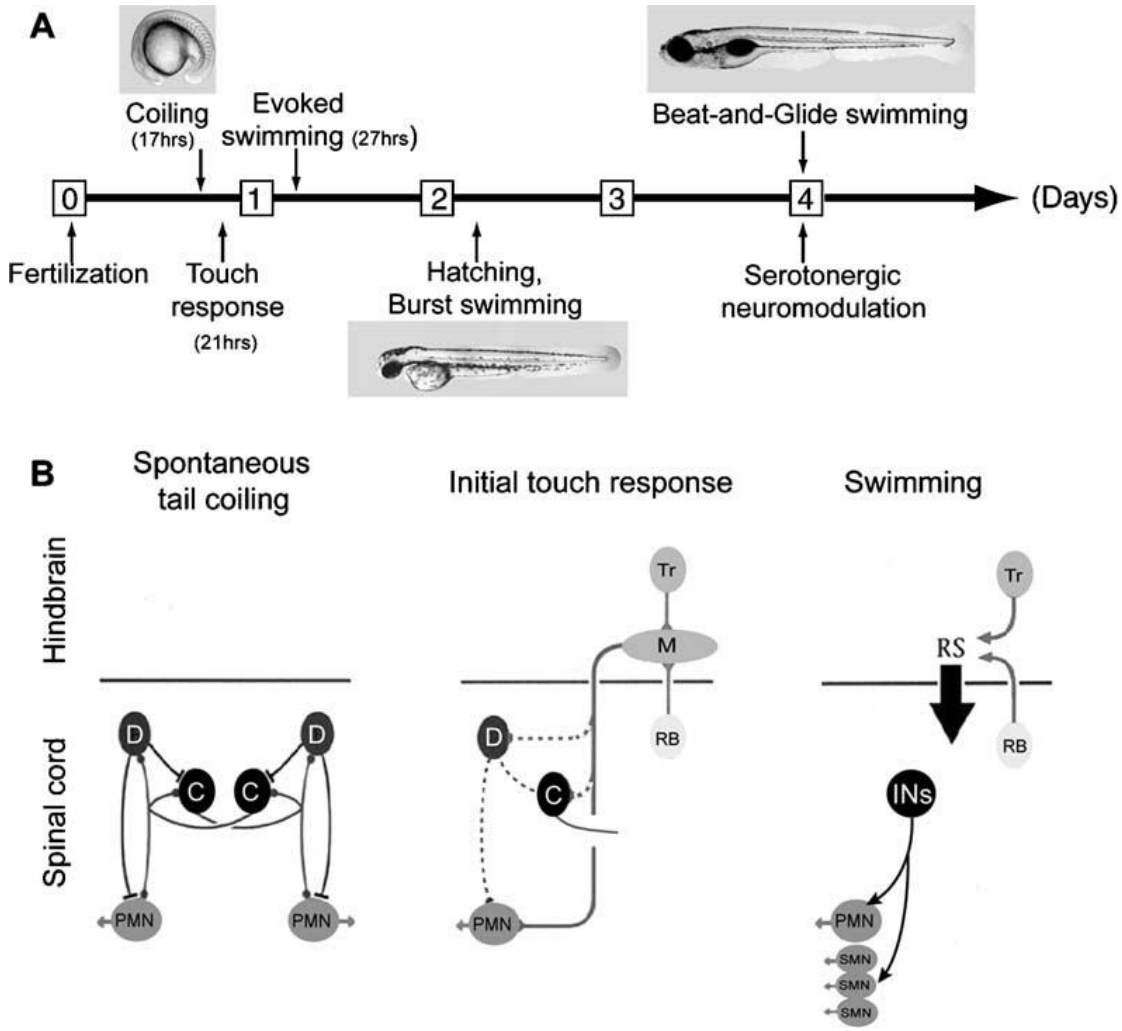

Figure 7. Changing behaviour and motor circuitry in larval zebrafish. (A) The chronological of motility patterns during development of the zebrafish. Images emphasize key stages of zebrafish embryo development. (B) Schematic diagrams of the possible neural building blocks underlying early motility patterns in the zebrafish. Left: the neural network active during spontaneous tail coilings (17 h) is limited to the spinal cord and includes only primary motoneurons (PMN) and a restricted number of interneurons (presented as D=descending interneuron and C=commissural interneuron. The network activity is based on electrical coupling. Center: the initial touch response (21 h) requires the activation of both hindbrain neurons (M=Mauthner, Tr=Trigeminal) and spinal neurons including the sensory RB neurons. Dashed lines represent hypothetical connectivity. At this time the synaptic response is mediated partly by glutamatergic synapses. *Right:* at 27 h the embryo can swim in response to touch. Swimming requires integration of both hindbrain reticulospinal neurons (RS) and spinal cord interneurons (INs) and secondary motor neurons (SMN). (Adapted from Brustein et al. 2003)

In *Xenopus* development, the characterisation of different behavioural sequences has similarly been described and requires close consideration. A behavioural classification was defined by Louise Muntz (1975), in which both structural (i.e. morphology of the nerves) as well as functional behaviour was used to determine five stages in the

developing *Xenopus* tadpole (see Table 1). This description starts with a non-motile phase, where there is no behavioural response to stimulation and the RB afferent fibres have only just started to leave the spinal cord. As development ensues, the tadpole acquires a greater ability to generate responses to stimuli, starting with slow homolateral contractions in the neck region at stage 22-24, developing into faster contralateral contractions and then onto early swimming at stage IV which is accompanied by the maximal amount of observed spontaneous contractions. The final stage, which the author refers to as free-swimming, details the emergence of a mature left-right alternating swimming pattern which can be evoked through stimulation and a concomitant decrease in spontaneous contractions around stage 32-42. This report is further supported by van Meir and colleagues (1989) who showed that *Xenopus* was able to generate forward locomotion by swimming from around stage 31-33 (van Mier et al. 1989). This description, however, raises a few concerns, the most obvious relating to the omission of the hatching phase at around stage 37/38 and the fact that the series ends prematurely – the ethological investigation of the tadpole should not end at stage 42. Whilst it is true that the animal is now capable of generating swimming, there is no mention of the native behaviour of the animal – how does the animal actually behave? Not simply, how may it act under stimulation? So, this classification does well to marry together the developing structure and functional output of the nervous system in the developing tadpole but lacks in its ability to define the final stage and leaves a large section of development between stage 42 and the start of metamorphosis without any mention.

Table 1. Summary of structural and functional development in *Xenopus* tadpoles. (Adapted from Muntz 1964; 1975)

Behavioural Stage	N/F Stage	Hours post-fertilisation	Structure of nerves	Behaviour
I. Non-motile	20-22	21-24	Short thick Rohon-Beard fibres leaving cord laterally	No response to any form of stimulus
II. Pre-motile	22-24	24-27	Rohon-Beard cells in lateral position with longer fibres reaching between myotomes	Responds with slow homolateral contraction in neck region to direct mechanical and electrical stimulation
III. Reflexogenic	24-27	26-31	Rohon-Beard cells with longer fibres reaching skin. Ventral fibres leaving cord	Responds to sensory stimulation with contralateral head flexure or coil. Spontaneous contractions observed
IV. S-flexure and early swimming	28-33	32-44	Rohon-Beard in dorso-lateral position. Peripheral sensory fibres to skin and longitudinal fibres in cord. Ventral fibres and ventral roots to myotomes	S-flexure takes form of a rapid flutter. Early swimming in short uncoordinated bursts. Maximum spontaneous contraction
V. Free-swimming	32-42	44-72	Rohon-Beard cells in dorsal position. Many sensory and motor in cord. Mauthner's neurones well developed	Much less spontaneous contraction observed. Larva swims away after any form of stimulation

It is difficult to accept therefore, the term free-swimming for a stage of development where the animal does not normally swim but is able to when stimulated and can do so without the restriction of the embryonic envelope. The emergence of apparently non-evoked free-swimming, or spontaneous swimming at the time of filter-feeding is therefore in need of further investigation.

1.2 Spontaneous Activity

Spontaneity is found throughout the nervous system, from the level of a synapse to the electroencephalogram taken from a human scalp. In essence, spontaneous activity can be described as the ongoing electrical activity within the nervous system that bears no obvious relation to sensory input or indeed motor output (McCormick 1999) and, colloquially, has the connotation of randomness. However, there are some reservations to this definition.

At rest, during our waking hours, our overall neural activity recorded from the scalp shows a regular pattern of activity from the cerebral cortex and was described by Hans Berger, originally in the form of the alpha wave associated with oscillations of 8-12 Hz with the eyes closed (Karbowski 2002). But, with eyes closed when asleep the regular pattern changes considerably to a wave form such as the lower frequency (between 1-4Hz) delta wave associated with slow wave or non-REM sleep (Dang-Vu et al. 2008). It is clear from such recordings, discredited for many years but now a mainstay of cognitive neuroscience research, that spontaneous activity need not be random and can show a defined frequency range and regular pattern.

Spontaneous activity can also be recorded from the ventral roots of isolated neonatal mouse spinal cords without the addition of excitatory agonists, although the stability of the rhythm is significantly reduced and such events are rare (Hinckley et al. 2005). Here, like in *Xenopus*, spontaneous activity refers to the motor output and is not just in reference to activity within the nervous system.

In different areas of neuroscience 'spontaneous' has been used, often out of desperation one might think, to describe either the type of activity (random) or the cause of that activity (unknown) and in many cases, such as the true origin of spontaneous transmitter release, it happens to be both. Nonetheless, for the purposes of the work

presented here spontaneous activity or spontaneous swimming will refer to locomotor related activity where the origin is not known but is believed not to be caused by the experimental conditions. Spontaneous activity although hard to define does appear to have an important role in development.

Developing neural networks, in both invertebrates and vertebrates, display waves of spontaneous activity that can be driven from individual pacemaker-like cells or result from emergent network activity. This developmental hyperexcitability is often attributed to: the depolarising action of the normally inhibitory GABA and glycine, due to a chloride reversal potential that favours chloride outflow rather than inflow when the conductance is activated; gap-junctions; extra-synaptic transmission and transient excitatory synapses (Blankenship and Feller 2010). By whatever mechanism, activity-dependent development has been implicated in the proper ontogenesis of developing networks (Zhang and Poo 2001). In the developing retina, spontaneous action potentials are found within the retinal ganglion cells and throughout their projection pathway in the lateral geniculate nucleus of the thalamus and the primary visual cortex (Wong et al. 1993; Hanganu et al. 2006). Thus, spontaneous activity is spread throughout the developing circuit, which may be important in building the selectivity of the retinotopic map.

In the developing auditory system in mammals, spontaneous activity has similarly been implicated in generating an internal sensory map. In an elegant study by Tritsch and colleagues (2007), dual patch clamp recordings within the organ of Corti inner hair cells and cells of the developmentally transient Kölliker's organ, revealed that spontaneous ATP release from the support cells onto the P2X receptors of inner hair cells, caused them to depolarise and produce action potentials. This occurred in rats prior to the opening of the auditory canal (c.a. 11-12dpf) and suggests that spontaneous activity in Kölliker's organ support cells, provide a synchronising effect on neighbouring inner hair

cell activity - which is important later in development at the onset of hearing where neighbouring inner hair cells respond together at different sound frequencies (Tritsch et al. 2007). This purinergic signalling during development ceases when that rats start to hear, suggesting it has a role in helping to build the functionality of the inner ear neural circuitry. But, can spontaneous activity have any direct functional role?

Breathing in mammals requires a resilient and flexible rhythm able to respond to the changing behavioural landscape throughout the day. The generation of the respiratory rhythm has been carefully characterised over many years and is still extensively researched. The pre-Bötzinger Complex and RTN/pFRG in the medulla of the brainstem have recently been shown to be important and at the centre of the excitatory kernel underlying the rhythm (Feldman and Del Negro 2006). Neurons within these areas utilise pacemaker currents, such as the persistent sodium current (INaP), in order to cause the membrane potentials to fluctuate between two quasi-stable levels and be able to generate regular bursts of action potentials (Rybak et al. 2007). Respiratory rhythm generation has also been investigated in the amphibian (*Rana catesbeiana*), where there is a switch from gill to lung breathing - interestingly NO appears to change in its role as a modulator during this transformation, inhibiting lung breathing in pre-metamorphic tadpoles but exciting lung breathing after metamorphosis – when the animal is an obligate air breather (Hedrick et al. 2005). Respiratory rhythm is an obligatory autonomic movement but is there place for spontaneous activity in locomotion.

In the medicinal leech (*Hirudo medicinalis*), a relationship between spontaneous activity and behaviour has been demonstrated, where spontaneous bursts in neurons of semi-intact preparations or isolated individual ganglia cause motor output – even in the absence of sensory input (Garcia-Perez et al. 2005; Garcia-Perez et al. 2007; Li et al. 2007). In this case, the spontaneous movements are irregular and not rhythmic but they

are characteristic of the animal's actual behaviour – which is not solely governed by reflexes and recruitment of the swimming CPG. In the zebrafish, however, evidence is slowly emerging that spontaneous motor activity can recruit the locomotor CPG and produce sustained rhythmic output.

In the zebrafish (*Danio rerio*), the larval developmental programme (see Figure 7) ensues outside in the external environment, similar to *Xenopus laevis* development, and behaviour along with morphology changes over time. In a seminal study, Thirumalai and Cline (2008) showed that there is a marked increase in spontaneous swimming between 3 and 5 dpf and critically that this can been seen in immobilised preparations as an increase in spontaneous fictive swimming (see Figure 8).

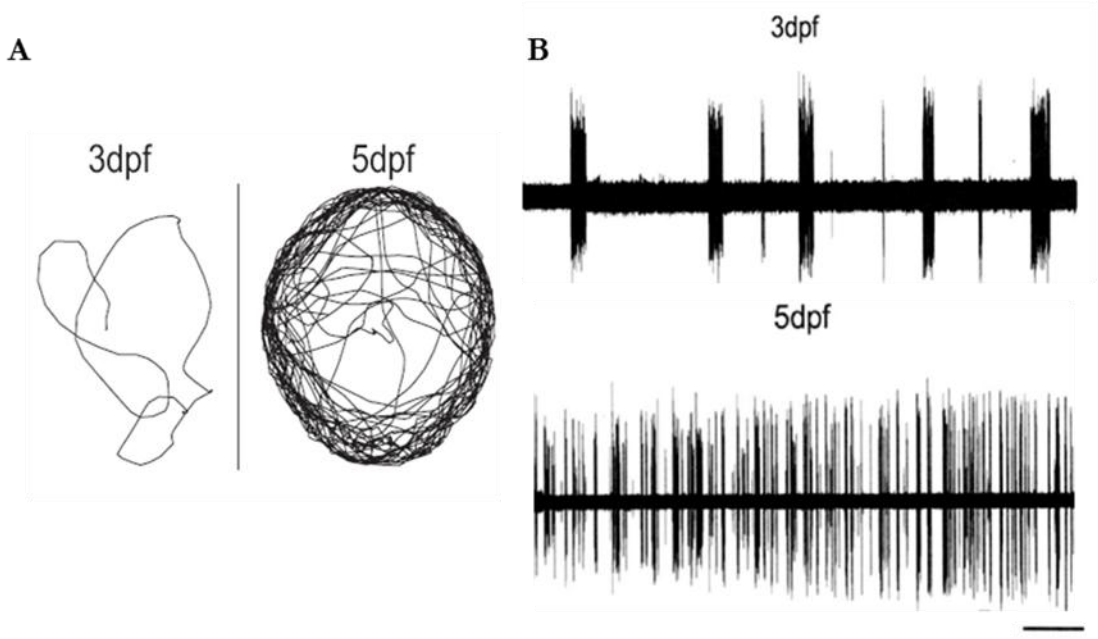

Figure 8. Changes in spontaneous behaviour and motor activity in the zebrafish. (A) Sample trajectories of spontaneous locomotion in the larval zebrafish at 3 and 5dpf. (B) Sample traces showing spontaneous fictive swimming motor pattern ventral root recordings at 3 and 5dpf (Scale Bar 1 min). (Adapted from Thirumalai and Cline 2008)

It is clear that this increase in spontaneous fictive motor activity matches the change in swimming behaviour and this suggests that there is a change in the 'internal

environment' of the animal's nervous system that occurs with development – that matches the new requirement of the animal to predate in the water column (Brustein et al. 2003; Thirumalai and Cline 2008). It is known that the isolated nervous system from metamorphic stages of *Xenopus laevis* produce a spontaneous motor output (see Figures 2 and 3) and it is known that at younger, early larval stages that spontaneous episode are rare (Li et al. 2010), so an important question remains - at what point does this change?

1.3 Aims of the present project

The project aims to describe the increase in spontaneous swimming behaviour produced by *Xenopus laevis* larvae as they enter a free-swimming existence at the onset of free-feeding. To complement this description, an experimental preparation will be developed in order to allow a physiological description of the neural control underlying the transition into a free-living animal. Specifically, the fictive swimming activity will be measured from animals at various stages of development around this onset and pharmacological manipulations used to start to determine the underlying physiology and neuromodulatory context of the pre-metamorphic, free-swimming tadpoles.

"You must work like an astronomer. Prepare for weeks, for months, if necessary, for years, until the method is working to perfection, then do one experiment, perhaps two-and publish your results"

Henry Hallet Dale[3]

Chapter II
Materials and Methods

2.1 Animals

All experiments were performed on either pre-feeding stage 37/38 to 44 or free-feeding stage 45 to 47 *Xenopus laevis* tadpoles, staged according to the Nieuwkoop and Faber normal tables (Nieuwkoop and Faber 1956). Pairs of adult frogs, selected from the laboratory breeding colony, were induced to mate by injection with human chorionic gonadotropin (1000 $i.u.ml^{-1}$; Sigma-Aldrich). The eggs were collected and reared in dechlorinated trays at temperatures between 17-23^{o}C to stagger their development in order that both pre-feeding and feeding stages would be available daily from a weekly injection rota. All procedures conformed to United Kingdom Home Office regulations regarding animal experimentation as described in the Animals (Scientific Procedures) Act, 1986.

2.2 Larval swimming behaviour

A single larva was placed in a shallow circular translucent dish (5cm diameter) on top of graph paper in HEPES saline [composition (in $mmoll^{-1}$): NaCl, 115; KCl, 2.5; $NaHCO_3$, 2.5; HEPES, 10; $MgCl_2$, 1; $CaCl_2$, 4; pH 7.4 with NaOH]. Swimming behaviour was recorded for 15 minutes per animal after a 2 minute acclimatisation

[3] Quotation taken from W.S. Feldberg (1970). The author notes the challenge presented by Henry Dale and is conscious of the continued need to improve in work ethic.

period, using a Casio EXILIM digital camera, 480 frames per second. Displacement (in cm) was calculated by summating, manually, the total displacement between each frame from the recording for the 15 min period.

The percentage of time spent swimming was calculated by observing the animal in the same manner as before but the time spent spontaneously swimming was taken as a percentage of the overall time.

2.3 Intact and semi-intact preparations

Intact preparation: Pre-feeding tadpoles (stage 37/38 – 44) were immobilised by scoring the dorsal and ventral tail skin several times with a tungsten needle and then placed in a solution of HEPES saline, containing 12.5μM α-Bungarotoxin, for approximately 30 minutes. Afterwards, animals were placed in a small (5cm diameter) shallow circular dish with HEPES saline to determine if they were in fact immobilised.

Semi-intact preparation: Free-feeding tadpoles (stage 45-47) were killed by a schedule 1 method; firstly anesthetised in ice-chilled HEPES saline, containing 230μg/ml 3-aminobenzoic acid ethyl ester (Sigma-Aldrich), and then were secured with insect pins in a glass Sylgard-lined Petri dish. The skin overlying the dorsal part of the brain was opened and the forebrain removed, followed by the destruction of the heart, with fine etched tungsten needles. The preparation was then placed, in a small (5cm diameter) shallow circular dish with HEPES saline (usually on ice) in order that swimming behaviour could return. Afterwards, the dorsal and ventral tail skin was subsequently scored with tungsten needles and the preparation transferred to an immobilising solution of HEPES saline, containing 12.5μM α-Bungarotoxin, for approximately 30 minutes. After immobilisation, the preparation was transferred back to the small circular dish ready for electrophysiological recordings.

2.4 Electrophysiological Recordings

Extracellular ventral-root recordings were taken from both intact and semi-intact preparations in the same manner. Immobilised preparations were transferred to a recording bath, circulating 100ml HEPES saline [composition (in $mmoll^{-1}$): NaCl, 115; KCl, 2.5; $NaHCO_3$, 2.5; HEPES, 10; $MgCl_2$, 1; $CaCl_2$, 4; pH 7.4 with NaOH] which was gravity-fed from a header tank into a Perspex chamber. Preparations were then secured, with two micro pins; one through the notochord at the level of the otic capsule and one approximately the 15th post-otic myotomal segment, onto a coated Perspex rotatable Sylgard^{TM} stage. The flank skin on the left and right sides removed with an etched sharp tungsten needle, leaving the myotomes below exposed and intact.

Glass suction electrodes were positioned over the inter-myotomal clefts to measure the fictive swimming by recording the activity of the MN axons that run in between the segments of axial muscle (see Figure 9). Suction electrodes were placed on the left and right side of the animal at approximately the level of the 5th - 14th post-otic inter-myotome. Clefts were numbered from the otic capsule in a rostral to caudal direction.

Signals were amplified using a differential AC amplifier (A-M Systems Model 1700, Carlsborg, WA, USA), digitized using an Axon Instruments Digidata 1322A data acquisition system and viewed on a PC computer using Axoscope software (Axon Instruments, Union City, CA, USA).

Electromyography (EMG) recordings were made following the same procedure, except the preparations were not immobilised with α-Bungarotoxin.

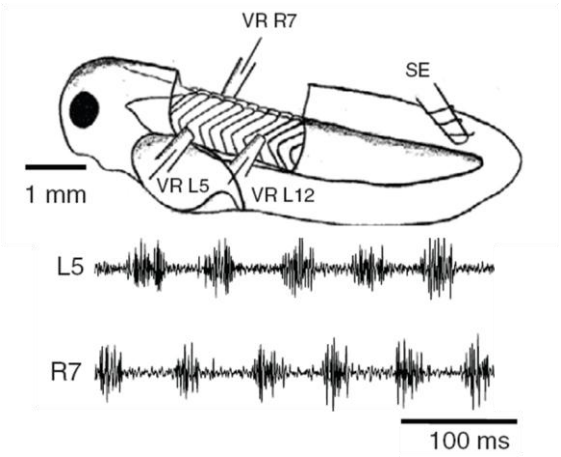

Figure 9. Schematic of a *Xenopus* stage 42 fictive recording preparation. Head pointing to the left and the skin over rostral myotomes removed. Suction electrodes placed over the inter-myotomal clefts, VR L5 and VR R7, sample ventral root bursts are shown with left-right alternation. VR L12 sample trace has not been included. In order to obtain episodes of swimming a stimulating electrode was placed on the intact caudal tail skin (SE). (Adapted from Sillar and Robertson 2009)

2.5 Data Analysis

The electrophysiological data were analysed using Dataview software (version 6.3.2, courtesy of Dr. W. J. Heitler), and all raw data transferred into Microsoft Excel spreadsheets and statistical analysis carried out in SPSS 17. Means of each condition compared with control (where N/F stages were used as the independent variable, stage 37/38 or all the pre-feeding stages, 37/38-44, were treated as the control) in a student's T-test or means were compared with a univariant ANOVA and a post-hoc Tukey's Honest Significant Difference. Data values are presented as means ± S.E.M, with a significance level of ($p<0.05$), unless otherwise stated.

Ventral root bursts were detected by setting a threshold cursor at the voltage level of maximum noise, during a quiescent period in the recording, and all periods where the events were larger were marked. Occasionally traces were rectified where the signal to noise level was low. Outliers were removed by setting a minimum onset time, usually

ventral root bursts had to be greater than 10 msec to be included, or were removed manually.

Spontaneous swimming activity was calculated as a percentage of the total time spent in fictive swimming compared with the overall time. Ventral root bursts usually occurred in episodes but episodes were of varying duration, both within and between preparations. However, ventral root bursts that occurred alone or in groups of less than three were not included in the analysis. In addition, the first 5 minutes of the recording were not included in the analysis to prevent any influence of the experimental procedure on the spontaneous motor output. Frequency and burst duration of fictive swimming was determined by sampling at least 30 cycles of ventral root bursts from episodes of fictive swimming activity selected at random from each experimental period. Frequencies higher than 30Hz and lower than 5Hz were removed from analysis.

2.6 Drug Application

For pharmacological manipulations, drugs applied throughout experiments were added to the header tank, usually after at least a 15-20 min control period, and perfused throughout the experimental setup. Drugs used throughout include: gabazine (SR95331) (10-20μM), bicuculine (4-20μM), DEA/NO (200-400μM), L-NAME (1-5mM), PTIO (1mM), riluzole (5μM) (4-10μM), veratridine (90nM), ouabain (5μM) and NMDA (100μM). Drugs were obtained from Sigma Aldrich. Final stock solutions were achieved by adding aliquots to the circulating solution.

"Given her deafness, the auditory part of the brain, deprived of its usual input, had started to generate a spontaneous activity of its own, and this took the form of musical hallucinations, mostly musical memories from her earlier life. The brain needed to stay incessantly active, and if it was not getting its usual stimulation..., it would create its own stimulation in the form of hallucinations."

Oliver Sacks, *Musicophilia: Tales of Music and the Brain* (2007).

Chapter III

Results: Part I

Basic properties of spontaneous swimming

3.1.1 Increase in spontaneous behaviour with development

Newly hatched *Xenopus laevis* tadpoles (stage 37/38) display very little spontaneous or endogenous locomotion but instead lead a dormant life, until they progressively acquire a more free-living existence at the onset of feeding at stage 45 - when the animal develops a gut (see Figure 10). The behaviour of animals was measured at stage 37/38 and stages 42 through to 47 (at the point the limb buds start to form (Nieuwkoop and Faber 1956)). A gradual increase in swimming motion was observed, shown as the natural log of displacement (ln(cm)) in the water column, from stage 45 (see Figure 10A). Compared with the level of spontaneous swimming observed at stage 37/38 (n=7, mean=2.46 ± 0.21 ln(cm)), which acts as a baseline or control, stage 45 (n=7, mean=5.8 ± 0.56 ln(cm)), stage 46 (n=7, mean=6.6 ± 0.4 ln(cm)), and stage 47 (n=7, mean=7.24 ± 0.23 ln(cm)) tadpoles showed a significantly higher level of spontaneous swimming ($p<0.01$). Figure 10B, shows swim trajectories drawn from the video footage at both stages 37/38 and 45, and the direct comparison revealed not only that stage 45 tadpoles spontaneously swam

significantly further than their stage 37/38 counterparts but the way in which they swim is different. Stage 37/38 tadpoles swim in a much more episodic fashion, the rare spontaneous swimming episodes have a clear beginning and end, whereas at stage 45 the tadpoles tend to swim around the dish and show a greater waxing and waning of swimming rather the binary distinction between swimming and not swimming observed in the younger pre-feeding tadpoles. By quantifying the time spent actively swimming, shown as a percentage of overall time at selected stages of development (see Figure 10C), the pre-feeding tadpoles at stage 37/38 ($n=7$, mean=1.6 ± 0.2%) which was again treated as the baseline, and stage 43 ($n=7$, mean=4.16 ± 2%) spent very little time spontaneously swimming. The free-feeding tadpoles stage 45 ($n=7$, mean=15.89 ± 3.2%, $p<0.05$) and stage 47 ($n=7$, mean=36.82 ± 5.2%, $p<0.01$) were significantly greater than the pre-feeding baseline in the time spent spontaneously swimming.

Overall, the behavioural data show a clear distinction between the amount of spontaneous swimming, in terms of distance, manner and duration, between two emerging groups of tadpoles between stage 37/38 and 44 which showed no significant difference between these parameters and the stage 45 to stage 47 tadpoles which showed a significantly greater spontaneous swimming distance travelled and time spent swimming compared with the stage 37/38 baseline. These distinctive groups also match the grouping of tadpoles into pre-feeding tadpoles (stage 37/38 – 44) and the free-feeding tadpoles from stage 45 onwards. There therefore appears to be a coincidence between a free-feeding existence and a free-swimming lifestyle. To further dissect out the properties of the free-swimming animal, a semi-intact preparation was developed in order to record fictive swimming from the older larval stages in order to better understand the characteristics of the motor pattern.

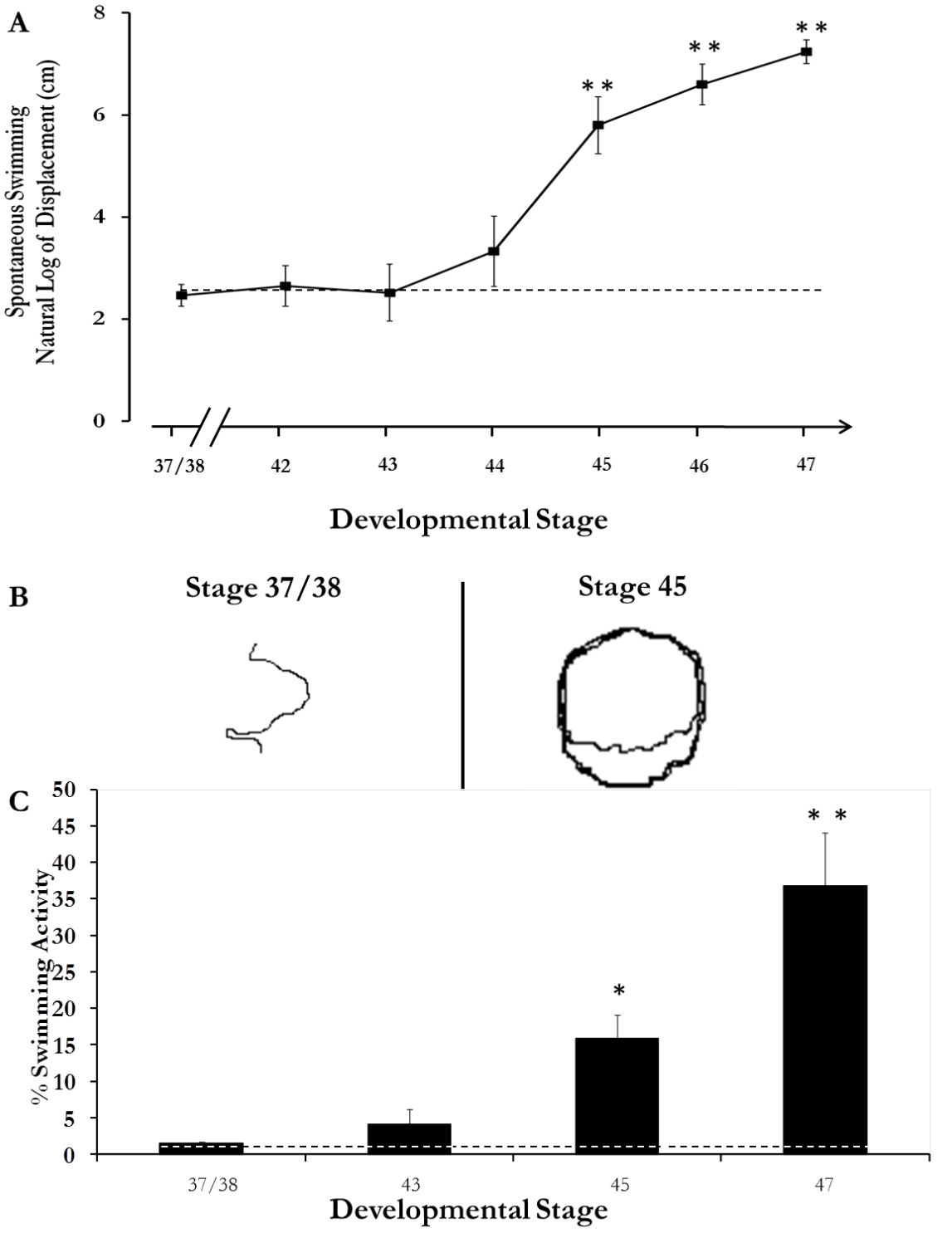

Figure 10. **Increase in spontaneous behaviour with development. A)** Line graph of natural log of displacement, showing an increase in spontaneous swimming at the onset of feeding (stage 45). **B)** Spontaneous swim trajectories at stage 37/38 and stage 45. **C)** Bar graph of percentage spontaneous swim activity at sampled developmental stages (* ,$p<0.05$; **,$p<0.01$).

3.1.2 Increase in spontaneous fictive swimming with development

Stage 45 animals were specifically selected for investigating free-swimming behaviour because this stage was the first to demonstrate a clear departure from the younger and more dormant larvae at the behavioural level of spontaneous swimming. Further experiments carried out at stages 46 and 47 used the same protocol.

Figure 11Ai, shows a schematic diagram of the stage 45 electrophysiological preparation, alongside the characteristic left-right alternation of the ventral root bursts expected of fictive swimming locomotion, recorded from glass suction electrodes (Figure 11Aii). This fictive swimming activity corresponds with the endogenous activity observed in Figure 10, as there is a spontaneous rhythm of fictive locomotion present that is intrinsic to the preparation (Figure 11Bi). This pattern can be compared over the same time-scale with the fictive swimming in the younger stage 37/38 animals (Figure 11Bii) which shows a single evoked episode of swimming and no spontaneous swimming bouts whatsoever. The extent of spontaneous fictive swimming, calculated as a percentage of overall time, shows an increase in spontaneous motor activity at stage 45 ($n=5$, mean=20.36 ± 15.4%, $p<0.05$) and stage 47 ($n=5$, mean=69.86 ± 23.5%, $p<0.01$) compared with the baseline stage 37/38 ($n=2$, mean=0.1 ± 0.02%) which was not significantly different than stage 43 ($n=4$, mean=1.4 ± 1.1%, n.s). This result is essentially similar to that presented in Figure 10C and shows that there is an increase in spontaneous fictive swimming from preparations derived from free-feeding stages.

Overall, this suggests that the newly developed preparation replicates the behavioural data favourably and is a useful experimental approach to investigating free-swimming activity in *Xenopus*. Furthermore, there appears to be a significant behavioural and physiological difference between preparations from pre-feeding (stage 37/38 – 44) and free-feeding (stage 45-47) tadpoles and this is further explored in the next section.

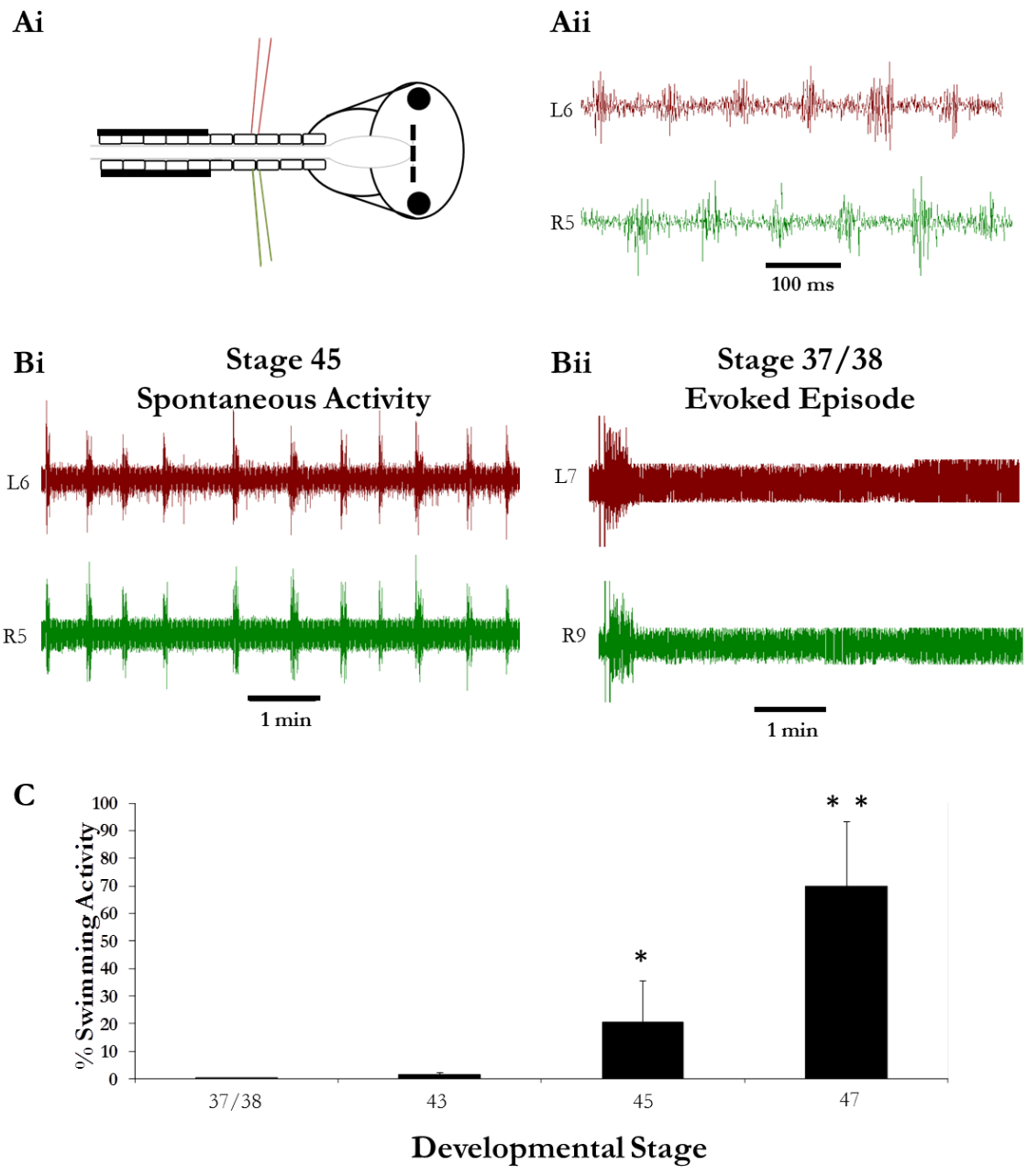

Figure 11. Increase in spontaneous fictive swimming over development. Ai) Schematic of stage 45 preparation. Dashed line shows the level at which the forebrain was removed in accordance with the Schedule 1 method. Suction electrodes left (shown in red) and right (shown in green). **Aii)** Ventral root burst from spontaneous fictive swimming showing left/right alternation. **Bi)** Sample traces of spontaneous fictive swimming at stage 45. **Bii)** Sample traces of an evoked swimming episode at stage 37/38. **C)** Bar graph of percentage fictive swimming activity (* ,$p<0.05$; **,$p<0.01$).

3.1.3 Differences between pre- and free-feeding fictive swimming

Following on from the behavioural differences in spontaneous swimming (see Figure 10) and the spontaneous fictive swimming recorded from pre-feeding and post-feeding (semi-intact) preparations (see Figure 11), further physiological differences in fictive swimming between the two groups were explored. It has previously been show that there is an increase in burst duration with development, between stage 37/38 and stage 42 (Sillar et al. 1992). To determine if there was a further increase in burst duration with development, the duration of ventral root bursts from evoked episodes of pre-feeding tadpoles (stages 37/38-44) was compared with that of ventral root bursts from spontaneous episodes recorded from the semi-intact preparations, representative of the late larval free-feeding stages (45-47). Figure 12Ai, shows sample traces of ventral root bursts from a pre-feeding preparation and a semi-intact free-swimming preparation. There was a statistically significant increase in burst duration (see Figure 12Aii) from pre-feeding (n=4, mean=16.1 ± 3.7msec) to free-feeding preparations (n=4, mean=38.7 ± 8.3msec, $p<0.05$). In Figure 12B, episode duration was measured from evoked episodes from pre-feeding stages and compared with spontaneous episodes from the semi-intact preparations (see Figure 12Bi). Figure 12Bii, shows a decrease from the pre-feeding preparations (n=4, mean=29.7 ± 5.1s) to the free-feeding preparations (n=4, mean=8.6 ± 10.1s, n.s.). Although this decrease was not statistically significant, there was considerable variability within episode durations in the spontaneous fictive swimming recorded from the semi-intact free-feeding preparations and this may have contributed to this result. Similarly, the fact that evoked pre-feeding episodes were compared with spontaneous free-feeding episodes may well have affected both episode and burst duration. In order to help clarify this conflict, evoked and spontaneous episodes were compared in free-feeding preparations (see figure 13).

Figure 13 compares burst and episode durations from free-feeding preparations that have either been evoked or have occurred spontaneously. There was no significant difference between free-feeding burst durations (see figure 13A) from evoked episodes (n=4, mean=33.69 ± 6.8msec) compared with those from spontaneous episodes (n=4, mean=38.7 ± 8.3msec, n.s.). Furthermore, there was no difference in episode duration (see Figure 13B) between evoked episodes (n=4, mean=13.78 ± 12.7s) and spontaneous episodes (n=4, mean=8.6 ± 10.1s, n.s.) and both were highly variable. This result suggests firstly, that the average burst duration is independent of whether bouts of fictive swimming occur due to a stimulus or spontaneously. Additionally, the results suggest that episode duration is highly variable, either when episodes are evoked or occur spontaneously, and therefore episode duration was not considered further as an effective measure of fictive swimming activity in the semi-intact preparations. The percentage of fictive swimming activity (see Figure 11C), where the activity was quoted as a percentage of overall time recorded, was used in preference. Further exploration of how evoked or spontaneous preparations may alter the nature of the fictive swimming recorded in the semi-intact preparations, was analysed by measuring the frequency of swimming – in order to determine if there are any changes across a period of activity.

The frequency of fictive swimming in semi-intact preparations was compared between evoked and spontaneous activity (see Figure 14). Evoked episodes of activity were found to have a stereotyped pattern of fictive swimming (see Figure 14A), where there was an initial rise in swimming frequency to a peak value (n=4, mean=26.1 ± 3.89Hz) which decreases across the episode to give a lower average swimming frequency (n=4, mean=20.02 ± 4.04Hz). In contrast, episodes of spontaneous activity (see Figure 14B) have multiple peaks of higher frequency swimming (n=4, mean=21.3 ± 3.49Hz), which regularly repeat (n=4, mean=1.4 ± 0.57s) within periods of activity, with an overall lower average swimming frequency (n=4, mean=14.75 ± 3.27Hz).

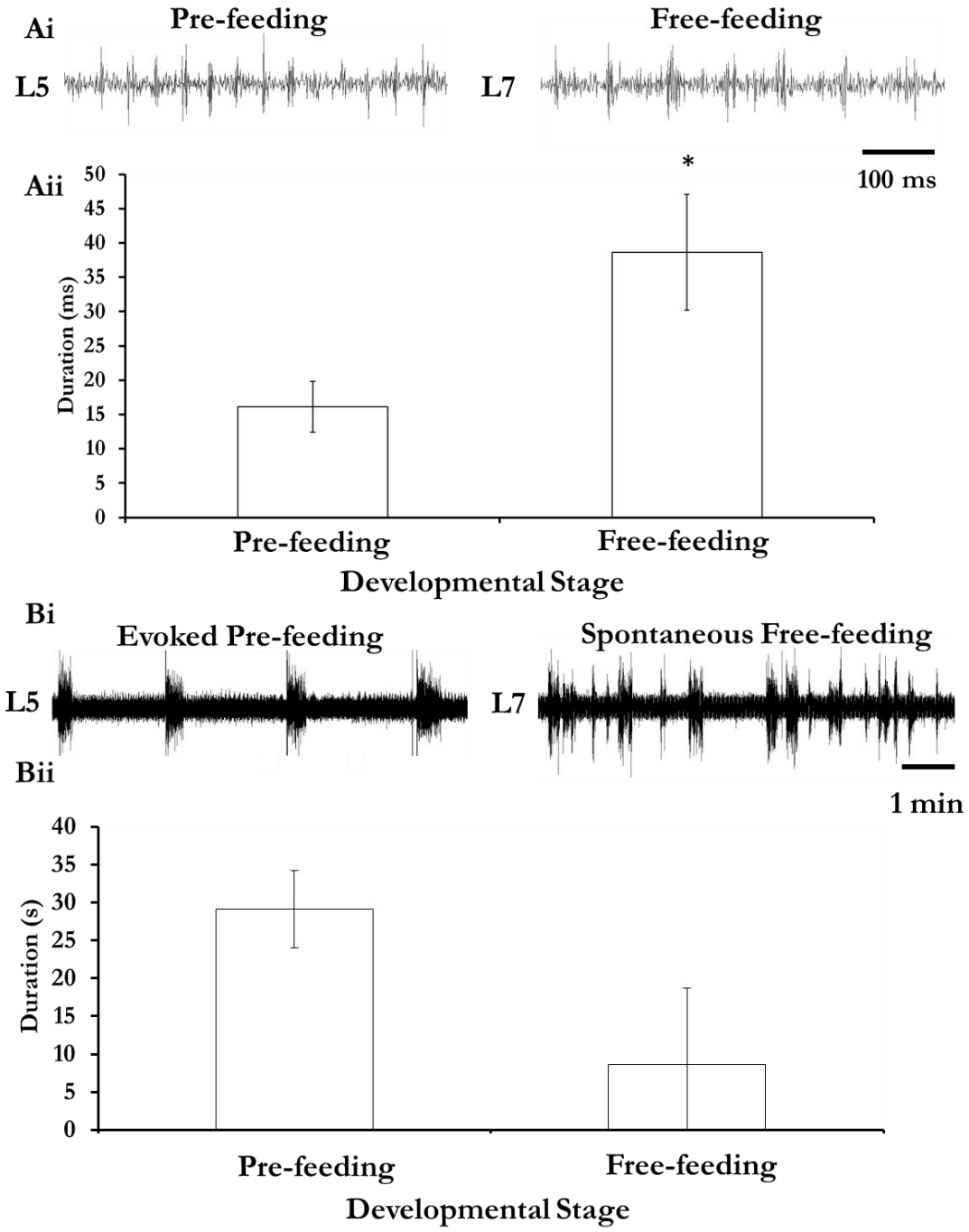

Figure 12. Changes in burst and episode duration with development. Ai) Sample ventral root burst traces from a pre-feeing preparation and a free-feeding semi-intact preparation. Scale Bar 100msec. **Aii)** Bar graph of ventral root burst duration shows a significant increase in burst duration with development (*, $p<0.05$). **Bi)** Sample traces of evoked pre-feeding episodes and of spontaneous free-feeding episodes. Scale Bar 1 min. **Bii)** Bar graph of episode durations shows a decrease in episode duration.

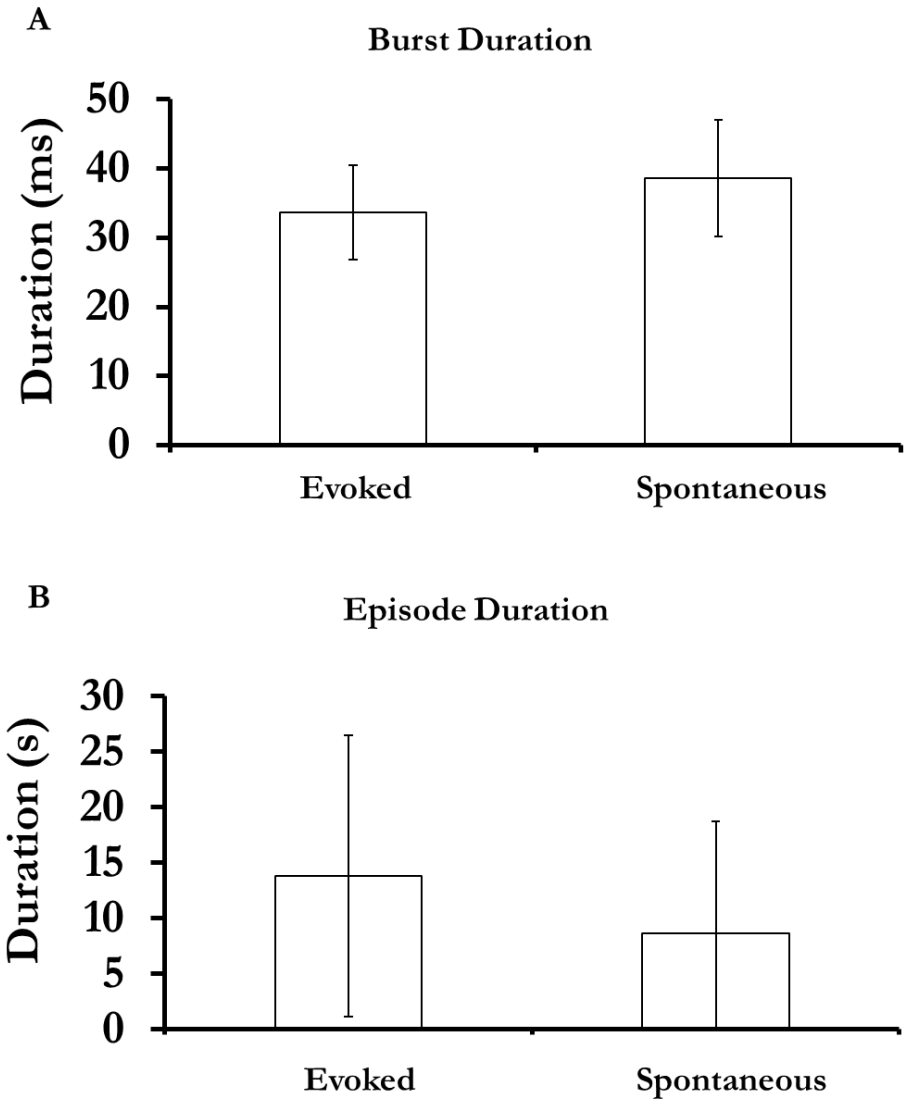

Figure 13. Evoked and spontaneous episodes in semi-intact preparations. A) Bar graph of burst duration in evoked (n=4, mean=33.69 ± 6.8msec) and spontaneous (n=4, mean=38.7 ± 8.3msec, n.s.) episodes shows no significant difference in .burst duration. **B)** Bar graph of episode duration in both evoked (n=4, mean=13.78 ± 12.7s) and spontaneous (n=4, mean=8.6 ± 10.1s, n.s.) shows no significant difference but does show a similar degree of variability in episode duration.

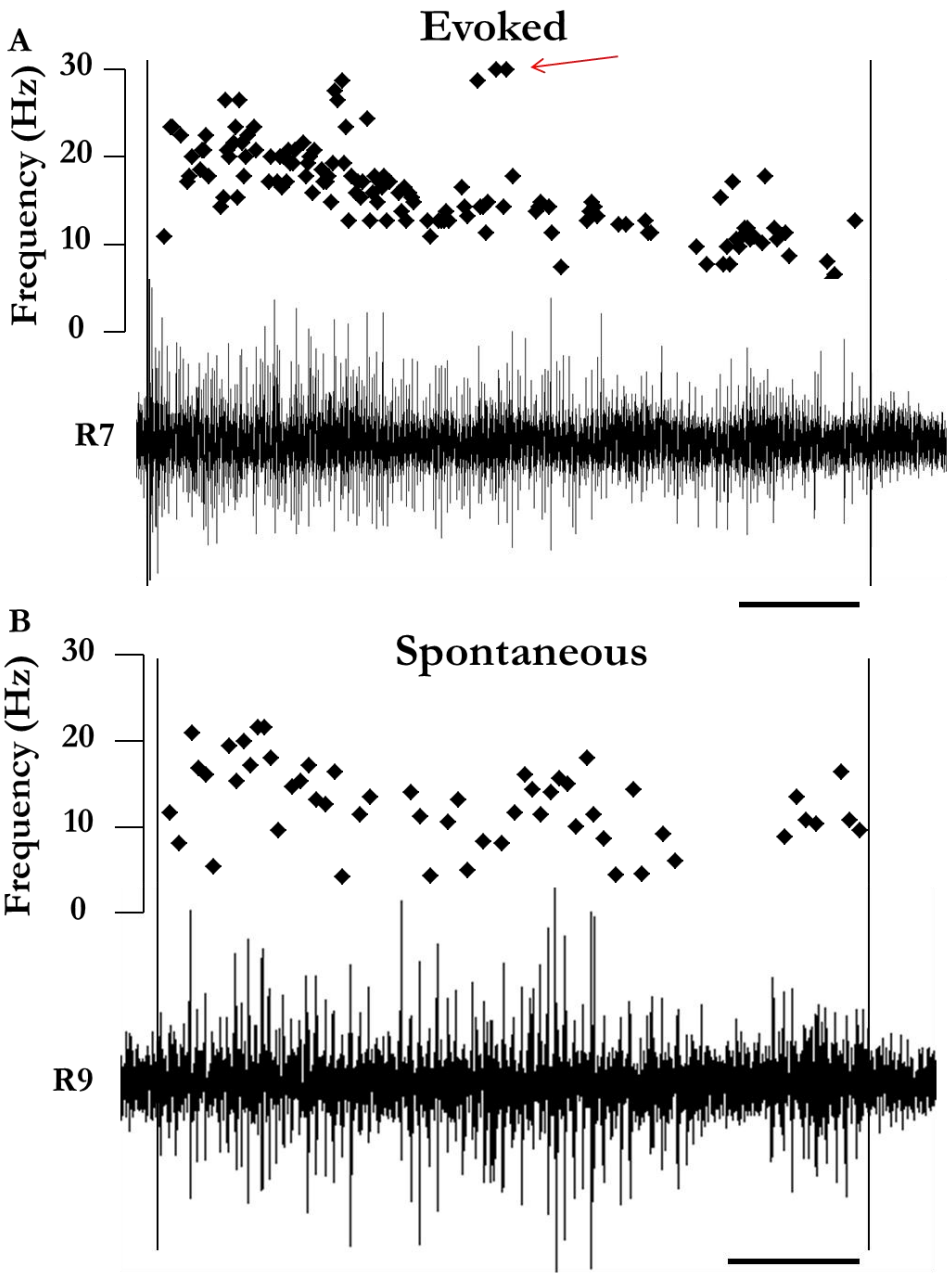

Figure 14. Frequency changes between evoked and spontaneous episodes. A) Sample trace of evoked activity with the distribution of fictive swimming frequency across the episode. There is a characteristic pattern of frequency across the period of activity, with a mean frequency (n=4 preparations, mean=20.02 ± 4.04Hz) which follows an initial increase in frequency. Accelerations of swimming frequency within the episode of activity can be observed (red arrow). Scale bar 10s. **B)** Sample trace of spontaneous activity with frequency distribution which shows a slower average frequency (n=4 preparations, mean=14.75 ± 3.27Hz) that than found in the evoked episode and shows cyclical periods of higher and lower frequency which repeat (n=4 preparations, mean=1.4 ± 0.57s). Scale Bar 5s.

The pattern of fictive swimming frequency was different between evoked activity and spontaneous activity. Although they share similar average fictive swimming frequencies and also show periods of higher swimming frequency, evoked activity only shows this period of more rapid fictive swimming at the beginning of episode whereas spontaneous activity show more modest increases in swimming frequency throughout periods of fictive swimming – creating a more sinusoidal appearance of activity. This suggests that spontaneous episodes are not merely the same as having evoked episodes one after the other. To help try to understand the origin of this spontaneous activity, EMG recording were performed to principally yield a better signal to noise ratio to improve the frequency analysis and additionally to try and determine the extent of any proprioceptive feedback on the pattern of evoked and spontaneous swimming.

3.1.4 Properties of EMG recordings

EMG recordings were made from semi-intact preparations, which were prepared exactly as before but preparations were not immobilised with α-Bungarotoxin and had muscle contractions (see Figure 15). Ventral root bursts of greater amplitude were present in EMG recordings (see Figure 15Ai) compared with fictive swimming but the burst durations of either evoked (n=4, mean=28.53 ± 3.48ms) or spontaneous (n=4, mean=28.46 ± 4.78ms) EMG recordings were similar and not significantly different from each other, or fictive burst durations (n=4, 38.68 ± 8.43ms) (see Figure 15Aii). A further comparison of spontaneous EMG swimming and spontaneous fictive swimming (see Figure 15B) revealed that there was no significant difference between the percentage of spontaneous swimming in the EMG recordings (n=4, mean=29.1 ± 13.64%) compared with spontaneous fictive swimming (n=4, mean=36.82 ± 15.2%). In addition, there was no significant difference between fictive swimming in a stagnant bath

preparation - where there was no circulating saline – and either the EMG or spontaneous fictive swimming recorded in the normal experimental setup (n=2, mean=25.07 ± 11.85%, n.s.). This result suggests that there is unlikely to be a major role played by any sensory feedback, from actively swimming, in the EMG recordings that would help generate the spontaneous swimming activity. The stagnant bath preparation was an attempt to determine whether any movement of saline may have caused a change or have contributed to the cause of the spontaneous motor activity. This activity is still present when the pump was turned off in the stagnant bath preparation, suggesting that centrally generated activity is the source of the spontaneous rhythm.

The pattern of swimming frequency was similar in both the evoked (n=4, mean=10.16 ± 1.9Hz) (Figure 16A) and spontaneous (n=4, mean=13.89 ± 2.85Hz) (Figure 16B) EMG recordings compared with that of the fictive swimming, although the EMG recordings had a lower swimming frequency.

Overall, EMG recordings provide a useful approach to studying the spontaneous swimming in the semi-intact preparations and are quantitatively similar to the fictive swimming recordings. In addition, it is becoming clearer that the origin of the spontaneous rhythm is dependent on central nervous activity, presumably at least somewhere between the mid-brain and the rostral spinal cord. To explore further the origins of spontaneous swimming, lesions were made to the brains of the semi-intact preparations and EMG recordings taken to determine how this may alter the spontaneous swimming EMG recorded.

3.1.5 Lesions to the midbrain retain spontaneous activity

Semi-intact preparations from free-feeding stages already have lesions to the brain, as the forebrain is removed under anaesthesia. To understand what role other brain areas may have in the spontaneous generation of swimming, more caudal areas of the brain were removed. Figure 17A, shows a schematic of the brain and rostral spinal cord in the free-feeding semi-intact preparation after the removal of the forebrain (grey lines). A sample trace of EMG spontaneous swimming in the normal semi-intact preparation is shown in Figure 17Ai. The schematic in Figure 17B shows the removal of both the midbrain and the forebrain with a sample trace of spontaneous EMG swimming shown in Figure 17Bi. Activity was still present in this preparation and suggests that the central generation of spontaneous swimming may not require the midbrain. The overall percentage of swimming activity, see Figure 17B, was reduced in this midbrain lesioned preparation (n=4, mean=17.04 ± 6.96%) but it was not significantly different from the normal semi-intact preparation (n=4, mean=29.1 ± 13.64%).

The removal of the entire brain, leaving only the spinal cord intact, shown in a schematic in Figure 18A - produced no spontaneous swimming activity and it was unable to evoke EMG swimming activity from this preparation, sample trace shown in Figure 18Ai. Activity could however be obtained from this spinal preparation with the application of the excitatory agonist NMDA (100μM), which washed in to generate near continuous swimming, Figure 18Aii. This suggests that the preparation was capable of generating a coordinated motor output but that without the presence of at least the hindbrain no spontaneous activity would occur. The bar graph in Figure 18B summarises the overall effect of lesions on spontaneous EMG swimming and compares the percentage of swimming activity in the different lesion conditions, along with the spinal preparation with NMDA (n=3, mean=54.57 ± 20.71%). Although no significant

difference was observed between forebrain and midbrain lesioned preparations, the trend was a decline in spontaneous EMG swimming, with the absence of any spontaneous activity in the hindbrain lesioned preparations. Overall, the lesion results lend further support to a central nervous system source for the spontaneous motor activity and suggest a role for the hindbrain in generating this spontaneous activity. To further develop an understanding of how this spontaneous motor activity may be controlled and generated centrally, a further pharmacological investigation of the fictive swimming was undertaken in Part II.

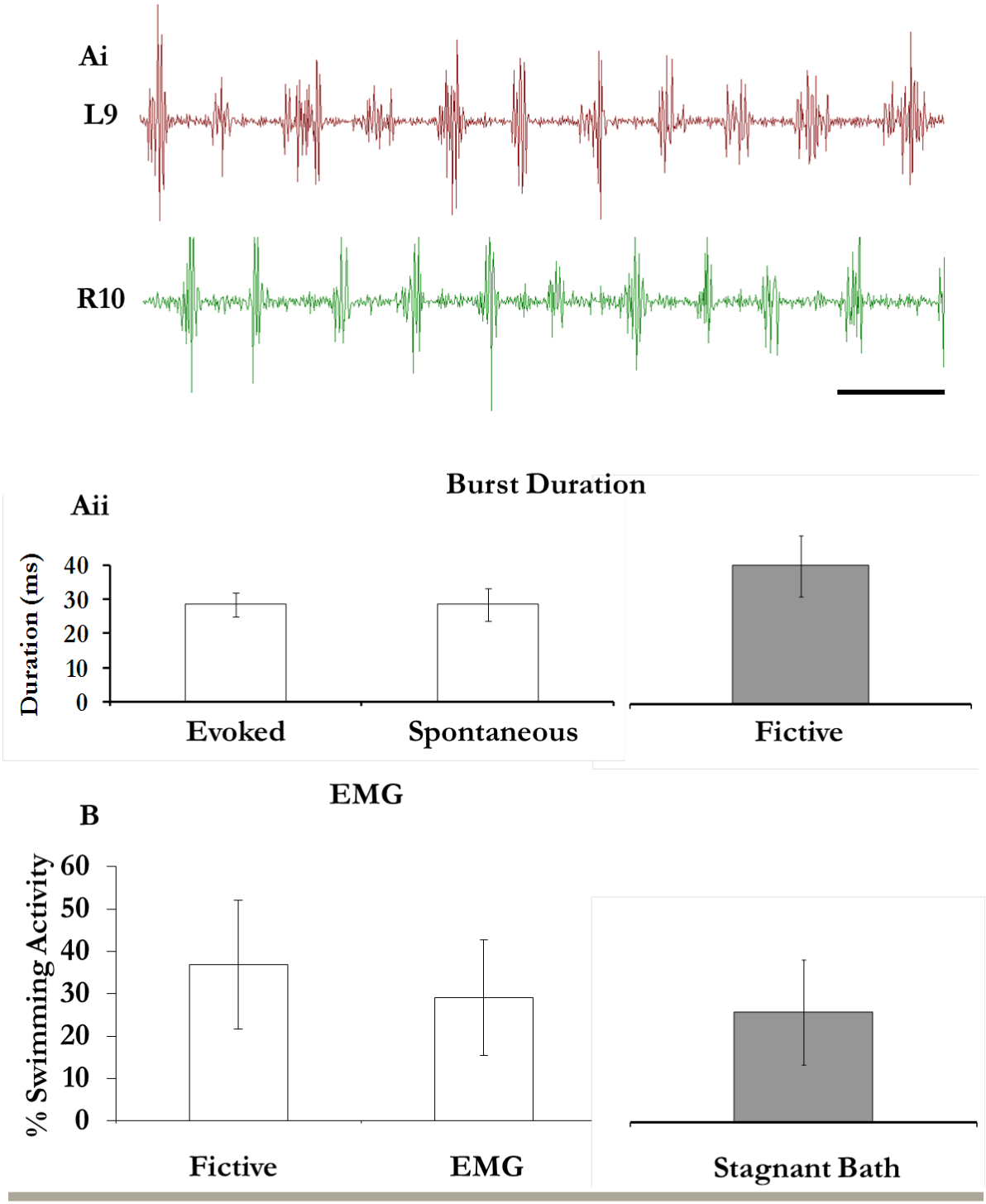

Figure 15. EMG recordings from semi-intact preparations. Ai) Sample traces showing left/right alternation of ventral root burst from spontaneous EMG recorded swimming. Scale Bar 100ms. **Aii)** Burst durations of either evoked (n=4 preparations, mean=28.53, +/-3.48ms) or spontaneous (n=4 preparations, mean=28.46, +/-4.78ms) EMG recordings showed no significant difference, or with that of fictive burst durations (grey bar graph). **B)** Bar graph of percentage swimming activity in fictive (n=4 preparations, mean=36.82, +/-15.2%), EMG (n=4 preparations, mean=29.1, +/-13.64%) and stagnant bath (n=2 preparations, mean=25.07, +/-11.85%,) which shows no significant differences between the groups.

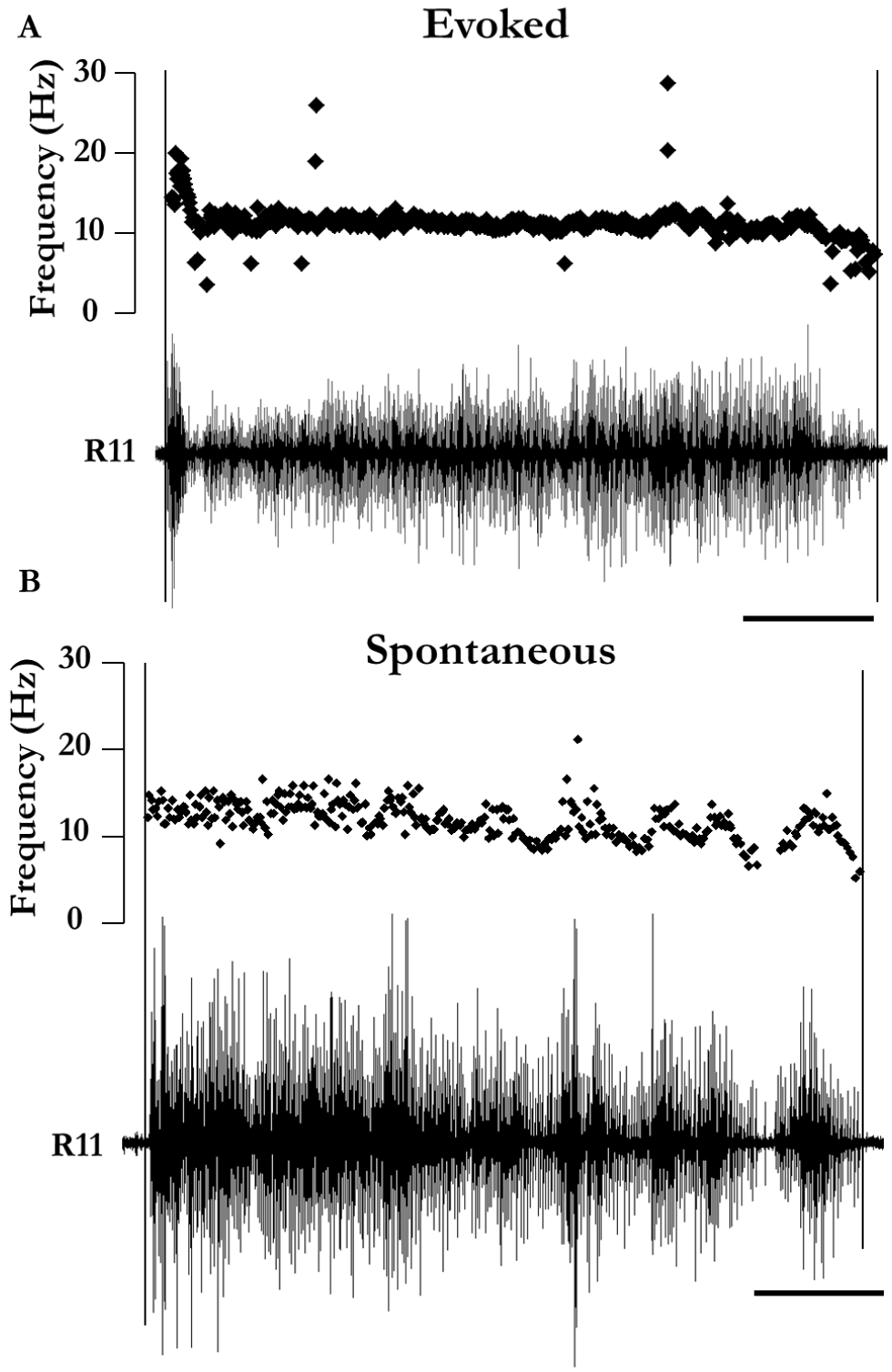

Figure 16. **EMG frequency analysis. A)** Sample trace of an evoked episode of EMG swimming activity with frequency distribution which showed a characteristic pattern of an initial increase in frequency which reduced to a lower average frequency(n=4 preparations, mean=10.16 ± 1.9Hz). Scale bar 10s. **B)** Sample trace of spontaneous EMG recordings with average frequency (n=4 preparations, mean=13.89 ± 2.85Hz). Scale Bar 10s.

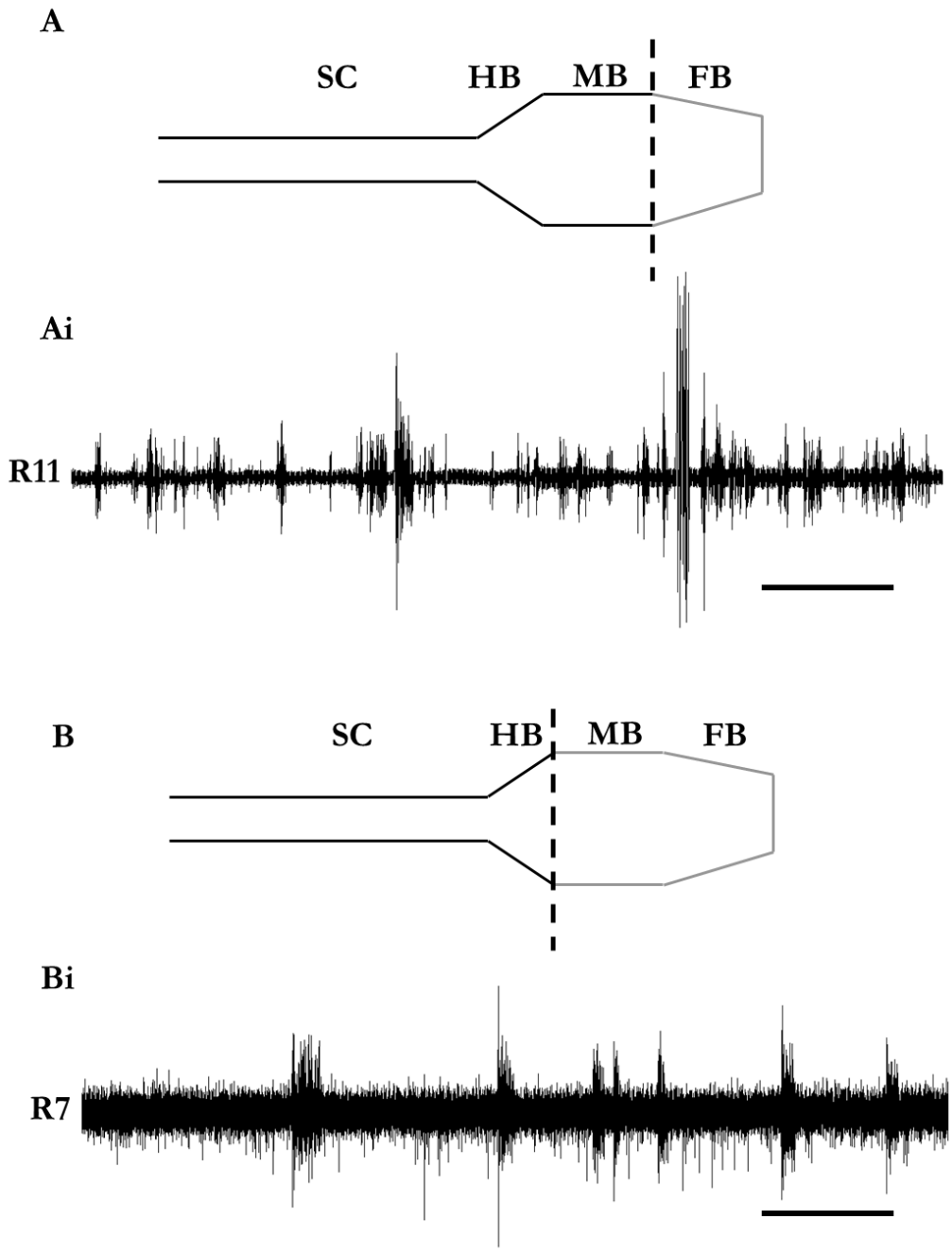

Figure 17. **Midbrain lesions retain spontaneous activity. A)** Schematic of brain and rostral spinal cord. Grey line shows the area of the forebrain removed. Dashed line shows the level of the lesion. **Ai)** Sample trace of EMG recording. Scale Bar 10s. **B)** Schematic of brain and rostral spinal cord, showing the removal of both the midbrain and forebrain outlined in grey. **Bi)** Sample trace showing spontaneous swimming EMG from a preparation bearing a lesion to the midbrain and hindbrain. Scale Bar 10s. SC: Spinal Cord. HB: Hindbrain. MB: Midbrain. FB: Forebrain.

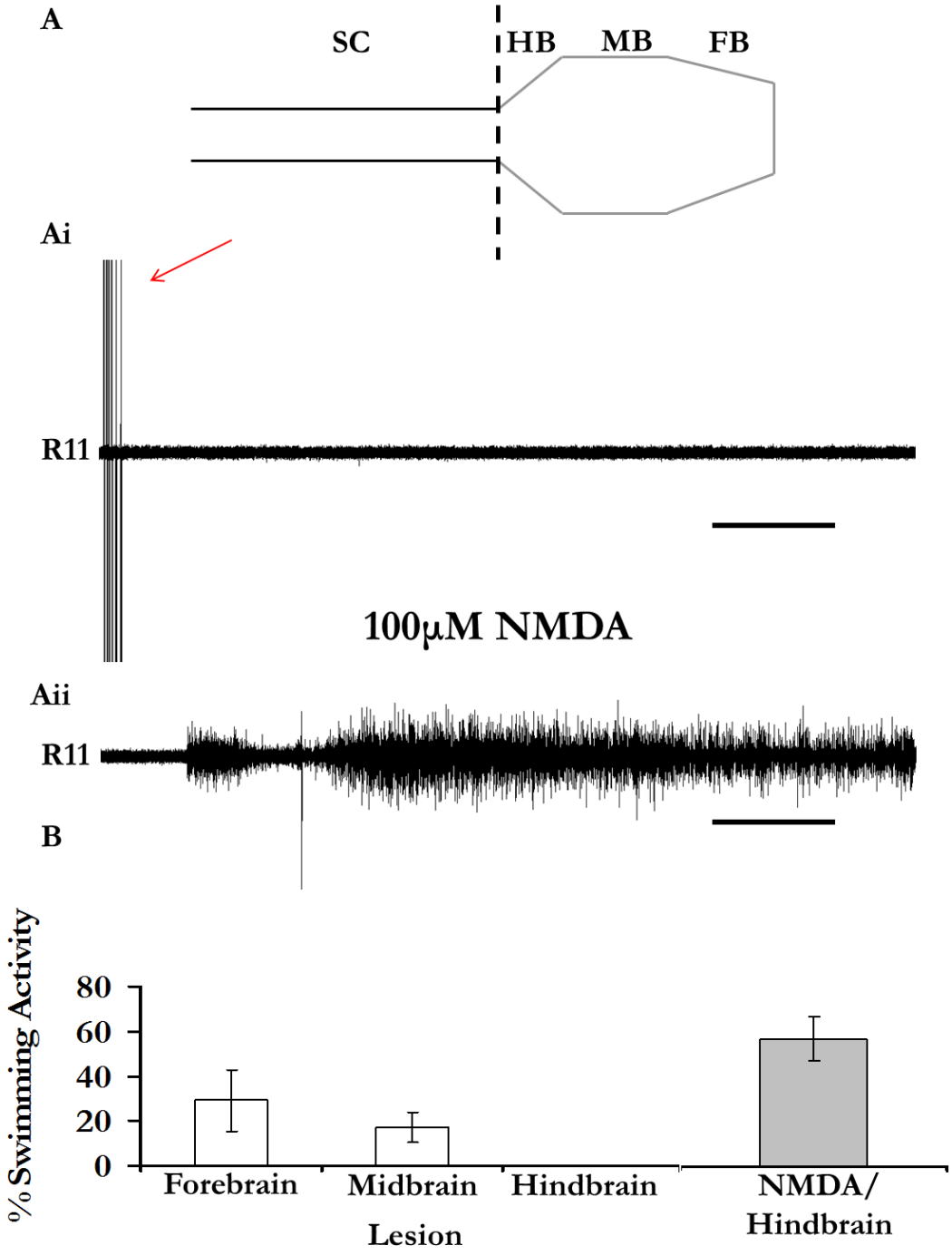

Figure 18. NMDA can rescue spontaneous activity. A) Schematic shows a lesion to the whole brain, spinalisation of the preparation. **Ai)** Sample trace showing no spontaneous EMG and no evoked episodes were produced either (red arrow shows stimulus artefact). Scale Bar 10s **Aii)** NMDA (100µM) applied to the hindbrain lesioned preparation restores EMG swimming activity. Scale Bar: 10s. SC: Spinal Cord. HB: Hindbrain. MB: Midbrain. FB: Forebrain. **B)** Shows bar graph summarising the effect of lesions on the semi-intact preparation spontaneous activity. The grey bar shows the effect of a hindbrain lesion, spinalisation, with NMDA (100µM).

Results: Part II

3.2.1 NMDA rhythm differs from spontaneous activity

In the first part of the results chapter, it was established that the semi-intact preparation taken from the free-feeding stages (45-47) with the forebrain and heart removed, produces spontaneous fictive swimming and that this activity was shown to be coincident with the increase in spontaneous swimming behaviour in the developing larval *Xenopus laevis*. Additionally, it was shown that this spontaneous activity could still be produced in the absence of the midbrain, and also without a circulating volume of saline, but not without the hindbrain. Completely spinalised preparations, where the brain is completed removed; do not show any form of spontaneous activity and activity can only be rescued by the application of the excitatory agonist NMDA to the preparations.

To further explore the excitatory effect of NMDA on the semi-intact preparation (with the hindbrain removed but the mid-hindbrain still intact), NMDA (100μM) was applied and the fictive swimming activity recorded from before and after NMDA application. Figure 19A, shows sample traces of control, NMDA and wash conditions, and shows that there is an overall increase in fictive swimming activity in the presence of NMDA (n=4, mean=42.57 ± 7.12%, $p<0.05$) compared with the control (n=4, mean=12.45 ± 5.95%) and wash (n=3, mean=21.74 ± 5.64%) conditions (see Figure 19B). Although NMDA increased the spontaneous activity, it was observed that the nature of the activity was different from that of the control spontaneous activity. Firstly, the frequency of the NMDA rhythm was much lower (n=4, mean=3.14 ± 1.71Hz) than the control fictive swimming rhythm (see Figure 20A and compare with Figure 14B) and had a much narrower range. Additionally, the NMDA rhythm was much more regular

(Figure 20B) and would repeat bouts of activity at regular intervals (n=4, mean=3.86 ± 2.18s) compared with control fictive swimming (n=4, mean=23.99 ± 18.63s). Furthermore, bouts of activity had a regular duration (n=4, mean=1.92 ± 0.92s) compared with the large variability observed in spontaneous episodes (see figure 13B). This suggests that maturing into a network capable of producing spontaneous bouts of swimming may not just utilise a straightforward increase in excitation to drive this change.

3.2.2 Inhibitory control of spontaneous activity

Inhibitory control of behaviour is important in earlier stages of *Xenopus laevis*, where spontaneous activity remains low. The role of inhibitory control was investigated in the semi-intact preparation.

Glycinergic inhibition was investigated (see Figure 21) by applying the glycine receptor antagonist strychnine (5μM) to the semi-intact preparation. Initially, strychnine caused an increase in swimming frequency (Figure 21B) before causing ventral root bursts to fuse resulting in a synchronised left and right bursts of high amplitude (Figure 21C). The bar graph in Figure 22A, shows the overall increase from a control (n=3, mean=14.75 ± 3.27Hz) to the higher average fictive swimming (n=3, mean=16.98 ± 2.17Hz, $p>0.05$) although not significant. Interestingly, the application of strychnine, even at this high concentration of 5μM, did not alter the overall level of spontaneous activity (n=3, mean=101.16 ± 3.78%), measured as a percentage of the control.

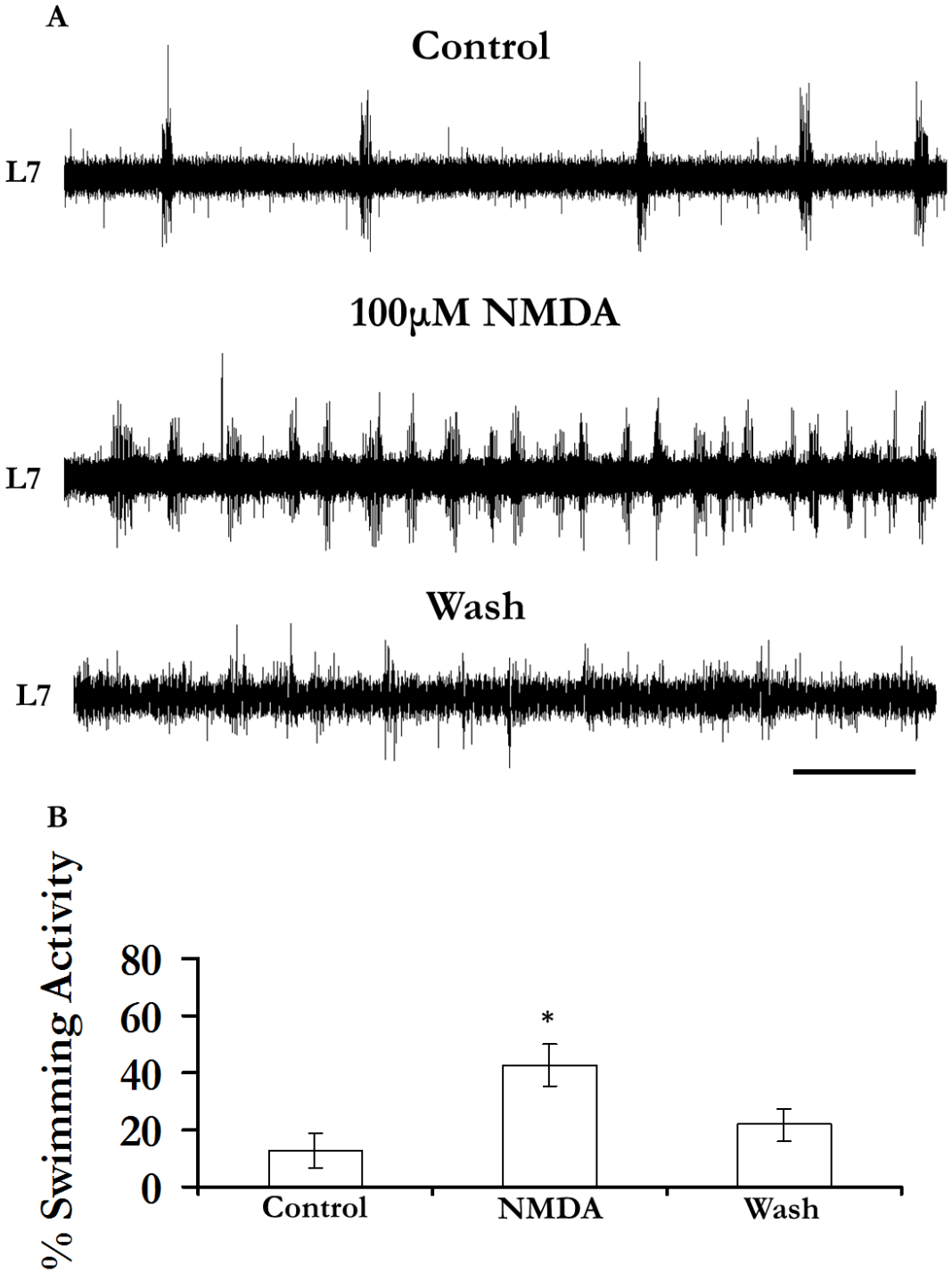

Figure 19. NMDA application increases spontaneous activity. A) Sample traces of spontaneous fictive swimming in control, NMDA (100μM) and wash conditions. Scale Bar 10s. **B)** Bar graph of percentage spontaneous fictive swimming activity, with control (n=4 preparations, mean=12.45 ± 5.95%) and wash (n=3, mean=21.74 ± 5.64%) were significantly different from the NMDA condition (n=4 preparations, mean=42.57 ± 7.12%, * $p<0.05$).

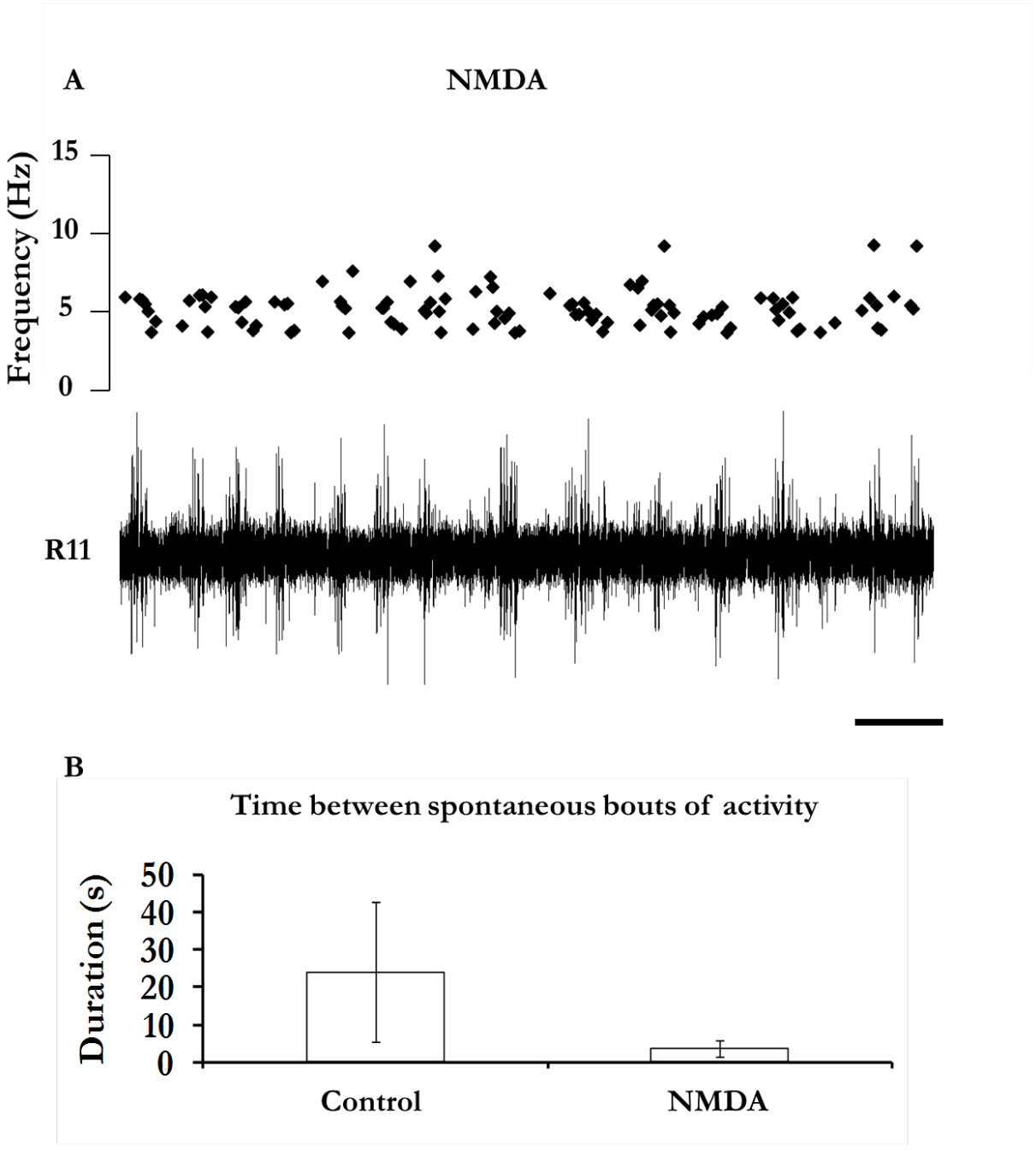

Figure 20. Spontaneous activity is distinct from NMDA rhythm. A) Sample trace with frequency distribution during activity, with an average frequency (n=4 preparations, mean=3.14 ± 1.71Hz). Scale Bar 20s. **B)** Bar graph shows the time interval between peaks of control (n=4, mean=23.99 ± 18.63s) and NMDA (n=4, mean=3.86 ± 2.18s).

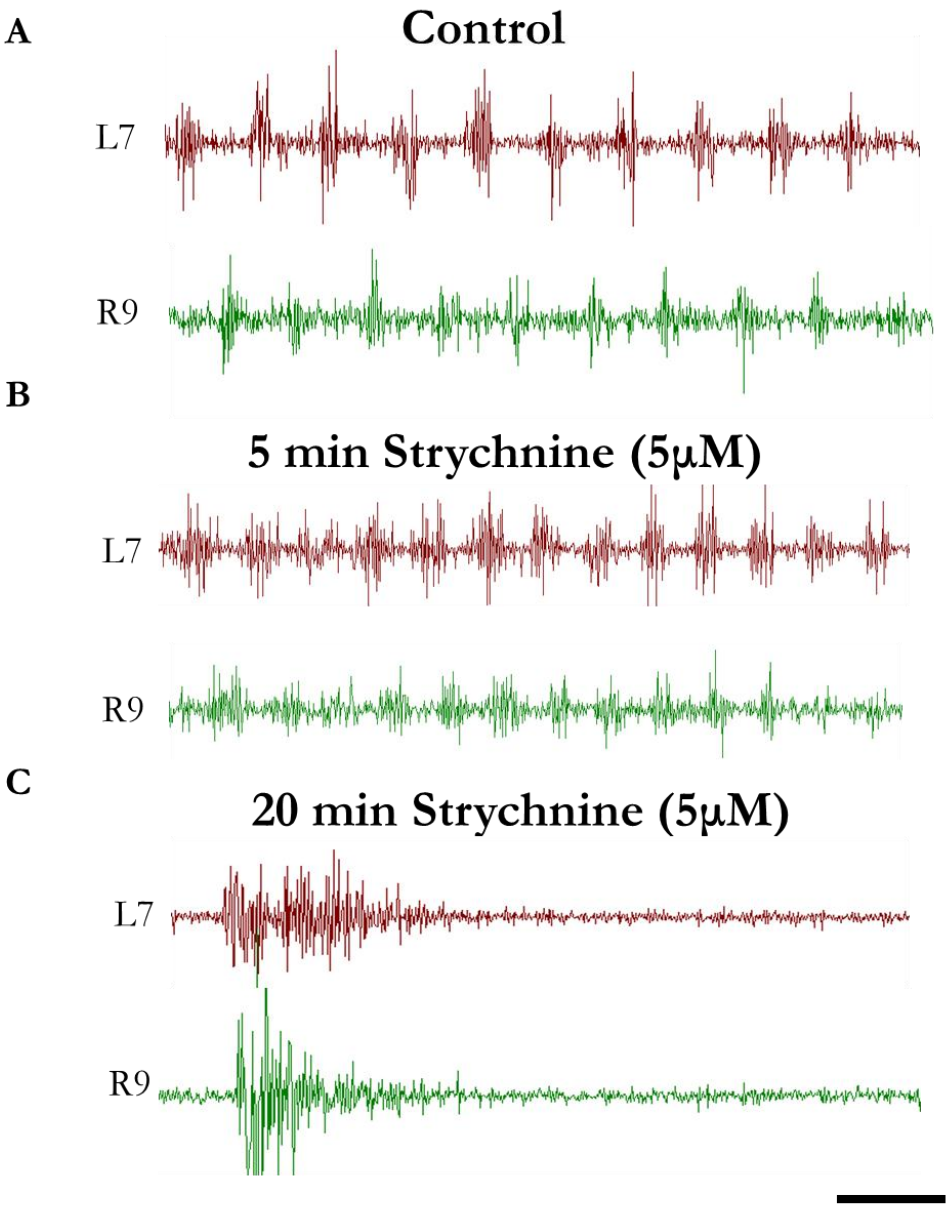

Figure 21. Strychnine synchronises but does not abolish spontaneous activity. A) Sample traces from a control showing left/right alternation of activity . **B)** After 5 minutes of strychnine (5µM) application left/alternation remains but the frequency has increased. **C)** Following 20 minutes of strychnine (5µM) bouts of fictive swimming were replaced with synchronous single bursts of activity. Scale Bar 50msec.

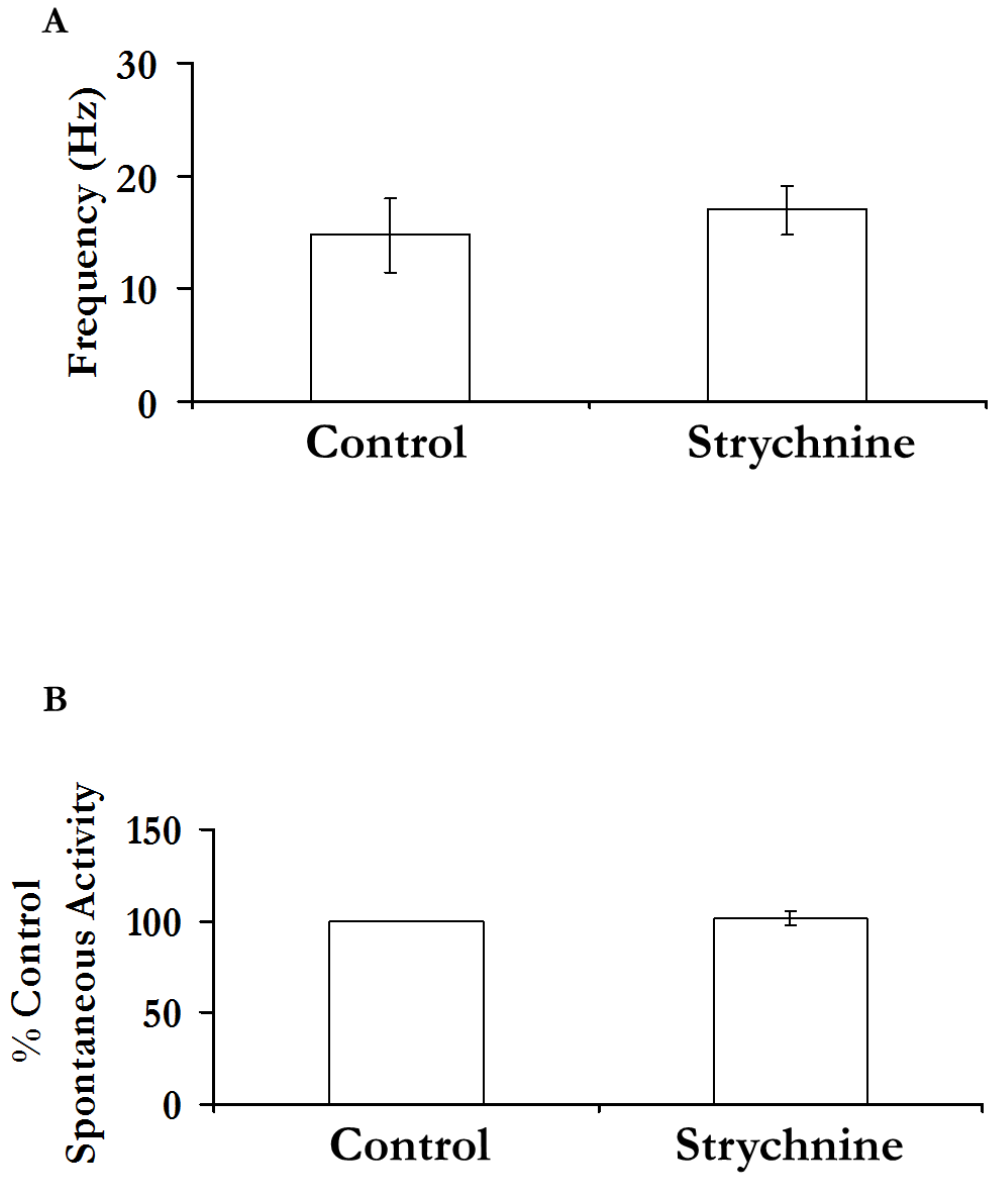

Figure 22. Strychnine application increases frequency but not percentage of spontaneous activity. A) Bar graph of average frequency in either control (n=3 preparations, mean=14.75 ± 3.27Hz) and strychnine (5μM) (mean=16.98 ± 2.17Hz). **B)** Bar graph of percentage spontaneous activity as a percentage of control (n=3 preparations, mean=100%) with strychnine (n=3, mean=101.16 ± 3.78%).

The role of GABAergic transmission was investigated in the semi-intact preparation by applying either bicuculline or gabazine (SR95531) both $GABA_AR$ antagonists. Bicuculline profoundly alters spontaneous activity by increasing activity and rescuing quiescent preparations (see Figure 23). The bar graph in Figure 23B, shows the average percentage spontaneous fictive swimming in control (n=4, mean=9.24 ± 8%), bicuculline (mean=33.34 ± 3.3%, $p<.05$) and wash conditions (mean=17.26 ± 12.1%). Disrupting GABAergic inhibition in the semi-intact preparation significantly increases spontaneous activity, suggesting that inhibitory control remains an important part of the network. Thus far, the role of greater excitation or reduced inhibition has been considered in contributing to the emergence of the spontaneous rhythm present in the semi-intact preparation. There is at least one other avenue that needs to be explored and that is the role of intrinsic neuronal properties in generating spontaneous network activity.

3.2.3 Possible role for a persistent sodium current

Neurons in networks generating spontaneous activity often have unstable resting membrane potentials due to currents in their membrane. The persistent sodium current is an example of a current which can contribute to a greater excitability of cells – by helping keep neurons depolarised after action potentials. The possible role of a persistent sodium current was investigated in the semi-intact preparation by using riluzole (5μM), a persistent sodium current blocker (Figure 24). Riluzole reversibly reduces and removes spontaneous motor activity. It was still possible to stimulate a bout of swimming in the presence of riluzole suggesting that the network was capable of fictive swimming.

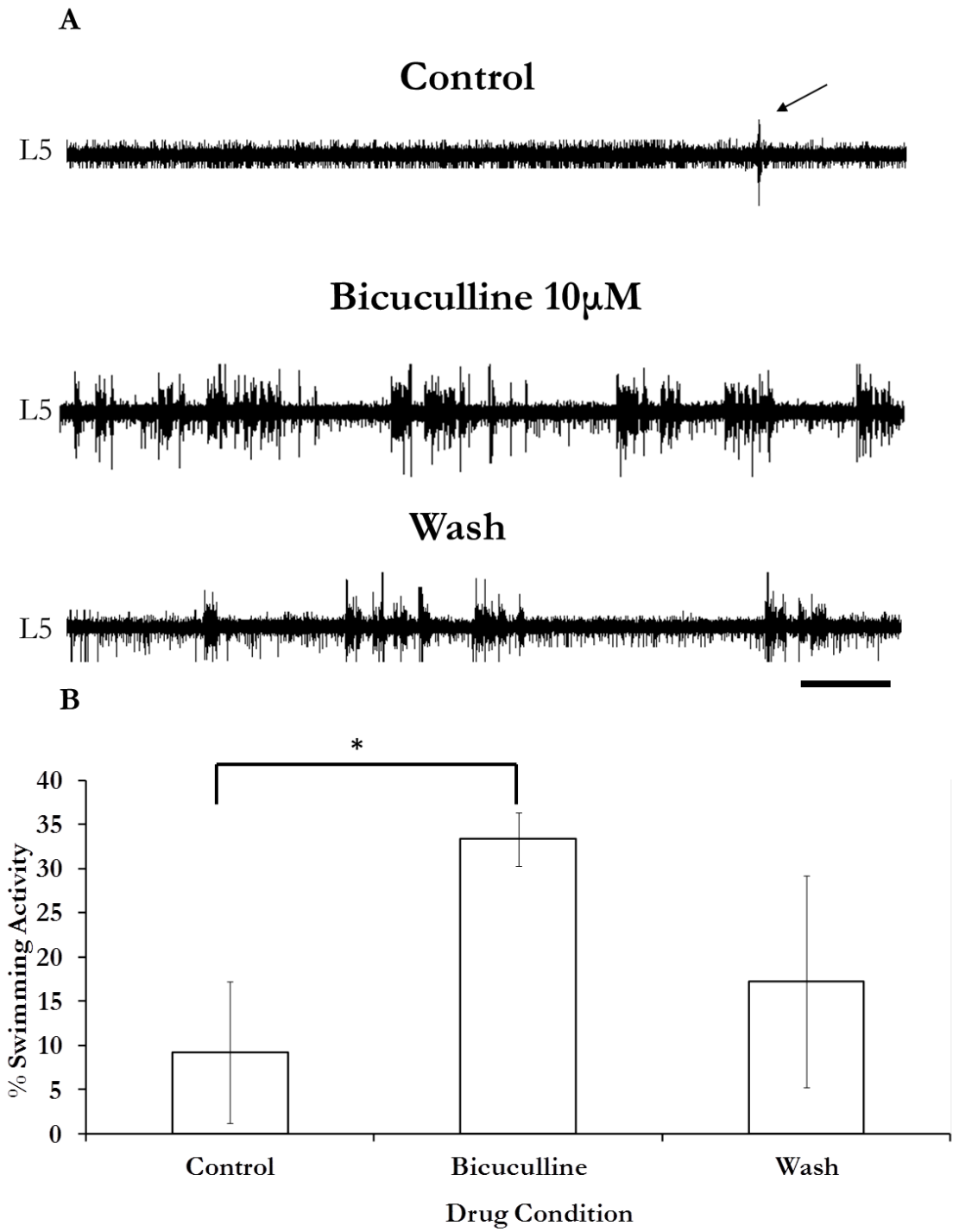

Figure 23. Blocking $GABA_AR$'s enhances spontaneous fictive swimming generation. A) Sample traces of control, bicuculline (10μM) and wash. Arrow in control shows a bout of spontaneous swimming activity. Scale Bar 1min. **B**. Bar graph of % spontaneous swimming activity, Control (n=4 preparations, mean=9.24 ± 8%), Bicuculline (mean=33.34 ± 3.3%, $p<.05$) and Wash (mean=17.26 ± 12.1%).

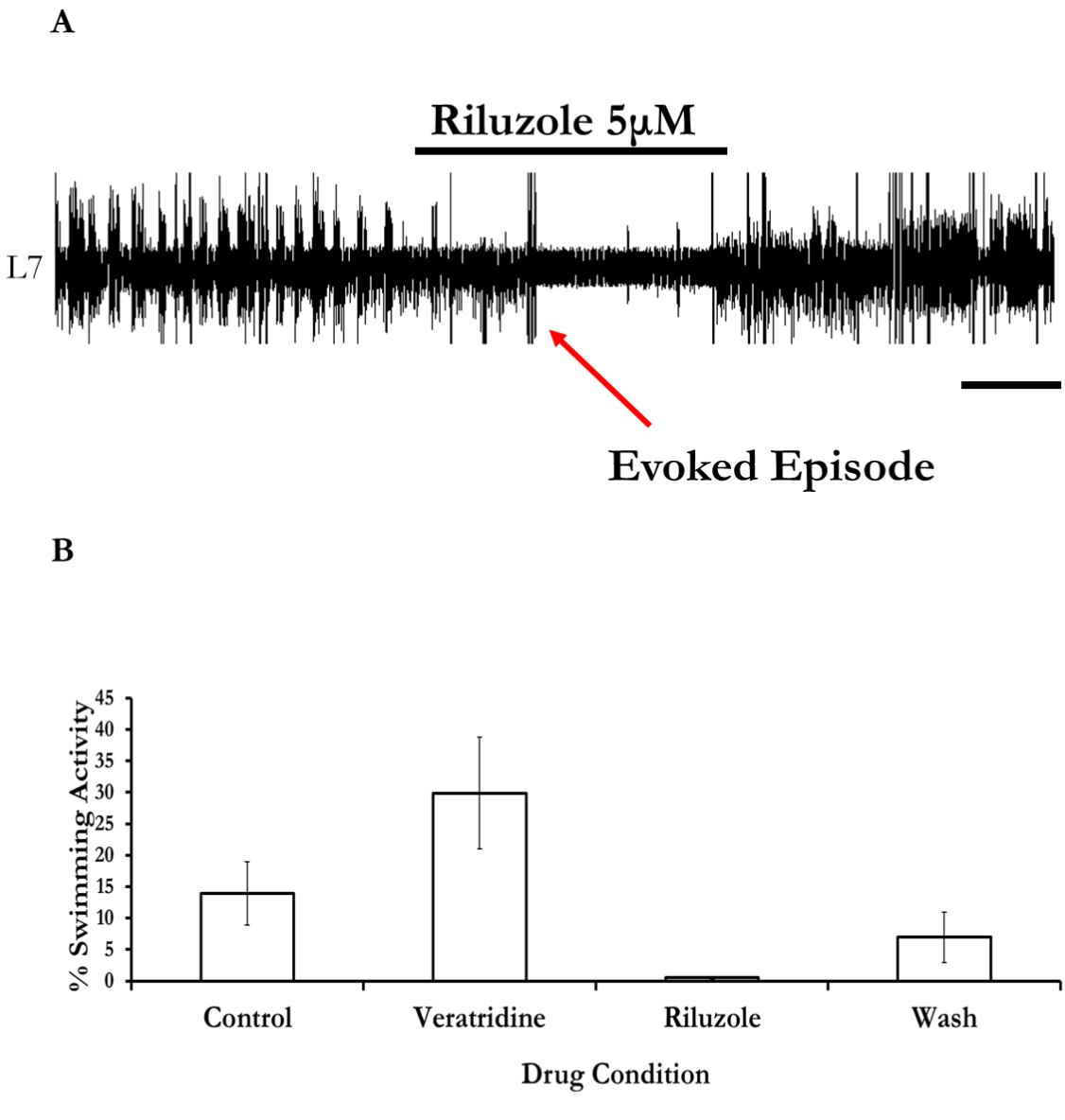

Figure 24. Possible role for a persistent sodium current in spontaneous rhythm generation. A Riluzole (5µM), a selective persistent sodium current blocker, reversibly reduces and removes spontaneous motor activity. It was still possible to stimulate a bout of swimming in the presence of riluzole (red arrow). Scale Bar 5 min. **B**. Bar graphs of spontaneous swimming activity in control, veratridine 90nM (selectively enhances the persistent sodium current), riluzole (5µM) and the wash.

In addition, the steroid derived alkaloid veratridine (90nM), which can potentiate the persistent sodium current, caused an increase in the percentage of fictive swimming (n=4, mean=29.8 ± 8.9%) compared with control levels of spontaneous activity (mean=13.9 ± 5%) (Figure 24B). Figure 24B, also shows that riluzole will remove this higher level of spontaneous activity (n=4, mean=0.5 ± 0.2%) and that the effect of riluzole can be washed out (mean=6.97 ± 4%) with spontaneous activity returning in the wash.

3.2.4 Ouabain and spontaneous activity

The cardiac glycoside ouabain, is a well know inhibitory modulator of the Na^+/K^+ ATPase. Recently, in *Xenopus*, the activity of this pump has been shown to be potentiated by ongoing fictive swimming activity, resulting in a usAHP in certain neurons which provides of form of short-term 'network memory' of past activity thereby influencing the length of subsequent episodes – an effect which ouabain can block (Zhang and Sillar 2012). The effect of ouabain (5μM) on spontaneous fictive swimming in the semi-intact preparation was explored and was found to increase the percentage of spontaneous activity from control (n=3 preparations, mean=22.16 ± 1.49%) to the higher percentage of time spent fictively swimming in ouabain (mean=37.42 ± 2.88%, $p<0.05$) which reduced during the wash (mean=8.88 ± 6.08%). This suggests that spontaneous activity in the older larvae may utilise a similar control over swimming through the regulating effect of the Na^+/K^+ ATPase.

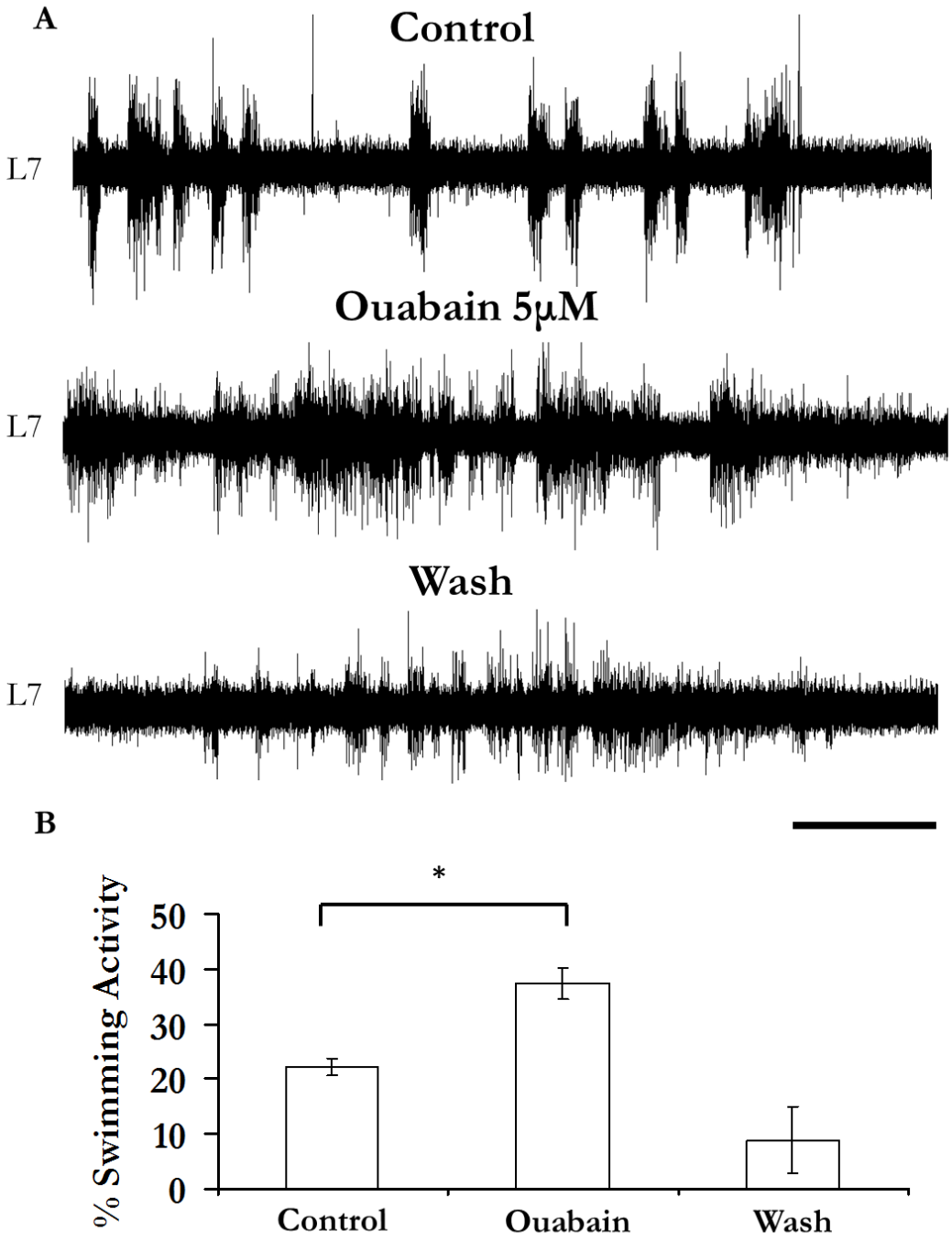

Figure 25. Ouabain causes an increase in spontaneous fictive swimming. A) Sample traces showing an increase in spontaneous swimming activity in the presence of ouabain (5µM) which then washes off. Scale Bar 1 min **B)** Bar graph of percentage spontaneous fictive swimming in control (n=3 preparations, mean =22.16 ± 1.49%), ouabain (mean=37.42 ± 2.88%, $p<.05$) wash (mean=8.88 ± 6.08%).

3.2.5 A switch in nitric oxide modulation

NO potentiates inhibitory synaptic transmission in the early larval stages of *Xenopus* before the start of free-swimming in the water column. It is known that nitrergic neurons are restricted to the brainstem at these younger stages and that NO acts as a metamodulator of the swimming CPG by acting through NA to increase mid-cycle inhibition, slowing swimming and by directly enhancing GABAergic inhibtition shortening episode length. In contrast, evidence is building which shows that NO appears to have an excitatory effect in older pre-metamorphic tadpoles. If the switch exists in the global effect of NO on the swimming CPG at what point does NO become an excitatory modulator of the motor output in *Xenopus*?

In the semi-intact preparation, the NO donor DEA/NO (200μM) was applied to determine the effects of NO on the spontaneous fictive swimming. DEA/NO significantly increased spontaneous swimming (n=7 preparations, mean=36.19 ± 3.2%, $p<.05$) from control conditions (mean=20.21 ± 2%) which partly reduced in the wash (mean=32.49 ± 5.2%). Whereas, the NO synthase inhibitor L-NAME (5mM) and the NO-scavenger PTIO (1mM) caused a reduction in the spontaneous activity from control levels (n=4, mean as a percentage of control levels=53.9 ± 7.9%, $p<0.05$) which showed a modest return to normal control levels of spontaneous activity (mean=63.3 ± 12.4%). Overall, the global effect of nitric oxide on the semi-intact preparation is to cause an increase in spontaneous activity and blocking its effects by L-NAME and PTIO caused a reduction in control levels of spontaneous activity.

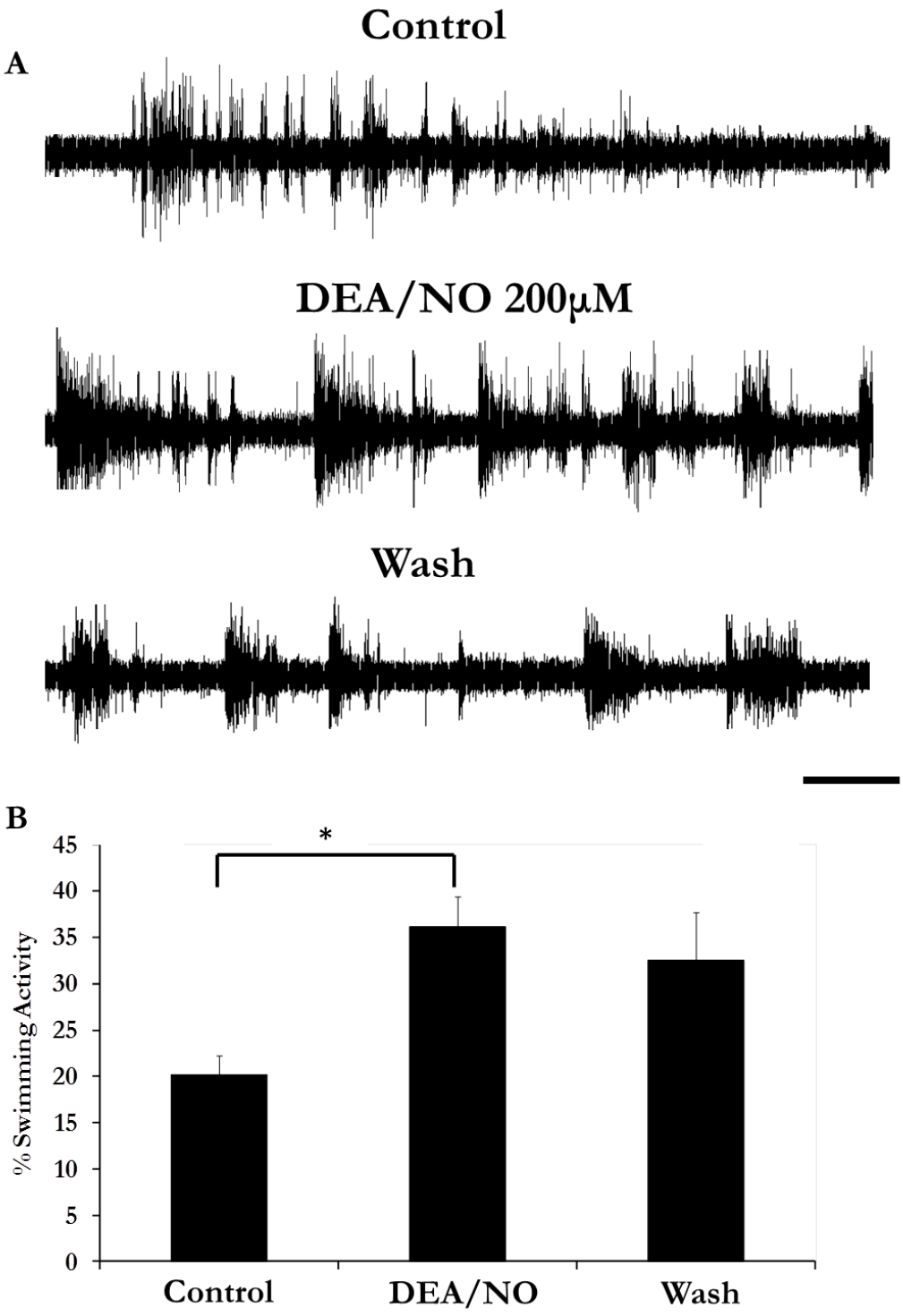

Figure 26. Nitric oxide increases spontaneous fictive swimming. A) Sample traces of spontaneous fictive swimming in control, DEA/NO and wash conditions. Scale Bar 1 min. **B)** Bar graph of spontaneous fictive swimming activity, control activity (n=7 preparations, mean=20.21 ± 2%) significantly increased spontaneous activity in the presence DEA/NO (mean=36.19 ± 3.2%) which showed a moderate decrease in the wash (mean=32.49 ±5.2%).

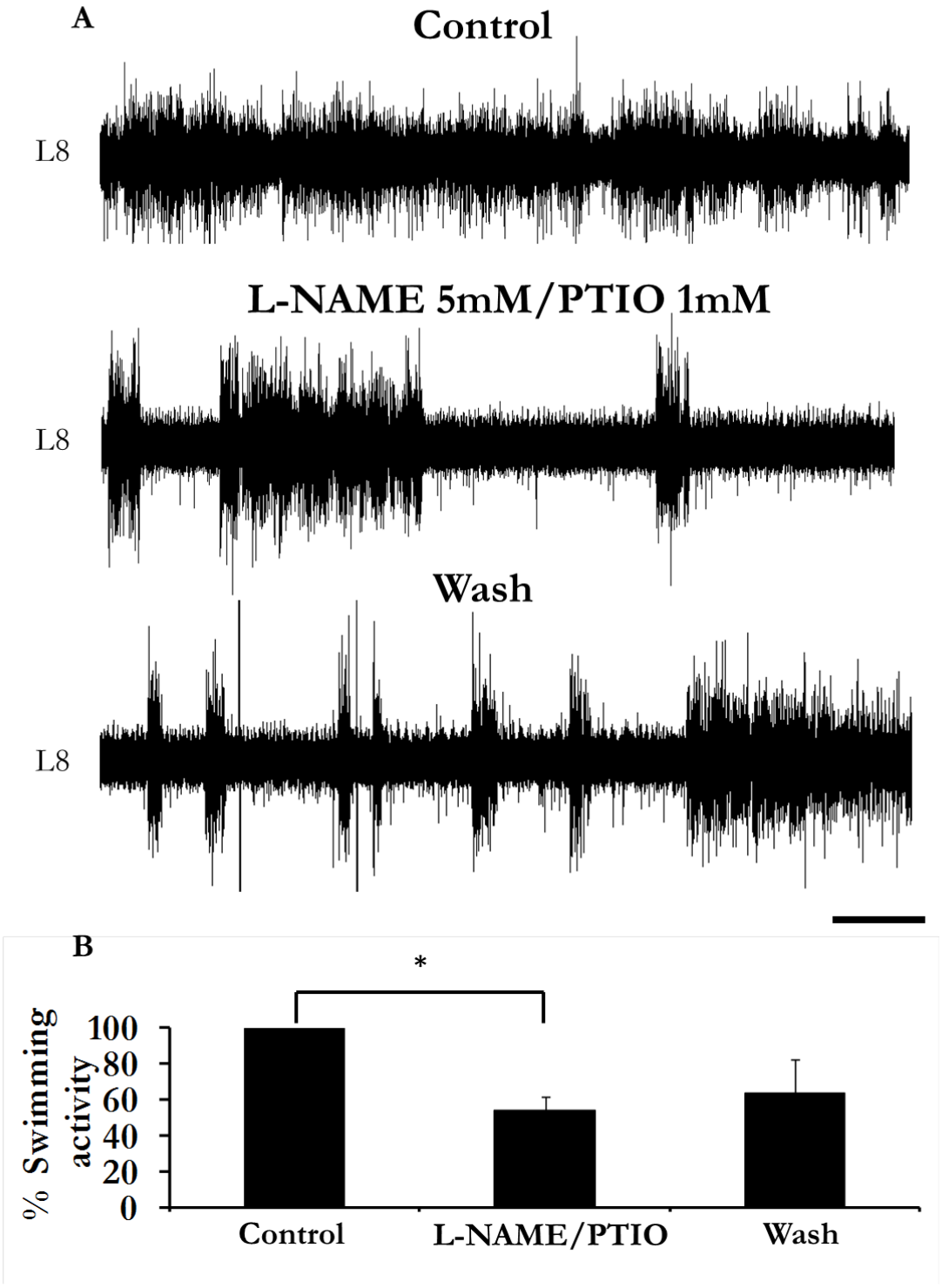

Figure 27. L-NAME and PTIO reduce spontaneous fictive swimming. A) Sample traces of spontaneous fictive swimming in control, L-NAME/PTIO and wash conditions. Scale Bar 1 min. **B)** Bar graph of spontaneous fictive swimming activity, as a percentage of the control activity (n=4 preparations) significantly decreased spontaneous activity in the presence of both L-NAME (5mM) and PTIO (1mM) (mean=53.9 ± 7.9%, $p<.05$) which returned in the wash (mean=63.3 ± 12.4%).

"Signs are small measurable things, but interpretations are illimitable … every sign is apt to conjure up wonder, hope, belief, vast as a sky, and coloured by a diffused thimbleful of matter in the shape of knowledge."

George Eliot, *Middlemarch* (1871-72)

Chapter IV
Discussion

4.1 Summary

This thesis aimed to determine the onset of free-swimming behaviour in *Xenopus laevis* tadpoles and to create a physiological preparation, in order to record, quantify and manipulate pharmacologically the fictive swimming obtained. Evidence has been presented that shows that there is an increase in spontaneous, free-swimming, behaviour in the larval animals that is coincident with the onset of free-feeding (ca. stage 45). Additionally, a preparation derived from free-feeding animals (stages 45-47) showed a coincident increase in spontaneous fictive swimming activity. This spontaneous activity is a considerable change in motor output compared with the relatively quiescent recordings from younger animals and therefore describes an important behavioural change in larval development, lying between the newly hatched tadpole and the beginning of metamorphosis.

The nature of this spontaneous fictive rhythm was investigated further and showed that the semi-intact preparations had longer burst durations than the early larval preparations but that episodes of activity were highly variable in the semi-intact preparations and this was true for both spontaneous and evoked episodes. Evoked episodes in the semi-intact preparations were however different in the pattern of

frequency across the episode compared with spontaneous periods of activity. This distribution of high initial frequency which then decreased to a lower level of swim frequency in evoked episodes was in contrast to the sinusoidal-like changes of periodic high frequency and lower average swimming frequency observed in the spontaneous bouts of fictive swimming. This distinction was similarly found in EMG recordings made from semi-intact preparations. Interestingly, these EMG recordings did not differ in their overall amount of spontaneous activity, suggesting that the spontaneous activity had a central nervous system origin and was not driven by sensory feedback.

EMG recordings also made it clear that the spontaneous activity did not rely on the midbrain, as activity persisted after the midbrain had been removed, but that the hindbrain was essential for the production of spontaneous activity. Hindbrain lesions removed all spontaneous activity and made it impossible to evoke episodes of activity using the stimulating electrode placed on the flanking tail skin of the preparation. Activity could only be recovered by the application of the excitatory agonist NMDA. These experiments raised the issue of what was the origin of the spontaneous activity and three major components were studied: excitatory, inhibitory and intrinsic neuronal properties of the spontaneously active fictive swimming network.

The NMDA rhythm was similar to the spontaneous rhythm but differed in some critical aspects. Spontaneous activity is much more variable than the NMDA rhythm in both its pattern of fictive swimming frequency and the percentage of time active. It was hypothesised that given the appearance of a more active preparation, inhibitory control may not play such an important role in the swimming network as it does at younger stages of *Xenopus* and yet blocking GABAergic transmission significantly increased the amount of fictive swimming, strongly suggesting that there is a background level of central GABAergic control over the emergence of spontaneous activity. Interestingly, although blocking glycine receptors with strychnine altered the left/right alternation of

the bursts of fictive swimming – unifying bursts on either side of the preparation – the overall amount of spontaneous activity did not change in the presence of a high concentration of strychnine (5μM). This suggested that the inhibitory role of glycine is important in the pattern of swimming but not in the emergence of the spontaneous activity. The possible role of intrinsic membrane properties was investigated by applying the persistent sodium channel blocker riluzole (5μM) which reversibly reduced and/or removed spontaneous fictive swimming, while veratridine (90nM) - an enhancer of the persistent sodium current - caused an increase in spontaneous fictive swimming. In addition, the Na^+/K^+ ATPase inhibitor ouabain reversibly enhanced the spontaneous fictive swimming activity suggesting that intrinsic properties of neurons within the late larval CPG may regulate the amount of spontaneous activity.

Finally, the role of NO in this spontaneously active network was investigated. NO was shown to enhance the amount of spontaneous swimming activity, as bath application of the NO donor DEA/NO (200μM) increased spontaneous activity, whereas application of the NOS inhibitor L-NAME and the NO-scavenger PTIO reduced the spontaneous fictive swimming.

Overall, this thesis has demonstrated that *Xenopus laevis* tadpoles do not simply increase in size between hatching and the start of metamorphosis. In fact, as this vertebrate develops, important changes in behaviour and physiology emerge around the onset of feeding that are worthy of continued investigation.

4.2 Behavioural significance of free-swimming

Initial descriptions of morphology and behaviour in *Xenopus* development, from Muntz (1975), did not extend beyond stage 42 and classified stages 33-42 as free-swimming. The experiments in this thesis directly challenge this description of *Xenopus* development and show that although stage 42 and other pre-feeding hatched larval animals are capable of swimming away from sensory stimulation, animals at these stages in fact show relatively little intrinsic swimming behaviour and this is also observed in physiological recordings which show very little spontaneous fictive swimming.

The transition to a more active self-motion described here in *Xenopus* has also similarly been described in the zebrafish (Thirumalai and Cline 2008) between 3dpf and 5dpf, curiously also at the same point that the animal begins to forage for food. A direct comparison of Figure 8A and Figure 10B shows that there is a great similarity between these species in the change in swimming trajectories – that is not revealed simply by noting the increase in spontaneous swimming observed. Both show a change from episodic swimming bouts, which have a characteristic beginning and end to activity, to one that implies near continuous motion. This new demand placed on the developing larva, that is to feed, challenges the organism to become more of an individual and with this comes a greater capacity for intrinsic behaviour.

Often though, the spontaneous behaviour of animals is overlooked, not least in *Xenopus*. In a study conducted by Böser and Horn (2006) on the effects of gravity on the development of the vestibular system in *Xenopus* tadpoles, failed record any spontaneous fictive swimming. Indeed, in Lambert and colleagues (2008) description of the development of the vestibulo-ocular reflex (VOR) in *Xenopus*, there was no reference made to the fact that the larvae are entering a free-swimming existence at the same time – and it is my view that this would have enhanced their description. They described an

unexpected delay in the onset of the VOR, specifically the horizontal angular VOR, which allows for the eyes to adjust for changes in head position due to self-motion. In stage 47 tadpoles, the semi-circular canals that underlie the reflex have developed and the necessary circuitry is present but the reflex doesn't occur until stage 49. The suggested explanation from Lambert and colleagues was that there was a physical constraint on the size of the semi-circular canals, which accompanied the particular increase in size in tadpoles from stage 46 to 48 (see Figure 6). The contribution of this reflex to gaze-stabilisation during swimming has recently been supplemented by work which has suggested a central origin of a feed-forward efference copy from the CPG to gaze-stabilising motor areas in the brainstem (Combes et al. 2008; Straka and Simmers 2012). At no point however, was it mentioned that during this period of increased growth of the larva (~stages 45-49) the animal actually becomes more active and indeed would be in need of a comprehensive and multifaceted vestibular system with mechanisms in place to help the newly active larva navigate. Indeed, before free-swimming the most likely requirement of swimming is to rapidly swim away from a noxious stimulus – the requirement for fine three-dimensional navigation would hardly seem to be the priority. Navigating for food on the other hand does require this and the newly active lifestyle predicts the advent of a more developed vestibular system. It is the view of the author that the behavioural context helps to demystify some of the confusion and speaks of the merit of being aware of the native behaviour of the animal.

Understanding the new behavioural context of the animal does indeed raise questions that would have perhaps not been considered without such an investigation. For example, what is to happen to the sensory system in the animal, how is it going to respond to near continuous motor-evoked self-stimulation? It is known that there are sensory gating mechanisms in place during reflex actions in *Xenopus* (Sillar and Roberts 1988) and during evoked fictive swimming in the younger larvae (Li et al. 2002).

Presumably this sensory gating becomes even more important at these later stages of development and this would be a further area of investigation. Furthermore, spontaneous activity could well be described as somewhat of a nuisance in recordings at the early stages of *Xenopus*, as the evoked recording procedure attempts to standardise the interval between episodes, which is disturbed by spontaneous episodes. Yet in the older larval animal, spontaneous activity takes on a new significance as the behaviour of the animal changes and spontaneous swimming becomes the norm.

4.3 The semi-intact preparation

The semi-intact preparation was devised by combining the original fictive swimming early larval preparation, created principally by Roberts and colleagues (Khan and Roberts 1982) with a schedule 1 method for removing the forebrain and destroying the heart developed for the later metamorphic stages – although the nervous system was not isolated, therefore providing a semi-intact rather than an *in vitro* preparation.

It was hypothesised that there would be a substantial increase in spontaneous fictive swimming in the semi-intact preparation compared with the early larval recordings and such an increase was indeed found. This gave two important outcomes, the first is that the preparation seemed to be suitably similar to the behaviour, in the way the younger evoked episodes were similar to the tadpoles swimming away from touch stimuli, and therefore lent confidence to the idea that the spontaneous fictive activity was a good experimental model for free-swimming behaviour. And, this would therefore allow questions to be addressed towards what the developmental changes might be that underlie this behavioural change. Secondly, the similarity of the recordings to the behaviour also suggests that the behaviour itself is central in origin – that is it comes from the central nervous system and is not just a hypersensitivity to environmental

stimuli for example. To give further support to the central origin of this activity, control recordings of spontaneous activity were made when the solution was not circulating by the action of the pump (see Figure 15B) in order to show that the action of the pump was itself not the origin of the spontaneous rhythm, although the recordings from the younger larva themselves act as a control, as you would expect them to also show the spontaneous rhythm if the pump was the cause. A recent article from Yvert and colleagues (2011) showed that the movement of artificial cerebrospinal fluid over a neonatal brainstem and spinal cord was the true origin of waves of rhythmic activity emanating from the hindbrain and propagating to the spinal cord – initiating ventral root bursts. In *Xenopus* however, there was no evidence that the pump itself was the source of the spontaneous activity. However, the Yvert article does raise some important questions about factors that can alter spontaneous activity, such as temperature and oxygen saturation within the recording apparatus. Throughout the experiments the saline used was at room temperature (~23^{O}C), and although this was checked by placing a thermometer in the header tank, no efforts were employed to maintain or control the temperature throughout the experiment. Furthermore, unlike the neonatal mouse *in vitro* preparation or the older *Xenopus in vitro* metamorphic larval preparation, no oxygen was perfused into the circulating solution. However, neither of these factors are likely to show rhythmic changes throughout the recording and therefore is unlikely to be responsible for generating the spontaneous activity, although they may contribute to the nature of the spontaneous activity. Future work would want to more strictly control these parameters.

Recordings from the semi-intact preparation showed an increase in burst duration compared with the early larval recordings. This occurs in a developmental context where burst durations were already increasing from stage 37/38 to stage 42 due to MNs firing multiple action potentials as a result of greater serotonergic modulation (Wedderburn

and Sillar 1994). Episodes of activity in the semi-intact preparation are on average smaller than the younger larval evoked preparation but display a large amount of variability and the inter-episode duration is also considerably more variable. Indeed, this was why the percentage of time spent spontaneously fictive swimming was taken as a measure of output rather than the number of episodes or the duration. This variability does reflect the swimming in the free-swimming animals which is much more continuous or periodic in nature. This variability could, however, also be due to the nature of the recording electrodes, where periods of activity were not recorded because the electrodes were placed too rostrally to record ventral root bursts. However, more rostral ventral roots were preferentially selected because they were easier to record from and in addition there was greater confidence that ventral root bursts at these positions reflected forward swimming locomotion and not the more postural rhythm present more caudally (see section 4.6 Future Directions for more details).

Finally, the EMG recordings that were made allowed for greater amplitude and signal to noise ratio and therefore a much easier analysis. EMG recordings also showed no significantly different amount of spontaneous activity suggesting that any sensory feedback through movement induced self-stimulation had little influence on overall generation of spontaneous activity. Taken together this shows that the semi-intact preparation replicated the behaviour well and was suitable for further analysis of the origins of the spontaneous activity and by inference the free-swimming behaviour.

A similar result was found in recordings from the isolated central nervous system of the bullfrog (*Rana catesbeiana*) which showed that EMG recordings were similar in their character to the fictive swimming recorded from ventral roots, which the authors argued gave support to their view that the activity was centrally generated and not the result of proprioceptive feedback (Stehouwer and Farel 1980). Interestingly this activity documented by Stehouwer and Farel (1980), was spontaneous activity in the larvae but

no direct connection was made between the spontaneous recordings and the behaviour, despite their use of EMG recordings. What they did do however was to perform mechanical lesions on their isolated larval preparation in order to determine what the possibly central origin of this activity was, if indeed it was not as they suspected dependent on the periphery. They showed that lesions to the caudal aspect of the optic tectum (a midbrain structure, in mammals is more commonly referred to as the superior colliculus) had no effect on the amount or character of the spontaneous motor output but physical transections progressively more caudally into the brainstem did progressively reduce the amount of spontaneous activity. In addition, they found that although activity decreased with progressive lesions into the brainstem, all other characteristics of the fictive motor output remained the same. Although some activity did persist even when the brain was totally transected, the suggestion from this report was that somewhere within this caudal brainstem was a region that dictated the emergence of the spontaneous activity. Evidence has been provided here in *Xenopus* larvae that there is a similar requirement for the caudal brainstem in generating spontaneous activity.

4.4 What is the origin of the spontaneous activity?

The lesions documented in this thesis were achieved through a physical transection with sharp tungsten needles and the major division of the developing brain, i.e. fore-midbrain junction, mid-hindbrain junction and the narrowing of the hindbrain at the otic vesicles, were used as landmarks for transection. Although the transections were traumatic and covered large areas of the brain, choosing these clear landmarks allowed for reliable lesions to be performed. Clearly, further work would try to refine and perform more specific lesions particularly within the hindbrain. The finding that removing the midbrain, leaving the hindbrain and spinal cord intact, did not significantly

alter the levels of spontaneous activity was very similar to the result published by Stehouwer and Farel (1980) discussed above and is consistent with numerous other findings throughout the vertebrates that the hindbrain is very important in producing spontaneous rhythmic activity (for review see Bass and Baker 1997; Garcia-Campmany et al 2010). In the adult zebrafish, a recent study by Kyriakatos and colleagues (2011) showed that electrical stimulation at the junction of the hindbrain and spinal cord produced sustained motor output and that the frequency of this swimming was similar to EMG recordings from a freely swimming animal and different from the NMDA induced swimming rhythm which had a much lower and fixed frequency range. In the semi-intact preparation, it was shown that evoked swimming activity, due to stimulating the caudal tail, in both fictive and EMG recordings showed a similar distribution of frequencies, although the average frequency in the EMG recording was lower than in fictive swimming. The native spontaneous activity was similar in both fictive and EMG recordings, on this occasion the average frequency range were very similar. Curiously, the pattern of activity was different though between the evoked and spontaneous activity in both fictive and EMG recordings, suggesting either that the swimming CPG is recruited by two separate mechanisms for self-motive and sensory evoked periods of activity or that evoked episodes of activity override the spontaneous swimming generator circuitry and produce a bout of high frequency activity that decreases in frequency down to lower frequency levels.

The role of the hindbrain in swimming generation and maintenance in hatchling *Xenopus* tadpoles has also been investigated in some detail. Li and colleagues (2006) reported that glutamatergic neurons within the caudal hindbrain, described as hindbrain dINs (hdINs), fired before all other CPG neurons and provided excitatory drive. Li and colleagues (2010) have recently shown that these hdINs are capable of sharing this excitation with other hdINs causing them to become rhythmically active – through

NMDA receptor activation. This helps to sustain activity in these neurons and provide drive to the CPG throughout episodes of activity. It is clear then that the hindbrain is vital to the production of spontaneous activity in the semi-intact preparation and the caudal hindbrain is important in generating and sustaining evoked episodes of activity in the younger larvae – through the action of the hdINs.

Spontaneous activity in developing nervous systems is often due to either the depolarising action of classical inhibitory transmission or unstable membrane potentials due to pacemaker currents or a combination of the two (Blankenship and Feller 2010). In larval *Xenopus* however, the chloride equilibrium potential is set such that fast inhibitory transmission normally causes hyperpolarising IPSPs. This would seem to suggest that neurons within the swimming CPG are more active and this may be due to changes in their integrative electrical properties triggered by the expression of new currents across their membranes. However, it is also known that inhibitory synaptic transmission is important in the evoked swimming activity in the early larval phase. And indeed evidence has been presented that the newly hatched larva as it hangs at rest from its cement gland has tonic background levels of GABAergic inhibition – a sensory driven GABAergic restraint (Lambert et al. 2004). So it could also be hypothesised that there is a reduction in this inhibition which heralds the emergence of much greater spontaneous motor activity in the semi-intact preparation. In this thesis an initial attempt to address these issues was presented.

From the lesion experiments it was clear that the excitatory glutamate receptor agonist NMDA rescued activity and it has been used in the early larval preparation to generate a fictive rhythm (Reith and Sillar 1998). Also, hdINs as discussed above excite neighbouring hdINs through activation of their NMDA receptors. Therefore, the role of NMDA was studied by adding this excitatory agonist to the semi-intact preparation. NMDA (100μM) induced rhythms in both the early larval and the semi-intact

preparations do share similar characteristics. The overall amount of activity increases and tends towards continuous motor activity but this activity is much slower than normal spontaneous swimming which is much more variable and fluctuating in frequency distribution and this is true for evoked episodes in the younger and semi-intact preparations as well. Clearly bath applying NMDA can create more activity but this global increase in excitation across the entire CNS doesn't seem to produce swimming activity that matches the spontaneous activity recorded. Similarly, in Reith and Sillar's work (1988) the increase in fictive swimming with NMDA might initially be confused with the later larval spontaneity but under closer inspection it doesn't match up, with swimming frequencies on average as low as 0.5Hz which are not found in the evoked swimming episodes of the early larva or the spontaneous fictive swimming of the semi-intact preparations. This suggests that while there may be regional increases in glutamatergic release the blanket increase in excitability alone will not recreate or enhance spontaneous activity.

The effects of strychnine (5μM) and bicuculline (10μM) on the larval swimming activity were distinct and bring up the important issue: namely that it is possible to affect the nature of the spontaneous activity without significantly changing the emergence of the rhythm. Given the GABAergic restraint placed on swimming activity in the younger early larva, it might be predicted that this will diminish in order to allow for a more active spontaneous swimming repertoire later in larval life. But, the results recorded in this thesis show that simply increasing global excitation doesn't create enhanced spontaneous activity and blocking $GABA_AR$'s with bicuculline was the most reliable method to increase and rescue spontaneous activity. This suggests that GABAergic inhibition continues to play an important role in regulating the emergence of spontaneous activity. In contrast, blocking glycinergic inhibitory transmission with strychnine resulted in no

significant changes in amount of overall spontaneous fictive swimming but rather altered the expression or the character of the rhythm.

This suggests that there may be layers of rhythm generators in the semi-intact preparation one which governs the rhythmic activation of the spinal motor network and the other the spinal swimming CPG itself.

An area of neurophysiology where a great many of these debates have already been discussed is that of respiratory rhythm generation. The persistent sodium current has been suggested to be important, along with many other cellular and network mechanisms, in generating this rhythmic motor output. Although, a comprehensive analysis of a cellular membrane current would require individual cell recordings – the extracellular ventral root recordings presented here are encouraging towards a possible role for an INaP in assisting the generation of the spontaneous rhythm. Riluzole as a drug is perhaps famous for two reasons; it is used in the treatment of amyotrophic lateral sclerosis and for its lack of specificity for blocking the persistent sodium current. Riluzole can also modulate glutamate release and therefore can act as global reducer of excitability and block persistent calcium currents (Lamanauskas and Nistri 2008). Therefore, it might be no surprise that riluzole so convincingly removed spontaneous motor activity in the semi-intact preparations. Except during this drug induced quiescence swimming activity could be evoked in the usual method through stimulation of the tail skin. This result imparts two point of interest, firstly that the sensory network (probably through the trigeminal nerve as the RB pathway will have degenerated by now and the dorsal root ganglion cells have yet to connect the periphery to the spinal cord) is not inhibited by the riluzole to significantly reduce synaptic transmission and secondly that the swimming CPG itself is still intact and rather like the earlier stages of the animal can produce swimming activity when stimulated but produces very little spontaneous activity. To further attempt to confirm that INaP may be contributing to the

spontaneous rhythm, the drug veratridine was used. Veratridine was used by Tazerart and colleagues (2008) to determine the role of INaP in generating pacemaker activities in the mammalian spinal CPG for locomotion, and was found to enhance the amplitude of INaP becoming statistically significant at 60nM and at this concentration veratridine did not alter the amplitude, threshold or duration of the action potential neither did it alter synaptic transmission. Veratridine also had effects at the ventral root level as well and caused a slowing of the NMDA/5-HT induced rhythm. Veratridine was chosen to be used in the semi-intact preparation to complement the experiments with riluzole. Veratridine enhanced the overall percentage of spontaneous swimming, activity which was then removed by application of riluzole. Although these results only suggest a possible role for INaP in the spontaneously active semi-intact preparation they certainly provide evidence in support of the view that perhaps somewhere in the later larval swimming CPG there are rhythmically active neurons, excitatory and inhibitory, able to generate activity in part because of a pacemaker current such as INaP.

The ability to modify the nature of future activity in light of past demands is an important and essential aspect of any nervous system. In a seminal study by Zhang and Sillar (2012), evidence is provided that is the first to show that the activity-dependent potentiation of the Na^+/K^+ ATPase activity can have a direct impact on the duration of future episodes of swimming. This unique form of network short-term memory matches the increase in pump activity, presumed to be the consequence of increased Na^+ inflow as the result of multiple action potentials from a previous episode of swimming, with a membrane potential hyperpolarisation (termed the usAHP) which modifies the duration of subsequent episodes. In effect longer intense episodes of activity are followed by shorter ones and shorter episodes allow for longer ones to follow (Zhang and Sillar 2012). At the stages of *Xenopus* discussed in this paper activity is evoked by stimulation and successive episodes of swimming are unlikely to occur. However, in the free-

swimming larvae where swimming activity is very common indeed this mechanism could prove invaluable, considering the requirement to sustain a large amount of time swimming. The role of the Na^+/K^+ pump was investigated in the spontaneously active semi-intact preparation by bath applying the Na^+/K^+ pump blocker ouabain. A similar effect to that of the younger larva was found in the spontaneously active swimming network, where ouabain reversibly increased the overall amount of spontaneous fictive swimming. What would be of particular interest looking forward would be to confirm that not only do neurons in the semi-intact preparation possess an usAHP, but also to establish which types of cells have them. At the younger stages, the dINs do not show an usAHP and it would be of particularly interest to determine whether such cells were to develop this characteristic. This very brief investigation has shown that this pump may well exert significant control over the amount of swimming and perhaps help to prevent bouts of activity going on too long without any respite and therefore contributes to the character of the spontaneous rhythm in a similar way as it does for the evoked activity in the younger animal.

4.5 A new role for NO in motor control

The observation that NO increases rather than decreases motor output in the semi-intact preparation, is one of the most interesting and clear findings documented in this thesis. In the early larva, NO selectively tunes and enhances inhibitory synaptic transmission, by acting directly on GABAergic neurons to bring about shorter episodes and acting through noradrenaline on glycinergic synaptic transmission, slowing swimming rhythm (McLean and Sillar 2002; McLean and Sillar 2004). However, the actions of NO are not exclusively inhibitory as NO will cause a depolarisation of motor neuron membrane potentials. NO itself acts principally through soluble guanylate cyclase

(sGC) and secondary messenger pathways in addition to interacting directly with proteins by nitrosylation of cysteine residues (Ahern et al. 2002). Its actions are not inherently inhibitory or excitatory and its production can be activity dependent or a background tonic level. NO can be said to be a master of all trades in neuromodulation.

A good example of NO's multifaceted neuromodulatory role would be to consider its effects on long term potentiation (LTP) within the hippocampus. Hippocampal LTP is one of the most widely investigated phenomena in the whole of neuroscience and involves the use-dependent increase in synaptic transmission and NMDA receptor activation within CA1 of the hippocampus and has been shown to be partly dependent on NO as a retrograde messenger – a phasic neuronal NO release in parallel with a background level of tonic endothelial NO release (Hopper and Garthwaite 2006).

During development in both vertebrates and invertebrates NO is involved in shaping networks in the nervous system. Recently, Bradely and colleagues (2010) showed that neuronal NOS is responsible for regulating the ontogeny of the axons of MNs within the immature spinal cord of the larval zebrafish. Similarly, in the bull frog breathing system NO shows a rather complex relationship to this rhythmic motor output. Initially, NO inhibits the gill and lung burst activity but as development progresses NO switches its effects and becomes excitatory towards lung ventilation (Hedrick et al. 2005). This change in the effect on NO on motor output over development is placed in the locomotor context by evidence which suggests that NO becomes an excitatory modulator of the metamorphic tadpole spontaneous rhythm generator (personal correspondence from Denis Combes, University of Bordeaux). There is a change in the expression of nitrergic neurons in *Xenopus* across development, particularly over the metamorphic period where NOS-positive cells progressively appear in the spinal cord in regions that flank the developing limb MN pools. This change occurs from around stage 48 and onwards to the metamorphic climax (Ramanathan et al. 2008). Given this change

in NO expression at this stage of development and the switch in the manner of locomotion from tail-based to hind-limb kicking propulsion, a plausible hypothesis would be to suggest that NO changes its overall effect on the motor network from an inhibitory to an excitatory agent at the onset of hind-limb development. However, the results of this thesis show in fact that the change appears to occur earlier in development – when the animal switches from a quiescent to a free-swimming animal.

In conclusion, what can be said about the origin and modulation of this newly acquired spontaneous activity in *Xenopus* tadpoles at the onset of feeding? Firstly, that it is unlikely that the activity is driven simply by a decrease in overall inhibition or by a global increase in glutamatergic synaptic transmission. Secondly, that the spontaneous activity is distinct from evoked episodes of activity. Thirdly, that inhibition through GABAergic synaptic transmission is important in regulating this activity. Fourthly, that glycinergic activity alters the nature of the bursts but does not govern whether spontaneous activity will occur or how much will occur. Fifthly, that cellular properties throughout the network such as pacemaker currents or the action of the Na^+/K^+ pump can modify the spontaneous rhythm. The schematic in Figure 28, attempts to combine these observed changes in the larval swimming network from the early larva to the spontaneously active late larval network.

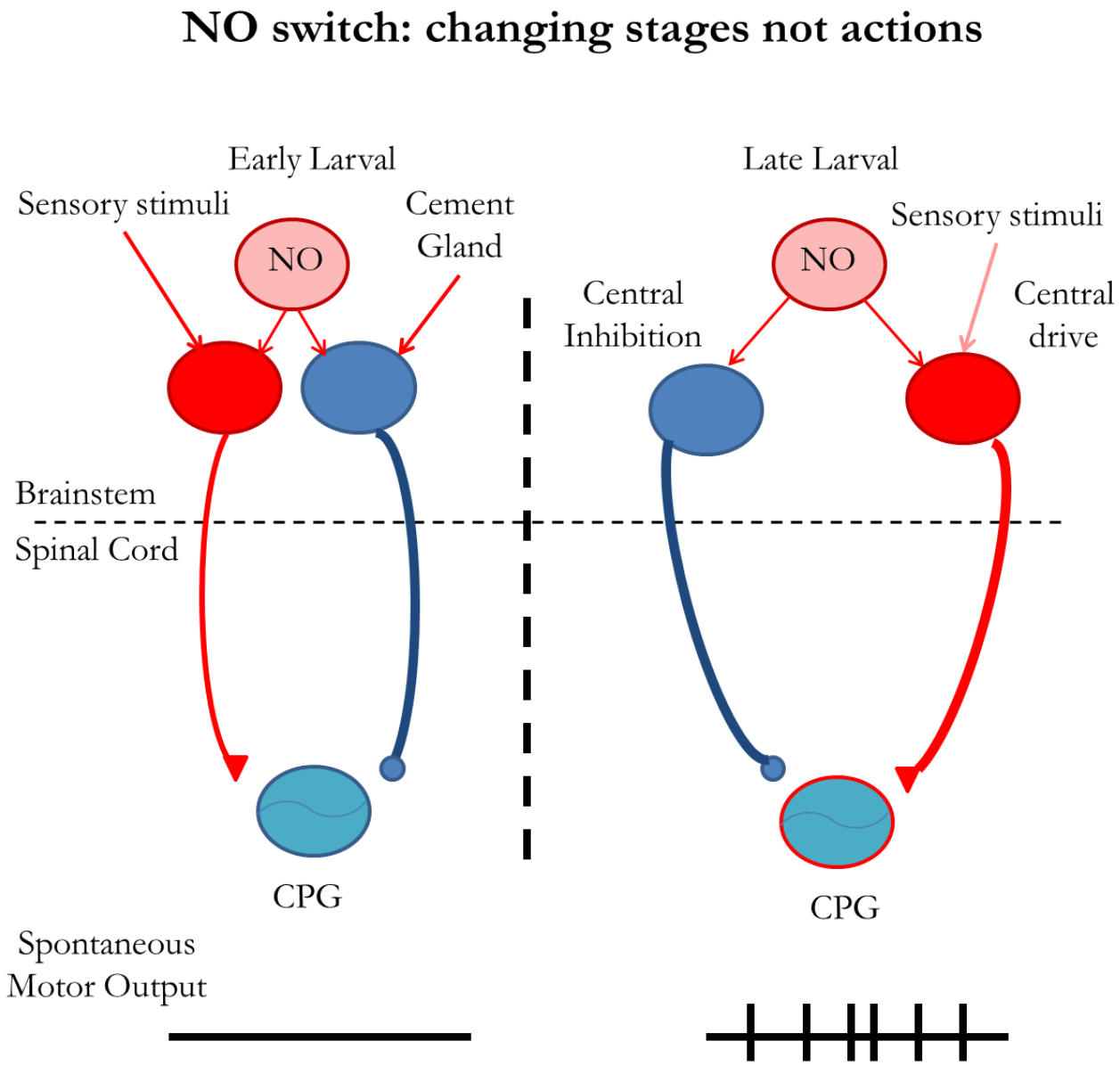

Figure 28. Possible model for changes in *Xenopus* larval motor network and switch in nitric oxide modulation. Early larval, shows a simplified swimming network in the early larva where spontaneous activity is essentially non-existent. Periods of evoked swimming activity can be produced through sensory activation of descending excitation (red circle and arrow). The predominant influence at this stage however is through the cement gland and descending inhibitory GABAergic restraint on the swimming CPG (blue circle and descending projection). **Late larval**, suggested late larval network where there is a substantial increase in spontaneous activity with an established central inhibition (blue circle and descending projection) and central drive (red circle and arrow) over the on-going CPG activity. Sensory stimulation can still drive motor output although this is not the primary source of motor output at this stage. The cement gland has degenerated, leaving central inhibition. NO is hypothesised to potentiate both excitatory and inhibitory inputs at both stages.

Taken together Figure 28 shows a possible model for the changes in the *Xenopus* motor network from early larva to free-swimming late larva and how NO might switch in its global effects on the network. The swimming network in the early larva is dominated by inhibitory control and it is really only with sensory activation that you can evoke activity. NO is suggested to potentiate this inhibition but has very little effect on excitation. At the later stages of larval life however, more spontaneous activity is present and the cement gland pathway has degenerated leaving in place a suggested central inhibitory pathway. Similarly, although the RB pathway for stimulation has been replaced, central excitation is proposed to have developed. The swimming network of the late larva is now dominated by a relationship of excitation and inhibition. In this new context, it is proposed that NO acts to potentiate both network but with the relative balance of the two networks favouring excitation, NO results in an enhancement of spontaneous activity. This is only one possible explanation of how NO might be causing two separate effects at two stages of *Xenopus* development separated by only a few days. It is with great excitement that we can look forward to future experiments to help determine how this new neuromodulatory context for the tadpole comes about.

4.6 Future Directions

The origin of the spontaneous motor activity described in this thesis and by inference the self-motive spontaneous free-swimming behaviour, still remains unclear and is the major question raised by this thesis that remains to be more extensively investigated. By employing single cell recordings, either in the semi-intact or in the later *in vitro* metamorphic preparations, new insights into the neuronal origin of this activity could be addressed. The advantage of using the *in vitro* metamorphic preparations is that split-bath experiments can be employed, because the tissue is larger and tougher, and drugs could then be added directly to the hindbrain and the effect on the spontaneous swimming observed. Particularly of interest would be to determine if applying riluzole and veratridine to the hindbrain produced the same reduction and increase respectively in spontaneous fictive swimming reported in this thesis. Additionally, the non-competitive antagonist of the NMDA glutamate receptor MK-801 could be used to investigate whether NMDA-driven oscillation in the hindbrain, possibly in presumptive hdINs at this stage, are present and required for spontaneous rhythm generation.

Another important area for further investigation is the caudal tail rhythm produced by *Xenopus* larvae. Not described in the work presented here, this caudal rhythm is very robust and develops at the same time as the spontaneous free-swimming behaviour but this motion appears to be more postural than locomotory. There also appears to be a strong relationship with this caudal activity and the recruitment of more rostral swimming CPGs into activity. The fact that swimming occurs in a rostral to caudal direction but can be recruited in a caudal to rostral direction is worthy of continued investigation.

The role of 5-HT in the developing larva has been shown to be paramount in the early larval stages 37/38 to 42 as serotonergic fibres descend from the brain and

progressively innervate more of the spinal cord transforming the CPG network as they process. Serotonin has been shown to modulate the persistent sodium current in rat MNs through the 5-HT2 receptor and could therefore contribute to repetitive firing in *Xenopus* as well.

In this thesis the importance of GABAergic control of spontaneous rhythm generation has been presented but further experiments involving agonist of the $GABA_AR$ could prove invaluable. Endogenous steroids are known to act on this receptor and modulate the response to GABA (for a review see Belelli and Lambert 2005). The steriod 5α-PREGNAN-3α-OL-20-ONE, is one of many stereoselective allosteric modualtors of the receptor and could be used to see if spontaneous rhtyhm generation decreased as a result of potentiating the presumed endogenous, or central, GABAergic control observed in the experiments in this thesis.

The physical lesions presented in this thesis could be made more precise, perhaps with the use of a vibratome, but excitotoxic lesioning could also be employed to try and directly target areas of interest within the hindbrain. This technique has already be employed with great success in behavioural neuroscience, specifically excitotoxic lesions to the posterior and not to the anterior pedunculopontine nucleus alter the self-administration of nicotine in rats (Alderson et al. 2006) and so there is a lot of potential for a variety of lesioning strategies to be implemented to help determine what regions of the hindbrain are necessary for the generation of the spontaneous rhythm.

Given that this newly acquired free-swimming existence is coincident with the onset of free-feeding in the larval *Xenopus*, an interesting direction to pursue would be to look at the role of dopamine modulation of the swimming network. Dopamine has been shown to be involved in the control of appetite and feeding behaviour in a variety of vertebrates and invertebrates. A recent article from (Marella et al. 2012) shows that dopaminergic neurons modulate response to tasting sucrose. Recent work in the

metamorphic stages of *Xenopus* has also suggested that dopamine may act as modulator of motor output. Given the strong connection with filter-feeding and locomotion in the larval animal the dopamergic system – which may develop at the onset of feeding as well – is the perfect neuromodualtory system to integrate the requirement to feed with motor output and is incredibly worthy of further investigation.

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