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Evolutionary Divergence in Developmental Strategies and Neuromodulatory Control Systems of Two Amphibian Locomotor Networks*

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Attempts to understand the neural mechanisms which produce behaviour must consider both prevailing sensory cues and the central cellular and synaptic changes they direct. At each level, neuromodulation can additionally shape the final output. We have investigated neuromodulation in the developing spinal motor networks in hatchling tadpoles of two closely related amphibians, *Xenopus laevis* and *Rana* temporaria to examine the subtle differences in their behaviours that could be attributed to their evolutionary divergence.

At the point of hatching, both species can swim in response to a mechanosensory stimulus, however *Rana* embryos often display a more forceful, non-locomotory coiling behaviour. Whilst the synaptic drive that underlies these behaviours appears similar, subtle inter-specific differences in neuronal properties shape motor outputs in different ways. For example, *Rana* neurons express N-methyl-D-aspartate (NMDA)/serotonin (5-HT)-dependent oscillations, not present in hatchling *Xenopus* and many also exhibit a prominent slow spike after-hyperpolarisation. Such properties may endow the spinal circuitry of *Rana* with the ability to produce a more flexible range of outputs.

Finally, we compare the roles of the neuromodulators 5-HT, noradrenaline (NA) and nitric oxide (NO) in shaping motor outputs. 5-HT increases burst durations during swimming in both *Xenopus* and *Rana*, but 5-HT dramatically slows the cycle period in *Rana* with little effect in *Xenopus*. Three distinct, but presumably homologous NO-containing brainstem clusters of neurons have been described, yet the effects of NO differ between species. In *Xenopus*, NO slows and shortens swimming in a manner similar to NA, yet in *Rana* NO and NA elicit the non-rhythmic coiling pattern.

Introduction

The generation of locomotory behaviours, such as walking, running or swimming relies upon the co-ordinated cyclical contraction and relaxation of antagonistic muscles, whose rhythmic activation is controlled by neuronal central pattern generators located within the central nervous system (CNS). The inherent ability of these neural networks to alter their output in response to a perpetually changing

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environment is crucial for an organism's survival and can be achieved by rapid and precise changes in both the cellular properties of individual neurons and their synaptic interconnections. For example, aquatic animals must be able to adjust their swimming speed and intensity, without compromising efficient movement through the water column. Therefore, the propagation of muscle contractions along the body should always be co-ordinated in such a way that an optimal body shape for propulsion is maintained. The traditional view that central networks are hard-wired can not fully account for the neural control of behaviour and more recently it has become clear that neuromodulators are able to confer a degree of flexibility.

It is clear that neuromodulation of motor behaviours occurs throughout the animal kingdom (for reviews see: Kiehn and Katz, 1999; Pearson, 1993). Some of the most fruitful investigations have derived from relatively simple organisms, where there is a realistic chance of gaining a detailed knowledge of the cellular and synaptic mechanisms that underlie changes in behaviour. Neuromodulation has now been investigated during a wide range of behaviours in both invertebrate and vertebrate organisms, from feeding in molluscs (*e.g.,* Morgan *et al.*, 2000), and digestion in crustaceans (*e.g.,* Selverston *et al.*, 1998), to the control of respiration in bullfrogs (Hedrick *et al.*, 1998) and locomotion in, for example, crayfish (*e.g.,* Pearlstein *et al.*, 1998) and lamprey (Buchanan, 2001). It is, however, important that parallels are drawn between different phyla and, in particular, between closely related species, to understand how such systems might have evolved. Careful comparisons between closely related species in relation to the different functions, and developmental emergence of homologous neuron groups may provide insights into how evolution sculpts different behavioural strategies from the underlying neural substrate. We have chosen the hatchling tadpole of the South African clawed frog *Xenopus laevis* as a model system in which to investigate the development and modulation of locomotion. There is now a detailed knowledge of the neuroanatomy (Roberts and Clarke, 1982; Roberts, 2000), physiology (Kahn and Roberts, 1982*a*, *b*) and development (Sillar *et al.*, 1992*a*, *b*; 1995*a*, *b*) of locomotion in *Xenopus*. Our recent focus has been on three neuromodulatory systems, the serotonergic, the noradrenergic and the nitrergic, which have a range of effects at different levels of locomotor control, but

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which collectively afford the animal a significant degree of flexibility (McLean *et al.*, 2000; Sillar *et al.*, 2002). In addition we have performed a series of comparative studies using embryos of the local species of tadpole, *Rana temporaria*. Despite being evolutionarily closely related, some interesting contrasts are now emerging about the subtle differences between the two species, which may be accounted for by differences in their evolution and habitats.

Methods

The methods used to examine the neural networks underlying the generation of behaviour in amphibian embryos have been well described previously (*e.g.,* Kahn and Roberts, 1982*a*; Soffe, 1991*a*), so only a brief description is given here. *Xenopus laevis* embryos (stage 37/38; Nieuwkoop and Faber, 1956) were obtained from an adult laboratory colony, whilst *Rana temporaria* embryos (stage 20; Gosner, 1960) were collected as spawn from local ponds. Animals of both species were kept at a range of temperatures (4–23°C) in order to control their rate of development. After being immobilised with the neuromuscular blocking agent α-bungarotoxin, animals were pinned onto a Sylgard elastomer-lined platform in an experimental chamber containing continuously recirculating frog ringer solution and electrophysiological recordings were made of neural activity appropriate to drive locomotion *in vivo.* Extracellular suction electrodes placed over the intermyotomal clefts along the body recorded activity from the ventral roots which lie within each cleft, whilst sharp microelectrodes were used to record from presumed motor neurons in the ventral region of the spinal cord. Pharmacological manipulations were performed by adding different agents to the perfusate.

Nitric oxide synthase (NOS) activity was detected using the nicotinamide adenine di-nucleotide phosphate (NADPH)-diaphorase technique. After being deeply anaesthetised and fixed in paraformaldehye the CNS of each animal was removed and incubated in the NADPH-diaphorase staining solution (0.1 M phosphate buffer containing 1 mg ml-1 β-nicotinamide adenine di-nucleotide phosphate, 0.1 mg ml-1 nitroblue tetrazolium and 0.3% triton X100) for 1–3 hours. Each CNS was then rinsed, dehydrated, cleared and mounted (see McLean and Sillar, 2000, 2001 for more details).

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Results

Morphological and ecological considerations

When embryos of *Xenopus laevis* and *Rana temporaria* hatch from their eggs they are clearly very morphologically different (*c.f.*, Fig. 1Ai,Bi). Not only are hatchling *Xenopus* smaller (~5 mm; c.f. 1 cm in *Rana*) and less pigmented, they are also fast and efficient swimmers from the point of emergence. The *Xenopus* tadpoles normally hang motionless from objects in the environment, such as the underside of a leaf, attached by the mucus-like cement that is exuded from a rostral gland (arrowed in Fig. 1Ai). Swimming can be reliably triggered by a mechanosensory stimulus and is characterised by rhythmic alternating flexions of the body at frequencies of 10 to 20 Hz, which propels the animal through the water column (Fig. 1Aii). There is little variability in motor burst durations from one cycle to the next and cycle periods gradually decline during the course of an episode. By contrast, *Rana* embryos at the equivalent hatching stage of development (stage 20; Gosner, 1960) are unlikely to swim at all unless given repeated and strong mechanosensory stimulation (Fig. 1Bii). Instead, they are normally motionless but will generate occasional spontaneous ''coiling'' (or ''flexing'') movements during which the body flexes arrhythmically and intermittently to either side. A slow bend of the body to one side, lasting a second or more may, or may not, be followed by a bend to the opposite side (Fig. 1Biii). Coiling in *Rana* is distinct from any behaviour displayed by *Xenopus*, although it is similar in some respects to ''struggling,'' in which forceful contractions of the myotomes also generate large scale, non-locomotory bends in the body (*cf.*, Soffe, 1991*a*, *b*). There are, however, clear differences because struggling is only initiated by repetitive sensory stimulation in *Xenopus*, such as when the tadpole is grasped, and unlike *Rana's* coiling behaviour, it is well-coordinated and rhythmic with tail to head propagation of activity (Soffe, 1991*b*, Fig. 1Aiii).

These differences in behavioural repertoire at the time of hatching may well be related to the different ecological and reproductive strategies of the two species. Adult female *Xenopus* lay their eggs singly, often under leaves, where they develop and hatch in

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approximately 2 days (at 23°C); *Rana*, however, lay large clumps of many hundreds of eggs each surrounded by a sticky gelatinous mass in which the developing animal grows and hatches after several days. It might be speculated that in contrast to *Xenopus*, the coiling behaviour is critical as a means of wriggling free and dispersing from the egg mass, whereas in *Xenopus* hatchling tadpoles are able to swim unrestrained immediately after hatching.

Network Output

Fictive correlates of these early behaviours can be recorded in embryos of *Xenopus* and *Rana* that have been immobilised in α-bungarotoxin (see methods section). Thus the complete behavioural repertoire of each species can be generated entirely by central neuronal networks and do not therefore depend upon sensory feedback for their production and maintenance.

In *Xenopus*, a single brief electrical or tactile stimulus to the tail skin will elicit a reflexive activation of motor neurons on the opposite side, often followed by an episode of swimming, during which motor neurons usually discharge a single action potential in each cycle (Fig. 1Aii). There is strict alternation in the activity of motor neurons on opposite sides of the body and, as in real swimming, the rhythm occurs at 10 to 20 Hz and propagates along the body with a brief rostrocaudal delay between segmental ventral roots. Repetitive stimulation will trigger fictive struggling in which motor neurons discharge bursts of action potentials in each cycle of a rhythm which, again like the real behaviour, alternates across the body and propagates in the opposite direction to swimming, from tail to head (Soffe, 1991*a*; Fig. 1Aiii). It is rare to observe any spontaneous motor discharges in *Xenopus* embryos.

By contrast, in immobilised *Rana* embryos, spontaneous motor activity is quite frequently observed. This activity is intermittent and highly variable, such as coiling behaviour, and it involves motor bursts of up to one second in duration (Fig. 1Biii). Bursts are never simultaneous across the body but they occur nearly simultaneously within a side, with a brief head to tail delay, in contrast to struggling in *Xenopus*. Sensory stimulation of the trunk or head skin can elicit a

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bend of the body to the side opposite the stimulus and, as in *Xenopus*, such responses can be followed by bouts of rhythmic swimming, although less reliably so. The coordination of swimming is similar between the two species. This is perhaps not surprising as the need to generate forward propulsion will rely on similar sequences of contractions of the myotomal muscles, as is the case for anguilliform swimming in other aquatic animals such as fish and lampreys (Wallen and Williams, 1984). However, the two swimming rhythms are quantitatively different: bouts of *Rana* swimming are normally far briefer and consist of fewer cycles; the rhythm is also much more variable in virtually all of its basic parameters, not least the duration of motor bursts which are commonly in the 50 to 100 millisecond range (cf. <10 ms in *Xenopus* embryos).

Synaptic Drive for Swimming

During swimming in both species, motor neurons are driven to fire impulses by excitatory depolarising synaptic inputs and they are inhibited during the inter-burst intervals by chloride-dependent, glycinergic synaptic inhibition. Evidence from both species supports the conclusion that the excitation derives from glutamatergic premotor interneurones and cholinergic homonymous motorneurones (Dale and Roberts, 1985; Roberts and Perrins 1994, 1995*a*, *b*; Perrins and Soffe, 1996). The only major difference appears to be that *Xenopus* motor neurons additionally receive an electrotonic component during the excitatory phase while *Rana* neurons do not (Perrins and Roberts, 1995*a*–*c*, Perrins and Soffe, 1996; but see Sillar and Simmers, 1994*a*). This difference may be important as the presence of strong electrical coupling in *Xenopus* may serve to tightly synchronise the firing of motor neurons in each cycle and which might account, at least in part, for the relatively stereotyped, single spike-per-cycle embryonic swimming rhythm. In *Rana* the lack of strong electrical coupling may mean that motor neurons are not so constrained to fire in synchrony during swimming, allowing for motor bursts to be more variable. It is not immediately obvious why such a difference is observed or required. The simplest explanation is perhaps their difference in size and the resulting biomechanical demands require a more robust motor pattern from *Rana*. The nervous system appears to accommodate this requirement via a suite of cellular and synaptic mechanisms.

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Electrical Properties of Motor Neurons

The reasons why *Xenopus* embryo motor neurons discharge a single action potential in each cycle of swimming are unclear. One possibility is that the set of ionic conductances at the time of hatching renders them incapable of firing more than one action potential, even when the excitatory drive is strong. The evidence in favour of this argument includes the fact that when recorded with sharp microelectrodes motor neurons will usually only fire a single impulse at threshold, regardless of how much supra-threshold depolarising current is subsequently injected (Soffe, 1987). However, sharp recordings from the same neurons at the hatching stage show that they *are* capable of firing bursts of action potentials during struggling behaviour (Soffe, 1991*b*, 1993). Whilst this indicates that the damage inflicted by the microelectrode cannot be sufficient to render neurons completely incapable of multiple firing, it raises questions as to why they normally fire only once during swimming when there is sufficient time to fire more. An alternative hypothesis, however, suggests that *Xenopus* embryo motor neurons are capable of multiple discharge during swimming but they only fire once due to the damage inflicted by penetration with a microelectrode which reduces their input resistance (Dale, 1995). Similar results for CPG interneurons have recently been obtained (Aiken *et al.*, 2003). On the basis of these findings, it has been argued that during swimming neurons discharge at a preferred firing rate, up to twice swimming frequency and that this rate is reduced during swimming by mid-cycle inhibition (Dale, 1995; Aiken *et al.*, 2003). As the tadpole matures, the same sharp electrode recordings, just 24 hours later in development, reveal that neurons become more excitable and can now fire multiply in response to current and during swimming (Sillar *et al.*, 1992*b*), a change accompanied by developmental alterations in the balance of ionic conductances (Sun and Dale, 1998). Interestingly, at the equivalent stage of development and under similar experimental conditions, *Rana* motor neurons will fire multiply both during swimming and in response to depolarising current injection. The functional consequences of the firing properties of individual neurons during behaviour at different stages and in different species is, however, unknown.

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An important cellular property possessed by a proportion of *Rana* motor neurons that may help to explain some of the differences between the two species is the presence of a prominent slow spike afterhyperpolarization (sAHP; Fig. 1Biv). The properties of this sAHP (Buchanan *et al.*, 1999), make it remarkably similar to the sAHP described in lamprey neurons (*e.g.,* Van Dongen *et al.*, 1986; Hill *et al.*, 1992; Meer and Buchanan, 1992; Buchanan, 1993; Cangiano *et al.*, 2002). It has a time to peak of around 10 to 15 ms and a duration of about 50–100 ms. (Buchanan *et al.*, 1999). Moreover, the sAHP in *Rana* is blocked by both apamin and 5-HT (Buchanan *et al.*, 1999), as is the case for lamprey motor neurons (Hill *et al.*, 1992; Meer and Buchanan, 1992). Presumably the current underlying the sAHP in *Rana* is a calcium-dependent potassium current (IKCa). Thus *Rana* and lamprey motor neurons are distinctly different from homologous neurons in *Xenopus* embryos in possessing a prominent sAHP. No such cellular property has been reported in *Xenopus* neurons, although voltage clamp studies indicate the presence of a small and extremely slow IKCa that has a time to half activation of several hundreds of milliseconds. It is thought that this current contributes to the termination of episodes of *Xenopus* embryo swimming rather than to the control of spike frequency within each cycle (Wall and Dale, 1995; reviewed in Dale and Kuenzi, 1997).

Intrinsic Oscillations

In a wide range of motor systems intrinsic oscillatory membrane properties are thought to contribute to the pattern of motor output. These oscillations result from the unusual properties of N-methyl-D-aspartate (NMDA)-type glutamate receptors, which are activated during locomotory activity. The NMDA receptor ionophore is blocked by Mg2+ ions in a voltage-dependent manner (Mayer *et al.*, 1984) and it is the regenerative, cyclical blocking and un-blocking of the ionophore which causes the membrane potential to oscillate between two quasi-stable voltage states; at rest and at some more depolarised level. However, although *Xenopus* embryo motor neurons possess NMDA receptors which are voltage dependent in the presence of magnesium and are activated during swimming activity (Soffe and Roberts, 1989), there is no evidence in recordings from the intact spinal cord that their activation leads to oscillatory membrane behaviour (Scrymgeour-

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Wedderburn *et al.*, 1997; but see, however, Prime *et al.*, 1999). In *Rana* neurons applications of NMDA also fail to induce membrane oscillations until 5-HT is added to the bathing medium (Fig. 2Ai,ii), which results in oscillations almost indistinguishable in waveform and time course to those induced by NMDA alone in lamprey neurons (Sillar and Simmers, 1994*b*; *c.f.*, Van Dongen *et al.*, 1986, Wallen *et al.*, 1989). This has led to the suggestion that there might be sufficient endogenous 5-HT in the adult and higher vertebrate spinal cord to gate the NMDA oscillations, a notion for which there is some experimental support (MacLean and Schmidt, 2001). Indeed, in both the cat and the turtle, membrane bistability, which supports the presence of long-lasting Ca^{2+}-dependent plateau potentials, also relies upon 5HT (Hounsgaard and Kiehn, 1989; Hounsgaard *et al.*, 1988). The studies on amphibians indicate a form of coupling between NMDA and 5-HT receptors, whose co-activation is a necessary step in the induction of membrane potential oscillations. Returning to the *Xenopus* embryo, the addition of 5-HT to the bathing medium, in the presence of NMDA still fails to induce oscillations (Fig. 2B). However, this is presumably due to the developmental immaturity of the *Xenopus* embryo, since one day later in development, at larval stage 42, 5-HT *is,* in the presence of NMDA, able to induce TTX-resistant oscillations that closely resemble those described in *Rana* and lamprey (Scrymgeour-Wedderburn *et al.*, 1997; Reith and Sillar, 1998; Fig. 2C).

The behavioural contributions of such intrinsic oscillations during locomotion, which have a cycle time of approximately 2 seconds, are still unclear. It has been proposed in *Xenopus* larvae that an oscillation might be triggered by a bolus of 5-HT released from the raphe during an episode which could then accelerate and intensify swimming activity over many consecutive cycles *i.e.,* during the depolarised plateau phase of an oscillation (Reith and Sillar, 1998). This idea is compelling, not least because when swimming is induced by bath applications of NMDA in stage 42 *Xenopus* larvae it is cyclically modulated in intensity and frequency with a period similar to the TTX-resistant oscillations. This ''harmonic'' modulation of swimming in the presence of NMDA does not occur in *Xenopus* embryos where intrinsic NMDA oscillations are not observed either (Reith and Sillar, 1998). In lampreys, the oscillations are thought to contribute to the cycle-by-

cycle fluctuations in membrane potential that occur during swimming due to the overlap of swimming frequencies and the frequencies of NMDA-induced, TTX-resistant oscillations. The synaptic inputs to motor neurons would then be in a position to trigger the onset and offset of an oscillation in each cycle and hence modulate the cycle frequency (Van Dongen *et al.*, 1986, Wallen *et al.*, 1989).

Species-Specific Modulation of Motor Networks

The effects of 5-HT, NA and NO on *Rana* and *Xenopus* neurons described below provide a tantalising glimpse of the way in which species-specific differences in neuromodulatory influences during development must somehow regulate the way in which a given behavioural repertoire is expressed. In this section we will compare the effects of different brainstem modulators on motor activity and motor behaviour in these two species.

Biogenic amines 5-HT and NA

In terms of the overall influence of 5-HT on swimming, this amine has broadly similar effects in *Rana* and *Xenopus*: in both species the duration of motor bursts increases relative to the cycle period (*i.e.,* the duty cycle). However, in *Xenopus* there is comparatively little effect upon the actual cycle period and, as a result, 5-HT leads to a relatively fast, intense version of swimming activity (Sillar *et al.*, 1992*c*). In *Rana* there is also an increase in the duty cycle but this is accompanied by a very dramatic slowing in swimming frequency (Woolston *et al.*, 1994). This response to 5HT in *Rana*, which can be mimicked by selective 5HT uptake inhibition (D. L. McLean and K. T. Sillar, unpublished observations), is very similar to the response to 5-HT observed in the lamprey swimming system when the spinal network is activated by NMDA or D-glutamate (Harris-Warrick and Cohen, 1985)

Whilst the effects of 5-HT in *Rana* and *Xenopus* are rather similar, the effects of NA are remarkably different between the two species. In *Xenopus*, NA slows swimming, has little effect on burst durations and hence decreases the duty cycle, the opposite effect to 5-HT in this species (McDearmid *et al.*, 1997, Fischer *et al.*, 2001). Thus,

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these two brainstem-derived aminergic modulators have opposing effects upon the spinal motor circuitry in *Xenopus* enabling one neural network to produce a wide range of locomotor outputs, from relatively fast, intense swimming (5-HT) to slower, weaker swimming (NA). In *Xenopus* there is evidence that endogenous activation of both 5-HT and NA receptors mimics the effects of exogenously applied amines (Merrywest *et al.*, 2002; Sillar *et al.*, 1995*a*) In *Rana*, surprisingly, NA has no significant effects on swimming (McDearmid, 1998), but instead triggers a non-rhythmic motor pattern that is indistinguishable from the coiling behaviour that occasionally occurs spontaneously (McLean and Sillar, 2003; McDearmid and Sillar, 1997).

Divergent nitrergic metamodulation

The free radical gas nitric oxide (NO) has profoundly different modulatory effects on motor activity in *Rana* and *Xenopus*. Intriguingly, the effects of NO (via bath application of the NO donor S-nitroso-N-acetylpenicillamine, or SNAP) and NA are similar within, but different between species. In *Xenopus*, NO has a net inhibitory effect on swimming, reducing episode durations and slowing swimming (McLean and Sillar, 2000), just like the effects of NA described above (McDearmid *et al.*, 1997; Fischer *et al.*, 2001). Whilst there are elements of overlap in the underlying inhibitory mechanisms of NO and NA, suggestive of a cooperative interaction between these two modulators, there are also some effects on the network that are unique. For example, NO produces a marked membrane potential depolarisation in motor neurons and a concomitant conductance decrease, (McLean and Sillar, 2002). NA appears to have neither of these effects, but does enhance post-inhibitory rebound firing (Merrywest *et al.*, 2003). In *Rana*, exogenously applied NO does not affect fictive swimming activity; instead, NO triggers a non-rhythmic motor pattern which is indistinguishable from the pattern either elicited by NA or recorded spontaneously in immobilised preparations (McLean *et al.*, 2001). Moreover, similar behavioural responses are observed when SNAP is added to a dish containing non-immobilised, intact *Rana* embryos, where it triggers bouts of tail coiling behavior (G. Mason and K.T. Sillar, unpublished observations). These movements are non-locomotory and involve large-scale bends of the body in which

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the whole animal coils towards the left or right sides at frequencies of around 0.5 to 1 Hz.

On the Sources and Ontogeny of Brainstem Modulators

We will now discuss the possible sources of 5-HT, NA and NO in the CNS of the two species, including when these sources appear during development and whether this provides a plausible explanation for the profound inter-specific differences in their behavioural effects.

5-HT immunocytochemistry indicates that at the time of hatching both *Rana* and *Xenopus* embryos possess a raphe nucleus in the hindbrain. However, in hatchling *Rana* the serotonergic system is arguably much more advanced since the innervation of the spinal cord by serotonergic axons by comparison with *Xenopus* is much more extensive (Woolston *et al.*, 1994). This difference may explain the more robust response to 5-HT during swimming in *Rana* as compared to *Xenopus*, although the receptor subtypes and targets of 5-HT, such as the sAHP, may also differ between the two species. There is less information on the distribution of neurons producing NA, although tyrosine hydroxylase staining suggests a group of neurons located more rostrally and dorsally in the isthmic region at the junction between the hindbrain and the midbrain, may correspond to the amphibian homologue of the locus coeruleus (Marin *et al.*, 1996). There is, however, no immunocytochemical evidence to suggest spinal neurons manufacture NA or 5-HT at these early stages of development in anuran amphibians (van Mier *et al.*, 1986; Woolston *et al.*, 1994; Sanchez-Camacho *et al.*, 2002).

The location and distribution of CNS neurons that display NADPH diaphorase activity (an indicator of nitric oxide synthase, or NOS), and are thus likely sources of NO, is illuminating. In *Rana* there are three clusters of neurons in the brainstem located in the isthmus, the raphe region, and the reticular formation (Fig. 3B). In *Rana* NADPH diaphorase labeling is also present in the marginal zones of the spinal cord, which presumably reflects descending axons, since there is no discernable intraspinal source of NADPH-diaphorase reactivity at this stage (McLean *et al.*, 2001). However, which of the three populations is the likely source is not yet known. In hatchling *Xenopus* embryos

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there are two prominent clusters of brainstem neurons in the isthmus and the reticular formation of the caudal hindbrain (Fig. 3A). However, there is no labeling of axons descending from these clusters into the spinal cord. Thus, there are some clear inter-specific differences in the distribution of nitrergic neurons and their axonal projections at the hatching stage. Whether or not these differences are adequate to explain the different behavioural effects of NO is open to debate. However, it is notable that at stage 42 in *Xenopus*, neurons of the raphe become NADPH diaphorase positive suggesting that all three groups of brainstem neurons of both species eventually use NO as a co-transmitter and are therefore homologous in terms of their transmitter phenotype. The caudal reticular group shows an interesting inter-specific difference, which may also contribute to the different behavioural effects in *Rana* and *Xenopus*. By stage 42 in *Xenopus*, this cluster comprises two sub-groups; a large ventral group and a less numerous group of more dorsally located neurons (McLean and Sillar, 2001). This latter group may correspond to the well-documented population of mid-hindbrain reticulospinal (mhr) neurons which constitute a descending GABAergic stopping pathway, which we have proposed from physiological experiments is facilitated by both NO and NA to prematurely terminate swimming (McLean and Sillar, 2002; Merrywest *et al.*, 2002). The function of the more ventral group, the only group to label in the reticular formation of *Rana*, is not known. However, these neurons appear not to label with antibodies against GABA or glycine and so are presumably excitatory (Dale *et al.*, 1986; Roberts *et al.*, 1987). The fact that in *Rana* the axons of brainstem neurons are NADPH-diaphorase positive may also be linked to the fact that NO and NA have an excitatory effect and are able to trigger motor activity in this species.

Summary and Conclusions

Rana and *Xenopus*, two closely related anuran amphibians, hatch at clearly different states of development, both in terms of their size and morphology and in terms of their respective behavioural repertoires. *Xenopus* hatchlings are relatively small (5 mm long) and are already efficient swimmers; *Rana* are much larger (1 cm) and swim less readily. Our recent comparative studies on spinal motor control suggest that these inter-specific differences are due to the way

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in which the late embryonic nervous system matures and is influenced by a variety of brainstem modulatory systems. We have attempted to link this to the ecological niches of the two organisms since *Rana* hatch from a large gelatinous eggs mass from which they must wriggle free, whilst *Xenopus* hatch from eggs laid singly.

The key systems that may account for these inter-specific differences in behavioural repertoire include the amines, 5-HT and NA, and the gaseous modulator NO. Our data have revealed that NA and NO have similar effects *within* each species but radically different effects *between* species. In *Xenopus* NA and NO modulate swimming through complex and sometimes parallel mechanisms (*e.g.,* facilitation of inhibitory synaptic transmission). However, in *Rana*, neither NA nor NO have any significant effect on swimming activity but instead they initiate a motor pattern believed to underlie the coiling movements that accompany hatching. Of particular interest in our attempt to explain the inter-specific differences in behaviour in terms of brainstem modulators is the observation that NO appears to be produced by three distinct and homologous clusters of neurons in the brainstem, some members of which appear to be homologous between the two species. In one of these species, for the most numerous caudal cluster in the reticular formation, NADPH diaphorase labels a dorsal sub-group in *Xenopus* which is thought to correspond to a well-described GABAergic population, the mhr neurons. The fact that in *Rana* this population is not labeled by NADPH diaphorase and that labeling extends into the spinal axons of brainstem reticulospinal neurons may well provide the explanation for the ability of NO to trigger coiling behaviour.

Whilst NA and NO have quantitatively different effects between species, 5-HT has similar but qualitatively different modulatory actions. In both species 5HT intensifies motor bursts during swimming but only in *Rana* does 5-HT have any significant effect of swimming frequency. It has been noted that the serotonergic raphe system is in a more advanced state in *Rana* compared to *Xenopus*, with a dense spinal projection already present at hatching. However, it is also clear that the cellular targets and effects of 5-HT on spinal motor neurons also differs between species. Thus, while 5-HT is able to induce NMDA receptor mediated oscillations in *Rana*, this induction process is absent

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in hatchling *Xenopus*, even though it develops in early larval stages. Also, *Rana* neurons possess a clear sAHP, similar to that present in lamprey motor neurons, which is an important target for serotonergic modulation, but which is apparently absent in *Xenopus* embryo motor neurons.

Notes

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Figure 1

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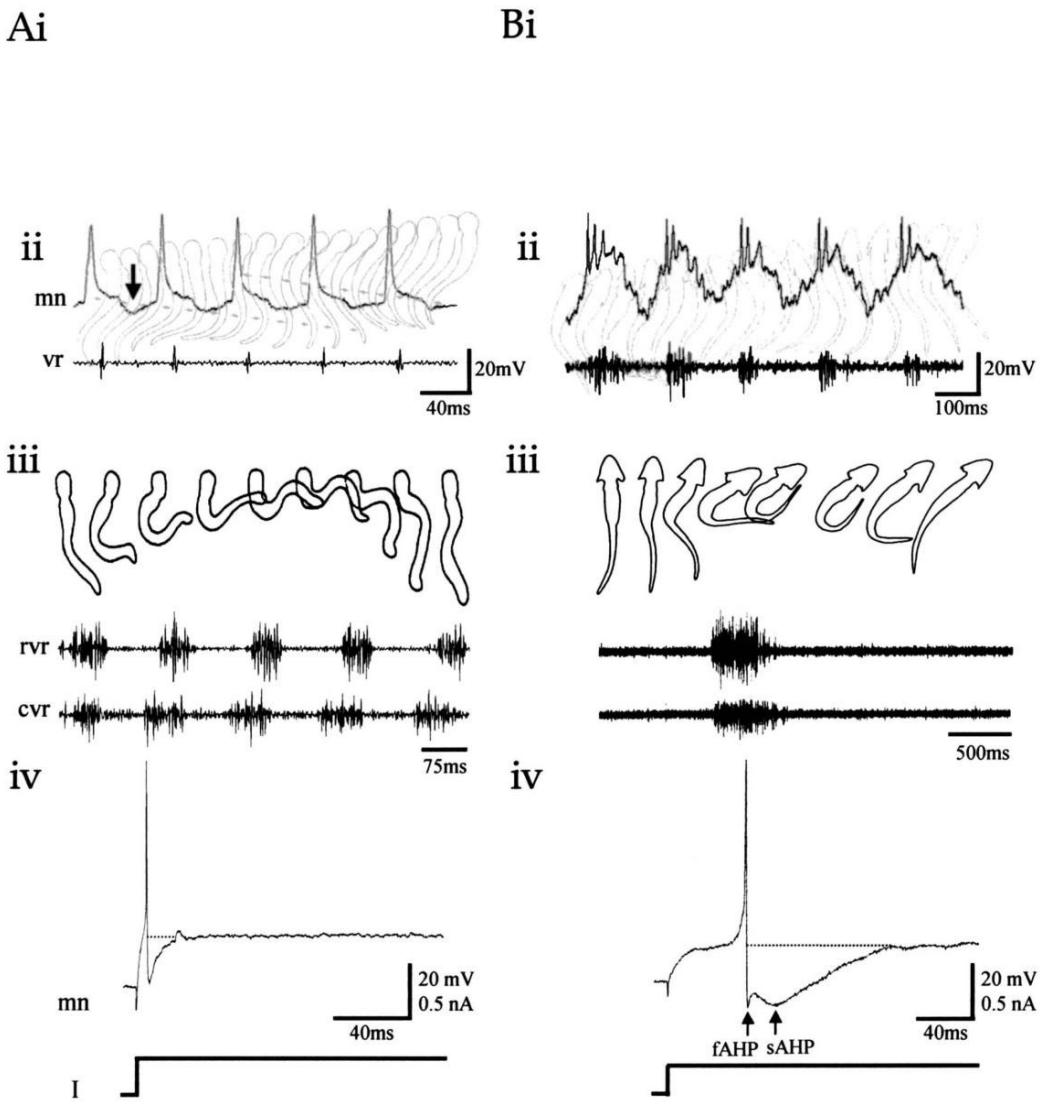

Behavioural repertoires of *Xenopus* and *Rana* embryos and their fictive correlates from ventral root recordings. (Ai, Bi) Stage 37/38 (Nieuwkoop and Faber, 1956) *Xenopus laevis* and Stage 20 *Rana temporaria* (Gosner, 1960) embryos. (Aii) In *Xenopus*, a brief mechanosensory stimulus initiates swimming, characterised by alternating head-to-tail flexions of the body (background silhouette of a single representative cycle) and underlaid by rhythmic synaptic drive comprising excitation and alternating mid-cycle inhibition (arrowed). (Bii) Swimming in *Rana* requires a repeated and strong stimulus, but is also characterised by alternating flexions of the longitudinal muscles. (Aiii) More prolonged or severe sensory inputs in *Xenopus* produce a struggling behaviour (silhouette), which like swimming is produced by alternating left and right muscle

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activation, but which progresses along the body from tail to head (lower traces). (Biii) Coiling in *Rana* is arrhythmic and is rarely sustained or prolonged. (Aiv) In response to injected depolarising current *Xenopus* motor neurons fire a single compound action potential which has no obvious sAHP. (Biv) By contrast *Rana* motor neurons fire an action potential which possesses a clear sAHP with a time to peak of 10–15 ms and duration of 50–100 ms. Vr = ventral root (r = rostral; c = caudal); mn = motor neuron; I = current.

Figure 2

NMDA/5-HT mediated oscillations are present in *Rana*, but not *Xenopus* embryo neurons. (Ai) In the presence of 0.5 μM TTX, application of 100 μM NMDA evokes a sustained depolarisation in *Rana* neurons, but does not induce intrinsic oscillatory activity. (Aii) Subsequent application of 5-HT induces oscillations that are similar to those described in other vertebrate motor neurons. (B,C) In the presence of NMDA and TTX, 5-HT hyperpolarises the resting membrane potential of both embryonic and larval *Xenopus* motor neurons. Subsequent injection of hyperpolarising current rarely elicits oscillations in embryonic neurons (B, lower panel), but often does in larval ones (C, lower panel).

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Figure 3

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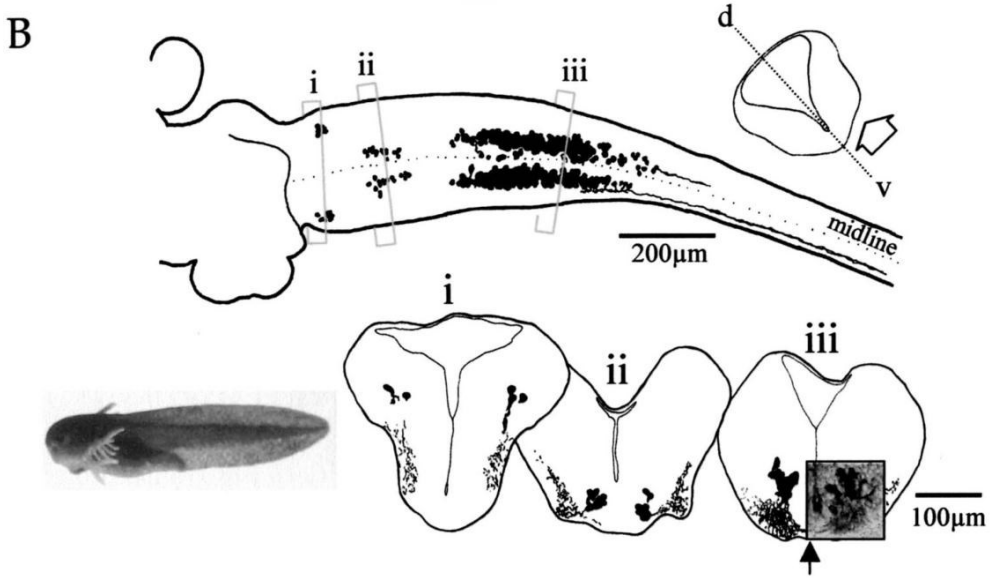
A



Similar patterns of NADPH-diaphorase staining in *Xenopus laevis* and *Rana temporaria* embryos. (A) Camera lucida schematic (top panel) of the excised, whole-mount CNS of a *Xenopus* embryo (photomicrograph) from the lateral view depicts the approximate regions (vertical lines) serial sections were made in the midbrain (i), locus coeruleus (ii) and reticular region (iii). (B) A similar camera lucida schematic (top panel) of the whole-mount CNS of a *Rana* embryo (photomicrograph) from a slightly oblique view (orientation given in accompanying inset, note midline) depicts the approximate regions (vertical lines) where serial sections were made in the locus coeruleus (i), raphe region (ii) and reticular region (iii). Arrow indicates crossing projections. d, dorsal; v, ventral.

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