THE GASEOUS MESSENGER MOLECULE, NITRIC OXIDE: A MODULATOR OF LOCOMUTOR MOVEMENTS DURING EARLY AMPHIBIAN DEVELOPMENT

David L. MacLean

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

2001

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by

David L. McLean
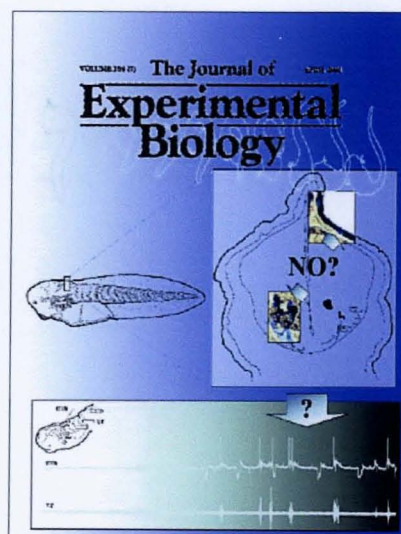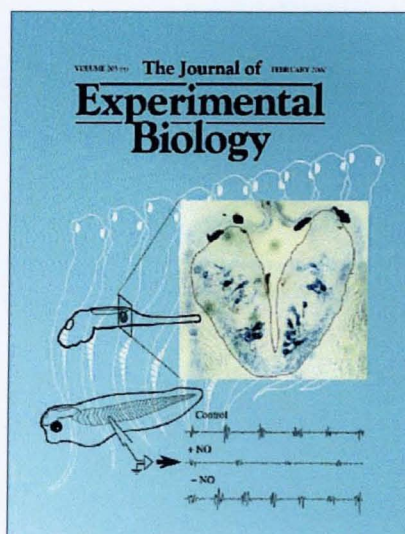
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DECLARATION

I, David Lindsay McLean, hereby certify that this thesis, which is approximately 42,000 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.

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I was admitted as a research student under Ordinance No. 12 in October 1997 and as a candidate for the degree of PhD in September 1998; the higher degree for which this is a record was carried out in the University of St Andrews between 1997 and 2001.

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I hereby certify that the candidate has fulfilled the conditions of the Resolutions and Regulations for the degree of PhD in the University of St Andrews and that the candidate is qualified to submit this thesis in application for that degree.

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ACKNOWLEDGEMENTS

There are two responses to which I have become well accustomed since starting my PhD and both are stated with the same air of disbelief. The first is "You work on what? Tadpoles?" and the second is, "You've been in St Andrews how long?" There are obviously a number of people I now have to opportunity to thank for this. Firstly, I'd like to thank my supervisor Keith Sillar, without whom I wouldn't have started this project, had a project to start in the first place, or enjoyed it quite so much. He's been a great support and inspiration. I'd also like to thank the lab, particularly Carolyn Reith, Simon (Mr Bond) Merrywest, Hanno (Hey come on boy) Fischer, Elly Hartis and Joe McDearmid. Their patience has been greatly appreciated. Also, I cannot thank Si, Joe or Keith enough for their input and contributions to the introductions and discussions of results, which have allowed me to publish a number of papers prior to thesis submission. Within the department I have to thank Jill McVee for histological guidance, Murray Coutts for computer wizardry and the guys in the Bute Photographic unit (Dave, Sean, Jim). Big thanks also go out to my family, who have been nearby throughout my PhD, and without whose love and support I don't think I could have made it. There are also a number of non-related people I need to thank for their financial abuse and alcohol support. In particular are the Gatty lot, a bunch of Bute folk, the Lade Braes posse, the Toll House gang, and a few Psychology stragglers. Listen, I'd like to name drop, but the risk of missing out someone is too great, and I'd rather not disappoint any potential pint buyers. So get over it. Lastly, I'd like to thank Kirsty Kemp, whose photograph I've used on the dedications page and whose company and laughter have made the past two years fly by.

Cheers you lot.

ABBREVIATIONS

5-HT	5-hydroxytryptamine or serotonin
ACh	acetylcholine
BDNF	brain derived neurotrophic factor
Ca^2	calcium ion
cGMP	3':5'-guanosine cyclic monophosphate
CNS	central nervous system
CPG	central pattern generator
GABA	γ-aminobutyric acid
DEANO	diethylamine nitric oxide complex sodium
DMSO	dimethyl sulphoxide
EDRF	endothelium derived relaxing factor
EPSP	excitatory post-synaptic potential
IPSP	inhibitory post-synaptic potential
K^+	potassium ion
KCl	potassium chloride
M	mol l^{-1}
mhr	midhindbrain reticulospinal
MS-222	tricaine methanesulfonate
NA	noradrenaline
NADPH	nicotinamide adenine dinucleotide phosphate
L-NAME	N^G-nitro-L-arginine methyl esther
NAP	n-acetylpenicillamine
NO	nitric oxide
NOS	nitric oxide synthase
nNOS, eNOS, iNOS	neuronal, endothelial, inducible NOS isoforms
NOS-IR	nitric oxide synthase immunoreactivity
L-NMMA	N^G-monomethyl-L-arginine
NMDA	N-methyl-D-aspartate
L-NNA	N^ω-nitro-L-arginine
PB	phosphate buffer
PBS	phosphate buffered saline
SNAP	S-nitroso-n-acetylpenicillamine
TTX	tetrodotoxin

LIST OF FIGURES

ABSTRACT

1. The free radical gas nitric oxide (NO) is now recognised as a ubiquitous and versatile signalling molecule and the investigation of its biological roles has involved a wide range of scientific disciplines in many different species. Yet despite this, its potential roles in the development of rhythmic motor activities in vertebrates have been largely ignored.

2. Physiological experiments recording extracellular ventral root output suggest that NO is playing an inhibitory role in the swimming system of *Xenopus laevis* larvae, shortening the duration of swim episodes and slowing swim frequency. Nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase histochemistry labelled three populations of neurons in the brainstem, which putatively co-localise NO with the aminergic neuromodulators serotonin (5-HT) and noradranaline (NA), and the fast descending inhibitory neurotransmitter, γ-aminobutyric acid (GABA). This suggests that the inhibitory role is supraspinal in origin.

3. Intracellular recordings from neurons presumed to be spinal motor neurons provide further evidence for the inhibitory influence of NO. My experiments suggest that NO potentiates both glycinergic and γ-aminobutyric acid (GABA)-ergic inhibition onto spinal motor neurons. The facilitation of the release of these inhibitory transmitters is consistent with the observed effects on swim frequency and swim episode duration, respectively. Additionally, NO appears to affect membrane properties, causing a pronounced membrane potential depolarisation and a decrease in membrane conductance. This suggests that NO shuts off a resting membrane conductance.

4. NADPH-diaphorase histochemistry was subsequently applied to determine the four dimensional expression of putative nitrergic neurons in the central nervous system and related structures. The developmental sequence of staining identifies groups and subgroups of interconnected interneurons, and provides further clues to their identity. NADPH-diaphorase labelling was also located in the eyes, skin and blood vessels, further confirming the validity of this staining technique for identifying nitric oxide synthase.

5. In the related anuran species, *Rana temporaria* nitric oxide donor drugs appear to have no affect on swimming, but instead reliably initiates a non-rhythmic "lashing" motor pattern similar to that elicited by dimming of the illumination. Interestingly the NADPH-diaphorase technique labelled three clusters of apparently homologous interneurons in the brainstem and additionally the inner layer of the skin was intensely stained, implicating a species-specific role for NO released from brainstem neurons.

DEDICATION

Things should be made as simple as possible, but not any simpler.

Albert Einstein

If the human mind was simple enough to understand, we'd be too simple to understand it.

Emerson Pugh

For my family.

THE GASEOUS MESSENGER MOLECULE, NITRIC OXIDE: A MODULATOR OF LOCOMOTOR MOVEMENTS DURING EARLY AMPHIBIAN DEVELOPMENT: A GENERAL INTRODUCTION.

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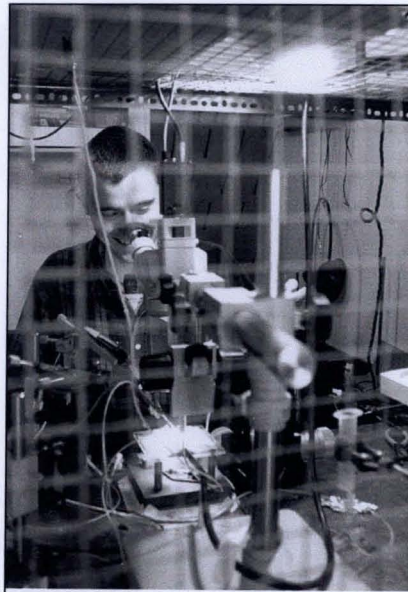
1.1. SUMMARY

Nitric oxide is now known to play a physiological signalling role in a variety of tissue types across a broad phylogenetic spectrum, making it a potentially ancient transmitter. As the research into the physiological actions of nitric oxide continues to intensify, one area that has largely been neglected is the contribution of nitric oxide to the co-ordination of developing locomotor movements. This has therefore been the goal of my research to date. The general introduction has been divided into sections covering: 1) nitric oxide, its discovery and its properties as a signalling molecule; 2) the properties and manipulation of the enzyme responsible for nitric oxide generation, nitric oxide synthase; 3) the methods of locating and the subsequent locations of nitric oxide synthase in the central nervous system, illustrating functional roles from recent literature; 4) nitric oxide's role in developmental processes; 5) the behavioural model used to analyse the involvement of nitric oxide in the development of co-ordinated movements, namely the tadpoles of the South African clawed frog, *Xenopus laevis* and the British common frog *Rana temporaria*; and finally, 6) the aims and direction of the subsequent chapters.

1.2. A BRIEF HISTORY

In the decade or so that has passed since nitric oxide (NO) was first identified as a biological molecule, a great deal of attention has been drawn to its actions within the central nervous system (CNS). The excitement followed the initial observation that endothelial cells synthesised a labile substance that acted as a potent smooth muscle relaxant (Furchgott and Zawadski, 1980). Termed endothelium-derived relaxing factor (EDRF), its identity remained unknown for several years, despite the fact that indirect evidence suggested that it might be NO. For example, the pharmacological profiles of NO and EDRF were virtually indistinguishable (Vincent, 1994). Conclusive proof only followed after the demonstration that cultured endothelial cells could be made to release NO in sufficient amounts to account for the biological activity of EDRF (Palmer *et al.*, 1987; Furchgott, 1988).

However, because of a general unfamiliarity with inorganic hydrophobic gases, many of the assumptions about the biological properties of NO have needed re-examination (Varner and Beckman, 1995). NO is usually described as short-lived, highly reactive and toxic. First of all NO is relatively long-lived in comparison to the time scale of many neural processes, such as synaptic transmission or action potential propagation. In fact, a 0.1 μM solution of NO has a half-life of approximately 80 minutes in phosphate buffer (Varner and Beckman, 1995). Consequently, other reactions must be responsible for the 4-6 second half-life observed *in vitro* and *in vivo* (Schuman and Madison, 1994). Secondly, NO reacts with a limited number of biological molecules, thus allowing it to diffuse into neighbouring cells readily. Like molecular oxygen, NO only becomes highly reactive and toxic following conversion to more strongly oxidising species (Janzen, 1994). Lastly, the toxicity of NO has been overestimated by many *in vitro* systems, due largely to the delivery agents being more reactive than NO itself (Varner and Beckman, 1995). For example, sodium nitroprusside is an excellent oxidant of sulfhydryls and also releases cyanide. Therefore secondary reactions and not NO are generally responsible for the reputation of NO as a short lived, highly reactive and toxic molecule.

These findings confirmed the suitability of NO as a legitimate, albeit unconventional, signal transduction molecule and as a consequence, research into the functions of NO in the CNS has intensified (McCall and Vallance, 1992). NO's reputation as an unconventional signalling molecule derives from its ability to diffuse from its site of production in the absence of any specialised machinery. It is also gaseous and membrane permeant so can bypass normal signal transduction routes that involve membrane receptors. Also, while on average only 2% of neurons contain NO in some systems, they are so highly branched that all CNS neurons are probably within only a few microns of an

NO source (Bredt *et al.*, 1990). This, combined with NO's unique signalling properties, makes it a potentially ubiquitous influence in the CNS. However, despite the proliferation of research in the CNS roles of NO, one area that has been largely ignored is NO's role within rhythmic motor systems, particularly with regard to the co-ordination of vertebrate locomotion. To study the possible influence of NO in locomotion, one must first examine whether the system contains NO and this requires an understanding of how NO is produced.

1.3. STRUCTURE AND MANIPULATION OF NOS

The enzyme responsible for the formation of NO in biological systems is known as nitric oxide synthase (NOS). Mammalian NOS consists of a family of three calmodulin-dependent biopterohaemoflavoproteins that can be functionally distinguished by their modes of regulation (Salerno *et al.*, 1996). There are two constitutive isoforms of NOS first identified in neuronal cells (nNOS) and endothelial cells (eNOS). The constitutive forms remain dormant until calcium/calmodulin binding is initiated by transient elevations in intracellular calcium ions (Ca^{2+}). Activation results from a displacement of the autoinhibitory control element located close to the calmodulin binding sequence in the flavin mononucleotide (FMN) binding domain (Salerno *et al.*, 1997a). Ca^{2+}-dependence means that NO release can be strictly controlled. The third isoform is an immunostimulant-induced NOS (iNOS) which is Ca^{2+}-independent, thus providing the continuous high output of NO needed for host defence (Marletta *et al.*, 1988). While all three isoforms are located within the CNS, it is interesting that the nNOS isoform represents the largest proportion of constitutive NOS activity in man (Knowles and Moncada, 1994).

All NOS isoforms contain haem and tetrahydrobiopterin (BH_4), as well as FMN and flavin adenine dinucleotide (FAD). The N-terminal region of NOS contains the catalytic domains, including the haem-binding region and the biopterin- and arginine-binding sites (see Fig. 1.1C). The consensus binding sites for nicotinamide adenine dinucleotide phosphate (NADPH), FAD, and FMN are all located in the carboxyl-terminal half of NOS. The chain reaction that results in NO production is illustrated diagrammatically in Figure 1.1A. The synthesis of NO begins with the semi-essential amino acid L-arginine. Within the reaction are two separate mono-oxygenation steps. After the first, the intermediate species N-hydroxyarginine results from a reaction requiring one oxygen molecule and one NADPH, in the presence of tetrahydrobiopterin. The second step results in the oxygenation of N-hydroxyarginine to form L-citrulline and NO (Knowles and Moncada, 1994). NO then freely diffuses from its site of production.

The structural and functional analysis of the NOS isoforms detailed above have been aided by studies with competitive inhibitors of NOS, which have in turn been used to elucidate the functional role of NO within biological systems. The majority of investigations aimed at producing clinically useful NOS inhibitors have concentrated on ligands that competitively bind to the arginine site. The most widely used arginine analogues are N^G-monomethyl-L-arginine (L-NMMA), N^{ω}-nitro-L-arginine (L-NNA), and N^G-nitro-L-arginine methyl ester (L-NAME). L-NMMA has been shown to be an inhibitor of all the isoforms of NOS with a K_i of approximately 1 μM (Knowles and Moncada, 1994). For the most part this inhibition is reversible, contrasting the inhibition produced by L-NAME and L-NNA, which is progressive and irreversible following covalent binding (Dwyer *et al.*, 1991). Additionally, L-NNA is selective for nNOS and eNOS isoforms, while L-NMMA is a more potent eNOS inhibitor. L-NNA inhibits NOS with a K_i of approximately 10 μM (Bland-Ward *et al.*, 1994) while L-NAME inhibits with a K_i of

approximately 1 mM (Torres *et al.*, 1997). A recent report suggested that the usefulness

of L-NAME and other alkyl ester analogues of arginine is limited *in vivo* due to a

muscarinic acetylcholine receptor antagonism at K_i values of at least 100 μM in mammals

(Knowles and Moncada, 1994). Caution must therefore be exercised when using these

compounds, to avoid non-specific effects.

The above mentioned isoform specificity of arginine analogues helps identify and

selectively manipulate NOS isoforms *in vivo*. Recently, electron paramagnetic resonance

(EPR) spectroscopy was used to investigate the origin of isoform specificity, by examining

the response of the eNOS and nNOS haem environment to arginine site ligands (Salerno *et*

al., 1997b; Migita *et al.*, 1997). It was found that the specificity of the respective

inhibitors results from the shape of the binding site and the modes of binding (Salerno *et*

al., 1996; Nishimura *et al.*, 1995). In particular: 1) the magnitude of the conformational

rearrangement of the arginine binding site-haem pocket region dictates the strength of

inhibition; the larger the rearrangement, the stronger the inhibition, and 2) each isoform has

a slightly different haem environment (Salerno *et al.*, 1997b).

Enzyme inhibitors however are only one of a number of compounds that can be used to

investigate the function of NOS. To begin with, NO can exert negative feedback on its

own production, by interacting with the haem moiety contained within NOS (Knowles and

Moncada, 1994). Another way of manipulating NO levels in biological systems is to

modulate the supply of one of either its substrates or cofactors. Not only can L-arginine be

used to reverse the effects of the arginine-based inhibitors (Torres *et al.*, 1997), but it can

be used alone to elevate the endogenous activity of NO. For example, the introduction of

1 M L-arginine to rat brainstem slice preparations *in vivo* resulted in an increased spike

frequency as measured from neurons of the nucleus tractus solitarius (Torres *et al.*, 1997).

NO can also be regulated in a more direct manner. Nitrovasodilators, as their name

suggests, have been used clinically in the past to regulate blood pressure. Their

vasodilatory effect results from the circulatory reaction of NO. The effects of 4

nitrovasodilators, nitroprusside (NP), S-nitroso-n-acetylpenicillamine (SNAP), 3-

morpholinosydnonimine chloride (SIN-1) and hydroxylamine were examined on 3':5'-

guanosine cyclic monophosphate (cGMP) levels in rat cerebellar slices (Southam and

Garthwaite, 1991). SNAP produced the most potent increase (50 µM) followed by

hydroxylamine (200 µM) and SIN-1 (1 mM). There was no maximal response from NP

at concentrations up to 10 mM.

NO released from these agents is subject to rapid inactivation in cerebellar slice

preparations, which imposes the necessity for concentrations to be high in order to

generate sufficient amounts of NO locally (Southam and Garthwaite, 1991). Calculations

based on the coefficient of diffusion, the rate of production and the derived half life

suggest that concentrations of 1 µM to 10 mM, when applied to the bathing medium,

would correspond to a local NO concentration range of 0.1 nM to 1 µM in the

extracellular spaces of the slice (Southam and Garthwaite, 1991). Repeated electrical

stimulation of afferent pathways in cerebellar slices resulted in concentrations of

extracellular NO estimated between 70-100 nM (Shibuki and Okada, 1991). Therefore

concentrations of NO donors between 0.1-1 mM would correspond to physiological

signalling concentrations of NO (i.e., 10-100 nM). Similarly, the concentrations of L-

arginine-based inhibitors needed to inhibit nitric oxide production are higher in intact

versus homogenised tissue, due to neural transport barriers. It has recently been shown

that L-NNA and L-NAME were 800 times less potent in intact versus homogenised retina,

necessitating concentrations in the 10-20 mM range (Wellard et al., 1995). Finally, NO

scavengers like oxyhaemoglobin or carboxy-2-phenyl-4,4,5,5-tetramethylimidazoline-1-

oxyl (carboxy-PTIO) can also be used to lower endogenous NO levels. These pharmacological tools therefore provide the means by which levels of NO can be manipulated physiologically, therefore aiding the exploration of the function of NO *in vivo*. The next step in the physiological understanding of NO function in the CNS, and therefore its potential role in locomotion, is the identification of locations and targets for NO action.

1.4. NADPH-DIAPHORASE AND THE FUNCTION OF NOS

Quite possibly the most controversial assumption regarding the location of NO in the nervous system is the accuracy of one of its anatomical markers. It reminded Steven R. Vincent (Vincent, 1995) of a conversation from *Through the Looking Glass*, by Lewis Carroll:

> *'I see nobody on the road,' said Alice*
>
> *'I only wish I had such eyes,' the King remarked in a fretful tone.*
>
> *'To be able to see Nobody! And at that distance too! Why, it's as*
>
> *much as I can do to see real people, by this light!'*

Over thirty years ago, "solitary active cells" were described that contained an extremely high amount of formaldehyde-resistant NADPH-diaphorase activity (Thomas and Pearse, 1961). This histochemical reaction was a result of diaphorase activity transferring electrons from NADPH to tetrazolium compounds, thus producing a visible blue formazan deposition (Bruning and Mayer, 1996). Only within the past decade has it been confirmed that the formaldehyde resistant metabolism of these neurons was in fact due to NOS (Hope *et al.*, 1991), and not one of a number of other enzymes that contain an NADPH site (*e.g.* cytochrome P-450 reductase; Vincent, 1994). This simple method therefore provides an accurate tool for the anatomical localisation of neurons generating NO.

NADPH-diaphorase histochemistry has already been shown to be a selective marker for specific populations of neurons in both the central (Vincent and Kimura, 1992) and peripheral nervous system (Grozdanovic *et al.*, 1992). However, many have their doubts as to its true usefulness as a marker for NO. Recently the relationship between NOS immunocytochemistry and NADPH-diaphorase histochemistry was investigated in the anterior and posterior pituitary of ovariectomized rats (Wang *et al.*, 1997). A clear dissociation of NADPH-diaphorase histochemical activity and nNOS immunoreactivity (NOS-IR) was demonstrated in the rat anterior pituitary, while the two activities co-existed in the posterior pituitary. However, many other studies have reported the co-existence of the two labelling techniques (see below).

Despite the robust and reliable NOS localisation method that NADPH-diaphorase histochemistry provides, care must be taken in the interpretation of results under certain conditions (Vincent, 1995). One of the main conditions is a pre-treatment with formaldehyde fixation (Buwalda *et al.*, 1995). Artefacts may arise if the fixation period is not long enough. Conversely, prolonged fixation can also induce NADPH-diaphorase activity in cells that are normally diaphorase negative (Ott and Burrows, 1999). NOS-IR can also be adversely affected by fixation, resulting in staining of many cells not expressing nNOS mRNA or NADPH-diaphorase (Vincent, 1995). Therefore any results obtained *via* NADPH-diaphorase histochemistry or NOS-immunoreactivity would have to be confirmed with *in situ* hybridisation. Neuronal NOS cDNA segments that correspond to NOS sequence positions can be amplified by reverse transcription-polymerase chain reaction using total brain RNA and then, *via* insertion into a specific plasmid site, can specifically localise NOS by targeting its mRNA (Iwase *et al.*, 1998). Due to the explosion of interest in this novel messenger, there is now detailed anatomical identification of NOS in the nervous system across a broad phylogenetic range of both

vertebrate and invertebrate species alike, including fish (Schober *et al.*, 1993, 1994; Cioni *et al.*, 1998; Villani, 1999), frog (Crowe *et al.*, 1995; Bruning and Mayer, 1996), turtle (Fernandez *et al.*, 1998), mouse (Arnhold *et al.*, 1997), hamster (Decker and Reuss, 1994), rabbit (Salter *et al.*, 1991; Lukáčová *et al.*, 1999), ferret (Cramer *et al.*, 1996), cat (Riche *et al.*, 1995), quail (Panzica and Garzino, 1997), chicken (Goureau *et al.*, 1997), sheep (Northington *et al.*, 1996), primate (Bredt *et al.*, 1991), human (Schoser *et al.*, 1997; Ohyu and Takashima, 1998; Blum-Degen *et al.*, 1999), lobster (Scholtz *et al.*, 1998), fruit fly (Wildemann and Bicker, 1999), locust, honey bee, cricket and cockroach (also reviewed in Bicker, 1998 and Ott and Burrows, 1999). However, one of the most detailed examinations of NO in the nervous system has been in the rat.

Recently the regional distribution of neuronal nitric oxide synthase mRNA in the rat brain was revealed by non-radioisotopic *in situ* hybridisation (Iwase *et al.*, 1998). The combination of digoxigenin-labelled RNA probes and paraformaldehyde fixed paraffin-embedded tissue sections gave high enough resolution to distinguish the densely stained cytoplasm and unstained nucleus in the neurons. Areas expressing nNOS RNA are summarised in Table 1. These distributions correlate with previously reported areas using the NADPH-diaphorase technique (Bredt *et al.*, 1991). Expression of nNOS mRNA within the brain was limited to discrete neurons and/or cell groups (eg., cell layer, nuclei, etc.; Iwase *et al.*, 1998), which suggests that NO plays a functioning role within each specific area. In the context of the normal functioning of the central and peripheral nervous system, NO has been implicated in osmoregulation in the subfornical organ (Rauch *et al.*, 1997), circadian rhythmicity in the suprachiasmatic nucleus (Decker and Reuss, 1994), visual transduction in the retina and perception in the cortex (Lopez-Costa *et al.*, 1997; Ostwald *et al.*, 1997; Cudeiro *et al.*, 1997), skeletal muscle contraction (Kobzik *et al.*, 1994), cardiovascular reflex control in the nucleus tractus solitarii (Ogawa

et al., 1995; Ma *et al.*, 1995), hormonal control in the hypothalamus (Wayman *et al.*,

1994), synaptic plasticity (Gally *et al.*, 1990), and memory related functions like long term

potentiation (LTP) and long term depression (LTD) in the hippocampus and cerebellum

(reviewed in Schuman, 1995). It is thought that NO mediates these diverse functions by

modulating the release of other more conventional neurotransmitters (Schuman and

Madison, 1994; see also below).

TABLE 1. Classification of areas expressing nNOS mRNA[1]

Olfactory/vomeronasal system
  Main olfactory bulb
    Glomerular cell layer
    Mitral cell layer
    Internal plexiform layer
    Granule cell layer
    (Isolated scattered neurons over the entire region)
  Accessory olfactory bulb
    Mitral/tufted cell layer
    Granule cell layer
Limbic and related system
  Amygdala
    Medial nucleus
    Anterior cortical nucleus
      Basomedial nucleus
    Posteromedial cortical nucleus
    Amygdalopiriform transition
  Hypocampus
    Dentate gyrus
    Ammon's Horn
    (Isolated scattered neurons over the entire region
  Bed nucleus of the stria terminalis
  Epithalamus
    Medial habenular nucleus
  Hypothalamus
    Supraoptic nucleus
    Paraventricular nucleus
    Ventromedial nucleus
    Premammillary nucleus
    Mammillary nucleus
    Supramammillary nucleus
  Cerebral cortex
  Corpus callosum
  Basal ganglia
    Caudate-putamen
    Accumbens nucleus
    Olfactory tubercle
    Islands of Calleja

Visual system
  Lateral geniculate nucleus
    Dorsal lateral geniculate nucleus
    Ventral lateral geniculate nucleus
  Superior colliculus
Auditory system
  Nucleus of the trapezoid body
  Inferior colliculus
Vestibular system
  Medial vestibular nucleus
  Prepositus hypoglossal nucleus
Gustatory system
  Nucleus of the solitary tract
Somatosensory system
  Dorsomedial spinal trigeminal nucleus
Thalamus
  Paraventricular nucleus
  Central medial nucleus
  Reuniens nucleus
Subthalamic region
  Zona incerta
Pontine nucleus
Cerebellum
  Cortex
    Granular layer
    Molecular layer
  Deep nuclei
Others
  Magnocellular nucleus of the posterior
commissure
  Midbrain central gray
  Laterodorsal tegmental nucleus
  Pedunculopontine tegmental nucleus
  Medulla reticular formation
    Dorsal paragigantocellular field
    Parvocellular reticular field
    Intermediate reticular field

[1] nNOS mRNA positive areas are classified based on anatomical location and/or major function of each area (copied from Iwase *et al.*, 1998)

The major target of NO identified in most tissues is soluble guanylyl cyclase (cGC; Schuman and Madison, 1994). sGC is a heterodimer that contains a haem region responsible for the NO-induced activation of the cyclase. The conformational change and activation of guanylyl cyclase results from NO binding to an iron ion (Fe^{2+}) in the porphorin ring of the haem which in turn pulls the Fe^{2+} out of the plane of the porphorin ring (Koesling et al., 1995). The resulting activation and rise of cGMP levels can then affect ion channel or phosphodiesterase activity, or activate cGMP-dependent protein kinases (see Fig. 1.1B).

Nitrergic modulation of synaptic function can be initiated by a variety of means. For example, the modulatory actions of NO occur via interactions with ion currents produced by ionotropic receptors coupled to ligand-gated ion channels. One such example is the N-methyl-D-aspartate (NMDA) receptor, which is normally activated by glutamate, the major excitatory amino acid neurotransmitter in the CNS. The NMDA receptor is important in certain forms of activity-dependent synaptic plasticity in the CNS (reviewed in Contestabile, 2000) and is therefore thought to be involved in cognitive processes, memory acquisition and learning. There is a general consensus that the downstream activation of NOS following stimulation of the NMDA receptor is linked to calcium influx through the integral receptor ion channel. The first and most obvious interaction between NO production and NMDA receptor function is demonstrated by a NO-cGMP mediated increase in glutamate release, triggered by the activation of striatal NMDA receptors (Bogdanov and Wurtman, 1997). NO participates in a positive feedback loop controlling glutamate release as a result of the influx of Ca^{2+} through NMDA channels which subsequently promotes NOS activation. The postsynaptic production of NO via NMDA receptors has also been implicated in vertebrate neuromuscular junction transmission (Grozdanovic and Gossrau, 1997). The chain of events begins with glutamate release

from the presynaptic motor nerve terminals, which binds to NMDA receptors in the post-

synaptic sarcolemma causing Ca^{2+} influx and the activation of nNOS associated with $\alpha 1$-

syntrophin on the sarcolemma. Once released NO acts as a diffusible retrograde

messenger, influencing transmission characteristics or modulating the phosphorylation of

proteins (see below). A similar mechanism is at work in the nucleus tractus solitarius,

where NO has a positive feedback effect on glutamate induced hypoxia through NMDA

channels (Ma et al., 1995). NO mediated facilitation of glutamate release is thought to

prevent abrupt changes in ventilation and stabilise 'breath-to-breath' ventilation during

hypoxia (Ogawa et al., 1995). Evidence has also accumulated illustrating a regulatory role

for NO in neuroendocrine function. Specifically, NMDA has been shown to stimulate the

release of α-melanocyte-stimulating hormone from the rat hypothalamus through the

release of NO (Wayman et al., 1994). The functional coupling of NOS and NMDA is

further ensured by their direct physical linkage with post-synaptic density proteins

(Brenman et al., 1996). Additional mechanisms of NO/NMDA interaction are at present

unclear. However, a pair of closely spaced cysteine residues that form a di-sulphide bond

on the extracellular side of the NMDA channel could be oxidised by NO, which would

result in a decrease of the current flow through the channel (Schuman and Madison,

1994). In this way, NO could also be negatively coupled to its own production via

NMDA receptors.

NO also modulates synaptic function by way of metabotropic receptors, which are coupled

to G-proteins. Muscarinic acetylcholine (ACh) receptors are one such example. The

result of muscarinic receptor activation by ACh is an activation of NOS by an increase in

intracellular Ca^{2+}. NO is known to be necessary for ACh-induced stimulation of cGMP

production in dorsal root ganglion neuronal cultures (Bauer et al., 1994). A similar

situation is known for mouse neuroblastoma cells, where NO is involved in the positive

feedback support of a cGMP-dependent Ca^{2+} current after activation by muscarinic

agonists (Mathes and Thompson, 1996). Relatively little is known however about

downstream NO-modulation in target neurons. It has been shown that NO can reversibly

inactivate tryptophan hydroxylase, the initial and rate limiting enzyme in the biosynthesis

of the neurotransmitter serotonin (5-HT) by selective action on cysteine residues within the

enzyme (Kuhn and Arthur, 1997). NO can also inactivate 5-HT itself, creating less

efficacious 5-HT dimers by nitration (Fossier *et al.*, 1999). NO can therefore have a direct

effect on transmitter release *via* metabolic precursors or transmitters themselves.

However, the stimulatory action of NO on Ca^{2+}-dependent neurotransmitter release is also

well documented, including transmitters like ACh, the catecholamines, and neuroactive

amino acids such as γ-aminobytyric acid (GABA) and glutamate (Ohkuma *et al.*, 1998a).

The evoked release of GABA can be the result of NO-induced membrane depolarization

through the activation of voltage dependent calcium channels, which result in calcium

influx which in turn activates the exocytotic neurotransmitter release mechanism (Ohkuma

et al., 1998a). This is an example of a direct effect without the involvement of the cGMP

cascade. Conversly, NO has also been shown to inhibit the secretions of catecholamines

by the activation of a Ca^{2+}-dependent potassium ion (K^+) channel in bovine adrenal

chromaffin cells (Chen *et al.*, 1998). In this example the activation was both cGMP-

dependent and -independent. NO has also been shown to antagonise angiotensin II (Rauch

et al., 1997) and adenosine (Ostwald *et al.*, 1997) release in the subfornical organ and the

retina respectively. The methods by which NO facilitates or debilitates transmitter release

in rats can therefore be classified as direct or indirect, affecting the neurotransmitters

themselves or various processes upstream.

1.5. DEVELOPMENTAL ROLES FOR NO

It is quickly becoming apparent that the role of neurotransmitters extends well beyond the developmental formation of correct wiring patterns for neural circuits, to other aspects of neurogenesis such as proliferation, survival, migration and differentiation of neurons (reviewed in Katz, 1999). For example, it has been suggested that NO functions as a retrograde messenger sub-serving use-dependent modification of synaptic efficacy during the establishment of properly ordered cortical connections (Bredt, 1995). However, alternative roles for NO have also been suggested including axonal guidance (Van Wagenen and Rehder, 1999), responses to neuronal injury and degeneration (Hu et al., 1997) and growth cone collapse (Hess et al., 1993; Ernst et al., 2000). The neuronal survival roles of NO potentially result from an interaction with neurotrophins, such as brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF). The neurotrophins have been implicated in the regulation of synaptic plasticity and are known to play an integral role in neuronal survival and proliferation. This includes installation of certain transmitter phenotypes in neurons and regulation of levels of transmitter production (Huber et al., 1995). In experiments with embryonic day 6 (E6) cultured rat spinal cord neurons the neurotrophins BDNF, NT-3 and NT-4 but not NGF, augmented numbers of NADPH-diaphorase positive neurons (Huber et al., 1995). This therefore indicates that the developmental regulation of the nitrergic phenotype in spinal cord neurons depends on the exposure to specific neurotrophins. Recent evidence also suggests that motor neuron apoptosis induced by trophic factor deprivation in vitro involves both increased NO production after the induction of neuronal NOS and augmented intracellular production of superoxide (Estevez et al., 1998). Activation of the NMDA receptor in neurons can lead to the formation of superoxide through oxidation of fatty acids, which in combination with NO via a diffusion limited reaction, forms the much stronger and more toxic oxidant peroxynitrite (Estevez et al., 1998). At physiological pH, peroxynitrite is sufficiently

stable to diffuse over several cell diameters to reach critical cellular targets. Peroxynitrite

initiates lipid peroxidation, and reacts directly with sulfhydryl groups and transition metals

to form a powerful nitrating agent (Varner and Beckman, 1995). In this case BDNF

supports motor neuron survival in part by preventing neuronal NOS expression.

Additionally, downstream proteins like Bcl-2 that act as anti-oxidants or inhibit free radical

production are known to regulate neuronal survival (Davies, 1995), suggesting an

important role for NO. Yet, NO has been shown to promote filopodial elongation (Van

Wagenen and Rehder, 1999) and is needed to stabilise retinal axon growth in combination

with BDNF (Ernst *et al.*, 2000). Furthermore, experiments with nerve growth factor

(NGF)-differentiated pheochromocytoma (PC12) cell lines illustrated an acquired

resistance to NO-induced necrosis accompanied by an expression of NOS (Wada *et al.*,

1996). These examples illustrate the contrasting, and at times contradictory,

developmental roles NO plays in different functional areas of the CNS.

Interestingly, during the development of the CNS, the expression of nNOS belies its

constitutive title and appears to be actively regulated, much like the inducible NOS

isoform. The convenient NADPH-diaphorase histochemical identification method has

aided the definition of two functions for NOS expression in development. The first is a

persistent expression in areas, as described above, where NO-modulation occurs in the

CNS throughout life. There is also a second more transient expression, in which NOS

appears and then disappears and where NO is thought to play a temporary role during

neurogenesis. For example, in motor nuclei of the foetal human brainstem there are two

chronological patterns of nNOS expression; one that appears during prenatal development

and then remains, and another, present only at a specific foetal stage (Gonzalez-Hernandez

et al., 1994). The distribution of NOS-IR structures in the rat brain at stages of early

embryonic neurogenesis (embryonic day 15 or E15) and active synaptogenesis (E19) has

recently been examined (Terada *et al.*, 1996). This study demonstrated that NOS-IR cells

are already present by stage E15 predominantly in the hypothalamus and pons. By stage

E19, NOS-IR cells located in the striatum, hypothalamus, pons and medulla had increased

in size and number. There were however very few NOS-IR positive cells in the cerebral

cortex and the cerebellum at these early developmental stages. In the lower brainstem the

pattern of NOS-IR distribution was similar to that found in the adult animal, indicating an

early role for NO in this area. A detailed NADPH-diaphorase histochemical study of the

development of the brainstem and spinal cord from developmental stage E12 through to

postnatal day 20 (P20; Takemura *et al.*, 1996) also illustrated a persistent increase in

NOS-containing cells in regions that are important for primitive physiological functions

like muscle tone, respiratory activity, circulatory tone, and sensorimotor reflexive

functions.

There are also examples of a transient NOS expression during development in discrete

populations of neurons during neurogenesis (Bredt, 1995). Between P10 and P20 during

the development of the visual cortex in the rat some NOS-positive neurons, especially in

the deeper layers, displayed symptoms of degeneration, like shrunken cell bodies,

corkscrew and twisted dendrites (Luth *et al.*, 1995). From P20 into adulthood NOS-

containing neurons were no longer visible. The distribution of NOS-containing neurons

was also studied in the paratenial nucleus throughout development using NADPH-

diaphorase histochemistry (Garcia-Ojeda *et al.*, 1997). A similar transient expression was

observed with a progressive increase in staining from P1 to P10, after which a sudden

decrease in the staining intensity and in the number of NADPH-diaphorase reactive

elements was detected. However, in contrast to the visual cortex, NADPH-

diaphorase/NOS-IR positive neurons did not exhibit any degenerating morphology.

Transient roles for NO are not only limited to neurogenesis, but have also been described in populations of neurons destined for apoptosis. In amphibian species, metamorphosis involves a dramatic reconfiguration of physiology, not the least of which involves tissue reabsorption and reorganisation (Fritzsch, 1990). Recently a comprehensive mapping of NOS-positive cells in the brain of adult *Xenopus laevis* frogs were obtained using both NADPH-diaphorase histochemistry and NOS-IR (Bruning and Mayer, 1996). By using both techniques, the usefulness of NADPH-diaphorase histochemistry as a marker for NOS in *Xenopus* could also be determined. As a result, comparisons of sections revealed that the same structures were stained by both methods. Unlike the rat, in adult *Xenopus* the main and accessory olfactory bulb did not contain NOS-positive cells. However, overall the NOS expression in sub-populations of neurons throughout the brain of the frog (summarised in Table 2) reveals certain similarities to the distribution pattern seen in other species. The expression of NOS is not strictly limited to a particular system and therefore may be involved in a variety of different processes in the amphibian brain. Yet, species-specific differences raise the point that NO may play different physiological roles in different species, rather than a functionally general role in the vertebrate brain (Panzica and Garzino, 1997). The distribution of NADPH-diaphorase reactivity in the spinal cord has also been examined in metamorphosing and adult *Xenopus* (Crowe *et al.*, 1995). The finding that motor neurons labelled NADPH-diaphorase positive is not unique to the frog and suggests that NO also has a persisting role throughout life in this species. However, NO was also observed in motor neuron populations that were destined to die (*e.g.* tail motor neurons) as well as populations that were newly generated (*e.g.* limb muscle motor neurons), suggesting a possible role in developmental and ageing processes (Crowe *et al.*, 1995).

TABLE 2. Classification of areas expressing NADPH-diaphorase staining[1]

Forebrain	Midbrain
Posterior telencephalon	Mesencephalon
Pallium	Tectum
Striatum	Tectal commisure
Nervus terminalis	Posterior commisure
Nucleus accumbens	Trigeminal nucleii
Anterior entopenduncular nucleus	Tegmental nucleii
Lateral amygdaloid nucleus	Anterodorsal tegmental nucleus
Preoptic nucleus	Posterodorsal tegmental nucleus
Diencephalon	Ventral tegmental region
Thalamus	(Isolated scattered neurons over the entire
Lateral anterior nucleus	region)
Posterior entopeducular nucleus	Magnocellular nucleus of the torus
Posterior lateral nucleus	semicircularis
Pretectal area	Brainstem
Tuberculum posterior	Metencephalon
Superficial ventral thalamic nucleus	Superior reticular nucleus (amphibian
Hypothalamus	homologue of the locus coeruleus)
Nucleus preopticus	Inferior reticular nucleus
Nucleus preopticus periventralis	Descending nucleus of the trigeminus
Infundibular nucleus	Cerebellum
Suprachiasmatic nucleus	Purkinje cells
	Others
	Arcuate fibres

[1]Areas positively stained for NADPH are classified based on anatomical location. Compiled from Bruning and Mayer, 1996; Rao *et al.*, 1997.

These examples suggest that while NO may be located in a majority of similar regions in the vertebrate CNS, there may also be species-specific roles for NO in central nervous function. One function that appears to be conserved between species, however, is NO's role in development. Yet, despite the exhaustive literature discussing NO's role in the development and function of the CNS and the clear presence of NO-containing neurons in regions associated with locomotion, a physiological role for NO in the co-ordination of locomotion has yet to be described. Indeed, physiological research into NO's role in the co-ordination of vertebrate locomotor networks has to date been limited to qualitative behavioural investigations in which NOS inhibitors were used to assess effects on gross locomotor activity (*c.f.* Nelson *et al.*, 1995; Dzoljic *et al.*, 1997). The resulting limitations of this work are in part due to the complexity of the circuits and brain regions involved in these higher vertebrates, even at early developmental stages. Therefore, it seems prudent

to find a simple yet reliable model to investigate the potential role for NO in vertebrate

locomotion.

1.6. THE DEVELOPMENT OF RHYTHMIC LOCOMOTION

The hatchling amphibian tadpole is now a well-established model for the study of the

neural networks underlying locomotion. There is therefore a substantial physiological and

anatomical backdrop against which to investigate the possible modulatory role of NO in

the development of swimming rhythmicity. The attractions of studying the development of

locomotor activity in tadpoles include the relative simplicity of the nervous system at early

stages of development, with only eight classes of differentiated spinal neuron (Roberts and

Clarke, 1982; Fig. 1.3A), and the rapidity with which the motor pattern controlling

swimming matures after hatching (Sillar et al., 1991). Another clear advantage is that

motor patterns are evoked via sensory pathways in intact animals and therefore provide a

closer to natural representation of behaviour than motor patterns evoked by

pharmacological agents or artificial brainstem stimulation. Specifically, "fictive" motor

patterns that would be appropriate to drive natural swimming movements can be recorded

from paralysed animals after a brief stimulus (e.g. light dimming or electrical current

pulses applied to the skin). In hatchling Xenopus laevis embryos (stage 37/38;

Nieuwkoop and Faber, 1956; Fig. 1.2Ai), fictive swimming is a fast (10-20 Hz) rhythm in

which ventral root activity alternates between the two sides of the spinal cord and

progresses from head to tail with a brief rostrocaudal delay (recently reviewed in Roberts

et al., 1998). There is relatively little variability in motor burst durations from one cycle to

the next and cycle periods gradually decline during the course of an episode (Fig. 1.2Aii-

iv). This stereotyped and relatively inflexible motor pattern changes dramatically after just

24 hours. By larval stage 42 (Fig. 1.2Bi) the swimming motor pattern is much more

variable, with burst durations and cycle periods fluctuating markedly within each episode

of swimming (Sillar *et al.*, 1991; Sillar *et al.*, 1992a; Sillar *et al.*, 1998; Fig. 1.2Aii-iv). It has been shown that the descending influence of serotonergic raphe neurons contributes to the increase in the intensity and duration of motor bursts in a stage-dependent fashion (Sillar *et al.*, 1992b; Sillar *et al.*, 1995a, b), which coincides with the ingrowth of serotonergic axons (van Mier *et al.*, 1986).

As for many other vertebrate spinal networks, the *Xenopus* motor pattern results from descending excitation combined with reciprocal inhibition (Fig. 1.2Aiii). The descending excitation is primarily glutamatergic (Dale and Roberts, 1984), however motor neurons themselves contribute to descending excitation *via* their own ACh release (Perrins and Roberts, 1994; Perrins and Roberts, 1995a, b) and electrical coupling (Perrins and Roberts, 1995a, b). This means that motor neurons are not simply passive output elements, but can contribute to the maintenance of pattern generation. During swimming, motor neurons receive a rhythmic on-cycle excitatory post-synaptic potential (EPSP) which underlies single (stage 37/38) or multiple (stage 42) spikes fired on each cycle (Fig. 1.2Aiii, Biii). Separating the EPSPs of consecutive cycles is a mid-cycle inhibitory post-synaptic potential (IPSP). These two potentials are superimposed on an NMDA receptor-mediated tonic depolarisation whose amplitude is related to the frequency of swimming. It is largest early in an episode when frequency is highest and falls as the frequency declines, eventually returning smoothly to the resting potential once rhythmic activity stops (Roberts and Alford, 1986).

The biogenic amines 5-HT and noradrenaline (NA) are important modulators of tadpole swimming activity that allow expression of the full range of motor output patterns (Sillar *et al.*, 1992b; Sillar *et al.*, 1998; McDearmid *et al.*, 1997). NA lengthens cycle periods and can reduce burst durations and intensities relative to cycle periods (McDearmid *et al.*,

1997), precisely the opposite effect to 5-HT. Therefore, these amines can modulate the

swim-generating network between two extremes of output, from slow weak activity (NA)

to fast intense activity (5-HT; Sillar *et al.*, 1998). A primary determinant of swimming

frequency is the strength of the mid-cycle glycinergic inhibition (Dale, 1995) and it is

thought that the aminergic modulation of spinal networks in *Xenopus* tadpoles is partly the

result of supraspinal systems that act upon this component of the synaptic drive. The

opposing effects of 5-HT and NA result from the differential presynaptic modulation of a

common neuronal target in the swimming rhythm generator, the release of glycine from

commissural interneurons (McDearmid *et al.*, 1997; Fig. 1.3Ci-iii, D). 5-HT depresses

the amplitude of mid-cycle glycinergic IPSPs during swimming whilst NA enhances them

(McDearmid *et al.*, 1997; Fig. 1.3Bi-iii). In support of this, reducing glycinergic inhibition

either with the receptor antagonist strychnine, or by using low chloride salines, produces 5-

HT-like effects, a facilitation of motor bursts and an increase in swimming frequency.

Synaptic connectivity is, however, not the only determinant of motor output. The

aminergic modulator 5-HT also affects intrinsic membrane properties of motor neurons,

which play a central role in motor pattern generation (see above). The co-application of 5-

HT and NMDA in the presence of tetrodotoxin (TTX) triggers membrane potential

oscillations in larval (Fig. 1.5Bi), but not embryonic (Fig. 1.5Ai), motor neurons. The

underlying mechanism in this system is thought to involve the cyclical voltage-dependent

blocking and unblocking of NMDA receptor ion channel by magnesium ions, *via* an as yet

undescribed 5-HT/NMDA receptor interaction (Sillar and Simmers, 1994). This confers a

region of negative slope resistance in the current/voltage relationship, causing the

membrane potential to oscillate between two quasi-stable levels. The expression of

oscillations in *Xenopus* spinal neurons is also developmentally regulated in a parallel

manner to the post-embryonic establishment of functional serotonergic spinal projections,

which suggests a possible role for the oscillations during maturation of the swimming

system. While the oscillations are too slow (less than 0.5 Hz) to contribute to the phasing

of each swimming cycle, it is possible that they may modulate swimming over many

consecutive cycles. Addition of NMDA to larval (Fig. 1.5Bii,iii), but not embryonic (Fig.

1.5Aii,iii) preparations produces continuous swimming, which is slowly and rhythmically

(2-4 s) modulated, with epochs of fast, intense bursts interposed between slower, weaker

activity. This slow modulation appears to be due to a superimposition of the intrinsic

oscillations upon the network drive for swimming as it, like the oscillations, disappears

after spinalisation, block of 5-HT receptors or removal of magnesium ions (Reith and

Sillar, 1998). It has yet to be proven however if these oscillations play a part in natural

swimming behaviour.

The eventual termination of an episode of swimming occurs either spontaneously, or in

response to sensory stimuli. The spontaneous termination of swimming is suggested to

involve a gradual build-up of adenosine, which is produced during motor activity and

decreases network excitability by blocking voltage-gated Ca^{2+} currents (Dale and Gilday,

1996). However, there are almost certainly a number of more behaviourally relevant

systems that facilitate the appropriate termination of swimming, not the least of which

involves the release of another fast descending inhibitory transmitter, GABA. There is a

tonic level of GABAergic inhibition that contributes to the synaptic drive for swimming

that could slow and eventually stop swimming activity (Reith, 1996; Reith and Sillar,

1999). However, swimming can also be terminated prematurely by GABA. Descending

GABAergic midhidbrain reticulospinal (mhr) neurons (Roberts et al., 1987) are activated

by primary afferents of the rostral cement gland and terminate swimming when the tadpole

encounters an obstacle (Boothby and Roberts, 1992b; Fig 1.4A, Ci-ii) by activating

$GABA_A$ receptors on spinal motor neurons (Boothby and Roberts, 1992a). However, the

reliability of the stopping response declines during larval development, coincident with

degeneration of the cement gland and by around stage 46, when the animal is free

swimming, both have disappeared (Boothby and Roberts, 1992b). Yet, preliminary

immunocytochemical evidence indicates that the mhr neurons are still present at stage 42

(Reith, 1996; Fig 1.4Bi-ii), when the stopping response is already substantially diminished

(Boothby and Roberts, 1992b). Also, episodes of swimming at larval stage 42 often

terminate with a barrage of apparently spontaneous GABA IPSPs which closely resemble

those evoked by cement gland stimulation at stage 37/38 (Fig 1.4Di-ii). This evidence

suggests that during larval development the mhr neurons are recruited into an intrinsic

stopping pathway (Reith and Sillar, 1999), thereby providing a route *via* which other

brainstem systems might regulate the duration of swimming episodes.

The basic principles of operation for vertebrate locomotor rhythm generation are similar in

the related anuran species, *Rana temporaria*. In other words important features like cycle

rhythmicity, alternation between sides, and longitudinal delay are conserved between the

two species. The rhythmic swimming that follows sensory stimulation may therefore be

generated by similar underlying mechanisms in both animals. There are however

developmental differences between the two species that should be addressed. One of the

more obvious differences is that *Rana* swimming is driven by a pattern of motor neuron

bursts closer to those seen in adult vertebrate locomotor drive than the single spike pattern

of *Xenopus* embryos. This could be the result of a relatively advanced state of

development of descending serotonergic projections compared with *Xenopus*. In *Xenopus*,

serotonergic raphe nuclei projections are still largely absent from the ventrolateral aspect

of the spinal cord. However the situation is quite the opposite in *Rana*, with ventral

projections well developed, comprising a dense plexus of fibres that extend along the

ventrolateral cord (Woolston *et al.*, 1994).

Given NO's prominent role in developmental mechanisms, it is perhaps surprising that there is yet to be an investigation into its putative role in the amenable developing motor systems provided by anuran tadpoles. The potential co-localisation of NO with a number of the transmitter systems already described in *Xenopus* and *Rana* certainly suggests a potential role for it in their development (see Table 2). For example, the distribution of catecholaminergic neurons in the brain of the frog has recently been determined thus permitting the definition of a cell group ventromedial to the nucleus isthmi as the anuran homolog of the locus coerulus (González and Smeets, 1993; Marín *et al.*, 1996). This location corresponds approximately to an area referred to in *Xenopus* as the superior reticular nucleus, which was strongly NADPH-diaphorase positive (Bruning and Mayer, 1996). Therefore an interesting functional role could be a co-localisation of NO with NA which is known to modulate locomotion at early developmental stages of this species (McDearmid *et al.*, 1997).

1.7. SCOPE OF THE PRESENT STUDY

In the following chapters I provide: 1) a description of the effects of NO on fictive motor activity in two related anuran species, where it terminates a motor pattern in *Xenopus*, but initiates one in *Rana*; 2) evidence that NO is potentiating GABAergic and glycinergic inhibition in the spinal cord of *Xenopus*, thereby shortening swim episode duration and slowing swimming frequency, respectively; 3) a detailed description of the development of NOS in the CNS in *Xenopus*, indicating that NO plays an early and persistent role in neural development; 4) a description of presumably homologous groups of brainstem neurons in *Rana* from which NO is exerting species specific effects; and 5) a general discussion placing these results in the context of recent findings and proposed future research directions.

A

B

C
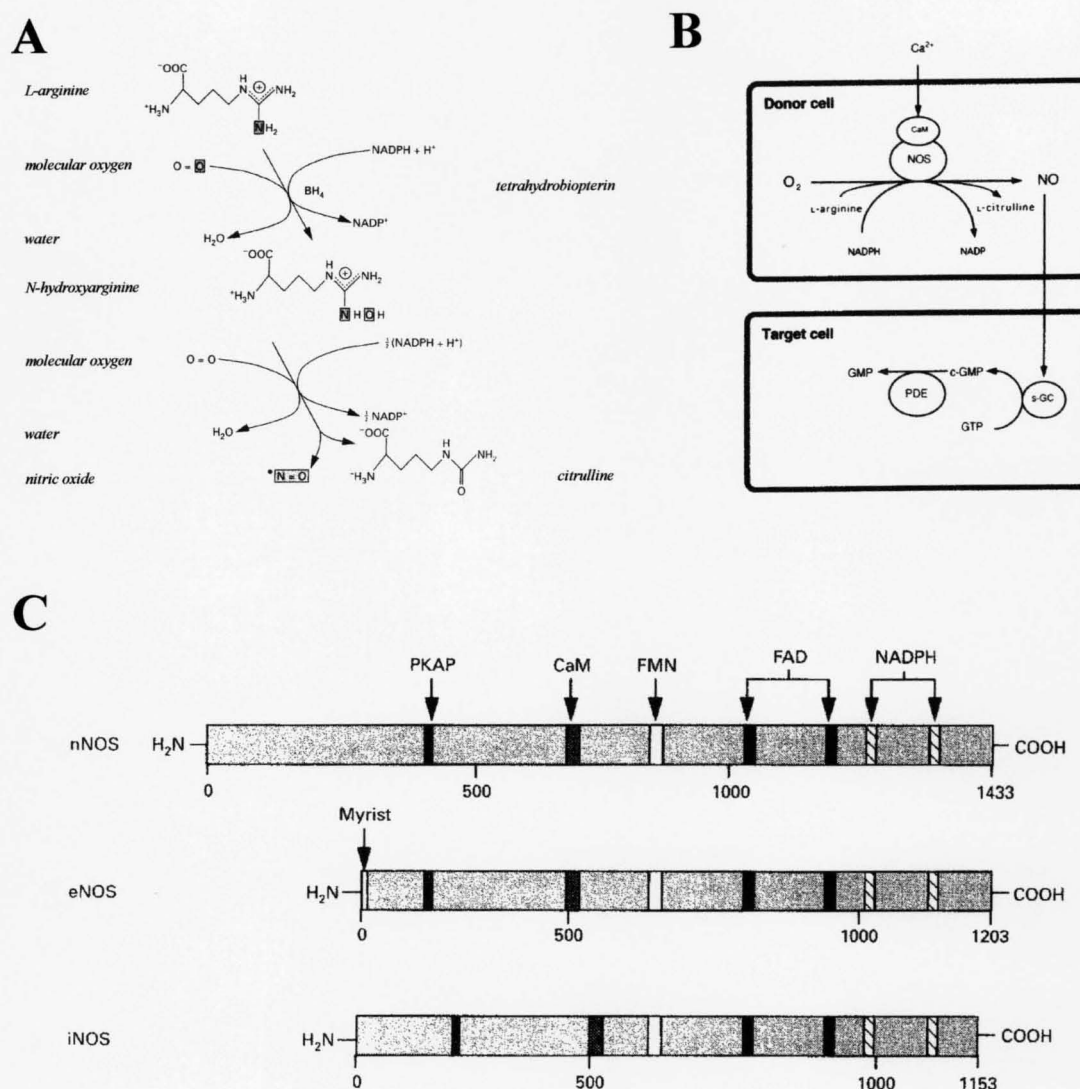
FIGURE 1.1. Examples of NOS structure, formation and activity. (A) The boxed O and N atoms show the origin of the constituent atoms of nitric oxide (NO). NO is produced by the oxidation of one of the terminal guanidino nitrogens of L-arginine resulting in the stoichiometric production of L-citrulline (taken from Knowles and Moncada, 1994). (B) Neuronal activity in the NO-donor cell leads to the influx of calcium, which stimulates *via* calmodulin (CaM) the nitric oxide synthase (NOS) enzyme. NOS catalyses the conversion of arginine to citrulline, which is formed stoichiometrically with NO. The reaction is NADPH-dependent, requiring it as a cofactor. In the target cell, NO binds to a haem moiety in soluble guanylate cyclase (s-GC), which consequently results in an elevation of cGMP. cGMP is down-regulated by phosphodiesterases (PDE), which are instrumental in impotence treatment (taken from Bicker, 1998). (C) This schematic diagram shows the lengths and consensus binding sites for NADPH, FAD, FMN and calmodulin (CaM) as well as the consensus PKA phosphorylation site (PKAP) and the N-terminal myristoylation site (Myrist; taken from Knowles and Moncada, 1994).

Ai stage 37/38

Bi stage 42

Aii

Bii

lmn

lvr

2 s 40 mV
100 ms

1 s 40 mV
100 ms

Aiii

Biii

Aiv
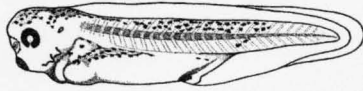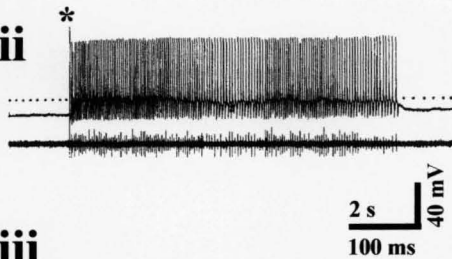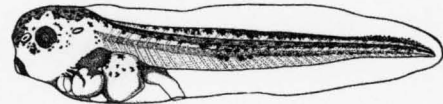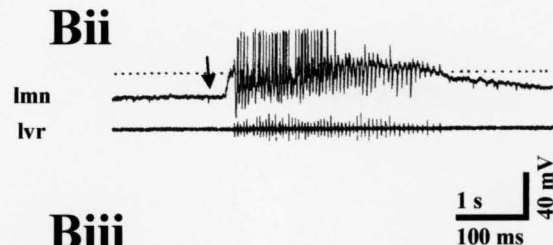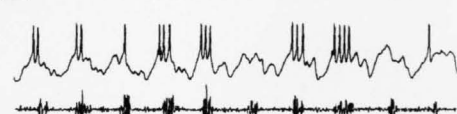
Biv
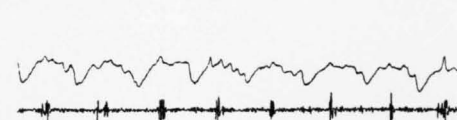
FIGURE 1.2. Post-embryonic development of multiple spike discharge during fictive swimming in *Xenopus laevis* tadpoles. (Ai) *Xenopus* embryo at stage 37/38 and its stereotyped fictive swimming rhythm (Aii-iv) as recorded intracellularly with a potassium acetate microelectrode from a presumed motor neuron on the left side of the animal. (Aii) Complete episode of swimming evoked by an electrical current pulse (at stimulus artefact; asterisk) to tail skin. Dotted line indicates level of tonic depolarisation sustained throughout episode. (Aiii, iv) Excerpts of activity from near the start (Aiii) and near the end (Aiv) of the episode illustrated in (Aii) on a faster time scale show a single impulse on every cycle, in time with ipsilateral ventral root discharge, and a prominent mid-cycle inhibitory post-synaptic potential. (Bi) *Xenopus* larva at stage 42 and its fictive swimming rhythm (Bii-iv) recorded extra- and intracellularly at similar locations to the embryo in A. Note similar sustained depolarization throughout episode (Bii), with up to four impulses per cycle (in phase with ipsilateral ventral root discharge) near the start of episode (Biii) but no spikes occurring near the end of the episode (Biv). Episode initiated by transient dimming of the lights (arrow in Bii). Adapted from Sillar et al., 1992.
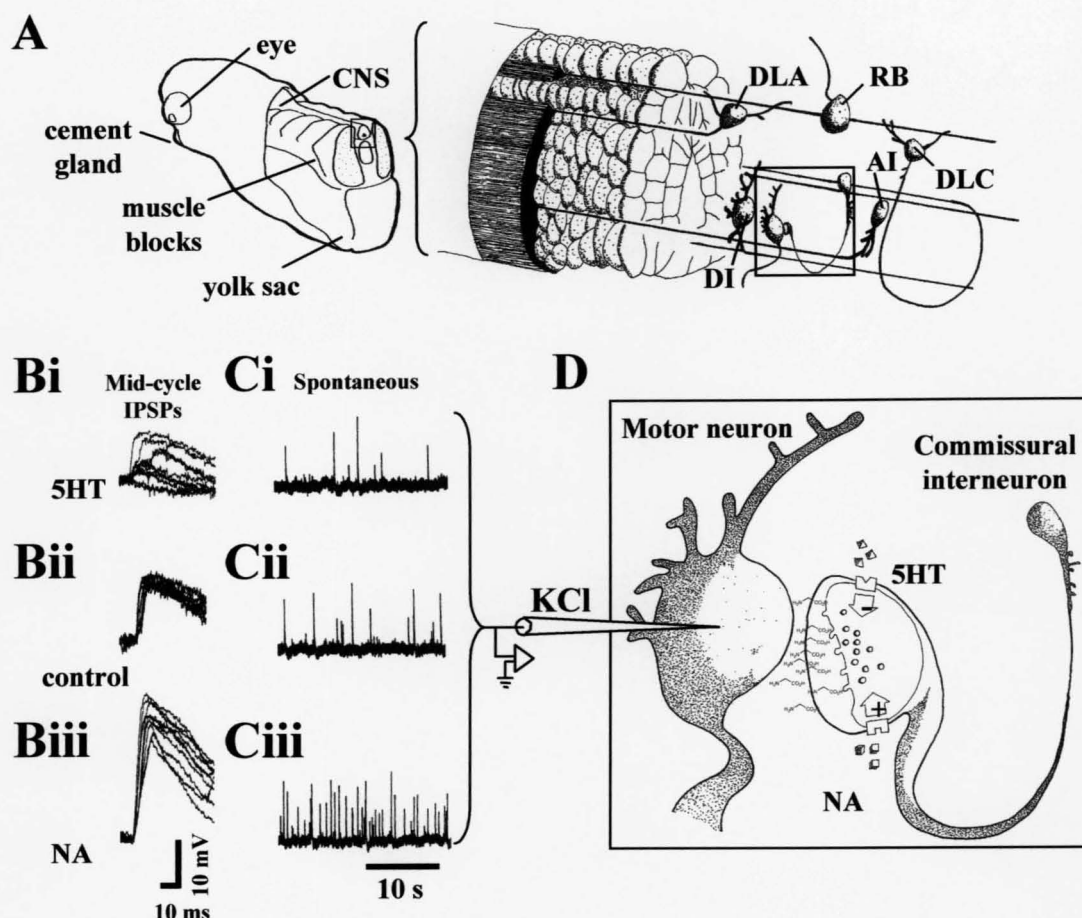
FIGURE 1.3. The biogenic amines 5-HT and NA differentially modulated glycinergic inhibition in the spinal cord of hatchling *Xenopus laevis* tadpoles. (A) Schematic illustration of the central nervous system (CNS) with respect to morphological landmarks, showing in detail the limited number of neuronal cell types in the spinal cord, including a Rohon-Beard sensory neuron (RB), a dorsolateral ascending neuron (DLA), a dorsolateral commisural neuron (DLC), ascending (AI) and descending (DI) interneurons and (in boxed area), a commissural interneuron and a motor neuron (see detail in D). (Bi) 5-HT depresses mid-cycle inhibitory post-synaptic potential (IPSP) amplitudes during fictive swimming and (Ci) reduces tetrodotoxin (TTX)-resistant spontaneous IPSP rate, as compared to controls (Bii, Cii). Conversely, NA (Biii) facilitates mid-cycle IPSP amplitudes and (Ciii) increases spontaneous IPSP rate, as compared to controls (Bii, Cii). Note that in this and subsequent figures intracellular recordings were made with potassium chloride (KCl) electrodes, which renders IPSPs depolarising due to chloride leakage. (D) A schematic enlargement of boxed area in (A) shows a single synaptic contact between a commissural interneuron and a motor neuron and illustrates the contrasting presynaptic effects of both amines on vesicular glycine release. The same voltage scale applies to both panels B and C. Diagram in A adapted from original drawing by SR Soffe, with permission. Figure taken from McLean et al., 2000.
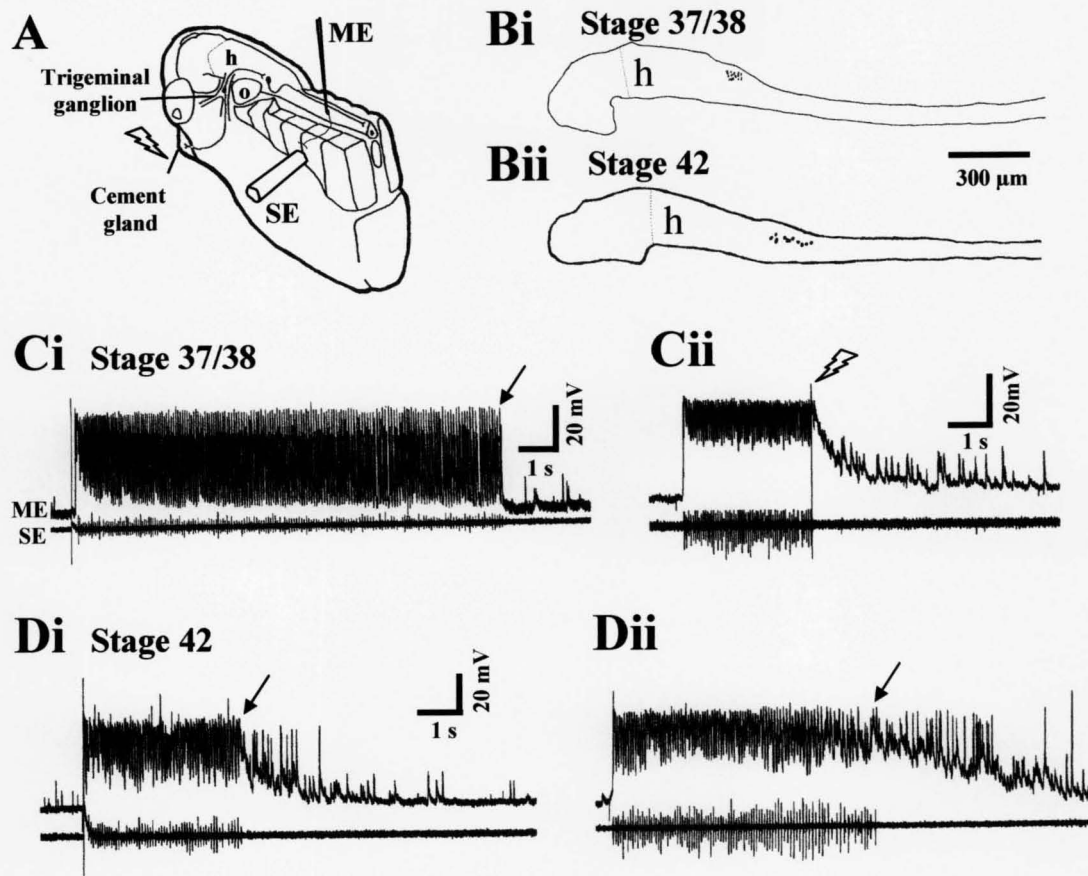
FIGURE 1.4. Termination of swimming episodes by $GABA_A$ receptor activation in embryonic and larval tadpoles. (A) Schematic illustration of the sensory pathway connecting the cement gland to GABAergic midhindbrain reticulospinal neurons located in the hindbrain (h) near the otic capsule (o). Fictive motor activity is measured from ventral roots between myotomal swimming muscles using glass suction electrodes (SE), with simultaneous intracellular recordings from motor neurons using a microelectrode (ME). (B) *Camera lucida* drawings of wholemount central nervous systems in (Bi) embryos and (Bii) larvae show location of GABA immunoreactive neurons. (Ci) Intracellular recordings from an embryo motor neuron show rhythmic activity during a swimming episode that terminates spontaneously (at arrow) with no obvious synaptic input. (Cii) Another episode terminates prematurely with a train of depolarising GABAergic potentials, following brief electrical stimulus (at lightning bolt) applied to cement gland. (Di,ii) At stage 42, swimming episodes often terminate spontaneously with a similar barrage of GABA IPSPs (at arrows) without stimulation of cement gland. Scale bar in Di also applies to Dii. Diagram (B) adapted from Reith, 1996, with permission. (C,D) adapted from Reith and Sillar, 1999. Figure taken from McLean et al., 2000.
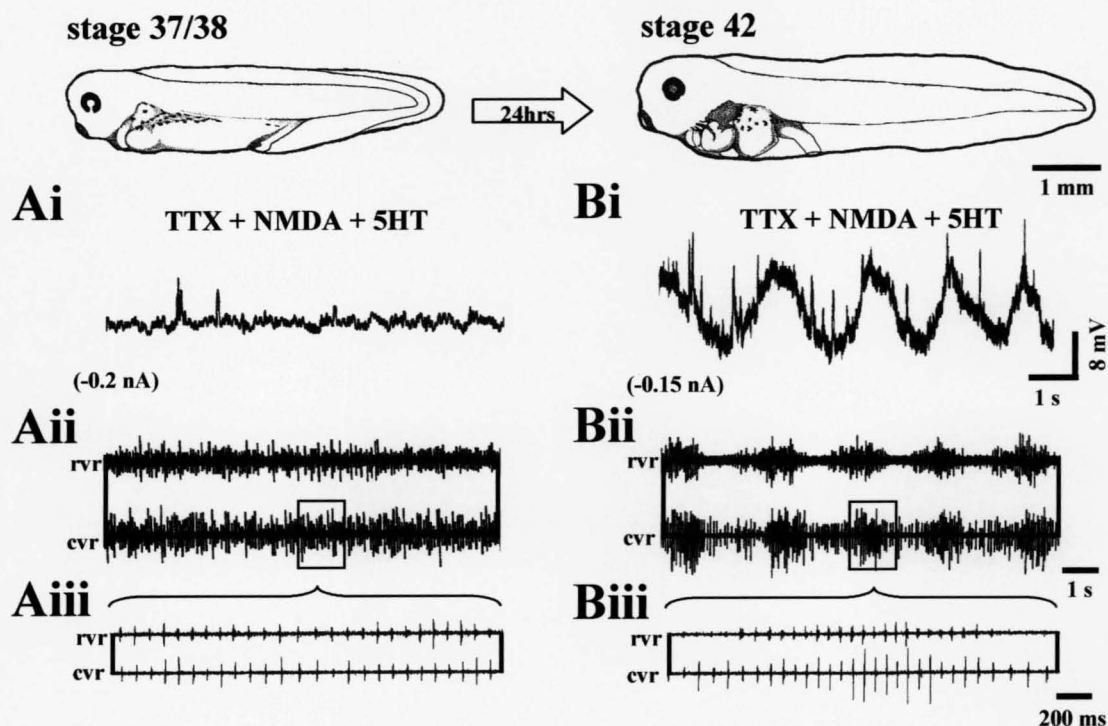
FIGURE 1.5. 5-HT-induced NMDA receptor-mediated intrinsic voltage oscillations are TTX-resistant, stage dependent and contribute to a slow rhythmic modulation of NMDA-induced fictive swimming in *Xenopus laevis* larvae. (Ai) intrinsic voltage oscillations in motor neurons are absent at stage 37/38 and NMDA-induced ventral root activity (rostral: rvr, caudal: cvr) is of relatively constant amplitude and frequency (Aii; expanded in Aiii)). (B) By stage 42, voltage oscillations are present under TTX, NMDA and 5HT (Bi) and NMDA-induced ventral root activity shows a pronounced waxing and waning in amplitude (Bii; expanded in Biii). In recordings Ai and Bi a negative holding current (amount in brackets) has been applied. Scale bars apply to panels A and B. Adapted from Reith and Sillar, 1998; Scrymgeour-Wedderburn et al., 1997. Figure taken from McLean et al., 2000.

THE DISTRIBUTION OF NADPH-DIAPHORASE LABELLED

INTERNEURONS AND THE ROLE OF NITRIC OXIDE IN THE

SWIMMING SYSTEM OF *XENOPUS LAEVIS* LARVAE[1]

2

2.1. SUMMARY

The possible involvement of the free radical gas NO in the modulation of spinal rhythm generating networks has been studied using *Xenopus laevis* larvae. NADPH-diaphorase histochemistry identified three putative populations of NOS-containing cells in the brainstem. The position and morphology of the largest and most caudal population suggested that a proportion of these neurons is reticulospinal. The possible contribution of nitrergic neurons to the control of swimming activity was examined by manipulating exogenous and endogenous NO concentrations in vivo with an NO donor (SNAP, 100-500 μM) and NOS inhibitors (L-NAME and L-NNA, 0.5-5 mM), respectively. SNAP decreased swim episode durations and increased cycle periods, whereas the enzyme inhibitors had the opposite effects. It is concluded from these data that the endogenous release of NO from brainstem neurons extrinsic to the spinal cord of *Xenopus laevis* larvae exerts a continuous modulatory influence on swimming activity, functioning like a "brake". Although the exact level at which NO impinges upon the swimming rhythm generator is yet to be determined, the predominantly inhibitory effect of NO suggests that the underlying mechanisms of NO action could involve modulation of synaptic transmission and/or direct effects on neuronal membrane properties.

2.2. INTRODUCTION

The labile and highly diffusible diatomic free radical gas, NO is now known to be widely

distributed and to play a diversity of roles in both vertebrates (see CHAPTER 1; reviewed in

Moncada and Palmer, 1991) and invertebrates (see CHAPTER 1; reviewed in Vincent,

1995). Within the CNS, NO is recognised as an important, albeit unconventional,

chemical messenger which is intimately involved with the regulation of synaptic function

in a variety of brain regions (see CHAPTER 1; reviewed in Schuman and Madison, 1994;

Vincent, 1994). The location of nitrergic neurons across a broad phylogenetic range has

been studied using both histological and/or immunocytochemical markers for NOS, the

synthetic enzyme that converts L-arginine to NO. One simple and reliable staining method

involves the NADPH-diaphorase histochemical reaction, because the enzyme responsible

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for this reaction is NOS (Hope *et al.*, 1991). This technique has revealed highly localised

staining in particular clusters of neurons and their processes in various locations that

extend to virtually all areas of the brain (see CHAPTER 1; reviewed in Vincent and Hope,

1992). Since this picture differs between species and can also alter at different

developmental stages in the same species, it seems likely that NO is involved in highly

specific behavioural roles, many of which are either poorly defined or not yet discovered.

In particular, nothing is known about the possible roles of NO in the control of the spinal

rhythm generating networks responsible for locomotion in vertebrates. For this reason the

distribution of nitrergic neurons and their role in a very simple vertebrate locomotor

system, that controlling swimming in newly hatched *Xenopus laevis* tadpoles, has been

chosen. This system has been reviewed in the first chapter, so only a brief account will be

given here. Swimming behaviour in these animals at the time of hatching (stage 37/38;

Nieuwkoop and Faber, 1956) involves the coordinated contractions of segmented

myotomal muscles that are distributed along each side of the body. The muscles on the

left and right sides contract in strict alternation during swimming while those on each side

contract in a head to tail sequence (Kahn *et al.*, 1982). This cycle of muscle contraction

creates back-propagating waves of body curvature that generate reactive thrust against the

water column and lead to forwards propulsion of the organism. Fictive swimming activity

involves the ventral root discharge appropriate to drive this swimming rhythm in

immobilised tadpoles and can be generated by a network of neurons located entirely within

the spinal cord, often called the swim central pattern generator (CPG). At these early

stages in development the spinal cord contains as few as eight types of differentiated

neuron (Roberts and Clarke, 1982) of which three are known to be involved in the

generation of rhythmic swimming activity (reviewed in Roberts, 1990). After the first day

of larval life (stage 42; Nieuwkoop and Faber, 1956), the swimming system is

dramatically transformed to produce a pattern in which motor neurons that used to fire in a single spike pattern per cycle at stage 37/38 (Soffe, 1990) can now discharge a burst of action potentials in each cycle of swimming (Sillar *et al.*, 1991, 1992a, b). This transition is dependent upon certain brainstem neurons with descending axonal projections to the spinal cord (Sillar *et al.*, 1995a, b). These raphe neurons modulate the output of the spinal locomotor circuitry *via* a range of serotonin-dependent actions (Sillar *et al.*, 1993) including changes in the strength of synaptic connections in the spinal cord (McDearmid *et al.*, 1997) and in the electrical properties of spinal neurons (Scrymgeour-Wedderburn *et al.*, 1997; Reith and Sillar, 1998).

In this chapter the possible contribution of descending projection neurons to the control of swimming is described and evidence that one such system involves the release of NO is presented. The distribution of nitric oxide synthesising cells is first examined in the CNS using NADPH-diaphorase histochemisty in cryostat-sectioned material and in wholemount preparations of the excised CNS. Next, the effects of NO are explored on the rhythmic motor pattern for swimming in paralysed tadpoles by manipulating the level of exogenous NO and the activity of endogenous NOS. These results are then discussed in the broader context of descending modulatory control systems in the vertebrate brainstem and it is argued that NO is used by brainstem neurons as a co-transmitter, which slows swimming through potentiation of inhibitory mechanisms.

2.3. MATERIALS AND METHODS

2.3.1. NADPH-DIAPHORASE HISTOCHEMISTRY

Xenopus laevis larvae (stage 42; pre-feeding) were obtained by induced breeding from an adult laboratory colony and staged according to Nieuwkoop and Faber (1956). For wholemount histochemistry, animals were deeply anaesthetised in tricaine

methanesulfonate (MS-222; 0.01-0.1%), and fixed at room temperature for 2-4 hours in ice-cold 4% paraformaldehyde. The animals were then rinsed in 30% sucrose in 0.1M phosphate buffer (PB; pH 7.4), and pinned through the notocord with finely etched tungsten pins to a Sylgard (Dow-Corning) platform within a Perspex dissecting bath filled with PB or phosphate buffered saline (PBS). Using fine needles, the skin was removed and the CNS was then carefully dissected out to the level of the 4th-6th myotome (as numbered from the otic capsule). Immediately after dissection, the CNS was transferred to 30% sucrose/PB and stored for up to 24 hours. The CNS was then pre-treated in 0.3% triton X100/PB to aid penetration of reagents. Subsequently the CNS's were transferred to the incubation medium for NADPH-diaphorase histochemistry [0.1 M PB containing: 1 mg ml^{-1} β-nicotinamide adenine di-nucleotide phosphate (reduced form; β-NADPH), 0.1 mg ml^{-1} nitroblue tetrazolium, and 0.3% triton X100] and kept at 37 °C in a humid chamber for 1-3 hours. All reagents were purchased from Sigma (UK).

The enzyme reaction was stopped by transferring the preparations to a fresh PB solution. The stained preparations were then dehydrated in a graded alcohol series (50, 70, 90, 95 and 100%), cleared in methylbenzoate and xylene, and mounted in DPX on cavity slides. The resulting preparations were studied and catalogued using a Zeiss Axiolab microscope equipped with a camera. Photographs were taken with an Olympus OM-4 Ti camera using Fuji 200 ASA film, and tracings of the resulting photographs were made on a Jessop Light Box. Drawings and cell counts were made with a *camera lucida* attachment. The results derive from 20 processed stage 42 central nervous systems.

For transverse sectioning, 12 animals were anaesthetised in MS-222, scored along the dorsal fin to aid fixative penetration and immersed in ice-cold 4% paraformaldehyde for 2-4 hours at room temperature. To remove aldehydes, tadpoles were transferred to 30%

sucrose/PB until they sank and then embedded in either rat brain or rat liver that had also

been fixed in 4% paraformaldehyde. Tissues were then frozen in a cryostat (Leica Jung

Frigocut 2800E; -18 °C) in a supporting cryomatrix and sectioned at 5-25 μm. The

cryostat-cut tissues were thaw-mounted directly on poly-L-lysine coated slides and

allowed to dry at room temperature for 1-2 hours prior to histochemical processing.

The protocol for the NADPH-diaphorase histochemical reaction was similar to that

described above for wholemounts. Briefly, the sections were incubated at 37 °C for 2-3

hours in the staining solution described above. The slides were rinsed in PB and distilled

water and then quickly dehydrated in a graded alcohol series. The slides were then cleared

in xylene and coverslipped in DPX. The resulting slides were studied and catalogued

using the equipment described above.

2.3.2. ELECTROPHYSIOLOGY

All experiments were performed on *Xenopus laevis* tadpoles at larval stage 42. Animals

were immobilised in approximately 10 μM α-bungarotoxin and secured in a chamber with

recirculating frog ringer (ionic composition in mM: 120 NaCl, 2.5 KCl, 2 CaCl$_2$, 1 MgCl$_2$,

2.5 NaHCO$_3$, 10 HEPES; pH 7.4). The experimental chamber was gravity fed from a 10

ml header tank *via* a stock bottle containing 100 ml of saline. L-NAME, D-NAME, L-

NNA, *n*-acetylpenicillamine (NAP; Sigma, UK) and *S*-nitroso-*n*-acetylpenicillamine

(SNAP; Chemistry Department, University of St Andrews) were first dissolved in distilled

water (L-NAME and D-NAME) or dimethyl sulphoxide (L-NNA, NAP and SNAP;

<0.01%) and then added to the stock bottle to achieve the desired final bath concentration.

SNAP was NO-depleted by keeping it at room temperature under light for 24 hours before

use. Animals were secured on their sides with fine etched tungsten pins (through the

notocord) to the Sylgard (Dow Corning) surface of a rotatable perspex platform housed

within the experimental chamber; one rostrally at the level of the otic capsule and another

caudally, about half way along the length of the body. The flank skin on the left side was

carefully removed to the level of the otic capsule using finely etched tungsten needles to

reveal the underlying myotomal swimming muscles (see schematic diagram in Fig. 2.3).

In experiments using NOS inhibitors, the first three myotomes on the left side were

removed to facilitate drug access (see schematic diagram in Fig. 2.4). Fictive motor

patterns appropriate to drive swimming behaviour were recorded in clefts between

myotomal swimming muscles using glass suction electodes (*ca.* 50-70 μm tip diameter;

c.f. Kahn and Roberts, 1982). In all experiments, episodes of fictive swimming were

evoked by a brief, 1 ms current pulse applied to the tail skin *via* a glass suction electrode.

Data were recorded, displayed conventionally and stored on videotape using a PCM

adaptor (Medical Systems Corporation). Hard copy records were made off-line using a

thermal plotter (Graphtec). Data were digitised with a CED 1401 and analysed off-line

using Spike 2 software and Dataview (courtesy of Dr William Heitler; available at

http://biology.st-and.ac.uk/sites/dataview). The 'Students' *t*-test was used to determine

statistical significance (P<0.05). The data presented here derive from experiments on 61

stage 42 larvae.

2.4. RESULTS

2.4.1. DISTRIBUTION OF NADPH STAINING IN *XENOPUS LAEVIS* LARVAE

In cross-sections of intact tadpoles, positive staining was located in structures external to

the CNS, the detailed description of which is not covered here (see CHAPTER 4). Briefly,

however, labelled areas included the walls of blood vessels, various cells in the retina and

the skin. These tissues are known to contain NOS in a variety of species (Salter *et al.*,

1991), therefore supporting the conclusion that the positive staining described here

corresponds with NO producing cells.

Within the CNS, intense labelling was found in three discrete bilaterally symmetrical

clusters of neurons located in the brainstem (Fig. 2.1A, B). Only a few cells were labelled

in the forebrain and no cell bodies were stained in the spinal cord (see CHAPTER 4). The

most numerous and most caudal population extended from the junction between the spinal

cord and hindbrain, rostrally for 200 to 300 μm and consisted of approximately 150

neurons (70-80 per side; Fig. 2.1Ci). The majority of neurons in this group had cell bodies

located ventrally, close to the neurocoel, extending ventrolaterally to mid-dorsal positions

(Fig. 2.1Bi). At more rostral locations this cluster split into two sub-groups with the

second, smaller population occupying a more dorsal position (Fig. 2.1Bi). All of the

neurons in the caudal hindbrain cluster had unipolar somata with mainly a single, but

sometimes a double, neurite that extended laterally into the marginal zone of the brainstem

where an extensive ramification of dendritic processes occurred (Fig. 2.1Bi). Many of the

neurons in this cluster had contralaterally projecting processes that could be detected as

they coursed beneath the neurocoel (Fig. 2.1Bi). Beyond the rostral extent of this

population there followed a region of the brainstem in which no staining of cell bodies

occurred. The next most rostral cluster occurred approximately 200 μm from the

hindbrain-midbrain boundary and extended caudally for approximately 100 μm. These

neurons, numbering approximately 80 (ca. 40 neurons per side; Fig. 2.1Cii), were

positioned ventromedially with neurites extending from the unipolar somata laterally into

the margins of the rostral brainstem (Fig. 2.1Bii). No evidence was found for crossing

projections in neurons belonging to this cluster. The most rostral brainstem cluster was

located very close to the midbrain-forebrain boundary and comprised approximately 40

somata (ca. 20 per side; Fig. 2.1Ciii). These tightly packed cells occupied a more dorsal

and lateral region of the brainstem (Fig. 2.1Biii). Their distinctive processes were

unilateral and consisted of very long primary neurites that projected ventrally, where they terminated in a plexus of dendritic processes in the ventrolateral marginal zone.

The topology of these three primary clusters of putative nitrergic brainstem neurons was clearest in wholemount preparations of the excised intact CNS (Figs. 2.1A, 2.2). From a lateral perspective the location of the three groups in the dorso-ventral plane is readily apparent (Fig. 2.1A). From the dorsal aspect it is clear that each cluster is bilaterally symmetrical (Figs. 2.1A, 2.2A), that the most caudal cluster represents the most numerous population and that the cells of this group give rise to processes that cross the ventral commissure beneath the neurocoel (Fig. 2.2B). The wholemount preparations also allow description of the axonal projections of these nitrergic neurons in the brainstem. The caudal group emanated axons that projected caudally in the marginal zone and reached only the rostral most segments of the spinal cord (Fig. 2.2C). Of course, these axons were only detected by the presence of NADPH-diaphorase reaction product and it is entirely possible that the axons of neurons in this cluster were both more numerous and more extensive in their innervation of the spinal cord (see DISCUSSION, section 2.5). Rostrally projecting nitrergic axons also emanated from this cluster and could clearly be seen (Fig. 2.2D) as they coursed within the marginal zones between the cell groups.

2.4.2. EFFECTS OF NITRIC OXIDE ON SWIMMING

The motor pattern during swimming provides four measurable features: 1) burst duration, which is the time that a recorded ventral root motor pool is active in each cycle; 2) cycle period, defined as the interval between consecutive bursts and measured from the onset of a burst in one cycle to the onset of the burst in the next cycle; 3) rostrocaudal delay, the delay between consecutive bursts as the motor activity progresses down the body; and 4) episode duration, which is the time the animal swims in response to a brief sensory

stimulus (see MATERIALS AND METHODS, section 2.3). SNAP, a stable analogue of endogenous S-nitroso compounds was used to increase levels of NO *in vivo* with a view to assessing its effects on these four parameters of swimming. The bath application of 100-500 μM SNAP produced a significant (*t*-test, P<0.05) and reversible decrease in episode duration in approximately 90% (13 of 14) of the experiments (Fig. 2.3A, B). There was also a profound lengthening of cycle period in approximately 80% (11 of 14) of experiments in the presence of SNAP (Fig. 2.3C, D). There was however no consistent effect observed on either burst duration or rostrocaudal delay. To investigate if the effect was specific to NO, experiments were conducted with NO-depleted SNAP, the inactive isomer of SNAP, NAP, and its vehicle dimethyl sulphoxide (DMSO). The bath application of either NO-depleted SNAP (50-100 μM), NAP dissolved in DMSO (100-500 μM), or DMSO alone (0.01-1%) produced no significant reduction in episode duration (*t*-test, P>0.05). The effect on cycle period in each case was highly variable. NO-depleted SNAP significantly decreased cycle period in 40% (2 of 5; *t*-test, P<0.05) of experiments, significantly increased cycle period in 25% (1 of 5; *t*-test, P<0.05) of experiments, and had no significant effect in 40% (2 of 5; *t*-test, P>0.05) of experiments. In approximately 27% (3 of 11; *t*-test, P<0.05) of experiments NAP significantly decreased cycle period, in approximately 36% (4 of 11; *t*-test, P<0.05) it significantly increased it, and in approximately 27% (3 of 11) there was no significant effect (*t*-test, P>0.05). DMSO decreased cycle period in 40% (4 of 10; *t*-test, P<0.05) of experiments, increased it in 30% (3 of 10; *t*-test, P<0.05), and had no significant effect in 30% (3 of 10; *t*-test, P>0.05).

The exogenous application of a NO donor cannot alone prove that endogenous NO plays a role in the tadpole nervous system. To demonstrate this, NOS inhibitors can be used to determine whether a reduction in endogenous NO produces any change in fictive

swimming activity. To this end, a series of experiments was conducted with both broad

spectrum (L-NAME) and more selective neuronal (L-NNA) NOS inhibitors. The bath

application of either L-NAME or L-NNA (0.1–5 mM) resulted in a significant (t-test,

$P<0.05$) increase in episode duration in approximately 65% (7 of 11) of L-NAME

experiments and approximately 55% (5 of 9) of L-NNA experiments (Fig. 2.4A, B). This

was also associated with a decrease in cycle period in approximately 70% (8 of 11) of L-

NAME experiments and approximately 75% (7 of 9) of L-NNA experiments (Fig. 2.4C,

D). The most consistent effects on episode duration occurred when control episodes were

relatively short. To control for the specificity of NOS inhibition, experiments using the

inactive form of L-NAME, D-NAME (5-10 mM) were conducted. There was no

significant increase in episode duration (t-test, $P>0.05$), and the effect on cycle period was

inconsistent, with 50% (3 of 6) increasing, approximately 16% (1 of 6) decreasing, and

approximately 33% (2 of 6) showing no significant effect (t-test, $P>0.05$). As one would

expect therefore, the inhibition of endogenous NOS produced precisely the opposite

effects to SNAP on episode durations and cycle periods.

2.5. DISCUSSION

The main conclusion of this series of anatomical and electrophysiological experiments is

that the free radical gas NO is generated by brainstem neurons and exerts a profound

influence upon the network of spinal neurons responsible for rhythmic swimming activity.

This is believed to be the first demonstration of a role for nitrergic transmission in the

function of a rhythmic locomotor network.

2.5.1. DISTRIBUTION OF NITRERGIC NEURONS IN *XENOPUS* LARVAE

The pattern of staining obtained using NADPH-diaphorase histochemistry, at least at this

early stage of development, is restricted to specific groups of neurons located in the

brainstem. There is strong precedence to suggest that this staining procedure labels

neurons that synthesise NO from one or more isoforms of NOS (see CHAPTER 1; Hope *et al.*, 1991). However, confirmation that the NADPH-diaphorase positive cells in *Xenopus* express a particular isoform of NOS will require subsequent immunocytochemical studies. Nevertheless, the present study has revealed labelling in precisely the same regions that NOS is normally found, for example in the walls of blood vessels (see CHAPTER 3). Moreover, a range of vertebrate neurons in similar locations in the CNS to those described here in the tadpole brainstem have been shown to be nitrergic (see CHAPTER 1; Schober *et al.*, 1994; Bruning and Mayer, 1996; Vincent and Kimura, 1992).

The structure and location of the neurons that stained in *Xenopus* larvae neurons indicate that they belong to particular subsets of brainstem neurons that may have been described previously and that may co-localise with other transmitters. For example, by far the largest population lay in the hindbrain and at least some of these neurons showed positive labelling in axons that extended to the rostral spinal cord. Presumably therefore these neurons are reticulospinal. A variety of neuron clusters in this region have been described using both standard anatomical tracing methods such as horseradish peroxidase labelling and immunocytochemistry using antibodies against different neurotransmitters (Roberts and Alford, 1986; Roberts *et al.*, 1987). These include the most rostral members of a population of descending excitatory interneurons that also extend along most of the length of the spinal cord. These interneurons have ipsilaterally projecting axons and are known to generate the excitatory drive for swimming by activating glutamate receptors on spinal motor and interneurons (Roberts and Alford, 1986). Commissural interneurons, which are glycinergic, provide the reciprocal mid-cycle inhibition during swimming (Dale *et al.*, 1986). Like the descending interneurons, the commissurals also extend rostrally from the spinal cord into the caudal hindbrain. However, neither of these two populations was

labelled in the spinal cord using the NADPH-diaphorase technique and it seems unlikely

that a sub-population would selectively express NOS only in the caudal hindbrain. Mid-

hindbrain reticulospinal (mhr) interneurons also occur in the region of NADPH-diaphorase

staining described here. They have been identified in *Xenopus* embryos using antibodies

directed against GABA (Roberts *et al.*, 1987) and they participate in a descending

GABAergic "stopping" response (Boothby and Roberts, 1992a, b). The mhr neurons are

activated following stimulation of the rostral cement gland and release GABA in the spinal

cord to terminate swimming when the embryo encounters an obstacle in its path. A

proportion of mhr neurons have decussating axons, like some of the NADPH-diaphorase

positive neurons described here (see Figs. 2.1Bi, 2.3B). Other hindbrain reticulospinal

neurons are likely to have excitatory functions in the spinal cord. However, NO often co-

localises with inhibitory transmitters (Vincent, 1995) and has been reported to evoke

GABA release (Ohkuma *et al.*, 1998a,b). While the transmitter phenotype of the reticular

NADPH-diaphorase neurons is yet to be determined, it seems plausible that they might

use GABA or perhaps glutamate as a fast descending transmitter, and NO as a co-

transmitter.

The next, most rostral, cluster of NADPH-diaphorase positive neurons is located ventrally,

close to the mid-brain hindbrain boundary and extends caudally for about 150 μm. This is

an area of the brainstem that substantially overlaps with a region that is known to be

occupied by serotonergic neurons of the raphe nucleus (van Mier *et al.*, 1986; Sillar *et al.*,

1995b). The serotonergic neurons and the NADPH-diaphorase positive neurons are

remarkably similar in overall morphology; the cell bodies are arranged as a column that

extends dorsally from the ventral commissure close to the boundary of the neurocoel (see

Fig. 2.1Bii). Primary neurites then project ventro-medially to the lateral margins of the

brainstem where they branch into a dense plexus of dendritic processes. Although

requiring confirmation, the present proposal, based on the location of this group and their

general anatomical features is that they correspond to serotonergic neurons of the raphe.

Since many raphe neurons have axonal projections that innervate the spinal cord, it is

conceivable that NO released from these neurons is somehow involved in the NO-induced

modulation of swimming described here. However the raphespinal projections in *Xenopus*

larvae are responsible for enhancing the intensity and duration of rhythmic motor bursts

during fictive swimming, the opposite effect to that produced by exogenous NO.

The most rostrally located cluster of NADPH-diaphorase positive neurons is more difficult

to correlate with previously described groups of brainstem neurons. They are positioned

more dorsally and laterally to the other groups, very close to the boundary between the

mid- and the hindbrain. They are fewer in number (approximately 20 cell bodies per side)

and have a very distinctive anatomy. These neurons are similar in morphology to both the

catecholaminergic neurons of the nucleus tractus solitarii identified in the larval stage of

the amphibian, *Pleurodeles waltlii* (González *et al.*, 1995) and the anuran homologue of

the locus coeruleus, the isthmic region, identified in adult anurans (Marín *et al.*, 1996).

Whether or not these diaphorase-positive neurons are also tyrosine hydroxylase

immunoreactive in *Xenopus*, has yet to be confirmed.

2.5.2. THE CONTRIBUTION OF NO TO THE CONTROL OF SWIMMING

The experiments described in this chapter provide the first evidence for intrinsic regulation

of vertebrate locomotor rhythm generation by NO. This evidence is based on the fact that

the NO donor SNAP and NOS inhibitors L-NAME and L-NNA strongly affect the

rhythmic motor output for swimming. While it is possible that the manipulations of NO

influence rhythmic output indirectly, for example *via* effects on the cardiovascular system,

this is unlikely as *Xenopus* larvae posses only a rudimentary vascular system at this early

stage of development. While blood vessels did display NADPH-diaphorase staining, there

were none detected within or close enough to the CNS to act as the source of NO. These

data instead suggest that endogenous release of NO from one or more subsets of the

NADPH-diaphorase positive brainstem neurons exerts modulatory control over spinal

locomotor circuitry. It has been argued above that the most likely group is the most

numerous and most caudally located cluster of reticular neurons. In principle at least, NO

released from brainstem neurons could affect spinal rhythm generation by acting directly

on spinal neurons (or their synaptic interconnections), or by altering the descending drive

to the spinal swimming circuitry. At present it is not possible to distinguish between these

two possibilities but both seem likely. Since the main target for NO is sGC, presumably

the NO-induced modulation of swimming is accomplished by cGMP-dependent

facilitation of synaptic transmission, in keeping with the results of previous studies on the

central nervous effects of NO (see CHAPTER 1; also reviewed in Schuman and Madison,

1994). Increased levels of extracellular NO following application of SNAP led to a

reduction in the duration of swim episodes and a diminution in the intensity and frequency

of swimming, consistent with either an increase in inhibitory synaptic processes or a

decrease in the excitatory drive for swimming. Of these two, non-mutually exclusive

possibilities, it is more likely that NO slows swimming by increasing inhibitory synaptic

transmission. This is because inhibitory synapses in the swimming system appear to be

much more variable and modulable than excitatory ones. For example, the amine NA has

a very similar effect to NO upon swimming, producing slow, weak activity. This is

accomplished by the presynaptic facilitation of glycinergic transmission (McDearmid *et*

al., 1997), although NA can also facilitate GABAergic transmission (McDearmid, 1998;

Merrywest and Sillar, 2000). GABA synapses in *Xenopus* tadpoles are also pre-

synaptically facilitated by a neuroactive steroid ($5\beta3\alpha$; Reith and Sillar, 1997) which again

weakens and slows down the swimming rhythm. In the next chapter the hypothesis that

NO affects swimming by facilitating descending GABAergic and mid-cycle glycinergic

inhibition at sites in the brainstem is tested.
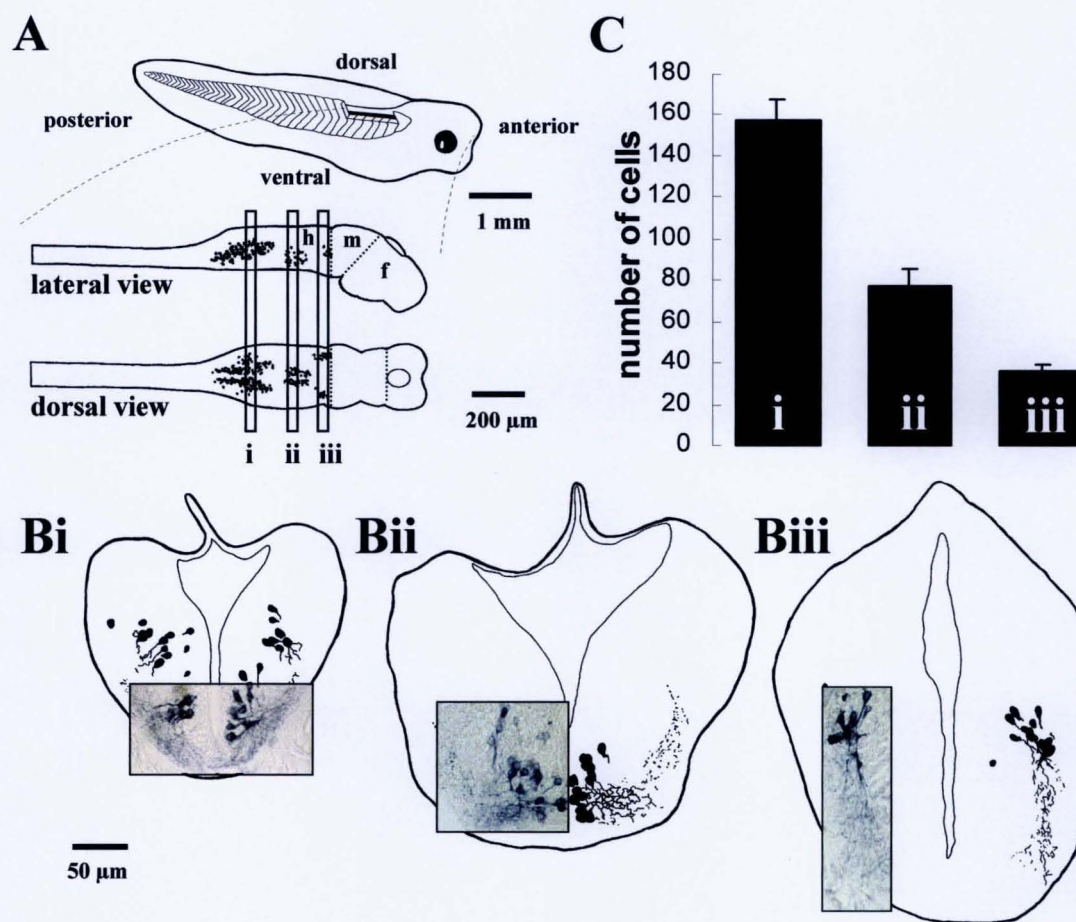
FIGURE 2.1. NADPH-diaphorase staining of neurons in the CNS. (A) Schematic diagram of stage 42 tadpole (top), with *camera lucida* drawings of the CNS (bottom). This illustrates the approximate location and orientation of the sections and lateral and dorsal perspectives of the three populations with respect to the hindbrain (h), midbrain (m) and forebrain (f). (B) Tracings from photographs of cryostat-sections with inset colour photographs of (Bi) the most caudal cluster, (Bii) the rostral hindbrain cluster and (Biii) the hindbrain/midbrain boundary cluster. (C) Cell counts of the total number of cells in each group. Error bars are standard error of the mean, N=8.
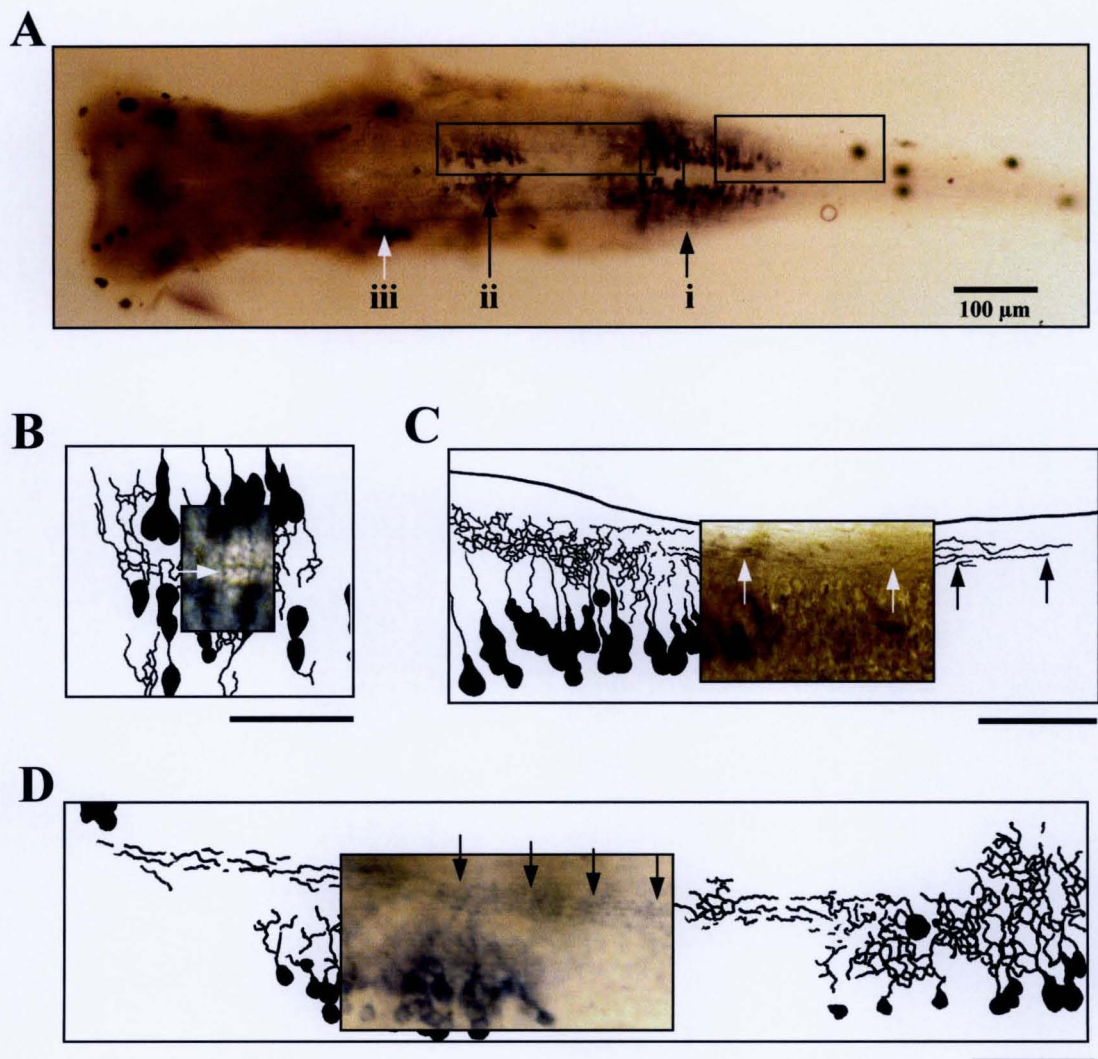
FIGURE 2.2. Dorsal perspective of NADPH-diaphorase stained neurons in the wholemount CNS. (A) Colour photograph of the CNS showing the relative positions of each bilaterally symmetrical population (i-iii; *cf.* Fig. 2.1C) and the approximate locations of the demarcated areas in B-D. Tracings with inset colour photographs from three different wholemount preparations illustrate (B) projections that cross below the neurocoel (white arrow), (C) faint tracts of projections that descend to the rostral-most segments of the spinal cord (white and black arrows) and (D) tracts projecting from the caudal-most cell group (black arrows). Scale bars for B-D, 50 μm.
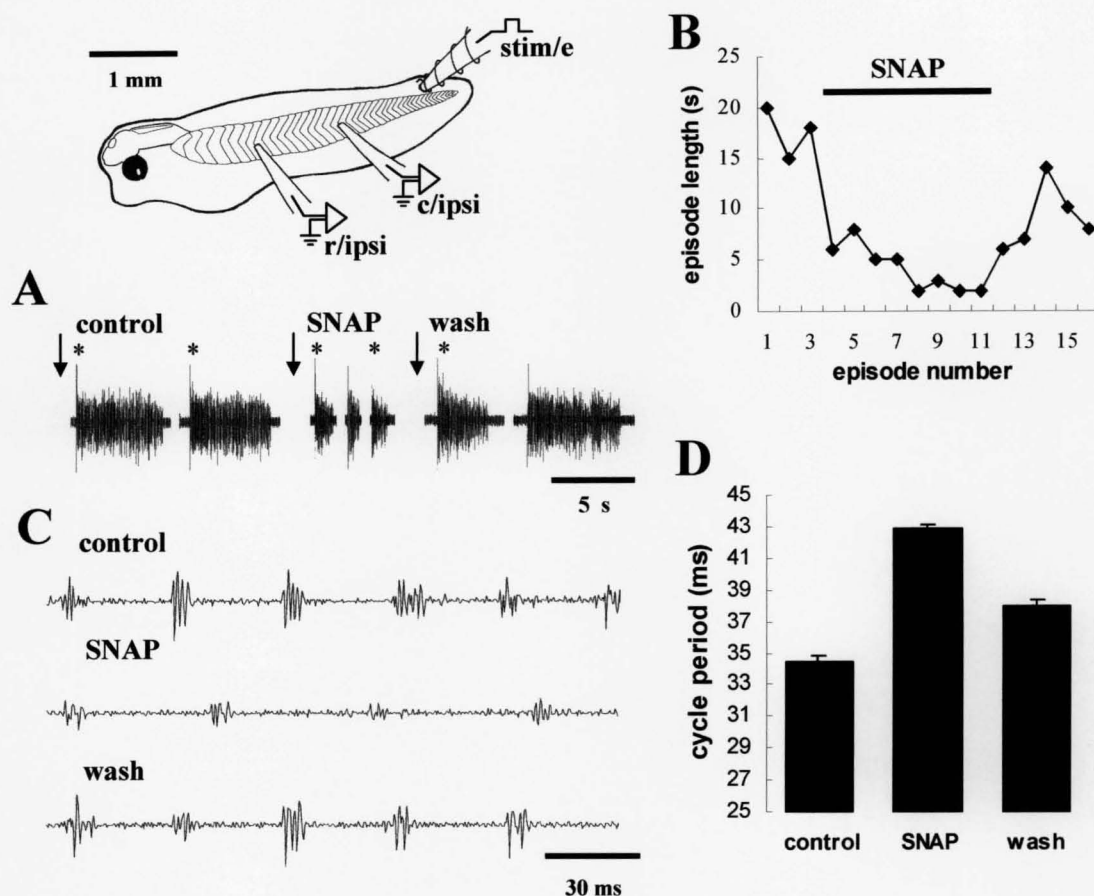
FIGURE 2.3. Effects of bath-applied SNAP on fictive swimming activity of *Xenopus laevis* larvae. Schematic diagram of a stage 42 experimental preparation illustrating the central nervous system in relation to the muscle blocks (see MATERIALS AND METHODS). c/ipsi, caudal recording electrode; r/ipsi, rostral recording electrode; stim/e, electrode used for stimulation. (A) Extracellular recordings of fictive swimming activity measured from the sixth post-otic intermyotomal cleft are shown on a slow time scale. Gaps between episodes of swimming represent a 30 s interval. The durations of episodes are shortened after 15 minutes of 300 μM bath applied SNAP and fully recover after a 30 minute return to control saline (wash). Asterisks indicate stimulation artefact. (B) Line graph illustrating the variation in episode duration over the course of an experiment. The bath application of 300 μM SNAP (black bar) shortens episode lengths reversibly. (C) The inhibitory effect of SNAP on cycle period can also clearly be seen on a faster time scale. (D) Histogram of the mean effect of SNAP on cycle period. Mean values of fictive swimming used for analysis comprise 20 cycles taken from near the onset of three different episodes under each experimental condition. Error bars are standard error of the mean.
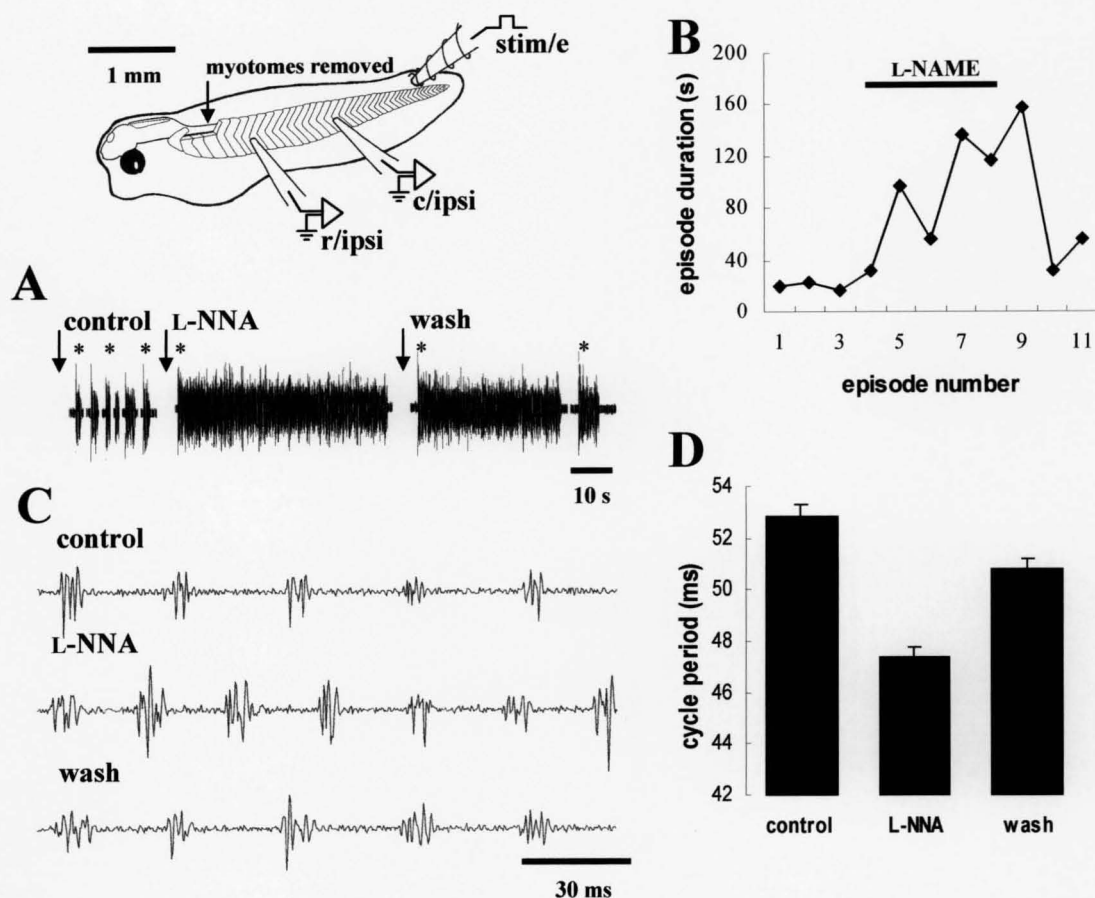
FIGURE 2.4. Effects of bath-applied L-NNA on fictive swimming activity.
Schematic diagram of stage 42 experimental preparation as described previously (see
Fig. 2.3) except with first three myotomes removed to facilitate drug access to
brainstem. (A) The bath application of 100 μM L-NNA substantially increases
episode duration after 45 minutes. This effect did not fully reverse after a 20 minute
return to control saline (wash). Asterisks indicate stimulation artefact. (B) Line graph
illustrating the variation in episode duration over the course of an experiment. The
bath application of 5mM L-NAME (black bar) increases episode lengths reversibly.
(C) Extracellular recordings of swimming activity recorded from the fifth post-otic
intermyotomal cleft show that L-NNA speeds and intensifies the swimming rhythm, in
contrast to the effects of SNAP (cf. Fig. 2.3C). (d) Histogram of the mean effects of L-
NNA on cycle periods. Error bars are standard error of the mean.

EVIDENCE FOR NITRERGIC FACILITATION OF SYNAPTIC

INHIBITION IN THE SPINAL LOCOMOTOR NETWORKS OF

DEVELOPING *XENOPUS LAEVIS* TADPOLES

3

3.1. SUMMARY

A potential link between NO and inhibitory neurotransmission mediated by GABA and glycine is investigated by recording extracellularly from ventral roots and intracellularly from presumed motor neurons in the ventral spinal cord of *Xenopus laevis* embryos (stage 37/38; Nieuwkoop and Faber, 1956) and larvae (stage 42) during episodes of stimulus-induced 'fictive' swimming. Evidence presented here based on extracellular recordings suggests that there is a stage-dependent increase in the reliability of SNAP (0.1-1mM) to modulate both swimming frequency and duration. This is corroborated by evidence from intracellular recordings, which also suggests that NO facilitates both glycinergic and GABAergic inhibition. In addition to effects on inhibitory transmitter release, a pronounced membrane depolarisation (*ca.* 5-10 mV) and conductance decrease (*ca.* 20%) are associated with the bath application of SNAP. The bath application of the inactive precursor, NAP (0.1-1 mM) and the vehicle DMSO (0.1-1.5%) had no effect on membrane potential, conductance or inhibitory synaptic transmission, suggesting that the affects on synaptic transmission and these membrane properties are in fact due to the actions of NO released from SNAP. At early developmental stages NOS does not appear to be present in the spinal cord (see CHAPTER 2) and therefore it is likely that the facilitation of inhibition by NO originates from the brainstem. If this is the case, then the witnessed effects of NO could be explained by the involvement of the aminergic modulators 5-HT and NA.

3.2. INTRODUCTION

The ability to shape locomotor movements to suit specific behavioural requirements is

important since it provides animals with the means to interact adaptively with their

environment. A simple example is the fast-start escape response, relayed through

Mauthner neurons located in the hindbrain, which enable fish (reviewed in Eaton *et al.*,

2001) and some species of amphibian tadpoles (Fox, 1981; Stehouwer, 1992) to react

quickly to potentially threatening environmental stimuli. However, in some cases a reflex

can manifest itself not as a reaction, but as the termination of one. One such example is

the stopping response of hatchling *Xenopus laevis* tadpoles. Embryos (stage 37/38;

Nieuwkoop and Faber, 1956) of this species will stop swimming when the rostral cement

gland contacts an obstacle. This allows the tadpoles to hang motionless, presumably to be less visible to predators. However, once the tadpoles become free swimming and filter feeding, simple foraging behaviour could elicit an inappropriate stopping response, unless this pathway disappeared. The synaptic pathways involved in this response are well described (Boothby and Roberts, 1992a, b) and involve fast descending inhibitory GABA release onto spinal motor networks from mhr neurons, which are in turn activated by cement gland afferent neurons. However, the reliability of this sensory afferent mediated response dramatically decreases during development (Boothby and Roberts, 1992b), coincident with cement gland degeneration, and by stage 42 the reflex response has all but disappeared.

It has recently been shown that the GABAergic IPSPs, reminiscent of those induced only by cement gland stimulation at stage 37/38, occur spontaneously at the end of swim episodes in larval tadpoles (stage 42; Reith and Sillar, 1999). Given the similarity of the two events, it has been proposed that the GABAergic termination of larval swimming also involves the mhr neurons, which are somehow incorporated into an endogenous stopping response as the animal matures (Reith and Sillar, 1999; McLean et al., 2000). Furthermore, the networks controlling swimming in Xenopus larvae are known to be controlled by another group of supraspinal systems, namely the aminergic brainstem nuclei that utilise 5-HT and NA (see CHAPTER 1). Together, these descending control systems could provide the means for the larval tadpole to not simply react to, but to interact with, its surroundings as it matures. Yet the developmental mechanisms controlling the precise timing and incorporation of these pathways into a mature larval swimming behaviour are not yet fully understood.

NO is a versatile signalling molecule that has been implicated in a number of developmental processes including synaptic plasticity (Wang *et al.*, 1995), growth cone collapse (Hess *et al.*, 1993), axon guidance (Van Wagenen and Rehder, 1999) and apoptosis (Estevez *et al.*, 1998). NO also acts as an important signal in the activity-dependent refinement of axonal projections and the stabilisation and strengthening of developing synapses (Gally *et al.*, 1990). NO has recently been shown to occur in neurons of the putative locomotive centres in the brainstem that affect locomotion early in *Xenopus* development (CHAPTER 2 and 3; McLean and Sillar, 2000). NO plays an inhibitory role in the modulation of swimming circuitry; shortening swim episode duration and slowing swim frequency (CHAPTER 2; McLean and Sillar, 2000). However, the way in which NO's inhibitory actions are accomplished have yet to be described. The two known fast inhibitory neurotransmitters that influence spinal swimming circuitry in *Xenopus* tadpoles originate from the GABAergic mhr neurons and glycinergic commissural interneurons, the latter being a common target for 5-HT and NA mediated neuromodulation. The shortening of swim episodes and slowing of swim frequency are consistent with a facilitation of GABAergic (Reith and Sillar, 1999) and glycinergic (McDearmid *et al.*, 1997) inhibition, respectively. However, direct physiological evidence linking NO with either of these fast acting inhibitory neurotransmitters in *Xenopus* tadpoles has yet to be provided.

The evidence provided here suggests that: 1) NO can shorten swim episode durations and slow swim frequency in embryos as well as in larvae, albeit not as reliably; 2) NO appears to have an effect on motor neuron membrane conductance; 3) NO facilitates both GABAergic and glycinergic transmission, *via* a presumed presynaptic influence on GABAergic and glycinergic output synapses; and 4) these effects on synaptic inhibition

appear to increase in reliability with development, which suggest that NO is working

through pathways that might also be developmentally regulated.

3.3. MATERIALS AND METHODS

3.3.1. EXPERIMENTAL PREPARATIONS

All experiments were performed on late embryonic (stage 37/38) and early larval (stage

42) *Xenopus laevis* tadpoles (see Fig. 3.1Ai, ii) obtained by induced breeding from an

adult laboratory colony and staged according to Nieuwkoop and Faber (1956). Animals

were first anaesthetised in 0.1-1% MS-222, immobilised in 12.5μM α-bungarotoxin

(Sigma, UK) and then secured in a chamber with recirculating frog Ringer solution (ionic

composition as described previously in MATERIALS AND METHODS section 2.3.2, but with 4

mM $CaCl_2$ for intracellular experiments). Fictive motor patterns appropriate to drive

swimming behaviour were also recorded as described previously. After removal of

overlying myotomes, intracellular recordings were made from neurons positioned in the

ventral quarter of the spinal cord (see Fig. 3.1B), where motor neurons predominate

(Roberts and Clarke, 1982), with microelectrodes pulled from 1 mm borosilicate glass

capillary tubing (CEI Harvard Apparatus) having DC resistances of approximately 100-

150MΩ when filled with 3M potassium chloride (KCl). KCl-filled electrodes were

selected to make chloride-dependent IPSPs strongly depolarising (see Fig. 3.1C) and

hence easier to measure. In addition IPSPs mediated by $GABA_A$ and glycine receptors

can be readily distinguished on the basis of their durations, as well as their

pharmacological sensitivities (Reith and Sillar, 1997). Also, during quiescent periods,

spontaneously occurring IPSPs can be distinguished according to their duration and their

pharmacological sensitivity (Reith and Sillar, 1997).

3.3.2. DRUGS

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 NAP (Sigma, UK) were made fresh daily and dissolved in 0.01% DMSO

(McLean and Sillar, 2000). Strychnine (Sigma, UK) and bicuculline (Sigma, UK) were

dissolved in distilled water and subsequently frozen at $-20°C$ in stock concentrations until

required.

3.3.3. DATA ANALYSIS

Data were recorded and displayed conventionally and stored on videotape using a PCM

adapter (Medical Systems Corporation). Hard copy records were made off-line using a

thermal chart recorder (Graphtec) or digital plotter (Gould). Data analysis was performed

off-line using the Spike 2 analysis software package (CED, Cambridge, UK). For each

experiment, three consecutive episodes of swimming activity were measured in control,

drug and wash conditions. For measurements within swimming episodes, the first 500 ms

of activity in each episode was ignored, to avoid possible influences arising directly from

sensory stimulation. Specific details regarding measurement parameters are given in the

appropriate figure legends. Data were pooled either as percentage changes from control,

in the case of mid-cycle IPSP amplitude and episode duration, or simply as raw data

points, in the case of spontaneous and terminating IPSPs, and cycle period. In both cases,

the 'Students' t-test was used to determine statistical significance between conditions

($P<0.05$) and unless stated otherwise, data are given as means ± S.E.M. Data expressed as

percentages were first arcsine transformed before statistical analysis. The data presented

here derive from experiments on 22 stage 37/38 embryos and 7 stage 42 larvae. In one

embryonic experiment a double application of SNAP was used to determine the

repeatability of the response (*i.e.* N=23 for stage 37/38).

3.4. RESULTS

The synaptic drive for swimming in hatchling *Xenopus laevis* tadpoles consists of a gradually declining depolarisation, upon which on-cycle fast excitation, triggering a single spike per cycle, and mid-cycle (glycinergic) inhibition are superimposed (Fig. 3.1C). This simple motor pattern matures in a rostrocaudal gradient coincident with the invasion of the spinal networks by descending serotonergic axons (van Mier *et al.*, 1986; Sillar *et al.*, 1995). The result is that only 24 hours later (stage 42; Nieuwkoop and Faber, 1956), motor neurons fire several action potentials per cycle in a much more variable motor pattern (Sillar *et al.*, 1992). The effects of NO on larval motor patterns are well documented (CHAPTER 2; McLean and Sillar, 2000). However due to the developmental differences between late embryonic and early larval swimming activity, I first investigated whether NO had the same effects at the earlier hatching stage. Bath application of the NO donor SNAP (0.1-1 mM) significantly (*t*-test; P<0.01) and reversibly reduced episode durations (Fig. 3.2Ai-iii) in 67% (8/12) of embryonic preparations (Fig. 3.2B). In the remaining preparations (4/12), there was no significant difference (*t*-test; P>0.05). NO could therefore shorten swim episode durations in embryos as it does in larvae, albeit not as reliably (*ca.* 90% of larval preparations; CHAPTER 2; McLean and Sillar, 2000). Similarly, NO could slow the frequency of swimming in embryonic preparations, as in 50% (6/12) of experiments (Fig. 3.2D), the bath application of SNAP was also associated with a significant (*t*-test; P<0.01) and reversible increase in cycle periods (Fig. 3.2Ci-iii). In the remaining 50% (6/12) of experiments there was no significant change in cycle period (*t*-test; P>0.05). In general, cycle period lengthening only occurred in concert with episode shortening, and not independently. However, and in contrast, 17% of embryonic experiments exhibited an episode decrease with no associated cycle period increase. This was also similar to, but not as reliable as, the effect witnessed in larvae (*ca.* 80%;

CHAPTER 2; McLean and Sillar, 2000). The increase in cycle periods indicates that SNAP

is not simply terminating swimming prematurely. If this were the case then measurements

at comparable periods of swimming activity would not differ significantly. Instead, cycle

periods are longer on a cycle-by-cycle basis in the presence of SNAP as compared to

controls (Fig. 3.2D). This evidence, combined with the increased reliability with which

SNAP affects episode durations (*ca.* decrease in 67% of embryos, 90% of larvae)

compared to cycle periods (*ca.* increase in 50% of embryos, 80% of larvae), suggests that

episode duration and cycle period might be mediated by separate nitrergic mechanisms.

In subsequent intracellular experiments, with both embryos (N=11) and larvae (N=7) the

effects of SNAP on membrane conductance and the synaptic drive for swimming were

examined in presumed motor neurons. In both embryonic and larval animals, a

pronounced 5-10 mV (average; 7.2 ± 1.4mV; N=8) membrane potential depolarisation

was observed shortly after the bath application of SNAP (Fig. 3.3A, B). To determine if

this effect was attributable to changes in the ionic conductance of the motor neuron,

hyperpolarising conductance pulses were applied during SNAP application in both

embryos (N=5) and larvae (N=5). In 80% of both embryonic (4/5) and larval (4/5)

experiments, there was an approximately 20% conductance decrease as compared to

control (Fig. 3.3C). In 20% of both embryonic (1/5) and larval (1/5) experiments, there

was no significant difference (*t*-test; P>0.05). The conductance decrease was still present

when the membrane potential was brought back to control levels (Fig. 3.3Aii, Bi) and can

therefore not be explained by the shift in membrane potential alone. In fact, one might

predict a depolarising shift in membrane potential would increase membrane conductance,

if large enough to open of voltage dependent channels (Scrymgeour-Wedderburn *et al.*,

1997). These data suggest that the membrane depolarisation is due to the closure of a

conductance to positively charged ions, of which K^+ is the most likely candidate (see

DISCUSSION, section 3.5).

Embryonic swim episodes normally terminate spontaneously, however in 55% (6/11) of

experiments episode durations were significantly (t-test; P<0.05) reduced by SNAP,

coincident with a significant increase (t-test; P<0.01) in GABAergic IPSPs at the end of

episodes (Fig. 3.4B). GABAergic IPSPs are easily identifiable on the basis of their

duration, as compared with glycinergic IPSPs (Fig. 3.4Aiii). This effect was even more

pronounced in larval animals, where 71% (5/7) of preparations showed a significant (t-

test; P<0.01) and reversible (Fig. 3.4B) increase in GABAergic IPSPs terminating

swimming (Fig. 3.4Ai, ii). These IPSPs are also known to be GABAergic as they

disappear in the presence of the $GABA_A$ receptor antagonist, bicuculline (Reith and Sillar,

1999), yet persist in the presence of the glycine antagonist, strychnine (Fig. 3.7D). For

example, in embryonic and larval experiments where SNAP potentiated terminating

GABAergic IPSPs, subsequent application of strychnine (1-2μM; N=3) could not block

them (Fig. 3.4Ci-iii).

SNAP also affected the amplitude of glycinergic mid-cycle IPSPs, although the effects in

embryonic preparations were not as consistent as in larval preparations. In 40% (4/10) of

embryos I detected no significant change (t-test; P>0.05), in 10% (1/10) there was a

significant decrease (t-test; P<0.01) and in 50% (5/10) there was a significant increase (t-

test; P<0.01) in mid-cycle IPSP amplitude (average; 37 ± 3.2%; N=150). In larvae

however there was a significant (t-test; P<0.01) and reversible increase (Fig. 3.5B) of mid-

cycle amplitude in 85% (6/7) of experiments (Fig. 3.5Ai, ii, iii). Mid cycle IPSPs are

blocked by strychnine, but not by bicuculline (Soffe, 1989) so they are likely to be

mediated by the inhibitory amino acid transmitter glycine, released from the synaptic

terminals of commissural interneurons (McDearmid *et al.*, 1997). Accordingly, SNAP

was unable to increase mid-cycle amplitudes in the presence of strychnine, in larval

preparations (Fig. 3.5C). Please note however, that due to the IPSP amplitude

measurement protocol (see Fig. 3.5 legend), complete elimination of the mid-cycle IPSP

still leaves a measurable tonic depolarisation, which is reflected in the histograms.

These effects of NO reflected in the inhibitory synaptic inputs to swimming were also

evident during quiescent periods in both embryos and larvae, when spontaneous

depolarising IPSPs are prevalent. For example, in the presence of TTX, strychnine and

bicuculline there is no spontaneous depolarising activity (Reith and Sillar, 1997). Within

the first 5 minutes of bath application, SNAP caused a profound and reversible (Fig. 3.6F)

increase in spontaneously occurring depolarising IPSPs in 82% (9/11) of embryonic (Fig.

3.6A, B, C) and 86% (6/7) of larval experiments. GABA (Fig. 3.6E) and glycine (Fig.

3.6D) IPSPs are known to occur during this time period and are easily distinguishable

based on their duration (Reith and Sillar, 1997). However, the nature of these IPSPs in

embryos and larvae was further investigated pharmacologically using strychnine (1-2µM;

N=6) and bicuculline (20-40µM; N=3). In control conditions both glycinergic and

GABAergic IPSPs were present (Fig. 3.7Ai). However, the bath application of 2µM

strychnine selectively eliminated glycinergic IPSPs (Fig. 3.7Aii) and the subsequent bath

application of 500µM SNAP induced a flurry of, presumably, GABAergic IPSPs (Fig.

3.7Aiii). Following the subsequent bath application of 40µM bicuculline in the presence

of strychnine, SNAP was unable to elicit any spontaneous activity (Fig. 3.7Aiv, B). In

embryonic and larval preparations where SNAP potentiated both GABAergic and

glycinergic inhibition, SNAP could also increase IPSP occurrence in the presence of either

bicuculline (Fig. 3.7Ci-iii, Di) or strychnine (Fig. 3.7Civ-vi, Dii).

The increase in IPSP occurrence suggests that NO is facilitating presynaptic release of GABA and glycine, rather than affecting the postsynaptic response. However, these series of experiments were purposely not carried out in the presence of TTX so the effects of NO on the synaptic drive for swimming could be examined. Therefore, it cannot be determined if effects on quiescent activity are pre- or postsynaptic or, more importantly, if they are the result of a manipulation of release machinery or the sodium-dependent activation of synaptic pathways. Yet, it is clear that NO is at least increasing the evoked release of inhibitory neurotransmitters, as witnessed by the effects on the synaptic drive of both embryos and larvae and that these effects appear to increase in reliability with development.

Finally, in order to control for any non-specific vehicle-mediated effects (c.f. Hedrick and Morales, 1999), experiments with DMSO (0.1-1%) and the inactive isomer of SNAP, NAP (0.1-1.25 mM) were carried out. The bath application of NAP (N=3) had no significant effects (t-test; P>0.05) on membrane potential (Fig. 3.8Ai, ii), membrane conductance (Fig. 3.8B) or spontaneous IPSP frequency (Fig. 3.8Ai, ii). Mid-cycle IPSP amplitudes (Fig. 3.8C) and GABA IPSPs occurring at the end of episodes (Fig. 3.8D) were similarly not significantly affected (t-test; P>0.05). Additionally, DMSO (N=4) had no pronounced effects on the synaptic drive for swimming as recorded from motor neurons (not illustrated), consistent with the lack of vehicle effects on the parameters of swimming in larvae (CHAPTER 2; McLean and Sillar, 2000).

3.5. DISCUSSION

These initial experiments using intracellular recordings suggest that NO has numerous effects on the spinal circuitry for swimming, including facilitation of transmission at both glycinergic and GABAergic synapses onto spinal motor neurons. The increases in

spontaneous glycinergic and GABAergic release are also reflected in facilitation of the

inhibitory synaptic drive during swimming in both embryos and larvae. Specifically, bath

applied SNAP can reversibly increase the amplitude of glycinergic mid-cycle IPSPs and

the incidence of GABAergic IPSPs at the end of episodes. The reliability of these effects

increases from stage 37/38 to stage 42. In combination, they result in a slowing and

shortening of swimming episodes, confirming interpretations of previous extracellular

findings (CHAPTER 2; McLean and Sillar, 2000). However at present it cannot be

determined at what level NO is acting, whether directly on GABAergic and glycinergic

synapses, or possibly upstream *via* modulation of the activity of aminergic neurons, which

then affect inhibitory synapses. The presence of NOS in brainstem populations and not in

the spinal cord at these stages suggests the latter is more plausible.

3.5.1. EFFECTS OF NO ON MEMBRANE POTENTIAL

The bath application of SNAP led to a pronounced and reliable membrane potential

depolarisation in all neurons tested. An unusual but frequently observed aspect of synaptic

inhibition early in development is that it appears to be depolarising (O'Donovan, 1989).

Although GABA is the main inhibitory neurotransmitter in the adult brain, during

development it excites and depolarises neurons through $GABA_A$ receptors (Wu *et al.*,

1992), due to the high chloride ion content of embryonic cells (Serafini *et al.*, 1995;

Rohrbough and Spitzer, 1996). The use of KCl-filled electrodes mimicked this situation

by leaking a high concentration of chloride ions intracellularly. Therefore SNAP-induced

GABA and glycinergic release would theoretically depolarise the motor neuron *via* an

efflux of chloride ions. However, the decrease in membrane conductance associated with

bath application of SNAP cannot be reconciled with the opening of ion channels.

Membrane depolarisation and conductance decrease is consistent with the closure of a

positively charged outward current, which implicates a K^+ conductance. There are several

outward K^+ currents, including fast and slow K^+ currents (I_{Kf} and I_{Ks}, respectively), a Na^+-

dependent K^+ current (I_{KNa}), and a slowly activating Ca^{2+}-dependent K^+ current (I_{KCa})

(Dale and Kuenzi, 1997). However, the relative contributions of these currents change

with development (Sun and Dale, 1998) and the membrane depolarisation and

conductance decrease described here are persistent at both developmental stages.

Furthermore, interference with these currents contributes to multiple spiking and spike

broadening, which leads to excitatory effects on swimming rhythm (Dale and Kuenzi,

1997). There is however a fourth, SK current which activates more slowly than the I_{KCa}, is

not subject to developmental regulation and is thought to contribute to the termination of

swimming episodes (Wall and Dale, 1995). NO interaction with K^+ channels is not

unprecedented (Ahern et al., 1999), however the exact nature and function of NO's

interaction with K^+ conductances will hopefully yield to further investigation.

3.5.2. THE FACILITATION OF GABA AND GLYCINE RELEASE

The activity-dependence of synaptic connectivity makes it tempting to suggest that

spontaneous motor activity might play a role in the development of locomotor behaviour

(Goodman and Shatz, 1993). An increase in inhibitory transmission during development

is associated with the expression of spontaneous activity and, in particular, GABA plays

an important role (Sernagor et al., 1995; O'Donovan et al., 1998; Fellippa-Marques et al.,

2000). The bath application of SNAP increased the frequency of both GABA and glycine

potentials occurring during inter-episode quiescent periods and this appeared to occur in a

stage dependent manner. However, it seems unlikely that inhibition is playing a similar

role in Xenopus embryos and larvae. Firstly, in larval Xenopus the swimming circuitry

normally develops in the absence of network activity (Haverkamp and Oppenheim, 1986).

In addition, SNAP induced effects were predominantly inhibitory, not excitatory.

Inhibitory events were identifiable based not only on their durations but also on their

pharmacological sensitivity (Reith and Sillar, 1997; see also Fig. 3.7). Also, there was no

obvious increase in spontaneous fictive swimming activity in the presence of SNAP.

Moreover, swim episode durations appeared to shorten and swim frequency decline

coincident with increased GABAergic and glycinergic activity respectively. This is

consistent with previously described inhibitory roles for GABA (Boothby and Roberts,

1992a,b) and glycine (McDearmid et al., 1997) in the swimming system of Xenopus

tadpoles and confirms that GABA is instead playing an inhibitory role at these stages of

development.

Recent evidence suggests that spontaneous and evoked transmitter release can be

regulated in parallel and that the frequency of miniature synaptic potentials can be used as

an indicator of the efficacy of evoked release (Prange and Murphy, 1999). This is

consistent with the SNAP-induced effects on both quiescent release and the synaptic drive

for swimming. Experiments in the presence of TTX are now needed to clarify at what

level NO facilitates inhibition.

3.5.3. THE NITRERGIC FACILITATION OF INHIBITION IS STAGE-DEPENDENT

It is clear from extracellular evidence that NO functions very much like a brake, slowing

down and then stopping swimming (McLean and Sillar, 2000). Therefore, it is reasonable

to assume that NO is having a net inhibitory effect on the spinal swim circuitry. This

effect, as illustrated in this chapter, appears to follow a developmental gradient, in that the

reliability with which NO modulates swimming behaviour increases as the animal

matures. This suggests that NO is not simply impinging upon spinal swim circuitry, which

is already in place at the time of hatching (Khan and Roberts, 1982), but rather is

performing its inhibitory role via pathways that are also developmentally regulated.

Interestingly, the relative contributions of GABAergic and glycinergic inhibition are not

fixed throughout development in the vertebrate CNS. In rat spinal motor neurons for

example, GABA mediated signalling is more potent embryonically and glycine-mediated

signalling is more dominant postnatally (reviewed in Singer and Berger, 2000), possibly

resulting from a transmitter phenotype switch during development (Berki *et al.*, 1995). A

shift from GABA to glycine transmission has also been described in the gerbil lateral

superior olive (Kotak *et al.*, 1998). There is a similar precocious potentiation of

GABAergic versus glycinergic transmission by SNAP. This is reflected in both its ability

to selectively increase the probability of GABA release at stage 37/38 (see Fig. 3.6) and

the higher probability of episode shortening (*ca.* 67%) versus cycle period lengthening (*ca.*

50%). The disparity of these effects also suggests that more than one modulatory

mechanism may be at work. Indeed, the modulation of glycinergic transmission does not

occur reliably until stage 42, when mid-cycle inhibition and quiescent release are both

potentiated by the bath application of SNAP. However, the progressive potentiation of

glycinergic release by SNAP makes it unlikely that a transmitter phenotype switch is

occurring.

There is now considerable evidence to suggest that the normal maturation and

development of locomotory circuitry depends on functionally intact descending inputs

(reviewed in Vinay *et al.*, 2000) at least some of which are nitrergic. It is therefore more

likely that higher centres, whose descending axons invade the spinal cord in a

developmentally regulated manner, are involved in the nitrergic inhibition described here.

In support, the number of nitrergic neurons appears to increase in strict developmental

sequence in the brainstem (see CHAPTER 4), in discrete populations that are not only inter-

connected, but could also have descending axons (CHAPTER 2; McLean and Sillar, 2000).

Therefore it would not be unreasonable to assume that NO is working in concert with

other, more conventional neurotransmitters.

The descending serotonergic innervation is not only causally linked to the maturation of the

swimming locomotor pattern in *Xenopus* (Sillar *et al.*, 1995b), but also appears to be

instrumental in the continuous modulation of swimming (Sillar *et al.*, 1992b). The

serotonergic effects work synergistically with another biogenic amine, NA on a common

cellular target, namely glycinergic commissural interneurons, which control the strength of

mid-cycle inhibition (see CHAPTER 1; McDearmid *et al.*, 1997). It is conceivable that NO

is modulating the release of these neuromodulators. For example, NO is known to inhibit

the synthesis of 5-HT from tryptophan hydroxylase (Kuhn and Arthur, 1994) and can

inactivate 5-HT itself (Fossier *et al.*, 1999). Additionally, in the amphibian tadpole of the

related anuran species, *Rana temporaria*, NA and NO both initiate qualitatively identical

motor patterns (McDearmid and Sillar, 1997) and in rat spinal synaptosomes NO

facilitates NA release (Li *et al.*, 2000). The combined promotion of NA release and

inhibition of 5-HT release would result in an increase in glycine transmission, which would

result in an increase in cycle periods, as described here.

The duration of swim episodes appears to be controlled at least in part by the activity of a

known GABAergic synaptic pathway, the mhr neurons (Fig. 3.9Ai, ii). If NO were

present in GABAergic mhr neurons, then activation of these neurons during a normal

stopping response could trigger the release of NO *via* Ca^{2+}-dependent pathways. NO

could then act in a retrograde manner on synaptic inputs from higher centres, re-enforcing

appropriate connections (Fig. 3.9Bi, ii). It would seem logical, given NO's use-dependent

nature, for it to act as an activity dependent molecule, incorporating the mhr neurons into

an endogenous swimming pathway. NO could not only promote GABA release

retrogradely but also anterogradely by promoting the fusion of synaptic vesicles (Meffert *et al.*, 1994, 1996). Either or both of these non-exclusive situations would certainly explain the facilitation of GABA by NO.

The effects described above in the presence of NO are consistent with the nitrergic facilitation of glycinergic and GABAergic inhibition to produce a decrease in swim frequency and a decrease is swim episode durations, respectively. This is corroborated by intracellular evidence recorded from presumed motor neurons in which the occurrence of glycinergic and GABAergic IPSPs increases in the presence of SNAP, as do glycinergic and GABAergic components of the synaptic drive for swimming.

3.5.4. CONCLUSIONS

Evidence has been provided that NO is a potential "higher order" modulator in the swimming circuitry of *Xenopus* tadpoles, in that it controls the function and release of more conventional neuromodulators and neurotransmitters, in a way that enhances inhibition. This function is consistent with the proposed location of nitrergic neurons in potentially serotonergic, noradrenergic and GABAergic neurons of the hindbrain (McLean and Sillar, 2000). While it is likely that these neurons are being actively regulated by NO production, the possible mechanisms and function of this modulation are at present unknown. However, these preliminary results, while still requiring further pharmacological investigation, provide important clues as to NO's role in motor control and generate yet another number of further questions. Most importantly, if NO is responsible for some transient developmental process in *Xenopus*, then its appearance should also be temporary as described in other systems (see CHAPTER 1).
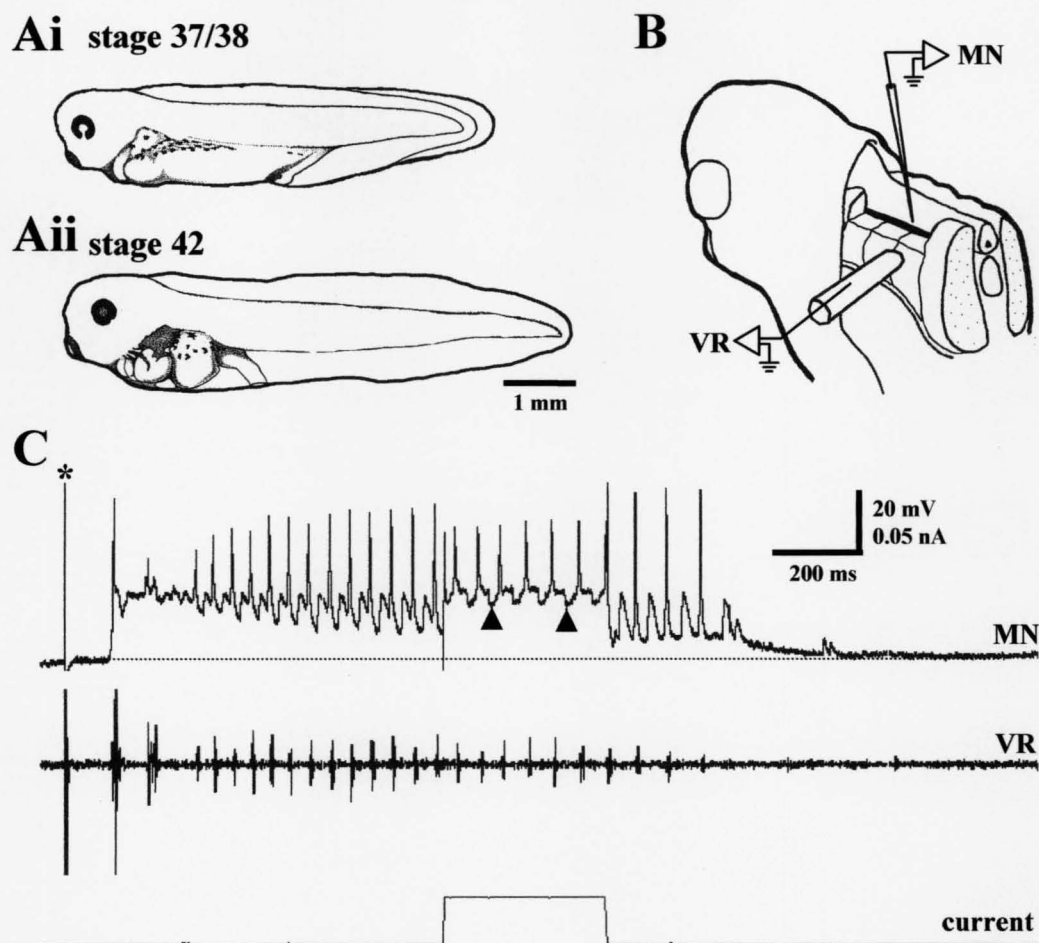
FIGURE 3.1. The embryonic and larval *Xenopus laevis* preparations. (A) *Xenopus* tadpoles at stage 37/38 (Ai) and stage 42 (Aii). (B) The experimental preparation (see MATERIALS AND METHODS) illustrating extracellular recordings from intermyotomal clefts (only one illustrated; VR) and an intracellular recording from a presumed motor neuron (MN). (C) The synaptic drive for swimming (MN) consists of a gradually declining tonic depolarisation, upon which on-cycle excitation (coincident with ventral root activity; VR) and mid-cycle inhibition (interposed between ventral root activity) are superimposed. KCl-filled electrodes can be used to sign reverse chloride-dependent inhibition, as sufficient amounts of chloride ions leak out of the electrode to cause depolarisation when chloride channels open. This is illustrated when a sufficient amount of positive current injection causes the inhibition to become hyperpolarising again. The stimulation artefact is asterisked.
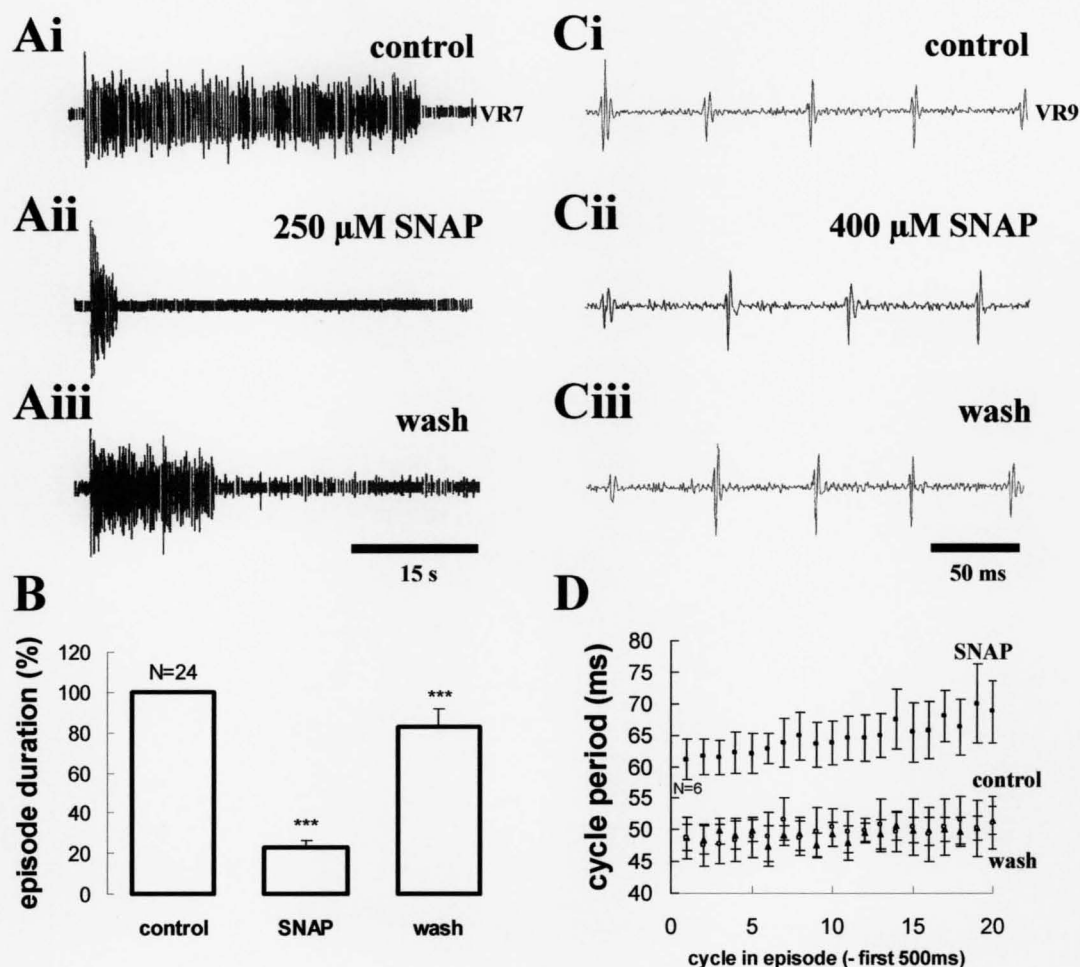
FIGURE 3.2. SNAP decreases swim episode durations and slows swimming frequency in stage 37/38 embryos. (A) Fictive swimming episode recorded on a slow time base before (Ai), during (Aii) and after (Aiii) 250 μM SNAP, illustrates the reversibility of the SNAP-induced effect on episode durations. (B) SNAP significantly decreases swim episode duration. For each experiment, 3 episodes of swimming activity were selected in control, drug and wash conditions. The graph illustrates the pooled data from the 8 experiments in which SNAP reversibly decreased episode durations (N=24) and are expressed as percentage difference from control values. (C) In a different preparation, fictive swimming activity recorded on a faster time base before (Ci), during (Cii) and after (Ciii) 400 μM SNAP, illustrates the reversibility of the SNAP-induced effect on cycle periods. (D) SNAP reversibly increases in cycle period. For each experiment, averages for cycle periods were calculated from a total of 60 measurements (20 measurements from 3 different episodes) under each condition, taken after the first 500ms of activity in each episode. This therefore avoided the possible influences arising directly from sensory stimulation and ensured equivalent points in episodes for statistical comparison. This graph illustrates on a cycle-by-cycle basis the pooled data from the 6 experiments in which SNAP reversibly increased cycle periods and is expressed as an average of raw data points (means ± S.E.M.; N=6) at equivalent points in the swim episode. (***, P<0.01).
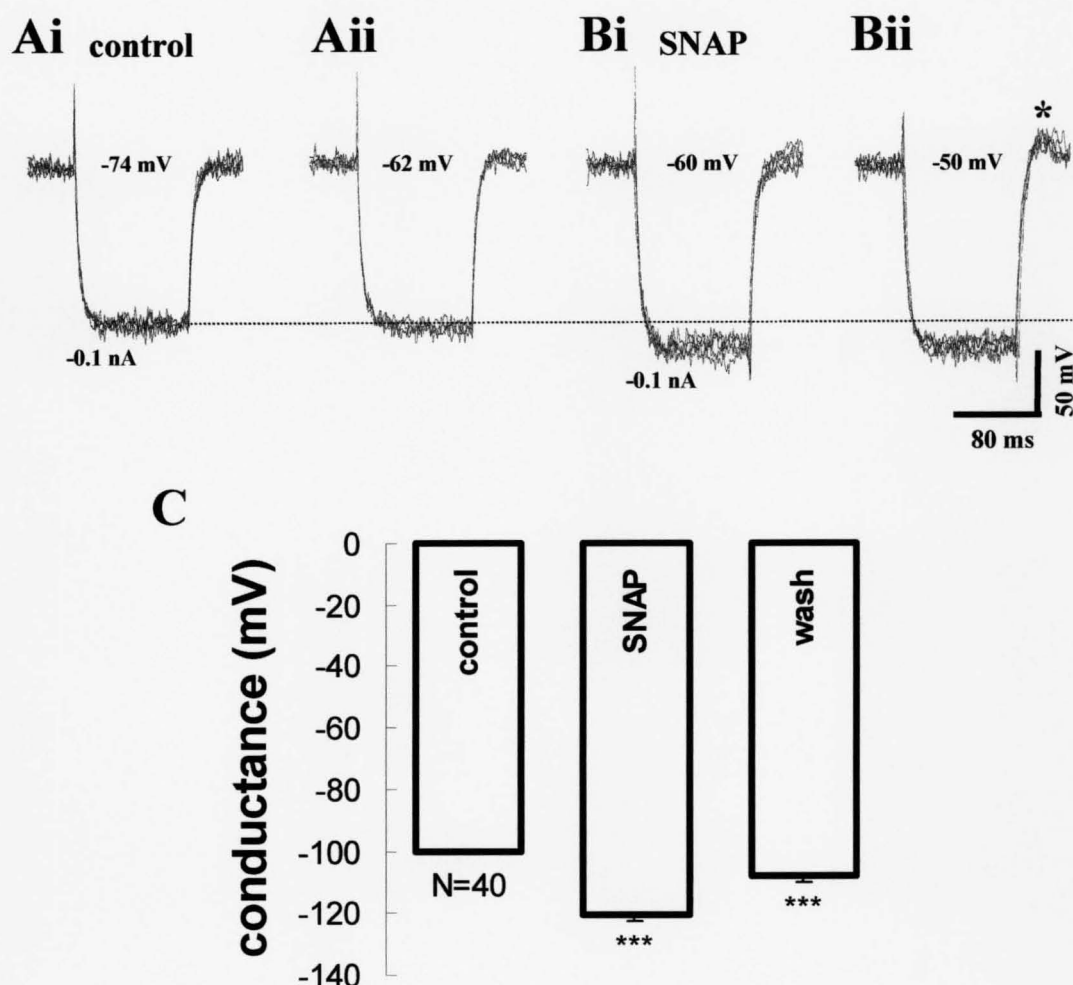
FIGURE 3.3. SNAP elicits a membrane potential depolarisation and a conductance decrease, which persists at equivalent levels of membrane potential. (A) Trains of five superimposed 100 ms hyperpolarising conductance test pulses with (Ai) and without (Aii) a 0.1 nA negative holding current show no pronounced decrease in membrane conductance, despite an 8 mV depolarisation. (B) Following the application of 1 mM SNAP, the membrane potential depolarises (compare values in Ai and Bi, Aii and Bii) and a pronounced decrease in membrane conductance is observed (Bi, Bii), that appears to be independent of membrane potential (compare values in Aii and Bi). Differing levels of membrane potential are aligned for ease of comparison (dotted line). Note that at depolarised levels a small rebound potential can occur (asterisk). This was also observed at similar membrane potentials in control conditions and therefore is unlikely to reflect a SNAP effect. (C) SNAP produced a significant (***, $P<0.01$) decrease in membrane conductance. For each experiment, averages for hyperpolarising conductance test pulses were calculated from a total of 5 measurements in each condition. The graph illustrates the pooled data from the 8 experiments in which SNAP reversibly decreased conductance (N=40) in both embryos and larvae and is expressed as percentage difference from control values. Error bars are S.E.M.
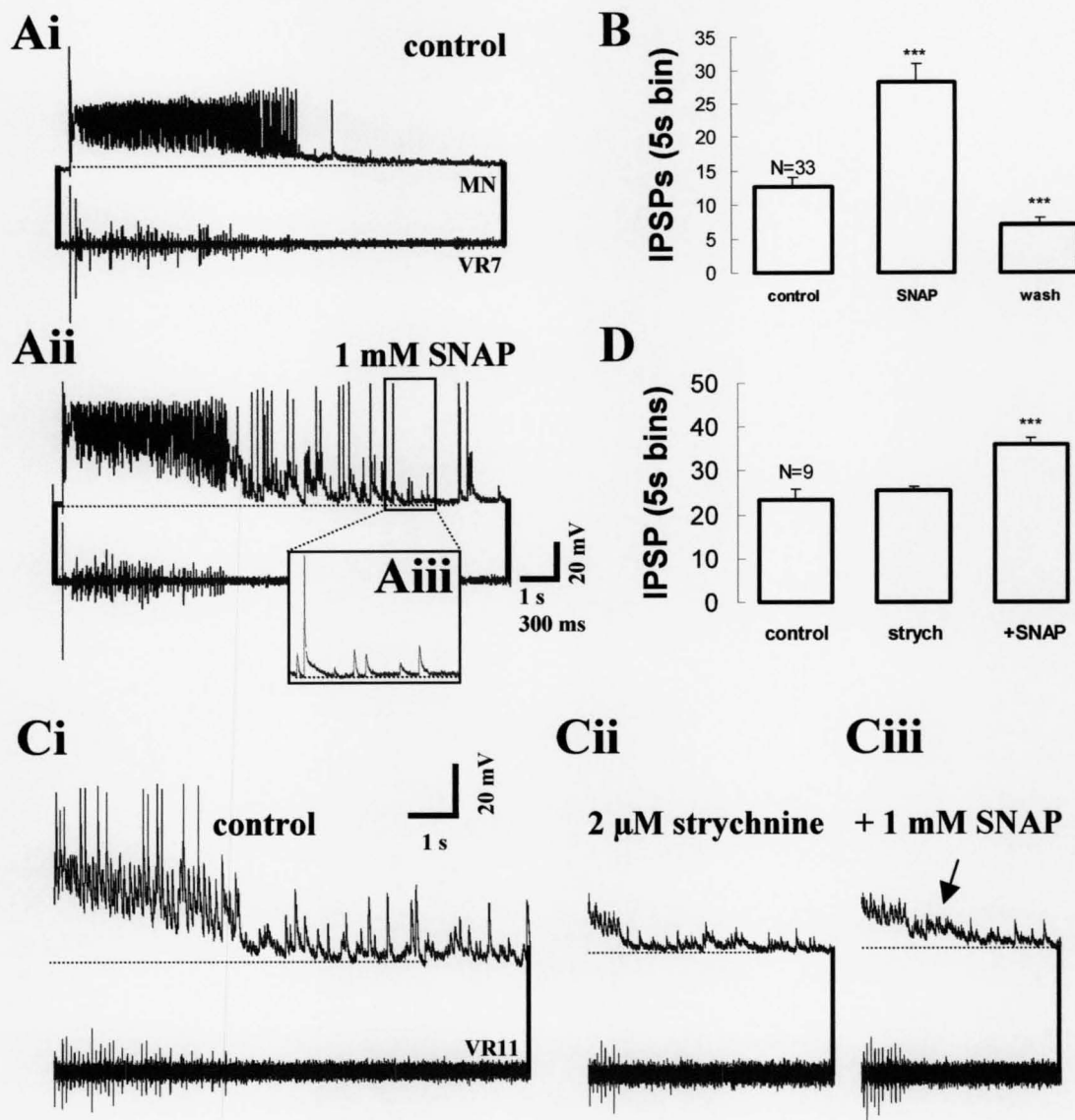
FIGURE 3.4. SNAP potentiates predominantly GABAergic IPSPs that terminate swim episodes. (A) An intracellular (MN) and extracellular (seventh ventral root; VR7) recording of fictive swimming on a slow time scale illustrates that swim episodes can often, but not always, terminate spontaneously in larvae (Ai), yet the subsequent application of 1 mM SNAP profoundly increases the occurrence of GABAergic IPSPs that can terminate swimming (Aii) and are distinguished based on duration (Aiii). (B) For each experiment, 3 episodes of swimming activity were selected in control, drug and wash conditions and IPSPs occurring 5 seconds after the end of each swim episode were measured. The graph illustrates the pooled data from 11 experiments in which SNAP reversibly increased GABAergic IPSPs in both embryos and larvae (N=33) and are expressed as averaged raw data points. (C) The facilitation of presumed GABAergic IPSPs by SNAP was followed by a test of its pharmacological sensitivity with the glycine antagonist, strychnine. Strychnine selectively (Ci) removes glycinergic inhibition but GABAergic inhibition (Cii) is still obvious, and is potentiated by SNAP (Ciii, at arrow). (D) A graph illustrates the pooled data from three experiments in which SNAP increased the occurrence of GABAergic IPSPs at the end of swim episodes in the presence of strychnine (N=9). Error bars are S.E.M, ***, $P<0.01$. Dotted lines represent the resting potential.
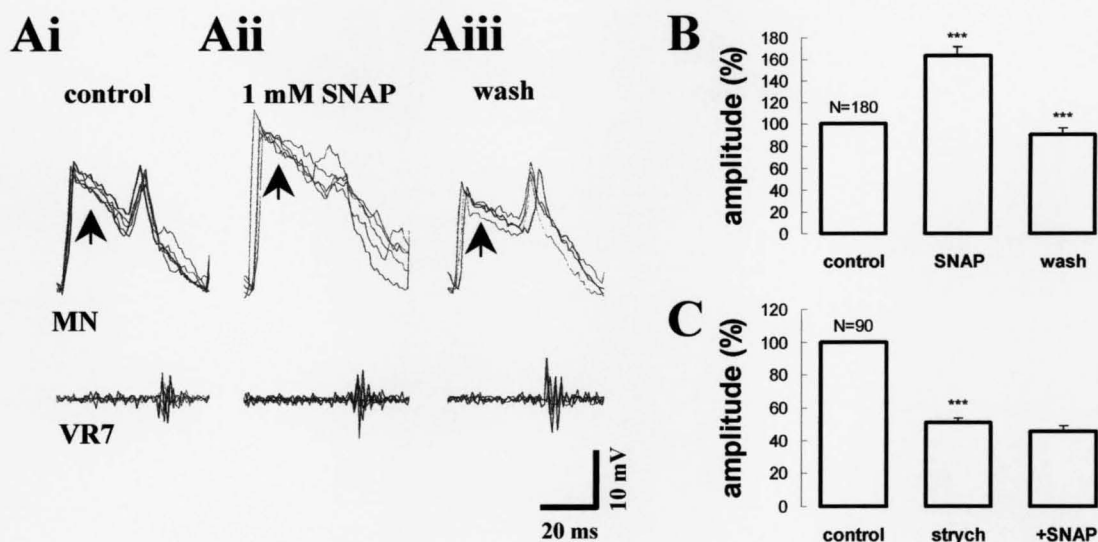
FIGURE 3.5. SNAP increases the amplitude of glycinergic mid-cycle IPSPs. (A) Trains of 5 consecutive superimposed cycles of intracellular (MN) and extracellular (seventh ventral root; VR7) activity displayed on a faster time scale. Note the mid-cycle IPSP (at black arrows) and the on-cycle EPSP. Note also the slight delay between the EPSP and the VR7 motor impulses, due to the more rostral location of the MN and the increased inhibitory shunting of the excitatory component of the synaptic drive. (Ai) The bath application of 1 mM SNAP results in a pronounced increase in mid-cycle amplitude (Aii), which is fully reversible upon return to control saline (Aiii). (B) SNAP produces a significant and reversible increase in mid-cycle IPSP amplitude. For each experiment, 3 episodes of swimming activity were selected in control, drug and wash conditions. Averages for mid-cycle amplitudes were calculated from a total of 30 measurements (10 measurements from 3 different episodes) under each condition by subtracting the resting membrane potential from the peak amplitude. In cases where spikes occurred, the peak amplitude was measured just after the spike. The graph illustrates the pooled data from the 6 experiments in which SNAP reversibly increased mid-cycle IPSP amplitude (N=180) in larvae and are expressed as percentage difference from control values. Note these effects were not as consistent in embryos, so their data are not included. (C) In three larval experiments, a facilitation of mid-cycle inhibition by SNAP was followed by a test of its pharmacological sensitivity with the glycine antagonist, strychnine. This graph illustrates the pooled data from three experiments in which SNAP was unable to increase the amplitude of mid-cycle IPSP in the presence of strychnine (N=90). Error bars are S.E.M. Note that strychnine does not completely eliminate the amplitude as a proportion represents the tonic NMDA-mediated depolarisation (see Fig. 3.1C). ***, $P<0.01$.
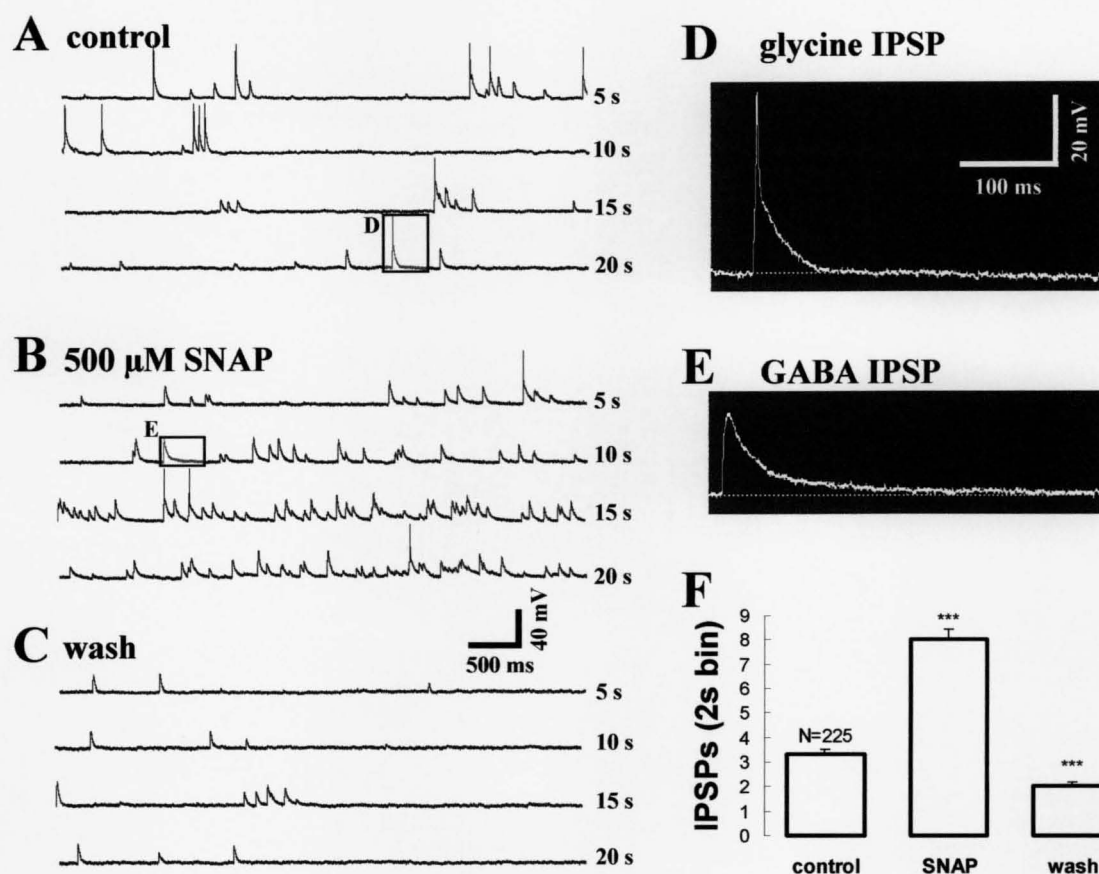
FIGURE 3.6. SNAP produces an increase in IPSP occurrence during quiescent periods. (A) During inter-episode quiescent periods, depolarising IPSPs can be distinguished that have previously been identified as TTX-resistant, spontaneous GABA and glycine release (Reith and Sillar, 1997) and can be identified based on their durations an pharmacological sensitivity (Reith and Sillar, 1997), as seen here in an embryonic preparation. (B) Shortly (2-3 min) after the bath application of 500 μM SNAP, there was a profound and (C) reversible increase in the occurrence of predominantly GABAergic IPSPs, which could be distinguished on the basis of their duration (compare D and E). (F) SNAP produces a significant and reversible increase in the occurrence of IPSPs. Averages of IPSP numbers were calculated from a total of 15 measurements (2s bins at 3 separate 10 second intervals) under each condition. The graph illustrates the pooled data from the 15 experiments in which SNAP reversibly increased IPSP occurrence in both embryos and larvae (N=225) and are expressed as raw data points. Error bars are S.E.M. ***, $P<0.01$.
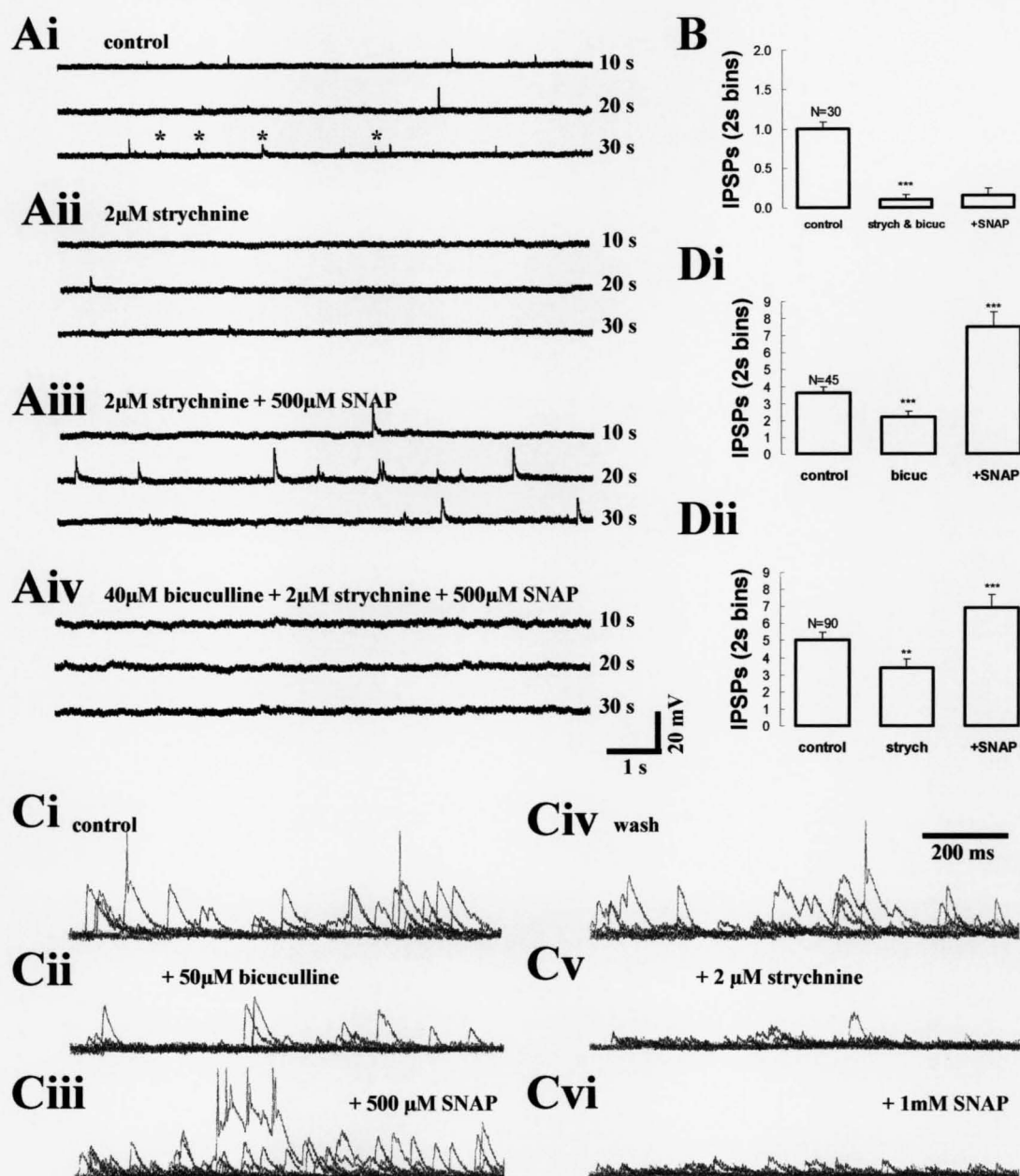
FIGURE 3.7. Pharmacological manipulations show that SNAP can increase both glycinergic and GABAergic IPSP occurrence. (A) In control conditions (Ai) both glycinergic and GABAergic (asterisks) IPSPs are easily distinguished on the basis of their duration, however glycinergic IPSPs are selectively eliminated by the bath application of 2 μM strychnine (Aii). The bath application of 500 μM SNAP increased the occurrence of GABAergic IPSPs (Aiii), which could be blocked by the subsequent application of 40 μM bicuculline (Aiv). (B) This graph illustrates the pooled data from two embryonic experiments, in which previous applications of SNAP could, but subsequent applications were unable, to increase IPSP occurrence in the presence of both strychnine (1-2 μM) and bicuculline (20-40 μM; N=30). (C) 10 s of overlapping quiescent activity at stage 42 illustrates that SNAP selectively increases glycinergic IPSPs (Ci-iii) in the presence of bicuculline and GABAergic IPSPs (Civ-vi) in the presence of strychnine. (D) These graphs illustrate the pooled data from (Di) 3 larval, 3 embryonic and (Dii) 2 larval, 1 embryonic experiments in which SNAP was able to increase IPSP occurrence prior to and in the presence of either strychnine (N=90) or bicuculline (N=45) alone. ***, P<0.01. See Figure 3.6 for analysis details.
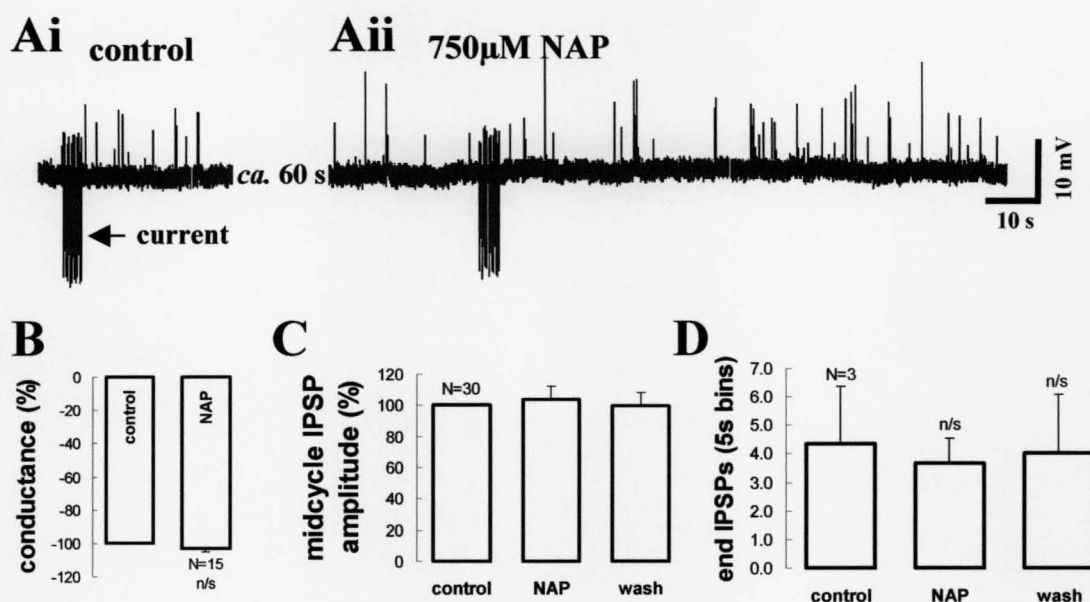
FIGURE 3.8. Synaptic effects of SNAP are due to the release of NO. (A) Quiescent synaptic activity illustrated on a slow time base before (Ai) and 30 seconds after (Aii) the bath application of 750 µM NAP. Note there is no pronounced effect on resting membrane potential or the occurrence of depolarising IPSPs. (B) Hyperpolarising conductance test pulses confirm that there is no significant difference (n/s) in membrane conductance before or after the bath application of NAP. Measurements are expressed as percentage changes from control in separate experiments, but are pooled together for the purposes of illustration in this graph (see Fig. 3.3). (C) The amplitude of mid-cycle inhibition is not significantly altered in the presence of 1.25 mM NAP, suggesting that the effects witnessed in the presence of SNAP are more likely to be the result of NO release.
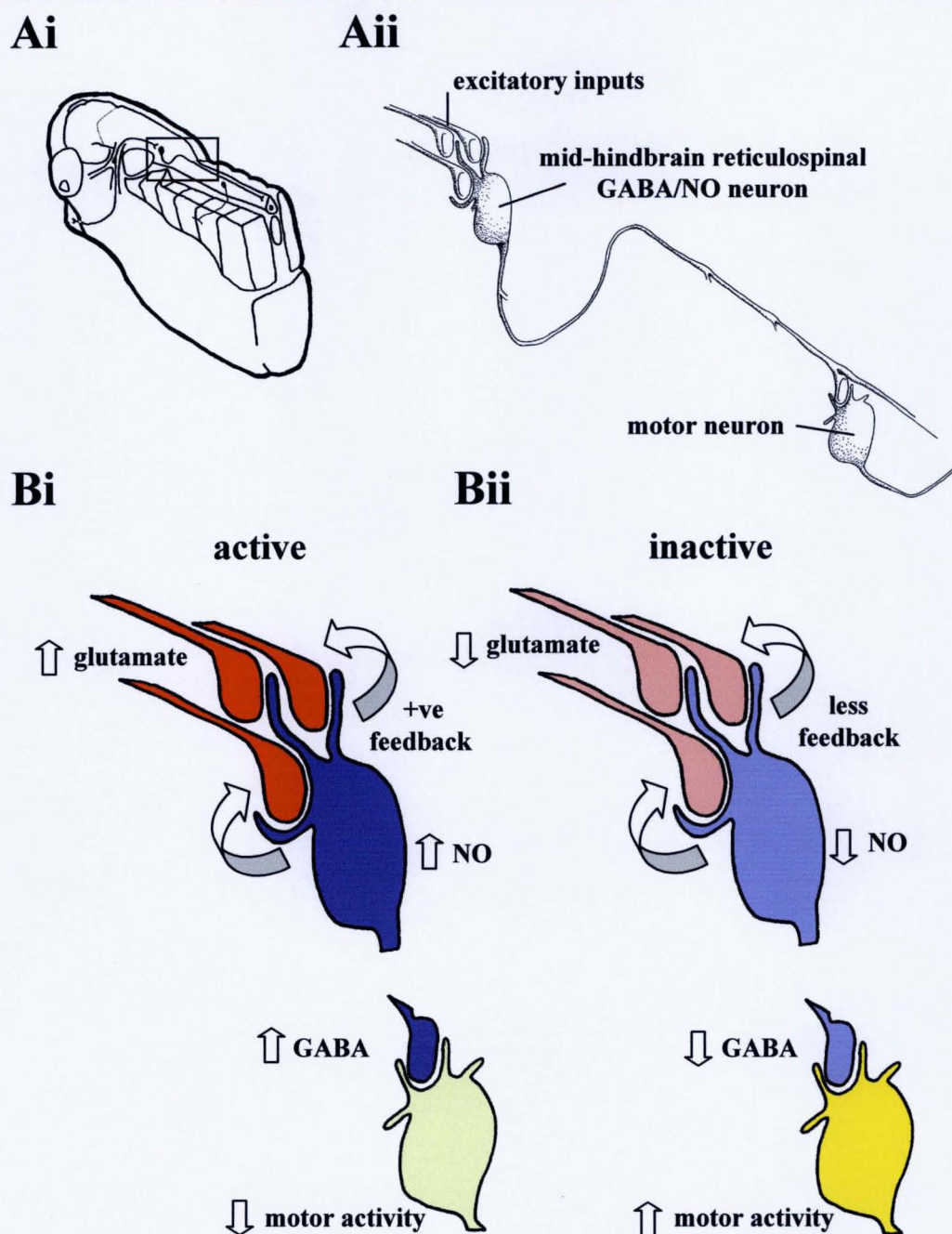
FIGURE 3.9. Activity-dependent model in which mhr neurons are incorporated into an endogenous stopping pathway. (A) A schematic drawing of an embryonic preparation (Ai) illustrating the cement gland pathway and (Aii) a close up of the synaptic connectivity. (B) Descending afferents synapsing on mhr neurons would replace embryonic cement gland afferents activating the mhr neurons in parallel. When active (Bi) mhr neurons would be active, NO would be released and the synaptic activity would be reinforced, terminating swimming. When inactive (Bii), there would be less feedback from NO, less mhr neuron activity and a resulting increase in motor activity.

THE SPATIOTEMPORAL PATTERN OF NADPH-DIAPHORASE REACTIVITY IN THE DEVELOPING CENTRAL NERVOUS SYSTEM OF PRE-METAMORPHIC *XENOPUS LAEVIS* TADPOLES[1]

4

4.1. SUMMARY

The progressive appearance of putative NOS-containing neurons has been catalogued in the developing CNS of *Xenopus laevis*. *Xenopus laevis* embryos and larvae were processed in wholemount and in cross section using NADPH-diaphorase histochemistry as a marker for NOS within the CNS. The temporal sequence of NADPH-diaphorase reactivity identified discrete groups and subgroups of neurons in the forebrain, midbrain and hindbrain on the basis of their morphology, location and order of appearance during development, a proportion of which appeared to be important in sensory processing and motor control. Staining also appeared at specific stages in the spinal cord, the retina and the skin. Following the appearance of labelling, NADPH-diaphorase reactivity continued in each of the cell groups throughout the stages examined. No evidence was found for staining that subsequently disappeared at later stages in any cell group, indicating a persistent, rather than transient role for NO in the *Xenopus laevis* tadpole CNS. These results are discussed in light of recent findings on possible roles for NADPH-diaphorase positive cell groups within the developing motor circuitry.

4.2. INTRODUCTION

The development of many anuran amphibians from egg to adult involves two substantial

changes in locomotor strategy. Non-motile embryos first develop into free-swimming

larvae, before the appearance of the hind limb buds signals the onset of a transition to bi-

or quadripedal locomotion. The ontogeny of these very different locomotory strategies

presumably involves major re-configurations of locomotor networks, either by adapting

pre-existing neural circuits or by creating entirely new ones. Most basic locomotor

patterns, like swimming and walking, involve the alternation between antagonistic motor

neuron pools. The underlying patterns of motor neuron activation are produced by rhythm

generating networks in the spinal cord (called central pattern generators or CPGs;

Delcomyn, 1980), which are organised as reciprocally antagonistic 'half-centres'

(reviewed in Kiehn *et al.*, 1997). Spinal CPGs are susceptible to descending modulation

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from a number of higher centres, which are also known to be instrumental in their

maturation (reviewed in Vinay *et al.*, 2000). It is therefore conceivable that systems out-

with the spinal cord are involved in the locomotory transformation during metamorphosis

and descending projections from the brainstem are possible candidates. While relatively

little is known about the influence of descending projections during the transition to limb-

driven locomotion in amphibians, much is known about their role in the coordination of

tail-driven locomotion, particularly within tadpoles of the South African clawed frog,

Xenopus laevis.

In the vertebrate CNS reticulospinal projections are among the first descending pathways

to develop from the brainstem to the spinal cord (reviewed in ten Donkelaar, 1982; also in

Vinay *et al.*, 2000), where they are known to contact spinal neurons (Nordlander *et al.*,

1985; van Mier and ten Donkelaar, 1989). In *Xenopus* embryos at stage 28 (Nieuwkoop

and Faber, 1956), only 30 hours after fertilisation, the first brainstem neurons project to

the spinal cord (van Mier and ten Donkelaar, 1984). At this time, spontaneous

uncoordinated motility gradually yields to rhythmic swimming movements, which generate

forwards propulsion. This swimming action is largely generated by synaptic inputs to

motor neurons that involve descending glutamatergic excitation (Dale and Roberts, 1984,

1985; Roberts and Alford, 1986) in combination with reciprocal glycinergic inhibition

(Dale *et al.*, 1986), each of which originates from spinal neuron populations that extend

into the brainstem. Brainstem neurons, particularly reticular neurons, not only initiate and

sustain swimming activity, but are also known to terminate it. The mhr neurons are

activated by primary sensory pathways in the rostral cement gland when the tadpole

encounters an obstacle and release the inhibitory neurotransmitter, GABA onto the spinal

swimming circuitry, activating $GABA_A$ receptors and terminating swimming (Boothby and

Roberts, 1992a, b). Thus, the descending influences of various brainstem populations are

responsible for the generation, maintenance and termination of the embryonic locomotor pattern. Furthermore, the ontogeny of the swimming rhythm, from stereotyped escape response to free swimming behaviour, is also known to involve specific brainstem nuclei (reviewed in McLean et al., 2000). For example, bath application of the biogenic amines, 5-HT (Sillar et al., 1992) and NA (McDearmid et al., 1997) can mimic the progressive maturation and modulation of swimming circuitry that follows the spinal invasion of serotonergic raphe (van Mier et al., 1986) and noradrenergic isthmic region (Marín et al., 1996; reviewed in Smeets and González, 2000) axons. However, because the spinal circuitry responsible for swimming behaviour is constructed well before hatching, this maturation of locomotory behaviour is presumably accomplished, not by creating new neural pathways, but by modifying pre-existing ones (Sillar, 1994). Yet, the mechanisms controlling the precise timing and organisation of descending projections in Xenopus tadpoles during this period are at present still not fully understood.

The free radical gas NO is transiently expressed in the developing brainstem and spinal cord of a variety of species, where it may function as an important neuronal signalling molecule (reviewed in Vincent, 1995). NO has also been reported to cause growth cone collapse and the retraction of axons (Hess et al., 1993; Renteria and Constantine-Paton, 1996) and has been linked to a number of neurotrophic factors (Lam et al., 1998; Klöcker et al., 1998; Estevez et al., 1998; Xiong et al., 1999; Ernst et al., 2000), suggesting that NO might be involved in the organisation of axonal projections during development. Recently, three bilaterally symmetrical populations of putative nitrergic neurons were identified in the hindbrain of Xenopus larvae using NADPH-diaphorase histochemistry (CHAPTER 2; McLean and Sillar, 2000). Pharmacological experiments have also provided evidence that NO released from these neurons plays an intrinsic modulatory role in the control of swimming in post-hatching tadpoles. However, it is not currently known at

what time the *Xenopus* brainstem populations begin to express NOS and whether this expression is transient or not. This chapter catalogues the appearance of NADPH-diaphorase reactivity (and therefore, presumably NOS; Bredt *et al.*, 1991; Hope *et al.*, 1991) from stage 28, when early locomotor movements begin, to stage 48, when the developing limb buds begin to grow. This investigation provides evidence that: 1) NOS expression in the CNS, as detected by NADPH-diaphorase histochemistry, starts at stage 29/30, progressively increases throughout development and plateau's between stages 45 and 47; 2) the sequence of NADPH-diaphorase reactivity identifies distinct groups, and subgroups, of neurons in the forebrain, midbrain and hindbrain; 3) the NADPH-diaphorase reactive groups are inter-connected by axonal processes; and 4) NOS expression is not restricted to the CNS, but also located in the eyes, skin and blood vessel walls.

4.3. MATERIALS AND METHODS

4.3.1. ANIMALS

Xenopus laevis tadpoles were obtained by induced breeding from an adult laboratory colony. Tadpoles were deeply anaesthetised with an overdose of MS-222 (0.01-0.1%) and fixed at room temperature for 45-180 minutes (see Table 3) in ice-cold 4% paraformaldehyde. The animals were then rinsed in 30% sucrose/0.1 M PB (pH 7.4) and processed either as wholemounts or as cross sections. Although processing was similar to that described previously (MATERIALS AND METHODS section 2.3.1), there are sufficient differences to warrant a brief description here.

4.3.2. WHOLEMOUNTS

Animals were selected (between stage 28 and 48; Nieuwkoop and Faber, 1956) and pinned through the notocord with finely etched tungsten pins to a Sylgard (Dow-Corning)

platform within a dissecting bath filled with either PB or PBS (pH 7.4, 0.01M). Using

fine needles, the skin was removed and the CNS was then carefully dissected out to the

level of the myotomes four to eight (as numbered from the otic capsule; Fig. 4.1A, B, C).

Immediately after dissection, the CNS was transferred to PB and then pre-treated in 0.3%

triton X100/PB to aid penetration of reagents. Subsequently the CNS's were transferred to

the incubation medium for NADPH-diaphorase histochemistry (see MATERIALS AND

METHODS, section 2.3.1) and kept at 37 °C in a humid chamber for 30-60 minutes. β-

NADPH was omitted as a negative control (N=4). The enzyme reaction was stopped by

transferring the preparations to 0.3% triton X100/PB. The stained preparations were then

quickly rinsed in distilled water, dehydrated in acetone, cleared in xylene and mounted in

DPX onto poly-L-lysine slides. All reagents were purchased from Sigma (UK).

TABLE 3. Size related fixation regime[1]

Stage	Size (mm)	Fixation (min)	Approximate (min)
28	3.8	43	45
29/30	5	56	60
31	5	56	60
32	5.1	57	60
33/34	6	68	70
35/36	6.3	71	70
37/38	6.8	77	80
39	7	79	80
40	7	79	80
41	7.2	81	80
43	8	90	90
44	9	101	100
45	9.5	107	110
46	10	113	120
47	12.8	144	150
48	16	180	180

[1]Stage 42 data are already presented (see CHAPTER 2; McLean and Sillar, 2000).

4.3.3. CROSS SECTIONING

Animals were selected and processed as above and then embedded in either rat brain or rat

liver that had also been fixed in 4% paraformaldehyde. Tissues were then frozen in a

cryostat (Leica Jung Frigocut 2800E; -18 °C) in a supporting cryomatrix and sectioned at

10-25 μm. The cryostat-cut tissue was thaw-mounted directly on poly-L-lysine coated

slides, rinsed in distilled water and air-dried at room temperature for 1-2 hours prior to

histochemical processing. The protocol for the NADPH-diaphorase histochemical reaction

was similar to that described above for wholemounts. Briefly, the sections were first

rinsed in triton X100/PB and then incubated at 37 °C for 60-120 minutes in the staining

solution described previously (CHAPTER 2). Subsequently, the slides were rinsed in

distilled water and air-dried at room temperature overnight. The slides were then cleared

in xylene and coverslipped in DPX.

TABLE 4. NADPH diaphorase histochemistry 'N' numbers[1]

Stage	Cross section	Wholemounts	Cell counts
28	-	4	4
29/30	3	7	3
31	2	3	1
32	4	3	2
33/34	2	8	4
35/36	-	3	4
37/38	9	16	2
39	-	3	3
40	5	3	3
41	3	3	2
43	2	10	3
44	3	5	2
45	4	6	3
46	-	15	3
47	3	8	4
48	-	8	4

[1]Stage 42 data are already presented (see CHAPTER 2; McLean and Sillar, 2000).

4.3.4. ANALYSIS

The resulting preparations were viewed immediately and catalogued using a Zeiss Axiolab

microscope equipped with a camera. Photographs were taken with an Olympus OM-4 Ti

camera using Fuji 200 ASA film. Tracings of enlarged photographs were made on a

Jessop Lightbox. Drawings and cell counts were made with a *camera lucida* attachment.

Many of the schematic drawings and images were made with the help of a Ricoh Digital

Camera (ROC-5300) and Photoshop Software, courtesy of Professor David M. Paterson,

University of St Andrews. Table 4 summarises the number of wholemounts and cross sections taken at each developmental stage.

4.4. RESULTS

4.4.1. PRE-HATCHING AND EARLY SWIMMING (STAGES 28 TO 42)

The first group of neurons to show NADPH-diaphorase reactivity appeared in the caudal hindbrain (hb; Fig. 4.1A) at stage 29/30 (Fig. 4.2). Consistent with recent findings in larval stage 42 animals (CHAPTER 2; McLean and Sillar, 2000), this was the group with the largest number of neurons at each stage of development (Table 5).

TABLE 5. Appearance of NADPH diaphorase reactive cells in the CNS[1]

	28	29/30	31	32	33/34	35/36	37/38	39	40	41	43	44	45	46	47	48
Forebrain																
scattered cells														+	+	++
optic group								+	+	+	+	+	+	+	++	++
diffuse patch												+	+	+	+	+
Midbrain																
caudal							+	+	+	+	+	+	+	+	+	+
dorsal															+	++
Hindbrain																
rostral								+	+	+	+	+	+	+	++	+	++
mid-rostral									+	++	++	++	++	+++	+++	+++
caudal																
primary	+	+	+	+	+	+	+	+	++	++	++	++	++	+++	+++	+++
secondary				+	+	+	+	+	++	++	+++	+++	+++/+	+++/+	+++/+	+++/+
tertiary									+	+	+	++	++	++	++	++
intermediary											+	+	+	++	++	++
quaternary													+	+	+	+
Spinal cord														+	+	+

[1]Subjective ratings of intensity and number of stained cells: +, 50 cells.

The NADPH-diaphorase reactivity appeared to mimic the development of these neurons in that it was at first restricted to the somata (see Figs. 4.2D, 4.3A, D), but subsequently appeared in the short projecting unipolar neurites (see Fig. 4.2E), which became more

intensely stained and branched as development proceeded (see photo overlays in Figs.

4.3G, M, 4.4E, 4.5Av, 4.6J). The appearance of NADPH-diaphorase reactivity was also

consistent with the appearance of newly generated neurons (van Mier and ten Donkelaar,

1984), namely large nuclei with yolk granules and little surrounding cytoplasm (see Fig.

4.3M). These characteristics were true for all the neurons stained with NADPH-

diaphorase. Up until approximately stage 33/34 (Fig. 4.2E, 4.3B, G) this group comprised

a single ventromedial cluster. However, it is clear that this caudal hb group comprises 3

distinct subgroups, which can be distinguished on the basis of their morphology (Fig.

4.3K, L, M) and on their appearance as the sub groups differentiate during development.

At stage 29/30 the primary caudal hb sub-group appeared at the level of the otic capsule

(Fig. 4.2Ai, ii, D). These neurons had cell bodies at a variety of levels up and down the

dorso-ventral axis (Fig. 4.2Bi, ii, E), from which arose ventrally projecting axons, (Fig.

4.2F, G) which then projected rostrally (Fig. 4.2Ci, ii, F, G, I). These projections did not

cross the ventral commissure below the neurocoel (Fig. 4.3K) and occupied a ventral

position in the marginal zone (Fig. 4.3J). A second caudal hb subgroup appeared at

approximately stage 33/34, 100-150 μm caudal to the otic capsule (Fig. 4.2Bii, E), whose

cell bodies were organised in a tighter, columnar fashion (Fig. 4.2Ci, ii, H, I) with

ventrolateral axonal projections to the marginal zones (Fig, 4.3C, M). These neurons,

however, had prominent contralateral projections (Fig. 4.3M) and comprised the largest

proportion of neurons in the caudal hb group (Table 5). Prior to the appearance of the

third caudal hb subgroup, but shortly after the second caudal hb subgroup, there appeared

a faintly labelled population located ventrally and medially in the forebrain (fb) at stage

35/36 (Fig. 4.3C, H). Coincident with this group was the appearance of another NADPH-

diaphorase reactive population, in the caudal midbrain (mb; Fig. 4.3B, E), near the mb/hb

border. The cell bodies were located mediolaterally and loosely distributed along the

dorso-ventral axis, with axonal processes extending ventrolaterally to the marginal zones,

as illustrated in Figure 4.4B at stage 41. The extensive ramification of axonal processes appeared to make contact with longitudinal processes already present in the marginal zones, which extended to the fb. Subsequently, at stage 37/38, a second population of neurons labelled in the rostral hb, near the mb/hb border (Fig. 4.3B, F). These tightly packed neurons were dorsally and laterally located and by stage 41 had pronounced ventral projections (Fig. 4.4C), which also appeared to contact processes already present in the marginal zones. Contralateral projections connecting this population bilaterally were apparent by approximately stage 44 (illustrated in Fig. 4.7G at stage 48).

Two more NADPH-diaphorase reactive groups were detectable between stages 39 and 40. A third caudal hb subgroup, morphologically distinct from the aforementioned subgroups, had dorsolateral cell bodies that extended 50-100 μm caudally from the otic capsule and ventral projecting axons (Fig. 4.3C, L). It could not be determined if these neurons had contralateral processes, as the secondary caudal hb sub-group was already in place. Coincident with the appearance of this group, a third group of neurons in the mid-rostral hb began to show NADPH-diaphorase reactivity (Fig. 4.3C, I). The cell bodies were ventrally located with ventrolateral projections that did not cross beneath the neurocoel, but rather projected both rostrally and caudally to the other cell populations. The projections were more medially and laterally located than the ascending projections of the primary subgroup (Fig. 4.3J). By stage 41 then, the three populations of NADPH-diaphorase reactive neurons that have been described previously in detail (CHAPTER 2; McLean and Sillar, 2000) were present.

4.4.2. FREE SWIMMING AND FEEDING (STAGES 43 TO 48)

By stage 43, three populations of NADPH-diaphorase neurons were present in the hb, increasing in cell number and rostrocaudal distribution (Figs. 4.4C, D, E, 4.5Aiii, iv, v). In

particular, the three caudal hb subgroups began to merge, so that all three could be distinguished in the same section (Fig. 4.5Av). Additionally, beyond stage 43, neurons interposed between the defined groups began to label with NADPH-diaphorase reaction product (Fig. 4.6Ai, ii, I) and display similar developmental staining profiles as described above; namely, faint and restricted to somata at first, then progressing to the axonal projections. Axonal processes within the marginal zones also displayed stronger reactivity (Fig. 4.5Bi, ii) and from stage 43 onwards staining became apparent in the marginal zones of the rostral spinal cord (Fig. 4.5Avi). Interestingly, contralateral processes were not as apparent in the caudal hb group as at earlier stages, instead NADPH-diaphorase staining became weaker and not as pronounced in the marginal zones of the brainstem (c.f. Figs. 4.5Av and 4.6J).

By stage 44, scattered cells with ventrally projecting axons began to label in the developing tectum mesencephali, located dorsally in the mb (Fig. 4.5Aii). There was also a pronounced labelling of axons in the marginal zones of the mb and fb (Figs. 4.6D, E, 4.7A, E, F), which were previously detectable at stage 39 (not illustrated). NADPH-diaphorase reactive tracts could also be seen across the optic chiasm (Fig. 4.7E). Scattered cells were also visible in the developing telencephalon, which had multiple processes, some of which appeared to project rostrally (Fig. 4.6B, 4.7B). Furthermore, the developing cells in the preoptic region, at the caudal end of the telencephalon appeared to move laterally at stage 42 (Figs. 4.3H, 4.4A, 4.5Ai, 4.6C, 4.7C). In addition to the scattered cells was a ventrolaterally positioned, discrete area of diffuse NADPH-diaphorase staining at the rostral junction of the developing diencephalon (Fig. 4.6D, 4.7D), which appeared at stage 44 (Table 5). By stage 45, the total number of cells appeared to plateau (Table 5). By stage 47, isolated neurons in the spinal cord also labelled (Fig. 4.8), with unipolar descending and ascending axons that projected to the

marginal zones (Fig. 4.8B, C). These spinal neurons were distributed unevenly along the spinal cord (Fig. 4.8A), beginning approximately 400 μm from the hb boundary, and appeared to occur in clusters and at irregular intervals of 500-800 μm as far back as the eighth myotome (Fig. 4.8E; schematised in Fig. 4.10A). A proportion of spinal neurons had a single axonal projection that crossed the ventral commissure from a mediolaterally located soma that lacked dendritic branches (Fig. 4.8C), suggesting that they may be inhibitory commissural interneurons.

At stage 48 the full complement of NADPH-diaphorase reactive populations described here were still present. No change in the brainstem populations was evident (Fig. 4.6F, G, H, J), with the exception of possibly a fourth, small, caudal hb subgroup, located between the mid-rostral hb group and the tertiary sub-population (not illustrated). Most notably, there was a pronounced increase in NADPH-diaphorase reactive neurons in the fb and mb after stage 42. The fb and mb populations are summarised in Figure 4.7, along with their inter-connecting processes. The developmental sequence of NADPH-diaphorase expression in the nervous system is summarised in Table 5. An overview of the NADPH-diaphorase staining pattern in the CNS during development is depicted schematically in Figure 4.10.

4.4.3. STAINING IS NOT RESTRICTED TO THE CNS

Although a detailed analysis of cells labelled for NADPH-diaphorase in structures outside the CNS is beyond the scope of the present study, there are three areas that warrant a brief description; namely the developing eyes, skin, and blood vessels. NADPH-diaphorase staining in the eyes began at stage 35/36 (Fig. 4.9A, B). This coincides with the differentiation of the retinal cell layers, when the three nuclear layers have formed, in between which are the inner and outer plexiform layers (Nieuwkoop and Faber, 1956).

By stage 42, rods and cones label and can be distinguished and by stage 47 and 48 their

appearance and relative numbers are the same as in the adult (Nieuwkoop and Faber,

1956). NADPH-diaphorase reaction product can be detected in the photoreceptor layer

(Fig. 4.9E) by approximately stage 44 (Fig. 4.9D), when a majority of the retinal cells

have their final structure. Diffuse bands of staining are obvious from stage 37/38 (Fig.

4.9B, C) in the inner and outer plexiform layers. Cell bodies can also be seen in the inner

nuclear layer by stage 47 (Fig. 4.9F). There was no vasculature detectable by NADPH-

diaphorase within the CNS of *Xenopus* at this stage of development, which corroborates

findings in older animals (Crowe *et al.*, 1995; Bruning and Mayer, 1996). However, this

was not the result of a failure to stain endothelial isoforms of NOS, as NADPH-diaphorase

reactivity was witnessed in the skin and the walls of peripheral blood vessels, located in

close proximity to the yolk sac (Fig. 4.9G).

4.5. DISCUSSION

This description of the developmental sequence of NADPH-diaphorase staining can

provide important clues to the function of NO during early *Xenopus laevis* development.

Staining was found in areas out-with the CNS (see Fig. 4.9) where NO is known to be

both located and play a functional role in amphibians, namely the skin (Neumann *et al.*,

1996), blood vessels (Knight and Burnstock, 1996) and the eyes (Savchenko *et al.*, 1997).

This lends further support to the proposal that NADPH-diaphorase labels NOS. The

development of staining in the brain broadly followed a caudorostral gradient, with some

notable exceptions; namely an early development of staining in the fb and mb (see Fig.

4.3E, H) and a later expression of staining in the spinal cord (see Fig. 4.8). This sequence

is consistent with the known development of reticular brainstem projections to the spinal

cord (van Mier and ten Donkelaar, 1984). However, if NADPH-diaphorase reactivity is

identifying populations of reticulospinal neurons, then its appearance is delayed with

respect to the synaptogenesis of these pathways. For example, reticular pathways are known to project well into the spinal cord by stage 28 (van Mier and ten Donkelaar, 1984), yet neurons in the caudal hindbrain do not begin to express NADPH-diaphorase staining until after stage 29 (see Fig. 4.2). While this could mean that NADPH-diaphorase reactive populations are not reticulospinal at all, it is more likely that they are, but NO's role in the function of these pathways excludes early developmental processes, such as axon pathfinding. A further property of NADPH-diaphorase reactivity is that it begins in the somata and subsequently appears in the axonal projections. This indicates that there is an expansion of NOS activity from somatic to axonal sites and more importantly, suggests that NO could potentially modulate both the inputs and the outputs of a particular neuron. Additionally, the extensive axonal inter-connections between the brainstem populations provide the potential for functional interactions during development, even though they might also have specific, unrelated roles.

The time course of NOS-expression could provide clues to its roles in development, since transient expression can only be associated with a transient function. For example, in motor nuclei of the foetal human brainstem there are two chronological patterns of NOS expression, one that appears during prenatal development and then remains, and another, present at only a specific foetal stage (Gonzalez-Hernandez et al., 1994). However, none of the NADPH-diaphorase reactive populations described here disappears at any stage, but rather the numbers of neurons in each group increases until stage 45. It would seem then that NO's roles in the developing CNS of Xenopus persist until at least metamorphic climax (ca. stage 52-60). The ultimate clue to its role in development would be provided by the functional identity and transmitter phenotype of the NADPH-diaphorase reactive populations. While double-labelling is required before any firm conclusions can be drawn,

the current understanding of NO in the CNS of *Xenopus laevis* allows us to make a

number of tentative suggestions.

4.5.1. NOS EXPRESSION IN ADULT *XENOPUS LAEVIS*

The extensive differentiation of the developing CNS often makes it difficult to relate

particular regions of larval to adult CNS. However, the CNS of amphibians is relatively

undifferentiated as compared to other vertebrates, making tentative comparisons between

late larval and adult animals possible. Despite being at early stages of development, the

NADPH-diaphorase reactive brain regions bear a resemblance to those described in a

study of the location of NOS in the brain of adult *Xenopus* frogs (Bruning and Mayer,

1996). While there were regions that did not correspond to any seen in premetamorphic

larvae, there were a number of cell groups that shared a similar locale and morphology. In

particular, large multipolar neurons were described in the dorsal and medial pallium of the

telencephalon (see Fig. 4.7B), strongly reactive tracts were observed at the base of the

telencephalon and diencephalon (see Fig. 4.7E, F) and across the optic chiasm (see Fig.

4.7E), there were a few positive cells in the preoptic nucleus (see Fig. 4.7C) and the

superficial ventral thalamic nucleus contained intense diffuse reaction product which

included the cell bodies (see Fig. 4.7D). In the midbrain a number of regions were

NADPH-diaphorase reactive (see Figs. 4.5Aii and 4.6F) and in the hindbrain, a cluster of

intensely stained neurons, ventromedial to the nucleus isthmi, was described (see Fig.

4.6G), as well as strongly stained neurons in the nucleus reticularis medius and inferior

(see Fig. 4.6J). These brain regions are particularly important in sensory processing and

motor control. In support, it has recently been demonstrated that NO profoundly affects

swimming in stage 42 *Xenopus* larvae (CHAPTER 2; McLean and Sillar, 2000). It was

hypothesised that the intrinsic nitrergic modulation of swimming originated from the three

populations of NADPH-diaphorase reactive populations located in the hindbrain at that

stage (CHAPTER 2; McLean and Sillar, 2000). The morphology and spatial distribution of

neurons within these populations suggested they might belong to (in caudorostral order):

the GABAergic mhr neurons (Boothby and Roberts, 1992a, b), the serotonergic neurons

of the raphe (van Mier *et al.*, 1986) and the anuran homologue of the noradrenergic locus

coeruleus, the isthmic region (Marín *et al.*, 1996). This is further supported by the

similarity of effects demonstrated by NO with the known aminergic and GABAergic

effects on the swimming rhythm (reviewed in McLean *et al.*, 2000). If they do indeed

correspond to at least a proportion of the regions described here, then it would appear that

they become NADPH-diaphorase reactive early in development and this persists

throughout metamorphosis into adulthood. While this continuous expression of NADPH-

diaphorase reactivity does not preclude an initial developmental role, it does indicate

ongoing functional nitrergic roles within the adult *Xenopus*, which begin quite early in

development.

4.5.2. POTENTIAL BEHAVIOURAL ROLES FOR NO IN DEVELOPMENT

The developmental transition from an aquatic to a terrestrial lifestyle necessitates dramatic

reconfigurations of amphibian physiology (Fritzsch, 1990), not the least of which involves

changes in locomotory behaviour (see Fig. 4.10B). Metamorphosis is usually associated

with extensive tissue loss, although there is also a certain amount of conservation and

generation, particularly in the CNS. For example, the large reticulospinal Mauthner

neurons, which are involved in fast start escape responses, are known to survive in post-

metamorphic frogs (Fox, 1981). Additionally, rostral primary motor neurons are retained

along with newly generated secondary motor neurons, which innervate limb musculature

(van Mier *et al.*, 1985). Given the suggested roles for NO during naturally occurring

neuronal death (Snyder and Bredt, 1992), it was recently hypothesised that it would only

be present in populations of neurons destined to die within the spinal cord of

metamorphosing *Xenopus* (Crowe *et al.*, 1995). However, there is a substantial

conservation of NADPH-diaphorase reactivity in adult and metamorphosing spinal

neurons, instead suggesting that NOS may be involved in processes other than apoptosis in

the spinal cord, which lends further support to NO's continuing function in the CNS of

Xenopus.

Given NO's location in regions associated with locomotion, together with it's profound

influence on swimming activity, it is conceivable that NO could be involved in the

reconfiguration of locomotor networks during metamorphosis. In vertebrates, the activity

of CPGs are initiated and maintained by brainstem locomotor regions, which are in turn

affected by more rostral structures (see INTRODUCTION, section 4.2). It has been suggested

that the hindbrain may be a critical region involved in the transition from one locomotory

strategy to another, in which the transition from swimming to stepping during

metamorphosis involves a disinhibition of pre-existing hindlimb stepping reflexes

(reviewed in Stehouwer, 1992). A role for NO in metamorphosis would not be

unprecedented, since NO has been implicated in this process in the marine snail, *Ilyanassa*

obsoleta (Froggett and Leise, 1999). In this case NO's involvement is associated with a

transient appearance of NADPH-diaphorase reactivity in CNS neurons of metamorphosing

larvae (Lin and Leise, 1996). Additional experiments examining the distribution of

NADPH-diaphorase reactive neurons in the brainstem of metamorphosing *Xenopus*

tadpoles are now needed to confirm the persistence of NADPH-diaphorase reactivity, to

clarify if a transient appearance also occurs during metamorphosis in *Xenopus*.

NO could also be involved in synaptogenesis in the brainstem. It is now widely recognised

that the pattern of adult synaptic connectivity is carved out of the developmental milieu

through processes of refinement, where appropriate connections survive and inappropriate

connections perish. One major tenet of synaptic refinement is the Hebbian principle of

activity-dependence, in which "neurons that fire together wire together" (reviewed in

Stent, 1973). NO has been linked to the refinement of synaptic connectivity where it

eliminates misguided targets, but maintains correct ones (reviewed in Contestabile, 2000).

These effects can vary between and within species in a concentration, location and time

dependent manner (Van Wagenen and Rehder, 1999). For example, NO inhibits neuronal

proliferation in tadpole retinal neurons (Renteria and Constantine-Paton, 1996) and in rat

dorsal root ganglion neurons (Hess *et al.*, 1993), yet promotes it in rat motor neurons

(Inglis *et al.*, 1998). This dual role for NO may depend largely on the condition of the

target neurons and the location of NOS, in which the expression of certain neurotrophic

factors may play a crucial role (Lam *et al.*, 1998; Klöcker *et al.*, 1998; Estevez *et al.*,

1998; Xiong *et al.*, 1999; Ernst *et al.*, 2000).

Interestingly however, the expression of NOS itself is also activity dependent and

increased Ca^{2+} levels not only activate NOS, but also induce the expression of NOS

(Baader and Schilling, 1996; Sasaki *et al.*, 2000). This activity-dependence is further

supported by the findings that NOS expression is present in the developing chick optic

tectum and that the level of expression correlates with the activity and presence of retinal

axons (Williams *et al*, 1994). Activity dependence may explain why the expression of

NADPH-diaphorase reactivity appears only after the network becomes active (stage

29/30) and not before, during synaptogenesis of the network (stage 28). The activity

dependence of NOS could also explain why only a discrete population of neurons

expresses NADPH-diaphorase reactivity. For example, the behavioural repertoire of

tadpoles between stages 28 and 48 is quite limited, consisting essentially of swimming and

filter feeding. Respiratory actions are needed for food intake (Stehouwer, 1992) and

respiratory centres are known to occur in the amphibian caudal hindbrain region (McLean

et al., 1995), where NO has been shown to play a functional role in adult amphibians (Hedrick *et al.*, 1998). It could therefore be that NO is involved in linking respiratory and locomotory brainstem regions by some form of activity-dependent mechanism.

4.5.3. CONCLUSIONS

Until the developing limbs become effective late in larval life (*ca.* stage 58 in *Xenopus*), axially based movements provide the amphibian larva's sole means of locomotion (Hoff and Wassersug, 1986). However, the rapidity with which this situation changes during metamorphosis, requires an equally rapid response from the CNS. Networks that must play roles at different developmental stages may be constrained from being dramatically altered, because changes that would be advantageous for one behaviour might be disastrous for another (Katz and Harris-Warrick, 1999). Take, for example, the simple escape response of anuran amphibians. In adult frogs there must be a simultaneous activation of contralateral locomotory muscles, so the animal can jump to safety. However, bilateral activation of swimming muscles would be inappropriate at early aquatic stages of development. It is thought that a weak electrical coupling of Mauthner neurons, which persists throughout metamorphosis, might account for the bilateral response in adults (reviewed in Stehouwer, 1992). Therefore subtle changes in the nervous system can cause large and important changes in the behaviour of an organism (reviewed in Kiehn and Katz, 1999). NO could potentially be a molecule with the versatility to accomplish this. At present, however, it is too early to conclude the precise role for NO in the developing CNS of *Xenopus* tadpoles. It has been shown here that NADPH-diaphorase reactive populations appear in the CNS early in development and persist to pre-metamorphic stages. Their developmental sequence provides a potentially important method to investigate the relative contributions of NOS-containing populations in developing locomotor systems. It is therefore hoped in the near future that this

experimentally amenable physiological system will yield important clues as to NO's role in

behavioural development.

A **stage 28**

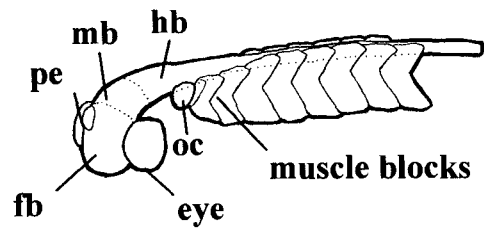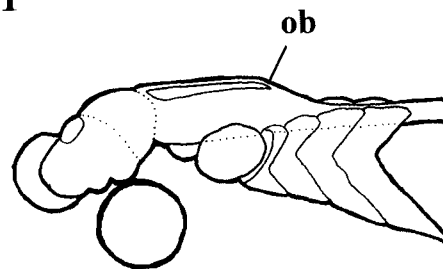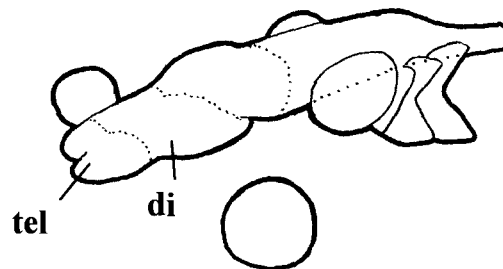**B** **stage 41**

C **stage 47**

FIGURE 4.1. Schematic drawings of the developing CNS at stages, (A) 28, (B) 41 and (C) 47, illustrating the morphological changes to the forebrain (fb), midbrain (mb) and hindbrain (hb) that occur with respect to anatomical landmarks. Abbreviations: otic capsule (oc), obex (ob), pineal eye (pe), telencephalon (tel) and diencephalon (di).
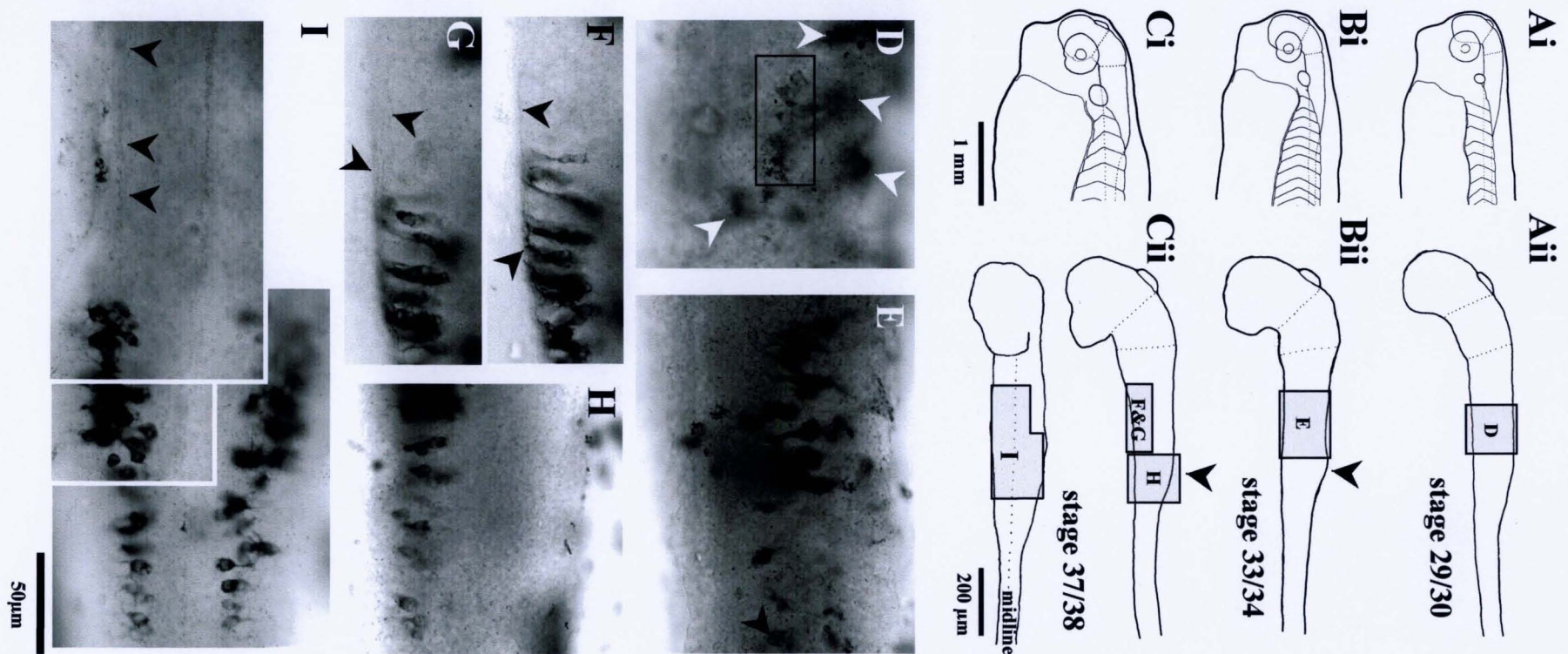
FIGURE 4.2. NADPH-diaphorase reactivity begins in the caudal hindbrain at stage 29/30 and can be divided into primary and secondary subgroups based on time course. (A-C) Schematic drawings of stage 29/30, 33/34, and 37/38 embryos illustrating the CNS in relation to the eye, otic capsule and the muscle blocks (Ai-Ci; see also Fig. 4.1) and a detail of the CNS illustrating the regions depicted below (Aii-Cii). The obex is at black arrowheads. (D) Photograph at 40x magnification illustrates the faint labelling of neurons in the caudal hindbrain (boxed area). Darkly pigmented melanophores are just out of the plane of focus (white arrowheads). (E) The primary subgroup is more darkly labelled at stage 33/34, with short ventrally projecting neurites. The secondary subgroup is just beginning to label at this stage (single black arrowhead). (F) By stage 37/38, ascending projections originate from the primary subgroup (black arrowheads). (G) Projections are also apparent in a different plane of focus (black arrowheads). (H) The secondary subgroup is now more darkly labelled at this stage. (I) Both populations are bilaterally symmetrical as viewed in a photographic composite from the ventral aspect (see Cii), and projections are visible as they course along the marginal zones (black arrowheads).
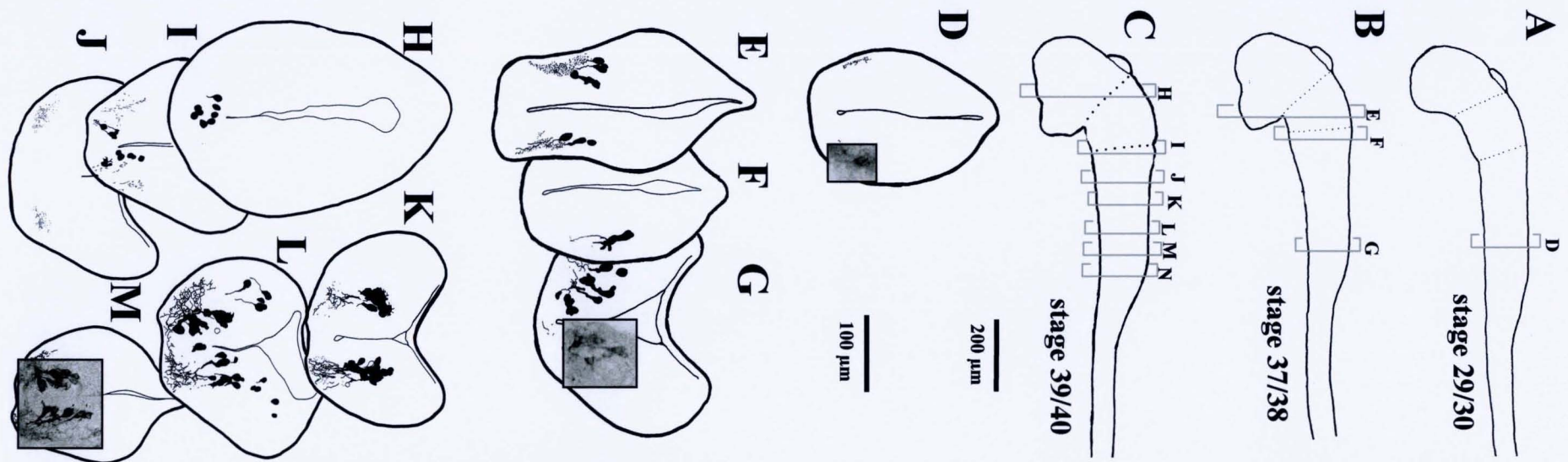
FIGURE 4.3. NADPH-diaphorase reactivity illustrated in cross section at early developmental stages shows the appearance of new populations in the forebrain, midbrain and hindbrain. Cross-sections in this and in subsequent figures were made at 20-25 μm. (A-C) Tracings of photographs taken at 10x magnification illustrate the approximate locations of sections in stage 29/30, 37/38 and 39/40 animals, as depicted below. (D) Single neurons in the caudal hindbrain are located ventrally, with staining restricted to the cell bodies, as illustrated with the photographic overlay. The subsequent development of these neurons is followed with photographic overlays in the remaining figures. (E-F) NADPH-diaphorase reactivity labels two populations that straddle the midbrain/hindbrain border, both with distinct morphologies. (G) The two subgroups of the caudal hinbrain population are identifiable based on their morphology as well as their sequence of appearance. The primary subgroup is more dorsal, with ventral projections, while the secondary subgroup has ventral cell bodies with ventrolateral projections, as illustrated with the photographic overlay. (H) A small population of ventrally located cell bodies appears in the forebrain at stage 39. (I) In addition to the primary and secondary caudal hinbrain subgroups and the rostral hindbrain group, another population of NADPH-diaphorase reactive neurons appears between stages 39 and 40 in the mid-rostral hindbrain. (J) Projections are visible in the marginal zones, which originate from rostral and caudal NADPH-diaphorase reactive groups. (K-M) The three caudal hindbrain subgroups are clearly represented by this sequence of sections taken at stage 40, with primary (see K), primary, secondary and tertiary (see L), and secondary (see M) subgroups identifiable based on their distinct locations and morphologies. Note in the photographic overlays that the multi-nucleated appearance of some neurons actually represents yolk granules.
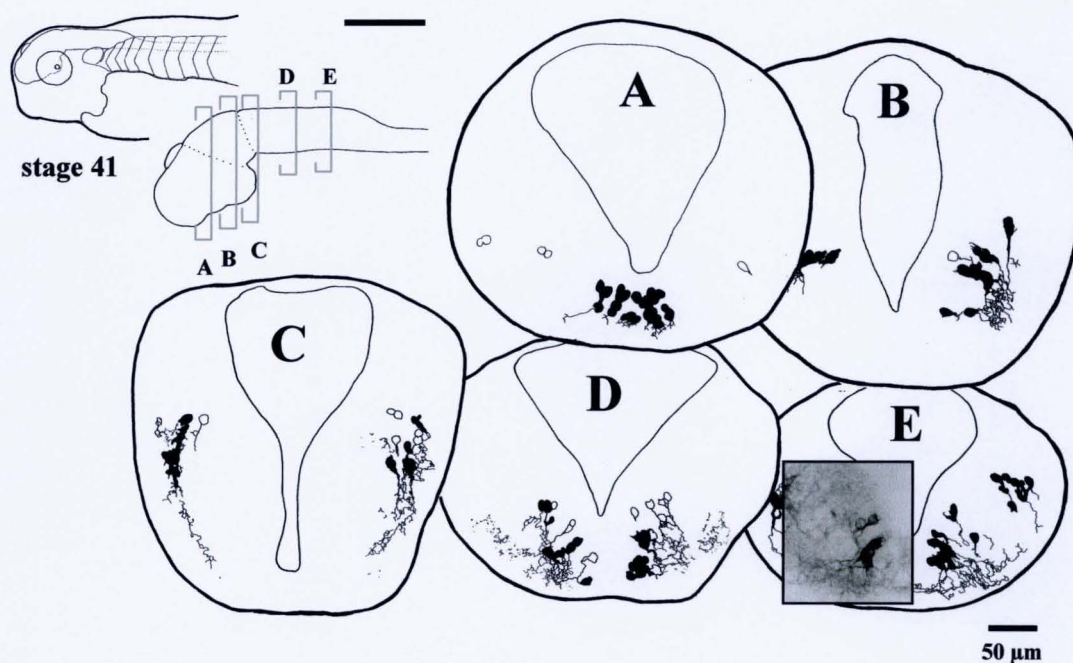
FIGURE 4.4. NADPH-diaphorase reactivity at stage 41. Staining can be seen in the forebrain (A), the caudal midbrain (B), the rostral hindbrain (C), the mid-rostral hindbrain (D), and the caudal hindbrain (E). This is the beginning of a dramatic increase in staining intensity, cell number (see Table 5) and processes. The approximate locations of the sections are indicated in the schematic insert. Scale bar, 1 mm (top schematic drawing) and 100 μm (bottom schematic drawing).
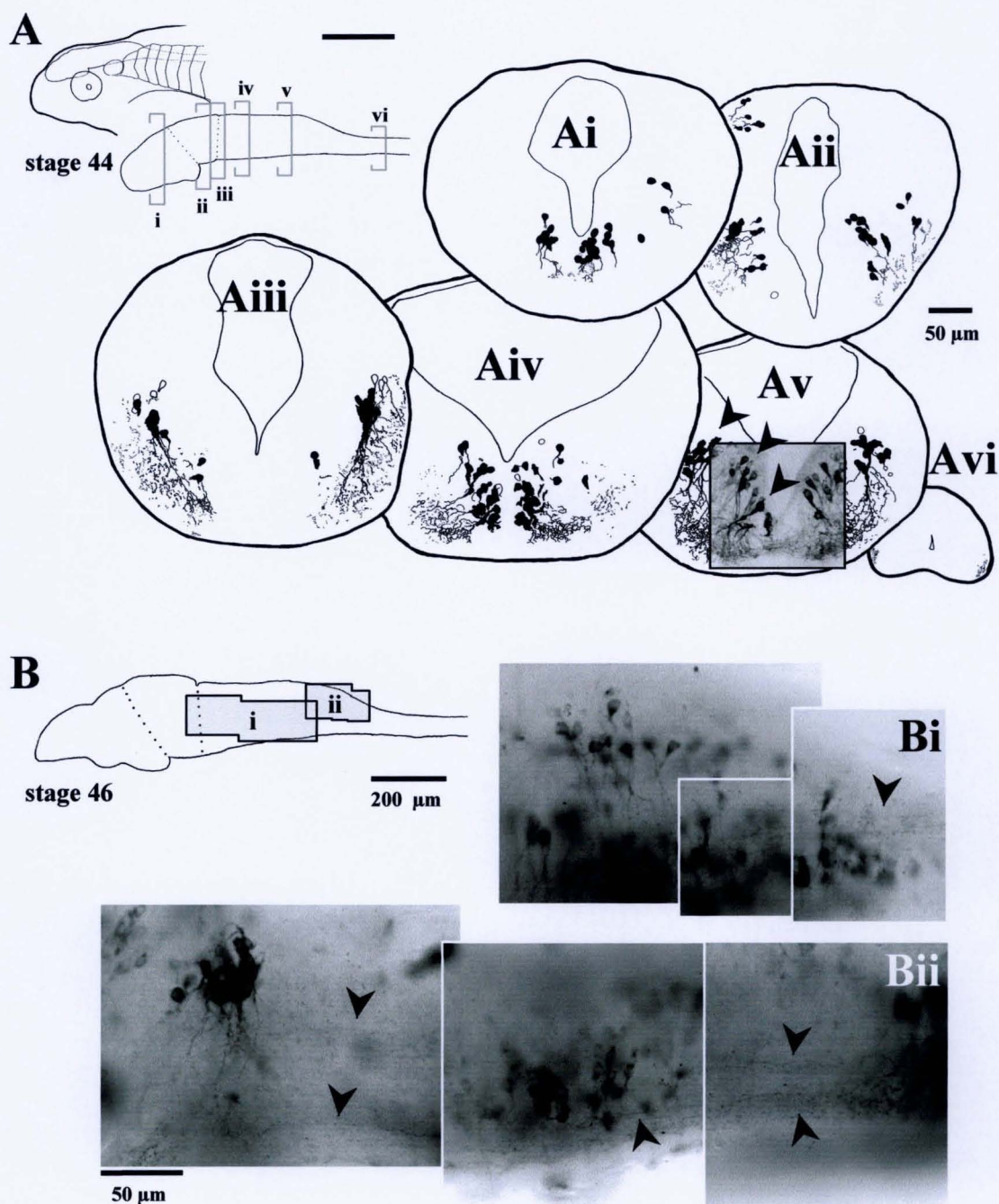
FIGURE 4.5. NADPH-diaphorase reactivity shows a rapid developmental increase. (A) By stage 44, there is a continued increase in staining in the aforementioned populations (see Fig. 4.4A-E for description), however there is also the appearance of cells in the dorsal midbrain (Aii), and pronounced axonal projections in the spinal cord (Avi). Scale bar, 1 mm (top schematic drawing) and 100 μm (bottom schematic drawing). Note in Av that the three subgroups of the caudal hindbrain population are distinguishable (at arrowheads). (B) At stage 46, illustrated as photographic composites in wholemount, the three hindbrain groups are still present, and caudal projections from the caudal hindbrain group (Bi, at arrowhead) and projections connecting the hindbrain populations (and possible midbrain and forebrain populations) can clearly be seen (Bii, at arrowheads).
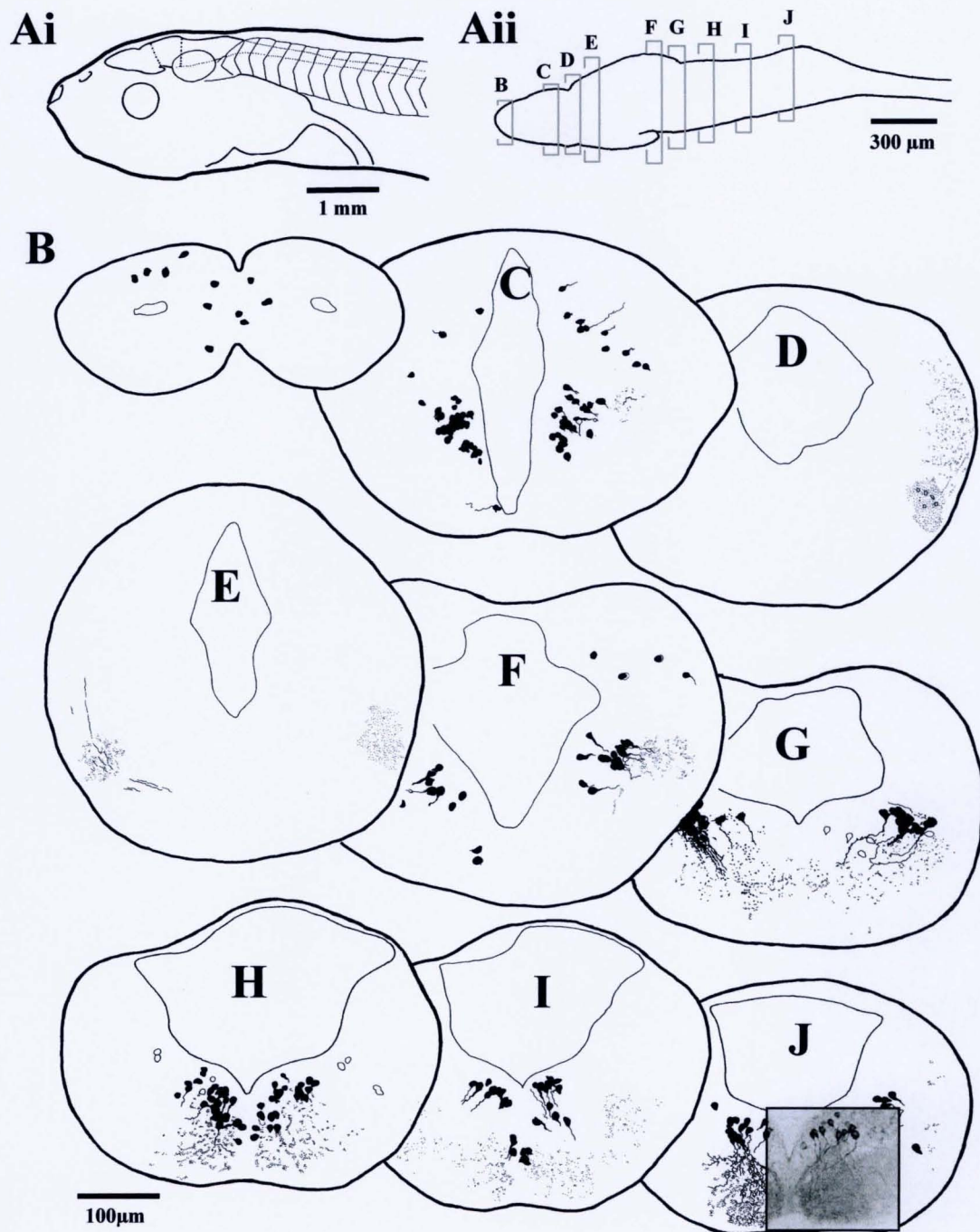
FIGURE 4.6. NADPH-diaphorase reactivity consistently labels the same populations late in larval life. (A) Schematic drawing illustrating the CNS in a stage 47 animal (Ai), and the approximate position of sections illustrated below (Aii). (B) Scattered neurons in the forebrain were apparent by stage 46 throughout the developing telencephalon. (C) A new population of forebrain neurons was located approximately 200-250 μm from the front of the forebrain, just before the beginning of the developing diencephalon. (D) In close proximity, but across the telencephalon/diencephalon border, was a discrete region of diffuse labelling not restricted to cell bodies. (E) The projections that coursed along the marginal zones are evident here in cross section. (F-J) Neuronal populations described in previous figures are also present at stage 47, however projections have become less intense, and contralateral projections are not obvious (see photo overlay in J).
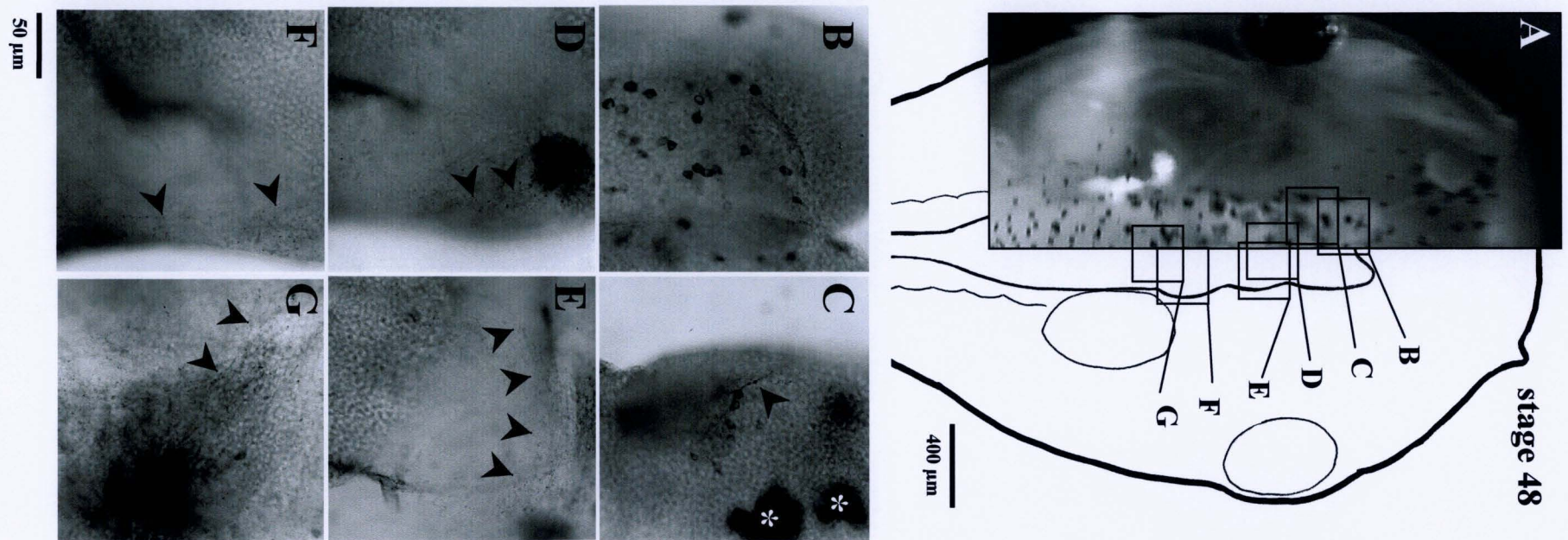
FIGURE 4.7. The forebrain and midbrain populations at stage 48. (A) A schematic drawing with photo overlay illustrating the approximate positions of the photographs detailed below. Note the darkly pigmented melanophores that cover the CNS before dissection and processing. (B) Scattered neurons in the telencephalon. (C) Neurons in the group described in Figure 4.5C appear to have ascending processes (black arrowhead). (D) The discrete region of diffuse staining with processes in the marginal zones (black arrowheads). Note the melanophores (white asterisks). (E) Contralateral processes that cross at the level of the telencephalon/diencephalon border (black arrowheads). (F) Ascending processes that cross the midbrain/hindbrain border (black arrowheads). (G) Contralateral processes that originate from the rostral hindbrain population are still present at this stage of development (black arrowheads).
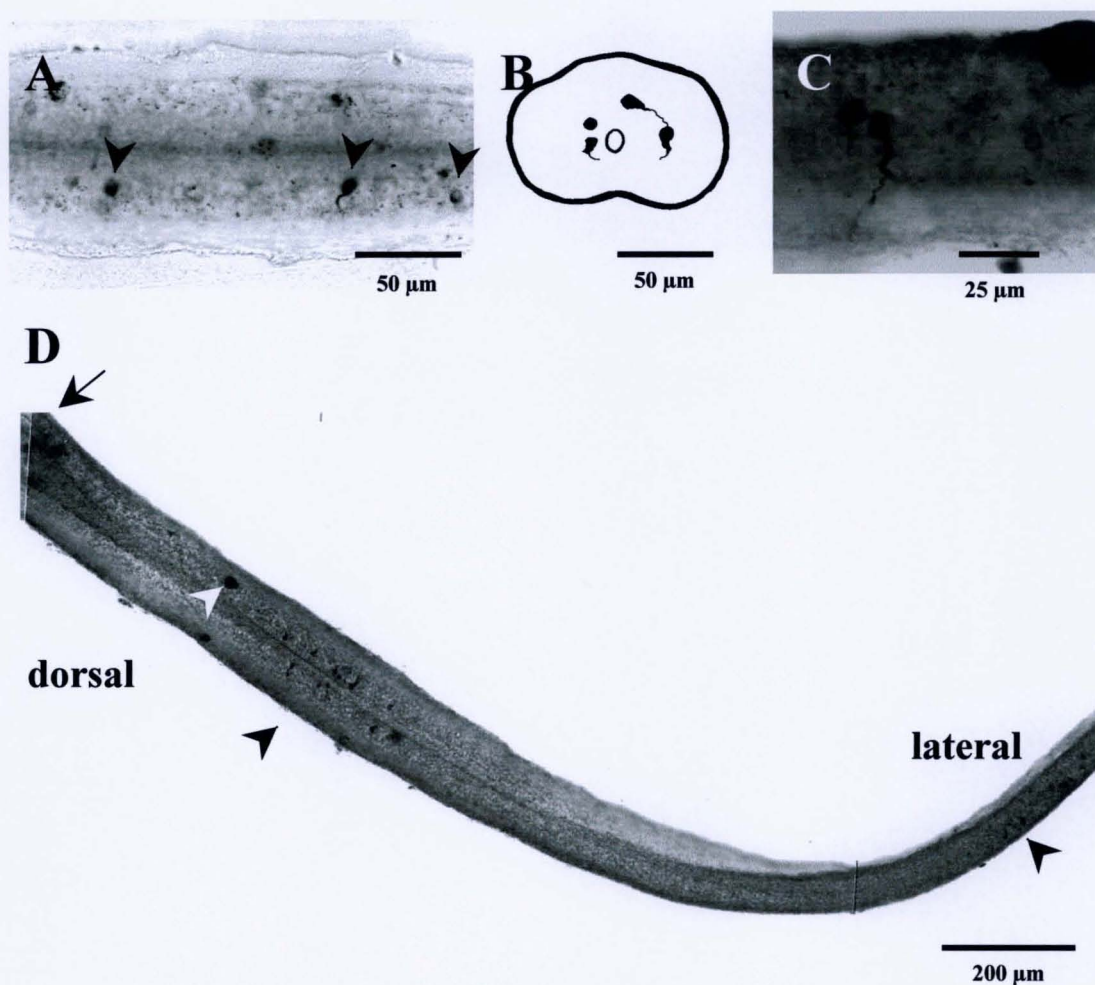
FIGURE 4.8. Neurons label with NADPH-diaphorase reaction product in the spinal cord. (A) At stage 47 faintly labelled neurons are present on both sides of the spinal cord as viewed from a dorsal aspect. Note the irregular interval at which they occur (black arrowheads). (B) In cross section the position of labelled neurons can be described as both dorsal and ventral, with ventrally projecting axons. (C) At stage 48 the NADPH-diaphorase reactivity is more pronounced, as illustrated in the dark ventrally projecting axon that crosses out of the plane of focus. (D) The spinal cord at 10x magnification further illustrates both the gap from the hindbrain boundary (at arrow) and the pronounced gap between groups of irregularly spaced NADPH-diaphorase reactive cells (at black arrowheads). Note that the spinal cord twists as you view from left to right (dorsal to lateral view). Note also the melanophore (at white arrowhead).
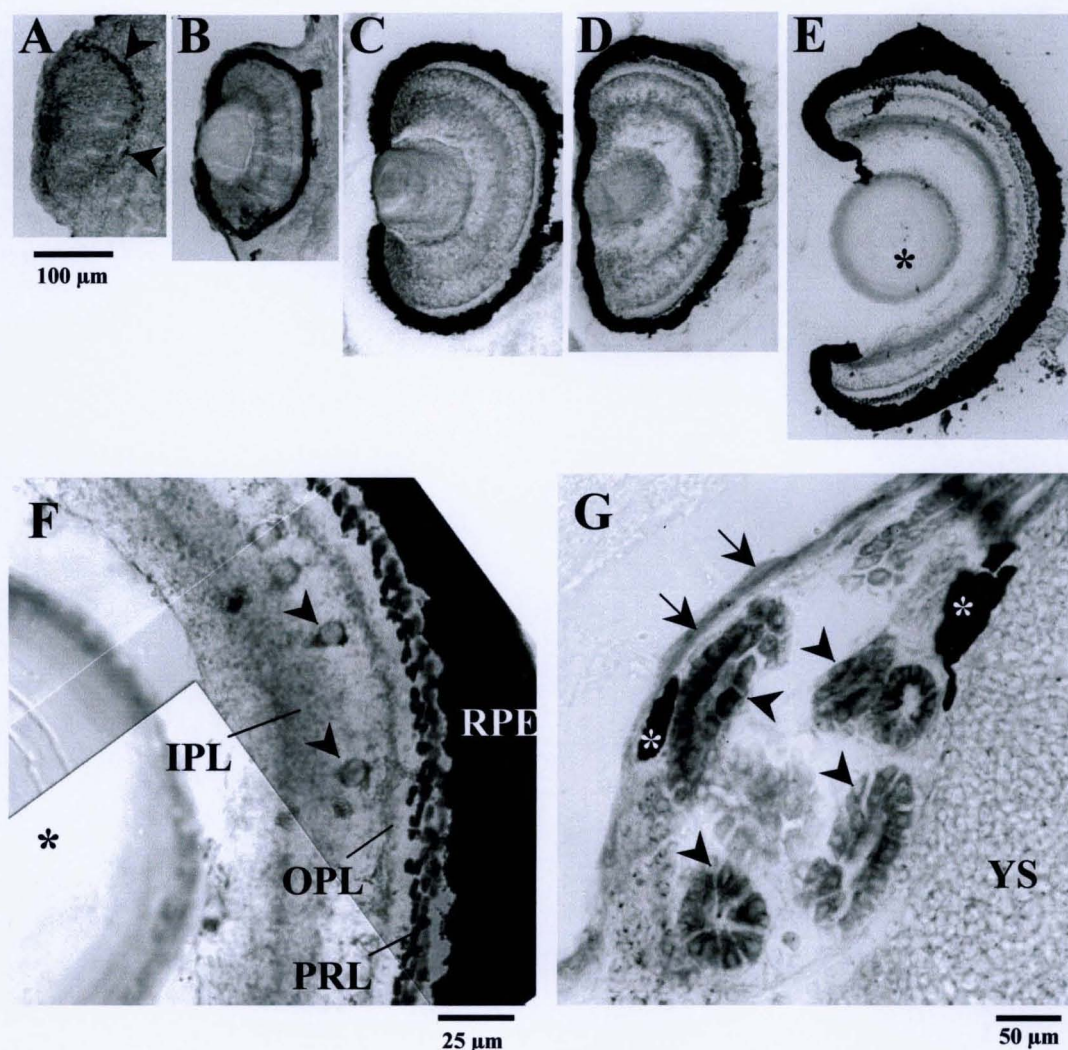
FIGURE 4.9. Areas outside the CNS also label with NADPH-diaphorase. (A) In the eyes at stage 32 there is no clear presence of NADPH-diaphorase reactivity, however the pigment granules that will eventually form the retinal pigmental epithelium (RPE) are obvious (at arrowheads). (B) At stage 37/38 two diffuse bands of staining in the inner and outer plexiform layer can be distinguished. (C) These bands are still present at stage 41 and by stage 44 (D) NADPH-diaphorase reaction product labels the photoreceptive layer at the back of the retina. (E) These three layers are clearer at stage 47. Note also the developmental maturation of the lens (black asterisk; also in F). (F) In a different preparation the diffuse banding of the inner (IPL) and outer (OPL) plexiform layers, cells in the inner nuclear layer (at arrowheads), and dark staining in the photoreceptive layer (PRL) is clearer. (G) The endothelial cells of blood vessels located below the muscle blocks in close proximity to the yolk sac (YS) also stained prominently for NADPH-diaphorase (black arrow heads), as did the epithelial cells of the skin (black arrows). Note the dark black pigmented melanophores (white asterisks). A-E, 10x magnification; F-G, 40x magnification.

A

stage 47

sc

stage 44

tect

hb

stage 33/4

stage 39/40

stage 29

stage 40

stage 37/38

stage 35/36

di

tegm

tel

stage 44

stage 39

stage 46

100 μm

B

| early swimming | hatching | free swimming | limb bud |

| embryonic period | | | larval period |

| 28 | 29/30 | 31 | 32 | 33/34 | 35/36 | 37/38 | 39 | 40 | 41 | 42 | 43 | 44 | 45 | 46 | 47 | 48 |

stage

FIGURE 4.10. NADPH-diaphorase staining and its relation to tadpole behaviour. (A) A schematic drawing summarising the approximate sequence and presence of staining in the telencephalon (tel), diencephalon (di), tegmentum (tegm), tectum (tect), hindbrain (hb) and spinal cord (sc) of the pre-metamorphic tadpole up to stage 48. (B) A diagram illustrating the major behavioural changes and the associated stages of development.
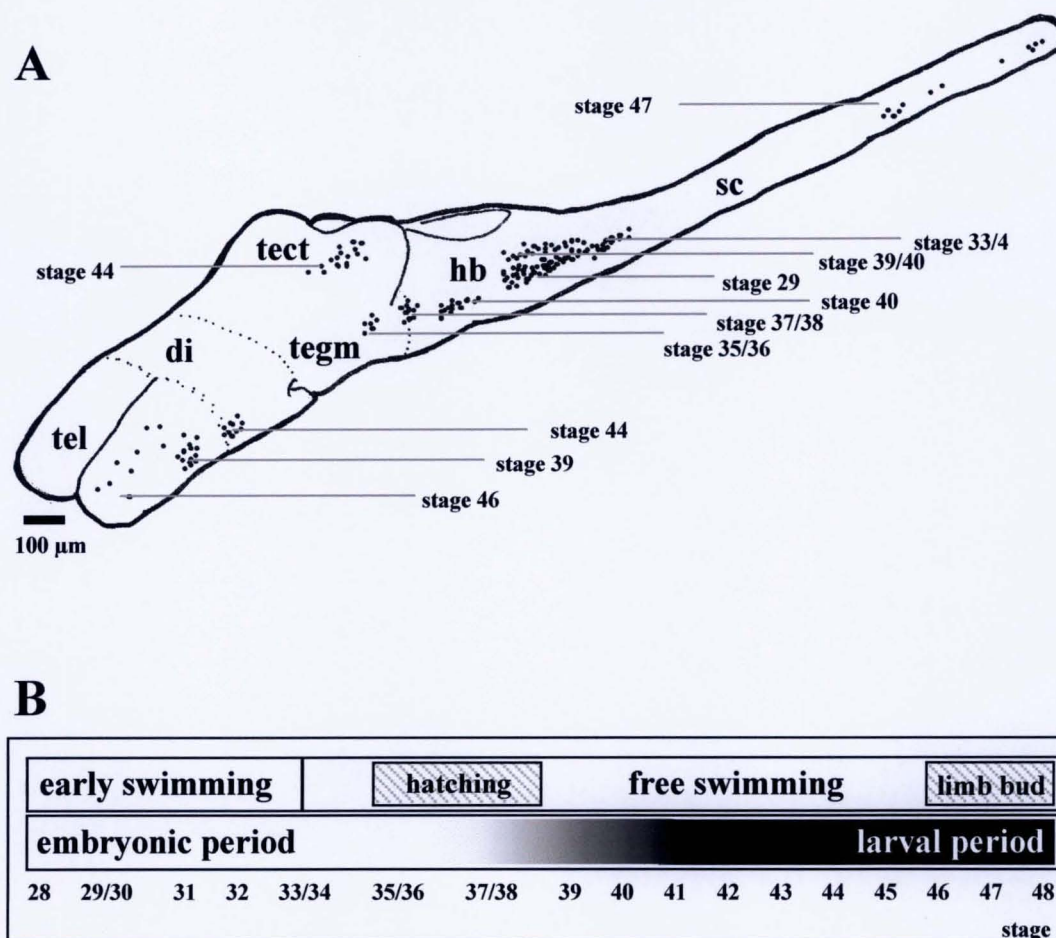
5.1. SUMMARY

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 effects of the NO donors SNAP (100-500µM) and DEANO (25-100µM) were examined on motor activity in *Rana temporaria* embryos. Immobilised *Rana* 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 ipsilaterally down the length of the body. The inactive precursor of SNAP, NAP at equivalent concentrations did not trigger motor activity. NO donors failed to initiate swimming behaviour and had no measurable effects on the parameters of swimming induced by electrical stimulations. The synaptic drive seen in motor neurons after dimming of the illumination was very similar to that induced by the NO donors. Intracellular recordings with KCl-filled electrodes also showed that inhibitory potentials were strongly depolarising suggesting that inhibition was chloride ion-dependent. NADPH-diaphorase histochemistry identified putative endogenous sources of NO in the CNS and the skin. Within the CNS, three populations of bilaterally symmetrical neurons were identified within the brainstem. Members of the brainstem populations had contralateral projections and many had axonal processes that projected to and entered the marginal zones of the spinal cord, suggesting they were reticulospinal. Additionally, preliminary data on their development are presented.

5.2. INTRODUCTION

Animals must be able to initiate and terminate motor patterns according to prevailing

behavioural and developmental requirements. Even among closely related species, the

expression and emergence of different motor behaviours 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 can 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

behaviours. For example, at the time of hatching, the tadpole of *Rana temporaria* (stage

20: Gosner, 1960) is capable of generating a well co-ordinated swimming motor pattern

(Soffe, 1991; Soffe and Sillar, 1991). However, fictive swimming in *Rana* 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 more developed descending serotonergic projections in *Rana*, as 5-HT is known to

facilitate motor burst generation in both species (Woolston *et al.*, 1994; Sillar *et al.*, 1993).

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 review, 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 inhibitory influence on the spinal swimming circuitry of hatchling

Xenopus tadpoles, presumably from populations of NADPH-diaphorase reactive neurons

in the brainstem (CHAPTER 2 and 3; McLean and Sillar, 2000).

The similarities and differences between these two species of amphibian tadpole, in

addition to the recent discovery of a role for NO in the swimming circuitry of *Xenopus*,

provided the impetus for the present study. The effects of NO donors on spinal motor

activity have been examined in the embryo of the frog *Rana temporaria*. In contrast to its

inhibitory role in *Xenopus*, NO has an excitatory role in *Rana*, initiating a motor pattern

qualitatively similar to the light dimming response. Using the NADPH-diaphorase

histochemical reaction as a simple, yet reliable, marker for the enzyme responsible for NO

generation, NOS, (Hope *et al.*, 1991), putative sources of NO have also been identified

within the CNS. Remarkably, three homologous populations of neurons with similar

developmental profiles stain in the CNS of both species, suggesting 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 (McDearmid and Sillar,

1997; McDearmid, 1998).

5.3. MATERIALS AND METHODS

5.3.1. ANIMALS

Rana temporaria spawn, available between the months of February and April, was

purchased from Blades Biological or collected from local ponds. Eggs were raised in

pond water at temperatures of 8-23 °C until they were of the correct experimental stage

(stages 17-22, Gosner, 1960; Fig. 5.1A).

5.3.2. ELECTROPHYSIOLOGY

Animals were prepared for experimentation as previously detailed (see CHAPTERS 2 and

3). Briefly however, animals were first anaesthetised in MS-222 (0.01-0.1%) before the

dorsal fin was nicked to facilitate neuromuscular block with α-bungarotoxin (*ca.* 12.5

μM). Animals were then pinned through the notocord to a rotatable Sylgard (Dow-

Corning) platform in an experimental bath through which 100 ml of frog Ringer solution

(ionic composition described previously in MATERIALS AND METHODS sections 2.3.2 and

3.3.1) was recirculated. 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 (*ca.* 50 μm tip openings) placed over ipsilateral (see Fig. 5.1B; L6 and

L8) intermyotomal clefts. Activity was initiated by applying a brief (*ca.* 1 ms) current

pulse (Digitimer DS2 isolated stimulator) to the tail skin using another glass suction

electrode (see Fig. 5.1B). The rostral 4 to 6 myotomes (as numbered from the otic

capsule) were removed, and intracellular recordings were made from presumed motor

neurons in the ventral spinal cord using electrodes filled with 3M KCl (resistances 100-

120 MΩ; see Fig. 5.1B). Penetrations were made using capacity overcompensation.

Electrophysiological data were recorded and stored conventionally, as described

previously (see MATERIALS AND METHODS sections 2.3.2 and 3.3.3). In experiments

investigating the effects of drugs on swimming, three episodes were analysed before,

during drug application and after washing in control saline. In order to quantify the

similarity of the NO-induced response to light dimming, five experiments were selected in

which 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. The

'Students' *t*-test was used to determine statistical significance ($P<0.01$). 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 NAP

(Sigma, UK) were either dissolved in distilled water or 0.01% DMSO (McLean and

Sillar, 2000). Diethylamine nitric oxide complex sodium (DEANO; Molecular Probes,

The Netherlands) was dissolved in distilled water.

5.3.3. ANATOMY

The anatomical processing for *Rana* tadpoles is sufficiently different to warrant a brief

description here. Animals were first staged according to Gosner (1960), anaesthetised in

MS-222, scored along the dorsal fin to aid fixative penetration, and fixed at room

temperature (20-22°C) for 1-2 hours in ice-cold 4% paraformaldehyde. The animals were

then rinsed in 30% sucrose in 0.1M PB (pH 7.4) to remove aldehydes, and processed

either as wholemounts or as cross sections. For wholemount histology, animals were

transferred to a dissecting bath containing PB and pinned to a Sylgard-coated platform.

The central nervous systems were carefully dissected out to the level of myotomes 6-8

using fine tungsten pins, immersed in the solution for NADPH-diaphorase histochemistry

(as described in MATERIALS AND METHODS section 2.3.1) and kept at 37 °C in a humid

chamber for 1-3 hours. They were then rinsed in PB and placed in 1% potassium

permanganate and then 2% oxalic acid (30 and 15min respectively) to remove the melanin

pigment. The wholemounts 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 hours prior to histochemical processing. The

slides were incubated in a solution for NADPH-diaphorase histochemistry and kept at 37

°C in a humid chamber for 1-3 hours. 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 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.

5.4. RESULTS

5.4.1. NO 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. 5.1C, D; Soffe, 1991; Soffe and Sillar, 1991). The first is a low frequency non-rhythmic "lashing" motor pattern characterised by long bursts of activity (*ca.* 0.5 - 1 second) which would not result in any net forward propulsion (Fig. 5.1C). 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. 5.1D). 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 of the experimental bath illumination (Soffe, 1991). No other motor patterns are known to be generated at this stage of development (Soffe, 1991). In order to confirm previously described effects of NO on fictive motor output (McDearmid and Sillar, 1997; McDearmid, 1998), the NO donors SNAP (N=6) and DEANO (N=2) 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. 5.2Ai, D) and occasional spontaneous motor bursts were observed in some preparations (Fig. 5.2Ai). Shortly after exposure to SNAP (100-500 µM; 1-2 minutes), non-rhythmic motor activity characterised by ventral root bursts of 400-500 ms duration was always observed (Fig. 5.2Aii, E). These bouts of activity typically lasted up to 10 minutes, although bout duration has not been quantified any further. However, the activity induced by SNAP rapidly disappeared upon returning

to control saline (Fig. 5.2Bi). In order confirm previous tests for possible non-specific

actions of SNAP (McDearmid, 1998), NAP (N=2), the inactive precursor of SNAP, was

bath applied to *Rana* embryos. NAP did not initiate any bouts of motor activity in the

same concentration range as SNAP (100-500 µM; Fig. 5.2Bii). These results indicate that

the ability of SNAP to induce motor output is due to its release of NO rather than any non-

specific actions of the donor molecule. Furthermore, experiments using another nitric

oxide donor, DEANO (25-100 µM), demonstrated that this NO donor also induced a non-

rhythmic pattern of motor discharge that was indistinguishable from the SNAP-induced

motor activity (Fig. 5.2C). The motor bursts induced by the NO donors were non-

rhythmic, in that they propagated rostrocaudally (Fig. 5.2E), but occurred at irregular

intervals (Figs. 5.2Aii, B, C). In addition, motor bursts never occurred synchronously

across the two sides of the spinal cord (McDearmid, 1998; McLean *et al.*, 2001).

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. 5.3Ai, ii) in

that no significant difference (*t*-test, P>0.01; N=6) was detected in burst duration (Fig.

5.3Bi), rostrocaudal delay (Fig. 5.3Bii) or cycle period (Fig. 5.3Biii) 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 NO's role in *Rana* is

distinct from that in *Xenopus* tadpoles where it has been shown that NO does not trigger

motor activity but has profound modulatory effects on ongoing fictive swimming

(CHAPTER 2; McLean and Sillar, 2000).

The similarity of 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 (t-test, P>0.01; N=5) was detected in either parameter (Fig. 5.4A,B) during the SNAP-induced and the dimming-induced, non-rhythmic motor patterns. Note, however, that there was a significant difference (t-test, P<0.001) in both burst durations (Fig. 5.4A) and inter-burst intervals (Fig. 5.4B) 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*.

5.4.2. SYNAPTIC DRIVE UNDERLYING NO-INDUCED MOTOR ACTIVITY

Intracellular recordings from motor neurons using KCl-filled microelectrodes revealed that the bursts of ventral root discharge that occurred either spontaneously, in response to dimming (Fig. 5.5A) or induced by SNAP (Fig. 5.5B) were accompanied by phasic depolarisations in motor neurons that were often, but not always, sufficient to trigger action potentials (McLean *et al.*, 2001). During the inter-burst intervals, periods of sustained membrane depolarisation usually occurred in the absence of any corresponding motor activity (Fig. 5.5B, small arrows). 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. The inter-burst inhibition is likely to be chloride ion dependent as it was sign reversed in intracellular recordings where KCl was used as the electrolyte (N=2, Fig. 5.5). In such recordings, periods of phasic excitation and inhibition were both characterised by bursts of impulses superimposed on a prolonged depolarisation, which often, but not always, crossed spike threshold (Fig. 5.5A, B). The use of KCl-filled electrodes also unveiled frequent depolarising potentials in the inter-burst intervals (*e.g.* Fig. 5.5C, D). Presumably these are spontaneously occurring IPSPs. Aside from these depolarising

events, there was no evidence of a tonic or sustained change in membrane potential caused

by the addition of NO donor drugs (McLean *et al.*, 2001). 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

(Fig. 5.5C, D) and had no effect on swimming in contrast to *Xenopus*.

5.4.3. DISTRIBUTION OF NADPH-DIAPHORASE STAINING IN *RANA* EMBRYOS

The CNS of *Rana temporaria* embryos is pigmented brown, partially obscuring the fine

localisation of the dark blue NADPH-diaphorase reaction product. Cross-sections were

therefore cut as thinly as possible (10-15 μm) in order to aid 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. 5.6A, 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.

NADPH-diaphorase staining in the CNS was almost entirely restricted to three bilaterally

symmetrical populations of brainstem neurons (Fig. 5.7). Only a few cells were labelled in

the forebrain at stage 20, and these will be discussed below. There was substantial

staining within the marginal zones of the spinal cord, (Fig. 5.7Aii) 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. 5.7Ai). A proportion of the neurons also had

contralaterally projecting processes that could be detected as they coursed beneath the neurocoel (Fig. 5.7Ai; arrowed). Rostral to this cell group there followed a region where there was intense labelling of projections in the marginal zones (Fig. 5.7Biii), followed by the next population of cell bodies located in the rostral hindbrain (Fig. 5.7Bii). 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 with long primary neurites that projected ventrally into the marginal zones (Fig. 5.7Bi).

The detailed topography of these cell groups became clear when the CNS was processed in wholemount (N=20). Since *Rana* embryos are darkly pigmented, the excised wholemount 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 did often diminish the staining of finer processes. However, in some cases (4 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. 5.7C), with cell bodies extending 300-400 μm (Fig. 5.7A, B), and axons projecting caudally into the marginal zones of the spinal cord (Fig. 5.7A). Rostral to this cell group there followed a 90-100μm region with intense labelling of projections in the marginal zones (Fig. 5.7B), 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. 5.7A, B, 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 and extended for 40-50 μm and comprised approximately 15-20 neurons (5-10 per side, Fig. 5.7A, B, C).

5.4.4. THE DEVELOPMENT OF NADPH STAINING IN *RANA TEMPORARIA*

Preliminary results suggest that NADPH-diaphorase reactivity may follow a similar developmental profile to that described for *Xenopus laevis* (see CHAPTER 4). The first neurons to show NADPH-diaphorase reactivity are located in the caudal brainstem at stage 17 (N=2; Fig. 5.8A). Subsequent development at stage 18 (N=2; Fig. 5.8B) shows axonal projections that extend into the marginal zones. Between stages 19 and 20 the hind/midbrain boundary and rostral hindbrain populations became NADPH-diaphorase positive, as previously described (Fig. 5.7). By stage 22 (N=2) there are populations in the midbrain (Fig. 5.8C, D) that show pronounced NADPH-diaphorase reactivity, as well as neurons in the spinal cord (Fig. 5.8F), the location and morphology of which suggests they may be motor neurons (Fig. 5.8E). Further histological experiments will need to be carried out to confirm that *Rana* and *Xenopus* have similar NADPH-diaphorase reactive populations of neurons appearing at equivalent developmental stages.

5.5. DISCUSSION

The results presented here provide evidence that NO can induce fictive motor activity reminiscent of that known to drive the strong body flexions in *Rana* embryos, which occur spontaneously, in response to an external stimulus and following dimming of the illumination (Soffe, 1991; Soffe and Sillar, 1991) and 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 and unaffected by the bath application of SNAP or DEANO (see Fig. 5.3).

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. 5.7). Collectively,

these results provoke intriguing questions including whether NO might be an endogenous

trigger for initiating hatching motor behaviour in *Rana* and, if so, whether it does so

directly or by enhancing the nervous systems responsiveness to other transmitters? In

addition, how can NO mediate different behavioural responses in *Rana* and *Xenopus*, two

closely related species, at equivalent developmental stages, when homologous nitrergic

cell groups are likely to be involved?

5.5.1. NADPH REACTIVITY AND PUTATIVE NITRERGIC CO-LOCALISATION

It seems reasonable to assume that NO is not the sole or principle neurotransmitter utilised

by neurons in the three brainstem clusters in *Rana* (CHAPTER 5; McLean *et al.*, 2001) or in

Xenopus (CHAPTER 2; McLean and Sillar, 2000). In *Xenopus*, the early development of

the nervous system has been sufficiently well described to allow tentative suggestions to

be made regarding the identity of potential co-transmitters in nitrergic neurons (McLean

and Sillar, 2000). Based on 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 that have been shown to be involved in

the termination of swimming (Boothby and Roberts, 1992a, b). 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 (González *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* 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* (McLean and Sillar, 2000). It is plausible that the homologies extend to the co-transmitters of these neurons as well. In support, immunocytochemical studies using anti-serotonin antibodies in *Rana* embryos (Woolston *et al.*, 1994) have revealed a population of brainstem neurons resembling in position and morphology both the raphe neurons of *Xenopus* (van Mier *et al.*, 1986) and the middle group of NADPH-diaphorase reactive neurons described for *Rana* in the present study (see Fig. 5.7Bii).

NO appears to play an excitatory role in *Rana* embryos, initiating a motor response rather than inhibiting one as it does in *Xenopus* (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 5-HT in the neonatal rat (Cazalets *et al.*, 1990, 1992; Kiehn and Kjaerulff; 1996) and in the rabbit (Viala and Buser, 1969). NA 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 NA 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.*, 1992b) or in spinal cats (Barbeau and Rossignol, 1990, 1991). It is possible, therefore, that NO functions as a co-transmitter, initiating a motor response in *Rana via* the 5-HT or NA systems. Indeed it has been shown in *Rana* that NA initiates a pattern of motor activity that is indistinguishable from that induced by NO (McDearmid and Sillar, 1997).

However, in *Xenopus* larvae, NO does not induce motor activity but rather inhibits fictive swimming. The very different actions of NO in each 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 product in the marginal zones of the spinal cord in *Rana*, as detected in cross-sections (see Fig. 5.7Aii). In *Xenopus*, processes project toward, but do not enter, the spinal cord. Therefore NO could predominantly be affecting higher centres in *Xenopus*, while acting additionally at the level of the spinal cord in *Rana*. Additionally, while distinct subgroups appear to make up the reticular group in *Xenopus*, the reticular group in *Rana* appears to only have one distinct morphology. Detailed examination of the developmental profiles of NADPH-diaphorase reactive populations in *Rana* may shed light on whether these are indeed homologous populations.

5.5.2. NO 'SELECTS' A NON-RHYTHMIC MOTOR PATTERN

It seems likely that non-rhythmic and rhythmic motor patterns in *Rana* 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. These 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.

The non-rhythmic activity occurs at the stage that *Rana* embryos normally hatch, suggesting that this may in fact represent a hatching behaviour. If NO is indeed the endogenous trigger for hatching behaviour in *Rana* 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. 5.2, 5.4, 5.5). 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* embryos is also pineal-mediated. Although we have obtained no evidence for NADPH-diaphorase reaction product in the pineal of *Rana* or *Xenopus*, there is evidence from other vertebrates such as the rat where exposure to constant light induces a marked decrease of NOS activity in the pineal gland (Schaad *et al.*, 1994). Perhaps more likely in *Rana* 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* embryos is rich in NADPH-diaphorase staining and is therefore a likely source of NO (see Fig. 5.6A). Due to its gaseous nature and the proximity of the skin to the CNS (see Fig. 5.6B), 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 in the NO-generating pathway exists (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, it has been shown here that NO can initiate non-rhythmic motor responses in the embryo of the frog *Rana temporaria*. The motor pattern induced by this neuromodulator comprises bursts of prolonged ventral root discharge that would be appropriate to drive the non-rhythmic body flexions first described by Soffe (1991). NO

appears to select this behaviour over rhythmic swimming, however it does not interfere

with the generation of swimming behaviour (see Fig. 5.3). For the moment, it is not clear

whether NO acts directly on spinal neurons or causes the release of another

neurotransmitter, but clearly NO is important in the control of motor output in vertebrate

motor systems in ways that are both species- and developmental-stage-dependent.
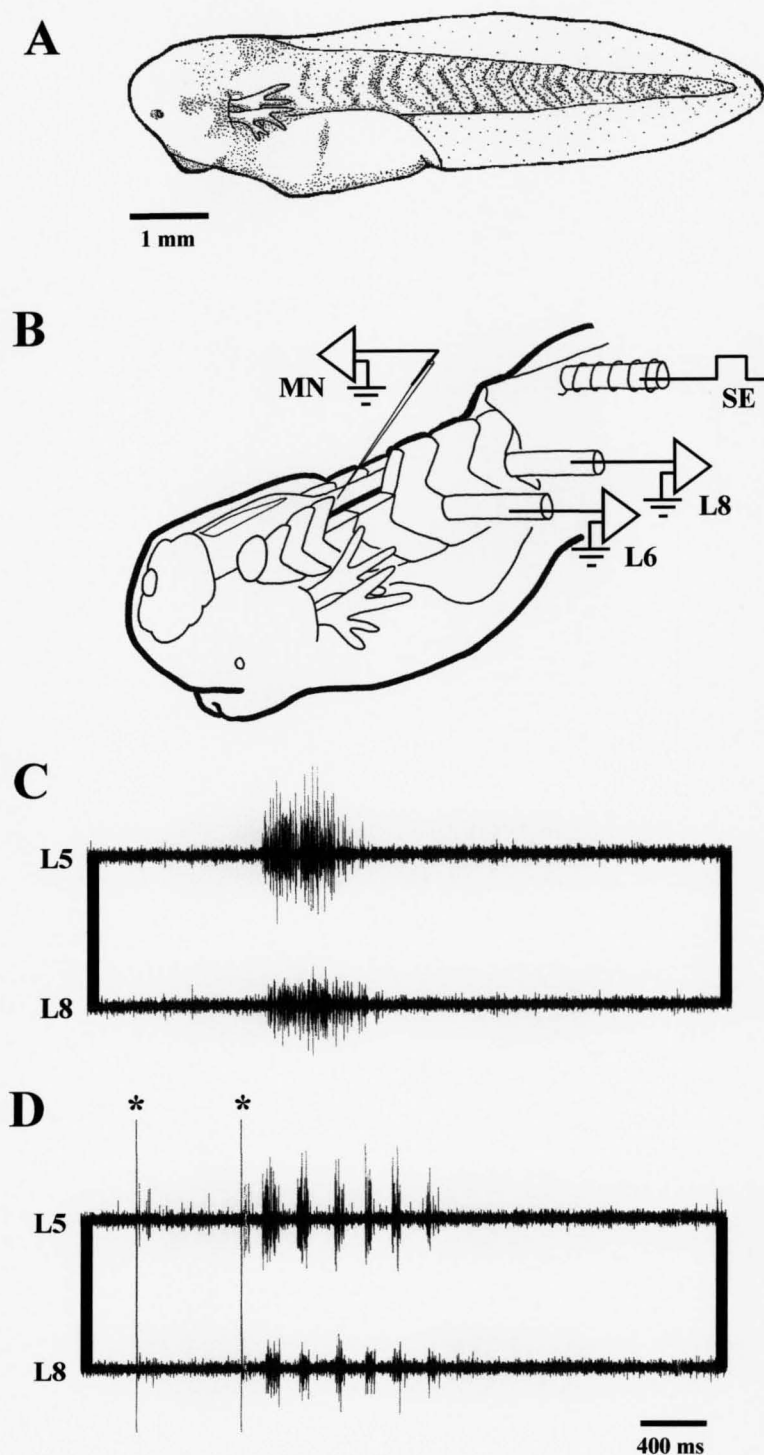
FIGURE 5.1. Two distinct non-rhythmic and rhythmic motor patterns in *Rana temporaria* embryos. (A) Stage 20 *Rana* embryo. (B) Schematic diagram of stage 20 experimental preparation illustrating the CNS in relation to the muscle blocks (see MATERIALS AND METHODS). MN, motor neuron; SE, suction electrode; 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.
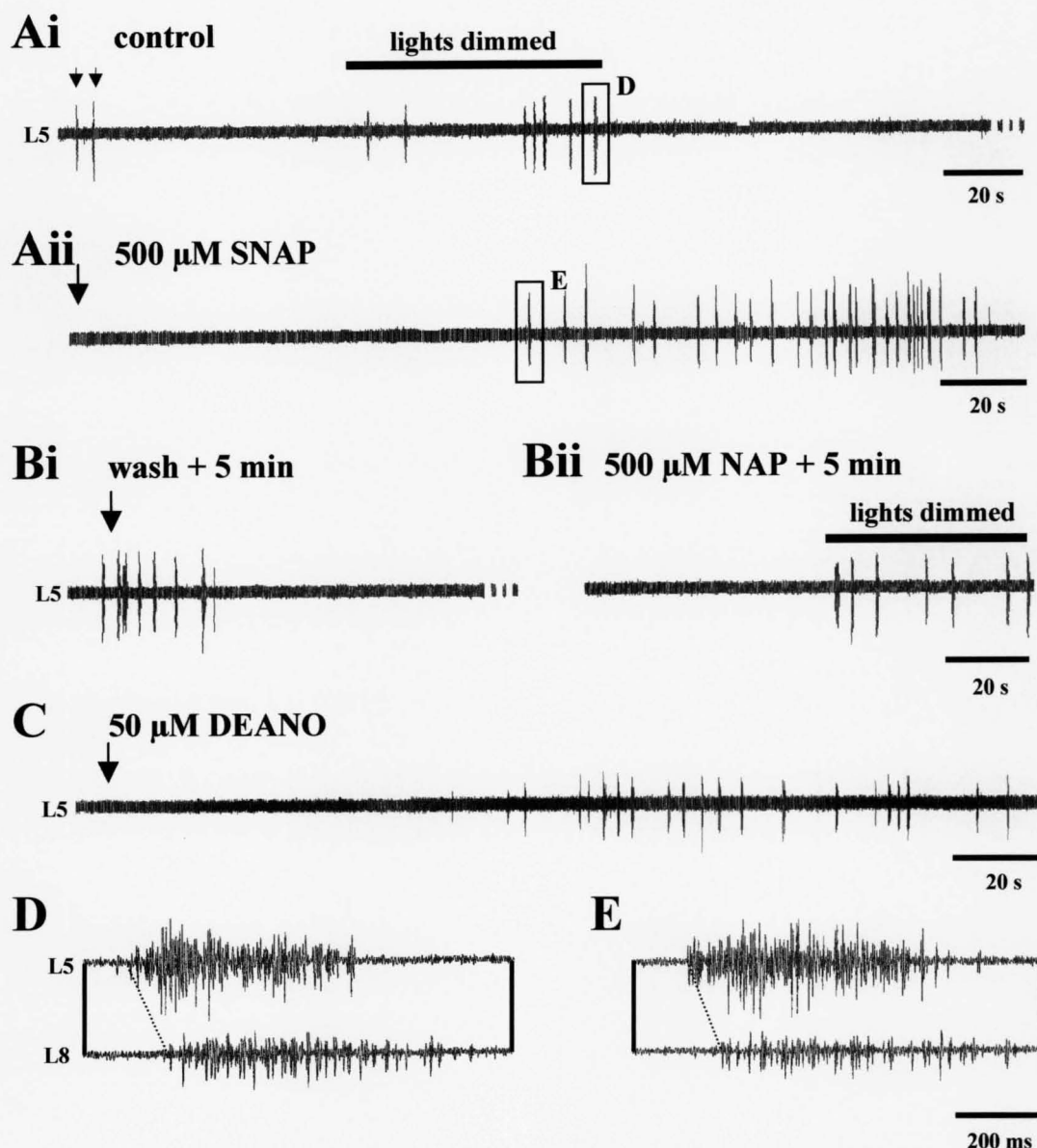
FIGURE 5.2. The NO donors SNAP and DEANO induce a non-rhythmic motor pattern in *Rana temporaria* embryos. (A) Motor responses recorded from L5 before (Ai) and after (Aii) bath application of SNAP (500μM, at large arrow). Note that activity can occur spontaneously, as indicated by the small arrows in 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, NAP (500 μM), did not initiate any motor activity at equivalent concentrations even after 5 minutes, however activity could be elicited by dimming the illumination. (C) In a different preparation, the bath application of DEANO (50 μM, at arrow) elicited similar motor responses to SNAP. For the purposes of illustration, only one ventral root recording is shown. The continuity of preparations is illustrated by breaks in the recording, which represents a 5 minute gap for Ai, Aii and Bi, Bii.
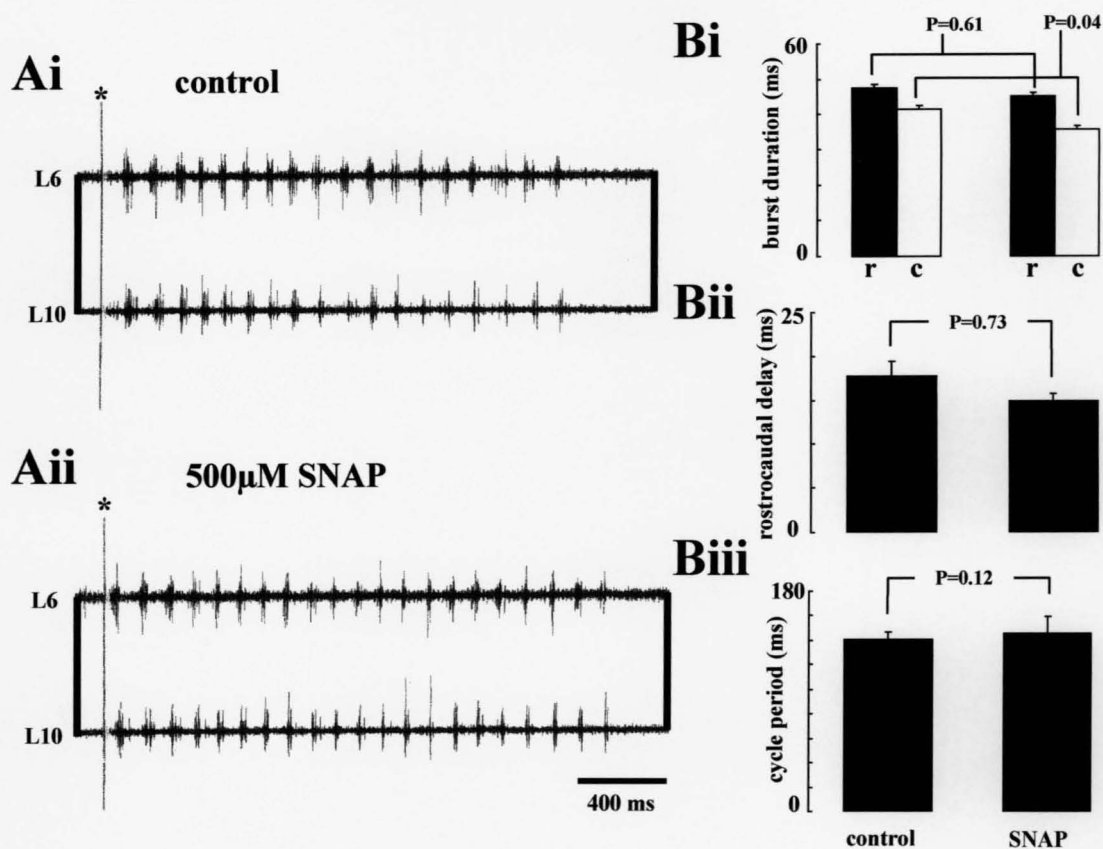
FIGURE 5.3. Fictive swimming activity is unaffected by SNAP. (A) Ventral root recordings made from L6 and L10 before (Ai) and 5 min after exposure to 500 μM SNAP (Aii) are indistinguishable. Stimulation artefacts are indicated by an asterisk. (B) Three full episodes of swimming (approximately 5-15 cycles per episode) were measured before, during and after the bath application of SNAP. No significant differences could be detected for (Bi) burst duration, (Bii) rostrocaudal delay or (Biii) cycle period. Values are means ± S.E.M., N=6 preparations. c, caudal recordings; r, rostral recordings.
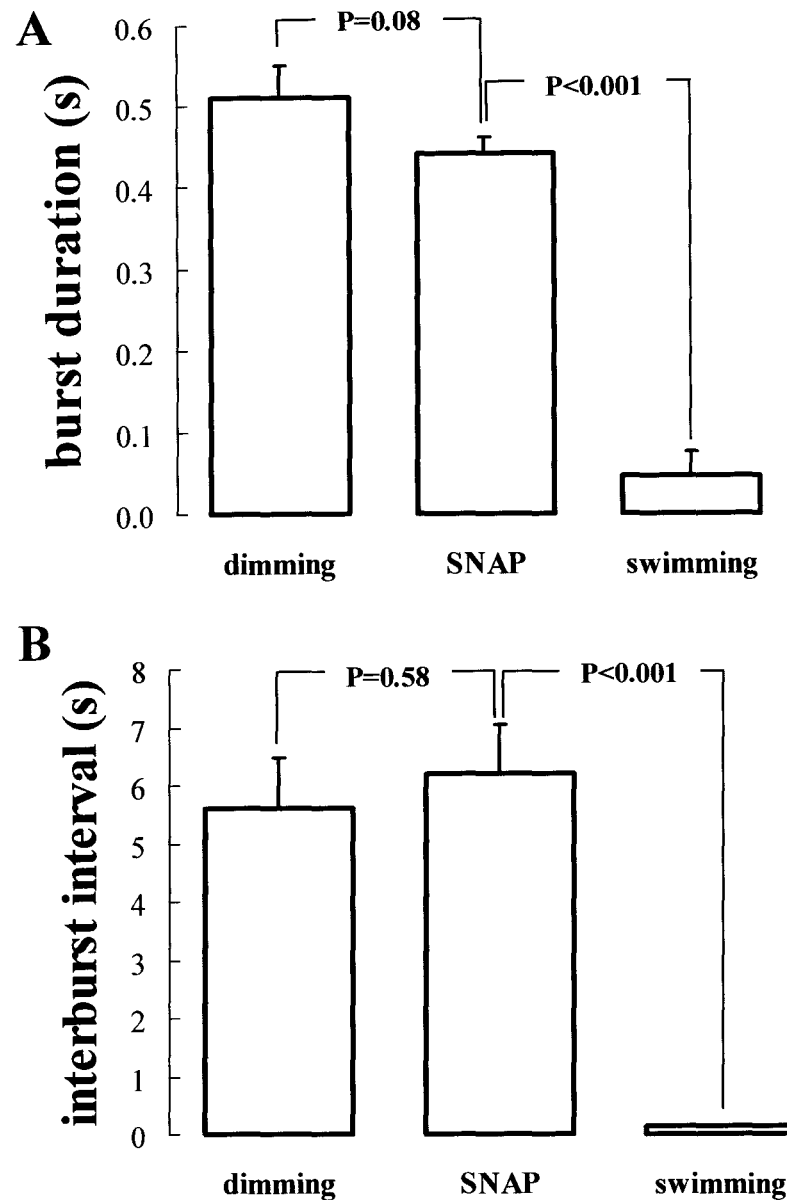
FIGURE 5.4. SNAP induces a non-rhythmic activity not significantly different from the dimming response. Measurements from five different preparations illustrate that the burst duration (A) and interburst interval (B) of non-rhythmic motor activity induced by SNAP (500 μM) are not significantly different 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.
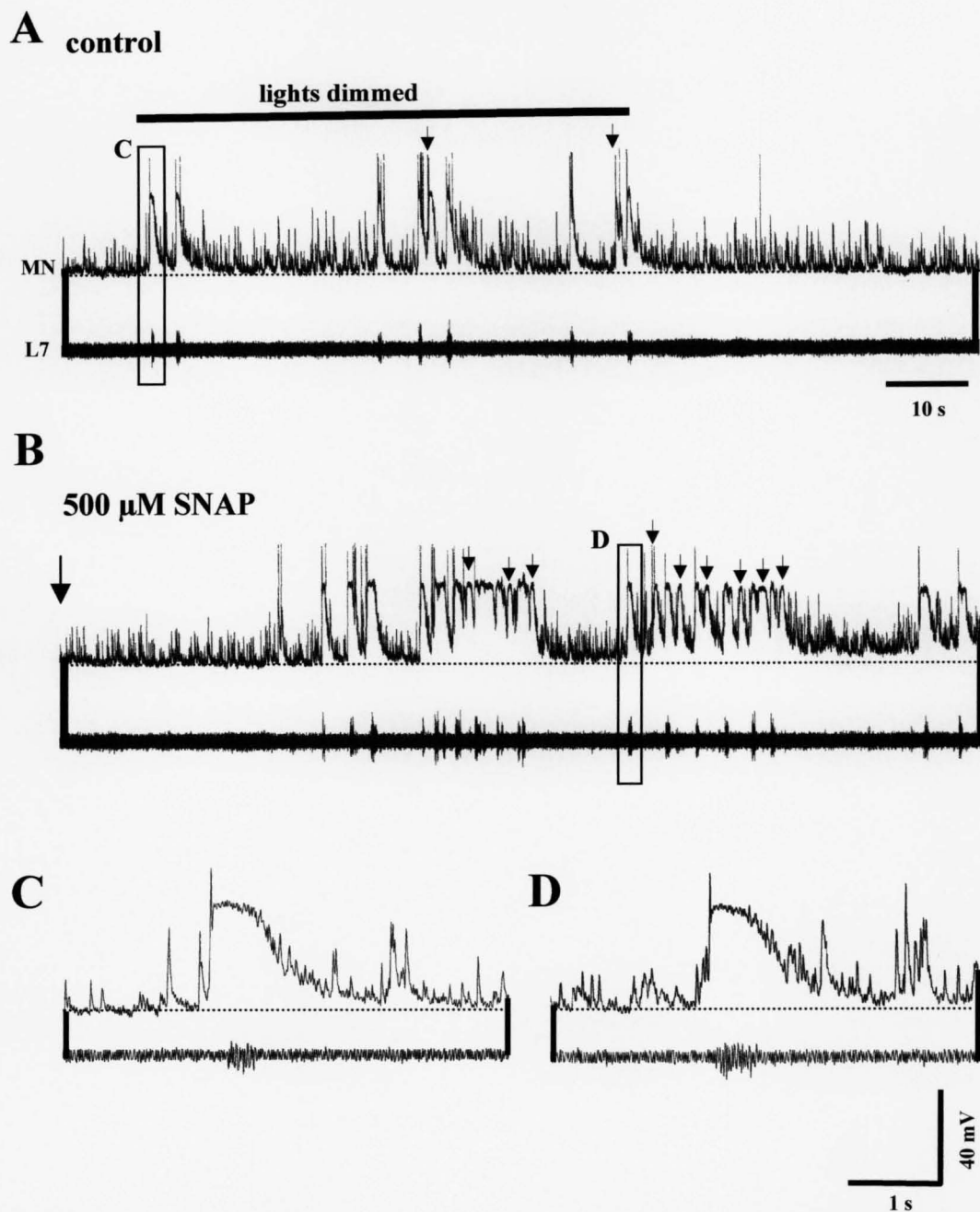
FIGURE 5.5. The synaptic drive under SNAP resembles the response to dimming of the illumination and inhibition is chloride ion-dependent. (A) Intracellular recording made with a 3M KCl-filled electrode from a presumed motor neuron (MN) located at the level of the third post otic cleft, which illustrates a dimming response. (B) The bath application of 500 μM SNAP (at large arrow) results in a pattern of activity similar to that seen with 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) 500μM SNAP are indistinguishable. Note that strongly depolarising potentials in the absence of ventral root activity (small arrows) correspond to sign-reversed inhibitory potentials, providing further evidence for chloride ion dependent inhibition.
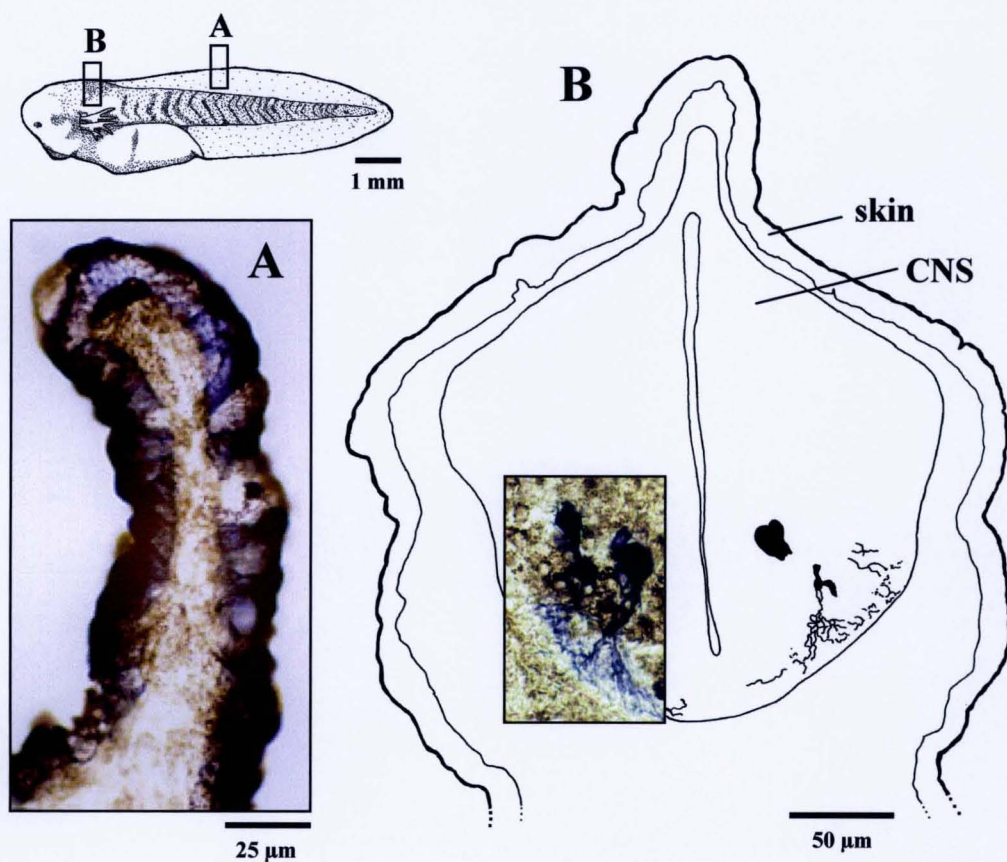
FIGURE 5.6. NADPH-diaphorase staining in the skin and the CNS. A schematic drawing that shows the approximate location of the cross sections of skin in the tail fin and the head (boxed). (A) NADPH-diaphorase staining in the inner layer of skin cells in the tail fin is visualised as a dark blue reaction 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 CNS illustrates the close proximity of the dark blue reaction product in the neurons of the CNS (colour inset) to the skin.
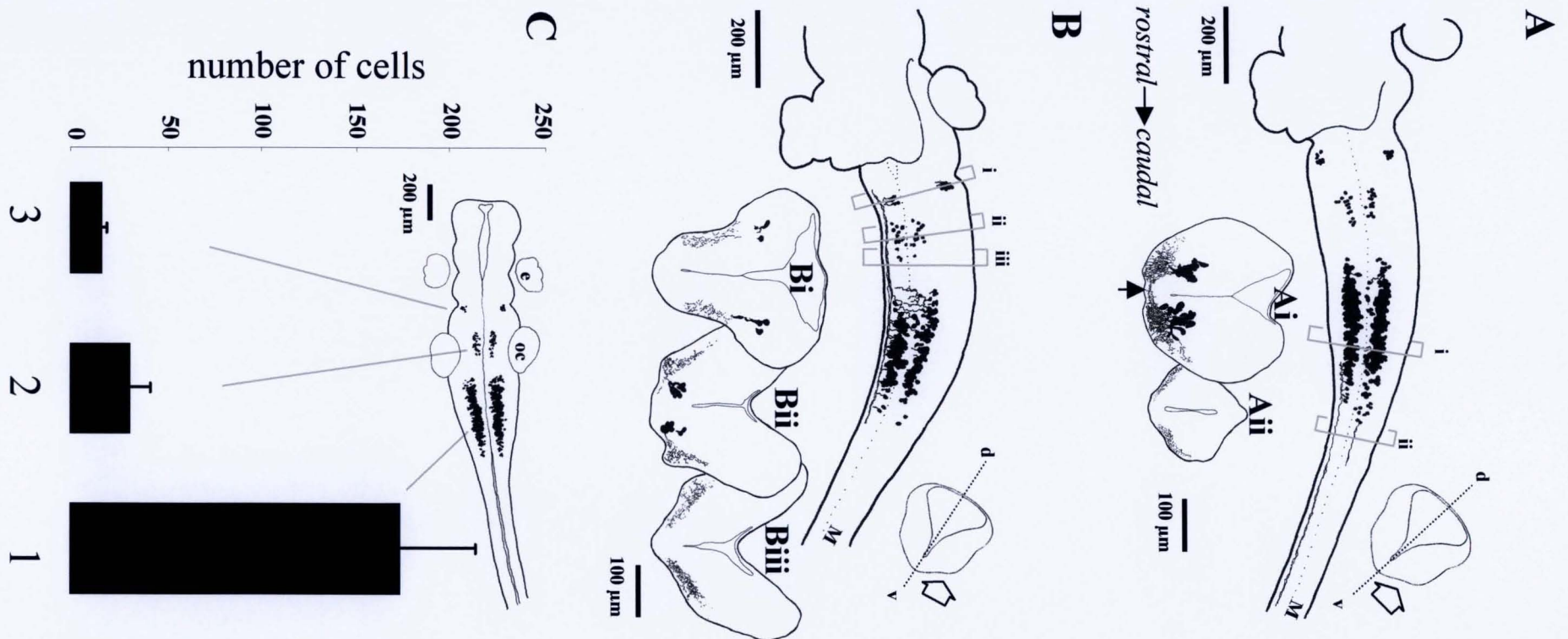
FIGURE 5.7. NADPH-diaphorase-labelled neurons in the CNS. (A) A wholemount preparation illustrated as a tracing at 10x-magnification showing descending projections well into the marginal zones of the spinal cord. Tracings of 10 μm sections illustrate the (Ai) caudal-most population (group 1) and (Aii) the descending projections within the marginal zones of the spinal cord. The arrow indicates contralaterally projecting processes. (B) A second wholemount illustrates ascending projections within the marginal zones of the brainstem that continue from group 1 to group 3 (see C). Tracings of 10 μm cryostat sections illustrate (Bi) the rostral-most population (group 3), (Bii) the mid-hindbrain population (group 2) and (Biii) the axonal tracts in the marginal zones of the brainstem. The rotation of both wholemounts 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 mean cell counts in each group 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., N=5 preparations.
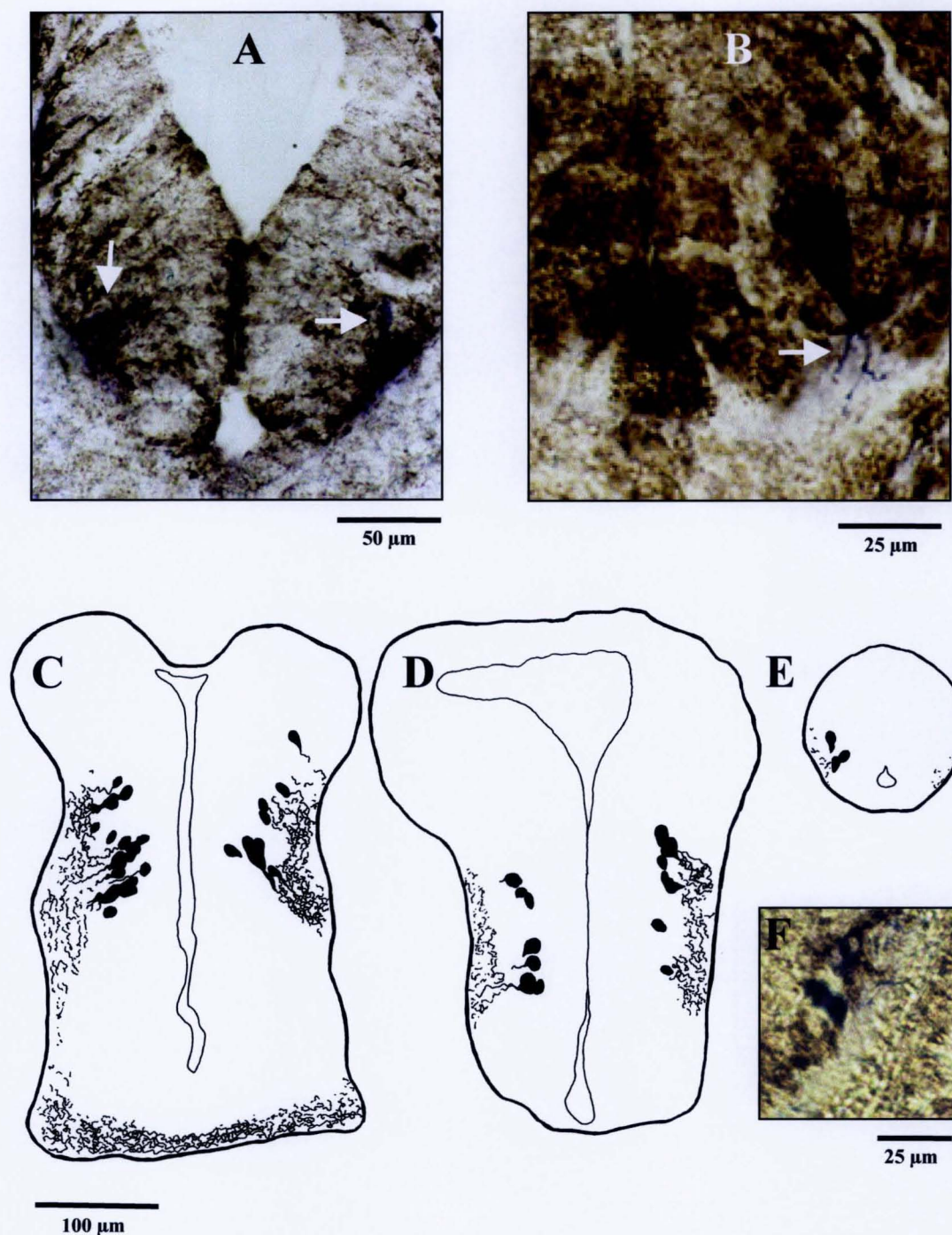
FIGURE 5.8. The development of NADPH-diaphorase staining in the CNS of *Rana temporaria*. (A) At stage 17, blue NADPH-diaphorase reaction product is seen in cell bodies located in the ventrolateral margins of the caudal brainstem (white arrows). (B) By stage 18 these neurons have developed axonal projections that extend to the marginal zones (white arrows). (C, D) At stage 22, two populations of NADPH-diaphorase reactive populations can be seen in the midbrain, with pronounced axons in the marginal zones. (E, F) NADPH-diaphorase reactivity can also be found in the spinal cord at stage 22.

THE GASEOUS MESSENGER MOLECULE, NITRIC OXIDE: A

MODULATOR OF LOCOMOTOR MOVEMENTS DURING EARLY

AMPHIBIAN DEVELOPMENT: A GENERAL DISCUSSION[1].

6

6.1. SUMMARY

The relative simplicity of the amphibian tadpole nervous system has been utilised as a model for exploration of the roles of NO in development and intrinsic modulation of vertebrate locomotion. This ubiquitous gaseous signalling molecule is known to play a crucial role in the developing nervous system, but until recently, it has not been directly implicated in the brain regions involved in motor control. NO appears to be produced by three homologous brainstem clusters in the developing motor networks of two closely related amphibian species, *Xenopus laevis* and *Rana temporaria* but, surprisingly, it plays contrasting roles in these species; terminating a motor pattern in *Xenopus* and initiating one in *Rana*. Given the hypothesised co-localisation of NO with conventional neurotransmitters, the potential interactions of nitrergic neurons with 5-HT, NA and GABA are discussed in these amphibian models. Also, future research directions will be discussed.

6.2. NITRERGIC HIGHER ORDER MODULATION OF MOTOR NETWORKS

Nitrergic modulation of neuronal circuitry occurs *via* a number of different mechanisms.

Most is known about its regulation of synaptic strength through its interaction with the

NMDA receptor (reviewed in Contestabile, 2000). NMDA-mediated NO generation can

produced a feedback increase in glutamate release, as illustrated in the olfactory bulb

(Kendrick *et al.*, 1997) where activation of NMDA-type postsynaptic receptors by

glutamate results in the influx of Ca^{2+}, increasing NO production, which then feeds back

onto the presynaptic terminal to enhance glutamate release. In certain pathological

conditions (*e.g.* ischaemia), it is this mechanism that is thought to contribute to glutamate-

mediated excitotoxicity (Resink *et al.*, 1996; also reviewed in Lincoln *et al.*, 1997). Under

normal circumstances, however, this situation is held in check, partly as a result of NO's

negative coupling not only onto its synthetic enzyme, NOS (Klatt *et al.*, 1992), but also

onto the NMDA receptor itself (Manzoni *et al*, 1992). In addition to direct action on the

NMDA receptor, NO has been shown to participate in the regulation of neuronal excitability in the rat paraventricular nucleus by activating a GABAergic inhibitory feedback loop, which dampens incoming excitatory signals (Bains and Ferguson, 1997). The retrograde effect of NO-facilitated glutamate release obviously relies on the post-synaptic target containing NOS. However, NO can not only regulate the input to a particular neuron, but also its output. For example, NO is known to stimulate calcium-independent neurotransmitter release from synaptosomes by promoting vesicle docking and fusion with the subsynaptic membrane (Meffert *et al.*, 1994, 1996). NO can also act in an anterograde fashion (see below). NO is therefore poised to mediate not only evoked, but also spontaneous transmitter release and can thus play a pivotal role in modulating the activity of neuronal circuitry.

Studies in the simple and accessible *Xenopus* tadpole model have so far revealed a number of brainstem interneurons that appear at early developmental stages and that are functional near the time of hatching (see CHAPTER 1). These populations contribute not only to the modulation of locomotor activity generated by spinal networks, but may also be involved in the maturation of these networks. Firstly, there are the mhr interneurons, which are GABAergic, utilise fast inhibitory synaptic transmission acting at $GABA_A$ receptors to terminate swimming and allow the embryo to hang motionless from objects in the environment (Boothby and Roberts, 1992b). In stage 37/38 embryos this pathway is normally only activated by contact of the rostral cement gland with those objects. However, coincident with the degradation of this sensory receptor structure, is the acquisition of an, as yet unidentified, endogenous stopping pathway. A second brainstem population is the raphe interneurons, the majority of which are serotonergic and for which there is evidence to suggest a direct involvement in the development and ongoing modulation of the larval motor system (Sillar *et al.*, 1992b, 1995b). This amine appears to

lead to relatively fast, intense swimming activity. A third, and least studied brainstem

population, may utilise another biogenic amine, NA and is located more rostrally in the

amphibian equivalent of the locus coeruleus, the isthmic region (Marín et al., 1996).

While the possible involvement of NA in locomotor network development has not been

explored thus far, its modulatory effects on swimming activity are essentially the opposite

of 5-HT, in that NA induces a slow and weak motor pattern. These directly contrasting

effects may be largely due to the differential modulation by the two amines of a common

spinal target, namely the presynaptic regulation of glycinergic synaptic strengths

(McDearmid et al., 1997). This does not exclude the possibility that each amine has its

own unique targets (e.g. 5-HT/NMDA oscillations; Scrymgeour-Wedderburn et al., 1997)

or that the two systems might interact with each other in the brainstem.

Thus, the brainstem of tadpoles, as in other vertebrates, contains a number of populations

of neurons, which are capable of exerting modulatory influence over the spinal circuitry to

produce a range of motor output patterns during swimming. Evidence presented in this

thesis has revealed an additional modulatory system that can be viewed as "higher order"

in that it may govern the activities of subordinate pathways, namely the nitrergic system

that utilises the signalling molecule, NO. The synthetic enzyme for NO appears to be

distributed in three discrete brainstem populations. For the purposes of discussion I will

assume NADPH-diaphorase histochemistry labels NOS, that NO is expressed in these

neurons as a co-transmitter with another more conventional molecule and that at least

some of the diaphorase-positive neurons have spinal projections. How do the three

nitrergic brainstem populations compare and overlap with the three populations described

above? The caudal hindbrain group overlaps significantly in morphology and location with

other reticulospinal populations that are known to play important excitatory and inhibitory

roles in motor control (Dale et al., 1986; Roberts and Alford, 1986; Roberts et al., 1987).

However, the more dorsal location and prominent contralateral processes of some members of this group suggest that a proportion correspond to the mhr interneurons. These neurons are the first to become NADPH-diaphorase reactive (see CHAPTER 4), and there appears to be not only a precocious increase in SNAP-induced GABA release over glycine (see CHAPTER 3) but also a developmental increase in the reliability with which NO decreases episode durations versus swimming frequency (see CHAPTER 3). This would not be entirely surprising or without precedence since NOS is known to co-localise with a range of transmitters including GABA (Vincent, 1995). In addition, NO is known to potentiate GABA release (Bains and Ferguson, 1997; Ohkuma et al., 1998a). These correlations and the known inhibition of Xenopus swimming by NO, suggests that nitrergic modulation could involve the enhancement of GABA release and this would explain the reduction in episode length by NO. If so, then co-transmitter modulation of conventional transmitter release must be occurring within the same neuron (c.f. Ohkuma et al., 1998b). Of course, given the highly diffusable and permeable properties of NO, it is also plausible that the modulation occurs between neurons in the brainstem cluster.

The rostral hindbrain population is similar in position and morphology to the serotonergic neurons of the raphe nucleus (van Mier et al., 1986). 5-HT's influence on Xenopus swimming is well-documented (Sillar et al., 1992b) and involves the enhancement of the duration and intensity of ventral root motor bursts. Since NO has the opposite effect, one might speculate that NO suppresses the activity of raphespinal serotonergic interneurons. In support of this idea, there are extensive axon tracts linking all three nitrergic brainstem clusters (McLean and Sillar, 2000) so that enhanced GABAergic inhibition in the caudal hindbrain could potentially reduce activity in the raphe in a feed forward manner. A similar situation occurs in the hypothalamus, where NA-induced NO release stimulates luteinizing hormone-releasing hormone (LNRH) release, coincident with GABA release

CHAPTER 6

onto LNRH-containing neurons, which subsequently inhibit LNRH release (Seilicovich *et al.*, 1995). The dualistic nature of this relationship means that NO can subsequently shut down the LNRH release that it initially stimulated. It also illustrates that NO can act anterogradely, as well as retrogradely. The later appearance of NADPH-diaphorase reactivity in the, potentially, aminergic populations (see CHAPTER 4), coupled with the delayed ability of SNAP to modulate glycine release consistently until stage 42 (see CHAPTER 3), certainly suggests that all three populations are necessary for the nitrergic modulation of swimming activity described here. It has been hypothesised that a single NO-containing neuron would be sufficient to signal within 200 μm (Trimmer, 1999). If this was the case in the tadpole then the distribution of NADPH-diaphorase reactivity within populations of neurons must reflect a benefit to each member of that population, and not the population as a whole. The striking locale of NADPH-diaphorase labelling in the brainstem of *Xenopus* indicates that, contrary to the emerging dogma that it acts as a global signal, NO can act with extreme precision in the development of neuronal circuitry

These possible correlations have important implications regarding co-localisation of NO with NA, particularly since these two very different signalling molecules have remarkably similar physiological effects on swimming in *Xenopus* (Fig. 6.1). These tentative proposals regarding co-localisation of NO as a metamodulator now await experimental confirmation using, for example, double immunocytochemical labelling for NOS with GABA, 5-HT and NA, together with physiological studies investigating the interactions between these interconnected modulatory systems.

6.3. EVOLUTION OF DEVELOPMENTAL MECHANISMS

The novelty of the findings on the distribution and role of NO in *Xenopus* tadpoles prompted an examination of NO function in a related amphibian embryo, applying the

potentially powerful comparative approach to developmental neuroscience. In *Rana*

temporaria embryos at equivalent stages of development (*i.e.* near the time of hatching),

NO is excitatory, triggering a slow non-rhythmic motor pattern (McLean *et al.*, 2001)

which is very similar to the response to light dimming (Soffe, 1991) and which is entirely

appropriate to facilitate the hatching process. However, the putative endogenous sources

of NO, namely the NADPH-diaphorase-labelled brainstem neuron populations, are

remarkably similar and presumably homologous to those in *Xenopus* larvae. In fact they

differ only in number and subgroup morphology, and in the extent to which NADPH-

diaphorase reaction product enters the marginal zones of the spinal cord (*c.f.* CHAPTER 2

and CHAPTER 5; also McLean and Sillar, 2000 and McLean *et al.*, 2001). Thus, NO has

apparently evolved contrasting roles in these two closely related species. Such species-

specific differences in neuromodulation are not without precedence (see DISCUSSION

section 5.5.1). Quite how NO release from these homologous brainstem populations in

Rana and *Xenopus* effects such different behavioural outcomes is an important unresolved

question. It could be that the caudal-most brainstem population in *Rana*, which lacks the

distinctive dorsal subgroup as described in *Xenopus* (*c.f.* Figs. 2.1 and 5.7), is therefore

missing a GABAergic component and is instead predominantly excitatory, perhaps

utilising glutamate (Roberts and Alford, 1986). The lack of GABAergic inhibition would

perhaps release some of the interconnected populations from inhibition, and could explain

the excitatory effects of NO. For example, the bath application of NA is known to elicit a

qualitatively identical non-rhythmic response in *Rana* embryos (McDearmid and Sillar,

1997; McDearmid, 1998). If the rostral-most population of neurons in the hindbrain does

correspond to the anuran homologue of the locus coeruleus, then perhaps both NO and

NA are facilitating glutamate release from the brainstem to the spinal cord. From an

ecological perspective, however, it is noteworthy that non-rhythmic body flexions are the

earliest form of behaviour produced by this animal, occurring when it is still clumped

together with several hundred siblings in a large egg mass (Soffe, 1991). In contrast,

Xenopus lay their eggs singly and hatchling tadpoles are efficient at swimming from the

moment they hatch. It is conceivable that in *Rana* it is beneficial for the hatching process

to be synchronised by an environmentally signal, such as light levels, for which NO is the

trigger. Whether or not this speculation can be verified, these findings certainly indicate

that NO plays an important but functionally different role in the developing motor nervous

system of anuran amphibians

6.4. FINAL COMMENTS

As with all scientific research, investigation often generates more questions that it answers.

The main aim of my work has been to explore roles of nitric oxide in the development of

rhythmogenesis. In doing so I have used histochemical techniques to locate the enzyme

responsible for its generation and electrophysiological techniques aimed at resolving the

mechanisms targeted by nitric oxide. The modulatory effects of nitric oxide in the CNS

are presumably dependent upon not only the identity and location of nitrergic neurons, but

also the specific targets of nitric oxide, of which there are many, including sGC (see

CHAPTER 1; also reviewed in Garthwaite and Boulton, 1995). Although my data implicate

facilitation of inhibitory synapses in nitric oxide function, the targets for nitric oxide in the

synaptic terminals now need to be resolved. This has been done to great effect in

invertebrate models, where the timing and appearance of nitric oxide sensitive cGMP has

been used as an anatomical marker for the onset of interactions between pre- and

postsynaptic targets (reviewed in Scholtz and Truman, 2000; *c.f.* CHAPTER 4). In

particular nitric oxide has been found to initiate a feeding behaviour in the gastropod

mollusc, *Lymnaea stagnalis* (Moroz *et al.*, 1993; Elphick *et al.*, 1995; *c.f.* CHAPTER 5),

modulate motor activity in the stomatogastric ganglion of the crab, *Cancer productus*

(Scholtz *et al.*, 1996, 1997; *c.f.* CHAPTER 2), and acts as an intracellular messenger in the

crustacean cardiac network (reviewed in Scholz and Truman, 2000; *c.f.* CHAPTER 3). The

obvious advantages of invertebrate models, in other words opportunities to compare

different mechanisms of NO/cGMP neuromodulation in the generation of discrete

behaviours, also apply to the simple and accessible vertebrate model provided by

amphibian tadpoles. Therefore, it is hoped in the future that this model system will

facilitate the systemic investigation of nitric oxide function.
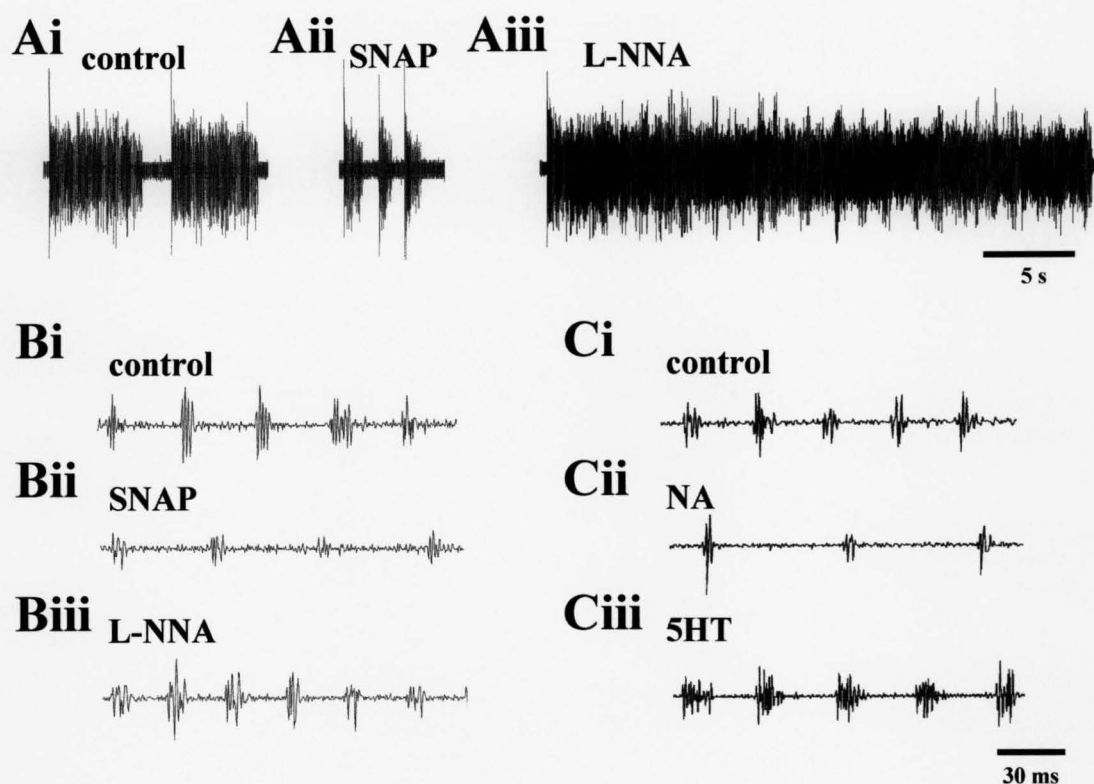
FIGURE 6.1. Comparison between nitrergic and aminergic modulation of larval fictive swimming. (A) Extracellular recordings of ventral root activity in (Ai) control saline, (Aii) 5-10 min after bath application of the NO donor SNAP (300 μM) and (Aiii) after application of the neuronal NO synthase inhibitor, L-NNA (100 μM). (B) Excerpts of swimming on a faster time scale illustrate the profound effect on cycle period (Bi) before and after (Bii) SNAP (300 μM) and (Biii) L-NNA (100 μM). (C) The bath application of (Cii) NA (3 μM) and (Ciii) 5-HT (2μM) modulate the control rhythm (Ci) in a similar way to SNAP and L-NNA, respectively. Scale bar in C also applies to B. A, B adapted from McLean and Sillar, 2000. C, adapted from McDearmid *et al.*, 1997. Figure taken from McLean *et al.*, 2000.

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