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INDUCTION OF A NON-RHYTHMIC MOTOR PATTERN BY NITRIC OXIDE IN HATCHLING *RANA TEMPORARIA* EMBRYOS

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Summary

Nitric oxide (NO) is a ubiquitous neuromodulator with a diverse array of functions in a variety of brain regions, but a role for NO in the generation of locomotor activity has yet to be demonstrated. The possibility that NO is involved in the generation of motor activity in embryos of the frog *Rana temporaria* was investigated using the NO donors *S*-nitroso-*n*-acetylpenicillamine (SNAP; 100–500 μmol l^{-1}) and diethylamine nitric oxide complex sodium (DEANO; 25–100 μmol l^{-1}). Immobilised *Rana temporaria* embryos generate a non-rhythmic 'lashing' motor pattern either spontaneously or in response to dimming of the experimental bath illumination. Bath-applied NO donors triggered a qualitatively similar motor pattern in which non-rhythmic motor bursts were generated contra- and ipsilaterally down the length of the body. The inactive precursor of SNAP, *n*-acetyl-penicillamine (NAP), at equivalent concentrations did not trigger motor activity. NO donors failed to initiate swimming and had no measurable effects on the parameters of swimming induced by electrical stimulation. Intracellular recordings with potassium-acetate-filled electrodes revealed that the bursts of ventral root discharge induced by NO donors were accompanied by phasic depolarisations in motor neurons. During the inter-burst intervals, periods of substantial membrane hyperpolarisation below the normal resting potential were observed, presumably coincident with contralateral ventral root activity. With KCl-filled electrodes, inhibitory potentials were strongly depolarising, suggesting that inhibition was Cl^--dependent. The synaptic drive seen in motor neurons after dimming of the illumination was very similar to that induced by the NO donors. NADPH-diaphorase histochemistry identified putative endogenous sources of NO in the central nervous system and the skin. Three populations of bilaterally symmetrical neurons were identified within the brainstem. Some of these neurons had contralateral projections and many had axonal processes that projected to and entered the marginal zones of the spinal cord, suggesting that they were reticulospinal.

Key words: nitric oxide, frog, *Rana temporaria*, motor pattern, swimming, NADPH diaphorase, histochemistry.

Introduction

Animals must be able to initiate and terminate motor behaviour patterns according to prevailing behavioural and developmental requirements. Even among closely related species, the expression and emergence of different patterns of motor behaviour during development can follow different temporal sequences, presumably to meet the demands imposed by their distinct ecological niches. However, the way in which motor networks are modified during ontogeny to enable species-specific temporal expression of different motor patterns is not fully understood. Neuronal inputs originating in the brainstem and descending to the spinal cord both modulate ongoing motor programs and prime, activate and suppress motor behaviour. As such, developmentally regulated ingrowth to the spinal cord by the axonal processes of brainstem neurons may be important for the temporal expression of different behaviour patterns. For example, at the time of hatching, the tadpole of *Rana temporaria* (stage 20: Gosner, 1960) is capable of generating a well-coordinated swimming motor pattern (Soffe, 1991; Soffe and Sillar, 1991). However, fictive swimming in *Rana temporaria* is relatively more mature than in hatchling tadpoles of the related anuran *Xenopus laevis* in that motor bursts are longer and more variable on a cycle-by-cycle basis (Sillar and Soffe, 1989). It has been speculated that this interspecific difference at equivalent stages of development is due to the greater development of descending serotonergic projections in *Rana temporaria* because serotonin (5-HT) is known to facilitate motor burst generation in both species (Woolston et al., 1994; Sillar et al., 1993).

The free radical gas nitric oxide (NO) is recognised as a ubiquitous signalling molecule that is intimately involved with

the regulation of synaptic function and developmental processes in a variety of brain regions (for reviews, see Schuman and Madison, 1994; Vincent, 1994), although rather less is known about its role in motor network function. Recently, however, NO has been shown to exert a potent influence on the spinal swimming circuitry of hatchling *Xenopus laevis* tadpoles (McLean and Sillar, 2000). At larval stage 42 (Nieuwkoop and Faber, 1956), NO has an inhibitory effect on the rhythmic motor output for swimming recorded in paralysed tadpoles, reversibly decreasing swim episode duration and increasing cycle period. NO is presumably generated in the brainstem of *Xenopus laevis* tadpoles by one or more of the three clusters of neurons that label with NADPH-diaphorase histochemistry (McLean and Sillar, 2000), although its site of action is not known.

The similarities and differences between the tadpoles of these two species of amphibian, in addition to the recent discovery of a role for NO in the swimming circuitry of *Xenopus laevis*, provided the impetus for the present study. We have examined the effects of NO donors on spinal motor activity in the embryo of the frog *Rana temporaria*. Our results show that, in contrast to its inhibitory role in *Xenopus laevis*, NO has an excitatory role in *Rana temporaria*, initiating a motor pattern qualitatively similar to the light dimming response. Using the nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase histochemical reaction as a simple, yet reliable, marker for the enzyme responsible for NO generation, nitric oxide synthase (NOS; Hope et al., 1991), we have also identified putative sources of NO within the central nervous system (CNS). Remarkably, three homologous populations of neurons stain in the CNS of both species, suggesting that the species-specific effect of NO potentially originates from similar sources. These collective findings are discussed with reference to species-specific differences in morphology and ecological niche. Some aspects of this work have been published previously in abstract form (McDearmid and Sillar, 1997).

Materials and methods

Animals

Rana temporaria spawn, available between the months of February and April, was ordered from Blades Biological or collected from local ponds. Eggs were raised in pond water at temperatures of 8–23 °C until they reached the correct experimental stage (stage 20; Gosner, 1960; Fig. 1A).

Electrophysiology

Animals were prepared for experimentation as previously detailed (Soffe, 1991; Soffe and Sillar, 1991). Briefly, animals were first anaesthetised in tricaine methanesulphonate (MS-222; 0.01–0.1 %) before the dorsal fin was nicked to facilitate neuromuscular block with α-bungarotoxin (approximately 12.5 μmol l^{-1}). Animals were then transferred to the experimental bath through which 100 ml of frog Ringer solution [composition in mmol l^{-1}: 115 NaCl, 2.5 KCl, 1 $MgCl_2$, 2.4 $NaHCO_3$, 10 Hepes, 2 $CaCl_2$ (for extracellular experiments), or 4 $CaCl_2$ (for intracellular experiments);

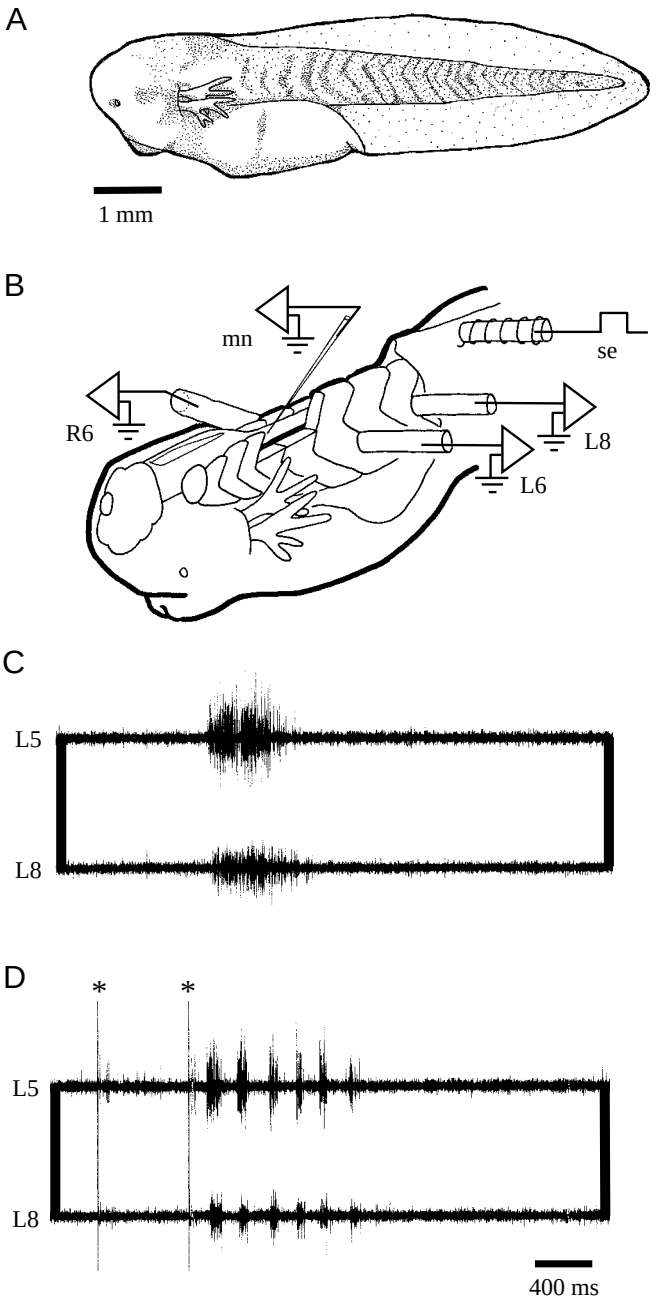


Fig. 1. Two distinct non-rhythmic and rhythmic motor patterns in *Rana temporaria* embryos. (A) Stage 20 *Rana temporaria* embryo. (B) Schematic diagram of stage 20 experimental preparation illustrating the central nervous system in relation to the muscle blocks (see Materials and methods). mn, motor neuron; se, suction electrode; R6, sixth post-otic cleft on the right (contralateral) side of the body. (C) A spontaneous motor burst recorded simultaneously from the fifth (L5) and eighth (L8) post-otic clefts on the left side of the body. Note that motor output occurs with a brief rostrocaudal delay. In contrast, swimming occurs at a higher frequency and is clearly rhythmic (D). Asterisks indicate electrical stimulation artefacts.

pH 7.4; 20–22 °C] was recirculating and were pinned through the notocord to a rotatable Sylgard (Dow-Corning) platform. The flank skin from around the level of the anus to the otic capsule was removed, and extracellular ventral root recordings were made using glass suction electrodes (approximately 50 μm tip openings) placed over either the ipsilateral (see Fig. 1B; L6 and L8) or contralateral (see Fig. 1B; R6) intermyotomal clefts. Activity was initiated by applying a brief (approximately 1 ms) current pulse (Digitimer DS2 isolated stimulator) to the tail skin using another glass suction electrode (see Fig. 1B). The rostral 4–6 myotomes (numbered from the otic capsule) were removed, and intracellular recordings were made from presumed motor neurons in the ventral spinal cord using microelectrodes filled with either 3 mol l^{-1} KCl (resistance 100–120 MΩ) or 3 mol l^{-1} potassium acetate (resistance 150–200 MΩ) (see Fig. 1B). Penetrations were made using capacity overcompensation. Electrophysiological data were recorded and stored onto videotape, using either a Vetter integrated video cassette format instrumentation recorder (model 420) or a Medical Systems Corp. A/D VCR adaptor (model PCM 4/8), and analysed off-line either manually or using the 'Spike 2' analysis software package (CED, Cambridge, UK). In experiments investigating the effects of drugs on swimming, three episodes were analysed before and during drug application and after washing in control saline. To quantify the similarity of the NO-induced response to light dimming, five experiments were selected in which *S*-nitroso-*n*-acetylpenicillamine (SNAP) and the dimming protocol were applied, and the first five ventral root bursts and burst intervals of non-rhythmic motor activity were analysed in each case. Drugs were bath-applied by adding known quantities to the stock bottle to achieve the desired final bath concentration. SNAP (Chemistry Department, University of St Andrews) and *n*-acetylpenicillamine (NAP; Sigma, UK) were dissolved in either distilled water or 0.01 % dimethylsulphoxide (DMSO) (McLean and Sillar, 2000). Diethylamine nitric oxide complex sodium (DEANO; Molecular Probes, The Netherlands) was dissolved in distilled water.

Anatomy

Stage 20 *Rana temporaria* tadpoles were anaesthetised in MS-222, scored along the dorsal fin to aid fixative penetration, and fixed at room temperature (20–22 °C) for 1–2 h in ice-cold 4 % paraformaldehyde. The animals were then rinsed in 30 % sucrose in 0.1 mol l^{-1} phosphate buffer (PB; pH 7.4) to remove aldehydes, and processed either as whole-mounts or as cross sections. For whole-mount histology, animals were transferred to a dissecting bath containing PB and pinned to a Sylgard-coated platform. The CNS of each animal was carefully dissected out to the level of myotomes 6–8 using fine tungsten pins, immersed in the solution for NADPH histochemistry [0.1 mol l^{-1} PB containing 1 mg ml^{-1} β-nicotinamide adenine di-nucleotide phosphate (reduced form; β-NADPH), 0.1 mg ml^{-1} nitroblue tetrazolium (NBT) and 0.3 % Triton X-100] and kept at 37 °C in a humid chamber for 1–3 h. Each CNS was then rinsed in PB and placed in 1 % potassium permanganate (for 30 min) and then 2 % oxalic acid (for 15 min) to remove the melanin pigment. The whole-mounts were then quickly dehydrated in acetone, cleared in xylene and mounted with DPX on cavity slides. Preparations in which NADPH was omitted from the staining solution were used as negative controls and resulted in a complete absence of staining (*N*=5; results not shown).

For transverse sectioning, the animals were embedded in either rat brain or rat liver that had also been fixed in 4 % paraformaldehyde. Specimens were then frozen in a cryostat (Leica Jung Frigocut 2800E; −18 °C) within a supporting cryomatrix and sectioned at 10–15 μm. The cryostat-cut sections were thaw-mounted directly on poly-L-lysine-coated slides and dried at room temperature for 1–2 h prior to histochemical processing. The slides were incubated in a solution for NADPH histochemistry and kept at 37 °C in a humid chamber for 1–3 h. The slides were then rinsed in PB and distilled water and dried at room temperature overnight. Finally, the tissue was cleared in xylene and coverslipped in DPX. All reagents were purchased from Sigma (UK). Anatomical preparations were analysed and catalogued using Zeiss microscopes equipped with camera and *camera lucida* attachments. Photographs were taken with an Olympus OM-4 Ti camera using Fuji 200 ASA film. Tracings of photographs were made with a Jessop Lightbox.

Results

Nitric oxide induces a non-rhythmic motor pattern

Rana temporaria embryos, immobilised during the stage at which they normally hatch (stage 20; Gosner, 1960), are capable of generating only two distinct motor patterns either spontaneously or in response to an external stimulus (Fig. 1C,D; Soffe, 1991; Soffe and Sillar, 1991). The first is a low-frequency non-rhythmic 'lashing' motor pattern characterised by long bursts of activity (approximately 0.5–1 s) that would not result in any net forward propulsion (Fig. 1C). The second is a high-frequency 'swimming' motor pattern characterised by comparatively short, rhythmically repeating bursts of activity that alternate across the body and propagate from head to tail, appropriate for forward locomotion (Fig. 1D). While both motor patterns can be evoked by electrical or mechanical stimulation to the tail skin (Soffe and Sillar, 1991), the non-rhythmic motor pattern is also consistently evoked by dimming the experimental bath illumination (Soffe, 1991). No other motor patterns are known to be generated at this stage of development (Soffe, 1991). To study the effects of NO on fictive motor output, the NO donors SNAP (*N*=17) and DEANO (*N*=6) were bath-applied to immobilised preparations. Before the bath application of SNAP, the ventral roots were silent, although motor activity could be elicited by dimming of the illumination (Fig. 2Ai,D) and occasional spontaneous motor bursts were observed in some preparations (Fig. 2Ai). Shortly after exposure to SNAP (100–500 μmol l^{-1}; 1–2 min), non-rhythmic motor activity

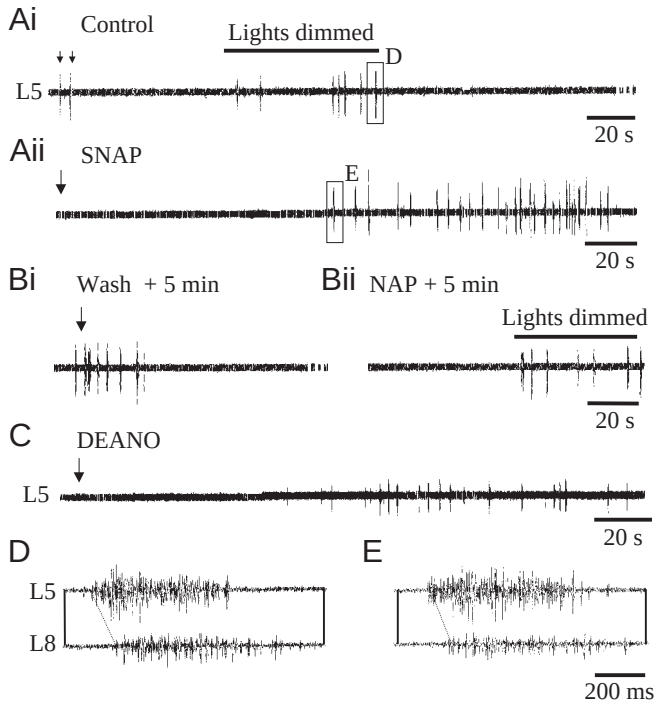


Fig. 2. The NO donors *S*-nitroso-*n*-acetylpenicillamine (SNAP) and diethylamine nitric oxide complex sodium (DEANO) induce a non-rhythmic motor pattern in *Rana temporaria* embryos. (A) Motor responses recorded from L5 before (i) and after (ii) bath application of SNAP (500 μmol l^{-1}, at large arrow). Note that activity can occur spontaneously, as indicated by the small arrows in Ai. Note also a characteristic rostrocaudal delay for both responses, illustrated on an expanded time scale in D and E. (Bi) The effect of SNAP is fully reversible upon return to control saline (at arrow), as illustrated in a different preparation. (Bii) Subsequent bath application of the precursor to SNAP, *n*-acetyl-penicillamine (NAP) (500 μmol l^{-1}), did not initiate any motor activity at equivalent concentrations even after 5 min; however, activity could be elicited by dimming the illumination. (C) In a different preparation, the bath application of DEANO (50 μmol l^{-1}, at arrow) elicited motor responses similar to those elicited by SNAP. For the purposes of illustration, only one ventral root recording is shown. The continuity of preparations is illustrated by the breaks in the recording, which represents a 5 min gap for Ai,ii and Bi,ii.

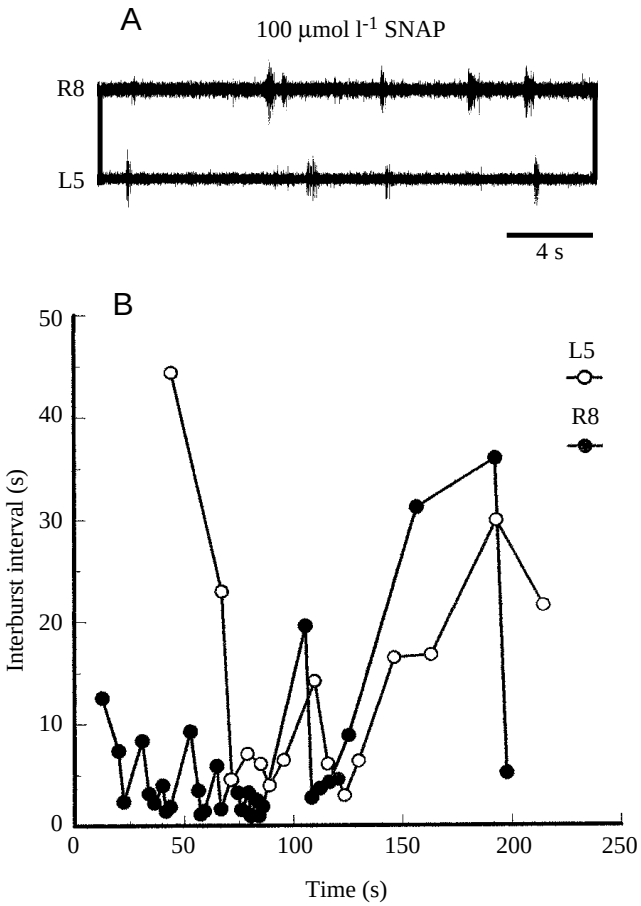


Fig. 3. Activity induced by *S*-nitroso-*n*-acetylpenicillamine (SNAP) occurs at irregular intervals and is never synchronous across the two sides of the spinal cord. (A) Ventral root recordings from R8 and L5 show that activity elicited by SNAP was always alternating and never synchronous across the body. (B) In a different preparation, the interval between each burst of ventral root activity recorded from L5 and R8 plotted against time for an entire episode of non-rhythmic motor activity induced by 100 μmol l^{-1} SNAP. Note that the ventral root discharge occurs at irregular intervals.

characterised by ventral root bursts of 400–500 ms duration was always observed (Fig. 2Aii,E). These bouts of activity typically lasted up to 10 min, although we have not quantified bout duration any further. However, the activity induced by SNAP rapidly disappeared upon returning to control saline (Fig. 2Bi).

To test for possible non-specific actions of SNAP, NAP (*N*=6), the inactive precursor of SNAP, was bath-applied to *Rana temporaria* embryos. Treatment with NAP did not initiate any bouts of motor activity in the same concentration range as SNAP (100–500 μmol l^{-1}; Fig. 2Bii). These results indicate that the ability of SNAP to induce motor output is due to its release of NO rather than to any non-specific actions of the donor molecule. Furthermore, experiments using another nitric oxide donor, DEANO (25–100 μmol l^{-1}), demonstrated that this also induced a non-rhythmic pattern of motor discharge that was indistinguishable from the SNAP-induced motor activity (Fig. 2C). The motor bursts induced by the NO donors were non-rhythmic in that they propagated rostrocaudally (Fig. 2E) but occurred at irregular intervals (Figs 2Aii,C, 3B). In addition, motor bursts never occurred synchronously across the two sides of the spinal cord (Fig. 3A; *N*=3).

Unlike the non-rhythmic motor response that always occurred upon exposure to SNAP, spontaneous fictive swimming activity was relatively uncommon in the presence of NO donors. However, episodes of swimming evoked by skin stimulation in the presence of SNAP were indistinguishable from those elicited in control conditions (Fig. 4Ai,ii) in that no significant difference (Student's *t*-test, $P>0.01$; *N*=6) was detected in burst duration (Fig. 4Bi), rostrocaudal delay

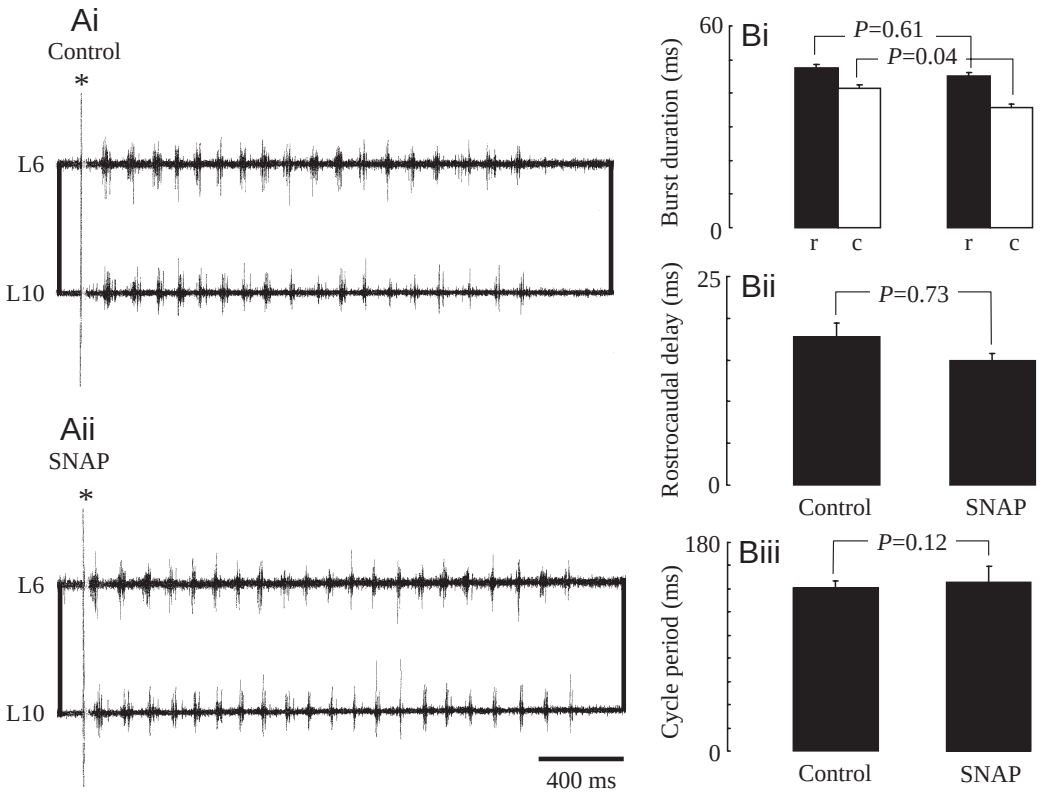


Fig. 4. Fictive swimming activity is unaffected by *S*-nitroso-*n*-acetylpenicillamine (SNAP). (A) Ventral root recordings made from L6 and L10 before (i) and 5 min after exposure to 500 μmol l^{-1} SNAP (ii) are indistinguishable. Stimulation artefacts are indicated by an asterisk. (B) Three full episodes of swimming (approximately 5–15 cycles per episode) were measured before, during and after the bath application of SNAP. No significant differences could be detected for (i) burst duration, (ii) rostrocaudal delay or (iii) cycle period. Values are means ± S.E.M., *N*=6 preparations. c, caudal recordings; r, rostral recordings.

(Fig. 4Bii) or cycle period (Fig. 4Biii) after the bath application of SNAP. Similarly, DEANO (*N*=2) did not obviously affect the fictive swimming rhythm (results not illustrated). These results suggest that the role of NO in *Rana temporaria* is distinct from that in *Xenopus laevis* tadpoles, where it has been shown that NO does not trigger motor activity but has profound modulatory effects on ongoing fictive swimming (McLean and Sillar, 2000).

The similarity between the dimming response and the NO-induced response was quantified by comparing the burst durations and inter-burst intervals associated with these two responses. No significant difference (Student's *t*-test, $P>0.01$; *N*=5) was detected in either variable (Fig. 5A,B) during the SNAP-induced and the dimming-induced, non-rhythmic motor patterns. Note, however, that there was a significant difference (Student's *t*-test, $P<0.001$) in both burst duration (Fig. 5A) and inter-burst interval (Fig. 5B) when the SNAP-induced response and rhythmic swimming were compared in the same preparations. This further illustrates the clear difference between rhythmic and non-rhythmic behaviours in *Rana temporaria*.

Synaptic drive underlying NO-induced motor activity

Intracellular recordings from motor neurons using potassium-acetate-filled microelectrodes revealed that the bursts of ventral root discharge that occurred spontaneously (Fig. 6Ai), in response to dimming (Fig. 6Aii) or induced by SNAP (Fig. 6B) or DEANO application were accompanied by phasic depolarisations in motor neurons that were often, but not always, sufficient to trigger action potentials (*N*=6; Fig. 6B,D). These depolarisations often included superimposed inhibitory postsynaptic potentials (IPSPs), sometimes large enough to cause the membrane potential to fall below its resting level (e.g. dotted line and arrowed in Fig. 6D). During the inter-burst intervals, periods of more sustained membrane hyperpolarisation usually occurred during which the membrane potential again fell below the resting level (Fig. 6B,C). This is consistent with the idea that activity on one side of the spinal cord produces reciprocal inhibition of motor neurons on the opposite side. Aside from these depolarising and hyperpolarising events, there was no evidence of a tonic or sustained change in membrane potential caused by the addition of NO donors.

The inter-burst inhibition is likely to be Cl^--dependent because it was sign-reversed in intracellular recordings where KCl was used as the electrolyte (*N*=10, Fig. 7). In such recordings, periods of phasic excitation and inhibition were both characterised by bursts of impulses superimposed on a

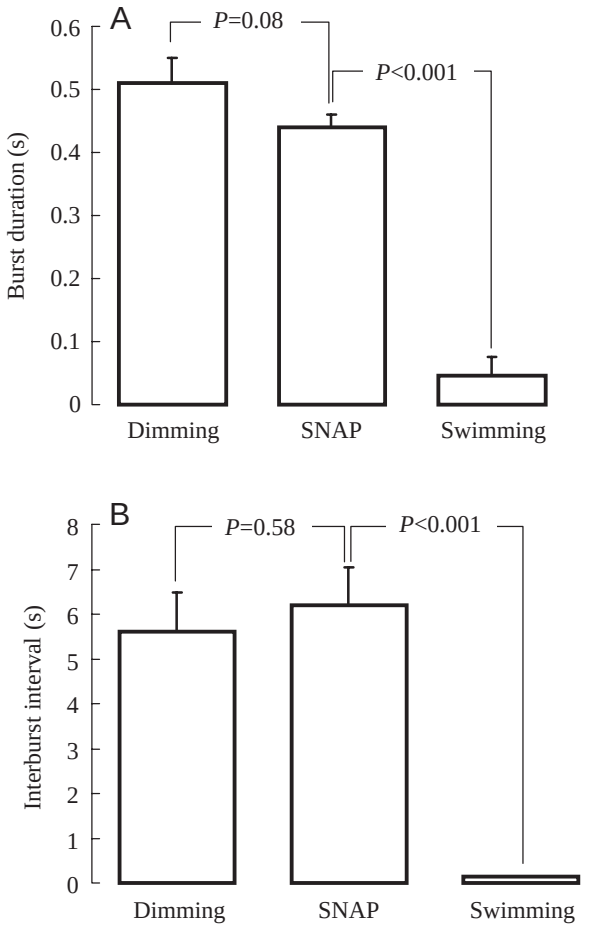


Fig. 5. *S*-nitroso-*n*-acetylpenicillamine (SNAP) induces a non-rhythmic activity not significantly different from the dimming response. Measurement from five different preparations illustrate that the burst duration (A) and interburst interval (B) of non-rhythmic motor activity induced by SNAP (500 μmol l^{-1}) are not significantly different from those of the dimming response. Note, however, that these variables measured for rhythmic swimming in the presence of SNAP are significantly different from both the dimming values and the non-rhythmic SNAP values. Values are means ± S.E.M.

prolonged depolarisation that often, but not always, crossed spike threshold (Fig. 7A,B). The use of KCl-filled electrodes also revealed frequent depolarising potentials in the inter-burst intervals (e.g. Fig. 7C,D). Presumably these are spontaneously occurring IPSPs that were less clearly detectable in recordings using potassium-acetate-filled microelectrodes, where the reversal potential is closer to the resting value.

In summary, the non-rhythmic motor behaviour induced by NO donors and the associated synaptic drive recorded from motor neurons resemble those produced by dimming of the illumination in control saline (Figs 6Aii,B, 7C,D).

Distribution of NADPH-diaphorase staining in Rana temporaria *embryos*

The CNS of *Rana temporaria* embryos is pigmented brown, which partially obscures the fine localisation of the dark blue NADPH-diaphorase reaction product. Cross-sections were therefore cut as thinly as possible (10–15 μm) to aid the identification of putative NOS-containing cells and processes. Staining was largely restricted to the CNS (*N*=13), although NADPH-diaphorase reaction product was clearly present in the inner of the two layers that form the skin (Fig. 8A,B). The epithelium is known to contain NOS in a variety of species (Salter et al., 1991), lending further support to the conclusion that the NADPH-diaphorase staining reported here corresponds to NO-synthesising cells.

The NADPH-diaphorase staining in the CNS was almost entirely restricted to three bilaterally symmetrical populations of brainstem neurons (Fig. 9). Only a few cells were labelled in the forebrain, and these will not be considered further. There was substantial staining within the marginal zones of the spinal cord (Fig. 9Aii), presumably corresponding to axons originating from NADPH-diaphorase-reactive neurons belonging to populations lying more rostrally in the CNS. The caudal-most population of NADPH-diaphorase-reactive neurons began near the spinal cord/hindbrain boundary. The neurons in this group had unipolar ventral cell bodies located close to the neurocoel, with processes extending ventrolaterally into the lateral margins of the brainstem, where an extensive ramification of processes occurred (Fig. 9Ai). A proportion of the neurons also had contralaterally projecting processes that could be detected as they coursed beneath the neurocoel (Fig. 9Ai; arrowed). Rostral to this cell group there followed a region where there was intense labelling of projections in the marginal zones (Fig. 9Biii), followed by the next population of cell bodies located in the rostral hindbrain (Fig. 9Bii). The cell bodies were located ventral and lateral to the neurocoel, and neuritic processes projected ventrolaterally into the marginal zones. No contralateral projections were observed. There was then a region where no staining of cell bodies occurred, but where the profiles of neuronal processes were clearly present in the marginal zones. The final rostral-most cluster of brainstem neurons was located close to the mid/hindbrain boundary. The tightly packed somata occupied a dorsolateral position and had long primary neurites that projected ventrally into the marginal zones (Fig. 9Bi).

The detailed topography of these cell groups became clear when the CNS was processed in whole-mount (*N*=20). Since *Rana temporaria* embryos are darkly pigmented, the excised whole-mount CNS required bleaching to remove the melanin pigment that would otherwise have obscured the visualisation of NADPH-diaphorase staining. Unfortunately, while the permanganate/oxalic acid bleaching process did not appear to affect cell body staining, it often diminished the staining of finer processes. However, in some cases (four out of 20), the ascending and descending processes were present. From the dorsal and lateral perspective, it was apparent that the caudal-most group was by far the largest population of neurons, numbering 75–100 per side (Fig. 9C), with cell bodies extending 300–400 μm (Fig. 9A,B) and axons projecting caudally into the marginal zones of the spinal cord (Fig. 9A). Rostral to this cell group there followed a 90–100 μm region

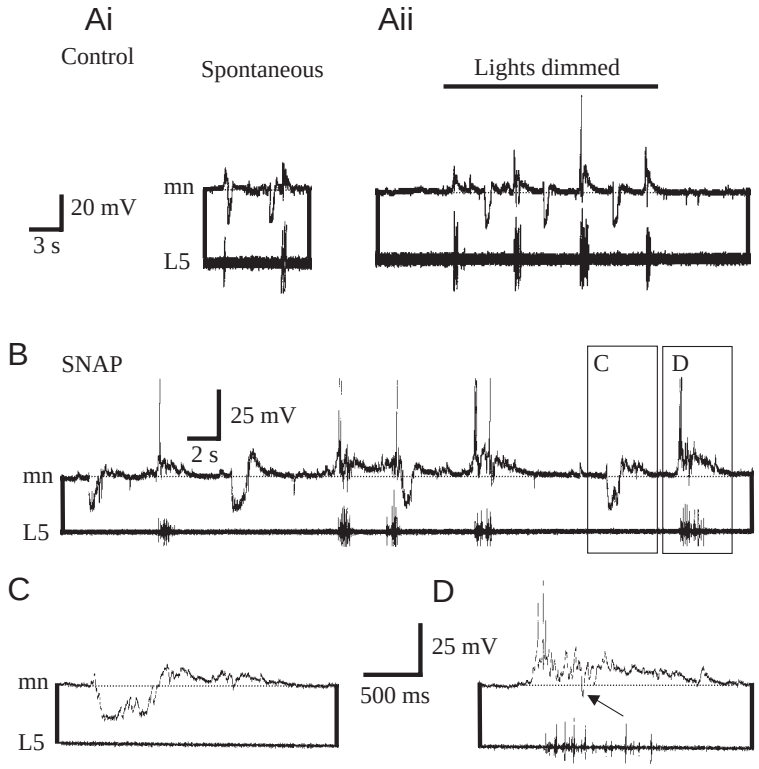
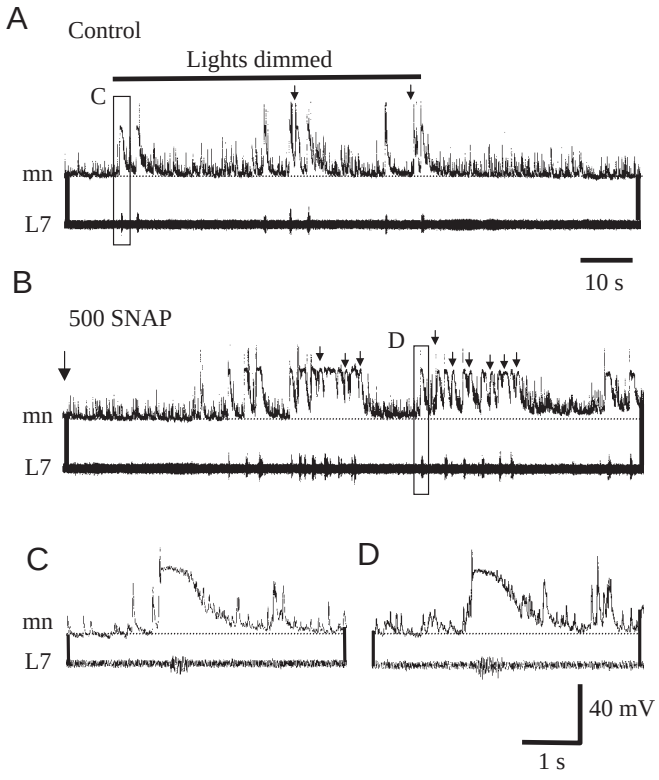


Fig. 6. Synaptic drive during *S*-nitroso-*n*-acetylpenicillamine (SNAP)-induced motor activity resembles the response to dimming of the illumination. (A) Intracellular recording with an electrode filled with 3 mol l^{-1} potassium acetate from a presumed motor neuron (mn). Bursts of ventral root discharge and the corresponding synaptic drive (recorded in L5) could occur spontaneously (i), but were more consistently evoked upon dimming of the illumination (ii). (B) Two minutes after the bath application of 100 μmol l^{-1} SNAP, the activity resembles phasic depolarisations and hyperpolarisations induced by the dimming of the illumination. (C) Hyperpolarising potentials in motor neurons occur when the ventral roots are silent, and (D) depolarising potentials occur in phase with the discharge from the ventral roots. Note some IPSPs are superimposed (arrow).

with intense labelling of projections in the marginal zones (Fig. 9B), followed by the population of neurons located in the rostral hindbrain extending for 90–100 μm and consisting of approximately 30 neurons (10–15 per side, Fig. 9A–C). There was then a 60–80 μm region where no staining of cell bodies occurred. Finally, the rostral-most population was located just rostral to the hind/midbrain boundary, extended for 40–50 μm and was made up of approximately 15–20 neurons (5–10 per side, Fig. 9A–C).



Discussion

The results presented here provide evidence that NO can induce fictive motor activity, reminiscent of that known to drive the strong body flexions that occur spontaneously in *Rana temporaria* embryos, in response to an external stimulus and following dimming of the illumination (Soffe, 1991; Soffe and Sillar, 1991) and which are also thought to facilitate the hatching process (Soffe, 1991). It should be noted that the non-rhythmic motor response predominated in the presence of NO donors; episodes of rhythmic swimming were both uncommon

Fig. 7. Inhibitory potentials are Cl^--dependent. (A) Intracellular recording made with an electrode filled with 3 mol l^{-1} KCl from a presumed motor neuron (mn) located at the level of the third post-otic cleft, which illustrates a dimming response. (B) The bath application of 500 μmol l^{-1} *S*-nitroso-*n*-acetylpenicillamine (SNAP) (at large arrow) results in a pattern of activity similar to that seen in response to the dimming of the illumination. On a faster time scale, the ventral root activity (L7) and the synaptic drive to motor neurons in response to (C) the dimming of the illumination in control saline and (D) 500 μmol l^{-1} SNAP are indistinguishable. Note that strongly depolarising potentials in the absence of ventral root activity (small arrows) correspond to sign-reversed inhibitory potentials, suggesting that inhibition is Cl^- dependent.

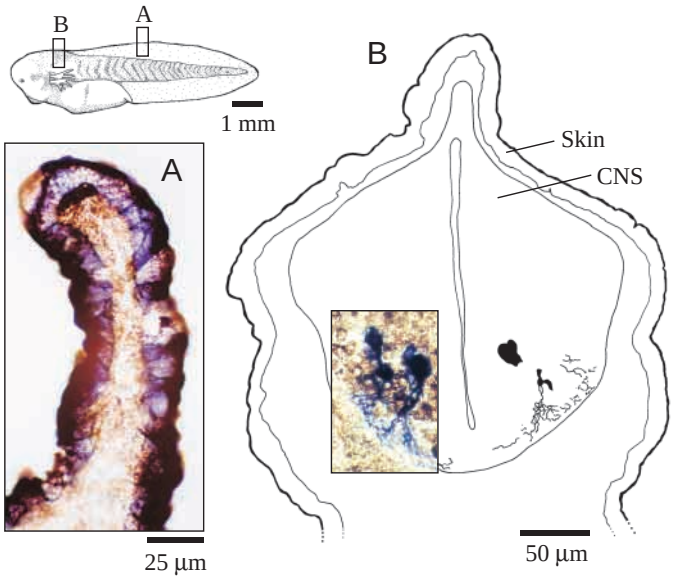


Fig. 8. NADPH-diaphorase staining in the skin and the central nervous system (CNS). A schematic digram that shows the approximate location of the cross sections of skin in the tail fin and the body (boxed). (A) NADPH-diaphorase staining in the inner layer of skin cells in the tail fin is visualised as a dark blue reaction product. Unfortunately, the outer layer of skin is too darkly pigmented to visualise NADPH-diaphorase reaction product. (B) A tracing of a cross section of the central nervous system illustrates the close proximity of the dark blue reaction product in the neurons of the central nervous system (colour inset) to the skin.

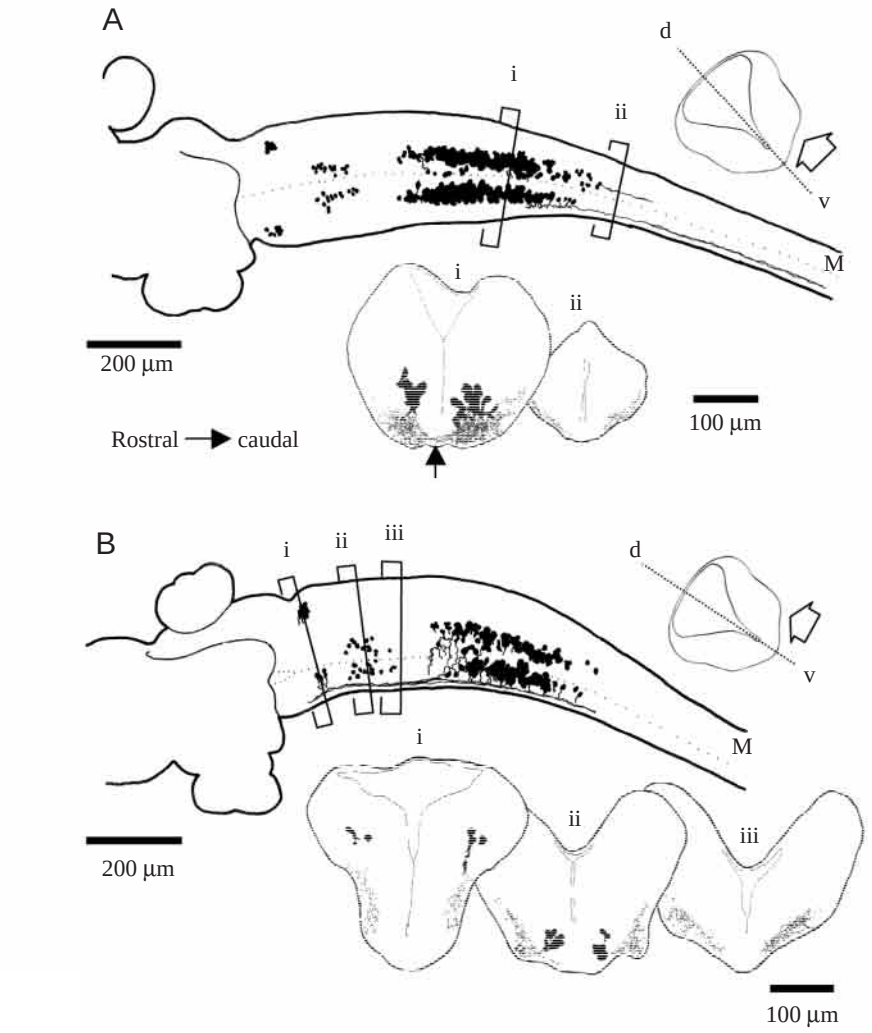
and unaffected by the bath application of SNAP or DEANO (see Fig. 4). Putative sources of NO have been identified in the skin and in three discrete clusters of neurons in the CNS using NADPH-diaphorase histochemistry (see Fig. 9). Collectively, these results provoke intriguing questions including whether NO might be an endogenous trigger for initiating hatching motor behaviour in *Rana temporaria* and, if so, whether it does so directly or by enhancing the responsiveness of the nervous system to other transmitters. In addition, how can NO mediate different behavioural responses in *Rana temporaria* and *Xenopus laevis*, two closely related species, at equivalent developmental stages, when homologous nitrergic cell groups are likely to be involved?

NADPH-diaphorase-reactive populations and putative co-localisation of NO with neurotransmitters

It seems reasonable to assume that NO is not the sole or principal neurotransmitter utilised by neurons in the three brainstem clusters in *Rana temporaria* (this paper) or in *Xenopus laevis* (McLean and Sillar, 2000). In *Xenopus laevis*, the early development of the nervous system has been sufficiently well described to allow tentative suggestions to be made regarding the identity of potential cotransmitters in nitrergic neurons (McLean and Sillar, 2000). On the basis of the relative location of each cluster and on the morphology of the neurons themselves, we have suggested that the caudal-most group includes the GABAergic mid/hindbrain reticulospinal neurons known to be involved in the termination of swimming (Boothby and Roberts, 1992). The next most rostral cluster has been compared with the serotonergic raphe nucleus. The most rostral cluster is more difficult to compare with known populations of brainstem neurons, but these neurons bear superficial similarities with both the tyrosine-hydroxylase-positive neurons of the nucleus tractus solitarii (Gonzalez et al., 1995) and the aminergic neurons of the isthmic region, the amphibian equivalent of the locus coeruleus (Marin et al., 1996). Double-labelling experiments are now needed to confirm these correlations.

Much less is known about the anatomy and the transmitter phenotypes of brainstem neurons in *Rana temporaria* embryos. However, the location and morphology of the three nitrergic neuron clusters strongly suggest that they are homologous with the neuron clusters described earlier using the NADPH-diaphorase technique in *Xenopus laevis* (McLean and Sillar, 2000). It is plausible that the homologies extend to the cotransmitters of these neurons as well. In support, immunocytochemical studies using anti-serotonin antibodies in *Rana temporaria* embryos (Woolston et al., 1994) have revealed a population of brainstem neurons resembling in position and morphology both the raphe neurons of *Xenopus laevis* (van Mier et al., 1986) and the middle group of NADPH-diaphorase-reactive neurons described for *Rana temporaria* in the present study (see Fig. 9Bii).

NO appears to play an excitatory role in *Rana temporaria* embryos, initiating a motor response rather than inhibiting one as it does in *Xenopus laevis* (McLean and Sillar, 2000). Serotonergic and noradrenergic neurons are involved in the induction of locomotion in a variety of species. For example, alternation between flexor and extensor motor neuron activity can be evoked by serotonin (5-HT) in the neonatal rat (Cazalets et al., 1990; Cazalets et al., 1992; Kiehn and Kjaerulff; 1996) and in the rabbit (Viala and Buser, 1969). Noradrenaline can also initiate stable locomotor activity in the cat (Barbeau and Rossignol, 1991) and can elicit either tonic motor activity or trigger a slow, irregular and often synchronous motor pattern in the neonatal rat preparation (Kiehn et al., 1999). However, it should be noted that the effects of noradrenaline and 5-HT in vertebrates are also sometimes species-specific. For instance, 5-HT modulates ongoing activity but does not initiate motor output in intact *Xenopus laevis* tadpoles (Sillar et al., 1992) or in spinal cats (Barbeau and Rossignol, 1991). It is possible, therefore, that NO functions as a cotransmitter, initiating a motor response in *Rana temporaria via* the 5-HT or noradrenaline systems. Indeed, it has been shown in *Rana temporaria* that noradrenaline initiates a pattern of motor activity that is indistinguishable from that induced by NO (McDearmid and Sillar, 1997). However, in *Xenopus laevis* larvae, NO does not induce motor activity but rather inhibits fictive swimming. The very different actions of NO in the two species could be due to the level at which NO impinges upon the spinal motor networks. For example, there is a notable difference in the amount of NADPH-diaphorase reaction



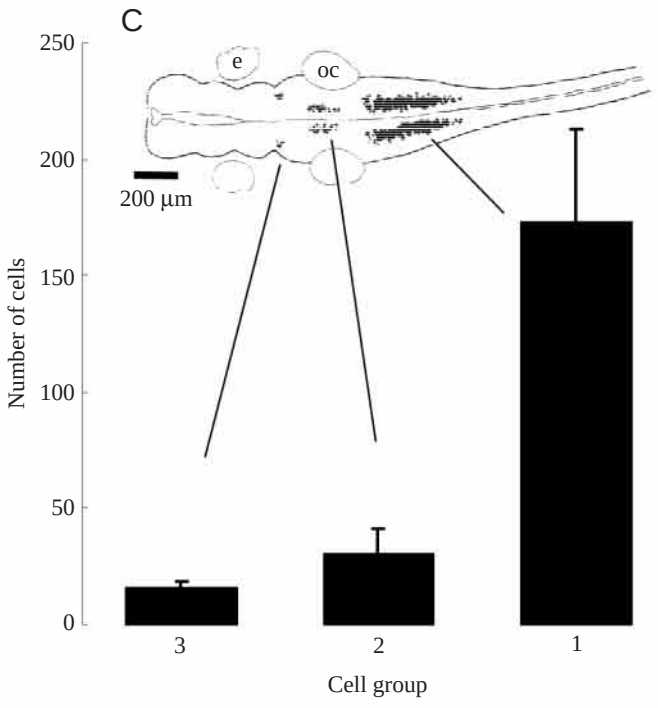


Fig. 9. NADPH-diaphorase-labelled neurons in the central nervous system. (A) A whole-mount preparation illustrated as a tracing at 10× magnification showing descending projections well into the marginal zones of the spinal cord. Tracings of 10 μm sections illustrate (i) the caudal-most population (group 1) and (ii) the descending projections within the marginal zones of the spinal cord. The arrow indicates contralaterally projecting processes. (B) A second whole-mount illustrates ascending projections within the marginal zones of the brainstem that continue from group 1 to group 3 (see below). Tracings of 10 μm cryostat sections illustrate (i) the rostral-most population (group 3), (ii) the mid/hindbrain population (group 2) and (iii) the axonal tracts in the marginal zones of the brainstem. The rotation of both whole-mounts is detailed as insets showing the dorsal (d) and ventral (v) orientation, and the rostral–caudal direction is indicated in A. The approximate levels of the sections are given, and the midline is shown as a dotted line labelled M. (C) A histogram of total mean cell counts in each group (*N*=5 preparations) includes a ventral *camera lucida* drawing that illustrates the relative positions of each cell group (1, 2 and 3) in relation to the eyes (e) and the otic capsules (oc). Values are means ± S.E.M.

product in the marginal zones of the spinal cord in *Rana temporaria*, as detected in cross sections (see Fig. 9Aii). In *Xenopus laevis*, processes project towards, but do not enter, the spinal cord. NO could, therefore, predominantly be affecting higher centres in *Xenopus laevis*, while acting additionally at the level of the spinal cord in *Rana temporaria*.

NO 'selects' a non-rhythmic motor pattern

It seems likely that non-rhythmic and rhythmic motor patterns in *Rana temporaria* are produced by the same, or at least elements of the same, neural circuitry in the spinal cord and brainstem, so the organism must possess a means of altering this circuitry to generate the required behaviour. Our data indicate that NO biases the circuitry to produce non-rhythmic over rhythmic activity, but it does not occlude the generation of rhythmic swimming activity. Indeed, the swimming response can still be triggered in the presence of NO donors by electrical stimulation of the skin so that the expression of the non-rhythmic pattern can be over-ridden by changing environmental conditions.

If NO is indeed the endogenous trigger for hatching behaviour in *Rana temporaria* embryos, what in turn causes NO release? The NO-mediated non-rhythmic motor pattern is indistinguishable from that produced in response to dimming of the illumination (see Figs 2, 6, 7). It could be that a reduction in light intensity is the environmental signal that releases endogenous NO, thereby initiating the behaviour and allowing the animal to hatch under cover of darkness. In the tadpole of *Xenopus laevis*, dimming of the illumination induces rhythmic swimming activity, a response that is mediated through the pineal eye (Roberts, 1978; Foster and Roberts, 1982), and it is possible that the light-dimming response in *Rana temporaria* embryos is also pineal-mediated. Although we have obtained no evidence for NADPH-diaphorase reaction product in the pineal organ of either species, there is evidence for pineal involvement from other vertebrates, such as the rat, in which exposure to constant light levels induces a marked decrease in NOS activity in the pineal gland (Schaad et al., 1994). Perhaps more likely in *Rana temporaria* tadpoles is the possibility that light dimming activates pineal afferents that, in turn, activate nitrergic brainstem neurons to trigger motor activity. However, sensory pathways within the skin could also play a role. The skin of *Rana temporaria* embryos is rich in NADPH-diaphorase staining and is, therefore, a likely source of NO (see Fig. 8A). Because of its gaseous nature and the proximity of the skin to the CNS (see Fig. 8B), NO synthesis in the skin could have a direct influence on the CNS *via* simple diffusion. There is evidence to suggest that a photosensitive step exists in the NO-generating pathway (Venturini et al., 1993), although in this case light rather than dark triggers NO release. Nevertheless, light-mediated changes in NO production within the skin could result in profound changes in NO levels within the CNS, which in turn may initiate motor activity.

In summary, we have shown that NO can initiate non-rhythmic motor responses in the embryo of the frog *Rana temporaria*. The motor pattern induced by this neuromodulator consists of bursts of prolonged ventral root discharge that would be appropriate to drive the non-rhythmic body flexions first described by Soffe (Soffe, 1991). NO appears to select this behaviour over rhythmic swimming; however, it does not interfere with the generation of swimming behaviour (see Fig. 4). For the moment, it is not clear whether NO acts directly on spinal neurons or causes the release of another neurotransmitter, but NO is clearly important in the control of motor output in vertebrate motor systems in ways that are both species-specific and developmental-stage-dependent.

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