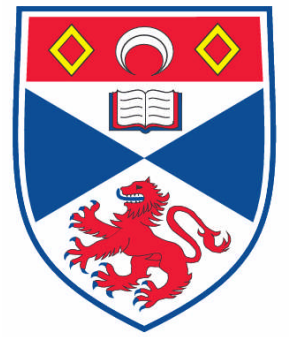
NITRIC OXIDE SYNTHASE AND THE CONTRIBUTION OF NITRIC OXIDE TO VERTEBRATE MOTOR CONTROL

Micol Ariella Molinari

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



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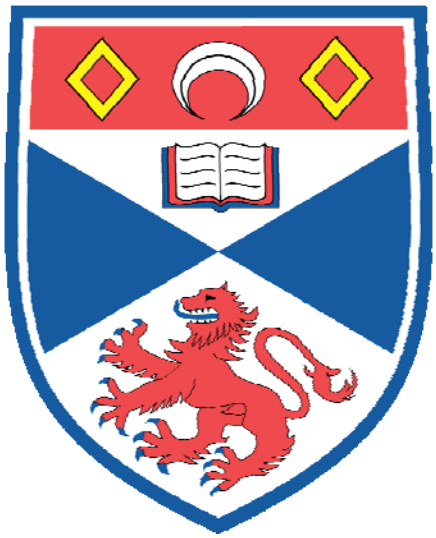

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Declaration

I, Micol Ariella Molinari, hereby certify that this thesis, which is approximately 35,000 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 in October 2005 and as a candidate for the degree of MPhil in October 2007; the higher study for which this is a record was carried out in the University of St. Andrews between 2005 and 2007.

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

Date _____ Signature of supervisor _____

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Contents

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Abbreviations

5HT	5-hydroxy-tryptamine; serotonin
BB:BA	benzyl benzoate : benzyl alcohol
Ca^{2+}	calcium ion
cGMP	3'-5'- cyclic guanosine monophosphate
ChAT	choline acetyltransferase
CNS	central nervous system
CPG	central pattern generator
DAB	3,3'-Diaminobenzidine
DMSO	dimethyl sulphoxide
EDRF	endothelium derived relaxing factor
GABA	γ-aminobutyric acid
HRP	horseradish peroxidase
LMC	lateral motor column
L-NAME	L-N^G-nitroarginine methyl ester
M	mol^{-1}
mhr	midhindbrain reticulospinal
Mg^{2+}	magnesium ion
MMC	medial motor column
MS-222	tricaine methanesulfonate
NA	noradrenaline
NADPH/NADPH-d	nicotinamide adenine dinucleotide phosphate/diaphorase
NBT	nitroblue tetrazolium
NMDA	N-methyl-D-aspartic acid
NMJ	neuromuscular junction
NO	nitric oxide
NOS	nitric oxide synthase
eNOS, iNOS, nNOS	endothelial, inducible, neuronal nitric oxide synthase
PB/PBS	phosphate buffer/phosphate buffered saline
sGC	soluble guanylate cyclase
SNAP	*S*-nitroso-*n*-acetylpenicillamine
T3/T4	thyroxine/triiodothyronine
TH	thyroid hormone
TR	thyroid hormone receptor

Abstract

I have explored the role of nitric oxide (NO) and the distribution of its synthetic enzyme in neurons, nNOS, in vertebrate motor control. The CNS of *Xenopus laevis* tadpoles was used primarily in this investigation as a developmental model of neural plasticity. During metamorphosis, spinal locomotor networks for axial-based swimming in the tadpole undergo dynamic reconfiguration in order to generate mature rhythmic motor patterns for limb-based propulsion in the frog.

Thyroid hormones (THs) orchestrate this change, possibly involving NO signalling in the CNS. Anatomical data were obtained from organotypic brainstem-spinal cord cultures using histochemical techniques. THs induced NOS expression in the spinal cord of stage 47, premetamorphic CNSs, a developmental stage when NOS is normally virtually absent in this region, after 3 days of organotypic culture. The pattern of NOS-expressing cells was found to be similar to that of more mature tadpoles, providing evidence that one developmental effect of THs in *Xenopus* CNS is to induce the expression of the NOS enzyme in a regionally specific manner.

The role of NO on the maturation of motor systems in the spinal cord was subsequently explored further, again using organotypic culture methods. *Xenopus* CNSs at stage 47 were cultured in the presence of the NO donor SNAP, or the NOS inhibitor L-NAME. Primary motor neurons in the spinal cord serve embryonic swimming; secondary motor neuron pools provide motor innervation to the limbs, but also to mature larval and adult axial musculature. During development, secondary motor systems eventually supersede the primary system. In my organotypic cultures at stage 47, L-NAME had no significant effect, but exogenous NO was found to cause an increase in the number of presumptive secondary motor neurons in the brachial region of the spinal cord, as indicated using choline acetyltransferase (ChAT) immunohistochemistry. This indicates that NO has a differentiating effect on secondary motor neurons in this region and provides evidence suggesting a maturational role of NO in the reconfiguration of spinal locomotor circuitry.

Finally, the presence of NOS in the spinal cord of the lamprey, a primitive vertebrate, was explored using histochemical techniques. The lamprey spinal cord is a well-studied model of rhythm generation by CPGs. NOS expression was found in motor and sensory cells involved in the generation and modulation of locomotion: motor neurons, dorsal cells, edge cells, and giant interneurons labelled for both NADPH-diaphorase and nNOS. This suggests that NO might exert a modulatory influence on rhythmic locomotion in the lamprey. Furthermore, my data indicate NO's role in motor control appeared early in vertebrate evolution, and that NO signalling in the CNS has been evolutionarily conserved. Taken together, data from *Xenopus* and lamprey suggest an important role for NO in the development and execution of vertebrate motor control.

For Alex, whose loving support makes everything possible.

1 Nitric oxide

1.1 Introduction to Nitric Oxide

The focus of this thesis is a small inorganic molecule that has received much attention in recent years: nitric oxide (NO) is a free radical gaseous chemical that has a widespread distribution in biological systems, so much so as to be considered ubiquitous. It all started in 1980, when Robert F. Furchgott's group identified in vascular epithelium a molecule responsible for mediating vasodilation. It was named endothelium-derived relaxing factor (EDRF) because of its source and for its effects on smooth muscle in blood vessel walls (Furchgott & Zawadzki, 1980). Within a few years it was in fact shown that EDRF is actually NO (Palmer, et al., 1987; 1988), which was rather surprising at first, considering that NO was generally thought to be a highly reactive environmental pollutant (Ripperton et al.,1970). Its discovery brought Furchgott the Nobel Prize for Physiology or Medicine, an honour he shared with Louis Ignarro and Ferid Murad, as their research served to confirm the function of NO as a signalling molecule in the cardiovascular system; Ignarro's work established that EDRF was actually NO (Ignarro et al., 1988) and Murad's group determined that the actions of nitroglycerine in smooth muscle tissue were mediated by the release of NO and subsequent activation of soluble guanylate cyclase (sGC) (Katsuki et al.,1977). Since the discovery of its role in blood vessel tone, NO and its synthetic enzymes, the nitric oxide synthases (NOSs), have been implicated in a whole range of normal and abnormal biological processes. As briefly elucidated above, NO has a role as a signalling molecule in the cardiovascular system, serving to control blood vessel tone. The contribution of NO to the maintenance of health does not stop here. As NO has been found to play an important role in the relaxation of smooth muscle (hence its vascular activity), it is therefore thought to be a key chemical signaller for the homeostasis of the gastrointestinal (GI) tract (Lamarque et al., 1996). Immunity may also be mediated by NO, for example, macrophages release NO upon invading pathogens, which causes their destruction (Green et al., 1990; MacMicking et al., 1997). Low level NO production in the skin may play a role in the maintenance of barrier function and

optimum blood flow in the microvasculature (Cals-Grierson & Ormerod, 2004). NO and NO synthase (NOS, its synthetic enzyme) are also found in nervous tissue in a variety of animals (for examples, see Bredt et al., 1990; Dawson & Snyder, 1994; Huang et al., 1997), where NO may exert a developmental influence as well as functional importance in healthy nervous systems. So far our understanding of NO in the nervous system has not developed to the extent that it has in the cardiovascular system. NO has been found to be involved in learning and memory (Bohme et al., 1993; Estall et al., 1993) due to its role in synaptic plasticity. Another area certainly worthy of investigation is the nervous circuitry that generates movement. In fact, NO has been implicated in various aspects of motor control from initiation (Rast, 2001) to modulation (Yanagihara & Kondo, 1996; Sillar et al., 2002*;* for a review see Del Bel et al., 2005). Movement and the ability to learn are fundamental in the survival of organisms, and just two of the reasons why studying NO/NOS in the nervous system is of paramount importance. The topic of this thesis therefore is the contribution of NO to motor control, with particular focus on the neural control of locomotion in the anuran amphibian *Xenopus laevis*, and the lamprey.

Despite its ubiquity in organisms and its role in health, this vital molecule has also been found to have a 'dark side'. Its production has been linked to inflammatory states such as ulcerative colitis (UC) (Rachmilewitz et al., 1995), and skin conditions like psoriasis (Cals-Grierson & Ormerod, 2004). NO is also implicated in nociception and the processing of various pain states. Chronic pain is an example of an abnormal pain state; by influencing spinal and sensory neuron excitability, NO/NOS may play a role in such pathophysiological pain (Luo & Cizkova, 2000). Another example of the pathophysiological roles of NO is in human erectile dysfunction (ED). NO from nerves and endothelia are crucial to penile vasodilation and erection, and in cases of ED, formation and action of NO is abnormal; reason for which NO-replenishing drugs like sildenafil can be used to effectively treat this condition (Cartledge et al., 2001). Much attention has also been drawn to the role of NO in carcinogenesis: NO and its derivatives can react with DNA in inflamed tissues and cause mutations (see Felley-Bosco, 1998). Indeed, NO has

been implicated in the progression, invasion, metastasis and angiogenesis of different cancers, including melanoma (see Ekmekcioglu et al., 2005, for a review).

Notwithstanding its contribution to abnormal health, NO's involvement in such pathophysiological states does make it a possible target for pharmacological treatment of such conditions: expression of iNOS (the inducible isoform) in tumour cells has been associated with suppression of tumorigenicity and regression of already-present metastases (Xie et al., 1996); cardiovascular disorders such as angina can readily be treated with NO-releasing drugs, and such NO-based remedies may be derived from botanicals (Achike & Kwan, 2003). In fact, such medicinal uses may have also opened up the exploration of NO activity in plants (for a review see Beligni & Lamattina, 2001). Research into the dual deleterious/beneficial nature of NO will certainly help us understand the implications of oxidative stress on tissues, and may help us live longer, healthier lives (for a recent review of free radical oxidation in health and disease see Valko et al., 2007). I have but scratched the surface of NO's biological roles, but I hope I have highlighted some of its essential functions in health and disease, and just how important and bewilderingly diverse the functions of nitric oxide are to life.

1.2 3 isoforms of NOS

NO is synthesized by the enzyme nitric oxide synthase (NOS), of which one or more isoforms exists in nearly all cells and tissues. Since NO is not regulated by conventional mechanisms of storage, release or reuptake, it is its biosynthetic enzymes that must be regulated to provide appropriate levels of the chemical (Bredt & Snyder, 1994). So far three NOS isoforms have been identified: eNOS, iNOS and nNOS.

1.2.1 Endothelial NOS (eNOS) or NOS-3

This isoform is stereotypically associated with endothelial membranes (see Forstermann et al., 1998, for a review) and eNOS-generated NO is heavily involved in mediating vasodilation, which it does by diffusing into the smooth muscle cells surrounding the lumen of the blood vessel and interacting with soluble guanylyl cyclase (sGC). sCG in turn generates the second messenger cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP). The soluble cGMP goes on to activate cyclic

nucleotide dependent protein kinase G (PKG), which phosphorylates various proteins that can regulate intracellular calcium concentrations, hyperpolarizing cells by activation of Ca^{2+}-dependent potassium channels, and subsequently affecting actin and myosin filaments that change the morphology of the cells, leading to an overall relaxation of smooth muscle tissue (see Tanaka et al., 2006). eNOS activity is not restricted only to vascular endothelial cells, but can also be found in platelets, where it regulates aggregation (Radomski et al., 1990). A role for eNOS in wound healing has been supported by experiments where inhibition of the eNOS enzyme results in impaired wound closure and angiogenesis (Lee et al.,1999), which makes sense considering the close association of the enzyme with vascular networks and their contents.

1.2.2 Inducible NOS (iNOS) or NOS-2

iNOS is found in the cytosol rather than being membrane-associated like eNOS (for a review see Sessa, 1994), and is expressed in many different cell types, including macrophages. It is believed that macrophage NO production is a key cytotoxic weapon of the immune system (MacMicking et al., 1997; Billack, 2006) in a variety of species (for examples see Ribeiro et al., 1993; Conte & Ottaviani, 1995; Lin et al., 1996), and it is thought that NO is toxic to invading pathogens due to its ability to interfere with DNA synthesis (Chien et al., 2004). However, like other components of the immune response, NO can be destructive as well as protective, due to the damage potential to normal host cells. iNOS NO production from macrophages has been found to suppress the activation of lymphocytes (Hoffman et al., 1990) and contribute to the inflammation of joints (McCartney-Francis et al., 1993) and pancreas (Burkart et al., 1992) amongst other disorders. In fact, NO has also been linked to the inflammation of respiratory airways that occurs in asthma (Payne et al., 2001) and iNOS expression is found to be up-regulated in asthmatic patients (Yates et al., 1996). In other words, the NO produced by the immune system can be linked to a variety of protective and pathophysiological states, but perhaps evolution has selected for this molecule as a nonspecific cytotoxic effector because its small size and lipophilic properties make it difficult for microbes to block its entry. Furthermore its prime targets (iron and sulfhydryls) are very

central in biochemistry and microbes or tumours would not be able to mutate to a fully resistant state (see Karupiah et al., 1993). Perhaps the cost of destroying some of the body's own cells is a price worth paying to secure the broad protection that NO production seems to provide against tumourous cells (Hibbs et al., 1987) and invading pathogens and parasites (Nathan & Hibbs, 1991). In any case, it becomes clear that a delicate balance in NO production is important for organisms in healthy states, and that perturbation of this balance can have severely negative consequences on the system.

1.2.3 Neuronal NOS (nNOS) or NOS-1

nNOS was the first isoform to be isolated and purified (Bredt & Snyder, 1990). Like iNOS, it is found in the cytosol. Originally identified in central nervous system (CNS) neurons (Bredt et al., 1990), nNOS has also been localized to other cell types such as skeletal muscle and kidney cells (Nathan, 1992; Bredt & Snyder, 1994). In neurons, NO is believed to be an important neurotransmitter and modulator of synaptic activity (for review, see Schuman & Madison, 1994), and has been identified as a key factor in the establishment of neural plasticity such as long term potentiation (LTP) (Schuman & Madison, 1991). Unlike most other neurotransmitters which generally only transmit information in one direction, from presynaptic to postsynaptic terminals, NO's small size and lipid-solubility allow it to readily enter cells by diffusion through membranes – it is not packaged in vesicles like other neurotransmitters, but diffuses from its site of production- thus it can have effects on many nearby cells, not only those separated by synaptic clefts (Schuman & Madison, 1994). On the other hand, NO has a short half-life (in experimental preparations where it is perfused over tissue, this is about 4 seconds (Moncada et al., 1989)), so its action is spatially restricted by its high reactivity, without the need for enzymatic breakdown or cellular reuptake (see Kelm, 1999, for a review). The synthesis and metabolism of NO is something that I will discuss in more detail later on. In addition to its functions as a neuronal messenger molecule, it has been established that nNOS-generated NO plays a major role in ischemic damage (cell death is likely a result of the conversion of NO to the highly oxidative peroxynitrite, $ONOO^-$) and glutamate neurotoxicity (Dawson

et al., 1996). Thus, the molecule has been shown to have essential as well as damaging influences in the CNS. Later in this thesis I will delve more deeply into the functions of NO in the nervous system.

This brief outline of the various isoforms of NOS shows that while a distinction between the three isoforms is certainly valid, it would be an oversimplification to associate any of them uniquely with neurons, endothelial tissue, or the immune system. eNOS has been localized to hippocampal cells in the brain (Dinerman et al., 1994) as well as being induced under certain conditions, like chronic exercise (Sessa et al., 1994), which implies a possible role of NO in the cardiovascular benefits associated with physical exercise. While there are data suggesting iNOS plays a role in the progression of malignant tumours (see Massi et al., 2001) there is also recent evidence that the constitutive forms, eNOS and nNOS (their genes are thought to be permanently expressed in cells, unlike the inducible isoform which relies on external regulating influences) can be expressed in certain kinds of tumour (Broholm et al., 2003; Cunha et al., 2006). Furthermore, as mentioned above, nNOS is not restricted to expression in neural tissue but has been found in systems like skeletal muscle and the epithelium of the bronchi and trachea (Kobzik et al., 1993; 1994). What this serves to highlight, therefore, is the incredible range of physiological influence that this simple molecule can have in a single organism, and that simply because each of its synthetic enzymes was discovered first in a particular tissue type, does not in any way imply it is restricted to that tissue. Research constantly attempts to refine roles for enzymes and bioactive chemicals, but it appears that the scope of NOS and NO instead continues to broaden as more is discovered.

1.3 Synthesis of NO

The synthesis of NO by NOS occurs via the same biochemical pathway for all 3 isoforms (at least in mammalian tissue), with two monooxygenase reactions occurring sequentially. In the first one, the guanidine nitrogen terminal of one molecule of the amino acid L-arginine is oxidized, using endogenous reduced nicotinamide adenine dinucleotide phosphate **(**NADPH) as a cofactor and converting L-arginine

to N^{ω}-OH-L-arginine. A further oxidation catalyzed by NOS and again using the electron-donor substrate NADPH results in NO and L-citrulline (see Griffith & Stuehr, 1995, for review).

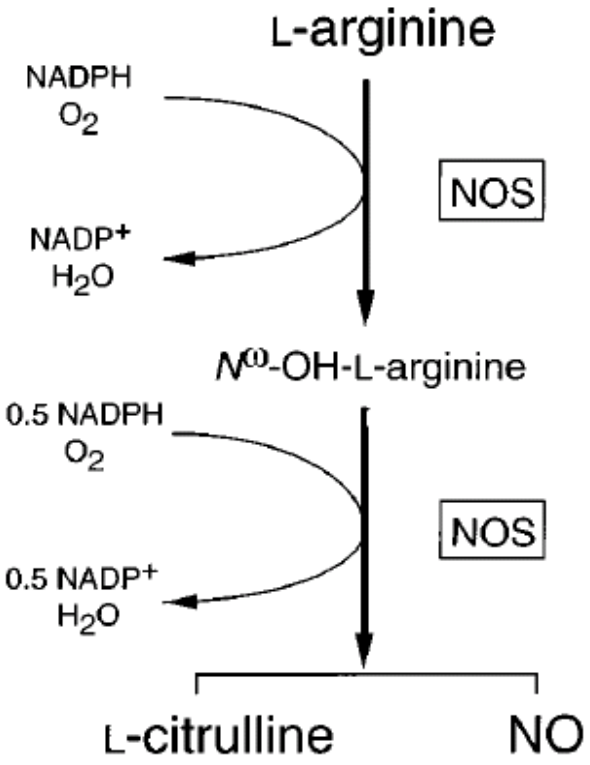

Figure 1. NO synthesis. Simple flow diagram outlining the formation of one molecule of NO after two sequential NOS-catalysed oxidations of L-arginine. The NADPH substrate acts as an electron donor for both oxidations. From MacMicking et al., 1997.

Aside from requiring NADPH and L-arginine, NO synthesis by NOS also depends on four cofactors and calmodulin (Bredt & Snyder, 1990). In fact, eNOS and nNOS activity depend on calcium/calmodulin levels, but iNOS is fully active at normal intracellular calcium concentrations so it is said to be the calcium-independent isoform. Activation of eNOS and nNOS depends on the cells receiving an appropriate chemical stimulus, for example acetylcholine for endothelial cells and glutamate in neuronal cells. By activating their respective receptors, the neurotransmitters cause the opening of receptor-operated ion channels, leading to an increased permeability of the membrane to calcium, and a subsequent rise in cytosolic Ca^{2+} levels (see Hecker et al., 1993). Calmodulin is a calcium-binding protein associated with all forms of NOS, and when calcium levels in a cell rise, the Ca^{2+}/calmodulin complex

binds to and activates the enzyme, which starts the process of NO synthesis. The Ca^{2+}/calmodulin complex is already strongly bound to iNOS no matter what the calcium level is in the cell, which makes iNOS appear insensitive to calcium levels (see Butler & Nicholson, 2003).

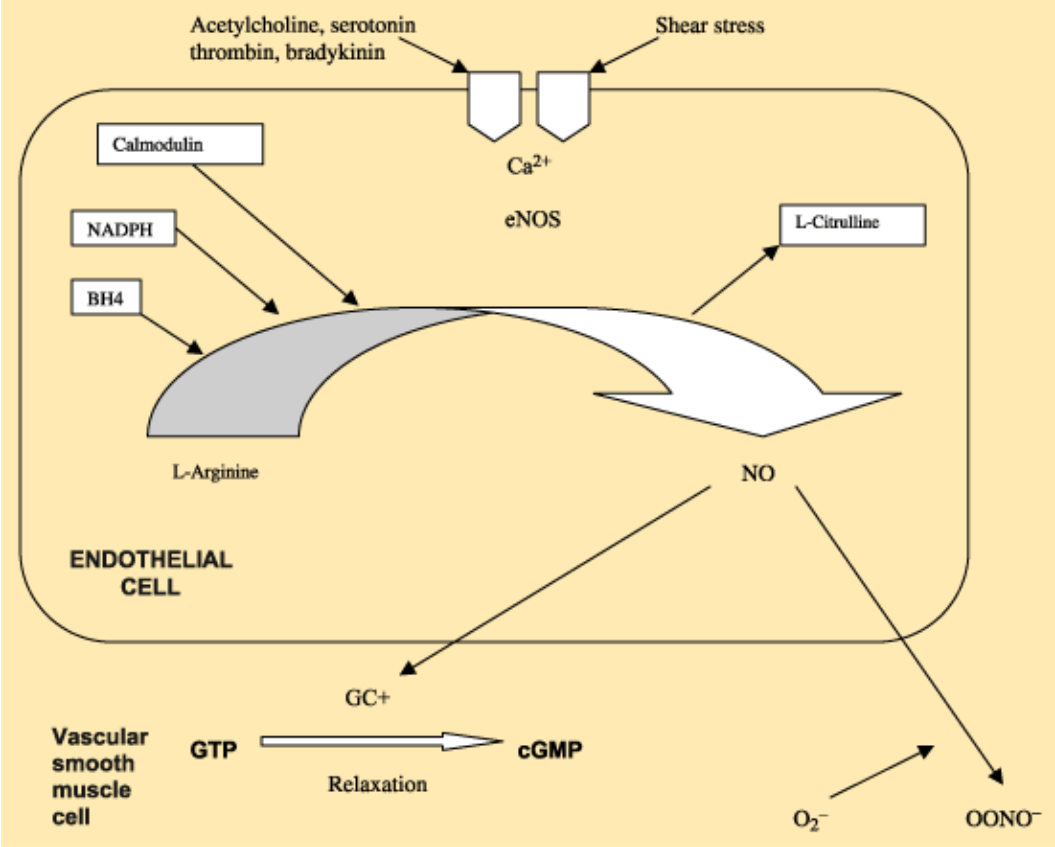

Figure 2. Influences to the NO-generating pathway in an endothelial cell. Image obtained from www.heartandmetabolism.org

It is also interesting to note that nNOS and eNOS produce NO only transiently whereas iNOS can produce NO for long periods once it is transcriptionally activated (Nathan & Xie, 1994). It can be speculated that the difference in availability of NO produced by each enzyme must be of biological significance, possibly linked to the tissues within which it may be acting, given its short half-life.

1.4 Targets of NO and mechanisms of NO action

Generally, NO has been viewed as exerting its effects through the 3'-5'-cyclic guanosine monophosphate (cGMP) signalling cascade, an association commonly referred to as the NO/cGMP

signalling pathway. As described above when discussing eNOS (though this pathway is not restricted to vasoregulation), NO causes the accumulation of cGMP in cells by binding to the heme group on the cytosolic sGC within the cell it diffuses into, causing its activation and the subsequent production of cGMP (Ignarro, 1990). The main effect of increased cGMP in cells is the potentiation of cGMP-dependent kinases like PKG which can cause a variety of different effects in the cell (see Schmidt et al., 1993) including phosphorylation of the NOS enzyme itself (Mullershausen et al., 2001), modulation of Ca^{2+} entry into the cell (Kwan et al., 2000), and stimulation of sGC (Ferrero & Torres, 2002), as well as selectively inhibiting phosphodiesterase (PDE), which acts to degrade cGMP (Rybalkin et al., 2002). In other words, through the activation of PKG, cGMP can modulate its own synthesis (by actions on NOS and sGC) and degradation.

It has been found that NO synthesized within endothelial cells can, by its actions on sGC, cause the formation of cGMP within those cells of origin as well as nearby cells (Luscher, 1991). As it is a common mediator of various physiological responses, the build-up of the second messenger cGMP provides an advantage in the NO signalling mechanism: the use of a single molecule (NO) to orchestrate different influences depending on the type of cell it diffuses into and the availability of targets, as well as biosubstrates, for the formation of cGMP; a widespread means of as-needed signal amplification. All this implies a complex system of complementary regulatory interactions between adjacent cells which could not be accomplished without the high diffusibility of NO through membranes and its short half-life (which limits the area of influence of a given NO-producing cell).

The NO/cGMP pathway is not the only mechanism of NO action. I have chosen to detail the cGMP pathway because it appears to be common as a signal transduction mechanism in cells. However it should be noted that NO also modifies proteins through S-nitrosylation (Lipton et al., 1993; Jaffrey et al., 2001), ADP-ribosylation (Brune et al., 1994) and by modulation of fatty acylation (for an example, see Hess et al., 1993). Furthermore, the activation of the NOS enzyme itself can be regulated by a variety of

different protein kinases (see Bredt et al., 1992), which means that there is flexibility in the NO-generating pathway, and much interaction between the second messenger systems.

Nitric oxide is thought to act as a retrograde messenger from postsynaptic neurons, that modulates transmitter release from presynaptic terminals, and this mechanism is believed to be of vital importance in the induction of synaptic plasticity like LTP in the hippocampus (Izumi & Zorumski, 1997; Malen & Chapman, 1997). The NO/cGMP pathway is important in this process of synaptic modulation (I will address this ahead), but the mechanisms mentioned above for post-transcriptional protein modification may also play a role in the modulatory effects that NO has on neurotransmitter release, possibly because the changed shape of the proteins prevents normal interactions between the synaptic vesicles and presynaptic terminal membranes during exocytosis (Meffert et al., 1994; 1996).

1.5 NO is involved in cell cycle processes

NO plays a role in various physiological and cellular processes, some of which I have described. This leads me to highlight some important cell cycle processes in which NO has been found to be implicated.

1.5.1 Apoptosis

Apoptosis is a type of programmed destruction of a cell, involving a series of orchestrated biochemical events (as opposed to necrosis which is usually due to acute tissue injury and results in noxious cellular debris and inflammation) and is involved in the control of tissue development (for a recent example, see Buss et al., 2006). Tumour suppression depends critically on apoptosis (Stuehr & Nathan, 1989) and deficits in apoptotic mechanisms may lead to uncontrolled cell proliferation, of which cancers are a major example (see Evan & Vousden, 2001; Garcia-Velasco & Arici, 2003). On the other hand, excessive apoptosis can lead to diseases of cell-loss like that found in ischemic damage (see Yu et al., 2001; Chan, 2001), therefore it becomes clear that like so many other physiological processes in the body, a suitable means of regulating apoptosis is key to the healthy functioning of the organism, as well as its correct development (see Meier et al., 2000).

NO is a cellular signal for apoptosis (see Brune, 2003). The tumour suppressor gene p53 has been implicated as a target during cell death execution in the apoptotic pathways mediated by NO (Ishida et al., 1993), revealing another cGMP-independent mechanism of NO action on tissues. The other apoptotic death pathway occurs through mitochondria which are one of the intracellular targets of NO (Moncada & Erusalimsky, 2002); there is evidence that inhibition of mitochondrial respiration is one of the pro-apoptotic effects mediated by NO (see Boyd & Cadenas, 2002; for a review see Pacher et al., 2007).

In fact, the biochemical actions of NO can often be attributed to reactive nitrogen intermediates (RNIs) that constitute different oxidation states and adducts of NO (see Stamler, 1994; Fukuto, 1995). Indeed, the formation of peroxynitrite (ONOO-) in cells, from the reaction of NO with another reactive species, superoxide (O_2^-) (Ischiropoulous et al., 1992), seems to be one of the key players in the damage that NO can wreak on cells (Grisham et al., 1999). Peroxynitrite can, over long periods of time, potentially oxidize and destroy cellular constituents and lead to the dysfunction of critical processes, disruption of cell signalling pathways, and bring about the death of the cell (Virag et al., 2003). As mentioned above, one of the oxidative targets of NO and peroxynitrite (see Radi et al., 1994) are mitochondria. Oxidative-stress induced disturbance of Ca^{2+} cycling (the normal Ca^{2+} uptake and release across the mitochondrial membrane) seems to be pivotal in mitochondrial inactivation, with the cycling becoming excessive and hence causing a loss of membrane potential and a leakiness of the inner mitochondrial membrane. This kind of damage to the mitochondria inhibits the synthesis of ATP and leads to the death of the cell. For a review of NO action on mitochondria see Richter, 1998.

A role for NO as a signal for apoptosis has certainly been established at this point, but it is complex. In fact, certain concentrations of NO and its congeners can suppress cell death by apoptosis (see Nicotera et al., 1997) for example in neuroblastoma cells (Ogura et al., 1997), once again highlighting the paradox of this tiny molecule and its enormous scope of influence.

1.5.2 Cell proliferation

Along with apoptosis, another mechanism that sculpts developing tissue is selective proliferation of cells. NO is believed to block the progression of the cell cycle in different cell types, thus acting as an inhibitor of cell proliferation and promoting differentiation (for review see Contestabile & Ciani, 2004; Villalobo, 2006). Programmed cell death (some of which was described above) sometimes results from exposure to NO after it has induced proliferative arrest, as research in cardiomyocytes and cancer cells has shown (Pignatti et al., 1999; Jarry et al., 2004), suggesting that one step for apoptosis is in fact the prior arrest of the cell-cycle. Investigations involving the up-regulation of endogenous NO production by increasing the availability of L-arginine demonstrated inhibited cell proliferation (Albina & Henry Jr, 1991; Hajri et al., 1998), as did experiments where iNOS expression was enhanced (Nunokawa & Tanaka, 1992; Punjabi et al., 1992). The brain of the South African clawed toad, *Xenopus laevis*, has been used as a model to study the proliferation/differentiation transition process of development, and it is interesting to note that inhibition of NOS in the brain results in larger brains with excess cell division and distortions in cytoarchitecture (Peunova et al., 2001). Different cells may become sensitive to NO at different points in their cycle (Sarkar et al., 1997; Tanner et al., 2000; Jarry et al., 2004). In other words, the sensitivity to the antiproliferative effects of NO is programmed and can differ among tissue types.

Alas, there seems to be no description of the activity of NO without reports of an opposing effect. Although inhibition is the main effect of NO on proliferation of different cell types, some experimental preparations supplying low exogenous concentrations of NO have resulted in the enhanced proliferation of cells such as keratinocytes (Krischel et al., 1998) and vein endothelial cells (Luczak et al., 2004), while supplying higher concentrations had the reverse effect, depressing proliferation. The point to note, however, is that these effects have generally been found *in vitro*, rather than *in vivo*.

1.5.3 Synaptogenesis

Synaptogenesis is the essential process that results in a specialized morphological structure between two synaptic partners, through which communication can take place usually by way of chemical

messengers. After a developing neuron reaches its final destination in the nervous system (following proliferation, migration and differentiation), it must establish contacts with its synaptic partners (for a review of synaptogenesis see Munno & Syed, 2003). Growth cones, the specialized structures located at the tip of a developing neurite projection, guide axons and dendrites toward their targets. They do this by responding to guidance cues from other cells and molecules present in their surroundings by repulsion or attraction (for a review see Mueller, 1999) and naturally, the correct integration of these cues is imperative in proper wiring of the nervous system. While transmitter-receptor interactions do appear important in some aspects of target cell recognition and synapse formation (see Lauder, 1993, for review), other studies have shown that synapse formation in the CNS can occur without the influence of transmitter-release machinery (for example, Verhage et al., 2000). As previously discussed, NO plays diverse physiological roles in many systems, and does not require receptors or release machinery. It can diffuse into and out of cells rapidly in three dimensions and has a short half-life, as well as being released upon excitatory stimulation of the postsynaptic terminal. One might speculate that these properties, and the fact that it can be produced in any cell, might make it a useful signalling molecule in the formation of communicative structures between, for example, neurons and glia. In fact, regionally and temporally specific patterns of NOS expression in the CNS are consistent with a role for NO in synaptogenesis and synaptic plasticity (see for example Ogilvie et al., 1995). Furthermore, studies in the locust suggest that NO sensitivity in developing neurons becomes manifest after the cell switches from the axon extension phase to that of maturation and synaptogenesis (Truman et al., 1996) and research in the developing chick suggests NO involvement in the pruning of ipsilateral projections in the visual system (Wu et al., 1994). More recently, NOS/NO has been implicated in the postsynaptic differentiation of the embryonic neuromuscular junction (NMJ) (Schwarte & Godfrey, 2004), and interestingly, as a signal for synaptic detachment after axonal injury in the mature mammalian CNS (Sunico et al., 2005), implying that synaptogenetic effects of NO are not restricted to embryonic tissues.

Taken together, there is much data supporting the idea that NO is involved in the formation, maintenance, and remodelling of functional synapses. It is worthy of mention that the three cellular processes outlined here, apoptosis, and proliferation, and synaptogenesis, are all vital to development and as such are active during embryogenesis and metamorphosis (see Su et al., 1999; Tissota et al., 2000), a topic which will be discussed in more detail later on.

1.6 The role of NO in the nervous system

By this time I have detailed some of the targets and sources of NO, and described some of its mechanisms of action in diverse systems. Much is known about eNOS and its role in vasoregulation and blood flow, and rather a lot of research has been carried out to investigate functional roles of NO in the immune system. However, neuronal NOS remains a largely new topic of discovery, one that could certainly be deepened to the benefit of medical science and our understanding of the nervous system. The brain is a rich source of NO synthesis, hence the first NOS to be cloned and purified was the constitutive neuronal isoform, nNOS, also named NOS1 for that reason (Bredt & Snyder, 1990; Bredt et al., 1990).

A great advance in nNOS research came when it was realized that the histological stain for NADPH-diaphorase (NADPH-d) corresponded to the distribution of nNOS (Dawson et al., 1991; Hope et al., 1991). This staining technique is still frequently used to characterize NOS activity in tissues, along with immunohistochemical processing that makes use of protein-specific antibodies to label cells that express NOS. Double-labelling procedures can also be employed, in order to identify areas of colocalization of NOS with neurotransmitters such as serotonin and acetylcholine (or their synthetic enzymes). Expression of NOS enzyme in the CNS has been identified in a broad range of species including birds (for example by Atoji et al., 2001), mammals (Mizukawa et al., 1989; Marsala et al., 1998), and amphibians (for example Munoz et al., 1996) to name but a few; with temporally regulated distribution (Foster & Phelps, 2000; McLean & Sillar, 2001; Lopez & Gonzalez, 2002; Ramanathan et al., 2006), supporting a role for NO in development/remodelling of the CNS.

Nitric oxide has, as detailed above in the section on targets and mechanisms of NO, multiple targets in cells. As a signalling molecule in the nervous system it has been identified as a modulator of synaptic activity between both neurons and glia (see Matyash et al., 2001). As a neurotransmitter, however, NO violates most of the criteria for classification as such: it is not stored in synaptic vesicles but simply diffuses from cells. It does not act at specific receptor types on adjacent cell membranes, but travels directly into cells and binds to a multitude of different target proteins, exerting its effects via a cascade of biochemical reactions. Finally, its inactivation comes not from enzymatic reuptake, but through reactions with other reactive species such as superoxide (see Gally et al., 1990).

1.6.1 Neurons and Glia

It has been suggested that a dynamic two-way communication exists at the synapses between neurons and glia, the support system surrounding neurons, by the fact that they release their own 'gliotransmitters' (see Volterra & Bezzi, 2002) when activated by neurotransmitters from the presynaptic neurons. Glia are therefore in a prime position to influence synaptic functioning (see Robitaille, 1998; Thomas & Robitaille, 2001). Recently described gliotransmitters are glutamate, which when released onto neurons is excitatory; ATP, which causes neuronal suppression, and d-serine (see Fellin et al., 2006; for review of d-serine see Wolosker et al., 2002), which is a co-agonist at postsynaptic NMDA-type glutamate receptors (Schell et al., 1995; Mothet et al., 2000). It is interesting to note that d-serine release from astrocytes has been implicated in the induction of long term potentiation (LTP) in hippocampal CA1 pyramidal cell synapses (see Yang et al., 2003). LTP in the hippocampus relies on NMDA receptor activation, so d-serine involvement doesn't come as a surprise, given the activity of d-serine at the NMDA receptor. The enzyme responsible for the production of d-serine in glia is glial serine racemase, which catalyses the conversion of L-serine to d-serine (Wolosker et al., 1999). Interestingly enough, it has recently been discovered that NO can influence the production/release of d-serine from glial cells by interacting with the serine racemase enzyme (Shoji et al., 2006), thereby affecting the production and release of the gliotransmitter and exerting a higher modulatory influence on a system

designed to regulate synaptic transmission in the first place. This makes in interesting link to the role of NO in LTP, which will be discussed ahead.

1.6.2 Synaptic Plasticity

Synaptic plasticity is an essential function of the nervous system, necessary for learning and memory formation, and the two major forms of synaptic plasticity underlying these processes are LTP and long-term depression (LTD). In LTP, synaptic transmission is enhanced for long periods of time after repeated coincident stimulation of pre- and postsynaptic terminals (for a review of novel mechanisms of coincidence detection at the synapse see Duguid & Sjöström, 2006). Ca^{2+} influx flowing through postsynaptic NMDA-receptor channels seems to be the minimum requirement for induction of such plasticity, but NO has also been found to play an important role in LTP (Huang, 1997; Hopper & Garthwaite, 2006) and LTD (Calabresi et al., 1999). The hippocampus has been identified as a major center of NO activity in LTP (for examples see Boulton et al., 1995; Hopper & Garthwaite, 2006), but NO is also found to mediate synaptic plasticity in the amygdala (see Schafe et al., 2005) and in the cerebellum, where LTP and LTD are linked to motor control and learning (see Lev-Ram et al., 1995; 1997). In fact, NO seems to function differently in different areas of the brain: as a retrograde messenger from the post- to the presynaptic cell in hippocampal synaptic plasticity (Schuman & Madison; 1991; Hawkins et al., 1998), whilst also serving as an intracellular messenger in the postsynaptic cell for certain types of synaptic potentiation (Ko & Kelly, 1999).

In the cerebellum, on the other hand, NO seems to be generated presynaptically and to act postsynaptically. Here, LTD of glutamatergic synapses from parallel fibres onto Purkinje cells (PC) has been studied extensively as a mechanism of cellular learning. It is found that the postsynaptic Ca^{2+} elevation (a result of depolarization of the cell membrane) coincident with presynaptically-generated NO, can cause long-term depression of this synapse. LTP can also occur, and it is necessary to allow the extinction of learned behaviours and their refinement. Experiments indicate that this NO-dependent LTP

may operate independently of the cGMP signalling pathway, and in fact does not require postsynaptic depolarization (Lev-Ram et al., 2002).

The actions of NO in synaptic plasticity are far from simple or universal. As a matter of fact, not all forms of LTP/LTD require NO as a retrograde signal (for example, see Alkadhi et al., 2001) so it is important to remember that NO isn't the *only* messenger molecule responsible for such synaptic plasticity. It is also worthwhile to note that nNOS may not be the only isoform responsible for NO production in the induction of LTP/LTD: research has suggested that eNOS can also contribute to the NO generated for synaptic plasticity (Kantor et al., 1996; Son et al., 1996). Recent work by Hopper and Garthwaite (2006) has in fact confirmed the different contributions that eNOS and nNOS make to LTP induction, by the tonic (eNOS) and phasic (nNOS) nature of their activity.

What this means is that the role of NO on synaptic plasticity may be effected at different times in different areas of the CNS, and this may hold particular importance for the synaptic plasticity that occurs during development. Furthermore, motor control starts in the nervous system, and since it has been shown that nerve cells make ample use of NO-signalling, it is a natural progression to assume that NO may have a function in motor control and locomotion. I have already briefly mentioned that NO is involved in motor learning, so it should come as no surprise that this molecule is also involved in the control and execution of motor actions. Vertebrate locomotion is discussed in the upcoming section, with particular attention to the role of NO in its expression, modulation, and, if the research on synaptic plasticity provides any precedent, on the development of spinal locomotor circuitry.

2 Vertebrate locomotion

2.1 Rhythmicity and central pattern generators

The ability of an organism to move within, respond to, and interact with its environment is one of the most obvious features of animal life; in terms of adaptive importance, locomotion serves escape, feeding/predation, and reproduction amongst other behaviours. Clearly then, an animal's capacity to locomote is central to its survival and dispersion. During locomotion, vertebrate movement occurs by way of rhythmic muscular contractions, which are stimulated by activity generated within the CNS, and modulated by inputs such as sensory feedback (for review see Harris-Warrick & Marder, 1991). The question of how the CNS produces specific patterns of impulses in motor neurons to generate coordinated movement has driven extensive research into the field of locomotor control.

Movements may fall into several different categories, such as reflexes (for example pupil dilation), fixed action patterns, and rhythmic motor patterns (see Hooper, 2001). The latter are responsible for repetitive locomotor behaviours such as walking (bipedal or quadrupedal), swimming, and flying. Almost 100 years ago, Brown (1914) suggested the existence of rhythmic central circuits that produced alternate flexion and extension of antagonistic muscles to drive walking. The idea that rhythmic motor programs are produced by spinal neural networks gathered force later on in the century and the term 'central pattern generator' (CPG) was coined in 1975 (Grillner & Zangger, 1975). It has now been established that these networks underlie the generation of most rhythmic motor patterns (for review see Marder & Bucher, 2001). Swimming is a commonly investigated mode of vertebrate locomotion, with the lamprey being a useful model for such studies. Indeed, the swimming circuits in the lamprey and the frog embryo are probably the best-decoded locomotor circuits so far, while recent research in zebrafish is rapidly catching up (McDearmid & Drapeau, 2006). Amphibians constitute the evolutionary transition from aquatic to terrestrial habitat, with correlated changes in locomotor strategy,

which makes them an interesting and useful model to study the development of locomotion from undulatory swimming to limb-based propulsion.

Locomotor activity can be studied quite reliably *in vitro* or in paralysed preparations. *In vitro* preparations have several advantages over *in vivo* approaches, such as the opportunity to apply drugs that cannot cross the blood-brain barrier, and the ability to manipulate the external bath medium as well as focus manipulations on discrete segments of the spinal cord (see, for example, Smith et al, 1988; Bonnot et al., 2002). While we cannot assume a perfect match between *in vitro* and *in vivo* motor activity, experiments have shown the rhythmic bursting in spinal preparations to be sufficiently similar or nearly identical to the rhythms generated in intact animals, for example in the lamprey (Wallen & Williams, 1984), reason for which it has been so extensively studied. Much of our understanding of locomotor networks has in fact developed thanks to spinal preparations of the lamprey, a relatively primitive agnathan vertebrate. I will now briefly examine this well-known model of a spinal locomotor network.

2.1.1 CPGs of the lamprey spinal cord

The lamprey locomotes using anguilliform swimming in which a wave of muscular contraction is propagated along the whole body. The muscular movements are generated by a neural network repeated in each of the approximately 100 spinal cord 'segments' that co-ordinate alternating contractions of the myotomes on the left and right sides of the body (see Grillner & Wallen, 1984), although strictly speaking, the spinal cord does not display anatomical segmentation. Interestingly enough fictive swimming can be induced in the isolated lamprey spinal cord even in preparations of only two or three segments (Buchanan & McPherson, 1995). In 1987, Buchanan and Grillner proposed a model of the lamprey locomotor network that has remained relatively unchanged since its publication. The model consisted of four types of neurons: **motor neurons**, the output elements; **excitatory network interneurons (EIN),** which provide ipsilateral segmental excitation to motor neurons as well as to the following cell types: **glycinergic crossed caudal interneurons (CC)** with contralaterally projecting axons

(mediating reciprocal inhibition between networks on opposite sides of the spinal cord) and **glycinergic lateral interneurons (LIN)** responsible for ipsilateral inhibition of the CC interneurons (Buchanan & Grillner, 1987). Other cell types have been characterized (such as dorsal cells, edge cells and giant interneurons) but show little or no rhythmic membrane potential activity during fictive swimming, so are not generally not included in the CPG model of the lamprey (Buchanan & McPherson, 1995). However, it must be pointed out that fictive preparations generally eliminate sensory inputs to the spinal cord, which might play a role in the apparent lack of rhythmogenicity of sensory cells such as the dorsal and edge cells *in vitro*.

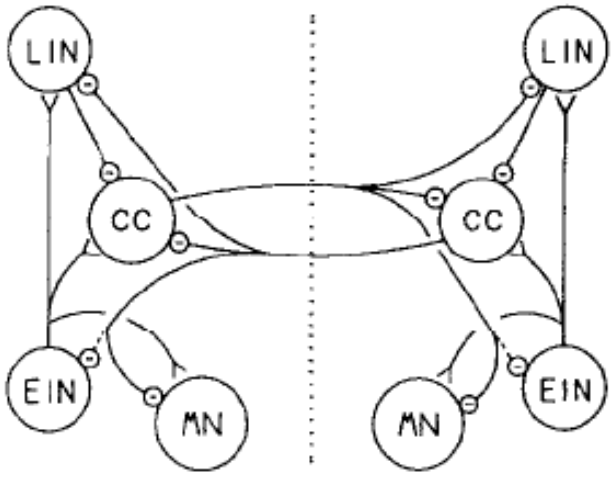

Figure 3. Model of the lamprey swim-generating spinal CPG. This representation shows synaptic connectivities of the proposed model (worked out using paired intracellular recordings). Note the reciprocal inhibition between CC neurons on opposite sides of the spinal cord. Coupling between segments allows for the rostrocaudal wave of activation underlying undulatory swimming. Adapted from Buchanan & McPherson, 1995.

I have chosen to briefly outline the swim network in lamprey because it is a well-understood model of a CPG and because motor control in the lamprey finds a place in this investigation; NO is an evolutionarily conserved signalling molecule (for invertebrate examples see Elofsson et al., 1993; Muller & Buchner, 1993; Tang et al., 1995, for a mammalian example) and a possible role for NO in lamprey motor control would substantiate this.

2.2 The development of locomotion

How the nervous system organizes the formation of functional rhythm-generating circuits during development is a major question in locomotion research. Identifying neurons that belong to CPG networks, exploring their synaptic connectivity and individual properties, and in particular studying motor patterns in animals at different developmental stages can provide clues to understanding the mechanisms in the development of dedicated spinal locomotor circuitry. As detailed above, the lamprey has been extensively used as a model system for the study of adult spinal locomotor networks (see also Cangiano & Grillner, 2005) and although CPG circuits are relatively well understood in this species, it is not well suited to developmental study, due to the difficulty of breeding lampreys in captivity. On the other hand, anuran amphibians and other metamorphosing vertebrates like the newt *Triturus*, provide an excellent model in which to investigate developmental changes in locomotion-generating circuits, as the animals make use of different motor patterns for locomotion at different stages of life and are easily bred in the lab. Along with the lamprey, the locomotor system of *Xenopus laevis*, in particular, is one of the best-understood models of rhythmic locomotor network function (for example see Roberts et al., 1998). Fascinating and dramatic changes occur in locomotor strategy over the development of *Xenopus*: axial-based body/tail undulations are employed for swimming in the tadpole, but once metamorphosis (which I will discuss in more detail later on) is complete, the animal switches to hindlimb-based propulsion. Underlying these different locomotor behaviours are two different rhythmic patterns: for tail swimming, in *Xenopus* as well as other species of frog, the spinal cord of the larva generates bursts of alternating activity in left and right ventral motor roots with a rostrocaudal delay of activation along the length of the spinal cord (see Stehouwer & Farel, 1980), much like in the adult lamprey, but hindlimb kicking is driven instead by bilaterally synchronous rhythmic bursting in the lumbar spinal cord (see Combes et al., 2004) which result in bilaterally synchronous cycles of flexion and extension in hindlimb musculature. What the development of amphibian locomotion implies, therefore, is that spinal locomotor networks must undergo functional and structural rearrangement. But metamorphosis is

clearly not the only example of developmental changes in locomotor rhythms or strategy: maturation of locomotor circuitry must necessarily occur in every vertebrate over development from embryo to adult- think of a human infant learning to crawl before developing the ability to walk. Such is an example of the qualitative changes in locomotor strategy that occur in many animals as the spinal systems mature to their normal adult configuration.

Few studies have managed to explore the development of CPGs, in part because of the intricacies of identifying CPG networks in minuscule embryonic preparations. Development of CPGs is likely to involve first of all the establishment of synapses between neuronal elements, followed by the acquisition of each element's intrinsic membrane properties, and thirdly, the appearance of extrinsic modulatory influences that control this target network (see Fenelon et al., 1998). In some cases, however, intrinsic properties (such as GABA synthesis) may develop in neural progenitors prior to synaptogenesis, suggesting a developmental role for the neurotransmitter before they it is required for synaptic transmission (Blanton & Kriegstein, 1991). The important point to remember is that the nervous system must generate appropriate behaviours for the requirements of the animal at all stages of development, and the neural circuitry responsible for such behaviours must, as development of the body progresses, also be susceptible to maturation, refinement and modulation in order to effect suitable motor control at later stages.

Basic synaptic connectivity between elements of the CPG appears early in development, as shown by the fact that coordinated motor outputs can be observed in preparations rather early in the developmental timetable, before all the appropriate musculature has developed for locomotion (Sillar, 1994). However, refinement of the connections must occur in order for full adult motor patterns to be expressed appropriately. Therefore, after synaptic wiring, the next step for development of the circuitry involves the tuning of cell's intrinsic membrane properties. Excitability of motor neurons and the presence of particular receptors in the spinal cord appear before birth in mammals such as the rat (see

Ziskind-Conhaim, 1988). The fact that in preparations, cells can be excited by exogenous neurochemicals such as bath-applied 5HT before the time that endogenous projections have descended into the spinal cord to their targets, suggests that the neurons' bioelectrical properties are specified before extrinsic modulatory influences come into play, and that postsynaptic receptor machinery may be present before the appearance of presynaptic stimulation (see Ziskind-Conhaim et al., 1993; Scrymgeour-Wedderburn et al., 1997). The neurotransmitters that mediate excitatory transmission may also change over development (as demonstrated in rat by Ren & Greer, 2003) and it has been discovered that the rhythmic release of these chemicals can guide neuronal pathfinding. It has been found, for instance, that blocking glycine and GABA-mediated synapses in the developing chick embryo causes pathfinding errors, misplaced motor neuron somata and altered patterns of spontaneous activity (Hanson & Landmesser, 2004), indicating that neurotransmitter activity is not only required for synapse stabilization and fine-tuning of circuitry, but also in the earlier stages where CPG networks 'wire up'.

There is so much still to be learned about the factors influencing the development of vertebrate spinal motor systems. While there are different means of investigating the development of motor CPGs, metamorphosis, particularly in amphibians, provides a fascinating opportunity to study the reconfiguration of motor circuitry, as it is necessarily associated with a complete reorganization of the animal's locomotor systems. This will be discussed in more depth in the third section of this introduction.

2.3 Studying the neural control of rhythmic locomotion

2.3.1 Electrophysiology

Locomotor behaviour can be studied using a variety of different *in vivo* and *in vitro* preparations. Intact preparations involve an awake animal moving, for example a mouse walking on a treadmill, and recordings being taken from flexor and extensor muscles during the generation of locomotor behaviour (for an example see Pearson et al., 2005). Semi-intact preparations can also be used when studying locomotion, for example the thoracosacral spinal cord with attached hindlimbs (Whelan et al., 2000). An advantage of using such a preparation over an isolated *in vitro* spinal preparation is that the neurogram

from the muscle nerves can be compared to data obtained from *in vivo* studies. On the other hand, isolated spinal preparations are especially useful in allowing researchers to identify the components of a neural network. One way to deduce the rhythmogenic role of a given neuron type is to alter the activity of the chosen cell within the circuit and examine how the output of the system is perturbed- this has been a useful way of mapping network connectivity in many invertebrates (see Marder & Bucher, 2001). Manipulation of these circuits may be easily accomplished: pharmacological and electrical stimulation can reliably activate the networks in *in vitro* preparations (for example see Bonnot et al., 2002) and are therefore commonly used techniques in the study of rhythmic locomotion. In vertebrates, much rhythmic locomotion depends on populations of interneurons, those electrically excitable cells that form the bulk of the circuitry mediating the execution of motor commands. Thus, much research into the control of locomotion has focused on interneurons in the spinal cord; by altering the excitability of an identified population of interneurons and monitoring the frequency of the motor output, it can be discerned whether the particular class of neurons affects the function of the CPG. Such methods of studying motor control have been extensively employed in vertebrates like amphibians and the lamprey (Roberts et al., 1986; Roberts & Sillar, 1990; Buchanan & McPherson, 1995, provide examples).

2.3.2 Immunohistochemistry/anatomy

The structure of neural networks has been elucidated also through immunohistochemical techniques that involve labelling particular proteins (e.g. enzymes) and amino acids (and hence, the cells that express them) in order to construct a three-dimensional image of the neural populations implicated in the control of locomotion. For example, antibodies against glycine have been used to support electrophysiological evidence that the neurotransmitter is involved in locomotion by showing that certain groups of cells release them endogenously (Roberts et al., 1988). Immunolabelling techniques can also be used to follow the developmental time course of a particular cell type expressing the protein in question, which can help to shed light on the factors that impact the formation of dedicated motor systems. Furthermore, immunolabelling can be used to verify the role of a particular neurotransmitter in

the development of motor networks, for instance by combining it with genetic knockdown techniques and then revealing what, if any, changes have occurred in the morphology or number of the targeted cells (see McDearmid et al., 2006). Therefore, in combination with other techniques, immunohistochemical labelling is an invaluable method of exploring particular cell types and their connectivity.

2.3.3 Molecular approaches

Genetic approaches which enable the manipulation or deletion of whole classes of spinal interneurons have allowed researchers to determine their role in pattern generation, for example, mice mutant for the Dbx1 homeobox gene (expressed by ventral interneurons) had disturbed patterns of hindlimb left-right alternation, suggesting that those interneurons are important for controlling left-right alternation (Lanuza et al., 2004). Knockout studies in mouse have also been helpful in elucidating the connectivity of spinal locomotor circuitry, demonstrating the importance of even a single gene (such as EphA4) in influencing the normal development of connections, and therefore of normal locomotive behaviour (Akay et al., 2006) . Another way of using genetic tools is to add neurons to circuits during development- something that has been attempted in zebrafish. In fact, studies have shown that duplicates of mauthner neurons can incorporate into and function in the developing escape circuit to produce a normal escape response suggesting that part of circuit evolution may involve the incorporation of duplicate cells (Liu et al., 2003; Hale et al., 2004). The types of experiments that can be carried out to investigate rhythmogenesis in the spinal cord have really expanded due to novel genetic techniques, and while electrophysiology still provides the vital insight into the connectivity and intrinsic properties of the cells that compose the networks, molecular approaches are currently opening up a whole new world of research into the development and control of locomotion. The continued development of novel techniques is invaluable for providing and substantiating evidence in the study of locomotor control, as is combining the different techniques in order to gain a deeper understanding of the physiological and anatomical basis of motor control.

2.4 Neurochemicals implicated in the control of locomotion

The main neurotransmitters implicated in the control of locomotion are glutamate, γ-aminobutyric acid (GABA) and glycine. Motor patterns produced by vertebrate spinal networks are the result of descending excitation, combined with reciprocal inhibition. While excitation is often glutamatergic (for an example see Nistri et al., 2006), it has been shown that other neurochemicals (for example, acetylcholine) contribute to the excitatory drive (Perrins & Roberts, 1995). Furthermore, the two inhibitory neurotransmitters GABA and glycine play distinctly different roles in modulating locomotor rhythms (Sillar et al., 2002; Hinkley et al., 2005; for a review in mammals see Kudo et al., 2004).

In *Xenopus laevis*, swimming (as in other swimming vertebrates) involves reciprocal mid-cycle inhibition (i.e. the inhibition of contralateral motor neurons when one side of the spinal CPG is active). Glycine is the major neurotransmitter released by commissural interneurons to produce mid-cycle inhibition (Dale et al., 1986)**.** These commissural interneurons project to contralateral regions of the spinal cord, are active during fictive swimming and produce inhibitory post-synptic potentials, IPSPs, in contralateral motor neurons (Soffe et al., 1984; Dale, 1985). GABA serves other functions in *Xenopus* locomotion, the major one of which is mediating an innate stopping mechanism in the swimming tadpole. When the tadpole's head encounters an obstacle, afferents from the cement gland trigger GABA release from reticulospinal neurons onto spinal neurons (see Bothby & Roberts, 1992; Perrins et al., 2002), thereby terminating motor output.

2.5 NO and metamodulation of locomotion

CPGs do not produce highly stereotyped, inflexible rhythms; flexibility is a fundamental requirement of neural networks. The ability to adapt, for example when sensory inputs impinge on the body, is essential in order to generate a rapid and appropriate response. Modulation of spinal networks is a major part of behavioural plasticity in locomotion (for an example in *Xenopus* see Sillar et al., 2002) and research supports the idea that such modulation comes about by the effects of a varied array of endogenous chemicals on the neural networks underlying rhythm generation, which leads me to discuss

the role of NO in the 'metamodulation' of locomotion. I have already delved into the characteristics of this messenger molecule earlier in this introduction, but now I will describe some of its acute effects on locomotor rhythms in preparations of *Xenopus* embryos, in order to explain why it is a 'metamodulator'.

In the presence of NO, as supplied by NO-donor drugs such as S-nitroso-*n*-acetylpenicillamine (SNAP), fictive swim patterns slow down and prematurely terminate indicating an inhibitory effect on locomotion. On the other hand, using drugs that impair NOS, the synthetic enzyme for NO, causes swimming to accelerate in frequency and prolongs the bouts of locomotor activity. In fact, NO has been found to exert effects on GABAergic as well as glycinergic inhibition in locomotion (McLean & Sillar, 2002). Bath applied NO has also been found to have a direct effect on motor neuron membrane properties in *Xenopus* fictive locomotion preparations (McLean & Sillar, 2004). NO, therefore, has the ability to specifically influence different neurotransmitter systems as well as the motor neurons that translate their signals, implying that the role it plays in locomotor control is complex (operating at different possible levels in the locomotor hierarchy) and versatile.

The location of NO sources is also of particular interest in the issue of metamodulation. The neurotransmitters GABA and glycine are modulated by the biogenic amines noradrenaline (NA) and serotonin (5HT) (McDearmid et al., 1997; see also Sillar et al., 2002). Interestingly, in the developing *Xenopus* embryo NOS is found in brainstem cells within or in close proximity to descending sources of 5HT, GABA and NA (McLean & Sillar, 2000, see also Lopez & Gonzales, 2002), therefore allowing speculation that at least some of the effects of NO on locomotor activity are due to NO 'metamodulation' of such descending aminergic systems (Sillar et al., 2002; McLean & Sillar, 2004). Anatomical evidence indicates that NOS expression appears in *Xenopus* spinal cord after stage 47 (McLean & Sillar, 2001; Ramanathan et al., 2006) suggesting that NO activity intrinsic to the spinal cord may not appear until later in larval life. However as described above, NO is found to influence swimming rhythms in the preparations used to study fictive locomotion, which range between stages 37/38 and 42.

This clearly indicates that exogenously applied NO has the ability to modulate target neurons in the brainstem or spinal cord even before the presence of endogenously produced NO. In terms of how endogenous NO may be affecting the release of inhibitory transmitters *in vivo*, the presence of NOS expressing neurons in the brainstem suggests a direct mechanism of facilitating GABA release from nearby cells in the midbrain-reticulospinal (mhr) group, which project to spinal motor neurons (Sillar et al., 2002). Nevertheless, as spinal neurons are not found to contain NOS activity until later larval stages, it is less likely that endogenous NO is acting directly on commissural interneurons at earlier stages to facilitate spinal glycine release. This supports the idea of NO also exerting its regulatory influence on the aminergic modulatory systems above GABA and glycine in the locomotor hierarchy.

In fact, NO has been found to interact with 5HT and inactivate it (see Fossier et al., 1999) and can also affect 5HT synthesis (Kuhn & Arthur Jr, 1996), substantiating the case that NO may be debilitating the activity of descending serotonergic neurons that affect inhibitory synapses. On the other hand, NA seems to be potentiated by NO; when drugs that block the NA pathway were used in fictive *Xenopus* embryo preparations, the effect of NO on swimming frequency (which is mediated by glycine) was influenced. No effect was observed in the duration of swim episodes (controlled by GABA) (McLean & Sillar, 2004). This implies that NO works via a noradrenergic pathway to control glycine release but directly promotes GABA release. In other words, by potentiating NA, but having the opposite effect on 5HT, NO could be causing increased glycine release from commissural interneurons that synapse onto spinal motor neurons, while simultaneously orchestrating GABAergic inhibition of the motor neurons by acting directly on the GABAergic mhr neurons (Sillar et al., 2002) . Metamodulation can therefore be said to be nitrergic regulation of those aminergic brainstem systems which influence spinal central pattern generation mediated by inhibitory neurotransmitters.

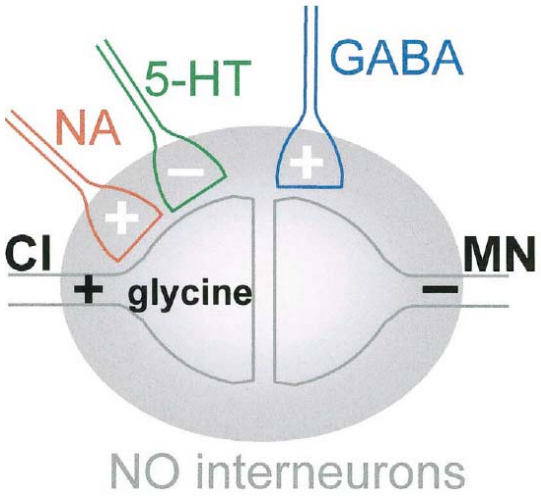

Figure 4. The proposed 'metamodulatory' influence of NO. NO could be facilitating glycine release from commissural interneurons (CI) onto motor neurons (MN) by exerting influence on upstream aminergic systems, but it could also be increasing GABA release from upstream sources onto motor neurons. From Sillar et al., 2002.

In summary, NO can play a role in the control of rhythmic locomotion by means of its modulatory effects on noradrenergic systems that influence inhibitory release, as well as by directly influencing inhibitory release of GABA onto motor neurons. As mentioned above, NO also changes the membrane properties of motor neurons themselves and therefore shows that its mode of action is through targets present in different cell types. The properties of NO/NOS seem to make it perfect as an modulator of neural firing: due to the dependency of nNOS activation on Ca^{2+} influx, NO generation seems to go hand-in-hand with cellular activity (see Salerno et al., 1997), also, as previously discussed, it can modulate neighbouring cells activity by affecting neurotransmitter release (see also Micheva et al., 2003), directly interacting with neurochemicals (see Fossier et al., 1999) or their synthetic enzymes (Kuhn & Arthur Jr, 1997). It also affects the output of a neuron by activating second messenger pathways, for example via interaction with sGC (Schuman & Madison, 1994). All this, combined with the fact that NOS is found to be expressed early in development in the CNS (for example in *Xenopus,* see McLean & Sillar, 2001), suggests the molecule is an important intrinsic modulatory component of spinal locomotor networks. Since the modulatory effects of NO on locomotion are not restricted to GABAergic potentiation, but also

due to potentiation of aminergic neuromodulators affecting glycine release, this posits NO at the top of a modulatory hierarchy, earning it the title of 'metamodulator' (Sillar et al., 2002; McLean & Sillar, 2004).

3 Metamorphosis

So far we have considered NO and locomotion in vertebrates. This section now deals with the amazing transformational process of metamorphosis, the resulting changes in morphology of an animal, and the subsequent adaptations in motor control. Metamorphosis is the process of an organism's biological development from juvenile (larva) to adult, characterized by a relatively abrupt change in the animals form through cellular differentiation and growth. While amphibians may be the most familiar example of animals that undergo metamorphosis, the process occurs in various other families of the animal kingdom, most notably insects and marine invertebrates. Many tissues are remodelled or developed during metamorphosis, including the nervous, digestive, and respiratory systems (see Pinto et al., 2003, for an example in insects; Schreiber & Specker, 2000, in fish; Schreiber et al., 2005, for amphibians). The metamorphic adaptations from larval to adult phenotype serve to prepare the animal for a change in habitat, niche and behaviour. For example, many anuran amphibians hatch from eggs in water and spend the larval part of their life cycle in this aquatic environment but move to occupy terrestrial predatory niches once they have developed limbs, lungs and other adult-specific features. In fact, there are three criteria that encompass the changes of metamorphosis (from Just et al., 1981):

1. There must be some change in form of non-reproductive structures between the time the embryo hatches and sexual maturity
2. The larval form occupies a different ecological niche from the adult- i.e. the change in form allows the species to exploit new ecological niches
3. The morphological changes that occur at the end of larval life depend on an external (eg. food supply, light) or internal (eg. hormonal) environmental cue

Furthermore, there are three major types of changes associated with metamorphosis:

1. The regression/resorption of tissues/organs that have only larval functions
2. The remodelling of larval organs/tissues to forms suitable for adult functions
3. The *de novo* development of adult-specific organs/tissues that are not required in the larval form

I will describe these in more detail later on, with specific reference to anuran amphibians. In these amphibians, there is also an alternate developmental strategy to metamorphosis, or biphasic development; namely direct development. Here, the young animal hatches from the egg as a miniature adult, and there is no free-living tadpole stage (see Dent, 1968). This seems to serve species that have adapted to an essentially terrestrial life, such as *Eleutherodactylus nubicola,* which lays its eggs in damp cavities under stones (see Shi, 2000). On the other hand, some biphasic species remain almost completely aquatic throughout their entire life cycle, such as *Xenopus laevis*. Other anuran species like *Rana temporaria*, the common frog, move out of their larval environment (water) when metamorphosis is complete. Many salamanders and toads also follow a similar developmental transition. Unfortunately the amphibian fossil record is very poor, but this two-part lifestyle can be viewed as an evolutionary remnant of those adaptations which allowed vertebrates to emerge from the ancestral waters and begin occupying the terrestrial niches. It is clear, therefore, that the developmental strategies of amphibians are fascinating and diverse, and serve as testament to the power of natural selection in generating diverse mechanisms for organisms to appropriately exploit the richness of ecological opportunities in the natural environment.

Metamorphosis throughout the animal kingdom is dependent on endocrine secretions (see Gilbert et al., 1980; Kikuyama et al., 1993; Heyland et al., 2004), but how these hormonal signals orchestrate such varied and complex changes in different tissues of the same organism is a subject of intense study. In fact, the hormonal control of metamorphosis provides an excellent model with which to study

vertebrate development, and due to the resulting changes in locomotor strategy, it is also a fascinating and valuable means of exploring the development of motor networks.

3.1 Thyroid hormone control of *Xenopus* metamorphosis

Metamorphosis can be looked at as a means to switch between two distinctive body forms: the larval one which grows and acquires food, and the adult one responsible for reproduction and dispersion (see, for example, Williams & Truman, 2005). In *Xenopus laevis* as in other amphibians, the hormones responsible for triggering metamorphosis are thyroxine (T4; 3,5,3',5'-tetraiodothyronine) and triiodothyronine (T3; 3,5,3'-triiodothyronine). Both are produced by the thyroid gland, but T4 can also be converted to the biologically active T3 by deiodinases in different tissues (Shi, 2000). The thyroid gland begins to fix iodine shortly after the animal starts free-feeding, at stage 45 (Cai & Brown, 2004); the hindlimb buds do not appear until stage 47 (Nieuwkoop & Faber, 1956) and the thyroid hormones (THs) are produced in rising levels from this stage as metamorphosis progresses; the thyroid gland develops full functionality around stage 53, with stages 60-63 showing the most elevated production and availability of hormones (see Figure 5). Stage 66 is considered the end of metamorphic climax, coincident with complete tail resorption, by which stage TH levels have fallen considerably (Leloup & Buscaglia, 1977).

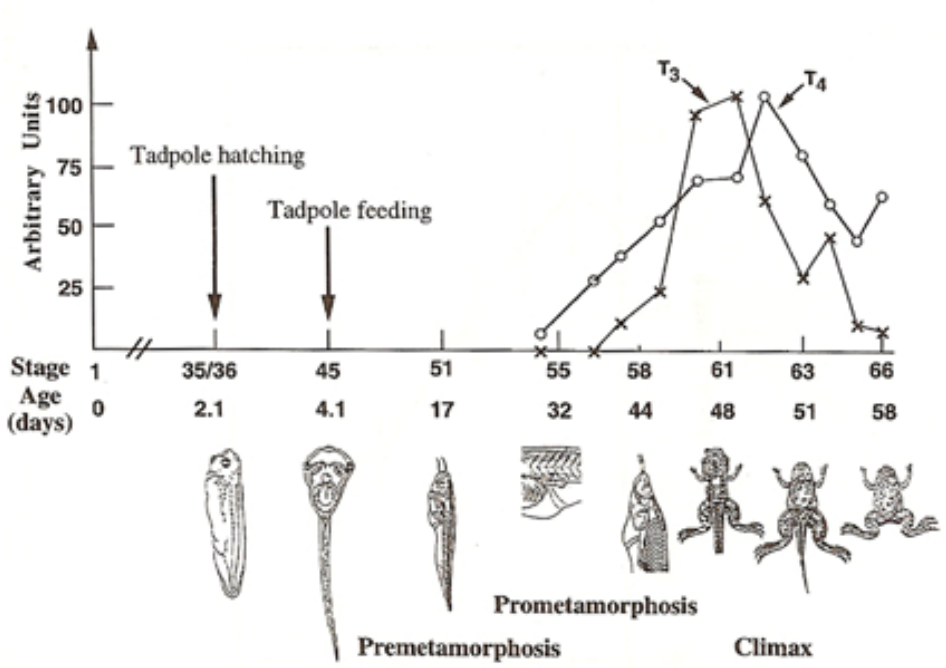

Figure 5. The correlation between stages in *Xenopus* development (according to (Nieuwkoop & Faber, 1956) and plasma thyroid hormone levels (from Leloup & Buscaglia, 1977). Note the falling concentrations of THs after stage 63. Figure from Shi, 2000.

Substantiating the claim that the thyroid gland produces thyroid hormones, and that these are responsible for orchestrating metamorphosis are experiments involving thyroidectomized tadpoles, which grow larger than normal but are not capable of metamorphosis (Allen, 1929). In addition, goitrogens (chemical inhibitors of thyroid function) have been used to block metamorphosis by impeding the synthesis of thyroid hormones (see Dodd & Dodd, 1976). Exogenous TH can reverse this action, providing unequivocal evidence that TH is the causative agent of metamorphosis in anurans.

Amphibian metamorphosis is also accompanied by changes in thyroid hormone receptor concentration and in the activity of deidodinase systems, both of which enhance the sensitivity of tissues to circulating T4 (Shi, 2000). A fascinating example of a deviation from the standard course of amphibian development is the Mexican Axolotl, *Ambystome mexicanum*. As a neotene, it normally completes its full life cycle without metamorphosing from its larval form. Anatomical metamorphosis can be induced with T4, but factors other than naturally low levels of T4 appear to be involved; in fact not only is there a

deidinace deficiency in the animal's tissues, there is also developmental decline in the number of T3 receptors that cells express (Galton, 1992), suggesting that mechanisms controlling neoteny (and metamorphosis) are more complex than just the presence or absence of THs. Deviations in amphibian development can be caused by disruptions in the thyroid axis, which includes nervous and peripheral tissues, at different levels (see Rosenkilde & Ussing, 1996).

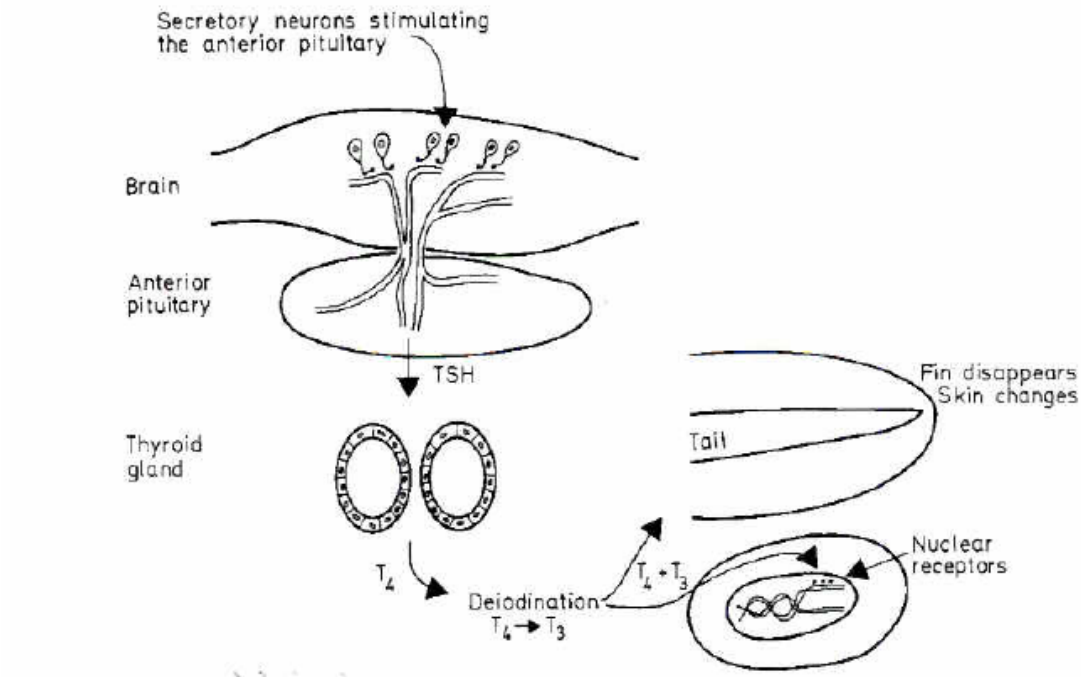

Figure 6. The amphibian thyroid axis. Disruptions at one or more levels ultimately influence the developmental outcome of the animal. From Rosenkilde & Ussing, 1996.

3.1.1 Changes in gene expression

Thyroid hormones are responsible for complex spatiotemporal alterations in gene expression through binding to thyroid hormone receptors (TRs) (see Marsh-Armstrong et al., 2004; Brown et al., 2005). Just in *Xenopus* limb buds, for example, over 120 genes are upregulated within the first 24 hours after induction of metamorphosis by thyroid hormone, while other genes in the tail are found to be slightly downregulated (Buckbinder & Brown, 1992). The idea that THs have varied effects in the organism depending on the tissue and availability of TRs is supported by the fact that *Xenopus laevis* has at least two types of receptor isoforms, TRα and TRβ (Yaoita et al., 1990) and they are expressed at

different times, with TRβ being expressed in response to rising TH concentrations (Wang & Brown, 1993) while TRα is expressed constitutively and before the appearance of TH (Elicieri & Brown, 1994). TRα is found at the highest levels in cells that proliferate upon treatment with TH, such as the limb buds, basal layers of the skin and subventricular zones of the brain (Berry et al., 1998). TRβ on the other hand, is highly induced in resorbing tissues like the tail, but at rather low levels in most proliferating tissues (Wang & Brown, 1993). In any case, TR mRNA has been found to be present in most tissues of the organism, particularly those scheduled to undergo metamorphic change, like the retina, digestive tract, and limb buds (Kawahara et al., 1991) and such differences in TR isoform expression suggest specific roles in metamorphosis.

As previously stated, the morphological and functional adaptations in bodily tissues/organs during anuran metamorphosis come about as a result of changes in the transcription of specific sets of genes induced by TH (see also Furlow & Neff, 2006). TH-bound TRs can activate or repress transcription of genes, but the majority of genes that have been studied in this respect are those that are up-regulated (Shi, 2000). The precise mechanisms of transcriptional regulation are beyond the scope of this thesis, but what this section serves to highlight is that a single chemical trigger (TH) can have widely differing, tissue-specific effects depending on the receptors those tissues express, and the timing of their expression. In this way, TH may be able to control multiple developmental programs simultaneously. This is of crucial importance to the animal undergoing metamorphosis; the limbs must be completely formed and functional before the tail is resorbed, and the lungs must be functional before the gills disappear (Furlow & Neff, 2006). In other words, the widespread rearrangement of diverse tissues and organs in the metamorphosing frog is orchestrated by a seemingly simple hormonal trigger, which induces a cascade of gene regulation and thus initiates tissue-specific genetic programs (for example in nervous tissue, see Denver, 1998, for a review). The result: the dramatic transformation of the aquatic, filter-feeding tadpole into a terrestrial predator, almost unrecognizable when compared to its larval form.

3.2 Morphological changes in anuran metamorphosis

In anurans the general metamorphic timeline starts with the development of hindlimbs, followed shortly by the forelimbs. The developing lungs begin to replace the gills as respiratory apparatus, the digestive system is remodelled to prepare for a carnivorous diet, the eyes migrate rostrodorsally, and finally, the tail is resorbed, resulting in a completely metamorphosed juvenile adult amphibian, with all the appropriate adaptations for a new behavioural lifestyle and, most likely, habitat (Dodd & Dodd, 1976). What is remarkable is the fact that these changes occur at distinct developmental stages to make the transition from tadpole to frog relatively smooth, and even within one organ, different tissues may respond differently to regulation by thyroid hormones.

3.2.1 Resorption

The tail and gills are two tadpole-specific organs that degenerate completely during metamorphosis. While necessary for survival of the larvae, these two organs are not needed by the adult frog, as locomotion is served by the limbs rather than by tail-swimming, and respiration is mediated by gas exchange not at the gills, but in the lungs. The tail consists of several major tissues- epidermis, connective tissue, muscles, notochord and blood vessels, yet despite the diversity in tissue types, major resorption in *Xenopus* begins around stage 62 and all tissues are resorbed by the end of metamorphosis (Nieuwkoop & Faber, 1956). Isolated tails cultured *in vitro* undergo resorption in the presence of TH (see Weber, 1967), providing evidence that tail resorption is under the control of TH and is organ autonomous. In fact, a tail transplanted to the body of another tadpole undergoes resorption simultaneously with the host tail, while the rest of the body continues normal development- neighbouring tissues do not affect the tail tissues response to TH. Similarly, transplanting an eye cup onto the tail does not cause it to degenerate when the tail resorbs- tissues seem to be genetically predetermined to respond to TH in a specific manner and only when TH levels are appropriate (Weber, 1967).

3.2.2 *De novo* construction

Arguably the most dramatic morphological change that occurs over anuran metamorphosis is the appearance and development of functioning limbs. Hindlimb bud formation and growth interestingly enough, do not require TH, as is shown by the fact that the limb buds appear and grow in size before the thyroid gland becomes fully functional at stage 53 (Dodd & Dodd, 1976). Most morphogenic changes occur between stages 54-58, when the hindlimb buds begin to differentiate and form toes as TH levels rise (Leloup & Buscaglia, 1977). Blocking TH synthesis has been found to prevent morphogenesis of the hind limb but not the development of the hindlimb bud (Allen, 1929; Dodd & Dodd, 1976). After stage 58, there is an increase in hindlimb size but little further morphological change. The forelimbs, on the other hand, develop slightly later than the hindlimbs and they are not visible as buds until stage 48 (Nieuwkoop & Faber, 1956). Growth is again independent of TH, but when endogenous TH becomes readily available at stage 53 the forelimbs undergo morphogenesis and digits are formed. By stage 58 the forelimbs break through the peribranchial sacs, and after this, growth in size occurs, much like for the hindlimbs after their complete morphogenesis (Shi, 2000).

3.2.3 Remodelling

Many organs are already present in the tadpole and require remodelling before they become adult-specific, such as the liver, nervous system, and intestine (Dodd & Dodd, 1976). Here I will limit myself to describing the changes in the nervous system, as changes in this likely coincide with developmental changes in rhythmogenetic capabilities of the spinal cord, and the neural control of locomotion is the main focus of interest in this thesis. Coincident with the changes that are occurring on other body systems, morphological alterations of the nervous system take place during metamorphosis. Brain regions associated with new tissues, such as the limbs, develop *de novo* just as the limbs do, whereas neural tissue dedicated to tissues that regress (for example the tail) also undergo degeneration (Kollros, 1981). It makes sense that developmental changes in body morphology are reflected in the morphology of the nervous system, with proliferation and differentiation (as well as degeneration) of

neurons occurring in association with new, remodelled, or resorbed tissues. The transformation of the nervous system also depends on TH, for example, inhibition of thyroid function leads to abnormalities in brain morphology such as thinner cerebellar walls and larger ventricles compared to metamorphosed control animals (Kollros, 1981).

During CNS remodelling, larval structures like Mauthner neurons are eliminated, as are the sensory and motor neurons supplying the tail (Dodd & Dodd, 1976; Fox, 1981; Kollros, 1981). The visual system undergoes an overhaul too; compared with the panoramic vision of the tadpole, binocular vision of the frog required for a predatory lifestyle necessitates changes in retinal circuitry and visual projections to the brain in the di- and mesencephalon (Hoskins, 1990). Other changes include development of the mesencephalic nucleus of the trigeminal nerve (Kollros & McMurray, 1956), the enlargement of the cerebellum (Gona et al., 1988), and importantly, the development of spinal cord segments associated with the limbs (Beadoin, 1956; Hughes 1966).

Clearly then, the CNS of the frog undergoes dramatic changes that reflect the function of not only new and remodelled organs, but also new inherent functions of the nervous system itself, such as a switch in locomotor strategy and visual capabilities. In mammals, TH exerts pleiotropic actions on the developing brain, influencing neuronal maturation, neurite outgrowth, synapse formation, timing of cell differentiation, and myelination (see Porterfield & Hendrich, 1993 for a review). It is not surprising, therefore, that the neurodevelopmental abnormalities caused by insufficient TH during fetal and neonatal development are found to result in postnatal behavioural abnormalities. This highlights the importance of TH in neurological development, something that is likely to have been evolutionarily conserved, and thus important in earlier vertebrates like amphibians as well. In fact, with TH action affecting genetic programs for transcription factors, cellular enzymes, structural proteins, and secreted signalling molecules (reviewed in Denver, 1998) it is not difficult to see how deeply impactful the presence or absence of TH can be to a developing nervous system.

3.2.4 Changes in locomotor control in amphibians

The way an animal moves through and interacts with its environment is crucial to its survival. Along with developing lungs and adult skin, forward-facing eyes and an adult-appropriate digestive system (all designed to respond to a new habitat, diet and respiratory behaviour), amphibian tadpoles must prepare for a major switch in locomotor strategy. In the larval phase of life, *Xenopus laevis* propels itself through its watery environment using axial-based movements that occur with bilateral alternations and in a rostro-caudal fashion. In this case the tail is the primary means of generating forward thrust. By the time it is a fully developed juvenile froglet however, *Xenopus laevis* has grown four limbs, undergone complete tail resorption, and locomotion is served by bilaterally synchronous hindlimb kicks. Electrophysiological research that makes use of *in vitro* brainstem/spinal cord preparations at different metamorphic stages has plotted the time-course of the emergence of limb motor circuitry in *Xenopus laevis* (Combes et al., 2004). Initially this secondary locomotor network is found to be present in the spinal cord but non-functional; recordings made from extensor/flexor limb motor nerves demonstrated the existence of spontaneous rhythmic motor bursts characteristic of hindlimb kicks, at stages when the hindlimbs are not actively used for propulsion (*in vivo* observations showed the legs are actually held swept back against the undulating tail). As development progresses the secondary locomotor nework becomes functionally attached to the axial network, then co-active but independent of the axial circuitry, and finally, after the axial network disappears at metamorphic completion (the tail will be fully resorbed), the limb circuitry remains as the only network driving locomotion in the froglet. The existence of a fascinating period in which the two locomotor patterns can occur simultaneously in the animal, before complete transition to the adult locomotor behaviour, implies that as the limbs grow and the tail regresses, a dynamic reconfiguration of rhythm-generating circuitry is happening in the spinal cord. The animal must continue to survive within its environment as the developmental alterations are occurring, and the spinal cord must continue to supply environment-appropriate excitation to the muscle groups responsible for movement at the same time as adult circuitry is assembled and gains functionality.

Remodelling of extant networks, deletion and *de novo* assembly of neural elements in the spinal locomotor circuitry are therefore likely to be occurring simultaneously during metamorphosis.

One of the changes in the nervous system over metamorphosis of anurans is the appearance of lateral motor column (LMC) neurons. The development of these neurons in the lumbosacral region of the nerve cord accompanies hindlimb development (see Beaudoin, 1956; Dodd & Dodd, 1976; Kollros, 1981) and therefore is associated with the subsequent changes in locomotor strategy. In fact, during embryonic and larval development of *Xenopus*, two different populations of motor neurons appear in the spinal cord (van Mier et al., 1985). Primary motor neurons innervate the axial musculature used for embryonic swimming, and are found to be present in the spinal cord prior to hatching. Secondary motor neurons serve the musculature (limbs and axial) over the course of development, during late metamorphosis, and thereafter, and are found to supersede primary motor neurons both in number and in function (Forehand & Farel, 1982; Nordlander, 1986). The LMCs, which innervate the limbs and are found only in spinal regions at limb levels (i.e. brachial and lumbar), are composed of clustered secondary motor neurons. The medial motor column (MMC), innervating axial musculature in the developed larva and adult frog (Forehand & Farel, 1982), is also composed of secondary neurons but located ventromedial to the LMCs and running the length of the spinal cord. The first neurons innervating the developing hindlimb buds can be traced into the spinal cord with horseradish peroxidase at stage 48. Van Mier and colleagues (1985) posited that circuit development proceeds first by the establishment of contacts between motor neurons and their target muscles, and only after that do the motor neurons make connections with other nerve cells within the CNS. It follows therefore, that the first appearance of secondary motor neurons that will form the LMCs would coincide with the emergence of the structure they must innervate.

TH mediates morphological and functional adaptations in tissues through changes in gene expression, and as mentioned previously, one of the results of this could be a change in availability and

synthesis of signalling molecules. In fact, evidence suggests that TH can upregulate the activity of NOS enzyme in tadpole tissues (Kashiwigi et al., 1999) and that this enhancement of NO generation may, through increased oxidative stress, activate cellular programs for apoptosis. I described earlier in this introduction that NO can have varying effects on cell processes like proliferation and synaptogenesis; tied in with the fact that TH can induce NOS expression, this suggests NO as an interesting signalling molecule that could play a role in the cellular processes that occur during metamorphosis. Might NO be involved in the development of secondary motor neuron populations that serve limb-based locomotion? This is one of the questions this investigation seeks to answer.

3.3 NO in development/metamorphosis

I have spent some time detailing the involvement of NO in cellular processes like apoptosis, synaptogenesis and proliferation. Moreover, metamorphosis necessarily involves large-scale changes in an organism's tissues by way of such cellular processes. Therefore, it is logical to suggest that certain metamorphic changes in tissues may involve NO signalling. In fact, NO is found to be involved in regulating the initiation of metamorphosis in some marine invertebrates (see Bishop & Brandhorst, 2003) and inhibition of NOS activity is found to promote metamorphosis of the marine snail (Froggett & Leise, 1999), the sea urchin (Bishop & Brandhorst, 2001) and ascidians (Bishop et al., 2001). The research has also shown that exogenous NO can suppress natural metamorphosis. Furthermore, inhibition of sGC or the chaperone HSP90, (another implicated component of the NO-based signalling system) induce metamorphosis of ascidian and urchin larvae (Bishop & Brandhorst, 2001; Bishop et al., 2001). Altogether what this suggests is an inhibitory regulating role for NO in invertebrate metamorphosis. In the developing amphibian brain, NOS is found to be expressed adjacent to zones of dividing neuronal precursors, and when NO is applied exogenously *in vivo*, it causes a decrease in the number of proliferating cells and in the total number of cells in the optic tectum (Peunova et al., 2001). NO is found to act as an antiproliferative agent in insect development too (see Kuzin et al., 1996). Such evidence suggests NO as a negative regulator of neuronal precursor proliferation during development, and

proposes an essential role for it in the control of the balance between cell proliferation and differentiation.

3.3.1 NOS expression correlates with metamorphic changes in sensorimotor systems

As stated previously, research has shown that one of the developmental alterations in the nervous system of anuran amphibians is a change in the expression of NOS (McLean & Sillar, 2001; see also Cristino et al., 2004). As has been made previously discussed, metamorphosis in the frog involves the substitution of undulating tail movements with limb-based motor patterns, and therefore requires functional and structural rearrangement of locomotor and sensory networks (Stehouwer & Farel, 1985). Separately from the acute effects that they may have on locomotion, endogenous neurochemicals can also contribute to the development of spinal locomotor circuits, as has been shown for 5HT in mouse (Cazalets et al., 2000; Branchereau et al., 2002). If the developmental release of neurochemicals influences spinal locomotor circuitry, then it is easy to see why the diffusible properties of NO, and its short-half life (as well as the fact that it is expressed in brainstem cell types proximal to the cell bodies of descending aminergic systems) make it an ideal candidate for regional influences on the development of motor systems. It has been found in *Xenopus* that while NOS expression is restricted to brainstem and higher brain areas before stage 47 (McLean & Sillar, 2001), after that it begins appearing in groups of cells in a temporally-specific way, starting with a region in the spinal cord below the brainstem, and then appearing also further down the cord at later stages (Ramanathan et al., 2006). Judging by the pattern of expression of this enzyme in the spinal cord, and the fact that it occurs at a time when limb buds are beginning to appear (and thus, dedicated circuitry is being assembled) it is easy to speculate that such changes in NOS expression could in fact correlate with the emergence of the limbs and their respective motor networks. Indeed, it has recently been shown that cells expressing NOS are initially excluded from spinal areas where the limb networks may be forming, suggesting that some of the metamorphic changes occurring in the nervous system may be mediated by NO (Ramanathan et al., 2006).

A role for NO in sensorimotor maturation has been suggested before. The dorsal root ganglia (DRG) (pair 3 for the forelimbs and 9-10 for the hindlimbs) supply a much larger peripheral target in the frog than in the tadpole, and therefore their neurons must be undergoing plastic remodelling in order to accommodate increased motor and sensory demands (Cristino et al., 2004). In fact, sensorimotor maturation in the DRG is found to be associated with a change in NOS expression. Limb DRGs experience a transient rise in NOS expression, which is down-regulated at the end of metamorphosis. More interesting still, tracing experiments showed that NOS is expressed by those DRG cells that elongate their axons towards the periphery at the time of the initiation of limb movements, which suggests that NO may be shaping *de novo* assembly of sensory elements in the metamorphosing frog. NO could also be involved in neuronal differentiation, as this same study found that NOS expression was concomitant with the transition from an immature to a mature cell phenotype in DRG neurons; in other words, with the establishment of functional connections. There is also research showing that NOS expression in the nervous system appears after or during processes of cell division and migration (Lopez & Gonzales, 2002). Combining this with evidence that NOS expression in supraspinal neurons controlling motor activity was found to be concomitant with the initiation of aquatic swimming (McLean & Sillar, 2001), it seems reasonable to suggest that NO synthesis might play different roles at different times in the developing nervous system, influencing the establishment and consolidation of synaptic connections as well as the functioning of mature circuitry.

3.3.2 TH affects NO signalling

Unpublished observations by workers in this lab have reported that T4 can induce metamorphic changes in the nervous system of *Xenopus laevis*, as established in organotypic culture of the spinal cord of stage 47 tadpoles (Ramanathan & Sillar, unpublished observations). Addition of T4 to the culture medium over 3 days and subsequent staining for NADPH-d has shown that NOS expression changes from a typical stage 47 pattern (brainstem neurons express reactivity but very few or no spinal neurons are found to do the same) to that characteristic of an older animal (NOS-reactivity appears in a cluster of

cells in the rostral spinal cord). This provides evidence that T4 can trigger metamorphic changes in the nervous system of the frog, and that one of those changes is an alteration in the expression of NOS. It has previously been shown that NOS is excluded from regions in the spinal cord where dedicated limb circuitry is forming, demonstrated by backfilling limb motor neurons with horseradish peroxidase. The cells expressing NOS (as indicated by NADPH-d reactivity) are found to occur in spinal cord regions between the areas where limb motor neurons are located (Ramanathan et al., 2006). NADPH-d reactivity is excluded from limb regions of the spinal cord, possibly for developmental reasons, and begins to appear in cells distributed along the entire length of the spinal cord only later, as the tadpole approaches stage 53. Based on these observations, it makes sense to consider NO as a possible mediator of metamorphic alterations in neural circuitry. Considering that NO has been found to regulate aspects of metamorphosis in invertebrates, and that in *Xenopus* TH influences NOS expression, it seems logical to propose that some of the metamorphic effects of TH could be effected by NO generation in tissues. Indeed, other research has suggested that thyroid hormones enhance NO production in cells, and that this may contribute to apoptotic degeneration of the tail (see Kashiwigi, 1999).

Since it has been established that NO and apoptosis play major roles in remodelling amphibian tissues during metamorphosis, and it has also been shown that NOS expression in the nervous system changes in response to the presence of thyroid hormones (unpublished observations), then it follows that NO could be assisting with the development of new circuitry dedicated to the sensorimotor innervation of the developing limbs. There is evidence for the appearance of populations of hindlimb secondary motor neurons in *Xenopus* spinal cord around stage 48 (van Mier et al., 1985), and seeing that the first spinal groups of NOS are also appearing at this stage, it is easy to speculate that there may be a correlation between the differentiation of secondary motor neurons in the limb regions and the expression of NOS in the 'interlimb' regions. NOS expression in particular regions of the spinal cord may serve an anti-proliferative role on motor neuron precursors, whilst its exclusion from the limb regions could allow motor neurons for the LMCs to proliferate unhindered.

4 The scope of this investigation

The roles of NO in the nervous system and neural plasticity are complex. NO can mediate motor neuron development (Kalb & Agostini, 1993) but has also been found to inhibit proliferation (see Villalobo, 2006, for a review) and impact synapse formation (Sunico et al., 2005). There is evidence for NO involvement in selective neuronal death following injury (Zhou, et al., 1999) as well as in apoptosis (Brune, 2003). Nitric oxide has been implicated as a metamodulator of locomotion and may play a part in the development of amphibian motor systems and their maturation (see McLean et al., 2000). For these reasons, NO is an important molecule to investigate with respect to its role in motor control. This will be done in a variety of ways. Since the lamprey is a particularly well-studied adult model of vertebrate locomotion, it will be of interest to explore NOS expression within the spinal cord, in an attempt to elucidate whether NO might be involved in the generation of rhythmic motor patterns. The use of this classical model makes sense because it has been extensively investigated and many of the cell types have been identified and their involvement in the generation of rhythmic locomotion has been established. Furthermore, the role of NO as a signalling molecule in biological systems seems to have been highly conserved over evolution and exploring the presence of NO in the motor systems of a more primitive vertebrate could substantiate this. NADPH-d histochemistry and nNOS immunohistochemistry will provide evidence on whether spinal cells known to contribute to motor control in the lamprey are also nitrergic. The amphibian nervous system, however, will contribute the bulk of this investigation. It is an excellent model through which to explore locomotion, as it evolves during metamorphosis, in order to verify whether NO plays any role in the development of dedicated limb motor circuitry.

Using organotypic culture of spinal cord tissue in combination with THs (to simulate the elevated concentration of TH during metamorphosis), and then with nitrergic drugs, the development of the limb motor circuitry will be investigated. The first set of experiments will serve to establish a suitable organotypic culture protocol, using THs to attempt to trigger metamorphic changes in the amphibian CNS *in vitro*. If alterations in spinal NOS-labelling can be viewed as developmental changes in the CNS,

then it sets the stage for the investigation of NO as a metamorphic signalling molecule. Subsequently, I will conduct a set of experiments that will test whether the presence of exogenous NO, or the inhibition of NOS, has any impact on the development of limb motor circuitry. These culture experiments will involve the addition of the NO-donor SNAP, or the NOS-inhibitor L-N^G-nitroarginine methyl ester (L-NAME) to the culture medium. If NOS expression and NO production in the 'interlimb' region of the spinal cord are preventing proliferation or differentiation of motor neurons in this area during metamorphosis, thus allowing the developmental progression of neural circuits only in those areas of the spinal cord destined to innervate the limbs, then supplying NO exogenously should inhibit the developmental progression of the motor circuits in the limb regions. The effects of the exogenous NO, as well as the NO-depleted state, on the developing motor neurons will be evaluated after the 3-day culture by using choline acetlytransferase (ChAT) immunolabelling to identify motor neurons and characterize any developmental changes in morphology and/or cell number. The spinal region of interest will be primarily the area that corresponds to the forelimbs, i.e. a length of spinal cord immediately caudal to the brainstem. This area develops during metamorphosis to display the forelimb LMCs, characteristic ventral horn groupings of secondary motor neurons that power limb movements (Fortune & Blackler, 1976). Overall, this investigation aims to substantiate evidence regarding the role of NO in locomotor control, with particular attention on its implications for the emergence of forelimb spinal motor circuitry. I hope to add to the ever-growing body of research on the varied functional and developmental roles of this deceptively simple molecule.

All *Xenopus laevis* tadpoles used in this investigation were obtained by induced breeding from an in-house adult laboratory colony, and raised in large glass tanks until the desired age. Wildtype tadpoles were generally used, with the exception of NADPH-d staining in skin, where albino animals were preferred. Tadpoles to be used for experiments were selected and staged, according to Nieuwkoop and Faber (1956). Prior to dissection, animals were anaesthetised in tricaine methanesulfonate (MS-222; 0.1%) in water; this immobilized them and allowed for accurate staging. When performing any dissection, the anaesthetized animals older than stage 42 were subjected to a Schedule I kill (as defined by the Animals Scientific Procedures Act of 1986) by destruction of the forebrain. All solutions used in this investigation are catalogued in Appendix 1.

1 Labelling NO/NOS

1.1 NADPH-diaphorase histochemistry

1.1.1 Wholemount *Xenopus* stage 37/38

Albino animals at stage 37/38 were selected and fixed whole for 2 to 3 hours in cold (4°C) paraformaldehyde (see Appendix 1 for composition). Tadpoles were washed (3 x 10min), immersed in freshly made NADPH-d staining solution (see Appendix 1) and incubated for 2 hours at 37°C. Once the stain had developed, they were washed (3 x 10min) in phosphate buffer (PB, see Appendix 1) to end the enzymatic reaction. Tadpoles were subsequently washed in distilled water (3 x 5min) and dehydrated in a methanol series (30min each in 25%, 50%, 75% and 100% methanol). Clearing of the tissue was done by transferring the tadpoles to a 1:1 mixture of methanol and benzyl benzoate: benzyl alcohol (BB:BA, 2:1) for 30 minutes, and finally into BB:BA for 30 minutes, before mounting in glass cavity slides using BB:BA as a mountant. Slides were stored in protective slide folders at 4°C; however, this is not indefinite storage as the BB:BA slowly clears out all staining in the tissue within 6 months.

1.1.2 Wholemount *Xenopus* CNS after organotypic culture

Wholemount *Xenopus* CNSs were stained for NADPH-d reactivity after 3 days in organotypic culture (see organotypic culture protocols ahead). Staining solution was made up fresh and the CNSs were immersed in it and incubated for 2 hours at 37°C, checking with the naked eye after one hour to see how the stain was developing (blue brainstem staining is particularly intense and can be seen even without a microscope, and functions as a positive control for this histochemical technique). After incubation, tissue was removed from the staining solution and washed (3 x 10min) in PB. CNSs were then washed in distilled water (3 x 2min) to remove salts, before dehydrating and clearing the same way as done for stage 37/38 tadpoles described above. CNSs were mounted individually using BB:BA in cavity slides and coverslipped for viewing. The mounting medium is a very effective clearant which can clear out the blue reaction product in projections and in faintly labelled cells, it was therefore very important to document all results immediately after processing. Due to building work in the Department, for extended periods during this part of my project an unusually high level of dust was present in the atmosphere at the time of culturing the tissue. While steps were taken to reduce dust contamination of the samples, unfortunately after NADPH-d processing this dust was visible as blue-stained debris coating the CNS. While it did not prevent accurate identification of stained neurons, removal of this debris proved impossible without damaging the underlying tissue.

1.1.3 Segments of wholemount lamprey spinal cord

Adult lamprey spinal cords were provided by Professor Sten Grillner at the Karolinska Institute, Stockholm. The tissue was fixed in 4% paraformaldehyde in Stockholm and delivered to St. Andrews stored in PB. The meningeal sheath was removed with forceps before processing. Segments of adult lamprey spinal cord were processed for NADPH-d histochemistry in much the same way as *Xenopus* tissue was, with the exception that prior to incubation in staining solution, the lamprey tissue was incubated in 0.3% PBTX (see Appendix 1) for 1 hour. This served to help permeabilize the tissue. Furthermore, the incubation at 37°C lasted 3 hours rather than 2, as the lamprey tissue was much thicker

than *Xenopus* CNSs. Dehydration times in the methanol series were also adjusted to account for this (45min in each methanol concentration), and after clearing in BB:BA, samples were mounted in the clearant.

1.1.4 Lamprey spinal cord sections

Segments of the fixed spinal cord were embedded in agarose (3% in PB) and freefloating sections were cut in a PB bath at 100µm on a vibrating microtome (Vibratome). Sections were then immersed in NADPH-d staining solution and incubated for 2 hours at 37°C. Stained sections were washed in PB (3 x 2min) and dehydrated, cleared and mounted the same way as for the wholemount lamprey segments, with the exception of flat glass slides being used rather than cavity slides.

Photography and analysis: All samples of *Xenopus* and lamprey tissue were examined on a Zeiss Axiolab compound microscope and photographed using a mounted A620 Canon digital camera. Images were collated using Adobe Photoshop.

For wholemount *Xenopus* at stage 37/38, stained cells were identified, and the pattern of staining compared to published work on skin and CNS NADPH-d labelling (McLean & Sillar, 2001; Wildling & Kerschbaum, 2007).

For wholemount *Xenopus* CNS, in addition to photographical evidence, results were also recorded by means of a Zeiss RA microscope with a camera lucida attachment (kindly loaned by Professor William Heitler of the School of Biology) that was used to draw the CNS labelling whilst omitting the surrounding debris. The spinal cord regions of the stained CNSs were examined for NADPH-d reactivity, and any labelled cells counted and described. Importantly, the brainstem acts as a positive control for the NADPH-d histochemical technique, as cells label intensely at all larval stages and therefore confirm that the staining procedure worked correctly. Data from experiments using the same concentration of hormone in organotypic culture were pooled to give a total n number for that concentration. Average

spinal cord cell counts for each concentration were compared against the average cell counts of their respective control samples: raw data (labelled cells per sample) were analyzed using InStat statistics software, with an unpaired t-test (Welch correction applied if necessary), or a non-parametric test (Mann-Whitney test) depending on whether the data from a particular group of experiments was normally distributed. The P value for my analysis was 0.05.

For lamprey spinal cord sections and segments of wholemount, labelled cells were described and identified according to published criteria (Selzer, 1979; Grillner & Wallen, 1984; Buchanan, 2001).

1.2 nNOS immunohistochemistry

1.2.1 wholemount lamprey spinal cord segments

nNOS immunohistochemistry was carried out on segments of wholemount lamprey spinal cord in order to corroborate evidence obtained from NADPH-d labelling. After careful removal of the meningeal sheath, 4-5mm segments of spinal cord were incubated in primary antibody against nNOS (rabbit anti-NOS1, Santa Cruz Biotechnology, 1:100, see Appendix 1) for 2 days at room temperature, washed in PB (3 x 10min) and incubated in CY3-conjugated fluorescent secondary antibody for 12 hours at room temperature (Goat anti-rabbit IgG, Jackson Immuno, 1:100) with constant gentle agitation. Samples were kept in the dark to preserve fluorescence of the secondary antibody. Finally, segments of tissue were washed in PB for 12 hours (changing solution every 4 hours) in order to reduce background fluorescence caused by nonspecific antibody binding, mounted in Citifluor AF2 (Citifluor) on glass cavity slides, and viewed immediately, or stored in opaque slide trays in the refrigerator for later viewing.

Photography and analysis: samples were viewed on a Leica confocal microscope system operating with a TRITC filter, i.e. an excitation spectrum of 568-633nm, appropriate for a range of red-emission fluorochromes. Images were obtained from 10µm optical sections and were captured and stacked using the Leica Confocal Software to create a more 3-dimensional view of nNOS labelling. Stained cells were

examined and described, and compared to the pattern of labelling observed for NADPH-d staining of lamprey spinal cord segments.

2 Labelling motor neurons

2.1 ChAT immunohistochemistry

Choline Acetyltransferase (ChAT) immunohistochemistry was used to label motor neurons, as these cholinergic cells contain high levels of ChAT. Various non-motor cells in the spinal cord, such as the sympathetic preganglionic neurons, can also show immunoreactivity for this enzyme (Kondo et al., 1985), but the topographical location in the spinal cord and shape of the labelled cells make identifying motor neurons quite straightforward. In order to characterize the morphological changes that occur in spinal motor neurons during metamorphosis, motor neuron ChAT labelling was compared at different developmental stages. Animals were selected for ChAT immunohistochemistry at stages **47** and **51**. Stage 47 is just prior to the appearance of the forelimb buds, as well as being the first time a hindlimb bud is visible. NOS expression in the CNS up until this stage is restricted mostly to the brain and brainstem region (McLean & Sillar, 2001; Ramanathan et al., 2006) with the possibility of a few cells being faintly labelled in the rostral spinal cord. Stage 51 is the stage at which spinal NOS reactivity is found in two distinct spinal clusters: between the limb regions, and caudal to the hindlimb region (Ramanathan et al., 2006). My goal was to characterize the changes in the morphology and number of motor neurons in the brachial region of the spinal cord at these two important developmental stages, a time during which NOS reactivity appears in the spinal cord and when NO may be exerting some kind of regulatory influence on the development and differentiation of the motor circuitry innervating the growing limb buds.

In order to characterize the morphology of brachial spinal motor neurons at various stages of development, the area of CNS from the brainstem to halfway between the forelimb and hindlimbs was paraffin-sectioned and processed for ChAT immunohistochemistry. This length of nervous tissue would include the area in which brachial motor circuitry should be developing. At later stages (e.g. stage 57), it

is easy to define the brachial region of the spinal cord, as the limb motor neurons begin differentiation to form the clearly identifiable brachial LMCs at stage 52/53, and differentiation is generally complete by stage 58 (Fortune & Blackler, 1976). However, in younger animals these groupings of secondary motor neurons have not yet appeared, and therefore it is more difficult to define where the brachial region begins and ends. The approximate lengths of the brachial spinal region have been measured by Fortune and Blackler (1976), in animals at stage 52 and older. In stage 51 animals, the brachial region has been described by Ramanathan and colleagues (2006) as extending approximately 600μm. This was helpful in knowing where to look in my sections of stage 51 animals. However, no data exists for the length of the future brachial region in younger animals. Since I used animals at stage 47, the CNS of which is smaller than in stage 51 tadpoles, I could only estimate that the forelimb region would extend less far along the spinal cord. To control for such differences and avoid confusion, I simply looked at the first 30 sections (cut at 9-10μm) of post-brainstem spinal cord. This was certain to include the area of spinal cord that would eventually innervate the forelimbs. I did the same for all animals sectioned, whatever the stage (keeping in mind of course that in older and larger animals, the forelimb region is bound to extend further than the area to which I restricted my examination). Forelimbs were chosen as a focus, rather than the hindlimbs, because at stage 47 the forelimb buds have not yet appeared (first time visible is normally stage 48), and the brachial motor columns are also conspicuously absent so any changes in this region would be more evident.

Comparisons of brachial motor neurons were carried out for stages 47 and 51 as previously detailed. Whole animals were fixed and embedded in paraffin. The CNS of younger animals, in particular, is very thin and delicate and simply sectioning the whole animal maintains tissue integrity as well as providing an easier way to identify the forelimb region of the spinal cord, based on macroscopic structures present on the tadpole's body, that are fortuitously aligned with the forelimb region.

2.1.1 *Xenopus* CNS sections stage 47 and 51

Animals were deeply anaesthetized with MS-222 and staged. Tadpoles were fixed in Carnoy's fixative (see Appendix 1) overnight at 4°C. Larger animals were fixed for up to 3 days. Samples were then embedded in paraffin and sectioned at 9-10µm onto coated glass slides (Histobond, Raymond Lamb). Cutting was done using a Microm microtome. After dewaxing and rehydrating, slides were clipped into Shandon trays for ChAT immunohistochemistry. Incubation in primary antibody (Goat anti-ChAT, chemicon, 1:100, see Appendix 1) took place at 4°C for 48 hours, followed by a washing step with phosphate-buffered saline (PBS, 3 x 5min). Secondary incubation (biotinylated anti-goat IgG, Vector Labs, 1:200) was carried out for 60 minutes at room temperature. A Vectastain standard kit (Vector Labs) was used after the secondary step. This involved the addition of the ABC conjugate for 45 minutes at room temperature. Adding a washing step between secondary and ABC steps proved unnecessary, however slides were washed in PBS (3 x 5min) after the final antibody incubation (ABC step). To develop the stain, sections were subsequently incubated in DAB + hydrogen peroxide (DAB staining kit, Vector Labs) for 2 minutes- this was done by addition of 200µl DAB/H_2O_2 solution to each slide while still in the Shandon tray. The addition of tap water to the slides for 5 minutes helped define the stained areas better, followed by a wash in distilled water to halt the chromogen formation. Slides were removed from their coverplates and passed through an ethanol series to dehydrate (2min each in 70% and 96% ethanol, then 10 minutes in 100% ethanol) followed by 10 minutes in Histoclear (Raymond Lamb), before they were mounted in Histomount (Raymond Lamb) and viewed.

2.1.2 *Xenopus* CNS sections after organotypic culture

After 3 days in culture (see below), samples were examined to confirm the tissue was still alive (tails were swimming after gentle probe stimulation). As for the culture experiments with thyroid hormones (which were stained for NADPH-d activity, described above), samples that appeared unresponsive were eliminated. Fixation took place overnight in Carnoy's fixative, and samples were paraffin embedded and sectioned much like whole animals (as described above). After labelling for ChAT

immunoreactivity (as described for sections of the whole tadpoles at stages 47 and 51), slides were mounted in Histomount and stored horizontally to dry before viewing.

Photography and analysis: ChAT-stained sections were viewed on a Zeiss Axiolab compound microscope. For the representative stage 47 and stage 51 sections, 30 sections were examined, and labelled cells counted every third section, distinguishing between primary and secondary motor neurons (see results for criteria used). The raw data (for each motor neuron type) was tabulated and subjected to a Mann-Whitney test (as the data was not normally distributed) using InStat software. The outcome of these tests verified whether or not there were any significant changes in numbers of primary and secondary motor neurons in the brachial spinal region between the two stages (representing progressive development). The p value for analysis of motor neuron number was 0.05.

For each of the cultured samples, 30 sections caudal to the brainstem were also examined, counting labelled cells every third section (a total of 10 sections counted per animal). Raw data, categorized as primary and secondary motor neurons, were tabulated and motor neurons of each type were analysed for all three conditions against each other, using a Kruskal-Wallis test with a multiple comparison post-test. This verified whether or not there was any significant difference in numbers of each motor neuron type between conditions (SNAP, control, L-NAME), $p = 0.05$.

To supplement these results, 2 slides for each condition were randomly selected and reviewed using a Zeiss Axioskope 2 plus microscope and photographed with an Axiovision 3.1, Carl Zeiss Vision Imaging System digital camera and associated Axiovision imaging software. This software allowed me to calculate the surface area of stained tissue in each of my sections. Area of staining (in μm^2) for each section examined was tabulated for all three conditions, and analyzed using a Kruskal-Wallis test with a multiple comparison post-test. Results of this analysis verified whether there was any significant difference in area of staining (i.e. amount of cholinergic tissue) between conditions. These data also offer a comparison against results obtained by counting cells.

3 Organotypic Culture

3.1 Organotypic culture with thyroxine/triiodothyronine

In order to assess TH-associated changes in spinal NOS, a method of culturing the CNS of mid-larval (stage 47) *Xenopus* was developed. After careful staging and selection, anaesthetised animals were pinned through the nostrils and lateral to the otic capsules using fine etched tungsten pins, into a Sylgard (Dow Corning) surface at the bottom of a glass petri dish filled with frog Ringer's solution (see Appendix 1). Using fine dissecting scissors and forceps, the forebrain was cleanly cut away from the rest of the CNS and destroyed. The remaining CNS was revealed by gentle cutting and peeling back of the skin and surrounding muscle, being careful to avoid damaging the underlying nervous tissue. The underside of the CNS was freed by delicately severing all the nerves and roots running beneath it into the tadpole's body using scissors. The CNS was dissected out this way until just caudal to the end of the tadpole's body, but leaving the tail intact and attached to the nervous tissue.

In each experiment, 5 animals were dissected for each condition (drug and control), transferred to their respective petri dishes containing culture medium (see Appendix 1), and pinned securely using extra-fine tungsten pins (Fine Science Tools), one through the midbrain and one through the muscle on either side of the tail. This held the CNS firmly in place in the dish, but allowed the tail freedom of movement to beat. Once they held 5 samples each, both dishes were placed in a small refrigerator maintained at 16-17°C. Solutions were re-circulated using a peristaltic pump to move culture medium from the larger reservoir into the dish and back. Culture medium feeding into each dish was replaced every 24 hours, with TH solution at the required concentration (50nM, 500nM and 1µM for T4 and 1µM for T3, see Appendix 1) being made up fresh daily from powder stored in the dark at room temperature. The experiment was allowed to run for 72 hours. Before fixation, samples were inspected to ensure they had survived *in vitro*- this was verified by gently touching the samples with a blunt probe and seeing whether the tails would swim. Samples that were obviously degraded/necrotic (e.g. tail curled up,

decomposing, or opaque) or completely unresponsive, even to more aggressive stimulation, were eliminated as they were assumed to have died. Approximately 1 in 4 samples were lost this way. All remaining viable tissue samples were fixed in cold (4°C) paraformaldehyde (2 to 3 hours at room temperature) and then washed (3 x 10min) in cold PB before staining for NADPH-d.

3.2 Organotypic culture with an NO donor and a NOS inhibitor

An organotypic culture method similar to that used in the TH experiments was used to investigate NO-associated changes in *Xenopus* motor systems. Animals were deeply anaesthetized and a minimal dissection was performed (see diagram below): after destruction of the forebrain (1), the dorsal surfaces of the midbrain, brainstem and the initial portion of the spinal cord were freed from surrounding skin and muscle (2). The cerebellum was removed to improve drug access to the CNS via the central canal. The remainder of the spinal cord was left encapsulated in the axial/tail musculature. The ventral surface of the CNS was left in contact with cartilaginous tissue below it, and the whole preparation was freed from the body by cutting around the otic capsules and just above the thoracic cavity, just until the end of the tadpole's body, where the hindlimb buds are developing (3). This dissection freed the CNS and tail from the rest of the body, but kept the tail muscles intact so that swimming could still be observed, confirming the preparations were healthy. Samples were pinned into Sylgard dishes using fine tungsten pins through the otic capsules, leaving the tail free to move.

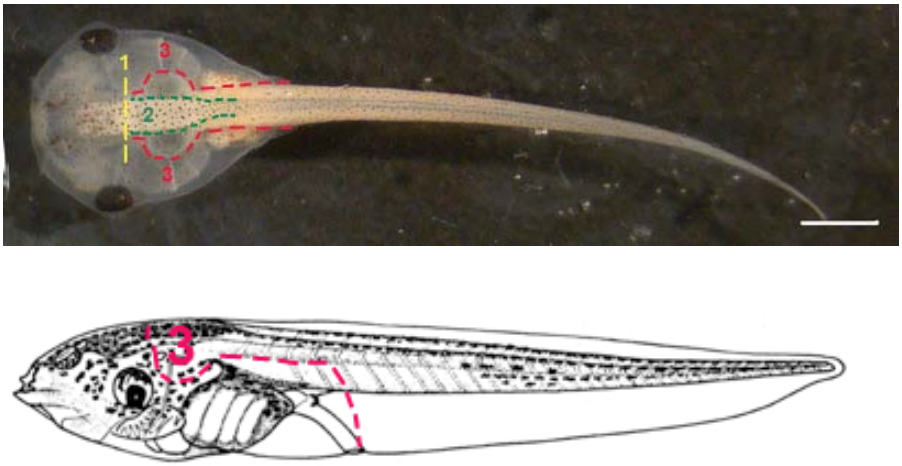

Figure 7. Dissection of stage 47 *Xenopus* for organotypic culture with nitrergic drugs. The dissection exposes only the rostral spinal cord, although as long as the cerebellum is removed, the culture medium should have access to the entire CNS via the central canal. Scale bar = 1mm.

Each experiment involved 5 animals in each of three conditions, two drug conditions (SNAP and L-NAME, both at 200µM) and a control. Experiments were maintained at 16-17°C, and culture medium was circulated by gravity-filling the petri dishes and using a peristaltic pump to remove solution back into the reservoirs. Over a 72 hour culture period, drugs were added afresh in bouts of 4 hours, interspaced with 4 hour bouts of fresh saline (culture medium with no drug or vehicle), excluding a 12 hour period of saline at night. This regimen meant that the preparations received drug 6 times over 3 days, for a total of 24 hours of drug application. An initial experiment with SNAP showed that when the drug was supplied continuously, with reapplication in fresh culture medium every 8 hours, the samples died well before the end of the experiment. Therefore the samples were given periods of drug-free saline in order to recover.

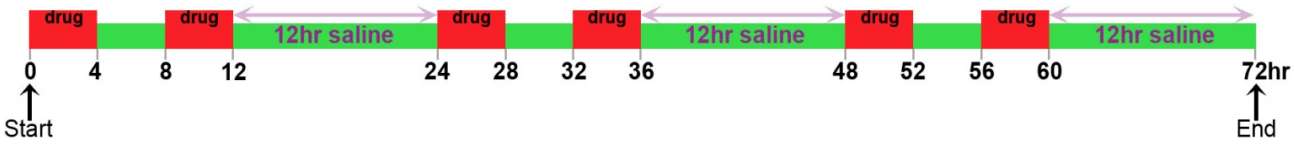

Figure 8. Administration schedule for organotypic culture with nitrergic drugs. Culture medium (saline) was replaced every time the drug was administered, and in between doses. Drugs were applied in 4-hour bouts.

The NO-donor drug SNAP was kindly provided by Professor Anthony Butler of the University of St Andrews Chemistry department. SNAP was made up in DMSO, but L-NAME (Tocris) was made up in

distilled water and when it was used in the culture medium, DMSO was also added to it to control for DMSO effects. Likewise, during the period of drug application, the control samples received saline containing DMSO at the same concentration as it was for either drug condition (0.2%).

1 Labelling NADPH-d in wholemount *Xenopus* stage 37/38

Intact albino tadpoles (n = 10) were processed for NADPH-d histochemistry (Figure 9). Characteristic blue staining was found in cells of the epidermis (Fig 9, B and C), in discrete clusters of neurons in the CNS (Fig 9, Ai-iii; cf. McLean & Sillar 2001), and in the cement gland (Fig 9, A) which appeared as a uniform, intense blue. Staining was also observed in parts of the vasculature, most notably the heart (Fig 9, A).

In the epidermis, labelling was distributed irregularly, usually in isolated oval/polygonal cells, but occasionally labelling was observed in clusters of two or more cells (see Fig 9, C). The cells measured approximately 15µm diameter, and were most easily observed in areas where only epidermis is present, such as the fin and the tail.

In the CNS, three clusters of neural staining were observed (Fig 9, Ai-iii), two small populations straddling the midbrain-hindbrain divide (isthmus), and a larger population in the caudal hindbrain. The labelled cells were unipolar, rounded or pear-shaped, had a diameter of 8-10µm, and most appeared to project neurites ventrally. Staining was extremely faint in the somata of the rostralmost group. Whilst the CNS had not been a particular focus of this experiment (I assumed dissection would be preferable when studying this structure), the pattern of CNS staining was repeatedly observed in all my samples.

My findings corroborate previously published work on NADPH-d labelling in the skin and CNS of *Xenopus* (McLean & Sillar, 2001; Wildling & Kerscbaum, 2007; Alpert et al., in press). This experimental protocol also provides an interesting opportunity to study neuronal populations in intact animals (see Discussion).

2 Induction of NOS expression *in vitro*

The aim of my organotypic culture experiments was to attempt to mimic aspects of neural plasticity occurring *in vivo*, in an *in vitro* situation by experimentally replenishing thyroid hormone. *In*

vivo, THs are transported via the bloodstream; at metamorphic climax the concentrations are around 10nM for T4 and 8nM for T3 (Leloup & Buscaglia, 1977). *In vitro*, the bloodstream route of access has been compromised, hence my use of much higher concentrations. At stage 47, NADPH-d labelling, while intense and widespread in the brain and brainstem, is virtually or completely absent in the spinal cord (Mclean & Sillar, 2001; Ramanathan, et al. 2006). Isolated, faintly labelled cells may be observed at stage 47, but generally, cells in the spinal cord of *Xenopus* larvae do not start labelling for NADPH-d until stage 48 with the appearance of a rostral cluster extending about 700-1200µm, as reported by Ramanathan and colleagues (2006). Figure 10 illustrates this schematically. In this investigation I selected animals at stage 47, and examined the pattern of NADPH-d staining after 72 hours in culture in control preparations and in sibling preparations maintained with either the hormone thyroxine (T4) or trio-iodothyronine (T3), at a range of concentrations.

2.1 NOS expression with T4 at 50nM

Figure 11 illustrates results of experiments using 50nM thyroxine. Brainstem NADPH-d staining was present and intense in all samples, as has been previously described for animals at premetamorphic stages (Mclean & Sillar, 2001), but its presence was noted primarily to verify whether the histochemical stain had worked and will not be the subject of further analysis. In drug-treated samples (n = 15) some degree of labelling of neurons in the spinal cord was seen in 7 samples. In all of these cases, staining in the spinal cord was restricted to a region located 300-450µm caudal to the brainstem and extending 250-400µm (Fig 11, Ci-Cii). Cell bodies were labelled on either side of the midline and were rounded or pear-shaped, unipolar, of diameter 5-10µm (Fig 11, Ciii). Projections could occasionally be traced for a few hundred microns in the tissue, but were not frequently seen. Approximately half (8 of 15) of the samples displayed characteristic NADPH-d staining only in the brainstem but a complete absence of staining in the spinal cord.

Control samples (n = 15), which did not receive any thyroxine over the culture period, showed labelling in only one sample. The rest displayed no NADPH-d labelling in the spinal cord, but cells in the

brainstem/hindbrain of all labelled with the characteristic pattern of premetamorphic tadpoles, although no difference was noted in staining intensity between control and thyroxine-treated CNSs (Fig 11, B; cf Cii).

Labelled cells in the spinal cord were counted for each sample and then control and drug groups were compared (Mann-Whitney test; $p < 0.05$). Spinal cords cultured in control conditions for 72 hours contained an average of **0.87 ± 3.36** stained cells per cord. Sibling preparations reared in 50nM thyroxine over the same period of time contained an average of **9.7 ± 12.9** stained neurons, a significant increase from the control condition (see Fig 11, D).

2.2 NOS expression with T4 at 500nM

For my next set of experiments, the concentration of T4 in organotypic culture was increased to 500nM. At this concentration (n = 5) spinal cord NADPH-d labelling was observed in 4 of the preparations. 3 out of 5 T4-treated samples displayed intense NADPH-d staining in 10-25 cells in a rostral spinal cluster, one T4-treated sample had only 3 labelled cells (which could place it in the 'normal' range for stage 47), and one showed no spinal labelling whatsoever. Figure 12 represents experiments using this concentration of thyroxine. Staining was observed in a cluster 300-550µm caudal to the brainstem/spinal cord divide, that extended between 270 and 350µm. Cells were pear-shaped or rounded, with the more intensely labelled cells displaying one or more projections (see Fig 12, Ci, Cii). These unipolar cells were located on either side of the midline but it was difficult to capture this distribution photographically as the CNSs tended to rotate in the slides. Most visible projections from the soma tended to travel ventrally. Some cell bodies were stained particularly intensely, appearing very dark blue. Projections were also more widespread in these samples compared to the 50nM treated samples, extending for distances of up to 80µm, but it was not always possible to associate the labelled projection with a given cell. Cells that labelled very intensely were more often observed to be pear-shaped than rounded.

Weak NADPH-d labelling in the spinal cord was observed in 3 of the control samples (n = 5) but this was restricted to only 1-3 stained cells in each cord, within the reported range of staining for stage 47 CNS (McLean & Sillar, 2001; Ramanathan et al, 2006).

Labelling was counted and analyzed (Welch-corrected, unpaired t-test; $p < 0.05$). Control cords averaged **1 ± 1.23** stained cells, whilst sibling preparations reared in 500nM thyroxine contained **11.6 ± 10.7** stained cells per cord, a significant increase from control (see Fig 12, D).

2.3 NOS expression with T4 at 1μM

The concentration of T4 was increased yet again, to 1μm for a third set of experiments, to see whether a dose-dependent effect could be observed with my organotypic culture protocol. At 1μm (n = 18) labelling was seen in 12 samples, of which 9 displayed staining in 10 or more cells (up to 39 for one particular sample). The staining was again localized to cells in a cluster, extending 250-400μm and situated 300-400μm caudal to the brainstem/spinal cord divide. Projections were visible extending from at least half the labelled cells, varying in length from 10-80μm, and again, most projections extended ventrally (see Fig. 13, Ci-Cii). The most intensely labelled cells appeared very dark blue and were almost always pear-shaped. The orientation of the spinal cord impacted the ease of tracing projections through the tissue (viewing the CNS laterally helped observe the ventral pattern) and most projections were no longer visible in tissue that was examined again after two weeks. Six T4-treated cords displayed no NADPH-d labelling.

Control preparations (n = 12) showed some spinal labelling in 6 samples, varying between 3 and 14 cells. The other 6 showed no labelling in the spinal cord (Fig 13, B). 12 out of 18 thyroxine-treated samples displayed NADPH-d labelling in the spinal cord, several showing numerous intensely labelled cells with multiple projections. This was not seen to be the case when examining control labelling: any NADPH-d reactivity was restricted to the cell bodies, which were faint-to-medium in intensity (data not shown).

NADPH-d labelling in control cords maintained in organotypic culture conditions over 72 hours was **3.25 ± 4.4** stained cells per cord. A significant increase (Mann-Whitney test; $p < 0.05$) in labelling was found in sibling preparations cultured with 1µm T4 over the same period, which contained **10.7 ± 10.9** labelled cells per cord. Figure 13, D represents these averages.

2.4 NOS expression with T3 at 1µm

For the fourth set of thyroid hormone experiments, I used triiodothyronine (T3) which is the more active derivative of T4. I was interested to see whether supplying T3 would cause the cultured tissue to respond differently, as there would be no need for de-iodination of the hormone in order to make it effective. Figure 14 illustrates these experiments. With 1µM T3 (n = 12) spinal cord labelling was seen in all samples except one. The staining in the spinal cord was localized to a rostral area extending up to 300µm, and positioned 300-500um caudal to the brainstem (Fig 14, Bi-Bii). As described above for T4, cells were pear-shaped or rounded, unipolar and irregularly organized on either side of the midline, however the CNS was drawn on its side due to rotation in the slide wells (Fig 14, Bii). Labelled projections were similar to experimental tissue with 500nM and 1µM T4, some extending up to 80µm.

Control samples (n = 6) showed faint labelling in 3 of the spinal cords, up to 7 cells (data not shown). Again, this is possible in stage 47 CNSs, but early stage 48 may also show such labelling, suggesting a possible error in staging (addressed in Discussion).

Control preparations maintained in organotypic culture conditions over 72 hours contained an average of **2.7 ± 3.2** stained cells per cord. A significant increase ($p < 0.05$; Welch-corrected unpaired t-test) in labelling was found in sibling preparations cultured in the presence of T3, which contained **9.3 ± 8.73** stained cells per cord. Figure 14, C represents this graphically.

2.5 Comparison of all concentrations

When analysed against their respective control group, spinal cords cultured in all concentrations of TH contained a significantly higher number of NADPH-d labelled cells. When numbers of labelled cells

for each concentration (raw data for 50nM, 500nM, 1µM T4 and 1µM T3) were analyzed against each other ($p > 0.05$, Kruskal-Wallis test), however, there was no significant difference between any of them. Increasing the concentration of hormone supplied, or even supplying a more potent one, did not cause a relative increase in the amount of NADPH-d labelling in the spinal cords. This lack of significantly different cell counts between concentrations of hormone used suggests that even at 50nM, I was operating at close to the top of the dose-response curve (see Discussion).

3 ChAT-labelling brachial motor neurons

3.1 Motor neurons of the brachial spinal cord at stage 47 and 51

The motor innervation of the trunk and brachial region of *Xenopus* follows a developmental progression, whereby the primary motor neurons are the earliest to develop, followed by the secondary motor neurons (Forehand & Farel, 1982; Nordlander, 1986, van Mier, 1986). The primary motor neurons project to trunk and tail myotomes, and are responsible for axial-based swimming- a behaviour that must be established by the time the embryo hatches. Secondary motor neurons on the other hand, develop with a delay, and the motor neuron pools dedicated to the limbs can first be observed in the spinal cord around stage 48 by application of HRP to the hindlimb bud (van Mier et al., 1985). I used the work of van Mier and colleagues (1985) to establish my criteria for identifying primary and secondary motor neurons. The most obvious difference between the two types is size; at stage 48 primary motor neurons in the rostral spinal cord vary between **25-35µm**, while secondary motor neurons are much smaller, between **10-15µm**. Their position in the ventral spinal cord can also differ, with primary motor neurons being located ventrolateral to the central canal, at the interface between the gray matter and marginal zones (white matter). Secondary motor neurons may have their somata closer to the matrix surrounding the central canal. ChAT immunohistochemistry was used to identify presumed motor neurons in the brachial spinal cord region of stage 47 and stage 51 tadpoles. The criteria above were used when distinguishing between motor neuron types. Results were compared in order to evaluate

differences that would imply developmental changes. It is worth noting that while care was taken when counting cells, a glancing section through a primary motor neuron could make it appear smaller and therefore cause it to be counted as a secondary motor neuron. This possibility will be dealt with ahead and in the Discussion section.

The first 30 sections of the spinal cord were taken and stained for ChAT immunoreactivity (see Figure 15). Labelled cells were counted every third section, thus aiming to examine 10 sections for each sample of stage 47 (n = 2 animals, 17 sections) and stage 51 (n = 2 animals, 9 sections). However, some sections were missing or unstained, and thus were excluded from analysis. For each sample, cells were only counted if they were clearly stained with a visible nucleus against the background. In particular, cells were counted as motor neurons only if they were located in the ventral half of the section, on either side of the central canal, an area representing the future ventral horns. Presumed primary motor neurons were large (up to 25µm diameter), intensely labelled, round, elliptical or semi-circular, and often with projections arising from the soma (see Fig 15, Ci, cf Dii). These projections were difficult to trace or quantify (other than to note their presence extending from the soma) as the sections were thin and thus did not allow three-dimensional tracing. Projections were more easily viewed at stage 51 than stage 47. Primary motor neurons were located at the interface between gray and white matter of the spinal cord, with the soma in the gray matter and projections extending into the surrounding white matter. Presumptive secondary motor neurons were smaller than presumptive primary motor neurons, rounded or elliptical, but also intensely labelled (Fig 15, Di) and with the occasional projection visible extending from the soma.

When examining sections, labelled presumed motor neurons were identified as primary or secondary (on the bases of previously established criteria), and the neuron counts for each type were compared between stages. Sections of stage 47 (n = 17) contained **1.18 ± 0.64** primary motor neurons per section; **0.53 ± 0.62** secondary motor neurons per section. Sections of stage 51 (n = 9) contained on

average **1.33 ± 0.71** primary motor neurons per section, not significantly different from the number of primary motor neurons at stage 47 (Mann-Whitney test; $p > 0.05$). However, the number of stained secondary motor neurons in sections of stage 51 was **1.67 ± 1.0** per section, a very significant increase when compared to the number of secondary motor neurons that stained in sections of stage 47 spinal cords (Mann-Whitney test; $p < 0.005$). Figure 18, H illustrates this graphically. This indicates that the primary motor neuron pool serving the axial musculature remains constant between stages 47 and 51, but new secondary motor neurons arise, implying that motor circuitry for the forelimbs or for the growing larval musculature is being assembled in the spinal cord during this period of development.

Apart from a difference in presumptive secondary motor neuron number, other differences were also noted in the pattern of ChAT staining between these stages: the shape of primary motor neurons in stage 47 sections tended to be more rounded (Fig 15, Ci-ii), compared to the narrower elliptical neurons at stage 51. Presumptive primary motor neurons at stage 51 also tended to show clearer projections from the soma (Fig 15, Dii) and ChAT immunoreactivity made more visible the dendritic arborisations extending into the gray matter, which appeared as very fine, faint brown branching (data not shown), although this was not quantified.

3.2 Motor neurons of the brachial spinal cord after organotypic culture

The motor neurons of the brachial region were examined after organotypic culture, when stage 47 CNSs were exposed, over 72 hours, to six 4-hour bouts of drug administration using either the NO-donor SNAP in DMSO, the NOS-inhibitor L-NAME in DMSO, or DMSO alone (control), as described in my Materials and Methods section. See Figures 16 and 17 for a visual comparison of ChAT labelling between control, SNAP, and L-NAME treated samples. As for the stage 47/51 comparison, presumed motor neurons of each type were counted in each section examined. Again, the first 30 sections after the brainstem/spinal cord divide were taken and processed for ChAT immunoreactivity, with cells being counted in every third section. The criteria to identify motor neurons were the same as described above. The primary motor neuron data were compared between all conditions, followed by a comparison of

secondary motor neuron counts for all conditions. These data are represented in Figure 18, G, allowing a comparison with data from cell counts for stages 47-51 (Fig 18, H) as described above.

3.2.1 Primary motor neurons

Control preparations (n = 10 animals, 95 sections) maintained in organotypic culture conditions over 72 hours contained **0.77 ± 0.63** primary motor neurons per section. Preparations reared in L-NAME (n = 7 animals, 68 sections) contained **0.96 ± 0.72** neurons per section; sibling preparations cultured in the presence of SNAP (n = 10 animals, 99 sections) contained **0.86 ± 0.76** neurons per section, neither of these values significantly different from control (Kursal-Wallis test, Dunn's post-test; $p > 0.05$), indicating no change in primary motor neuron pool after treatment with nitrergic drugs.

3.2.2 Secondary motor neurons

Control organotypic culture preparations contained **0.51 ± 0.70** secondary motor neurons per section, whilst sibling preparations cultured in L-NAME contained **0.60 ± 0.78** neurons per section, not significantly different from control (Kruskal-Wallis test; $p > 0.05$). However, in preparations reared in SNAP there was a highly significant increase ($p < 0.001$), in the number of secondary motor neurons in each section compared (Dunn's multiple comparison test) to either control or L-NAME conditions. Figure 16, Di, illustrates a section containing a presumptive primary and secondary motor neuron. SNAP-treated preparations contained **1.34 ± 1.06** secondary motor neurons per section. See Figure 18, G, for a graphical comparison of both motor neuron types for all conditions.

3.3 Surface area measurements to quantify ChAT staining

Due to the subjectivity of counting cells and possible errors made in distinguishing between primary and secondary motor neurons, a different method of quantifying ChAT immunoreactivity was applied to some CNS sections after organotypic culture. Software (Axiovison, see Methods) was used to measure surface area of stained tissue in sections, which could include cells and their projections. I did not distinguish between secondary and primary motor neuron staining in this case, because the overall area of staining would be quantified. I traced around localized staining in each section examined, to

calculate an overall total area for each section (see Figure 18). This method also took into account cholinergic staining in the ventral horn area that was not immediately identified as a motor neuron, but rather, due to the possible presence of neural projections in cross-section. In the preceding sections, when counting cell bodies I did not quantify such staining, so it was excluded it from my analysis. For surface area measurements I analyzed 2 animals (20 slides) per condition using random selection.

In sections of control preparations, (Fig 18, D; n = 2 animals, 20 sections) the average ChAT-stained area was **119.27 ± 52.67 μm^2**. Sections of preparations reared in L-NAME (Fig 18, C; n = 2 animals, 20 sections) contained an average stained area of **104.41 ± 45.34 μm^2**, which did not differ significantly from control (Kruskal-Wallis test, Dunn's multiple comparison test; $p > 0.05$). However, in sections of SNAP-treated preparations (Fig 18, E; n = 2 animals, 20 sections) there was an extremely significant increase ($p < 0.0005$) in average area of ChAT staining; **257.05 ± 142.46 μm^2** (see Fig 18, F). A comparison can be made with data obtained by cell counts (Fig 18, G) which shows a highly significant increase in the number of presumptive secondary motor neurons in SNAP-treated tissue compared with control. Data obtained by measuring the extent of ChAT staining appears to support an increase in motor neuron numbers in SNAP-treated samples. Finally, Figure 18, H shows average number of presumptive primary and secondary motor neurons labelling in sections of the brachial spinal cord at stages 47 and 51. Secondary motor neurons are found to increase significantly during this period of development, providing support for the suggestion that an increase in secondary motor neurons (an effect which was also observed in SNAP-treated samples) is indicative of ongoing motor system development.

4 NOS in the spinal cord of the lamprey

4.1 NADPH-d labelling

Segments of lamprey spinal cord (n = 4) were processed for NADPH-d histochemistry. In two of the preparations, no staining was observed (data not shown). Staining was observed in numerous cell types in the other two preparations. Cross-sections of the spinal cord were made (from a fifth segment

of the spinal cord) to supplement this data. Sections were cut at 100μm but the tissue thickness did not seem to impede identification of single cells.

Many neuron types of the lamprey spinal cord have been identified anatomically (Selzer, 1979; Buchanan, 2001), and their roles in generating locomotion described (Grillner & Wallen, 1984), thus providing a wealth of descriptive information with which the NADPH-d labelled neurons in my samples could be compared. The spinal cord of the lamprey is dorsoventrally flattened, like a ribbon, and can be subdivided into 7 longitudinal columns starting with the outermost known as the **lateral** columns (see Fig 19, A). Medial to the lateral columns are the areas known as the **gray** columns. On either side of the central canal are the **medial** columns. After processing for NADPH-d activity, a variety of cells was found to stain. In segments of wholemount, starting from the lateral columns; large, multipolar cells were found to stain heavily and were distributed irregularly along the length of the spinal cord segment, in the lateral margins (see Fig 19, E). These cells were situated close to the lateral edges of the cord and were heterogenous in shape. Large main dendrites were found to extend from the somata for over 50μm, and these projected laterally towards the edge of the spinal cord. These cells were identified as **edge cells,** a type of intraspinal proprioceptor that responds to stretching of the spinal cord tissue (Grillner et al., 1984). **Bipolar** neurons in the lateral margins, with one neurite extending towards the margin and the other one extending medially were also found to stain, albeit relatively faintly (see Fig 19, E).

In the gray columns, intense labelling was found in the cell bodies of a number of different cell types. **Motor neurons** were visible in two broad parallel bands of spindle-shaped or narrow triangular cells, transversely oriented (Fig 19, C). These cells were organized in clusters running the length of the gray columns (Fig 19, B) and measured 20-30μm through the long axis. They were identified by comparison to previous work on neuronal classification in lamprey spinal cord (Buchanan, 2001). Located in the lateral margin of the gray column and in close proximity to the motor neurons, were the stained **lateral** cells. These had larger but similarly shaped somata to the motor neurons with which they are

interspersed, and have very large, lateral pointing dendrites (Fig 19, C). **Giant interneurons** are also found to label intensely (Fig 19, F). These are in the medial margin of the gray column and are large, with multiple dendrites. Again, these were identified based on previously published lamprey data (Selzer, 1979; Buchanan, 2001).

In the medial columns, running the length of the cord segments, **small spindle-shaped** bipolar neurons were found to stain as well (see Fig 19, D). Also labelled in the medial columns were large circular cells identified as **dorsal** cells (Fig 19, D). These large cells (40μm diameter) were distributed irregularly along the length of the cord segment and are a type of primary sensory cell which respond to pressure on the skin of the lamprey (Buchanan, 2001). Furthermore, NADPH-d labelling of a lesser intensity was also found in **ependymal** cells lining the central canal (see Fig 19, D). These cells are small, columnar and arranged in a single or possibly a double layer around the lumen of the central canal. Cross-sectioning makes this arrangement easier to observe (Fig 19, A), although the cells described above were observed primarily in segments of wholemount spinal cord. Finally, also visible in cross section is a region of staining located ventrally in the lateral margin. This does not appear localized to a specific cell type, but is an area where blue labelling appears as a general tinge.

4.2 nNOS immunohistochemistry

NADPH-d histochemistry is generally a reliable stain for the presence of NOS, however due to the possibility that it can yield false positives (for example in cases where tissue is overfixed or incubated for an excessive period of time), nNOS immunohistochemistry was used to corroborate initial findings. Segments (n = 2) approximately 4mm in length were incubated with antibodies against nNOS. With confocal microscopy the pattern of immunofluorescence was found to be very similar to the pattern of NADPH-d labelling just described (see Figure 20; cf Figure 19). Cells in the gray column labelled brightly, and appeared similar in shape and size to the cells labelling for NADPH-d in the gray columns. However, in some ways the immunolabelling was less clear than NADPH-d staining, and it was more difficult to distinguish between cell types in this area (Fig 20, A and B). Taking optical sections at 10μm starting with

the dorsal surface of the segment made it easy to distinguish populations of cells located dorsally; **dorsal** cells were immediately evident in the medial columns also because of their size and intensity of labelling (Fig 20, C). **Edge** cells, which also fluoresced with particular intensity, were the other most obvious feature of the labelled spinal cord segments (Fig 20, D). Also staining for nNOS were the large **bipolar** neurons that project laterally towards the margin and medially towards the gray columns. These, as described above for NADPH-d, are interspersed with the edge cells (Fig 20, D). While brightfield microscopy has the advantage that the plane of focus is more easily controlled, therefore allowing for rapid focusing in both ventral and dorsal directions, the benefit of confocal microscopy is that optical sections can be stacked to create an image of labelling throughout the entire depth of a thick tissue sample. In this case, labelling in the edge cells was much more evident than it was for NADPH-d histochemistry, with superior detail of the laterally-tending projections (Fig 20, D; cf Fig 19, E).

Overall, the pattern of labelling observed for nNOS immunohistochemistry was found to closely resemble that of NADPH-d labelling in the spinal cord of the river lamprey.

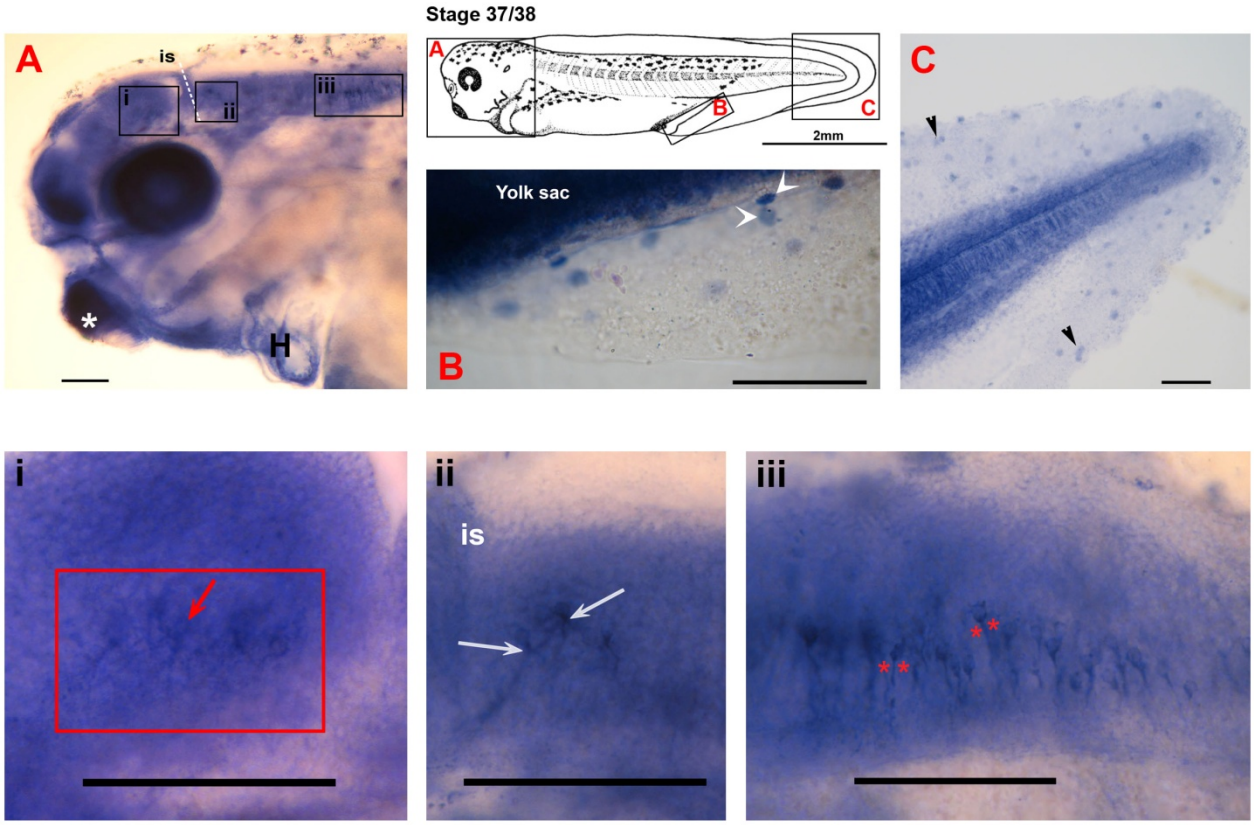

Figure 9. NADPH-d labelling in wholemount albino *Xenopus* stage 37-38. Inset drawing is a representation of stage 37/38 tadpole, adapted from Nieuwkoop and Faber (1956). **A-C** illustrate higher magnifications of boxed areas in inset. **(A)** NADPH-d labelling is visible in three areas of CNS (boxes i-iii) and in cement gland (white asterisk). The isthmus, the midbrain-hindbrain boundary, is represented by a dashed white line (is). The heart (marked H) also labels for NADPH-d. **(i)** Neurons in caudal midbrain show faint labelling in projections (within red box). Somata are difficult to identify (red arrow), because the staining is so weak. **(ii)** Cells label in rostral hindbrain, just caudal to isthmus (is). Labelling in somata is more distinct than in area (i), but still faint. Projections appear to branch off close to cell bodies (white arrows). **(iii)** Cells in caudal hindbrain form largest and most distinct population. Intensity of NADPH-d staining is slightly greater than in areas (i) and (ii). Cells appear unipolar and rounded/pear-shaped. Many projections run ventrally and some appear to travel a short distance before branching (red asterisks on either side). **(B)** NADPH-d labelling (white arrowheads) in epidermis at caudal end of yolk sac (marked). Staining is easiest to identify in areas where only epidermis is present. Note irregular punctate pattern and varying intensity of staining. **(C)** NADPH-d labelling in tail epidermis. Most labelled cells are isolated but occasionally two or more labelled cells may be clustered (black arrowheads). Scale bars = 100µm for all images, except in inset drawing.

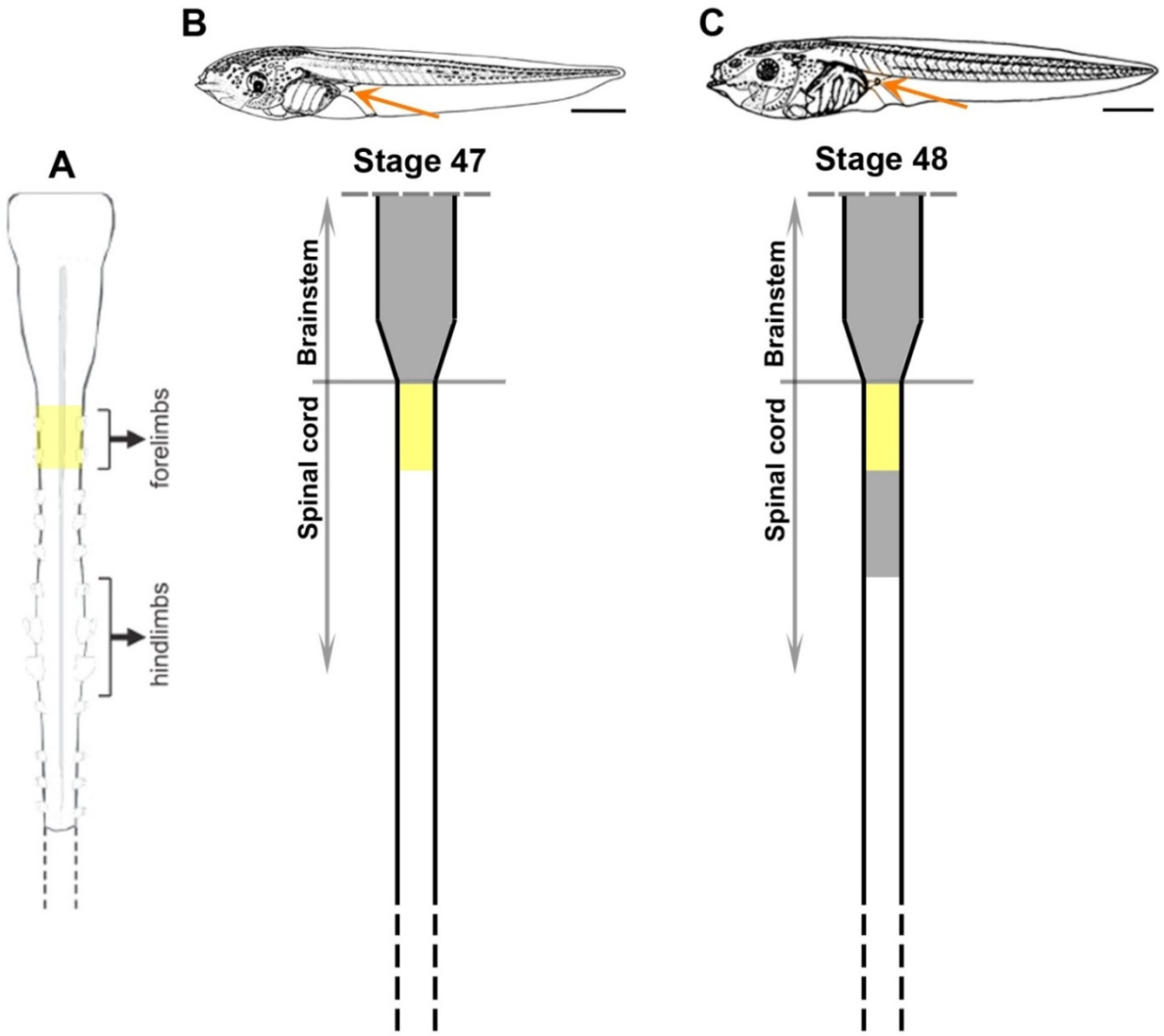

Figure 10. Development of NADPH-d labelling in *Xenopus* spinal cord between premetamorphic stages 47 and 48. (A) Schematic of brainstem and spinal cord showing relative positions of forelimbs and hindlimbs (adapted from Ramanathan et al, 2006). **(B)** and **(C)** compare regions of neuronal NADPH-d labelling in CNS of *Xenopus* at stage 47 **(B)** and 48 **(C)**. Drawings illustrate morphology of *Xenopus* at stage 47 and 48 (adapted from Nieuwkoop and Faber, 1956). Red arrows indicate hindlimb buds, visible for the first time at stage 47 and more distinct by stage 48. Scale bars for both = 2mm. Schematics below represent dissected CNS (brainstem and spinal cord). Gray shading denotes regions within which cell bodies stain for NADPH-d, indicative of the presence of NOS. The pattern of brainstem staining is typical of premetamorphic tadpoles (McLean and Sillar, 2001; Ramanathan et al, 2006) and does not change significantly between stages 47 and 48, however this developmental period is marked by the appearance of a cluster of NADPH-d stained cells in the rostral spinal cord (Ramanathan et al, 2006). This cluster is located caudal to the brachial region of spinal cord (shaded in yellow), where forelimb motor circuitry is developing. The forelimb bud also emerges at stage 48.

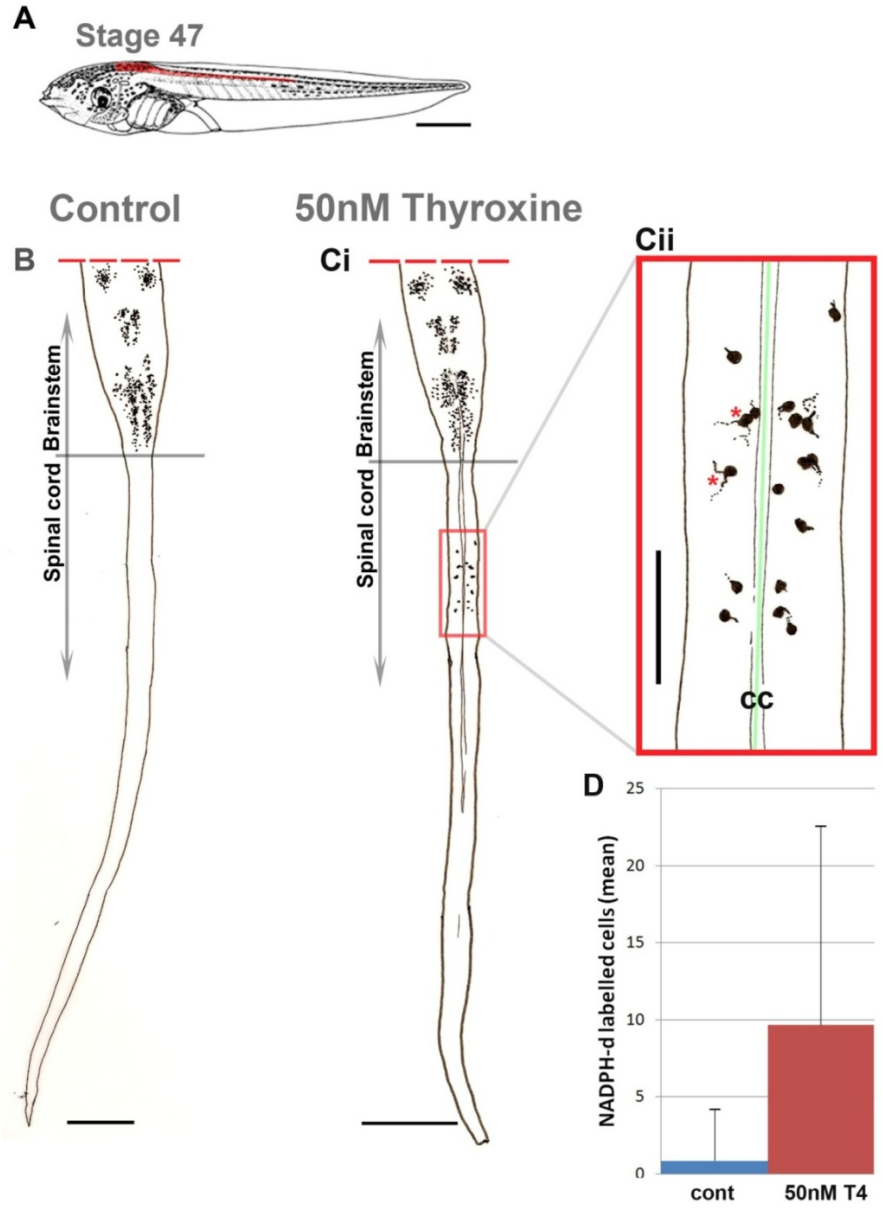

Figure 11. NADPH-d labelling in wholemount *Xenopus* spinal cord after organotypic culture with 50nM thyroxine. **(A)** Schematic of stage 47 animal prior to dissection (adapted from Nieuwkoop and Faber, 1956). Highlighted in red is the part of the CNS that was used for organotypic culture. Scale bar = 2mm. **(B)** Camera lucida drawing of representative CNS from control group. NADPH-d labelling of cells is represented by black dots. There is staining characteristic of stage 47 in the brainstem. Note absence of labelling in spinal cord. **(Ci)** Camera lucida drawing of CNS from thyroxine-treated group. Brainstem staining closely resembles that of control sample, however cells of the spinal cord were found to label for NADPH-d in a rostral cluster approximately 200μm caudal to brainstem/spinal cord divide. The cluster extends approximately 250μm, after which no labelled cells were seen. Scale bars for B and Ci = 200μm. **(Cii)** Enlargement of rostral cluster in (Ci). Stained cells are round or pear-shaped, irregularly dispersed on either side of midline (green line) and some have stained projections (red asterisks). The central canal (cc) was visible in this preparation because of its orientation. Scale bar = 100μm. **(D)** Graph representing average number of NADPH-d stained cells in spinal cord for control (blue) and 50nM thyroxine (red) groups. Error bars show standard deviation. Brainstem staining was not included in the analysis as it did not appear to differ from the characteristic pattern of premetamorphic tadpoles.

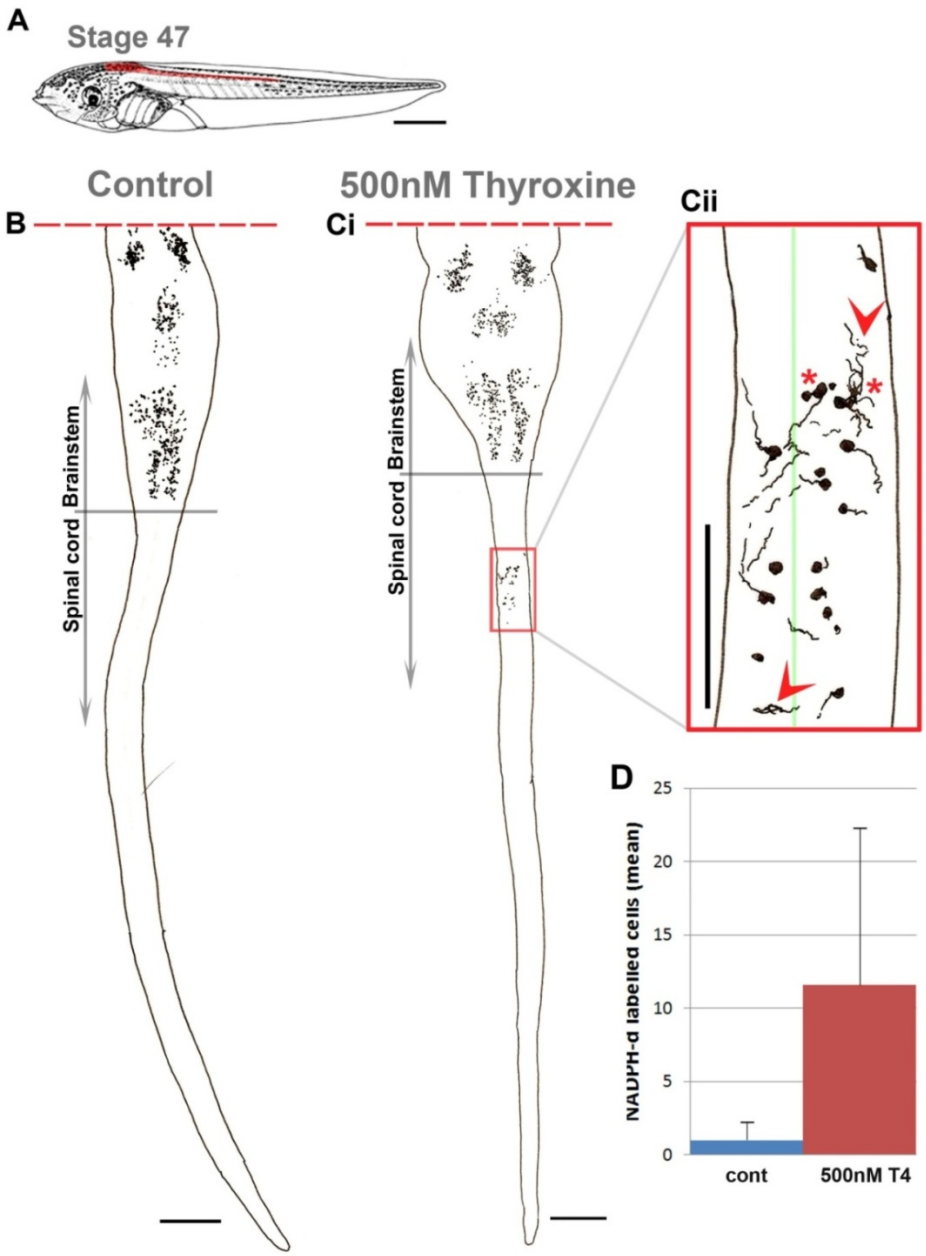

Figure 12. NADPH-d labelling in wholemount *Xenopus* spinal cord after organotypic culture with 500nM thyroxine. **(A)** Xenopus at stage 47. Scale bar = 2mm. **(B)** Camera lucida drawing of CNS from control group. NADPH-d labelling of cell bodies (black dots) is visible in the brainstem and resembles typical pattern of labelling seen in premetamorphic tadpoles. In this sample, no labelling is present in the spinal cord, typical of control tadpoles at stage 47. **(Ci)** Camera lucida drawing of representative sample from thyroxine-treated group. Labelling in the spinal cord is found in a rostral cluster about 270µm after brainstem/spinal cord divide, extending approximately 250µm. Scale bars for (B) and (Ci) = 200µm. **(Cii)** Enlargement of rostral spinal cluster shown in (Ci). Labelled neurons are rounded or pear shaped, and many stained projections (red arrowheads) are visible. Some cells appear to cluster close together (red asterisks). The midline (green line) is slightly off-center due to rotation of spinal cord after mounting on cavity slide. Scale bar = 100µm. **(D)** Graph representing average number of NADPH-d stained cells in the spinal cord for control (blue) and thyroxine (red) groups. Error bars show standard deviation. Brainstem staining was not included in the analysis.

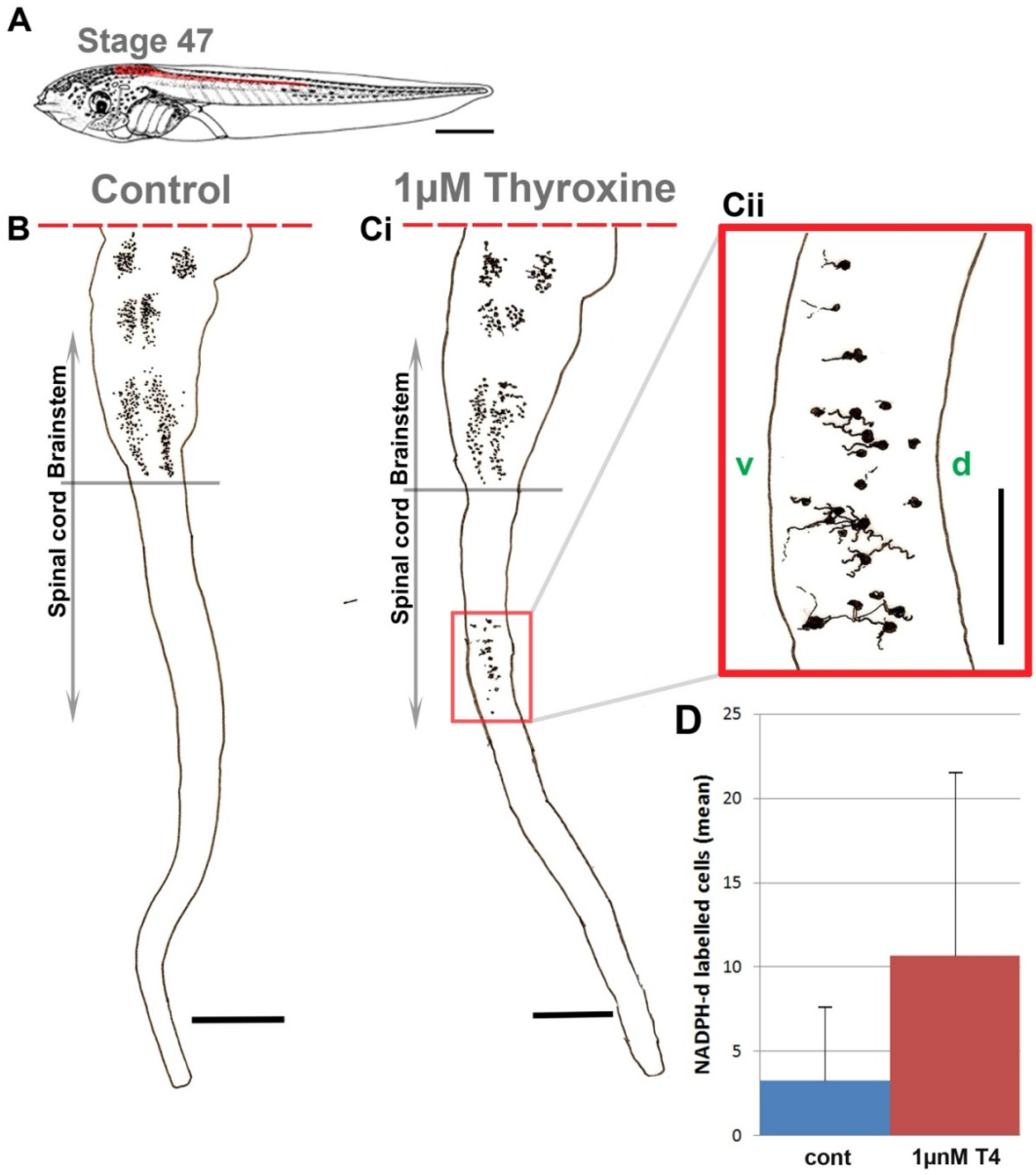

Figure 13. NADPH-d labelling in wholemount *Xenopus* spinal cord after organotypic culture with 1µM thyroxine. (A) *Xenopus* at stage 47. Scale bar = 2mm. **(B)** Camera lucida drawing of representative spinal cord from control group. Typical of animals at stage 47, no labelling is found in the spinal cord, but pattern of brainstem staining (black dots) reflects normal NADPH-d labelling of premetamorphic tadpoles. **(Ci)** Camera lucida drawing of sample from hormone-treated group. NADPH-d labelling in the spinal cord appears in a cluster approximately 300µm caudal to the brainstem. Scale bars for (B) and (Ci) = 200µm. **(Cii)** Enlargement of rostral cluster of NADPH-d labelled cells. The spinal cord of this preparation rotated when mounted in the cavity slide, so ventral (v) and dorsal (d) surfaces of the tissue are marked. Cells stained are rounded or pear-shaped, with many of the somata located in the ventral half of the cord, and some located dorsally (cf. Ramanathan et al., 2006). Most labelled projections from the cells travel ventrally. The cluster extends for about 250µm, after which no more labelled cells are observed. Scale bar = 100µm. **(D)** Graph shows average number of cells labelling for NADPH-d in the spinal cord of control (blue) and T4-treated (red) samples. Error bars show standard deviation. Brainstem staining was not included in the analysis.

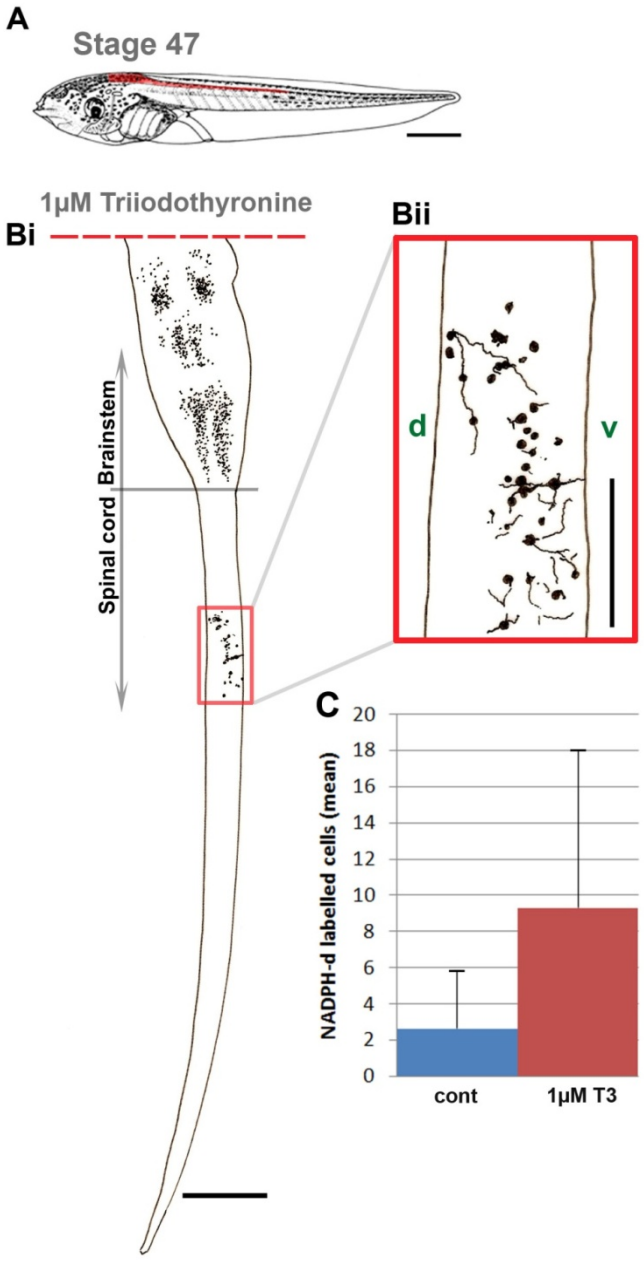

Figure 14. NADPH-d labelling in wholemount *Xenopus* spinal cord after organotypic culture with 1µm triiodothyronine (T3). **(A)** *Xenopus* at stage 47. Scale bar = 2mm. **(Bi)** Camera lucida drawing of representative sample from 1µM T3-treated group (control data not shown). NADPH-d labelling of cell bodies (black dots) is visible in the brainstem and in a rostral cluster in the spinal cord. Scale bar = 200µm. **(Bii)** Enlargement of rostral spinal cluster of labelled cells. Due to rotation of the spinal cord in the cavity slide, the enlarged region is depicted side-on. The ventral (v) and dorsal (d) surfaces of the sample are marked. Many of the cells appear to be located medially in the cord. Projections are observable on many, but not all, labelled cells. Scale bar = 100µm. **(C)** Graph represents average number of labelled cells in control (blue) and T3-treated (red) preparations. Error bars show standard deviation. Brainstem labelling was not included in the analysis.

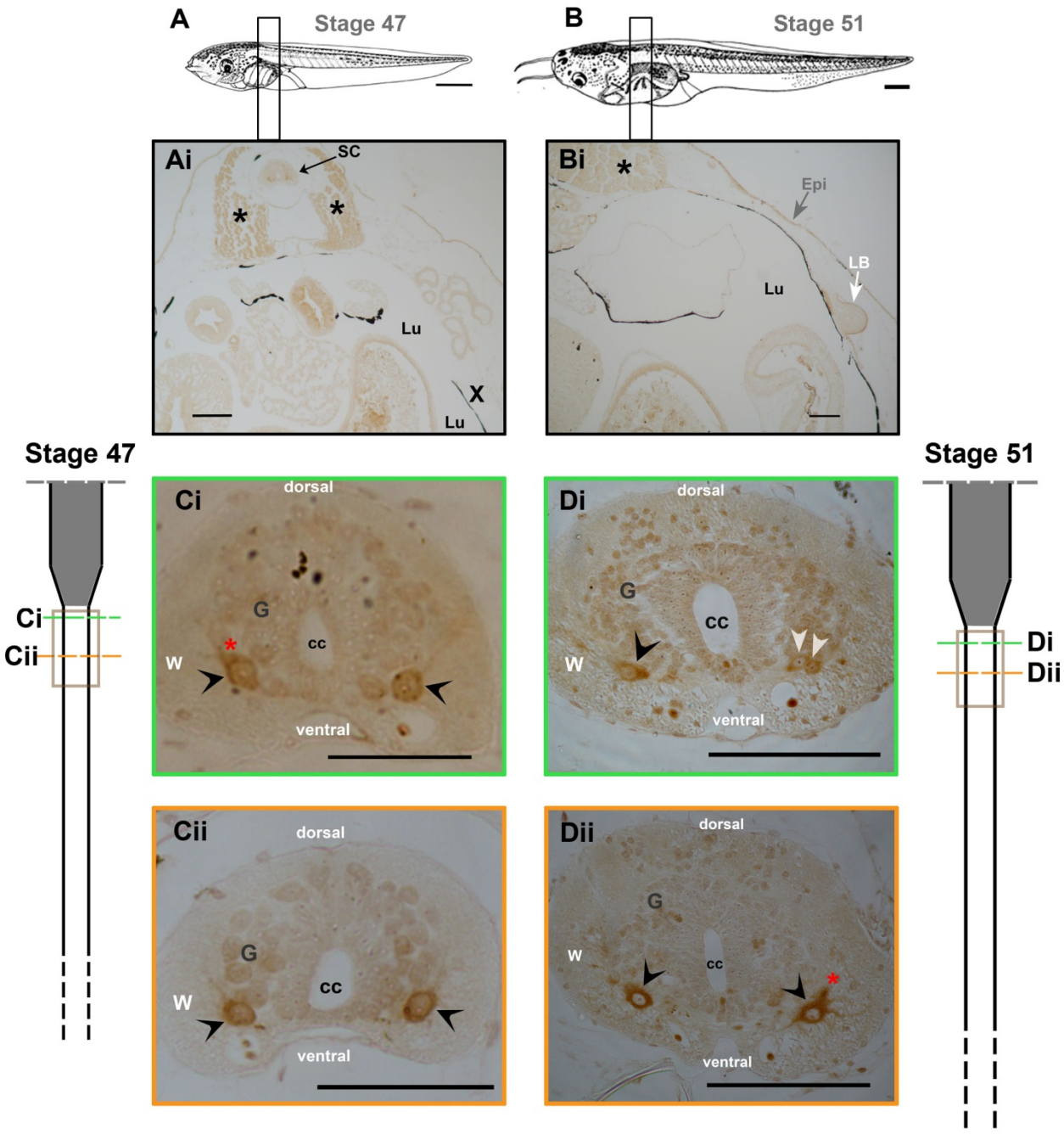
A
Stage 47
B
Stage 51
Ai
SC
Lu
X
Lu
Bi
Epi
LB
Lu
Stage 47
Stage 51
Ci
Cii
Di
Dii
dorsal
ventral
G
cc
W

Figure 15. Comparison of ChAT labelling in brachial motor neurons of *Xenopus* at stage 47 and 51. Drawings **(A)** and **(B)** represent animals at stages 47 and 51 respectively (adapted from Nieuwkoop & Faber, 1956). Boxed areas approximate the brachial region, from which cross-sections were taken. Scale bars on each = 2mm. **(Ai-Bi)** are representative cross-sections taken through the brachial region of the whole animal. **(Ai)** Cross-section through the brachial region at stage 47; includes spinal cord (SC) surrounded by trunk musculature (black asterisks), as well as the lumen of the tadpole's body (Lu). Note absence of forelimb bud, which at later stages is found to be developing exterior to the body cavity and under the dermis, in the area marked X. Scale bar (bottom left) = 200μm. **(Bi)** Cross-section at stage 51. The developing forelimb bud (LB) is marked. At this stage it is about 100μm in diameter and visible *in vivo* as a tiny translucent white sphere. Epi denotes the tadpole's epidermis. As in (Ai), trunk musculature is marked with a black asterisk. Scale bar = 100μm. **(Ci-ii)** Cross-sections from brachial region of stage 47 spinal cord showing ChAT-labelled presumptive motor neurons. See Methods for defining criteria. The schematic to the left represents the CNS at that stage (brainstem is shaded gray) and shows the level of the spinal cord at which the sections (Ci-ii) were taken. The brown box was presumed to be the brachial area, extending approximately 300μm. In both (Ci) and (Cii) neurons labelled are presumptive primary motor neurons (black arrowheads), located in the ventral spinal cord and lateral to the central canal (cc). It is difficult to see projections off the soma, note the cell marked with a red asterisk (this was the case with all sections examined). White matter (W) and gray matter (G) are also marked. Scale bars = 100μm. **(Di-ii)** Representative cross-sections of the brachial spinal cord at stage 51. Again, the schematic (to the right) represents the CNS and the level at which the sections were taken. **(Di)** Cells labelling for ChAT include presumptive primary (black arrowhead) and secondary (white arrowheads) motor neurons. **(Dii)** Cross-section containing only presumed primary motor neuron ChAT-labelling (black arrowheads). Note the more obvious projections off the soma (red asterisk) compared to stage 47. Scale bars for (Di-Dii) = 100μm.

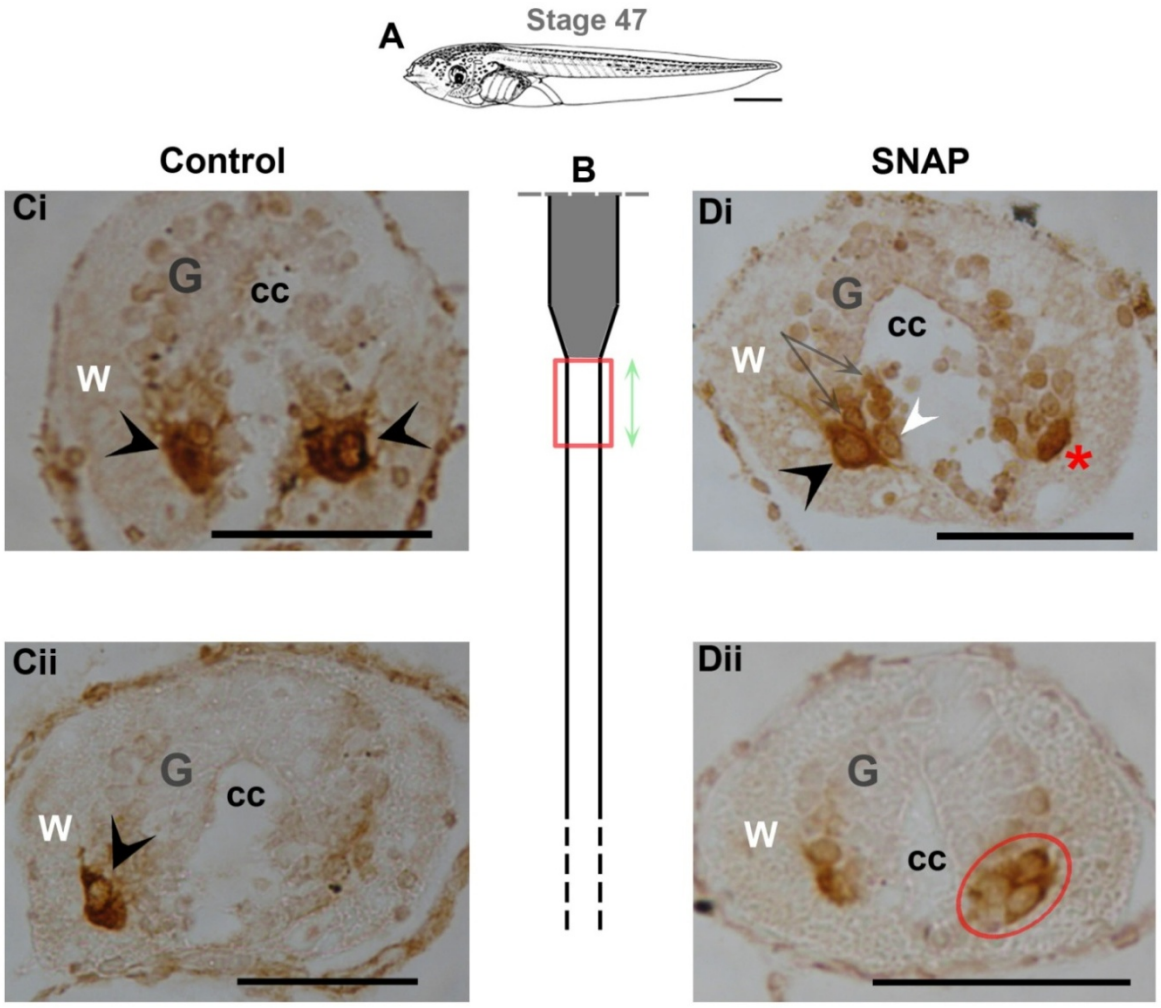

Figure 16. ChAT labelling in the brachial region of *Xenopus* spinal cord after organotypic culture with the NO-donor SNAP. (A) Representation of *Xenopus* at stage 47 (from Nieuwkoop & Faber, 1956), the stage at which the CNS was cultured with SNAP. Scale bar = 2mm. **(B)** Schematic of dissected/cultured CNS. Brainstem is shaded gray. The brachial region, from which cross-sections were taken and stained for ChAT immunoreactivity after the 72-hour culture period, is highlighted (red box, green arrow). **(Ci-Dii)** Selected sections from brachial region of spinal cord. White matter of spinal cord is marked W. Gray matter, where cell bodies are located, is marked G. Central canal in all is marked cc. **(Ci-Cii)** Cross-sections from control group. Presumptive primary motor neurons (black arrowheads) are found to stain for ChAT immunoreactivity, and are located at the boundary between white and gray matter of spinal cord. **(Cii)** Section showing a single large presumptive primary motor neuron labelling intensely for ChAT (black arrowhead). A dendrite is visible extending dorsally into the white matter. **(Di-Dii)** Cross-sections from SNAP-treated group. **(Di)** ChAT immunoreactivity is found in a presumtive primary motor neuron (black arrowhead), which stains intensely. A presumptive secondary motor neuron (white arrowhead) is found medial to the primary neuron. Note difference in relative sizes. Dendrites are visible extending into the white matter from the soma of the primary motor neuron. Staining is also often observed in the region surrounding the motor neurons (marked by gray arrows) although it cannot be attributed to cell bodies. Note that a glancing section through a primary motor neuron (red asterisk) could easily be mistaken for a secondary motor neuron due to its size. Such staining was not included in analysis. **(Dii)** Cross section showing clustering of presumed primary motor neurons (circled in red). On the left of the section, staining cannot be identified clearly as a primary or secondary cell, and such staining was therefore was not included in the analysis. Scale bars for all = 100μm.

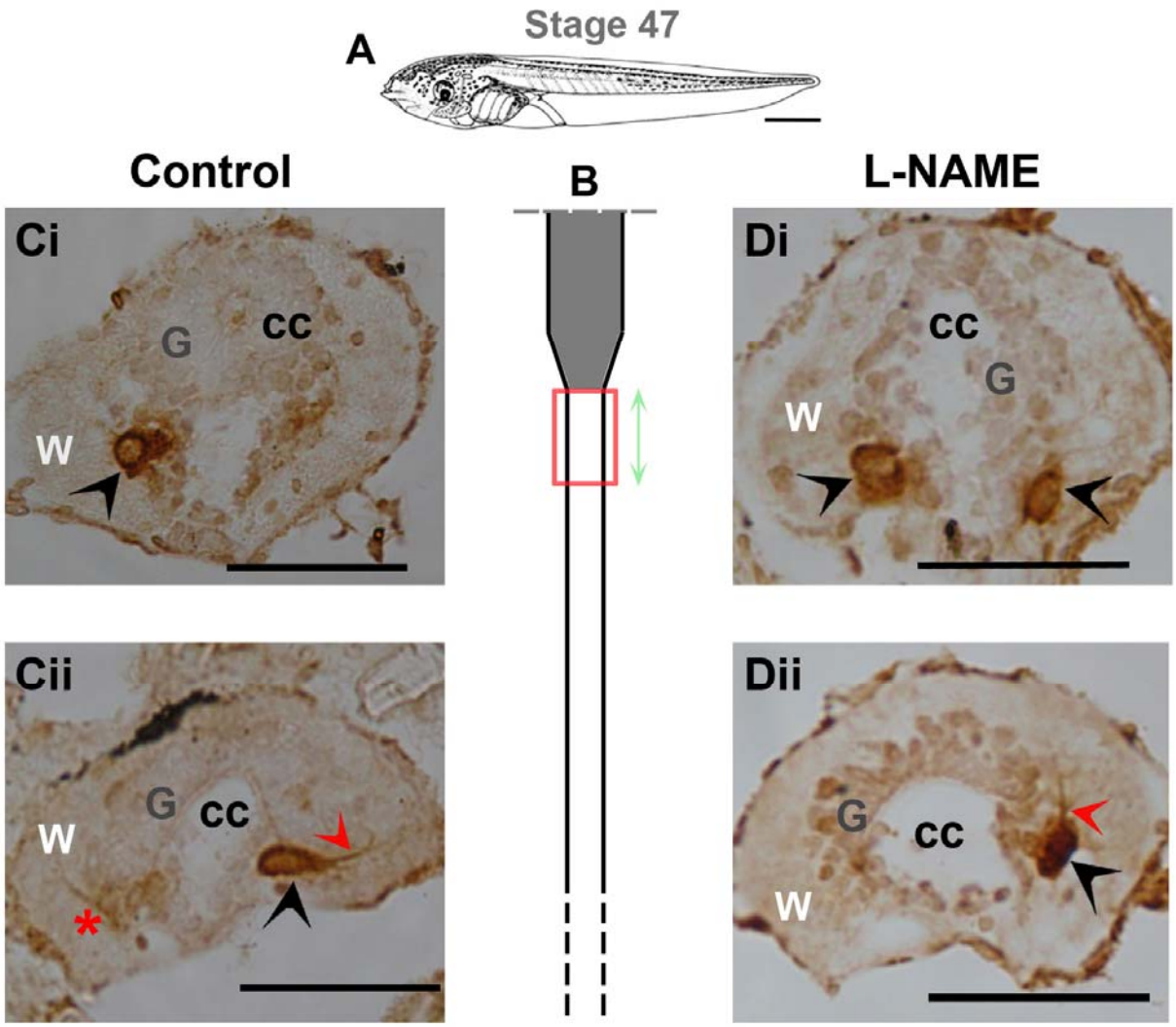

Figure 17. ChAT labelling in the brachial region of *Xenopus* spinal cord after organotypic culture with the NOS-inhibitor L-NAME. (A) *Xenopus* tadpole at stage 47. Scale bar shows 2mm. **(B)** Schematic of dissected/cultured CNS. Brainstem is shaded gray. Brachial region is highlighted. **(C-D)** photomicrographs of selected sections from brachial region of spinal cord. The central canal in all is marked cc. **(Ci-Cii)** Cross-sections from control group. ChAT labelling is found particularly in presumed primary motor neurons (black arrowheads), which are located at the boundary between gray matter (G) and white matter (W) of spinal cord. **(Cii)** shows a presumed primary motor neuron (black arrowhead) with projections tending dorsolaterally (red arrowhead). Projections from another presumptive motor neuron can be seen on the left (red asterisk). **(Di-Dii)** A similar pattern of ChAT labelling is found in L-NAME sections. Primary motor neurons (black arrowheads) are found to label intensely, but presumptive secondary motor neurons in L-NAME treated samples are rare, as in control samples. **(Dii)** Cross section containing a single presumptive primary motor neuron (black arrowhead). The soma is located at the edge of gray matter, with projection (red arrowhead) extending dorsally into the white matter. Scale bars = 100μm.

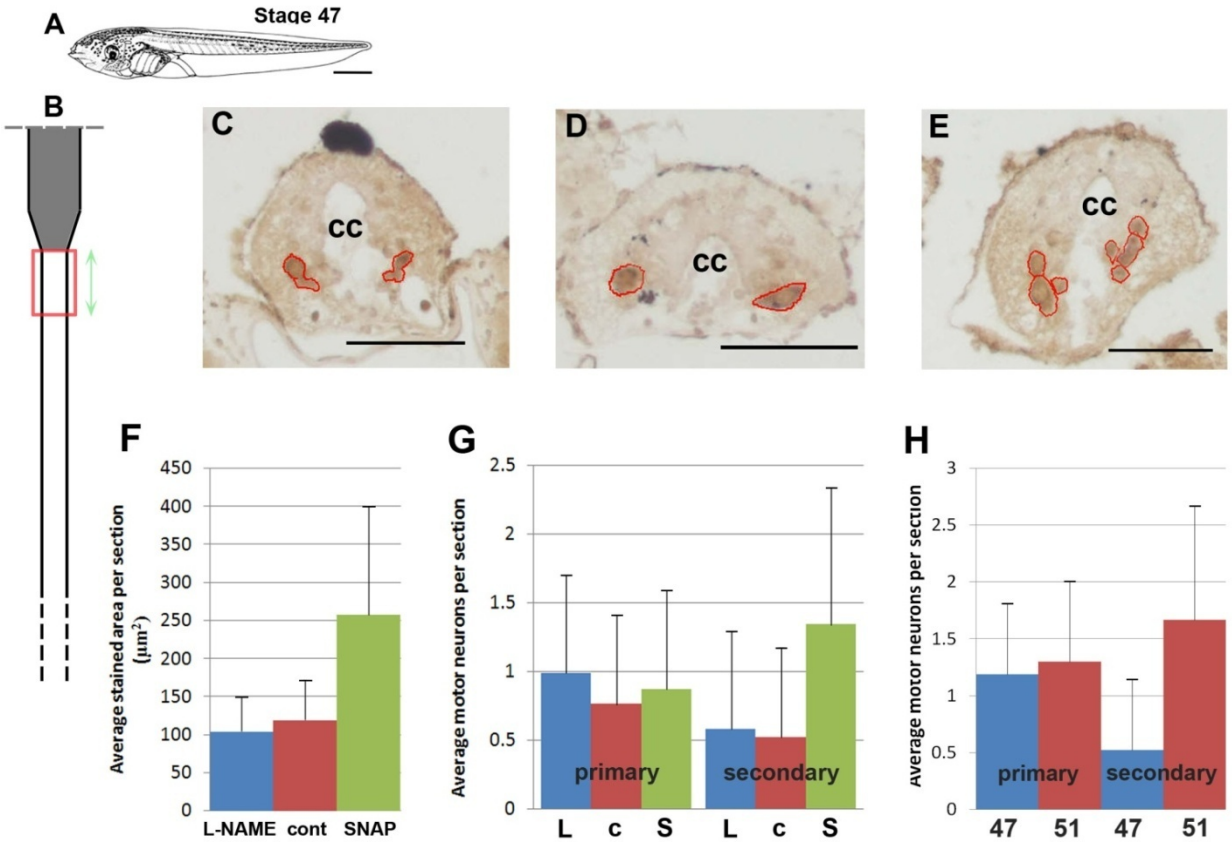

Figure 18. Extent of ChAT staining in sections of the brachial region of *Xenopus* spinal cord after organotypic culture with SNAP and L-NAME. **(A)** Representation of stage 47 *Xenopus* tadpole. Scale bar = 2mm. **(B)** Schematic of the portion of CNS which was dissected for organotypic culture experiments. **(C-E)** Photomicrographs of cross-sections from each experimental group, showing stained areas circled in red. The central canal is labelled cc in all three sections, and scale bars = 100µm. **(C)** sample section from L-NAME treated cord. **(D)** Sample section from control group. **(E)** Sample section from the SNAP-treated group. Areas of staining were calculated using Axiovision software. **(F)** Chart representing average area of staining per section, for each condition. Chart includes data from 2 animals for each condition. There is no significant difference in stained area between L-NAME and control, however there is a highly significant increase in staining for SNAP-treated preparations compared to control. **(G)** Chart comparing number of stained motor neurons of each type (obtained by counting method) for each condition. Chart includes data from all samples. Extent of staining (F) is in support of an overall increase in secondary motor neurons in SNAP-treated tissue. **(H)** Comparison of primary and secondary motor neuron numbers for stages 47 and 51 (chart includes data described in Figure 15). Secondary motor neurons increase during this period of development. (G) reflects a similar trend for secondary motor neurons in SNAP-treated tissue. Error bars for all show standard deviation.

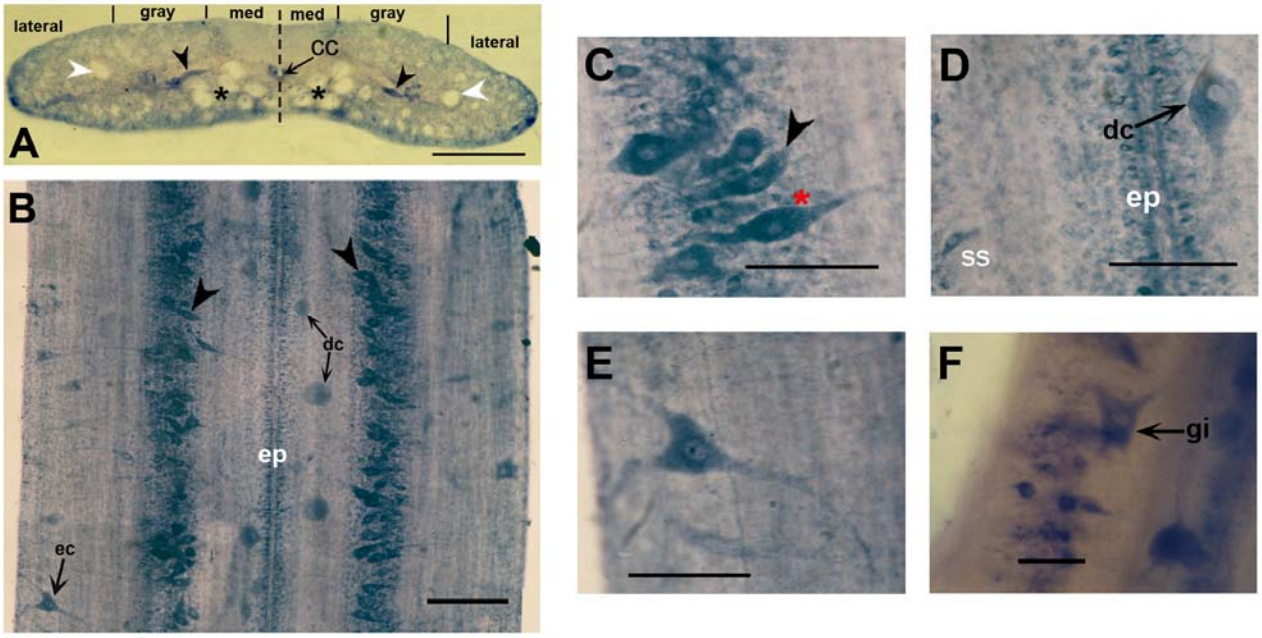

Figure 19. NADPH-d labelling in the spinal cord of the river lamprey. (A) Cross section of lamprey spinal cord, cut at 100µm thickness, showing subdivisions of spinal cord: lateral columns, gray columns, medial columns (med) and central canal (cc). The midline is marked (dashed line). Columns are divided by the type of tissue they include. Lateral columns contain many axons, but edge cells can be found to label for NADPH-d in these areas (see (B)). Axons of the Mauthner neurons are marked with white arrowheads. Gray columns contain the cell bodies of various neuron types. The somata of motor neurons (examples denoted by black arrowheads in both (A) and (B) are found to label intensely in this area. Descending axons of Muller cells are found in the ventral cord (surrounding black asterisks). Blue labelling was also observed in the ventral part of the lateral margins. **(B)** Wholemount segment of spinal cord viewed dorsally. Edge cells (ec) are located in the lateral columns and have long dendrites projecting towards the edge of cord. Dorsal cells (dc) are large, rounded, and irregularly distributed along the medial columns. Ependymal cells surrounding the central canal are marked ep. Scale bars for both (A) and (B) = 200µm. **(C-F)** Main types of cell identified by previously published work (Selzer, 1979; Grillner & Wallen, 1984; Buchanan, 2001). **(C)** Detail of gray column. Cells labelling are thought to be motor neurons (example black arrowhead), but the large bipolar spindle shaped cell (red asterisk) could be a lateral cell. Their dendrites are known to extend across the entire width of the cord. **(D)** Detail showing a dorsal cell (dc) medial to the central canal. Note that ependymal cells (ep) surrounding the central canal show NADPH-d reactivity. Small spindle-shaped cells (ss) in the medial columns were also found to label for NADPH-d. **(E)** Enlargement of edge cell. A narrow, oblique cell ventral to it is also seen to label faintly. **(F)** Giant interneuron (gi). These cells are found in the gray columns and are a type of primary sensory cell. Scale bars for images C-F = 100µm.

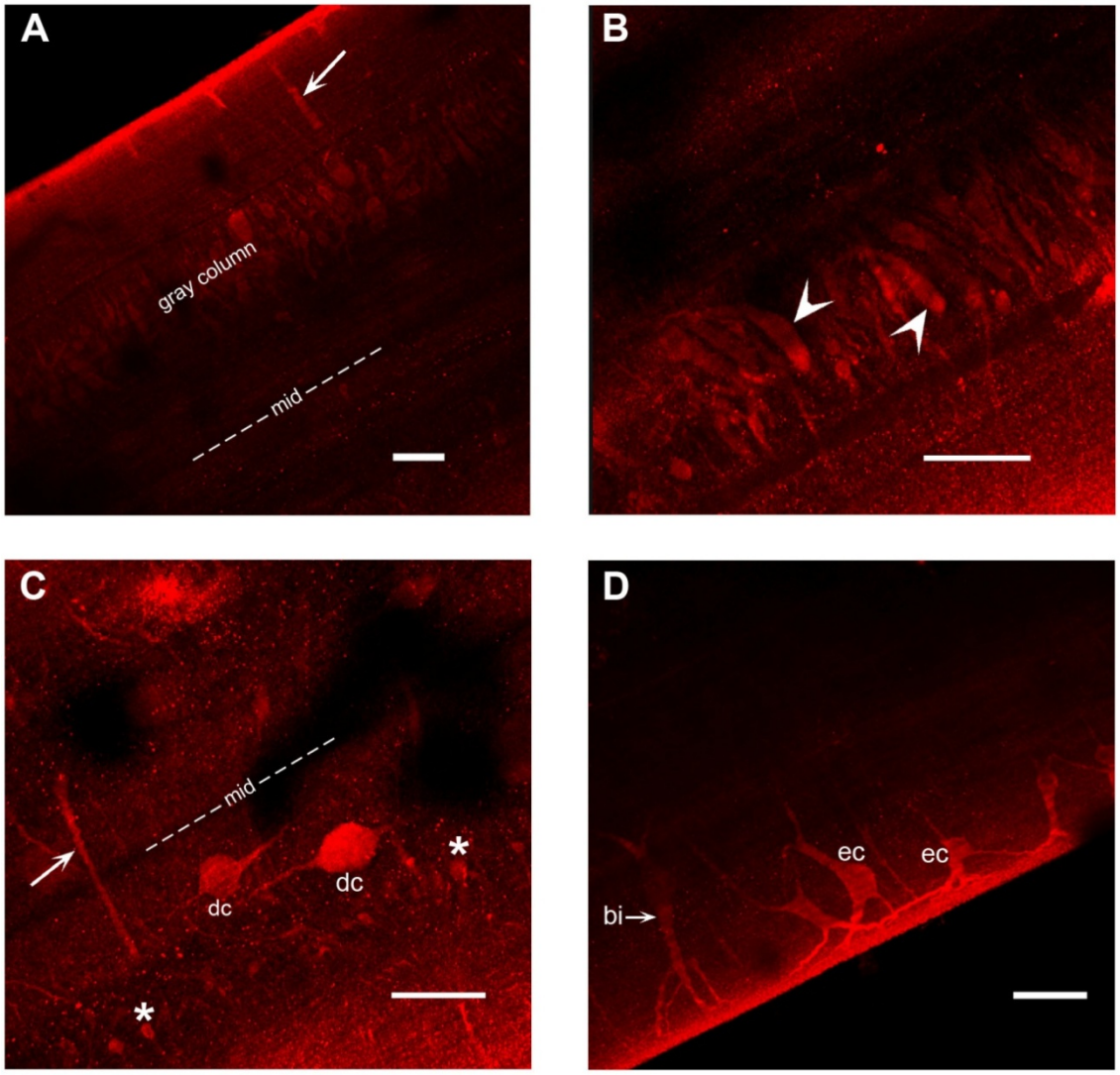

Figure 20. nNOS immunoreactivity in the spinal cord of the river lamprey. Images obtained with confocal microscopy (see Methods). **(A)** One of the gray columns is visible in this image. Midline is marked (mid). Cells are found to label for nNOS in a pattern very similar to that observed with NADPH-d staining. A projection extending laterally is visible (white arrow) although it is not clear which cell it is associated with. **(B)** Detail of gray column showing presumed motor neurons (white arrowheads). Note shape and size of labelled cells closely correlates with that of labelled cells in spinal cord processed for NADPH-d histochemistry. **(C)** Dorsal cells (dc) in the medial column label for nNOS. Dark spots on image are the result of gaps in the optical section, which was 10μm thick and close to dorsal surface. The midline is visible (mid) and is crossed by a brightly labelled projection (white arrow). Cells marked with a white asterisk are located dorsally in the gray column. **(D)** A stacked series of optical sections of lateral margin of spinal cord segment, showing edge cells (ec) and a bipolar cell (bi). The bipolar cell appears to show a lesser intensity of nNOS immunoreactivity than edge cells, similar to the degree of NADPH-d labelling of bipolar cells when compared to edge cells. Dendrites of edge cells are clearer with nNOS immunohistochemistry than with NADPH-d labelling. Scale bars = 100μm for all.

1 Summary

The scope of this investigation was to explore the contribution of NOS and NO to locomotion. In particular, a role for NO in the development of motor circuitry in the spinal cord of the anuran *Xenopus laevis* was investigated.

The central part of this thesis was based on experiments in which isolated brainstem-spinal cord preparations were maintained in organotypic culture with or without a given drug, and results were assessed *via* histological staining. Firstly, thyroid hormones were used to trigger developmental changes in NOS expression in the spinal cord in *in vitro* culture preparations at stage 47. NADPH-d histochemistry was employed to evaluate the change in NOS-expression in the preparations. Treatment with thyroid hormones caused a significant increase in the amount of NADPH-d labelling in the spinal cord after 72 hours in culture; this resulted in a pattern of staining that appeared similar to that of the CNS of *Xenopus* at stage 48. Earlier work (Ramanathan et al., 2006) has demonstrated a regionally- and temporally-specific appearance of NOS-expression in the CNS; of particular interest is the absence of NOS-labelling in the brachial and lumbar regions of the spinal cord during premetamorphosis. Combined with my evidence that applied thyroid hormones trigger changes in NOS-expression in the CNS, this information suggests that NO/NOS may be exerting an influence on the development of adult motor systems, i.e. the motor circuitry innervating the limbs.

The concept that NO might be playing an important role in the plasticity of the developing nervous system was also explored: nitrergic drugs (the NO-donor SNAP and the NOS-inhibitor L-NAME) were used to investigate the impact of NO on motor neurons of the spinal cord, again under *in vitro* conditions. Labelling of motor neurons was carried out *via* ChAT immunohistochemistry, as vertebrate motor neurons are cholinergic. Labelled cells were categorized as primary or secondary motor neurons based on their size and location, and counted. Due to the possibility of misidentifying cells, an alternative

method of quantifying ChAT-staining was also employed, to measure the actual area of stained tissue in sections, regardless of the type of cell. This method took into account the cholinergic staining that could not be attributed to a specific type of cell, (e.g. in processes) and was used to corroborate evidence from cell counts. SNAP-treated tissue was found to contain significantly more secondary motor neurons than control or L-NAME treated preparations; the area of staining in SNAP sections was also found to be significantly greater than in control or L-NAME sections. This suggests a pro-developmental role for NO in the assembly of spinal motor circuitry, because whilst primary motor neuron pools (which remained constant throughout all experimental conditions) innervate embryonic muscles and generate the early movements of the tadpole, it is the appearance of the secondary motor neurons that marks the addition of new musculature (for the limbs, the growing tail, and the trunk) during development (see van Mier, 1985; Nordlander, 1986).

The development of motor systems in the metamorphosing spinal cord is not restricted to the limb regions. The lateral motor columns, LMCs, (also known as the ventral horns) in both the brachial and lumbar regions of the spinal cord are composed of tightly clustered secondary motor neurons, and develop to supply motor innervation to the limbs. However, the tadpole also grows in size whilst the limbs develop, and the addition of new tail myotomes has been linked with the appearance of more secondary motor neurons in the spinal cord (Nordlander, 1986). These motor neurons are not part of the LMCs. The development of the axial/trunk musculature (like the tail, necessary for swimming) in the larval stages is also associated with the appearance of more secondary motor neurons. Therefore, the overall increase in the number of secondary motor neurons in the spinal cord may be seen as a sign of motor system maturation and developmental plasticity, as new neuronal elements are assembled to innervate the developing musculature. Thus, an increase in the number of stained secondary motor neurons (or an increase in the overall amount of cholinergic staining) in my SNAP-treated preparations suggests that NO *in vitro* may be having a differentiating effect on the motor circuitry in the spinal cord.

Finally, work on the adult river lamprey, *Lampetra fluviatilis*, served to demonstrate the presence of NOS in the spinal cord of an adult, but relatively more primitive, vertebrate. A variety of different cell types were found to display NOS-reactivity; of particular interest were those cells believed to be involved in the control of body movement, which included edge cells, motor neurons, and dorsal sensory cells. These cells were found to label for nNOS as well as NADPH-d, suggesting that NO's presence as a signalling molecule in the nervous system has been conserved over evolution, and that it may play a role in motor control in the lamprey. NO is known to play a modulatory role in controlling locomotor rhythms in *Xenopus*, and the presence of NO in locomotor cells of the lamprey could potentially be attributed a similar purpose.

2 NOS-expression is widespread in *Xenopus* embryos

An initial experiment to explore NADPH-d in intact albino tadpoles was carried out in order to refine the basic technique of NADPH-d labelling as a marker for NOS, used throughout most of this investigation. The NADPH-d technique can be capricious and the results have not always been found to overlap with NOS immunohistochemical methods (see Wörl et al., 1994). Furthermore, there is evidence that the resulting stain depends on the handling of the tissue (Bernstein et al., 2004). In fact, during my experimentation with older larval stages, I found that NADPH-d was undetectable in sections of paraffin-embedded CNS (data not included in this thesis), but clearly visible as blue-stained cell bodies in agarose-embedded tissue of the same type. For this reason, a strict experimental protocol was followed for NADPH-d staining; intact embryos were fixed in freshly-made 4% paraformaldehyde, a fixative to which NADPH-d/NOS enzyme is resistant. The tissue was washed carefully to help reduce fixation artefacts, and incubated for no longer than 2 hours. The samples were checked after 90 minutes, to ensure that the incubating solution was turning pale purple and that the tissue was developing the characteristic blue stain. If this was not the case, it was assumed the technique had not worked on those samples, possibly due to poor fixation of the tissue. However, all the intact albino embryos processed stained successfully.

I also avoided incubating the samples too long, because the blue stain develops progressively and, unless washed off carefully, continues darken the tissue until it is very dark blue and no structures can be distinguished.

Despite these reservations, the NADPH-d histochemical technique has come to be accepted as an effective means of labelling specific areas where the NOS enzyme is present. In the nervous system, workers have found an excellent degree of colocalization of NADPH-d labelling with nNOS immunoreactivity (for example, see Dawson et al., 1991) and the use of NADPH-d as a specific marker for cells expressing NOS has received support (Hope et al., 1991). Moreover, Ramanathan et al. (2006) have demonstrated a very close correlation between NADPH-d and nNOS immunolabelling in neurons of *Xenopus* spinal cord during metamorphosis. Therefore for the purpose of this investigation, the NADPH-d staining may be referred to as NOS-reactivity, or NOS expression.

2.1 NOS expression in the CNS

Staining in the CNS closely resembled the pattern of labelling reported in wild-type animals by McLean and Sillar (2001). The staining was observed side-on and therefore appeared as three clusters. McLean and Sillar (2001) carried out an analysis of NADPH-d staining during embryonic and larval development, and thus charted the temporal appearance of each group of cells; NADPH-d reactive cell populations in the brainstem appear progressively and are then retained during larval development. In fact, stained cells increase in number until stage 45 (McLean & Sillar, 2001), and not long after, NADPH-d/NOS reactivity begins to appear in the spinal cord (Ramanathan et al., 2006). This suggests that the roles NO plays in the developing CNS are not transient, but persist until metamorphic climax. McLean and Sillar (2001) show that staining in the brainstem generally develops along a caudorostral gradient, and highlights that the caudalmost group of cells could be part of the reticular formation, involved in the initiation and modulation of locomotor rhythms.

At stage 28 the first descending fibers from the reticular formation in the brainstem appear in the rostral spinal cord (van Mier & ten Donkelaar, 1984). NOS expression, however, appears for the first time in the caudal hindbrain at stage 29/30 (McLean & Sillar, 2001). This slight delay does not exclude NO from having important developmental effects on the reticular projections to the spinal cord, although early processes like axonal pathfinding and synaptogenesis within these projections are likely to have occurred already by the time NOS-reactivity develops (McLean & Sillar, 2001). The tadpole's swimming behaviour begins around stage 29/30, (coincident with the first appearance of brainstem NOS) and in fact, a modulatory role for NO in locomotion has been demonstrated (McLean & Sillar, 2000; 2002; 2004). The motor demands of embryonic tadpoles are restricted primarily to swimming, so while the caudalmost cluster of NADPH-d neurons observed in my preparations could be part of the reticular formation, the more rostral clusters may be involved in controlling other aspects of the embryo's survival, or may indicate newly generated/active neurons (see McLean & Sillar, 2001). NOS expression in the brainstem increases during development, and the reason for this may be that NO's modulatory role on motor control persists. In fact, NOS is shown to be activity-dependent, with elevated levels of Ca^{2+} activating NOS and even inducing its expression (Sasaki et al., 2000). Therefore it could be that the amount of NOS expression in the brainstem is proportional to the sensorimotor capacities of the animal at that stage, and that elevated NOS levels in cells indicate the increased activity in the areas responsible for sensorimotor processing. McLean (2001) also suggests a possible role for NO in local synaptogenesis in the brainstem. His study involved charting the chronological appearance of NOS-staining in the developing tadpoles and thus could speculate on the role of NO according to what developmental processes are also known to be occurring at each stage. My histological experimentation explored albino animals only at stage 37/38, and therefore does not provide additional evidence for developmental roles of NO in the brainstem. However, my experiments demonstrate the utility of using albino animals to study NOS expression in the CNS without the need to perform any dissection at all.

2.2 NOS expression in the skin

McLean and Sillar (2001) also reported evidence for NOS expression in the skin of *Xenopus* in cross-sections. The possibility of NO being produced in the skin is further supported by more a recent report that NO/NOS is present in *Xenopus* tadpole skin (albeit at later stages in development) as studied using NADPH-d histochemistry and DAF-2 DA labelling (Wildling & Kerschbaum, 2007). Alpert et al. (in press) also report findings of NOS expression and NO production in the skin of *Xenopus* embryos. In my samples, the distributed punctate pattern of staining observed in tadpoles at stage 37/38 is similar to the preliminary observations reported by McLean and Sillar (2001), and also resembles the patterns reported by Wildling and colleagues (2007). Furthermore, this substantiates evidence (Alpert, 2006; Alpert et al., in press) that NO is produced in the skin of *Xenopus* at stage 37/38. An interesting property of the embryonic skin is its electrical excitability, whereby an electrical impulse can travel the length of the body by passing through epidermal cells *via* gap junctions. Alpert (2006) showed that NO plays a role in the modulation of this 'skin impulse' in the young tadpoles; the impulse may form part of the animal's escape response to predators. NO has also been found to play a functional role in the skin of other amphibians such as the toad *Pleurodema thaul*, where it may increase ion transport in the skin (Neumann et al., 1996). In human skin, NO has been suggested to be involved in maintenance of barrier function and regulation of blood flow rate in the microvasculature, as well as in the skin's response to wounding and environmental stress such as UV irradiation (for review, see Cals-Grierson & Ormerod, 2004). It is possible that the NO generated by the NOS present in epidermal cells of embryonic *Xenopus* fulfils more than one single adaptive function. My findings are in full support of the conclusion that NOS is expressed in some epidermal cells of *Xenopus* at stage 37/38.

2.3 NOS expression in the heart

The presence of NOS in the heart cannot be clearly ascribed to individual cells but this seems likely. Sectioning of the heart might provide a better way of identifying the morphology and location of cardiac cells expressing NOS, but my animals were intact and therefore labelling in the heart was

observed as a general blue stain on what appeared to be the surface of the heart. The endocardial endothelium (EE) lies between the luminal blood and the underlying cardiac muscle and therefore surrounds the surface of the heart. It has been suggested that eNOS in the EE of *Xenopus* produces NO, which diffuses into the underlying myocytes and controls mitochondrial function and O_2 consumption in the tissues (Adler et al., 2004). NO from the EE has also been found to regulate myocardial contractions (Sys et al., 1997); in other words NO plays an important role in metabolism of the heart muscle cells. It is likely therefore that the blue NADPH-d staining in the heart of my preparations is due to the presence of eNOS in the EE, and that its regulatory role in cardiac function is being played out at this developmental stage.

2.4 NOS expression in the cement gland

The cement gland of *Xenopus* embryos activates a GABAergic stopping pathway that terminates swimming when contact is made with an obstacle; movement detector neurons that innervate the head skin and the cement gland project to GABAergic midhindbrain reticulospinal (mhr) neurons, which in turn project into the spinal cord and inhibit motor neurons of the swimming CPG (Boothby & Roberts, 1992). NADPH-d staining in the cement gland was visible as a uniform, deep blue stain but again, individual cells could not be distinguished. The possibility that NO is being produced in this area allows speculation that its role may be to modify the sensitivity of this stopping mechanism. The excitability of movement detector neurons could be influenced by the release of NO in the cement gland, by NO's actions on the membrane properties of the cells, or NO may modulate the cells output to GABAergic mhr neurons. The GABAergic system has in fact been shown to be the target of modulation and the presence of NOS in the brainstem at stage 37/38 suggests that it can directly facilitate GABA release from nearby mhr cells (Sillar et al., 2002).

It is possible that increased pressure on movement detector neurons could cause the Ca^{2+}-dependent activation of NOS in the cement gland. The NO released could in turn elevate excitability in these same movement detector neurons, affecting the intensity of the signal down to the mhr. An

increase in descending excitation could, for example, cause the activity-dependent activation of brainstem NOS, which, through NO's potentiation of descending GABAergic inhibition onto spinal cells, could enhance the tadpole's stopping response and help terminate swimming. Possibly, cement gland NOS is an activity-dependent way to connect to lower regions of NOS expression. At each 'node' in the pathway the transmitted signal has the potential for amplification by the actions of NO.

The presence of NADPH-d in the cement gland suggests the expression of NOS at this stage of development. However, only an overlap of the pattern of NADPH-d labelling with NOS immunoreactivity could confirm the presence of NOS in the cement gland. If NOS immunodetection methods confirmed the presence of the enzyme in the cement gland (and possibly allowed localization to the movement detector neurons), it would become relevant to explore how the movement detector neurons of the cement gland actually respond to NO. A suitable *in vitro* preparation would allow for this to be investigated using NO-donors.

2.5 Other considerations

In albino tadpoles at stage 37/38, staining was found in the skin, CNS, heart and cement gland. The skin of wild-type *Xenopus* is covered in melanophores, pigment cells which make it difficult to clearly observe NADPH-d staining, so albino tadpoles were used instead. The relative lack of pigmentation in the tissues of albino embryos made staining in the skin and internal structures more evident. The animals I used for these experiments were intact; I did not dissect the CNS from the preparations, yet it is apparent that the staining solution penetrated the animals adequately. My technique provides a very useful protocol for labelling NADPH-d in *Xenopus* embryos because it requires minimal preparation of the tissue aside from fixation, and therefore could be used in teaching labs where time is a constraint for experimentation. Students also need not have dissection skills to prepare the tissue, and results can be quantified with a good light microscope. Future research could include this technique in experiments to assess the impact of environmental conditions (e.g. light levels) on NOS expression in *Xenopus* embryos,

without the need for dissection and reducing the risk of excessive tissue processing that might affect NOS/NADPH-d labelling.

3 Thyroid hormones trigger a developmental change in NOS expression in the cultured CNS

The development of NADPH-d labelling in the spinal cord of premetamorphic and metamorphosing *Xenopus* has been charted by McLean and Sillar (2001) and Ramanathan et al. (2006), who reported that while at stage 47 NADPH-d labelling is essentially restricted to the brainstem, the first small, isolated cluster of NADPH-d/NOS stained spinal neurons appears in the rostral region of the spinal cord at stage 48. In my organotypic culture experiments using thyroid hormones (THs), the spinal cords of drug-treated CNSs displayed significantly more NADPH-d positive cells than controls. This increase in spinal labelling after the culture period, in tissue that had been dissected at stage 47 when NOS expression is virtually absent in the spinal cord, resembled the more mature pattern of NADPH-d/NOS-labelling normally typical of stage 48 animals. One interpretation of this finding is that NOS expression is triggered by circulating TH, which is known to orchestrate metamorphic changes throughout the organism, including the CNS.

The deiodinases (D1, D2, D3) are a family of enzymes responsible for the activation and inactivation of THs. D2 deiodinates T4 to generate T3, while D3 removes a second iodine molecule to inactivate T3. Less is understood about the role of D1 (reviewed in Bianco & Kim, 2006). Both T4 and T3 enter cells via transporters, by either high- or low-affinity binding to proteins on the plasma membrane. The former mechanism of entry is energy-and Na^+ dependent, the latter is not (see Hennemann et al., 2001, for a review). Once inside cells, T4 is converted to T3 *via* the D2 pathway, which increases the cytoplasmic concentration of T3; alternatively T3 concentration can be lowered by its reversion to T4 by D3 at the plasma membrane. Overall, the deiodinases serve to alter TH signalling within a cell by modulating cytoplasmic T3 concentration and hence thyroid receptor saturation (Oppenheimer, 1979).

What can be interpreted from my positive results using T4, is that the applied TH was transported into cells, where the D2 deiodinating pathway for conversion to T3 must still have been functional *in vitro*. Deiodinase activity is present in the tadpole's tissues (including the CNS) by stage 30 (Morvan Dubois et al., 2006) and my results suggest that deiodinase activity was preserved in the cultured CNSs.

Organotypic cultures of *Xenopus* tails with thyroxine have demonstrated *in vitro* tissue resorption during the culture period (Weber, 1967), implying that T4 can be converted to T3 in peripheral tissue *in vitro*. In a similar way, organotypic cultures of hindlimb buds and intestine (Ishizuya-Oka & Shimozawa, 1991; Tata et al., 1991) have shown metamorphic effects of THs *in vitro*, all of which support the idea that THs orchestrate metamorphosis in an organ-autonomous way. This, however, is the first time THs have been described to induce developmental changes in *Xenopus* CNS *in vitro.*

3.1 Comparison with labelling at stage 48

As described by Ramanathan and colleagues (2006), a cluster of NOS-positive neurons develops in the rostral spinal cord of *Xenopus* around stage 48. By stage 51, two clusters of NOS-positive neurons are found in the spinal cord, interspersed with regions devoid of NOS-labelled cells. HRP backfilling of the limb buds at this stage showed that limb motor neurons are found in the regions devoid of NOS-positive cell bodies, thereby allowing speculation that NO plays an inhibitory role in the 'interlimb' regions by preventing neurogenesis and thus allowing the proliferation of motor neurons destined to innervate the new appendages (Ramanathan et al., 2006). A cross section through the rostral NADPH-d spinal cluster at stage 51 showed the bilaterally symmetrical arrangement of the stained cells in three main zones: a dorsal group, showing weak NADPH-d reactivity; a medial group, with round or pear-shaped somata and primary neurites that projected ventrally; and a ventral group, which is composed of commissural cells that have labelled projections crossing the ventral midline (Ramanathan et al., 2006). In *Xenopus* neurons, NADPH-d reactivity is found to develop first in the soma, and then in the processes (McLean & Sillar, 2001), so the difference in the degree of NOS-labelling in the cells described by Ramanathan et al. (2006) suggests a different developmental timetable for the expression of NOS in each cell group.

While it is important to remember that the description of these three groups comes primarily from sections of a stage 51 spinal cord, whereas my preparations display labelling more similar to that of a stage 48 wholemount, the work of Ramanathan and colleagues (2006) provides some interesting information for comparison. In some of my samples, the spinal cord was rotated during mounting so that the distribution of NADPH-d labelled cells could be seen from the side. This allowed me to observe their dorsoventral positioning. Most of the labelled cells were found to be concentrated medially, with only a few located dorsally and ventrally. The darkest cells were pear-shaped, with extensive projections, and were often in the ventral half. This supports the idea that the NADPH-d labelling in the rostral spinal cluster develops in spatially-specific cell groups, and that labelling in the projections may hint at a different developmental timetable for the expression of NOS in cells of the cluster. Such dark, pear-shaped cells were never observed in controls, which, if any cellular labelling was present, stained much less intensely, and did not have projections. This provides further indication that the THs triggered a precise developmental timetable of NOS expression in these neural groups *in vitro*.

Functional identification of the cells was beyond the scope of this investigation, but my results lend support to the idea that the rostral spinal NADPH-d cluster is composed of cells distributed in dorsal, medial and ventral groups, and that different classes of cells express NOS in a precisely sequenced order. NO has been found to modulate neurotransmission, as well as the electrical properties of the cells that express NOS (McLean & Sillar, 2002), and the presence of NOS in the soma of cells, as observed in my preparations, indicates its potential influence on inputs to the cell body. However, as NOS-expression appears in the projections of older cells, it is possible that with maturity, the cell also acquires the ability to modulate its output (McLean & Sillar, 2001), as well as inputs received on the axon or dendrites. Furthermore, the very dark staining in the somata observed in my samples could be interpreted to indicate elevated levels of NOS-expression in those cells, another suggestion that the cells may have reached a more mature state.

3.2 Possible roles of NO in the spinal cord

The developmental progression of NOS expression in the spinal cord of metamorphosing *Xenopus*, as charted by Ramanathan and colleagues (2006), could reflect the capacity for nitrergic modulation of locomotor rhythms or for the developmental reconfiguration of motor circuitry, or both. The rostral cluster of NOS neurons appears at stage 48; NOS neurons in the caudal spinal cord appear at stage 50 and by stage 51 they have formed a distinct cluster. The neurons that label are found not to be motor neurons, however they may be part of the premotor circuitry, such as glycinergic commissural interneurons which are responsible for reciprocal inhibition and bilateral alternation of locomotor circuitry on either side of the cord (Soffe et al., 1984). Could it be that neurons initially express NOS in the trunk and tail regions of the spinal cord (the 'interlimb' regions) because they are modulating the larval swimming rhythm generated by the CPGs in these regions? Perhaps NOS is also regulating the assembly of larval motor or premotor circuitry for the developing trunk and tail musculature these regions innervate? NOS in the hindlimb region appears around stage 54, and the hindlimbs gain motility at stage 56 (Nieuwkoop & Faber, 1956). Again, might the appearance of NOS relate to the new functionality of the limb circuitry? This would imply that the appearance of NOS in the first rostral cluster at stage 48 reflects developing motor circuitry in that area, or the capacity of the locomotor circuitry of this region to receive modulation by NO.

One point to note is that NOS-reactivity has not been found in motor neurons of *Xenopus* larvae (Lopez & Gonzales, 2002; Ramanathan et al., 2006), so if the NO is modulating locomotion and/or network plasticity, it may be doing so via the premotor circuitry. The appearance of NADPH-d labelled cells in the rostral cluster does suggest they may belong to the spinal CPGs (Ramanathan et al., 2006), but they are clearly not motor neurons. NO has in fact been associated with arresting cell proliferation (for review see Villalobo, 2006) as well as with the differentiation and maturation of motor neurons (Kalb & Agostini, 1993). NO has also been implicated in the refinement of synaptic connections (Wu et al., 2001). An experiment to compare motor neurons in the region of the rostral spinal cluster at stage 48

with an earlier stage (for example, stage 46) would therefore help to elucidate whether motor neurons of that region change after the appearance of NOS, thus demonstrating whether NO is exerting some developmental influence on the motor circuitry of this area.

3.3 Effect of TH concentrations

T4 is light sensitive, and my use of increasing concentrations of TH was an attempt to offset both the instability of the hormone in solution, and the reduced accessibility of drugs to the CNS due to disruption of the vasculature *in vitro*. All of the hormone concentrations used in organotypic culture (50nM, 500nM, 1µM T4 and 1µM T3) caused a significant increase in the amount of NADPH-d labelling in the drug-treated spinal cords compared to controls. However, increasing the concentration of circulating T4 did not cause a relatively greater expression of NOS in the cultured CNSs. Nor was T3 at 1µm found to cause a relatively greater response in the cultured tissue, even though it is the more active TH. In fact, T3-treated preparations displayed no more labelling than T4 preparations, although both displayed significantly more labelling than controls. In summary, a dose-dependent effect was not observed by increasing the concentration of thyroxine, or by using the more potent T3. This suggests that I was operating at close to the top of the dose-response curve. Indeed, physiological concentrations of these hormones in the metamorphosing tadpole are much lower than what I applied, peaking *in vivo* at about 10nM for T4 and 8nM for T3 (Leloup & Buscaglia, 1977). Plasma membrane TH transporters have nanomolar values for affinity, so it is highly possible that the high concentrations I used caused a saturation of either TH transporters, TH receptors, or both, and the system was operating at its response limit.

3.4 Limiting factors

NADPH-d labelling was virtually or completely absent in some of the TH-treated preparations. It is important to recall that in all samples (control and hormone-treated), brainstem NADPH-d staining was always found to be present after the culture period. This was the criterion used to ensure that the preparations had been viable throughout the entire 72 hours, as enzymatic activity is diminished in

degraded tissue and does not result in reliable NADPH-d labelling. Furthermore, the tail of all preparations was verified to be beating at the end of the experiment. If it did not respond after tactile stimulation with a blunt probe, the sample was eliminated from the experiment. Necrotic tissue was easily spotted as opaque, curled up and degraded, possibly due to insufficient local oxygen supply to the tissue. Therefore, it is unlikely that CNSs showing brainstem, but no spinal labelling, had been degraded. Rather, it is likely that some unknown factor(s) prevented the tissues from responding to the circulating hormone.

T4 and T3 gain access to the intracellular milieu via transporters in an energy-dependent process (reviewed in Hennemann et al., 2004). ATP production and Na^+ availability could therefore have been a limiting factor; while the concentration of ions such as calcium and chloride in the culture medium attempted to mimic physiological levels, it is possible that the quantity of nutrient (glucose) available to my preparations in the culture medium could have affected the rate of hormonal access. Also, I bubbled carbogen, a 95:5% mixture of oxygen and carbon dioxide, through my culture reservoirs- although I did not measure the pH of my culture medium, it is also possible that this may have affected the rate of uptake or conversion of T4 to its active form T3. Another factor to bear in mind is that enzymatic activity is temperature-dependent. I maintained my preparations between 16 and 17°C, because tissue survival was compromised at higher temperatures. The lower temperature could have slowed down the conversion of T4 to T3. It is entirely possible that if the culture period were extended, the degree of NADPH-d labelling in hormone-treated spinal cords would also increase.

3.5 Difficulties in developmental staging

Development does not proceed as clear-cut steps, but is a gradual process, and this could have led to errors in staging of the animals I used. The two main criteria I used in staging the tadpoles were the presence of the forelimb bud, and the size of the hindlimb bud. Stage 47 tadpoles were identified therefore by the absence of forelimb buds (the first appearance is normally at stage 48) but the presence of a very small hindlimb bud (which at stage 46 has not yet appeared). It should therefore have been

quite straightforward to select animals at stage 47; at its first emergence, however, the forelimb bud is miniscule and translucent, making it necessary to manipulate the lighting in order to verify its presence. Often animals may be 'between stages' and an animal that was just past stage 47 but not visibly a stage 48 could have been mistaken for the younger stage. Furthermore, the hindlimb and forelimb buds do not always maintain the same relative rate of development, so there may be animals in which the forelimb bud has just appeared, but the hindlimb bud is particularly small (making it appear to be a stage 47). Errors in staging can translate to differences in the amount of NOS-labelling observed in each spinal cord; with slightly older animals containing more NOS-expressing cells. While occasionally, a few NADPH-d labelled neurons are found in stage 47 spinal cords (McLean & Sillar, 2001), it is possible that some of the CNSs showing more labelled spinal cord cells may have been slightly more developed than the other animals. It takes approximately 2 days for an animal kept at room temperature to develop from stage 47 to stage 48 (Nieuwkoop & Faber, 1956). Therefore, if the forelimb bud can escape detection, and the hindlimb bud suggests a stage 47, it is easy to see how I could have selected the occasional animal at stage 48 rather than 47. In any case, this possibility stands for the entire course of my experimentation and under all conditions, which counterbalances the occasional minor error in staging. As my analysis reveals, there is a significant difference between labelling in control and drug conditions, demonstrating that there was a drug effect for all concentrations used.

3.6 Other considerations

The rostral spinal cluster of NADPH-d positive cells at stage 48 extends approximately 700-1200μm (Ramanathan et al., 2006), but labelling in my samples in organotypic culture never extended more than 400μm. Nevertheless, there is reason to believe that the region that labelled in my experiments is the same region that Ramanathan and colleagues (2006) described. One reason I propose for the difference in the extent of the rostral region is that samples are subject to varying degrees of tissue shrinkage over the course of experimentation. My CNSs were maintained *in vitro*, and I observed that after the 3 days the tissue often appeared more opaque and shorter in length, occasionally even

pulling free from the pin through the soft brainstem, as the tissue retracted towards the tail (which was pinned much more firmly through the muscle). More tissue shrinkage was observed after processing for NADPH-d histochemistry, most likely due in part to the treatment with paraformaldehyde for fixation, and to the dehydration in methanol. After processing, CNSs were always noticeably smaller than freshly dissected CNSs, suggesting tissue shrinkage played a large part in the size difference between rostral NADPH-d positive spinal region as measured in my samples, and that measured by Ramanathan and colleagues (2006). Another reason for the difference when considering the pattern of NADPH-d labelling in tadpoles at stage 48, is that they have a slightly larger CNS than stage 47 animals. On the other hand, the pattern of labelling I describe is found in spinal cords that had been dissected at the smaller stage 47. Furthermore, the appearance of NADPH-d staining in stage 47 spinal cords when treated with thyroid hormones, as demonstrated by my experiments, does not mean the spinal cords displayed a pattern identical to that of stage 48 animals. What it does show, is that thyroid hormones can cause the emergence of NADPH-d staining in the cells of the spinal cord at a stage that normally does not have NOS-producing cells in that region. That this resembles the pattern of staining in more mature animals is very encouraging, and strongly suggests that thyroid hormones trigger metamorphic changes in the CNS *in vitro*, but does not mean that the CNS has developed into the equivalent of a stage 48 CNS. Verifying this would take more than simply exploration of NOS activity.

My results raise a number of possibilities for future experimentation: as mentioned above, it would be interesting to explore the distribution and morphology of motor neurons in the spinal region where the rostral NADPH-d cluster develops, comparing early stages before the NOS-expression appears, with later stages where the labelling is intense. This could help elucidate the role of NO in this region. Also, it would be interesting to culture spinal cords at slightly older stages, such as stage 49, when still only one rostral cluster of NOS-expressing neurons is present, and investigate whether applied thyroid hormones cause the development of a more mature staining pattern, i.e. the appearance of a second cluster of

NOS-positive neurons, as is typical of stage 50/51. This might reveal whether the metamorphic effects of THs are stage-dependent.

This first set of organotypic culture experiments demonstrates that one of the metamorphic effects of TH on the CNS is the appearance of NOS-expressing neurons in the spinal cord, in a pattern of labelling similar to that of a more mature spinal cord. This induction of NOS expression *in vitro* may be limited by a variety of factors, including saturation of the TH receptors and ATP availability. The presence of NO in the spinal cord may serve a modulatory role on locomotor rhythms, or may influence the reconfiguration of spinal motor circuitry, or both.

4 NO plays a role in the maturation of spinal motor circuitry

4.1 Changes in spinal motor neurons during development

The spinal cord of the *Xenopus* tadpole is an important model of neural plasticity; the motor patterns produced by the tadpole change during development with the appearance of the limbs (Combes et al., 2004), and underlying this switch in motor behaviour is a dynamic reconfiguration of spinal locomotor networks. The primary motor system, which is used for tail-based swimming behaviour from the time of hatching, is assembled in early embryogenesis. The secondary circuitry appears with a delay and develops during larval life, and with the appearance of the limb buds. The two systems are driven by different pools of motor neurons. 70% of primary motor neurons are incorporated between the embryonic stages 10-21, and innervate the axial musculature (van Mier, 1986), whilst over 50% of secondary neurons are born between the premetamorphic stages 46-50. Van Mier (1986) also reports that the rates of generation of new motor neurons in the spinal cord change over development, with the rate of generation of new primary motor neurons reaching a peak at stage 15 and undergoing a steady decrease towards stage 50, at which point the rate of production of primary neurons is close to zero. During larval stages, however, the animal grows in size and new muscle groups are added; the tail lengthens by addition of myotomes and the limb buds appear. These developing structures become

innervated by the secondary motor neurons. The development of the lateral motor columns (LMCs) in the brachial and lumbar regions is also marked by the increase of secondary motor neurons (Fortune & Blackler, 1976), which appear as bilaterally symmetrical clusters in the ventral spinal cord. The medial motor column, providing motor innervation to the axial musculature of the larval tadpole and adult frog, is also composed of secondary motor neurons (Forehand & Farel, 1982). Furthermore, as the tail of the tadpole grows in length, additional secondary motor neurons appear in the caudal region of the spinal cord, that project to new tail myotomes (Nordlander, 1986). In all cases, the appearance secondary motor neurons indicates the ongoing maturation of motor systems.

Motor neuron differentiation (as described in birds and mammals) is the process by which a neuroepithelial cell (surrounding the central canal) transforms into a motor neuron; first it loses its apical process, migrates laterally and develops an axon. Then the cell reorients itself with its long axis positioned dorsoventrally and dendrites start forming when the axon makes contact with its target, beginning with the apical dendrite (in Nordlander, 1986). In my organotypic culture experiments with T4 and T3, THs were found to trigger an increase in NOS-expression in the spinal cord, which can be identified as a developmental change and a sign of neural plasticity. This appearance of NOS was postulated to play a role in the development of limb motor circuitry; therefore, in my experiments with nitrergic drugs in organotypic culture of *Xenopus* CNS, the aim was to explore whether the presence or absence of NO had any observable effects on the motor neurons of the brachial spinal cord, as measured by choline acetyltransferase (ChAT) immunohistochemistry. First of all, however, I examined the appearance of motor neuron labelling in sections taken from the brachial region of tadpoles at stage 47 and stage 51, to investigate the normal changes in the motor neurons that occur during this developmental period.

4.1.1 Increase in secondary motor neurons is a characteristic of developing motor systems

Primary and secondary motor neurons in *Xenopus* spinal cord have been described and compared by van Mier et al. (1985) and can be distinguished on the basis of their soma diameters. The approximate diameter of primary motor neurons around stage 48 is 25-35μm, while secondary motor neurons labelled from the hindlimb bud are smaller, measured around 15μm. Primary motor neurons are always appreciably larger than their secondary counterparts in any sample. It is worth noting that van Mier used horseradish peroxidise (HRP) to label motor neurons in his experiments, while I stained for ChAT-immunoreactivity. The HRP method is preferred for highlighting projections and dendritic arborisation of cells, but it must be applied to live animals, and only motor neurons innervating the musculature to which it is applied are labelled. I chose ChAT-labelling as a method to stain motor neurons because it could be carried out on fixed, sectioned tissue and it is more reliable for staining all the motor neurons in a chosen region of the spinal cord.

At stage 47, NOS-labelling in the spinal cord is 'virtually or completely absent' (McLean & Sillar, 2001), but by stage 51, two distinct clusters of NOS-labelled cells can be observed (Ramanathan et al., 2006). These stages were therefore taken to represent key stages in the development of the pre-metamorphic spinal cord. Motor neurons of the brachial region were examined at stage 47 and 51, and compared. This was done by sectioning the first 300μm of the spinal cord and counting motor neurons labelling for ChAT-immunoreactivity. No significant difference was found in the number of labelled primary motor neurons per section between stage 47 (about 1.2 labelled primary motor neurons) and 51 (about 1.3). However, the number of secondary motor neurons labelling in sections of the brachial region was significantly greater in stage 51 than stage 47 (0.5 at stage 47, increasing to about 1.7 at stage 51), suggesting that during this developmental period, the secondary motor neuron pool in the brachial spinal cord increases, but the primary motor neuron pool has not undergone any significant change. The brachial motor columns (also referred to in this thesis as brachial ventral horns, or lateral motor

columns, LMCs) do not begin to differentiate until stage 52/53 (Fortune & Blackler, 1976). These secondary motor neuron pools will eventually provide motor innervation to the forelimbs, and were not observed in any of my samples, which were at younger stages. As highlighted above, the ongoing maturation of motor systems in the tadpole is not exclusive to the motor circuitry for the limbs; it also includes the appearance of secondary cells for the developing trunk and tail of the tadpole (see Nordlander, 1986). Eventually, during development, the secondary motor circuitry supersedes the primary one, although it is not clear whether the primary neurons degenerate entirely or simply fulfil lesser motor roles (van Mier, 1986). What can be deduced by comparing motor neuron numbers between stages 47 and 51, is that while stage 51 is still too early for the brachial motor columns to be observed, the increase in secondary motor neuron number reflects the assembly of more mature motor systems, and can be attributed to the developing secondary circuitry of the medial motor column, which is located ventrally to the LMCs (see Crowe et al., 1995), and provides motor innervation to the neck and trunk of the rapidly growing tadpole.

4.2 SNAP-related increase in secondary motor neuron number in the brachial spinal cord

Xenopus CNSs were dissected at stage 47 and cultured in the presence of the NO donor SNAP, to explore the influence of NO on motor circuitry of the spinal cord. After 72 hours, the preparations were fixed and processed for ChAT immunohistochemistry to determine differences in the pattern of motor neuron labelling. The average number of labelled primary motor neurons did not change significantly from control. SNAP-treated samples did however show a significant increase in the number of secondary motor neurons found to label for ChAT (an average of about 1.3 secondary motor neuron per section) compared to controls (an average of 0.5 secondary motor neurons). It seems likely that the SNAP caused this difference in my *in vitro* preparations, allowing speculation that the NO liberated into the culture solution was able to access the cells and induce some form of neural plasticity. An experiment using NO-

depleted SNAP would substantiate the notion that the NO released from the breakdown of SNAP is responsible for causing the increased number of secondary motor neurons.

NOS-expressing cells do not appear in the brachial spinal cord until about stage 53/54 (Ramanathan et al., 2006). With the application of the NO-donor *in vitro*, therefore, I created an NO-rich environment at a stage in which NOS is not normally present to exert a local influence in the spinal cord. However, the increase in the number of secondary motor neurons suggests that NO had a differentiating effect on motor cells. The mechanism of such action was not explored as it was beyond the scope of this investigation, but further experimentation could include the application of NO along with an inhibitor of sGC, which transduces the NO signal by the production of cGMP and the initiation of a signalling cascade (Ignarro, 1990). It could be that endogenous NO exerts a regionally-specific influence on motor systems by affecting only those cells that can respond to the NO signal, for example, sGC-containing cells. Therefore, operating on the downstream effectors of NO (which can also include the activation of tumor suppressor genes, see Gibbs, 2003), would help elucidate the mechanism by which NO might exert its developmental influence.

NO has in fact been implicated in proliferative arrest and in coupling the switch from proliferation to differentiation during neurogenesis (Gibbs, 2003; Contestabile & Ciani, 2004; Villalobo, 2006, provide reviews). The expression of NOS in response to growth factors has been observed in cultured neuroblastoma cells, where subsequent generation of NO halts proliferation and induces differentiation. This inhibitory effect on proliferation has been demonstratred *in vivo* in both vertebrate and invertebrate nervous systems, suggesting an evolutionarily conserved mechanism for regulating cell division and the acquisition of a mature phenotype. A variety of mechanisms of NO action have been proposed, and it has become clear that the process is not necessarily dependent on cGMP (Villalobo, 2006). NOS has also been implicated directly in the development of motor neurons, where it is reported that NO is important for the expression of Cat-301, a molecular marker of maturation (Kalb & Agostini, 1993). Administration of NOS inhibitors is found to inhibit the expression of Cat-301 immunoreactivity in

spinal motor neurons of young rats. This study also shows that while the acquisition of a mature molecular phenotype for a motor neuron may depend on the activity of the NOS enzyme, the maintenance of the adult molecular phenotype of the cell is NOS-independent, implying that there is a period of sensitivity of the motor neuron to NO. Indeed, they report the presence of NOS-positive cells adjacent to motor neurons (but no NOS expression in the motor neurons themselves) during this critical period of sensitivity to NOS-inhibition (Kalb & Agostini, 1993). It is interesting to speculate then, that the spatiotemporal expression of NOS in the *Xenopus* spinal cord may also reflect a critical period during which motor neurons are susceptible to NO-induced plasticity.

NO's role in the maturation of motor circuitry could be mediated by the activity-dependent expression of NOS in premotor interneurons that project to the motor neuron pools. The premotor circuitry integrates supraspinal and sensory input and could potentially regulate the plasticity of motor neurons by expressing NOS/releasing NO in a specific spatiotemporal pattern. Furthermore, the decline of NOS-expression in the spinal cord of *Xenopus* after metamorphic climax could serve to limit the time frame during which NO exerts its developmental influence in the spinal cord; after the completion of motor innervation of adult musculature (e.g. the limbs), the motor circuitry's mature configuration could remain stable without the need for NO in the maintenance of the mature phenotype, if the critical period of sensitivity to NO has passed. Insect motor neurons also exhibit NO-sensitivity. Here, the critical period appears to be after axonal outgrowth, during maturation and synaptogenesis, with sensitivity ending as synaptogenesis nears completion (Truman et al., 1996). The appearance of secondary motor neurons in the spinal cord of my samples cultured in the presence of an NO donor suggests that NO may play a role in the differentiation of motor neurons (and there is support for such a role, see Gibbs, 2003), corroborated by the fact that there is also evidence for a direct maturational role of NO (Kalb & Agostini, 1993; Truman et al., 1996). This allows me to speculate that the appearance of NOS in the aforementioned spinal cord clusters serves an important role in the switch from proliferation to active differentiation of motor cells, and it is spatiotemporally regulated to induce motor circuit plasticity only

during that period of neuronal sensitivity to NO. However, the observed response of the spinal cord to NO *in vitro* suggests cells of the brachial region are sensitive to NO (and therefore, susceptible to plastic changes) even before the expression of NOS in that region. In fact, NO has been found to modulate locomotor rhythms in *Xenopus* embryos, long before the appearance of NOS in the spinal cord (McLean & Sillar, 2002; 2004) which lends supportive evidence for NO-sensitivity before endogenous NO production in the spinal cord.

4.3 L-NAME did not have an effect on motor neuron number *in vitro*

The NOS-inhibitor L-NAME was also used in organotypic culture, to investigate whether an NO-deprived condition would influence motor cells in the brachial spinal cord. At stage 47, when the animals were dissected, NOS is normally found in specific groups of neurons in the brainstem (McLean & Sillar, 2001), but blocking it using L-NAME did not appear to have an effect on the number of primary or secondary motor neurons labelling in spinal cord sections. NOS inhibition in adjacent cells is found to inhibit the development of motor neurons (Kalb & Agostini, 1993), but it is worth noting that at stage 47, NOS-expression in the spinal cord is virtually absent (McLean & Sillar, 2001). At embryonic stages the brainstem source of NO is physically closer to potential motor targets in the spinal cord because of the reduced size of the CNS, but as the animal grows and the CNS lengthens, this brainstem NO source and its targets become more distant. If this is considered with respect to NO's restricted sphere of action in the immediate area it is produced, it suggests that motor neurons in the spinal cord are unlikely to be receiving NO signals from brainstem cells at stage 47. Therefore, using a NOS-inhibitor to block the production of NO in the brainstem would not necessarily translate to an observable difference in the motor neurons of the spinal cord *in vitro*. However, NOS-expressing cells in the brainstem might have important developmental influence on spinal motor circuitry later on in development. What can be gleaned from my results is that under my experimental conditions, the application of a NOS-inhibitor did not appear to cause significant changes in the brachial motor circuitry, but this must not interpreted as a lack of NOS involvement in the development of spinal motor circuitry. My culture period was 3 days;

organotypic cultures can be maintained viable for much longer (see Weber, 1967), and it is entirely possible that the cellular response to the effects of blocking NOS have a 'lag-time'. Furthermore, my results using SNAP suggest that NO does promote the development of motor neurons; NO in the spinal cord *in vivo* is likely to be produced by the NOS-immunoreactive cells that appear in a spatiotemporal pattern.

4.4 SNAP caused an increase in ChAT-immunoreactivity

An effect that I regularly observed in sections of my SNAP-treated tissue, but not with L-NAME or in controls, was a distinctive patterning of ChAT-immunoreactivity in the ventral spinal cord that could not always be attributed to cell bodies (see Fig 16, Di). Therefore, when counting presumptive motor neurons in my sections, I excluded this labelling from the analysis. Another point to mention is the possibility of identifying a glancing section through the soma of a primary motor neuron as a smaller secondary motor neuron (see Fig 16, Di) and although care was taken to only count cells if they had a visible nucleus, the distinction between motor neuron classes could be subject to ambiguity. An alternative method of quantification that could take these facts into consideration was therefore used on two samples from each experimental condition; to quantify the overall area of staining, the area of ChAT-immunoreactivity was digitally measured in sections and compared between conditions. Data from these measurements support an increase ChAT-positive cells (which I have presumed to be motor neurons) in SNAP-treated tissue; the average surface area of labelling in L-NAME and control samples is not found to differ significantly, however, SNAP-treated samples show approximately double the area of staining of control or L-NAME samples. If, as described above, the primary motor neuron pool does not appear to vary between conditions, but there is an increase in secondary motor neurons in SNAP-treated tissue, then my measurements provide additional evidence that NO induced some form of plasticity in the spinal cords maintained in organotypic culture. It is also worth noting that much of this cholinergic staining was located surrounding the central canal and the labelled motor neurons. It is conceivable that the applied NO induced ChAT-expression in cells before they had migrated successfully from the

neuroepithelial layer to their appropriate location, but additional experimentation will be needed to test this idea directly.

4.4.1 Possible roles for endogenous NO

I chose to monitor the changes in motor neuron number in the brachial region of the spinal cord because it allowed me to maintain consistency in cutting (I always took the first 30 sections caudal to the brainstem) and because it is the region in which the brachial ventral horns, or motor columns, develop. Thus it is an appropriate region to study in order to chart the assembly of limb circuitry. What has become clear is that while NO appeared to promote the development of secondary motor neurons in this region, it did not induce the appearance of the brachial motor columns. It is important to remember that the observed effect of NO in my preparations (i.e. an increase in secondary motor neurons) does not automatically translate to the actual effect of endogenous NO generated by NOS when it appears in the spinal cord neurons. It could be that NO in the 'interlimb' and caudal spinal regions (the two main clusters of NOS-reactive cells) halts the proliferation of motor neuron precursors and promotes the differentiation and synaptogenesis of secondary circuitry dedicated to the axial/tail musculature, but is excluded from the brachial and lumbar region to allow the formation of the motor columns by migration and proliferation of those cells before they must acquire a mature phenotype. When NOS does appear in these regions, by negatively regulating proliferation and signalling the switch to differentiation it could then be exerting an effect on the maturation and synaptogenesis of these neurons. Muscle has been found to contain NOS (Grozdanovic, 2001), so it could be that an NO signal at the neuromuscular junction also acts retrogradely on the motor neurons. Furthermore, there is evidence for the involvement of NOS in the differentiation of the neuromuscular junction in *Xenopus* embryogenesis (Schwarte & Godfrey, 2004), so it is clear that the roles of NO/NOS in the development and refinement of locomotor circuitry are diverse and far-reaching; in fact the widespread presence of NOS implies that various NO influences may operate in concert to halt neural proliferation, induce differentiation, and

refine synaptic connectivity as the motor circuitry acquires a mature configuration, and the developmental expression of this enzyme suggests these processes are under strict temporal control.

4.5 Other considerations

Throughout my experiments, I employed ChAT immunohistochemistry to identify motor neurons. The development of the protocol for ChAT labelling involved multiple aspects of histology. It was important to find the most appropriate fixative for the nervous tissue that would also work well for this antibody, a suitable sectioning technique, and optimization of the incubation times for sections. As it turned out, overnight immersion in Carnoy's was the preferred fixation method, followed by paraffin embedding and sectioning. The antibody incubations were optimized for room temperature processing and the result was visible red-brown cholinergic staining that contrasted against the background labelling. Although paraffin embedding is not the generally preferred method for antibody labelling due to the high temperatures the specimen is exposed to, this came after many failed attempts at labelling motor neurons for ChAT using nervous tissue that was fixed in paraformaldehyde and agarose-embedded. Sections of 100μm (cutting thinner made the agarose shatter, destroying the section) were incubated free floating, for much longer incubation times. This did not result in good quality staining; the paraformaldehyde left tissue soft and breakable, and many sections were lost or damaged. While the motor neurons did show ChAT immunoreactivity, the thickness of the sections prevented resolution of single neurons in a stained area (more than one neuron and its projections might be stacked in the same area). Furthermore, the long incubation times resulted in a greater degree of nonspecific staining. Altogether this did not provide suitably clear and quantifiable staining so I attempted embedding in egg-yolk, with the intention of cutting thinner sections that were more resistant to cutting and the incubations. While sections made with egg yolk as an embedding medium could be cut slightly thinner (60μm) without shattering, they did not stain at all for ChAT immunoreactivity. This could be because when embedding with egg yolk, the fixed tissue had to remain in the embedding mold surrounded by the egg while the paraformaldehyde vapours slowly cross-linked the proteins in the yolk. This long exposure

to paraformaldehyde could have overfixed the nervous tissue; overfixation can cause antibody binding to be weak or entirely absent (Werner et al., 2000) and this could explain why I did not have success with this procedure. In any case, it was found that the most reliable method for labelling ChAT in sections was to paraffin-section Carnoy's-fixed tissue and use Shandon Coverplates and incubation trays to stain the prepared slides. The advantages of this system were that I could more efficiently use the antibodies and not risk damaging the sections on the slides between incubations, as the coverplates remain on the slides until after the final step when DAB chromogen is added and developed.

Another point worth considering is that SNAP was applied in 4-hour bouts interspersed with periods of fresh saline for recovery, and this protocol was chosen because the preparations died when supplied with SNAP continuously. For future experimentation, the drug could therefore be administered in lower concentrations, and this could allow drug application to be maintained throughout the culture period. A longer culture period could also be explored. Overall, my work provides evidence for a central role of NOS/NO in the development and maturation of secondary motor systems in the CNS of metamorphosing *Xenopus*. NOS *in vivo* could be operating in a spatiotemporally relevant distribution to release NO and influence the differentiation and possibly the synaptic connectivity of spinal motor circuitry, and my work provides a platform for future experimentation in this field.

5 NOS is expressed in the spinal cord of the lamprey

NADPH-d histochemistry and nNOS immunohistochemistry demonstrated that several classes of neurons in the lamprey spinal cord express NOS. Some of these are believed to be involved in motor control: the edge cells, motor neurons, dorsal sensory cells and giant interneurons. Also labelled were ependymal cells surrounding the central canal, bipolar cells projecting to the lateral margins of the spinal cord, lateral cells with somata in the gray columns, and small spindle-shaped cells in the medial columns. This is the first time NADPH-d/nNOS expression has been described in the spinal cord of the river

lamprey *Lampetra fluviatilis*. In keeping with the main focus of this investigation, I will discuss only those cells that are thought to play a role in the generation and regulation of rhythmic locomotion.

5.1 NOS may play a role in integrating sensory input

Edge cells are a type of intraspinal mechanoreceptor (Grillner et al., 1984) that receive rhythmic excitation and inhibition from the central pattern generator network during the ipsilateral and contralateral muscular contraction, respectively (Vinay et al., 1996). Importantly, they are depolarized upon stretching of the lateral margin of the spinal cord, which occurs when the curvature of the spinal cord changes as the lamprey's body bends during swimming or turning. It has been reported that edge cells stain for GABA immunoreactivity (Batueva et al., 1990) as well as for glycine, suggesting colocalization of these two neurotransmitters (Shupliakov et al., 1996). NO has been found to play a modulatory role on swimming in *Xenopus* embryos; by acting to potentiate the effects of the neurotransmitters glycine and GABA and thereby exerting a form of control over the locomotor rhythm, it may provide the system with a means of flexibly adapting its output (Sillar et al., 2002). If NO is an effective modulator of other neurotransmitter systems, in particular GABA and glycine, then its production in edge cells, which are subject to oscillating excitation in time with the output of the CPG (Vinay et al., 1996), could serve a role in regulating the response to stretching in the spinal cord in situations that require complex modulation of locomotor rhythms, such as swimming in turbulent water flow where the force of the water may impinge on the lamprey's movement.

Dorsal cells also label for NADPH-d/NOS, and are visible as large, rounded cells located dorsally in the medial columns, on either side of the central canal. These primary mechanosensory neurons correspond to the Rohon-Beard cells of *Xenopus* tadpoles (Clarke et al., 1984). Dorsal cells in lamprey respond to either touch or pressure, and the presence of low-voltage activated calcium channels in touch cells (but not in pressure cells) (Christenson et al., 1993) suggests that the NOS-expression I observed may be labelling in just one or the other type of dorsal cell. nNOS is activated in a Ca^{2+}-dependent manner (Bredt & Snyder, 1990), and its presence could potentially be exclusive to the touch-

sensitive dorsal cells, where the activity-dependent release of NO could serve to lower the threshold of excitation, thus providing a way of sensitizing the cell after stimulation and possibly allowing for a heightened escape response.

Giant interneurons were also found to label for NADPH-d. While I did not find any such cells labelling for nNOS, this could be because there are only about 20 giant interneurons in the entire spinal cord (see Selzer, 1979) and it is very possible segments processed for nNOS simply did not include any. Processing lamprey CNSs in wholemount would be a worthwhile experiment to map the relative positions of NADPH-d/NOS expressing cells in the spinal cord, since some cell types can occur primarily in a specific region (e.g. giant interneurons are found in the caudal half of the spinal cord), whereas using separate segments of spinal cord could not provide such information. Giant interneurons can respond to mechanical stimulation of the fin and body skin and are considered 'second order sensory relay neurons' which do not receive rhythmic synaptic drive during swimming (see Buchanan, 2001). NOS in giant interneurons could serve to facilitate/potentiate the relay of information between cells of this type, but it could also be involved in the cell's response to mechanical stimulation by activity-dependent release of NO.

5.2 NOS may be involved in flexibility of motor output

The somata of motor neurons stain for NADPH-d/nNOS in the spinal cord of the lamprey, and appear in clusters within the gray columns. NOS has not been reported in motor neurons of metamorphosing amphibians (Gonzalez et al., 2002; Ramanathan et al., 2006), except for one report describing motor neurons in *Xenopus* nitrergic (Crowe et al., 1995). This instance, however, could have been due to the long fixation times used in the study, which have been reported to result in non-specific staining. NOS expression can be induced in motor neurons of mammals in response to a variety of bodily insults and injury, and it is speculated to have either a neuroprotective effect (He et al., 1997) or be implicated in the neurotoxic death of the motor neurons once they have been deprived of their target (Wu & Li, 1993), but in my samples of adult lamprey spinal cord, motor neurons appeared to label for

NOS without evidence of injury. Incidentally, I did not explore the distribution of NADPH-d/NOS in the larval lamprey, and NOS-expression in lamprey may also follow a temporally-specific pattern, like in *Xenopus*. It would be interesting to use larval lamprey spinal cords and compare the pattern of NOS-expression with that of the adult spinal cord, to establish whether cells express NOS as a function of maturity or connectivity, or whether expression of the enzyme is developmentally conserved. Another important point is that motor neurons in the spinal cord innervate different muscles, i.e. the myotomes of the body wall, or muscles of the midline fins (see Buchanan, 2001). It could therefore be that the motor neurons labelling for NADPH-d/NOS are those that innervate a specific group of muscles. An experiment to investigate this could be carried out by application of HRP to either the myotomes or the fins, followed by fixation of the tissue, and processing for HRP as well as NADPH-d histochemistry to verify an overlap in the staining. In any case, it has been shown that myotomal and fin motor neurons receive different patterns of inputs during fictive swimming (for review, see Buchanan, 2001); fin muscle contractions alternate with contractions of ipsilateral myotomes, and NO/NOS could play a role in the modulation necessary to achieve this alternating pattern. NO has in fact been found to alter the electrical properties of the membrane of motor neurons in fictive *Xenopus* preparations (McLean & Sillar, 2002) and it is conceivable to suggest that it may play a similar role in lamprey. Appropriate experiments could be carried out to verify this.

5.3 Addressing failed staining

Two out of four spinal cord samples processed for NADPH-d did not develop any blue staining, although the staining solution developed the characteristic pale purple colour in the incubator (indicating that the formazan-generating reaction was occurring). This could have been a fixation problem; my samples were not fixed in-house, but were delivered from Stockholm as 1cm long segments stored in phosphate buffer. These lengths of spinal cord were split into two halves for staining. Samples that stained came from the same piece of spinal cord, as did the samples that did not stain. This suggests that the samples may have been treated differently, with one piece possibly being fixed sub-optimally,

i.e. under-fixed, or fixing was delayed too long, which would allow enzymatic degradation (Werner et al., 2000).

5.4 Other considerations

NADPH-d and nNOS immunohistochemistry provide some interesting information suggesting the presence of NO in the lamprey spinal cord. The expression of NOS in a variety of cells involved in motor control could imply that NO provides a degree of modulation at every 'node' of the circuit, possibly allowing for more behavioural flexibility. The expression of NOS in various sensory cells suggests that NOS may play a role in integrating sensory input to the spinal cord, which is essential to the animal's survival. Furthermore, the presence of NOS in motor neurons suggests that NO may also act to regulate the output of the motor circuitry. Double labelling experiments would help elucidate what neurotransmitters NO is co-expressed with, in each cell type. Altogether, the presence of NOS in various cells involved in the generation of locomotion suggests a role in the integration of activity in these circuits; activity-dependent increases in NOS activity could release NO as a 'control switch' to permit the system's rapid response to changing motor demands. Therefore, the proposed role of NO as a modulator of locomotor rhythms in *Xenopus* may also be the case in the river lamprey, a phylogenetically older vertebrate. Histochemical studies have in fact suggested a biological signalling role for neuronal NO across major phylogenetic boundaries including annelids and arthropods (Eloffson et al., 1993) implying that the gene for neuronal NOS appeared very early on in evolution. While my results cannot provide evidence for the physiological role of NO in the lamprey nervous system, the patterns of labelling observed with both NADPH-d histochemistry and nNOS immunohistochemistry suggest that a complex NO signalling system and NO-sensitivity are present even in early vertebrates and may have been conserved for the purpose of behavioural flexibility and functional adaptation. That NOS appears in various cells related to the generation of rhythmic locomotion and the integration of sensory input, substantiates the notion that NO plays an important modulatory role in vertebrate motor control.

6 Concluding remarks

This investigation set out to explore the role of NOS and NO on motor control, using techniques of immunohistochemistry and organotypic culture, which provided anatomical data for interpretation and discussion. Many of my experiments involved organotypic cultures of *Xenopus* CNS. The appearance of NOS in spinal neurons during metamorphosis suggests that the expression of the enzyme is triggered by thyroid hormones. In fact, *in vitro* application of thyroxine and triiodothyronine to CNSs triggered the induction of NOS-expression in the spinal cord. Subsequently, it was discovered that the presence of exogenous NO could trigger the differentiation of secondary motor neurons *in vitro,* providing evidence for a maturational role of NO in the development of secondary motor systems in the spinal cord. Research supports an anti-proliferative and pro-differentiation role for NO in the development of the nervous system, and the spatiotemporal specificity of NOS expression in metamorphosing *Xenopus* CNS indicates that NO's influence in the assembly of spinal circuitry may be strictly regulated by hormonal influences during development. I suggest that the exclusion of NOS from the limb regions of the spinal cord may serve to allow proliferation of secondary motor neurons dedicated to the limbs, whereas its appearance in the 'interlimb' and tail regions of the spinal cord could signal secondary motor neurons of the medial motor column (innervating more mature axial musculature) to halt proliferation and induce them to differentiate. Thus, the decline in spinal NOS expression reported after metamorphic climax could serve to limit the time during which NO can exert its developmental influence on the motor circuitry.

NO has been implicated in many aspects of cell development, including proliferation, differentiation, and apoptosis. It is an enigmatic molecule and at times, research into it appears to provide contradictory findings. It has been associated with various aspects of cellular function and dysfunction, and perhaps it is precisely this dual nature that attracts so much scientific interest in NO. This investigation has demonstrated a positive regulatory role for NO on the maturation and reconfiguration of spinal motor circuitry. While my research cannot provide information on the

mechanisms by which NO exerts its maturational effects on motor neurons, it does provide an opportunity to ask important questions. Is NO necessary and sufficient to elicit specific forms of neural plasticity? Does this developmental effect apply to other vertebrates? Does the role of NOS change after motor circuitry has undergone structural and functional reconfiguration? My work on the lamprey provides evidence that neuronal NO signalling was present early on in vertebrate evolution and that one of its roles may be to exert modulatory control over the locomotor rhythms generated by spinal CPGs. Motor control is an essential aspect of an animal's response to its environment. The identification of a developmental role for NO/NOS in the spinal cord of *Xenopus*, supplemented by data that suggest a possible modulatory role on rhythmic locomotion in lamprey, provides evidence that NO exerts both chronic and acute effects on locomotor rhythm generation and synaptic plasticity, firmly placing NO as an important regulator of vertebrate motor control.

Appendix 1

HEPES saline

For 1litre: 6.72g NaCl 0.186g KCl 0.2g $NaHCO_3$ 2.38g HEPES

Dissolved in 1l distilled water, then buffered to pH 7.4

Frog Ringer's solution

For each 100ml HEPES saline, add: 1.0ml 0.1M MgCl2 0.2ml 1.0M CaCl2

0.1M Phosphate Buffer

PB was made up in batches of 5l and stored in a clean aspirator at room temperature

To 5l distilled water, add:

6.554g sodium dihydrogen orthophosphate

28.74g di-sodium hydrogen orthophosphate (anhydrous)

Stir until completely dissolved and pH to 7.4

4% Paraformaldehyde fixative

For 100ml: 4g paraformaldehyde (Analar) is dissolved in 36ml distilled water by gently heating on a magnetic stirrer/hotplate in a fume hood. 1M NaOH is added dropwise to fully dissolve, making sure the solution does not heat above 56°C. The paraformaldehyde solution is then filtered into a 100ml measuring cylinder containing 36ml PB, and made up to 100ml with distilled water. Fixative made this way is stored at 4°C, pHed to 7.4 when cool, and made up fresh daily.

Nitroblue Tetrazolium (NBT) solution

5mg powdered NBT is dissolved in 0.5ml 0.3% PBTX. This is kept at 4°C and can be used for up to 3 days.

NADPH-d staining solution

For 5ml: dissolve 5mg NADPH in 4.95ml 0.3% PBTX and add 50µl NBT solution. Filter to avoid precipitate and use within 15 minutes. Staining solution is always made up fresh as required.

0.3% Triton-X100 in PB (PBTX)

1ml Triton-X100 is added to 9ml distilled H20 to make up a 10% solution. 1.5ml of this solution is added to 48.5ml PB to make up 0.3% PBTX which is used for nNOS immunohistochemistry as well as for the NADPH-d staining solution.

Incubating medium for nNOS immunohistochemistryTo 0.3%PBTX add:

5% normal goat serum (Jackson Immuno)

3% bovine serum albumin

antibody at required concentration

Carnoy's fixative

60% ethanol (analar)

30% chloroform

10% acetic acid

Phosphate Buffered Saline (PBS)

Dissolve 0.9g NaCl for each 100ml PB. pH to 7.4 and store at 4C

0.1% Triton-X100 in PBS (PBSTX)

1ml Triton-X100 is added to 9ml distilled H20 to make up a 10% solution. 0.5ml of this solution is added to 49.5ml PBS to make up 0.1% PBSTX which is used for diluent for ChAT immunohistochemistry.

Incubating medium for ChAT immunohistochemistry

To 0.1% PBSTX add:

1% horse serum

Antibody at required concentration

Organotypic culture medium

To each litre of Ringer's solution add:

10ml penicillin-streptomycin solution (Invitrogen)

1.0g D-glucose

Thyroxine (T4) stock solution

1mM stock solution made up by adding 3.885mg L-thyroxine to 5ml HEPES saline and adding a drop of 1M NaOH to fully dissolve. This was added as needed to the culture medium to make a working solution. For a concentration of :

50nM, add 5µl per 100ml

500nM, add 50µl per 100ml

1µM, add 100µl per 100ml

Triiodothyronine (T3) stock solution

1mM stock solution made up by dissolving 3.255mg triiodothyronine in 5ml 1M NaOH. For a working concentration of 1µm, add 100µm per 100ml culture medium.

SNAP stock solution

1mM stock solution was made up by dissolving 22mg SNAP in 1ml DMSO. This was aliquoted out and frozen for up to a week, and added to culture medium to make a working concentration of 200µM.

L-NAME stock solution

26.9mg of L-NAME were dissolved in 1ml distilled water to make a 1mM stock solution. This was added to the culture medium to make a working concentration of 200µM. DMSO was also added to the culture medium for a concentration of 0.2%.

Unless otherwise specified in the materials and methods section, all reagents used were purchased from Sigma-Aldrich.

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