Functional roles of nitric oxide signalling during zebrafish nervous system development

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Abstract

Nitric oxide (NO) is a highly diffusible signalling molecule that serves a wide range of physiological functions. During development, biosynthetic enzymes for NO are often expressed in nascent neurons, although little is known of how this molecule regulates *in vivo* nervous system development. This thesis aims to address this problem by examining the functional roles of NO signalling in the zebrafish embryo, focusing specifically on nitrergic regulation of spinal locomotor network assembly.

The first aim of this study is to characterise the spatiotemporal distribution of NO synthase 1 (NOS1), the enzyme responsible for NO biosynthesis in the zebrafish nervous system. NOS1 transcript and protein was observed in discrete regions of the brain as well as a distinct class of spinal interneuron from early stages of embryonic development.

The second aim was to examine functional roles of NO signalling during *in vivo* spinal cord development. Using molecular antisense and pharmacological approaches, NO levels were disrupted during early life and the consequences to spinal circuit maturation assessed. NO was found to specifically regulate the growth of spinal motoneurons that innervate axial trunk muscles. Abrogation of NO signalling dramatically increased the number of motor axon branches formed within the muscle across the first three days of life whilst exogenous elevation of NO levels had the opposite effect.

The third aim was to determine downstream signalling pathways underpinning NOs effects. Pharmacological studies revealed that NO regulates motoneuron branching through the cyclic guanosine monophosphate pathway and subsequent analysis revealed that this pathway can modify neuromuscular synapse density, with high NO levels suppressing synaptogenesis and retarding locomotor maturation and low NO levels having the converse effect.

In summary, the work presented in this thesis identifies a novel and important role for NO signalling, demonstrating that it functions to sculpt neuromuscular synapse assembly and modify locomotor maturation.

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List of abbreviations

Acetylcholine (ACh)

Acetylcholine receptors (AChRs)

Adenosine diphosphate ribosyl cyclase (ADP-ribosyl cyclase)

Alkaline phosphatase (AP)

Alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA)

Amacrine cells (AC)

Amyotrophic lateral sclerosis (ALS)

Anterior (A)

Antisense morpholino oligonucleotide (AMO)

Brain derived neurotrophic factor (BDNF)

Branches per fascicle (bpf)

Ca^{2+}/CaM-dependent protein kinase (CaMK)

Ca^{2+}-induced Ca^{2+} release (CICR)

Calmodulin (CaM)

Carboxy-PTIO, potassium salt (c-PTIO)

Carboxy-terminal PDZ ligand of NOS1 (CAPON)

Caudal primary motoneuron (CaP)

Central nervous system (CNS)

Cerebellar plate (CeP)

$CH_3N[N(O)NO]^-(CH_2)_3NH_3^+$ (NOC13)

Circumferential ascending (CiA)

Circumferential descending (CiD)

Commissural bifurcating longitudinal (CoBL)

Commissural local (CoLo)

Commissural primary ascending (CoPA)

Commissural secondary ascending (CoSA)

Cyclic adenosine diphosphate ribose (cADPR)

Cyclic guanosine monophosphate (cGMP)

Cyclic nucleotide-gated (CNG)

Diethylenetriamine/nitric oxide adduct (DETA NO)

Digoxygenin (DIG)

Dinitrogen trioxide (N_2O_3)

Dorsal (D)

Dorsal longitudinal ascending (DoLA)

Dorsal longitudinal fasciculus (DLF)

Dorsal root ganglion (DRG)

Dorsorostral cell cluster (DRC)

Embryonic day (E)

Embryonic red (ER)

Embryonic white (EW)

Endothelium-derived relaxing factor (EDRF)

Ethyl 3-aminobenzoate methanesulfonic acid (MS-222)

Extracellular matrix (ECM)

Flavin adenine dinucleotide (FAD)

Flavin mononucleotide (FMN)

Focal laser induced photolysis (FLIP)

Glial cell line-derived neurotrophic factor (GDNF)

Guanosine-5’-triphosphate (GTP)

Horizontal myoseptum (hs)

Hour (hr)

Hours post fertilisation (hpf)

Hydrogen peroxide (H_2O_2)

Hydroxyl radical ($OH^{\cdot}$)

Hypothalamus (Hy)

Inhibitory postsynaptic potentials (IPSPs)

Inner plexiform layer (IPL)

Ipsilateral caudal (IC)

Kolmer-Agdur (KA)

Lateral geniculate nucleus (LGN)

Long term depression (LTD)

Long term potentiation (LTP)

lookup table (LUT)

Medial longitudinal fascicle (MLF)

Medulla oblongata (MO)

Membrane associated guanylate kinase (MAGUK)

Micrometre (µm)

Micromolar (µM)

Microtubule-associated protein (MAP)

Middle primary motoneuron (MiP)

Minute (min)

Multipolar commissural descending (MCoD)

Muscle-specific kinase (MuSK)

Nanomolar (nM)

Nerve growth factor (NGF)

Neural cell adhesion molecule (NCAM)

Neurolin (DM-GRASP)

Neuromuscular junction (NMJ)

N^G-monomethyl-L-arginine (L-NMMA)

Nicotinamide adenine dinucleotide phosphate (NADPH)

Nitrate ion (NO_2^-)

Nitric oxide (NO)

Nitric oxide synthase (NOS)

Nitro blue tetrazolium (NBT)

Nitrogen dioxide (NO_2)

Nitrosonium ion (NO^+)

N-methyl D-aspartate receptor (NMDAR)

NOS1-interacting DHHC domain-containing protein with dendritic mRNA" (NIDD)

Notochord (Nc)

N_ω-nitro-L-arginine (L-NOARG)

N_ω-Nitro-L-arginine methyl ester hydrochloride (L-NAME)

Olfactory placode (OP)

Pallium (P)

Para-(chlorophenylthio)-guanosine 3', 5' cyclic monophosphate (pCPT-cGMP)

Paraformaldehyde (PFA)

PDZ domain (post synaptic density protein-95, discs large, ZO-1 homology domain)

Peripheral nervous system (PNS)

Peroxynitrite ($ONOO^-$)

Phosphate buffered saline (PBS)

Phosphate buffered saline with TritonX (PBSTX)

Post synaptic density (PSD)

Posterior (P)

Posterior tuberculum (PT)

Post-optic commissure (POC)

Pre-optic region (PO)

Protein kinase A (PKA)

Protein kinase G (PKG)

Reactive oxygen species (ROS)

Revolutions per min (rpm)

Rhodamine-conjugated α-bungarotoxin (Rh-α-BTX)

Room temperature (RT)

Rostral primary motoneuron (RoP)

Ryanodine receptors (RyR)

Seconds (s)

Secondary motoneuron (smn)

S-nitroso-*N*-acetyl-D, L-penicillamine (SNAP)

Sodium nitroprusside (SNP)

Soluble guanylyl cyclase (sGC)

Spinal cord (SC)

Standard error of the mean (S.E.M)

Subpallium (SP)

Tegmentum (Tg)

Thalamic (Th)

Thrombospondin repeat (TSR)

Unipolar commissural descending (UCoD)

Variable primary motoneuron (VaP)

Ventral (V)

Ventral longitudinal descending (VeLD)

Ventral medial (VeMe)

Ventral nitrergic (VeNi)

Ventral serotonergic (VeSe)

Ventrocaudal cell cluster (VCC)

Ventrorostral cell cluster (VRC)

Z)-1-[2-(2-aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA-NONOate))

α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (NMDA)

(6R)-5,6,7,8-tetrahydrobiopterin (BH_4)

$(CH_3N[N(O)NO]^-(CH_2)_3NH_3^+$ (NOC13)

(*Z*)-1-[2-(2-aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate DETA NONOate

1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ)

2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO)

3-(2-hydroxy-1-methyl-2-nitrosohydazino)-*N*-methyl-1-propanamine (NOC-7)

3', 5'- cyclic adenosine monophosphate (cAMP)

3-bromo-7-nitroindazole (3B-7-NI)

3-morpholinosydnonimine (SIN-1)

5-Methyl-10, 11-dihydro-5H-dibenzo [a, d] cyclohepten-5, 10-imine (MK-801)

6-anilino-5, 8-quinolinequinone (LY83583)

7-nitroindazole (7-NI)

8-(4-Chlorophenylthio)-guanosine 3', 5'-cyclic monophosphate sodium salt (cGMP)

8-bromo-cyclic adenosine diphosphate ribose (8-Bromo-cADPR)

8-bromo-guanosine 3', 5' cyclic monophosphate (8-Br-cGMP)

9, 21-dehydro (Ry)

Table of Contents

List of figures and tables

Figures

Tables

1. Introduction

The principal aim of this thesis is to delineate the roles of nitric oxide (NO) during zebrafish spinal cord development. Specifically this thesis aims to determine the ontogeny and distribution of neuronal NO synthesising machinery in the zebrafish trunk, the consequences of disrupting NO signalling during motoneuron development and maturation of motor behaviour, and the downstream signalling pathways underpinning NO effects. This introduction will be broken down in to three main sections. The first will provide a brief introduction to NO, outlining its synthesis and physiological roles, particularly within nervous tissue. The second section will introduce the zebrafish as a model for neurodevelopmental studies, detailing key steps during zebrafish spinal cord development, with a specific focus on the generation and growth of spinal motoneurons. Finally, a brief overview of the experimental aims of this thesis will follow.

1.1 Introduction to nitric oxide

Nitric oxide (NO) is a small, simple diatomic molecule that is both highly reactive and freely diffusible. Although NO is now widely recognised as a key signalling molecule in wide a range of biological tissues, no less than 40 years ago it was regarded as nothing more than an environmental pollutant. The discovery that NO had important roles in biological tissue began in the 1970s when Ferid Murad and colleagues were exploring the pharmacological actions of nitroglycerine, a potent vasodilator that had been used to treat angina since the late 1870s (for review, see Marsh and Marsh, 2000). Murad, who was interested in elucidating how nitroglycerine

affected vascular tone, had found that nitro- and nitroso-containing compounds triggered vasodilation by increasing levels of biological messenger cyclic guanosine-5'-monophosphate (cGMP) in the vascular endothelium (Katsuki et al., 1977). This response appeared to require the liberation of NO which in some way acted to modify cGMP levels. This subsequently led Murad to propose in 1977 that NO may act as an endogenous biological signalling molecule, although there was no experimental evidence at the time to support this hypothesis (Arnold et al., 1977).

Working independently of Murad, another group led by Robert F. Furchgott, was attempting to identify the endogenous signalling pathways underpinning relaxation of the vascular endothelium. Furchgott and colleagues had demonstrated that acetylcholine (ACh) caused dilation of the rabbit thoracic aorta only if intact vascular endothelial cells were present (Furchgott and Zawadzki, 1980). These cells appeared to relax smooth muscle by releasing an as yet unidentified endogenous and diffusible factor, which Furchgott coined "endothelium-derived relaxing factor" (EDRF; Furchgott and Zawadzki, 1980, Cherry et al., 1982). The identity of EDRF became a focus of research laboratories across the world wishing to understand how endogenous signalling controls blood vessel tone and how this might be manipulated in cardiovascular diseases. By 1987, investigations into the nature of EDRF, led principally by Louis Ignarro's research group, had generated some curious observations that suggested a role for labile nitrso compounds in mediating EDRF signalling responses. First, it was demonstrated that EDRF, like nitroglycerin, acted via cGMP (Gruetter et al., 1979). Second, it was found that not only did inorganic nitrite mimic

the EDRF response but haemoglobin, which binds nitrso compounds, inhibited it (Stewart et al., 1987, Ohlstein et al., 1979, Gruetter et al., 1979).

A common thread was emerging between Murad's investigations into the pharmacological actions of nitro-based vasodilators and the biological actions of the endogenous vasodilator EDRF. Both appeared to act in an identical manner, through liberation of a reactive nitroso-based chemical that activated cGMP synthesis. Subsequently, in 1986, Furchgott and Ignarro attended the same conference in Rochester, Minnesota where they independently proposed that EDRF was in fact the free radical gas NO. This led to a flurry of research which culminated in the demonstration by Salvador Moncada's group that NO was generated by vascular endothelial cells: using chemiluminescent methods previously designed for detecting nitrate levels in food, Moncada showed that bradykinin caused release of small quantities of NO from the endothelium (Palmer et al., 1987). This was quickly followed by subsequent publications from Ignarro's group, which confirmed that the properties of EDRF were identical to NO (Ignarro et al., 1987b, Ignarro et al., 1987a). As a consequence of these discoveries, interest in the biological functions of NO grew tremendously and shortly thereafter, it was discovered that NO signalling had physiologically relevant effects on a wide range of tissues and organs (De Sarro et al., 1991, Shibuki, 1993, Garthwaite, 1991, Solomonson, 1991, East and Garthwaite, 1991; Liew and Cox, 1991; Snyder and Bredt, 1991).

Although it rapidly became accepted that NO was an endogenous biological signal, one key problem remained: evidence for an endogenous synthesis mechanism was lacking. The search for an NO synthetic enzyme began. The first lead came from

the discovery by Moncada and colleagues showing that when radioactively labelled L-arginine was added to vascular endothelial cells, radioactive NO was liberated (Palmer et al., 1988b, Palmer et al., 1988a); see also (Schmidt et al., 1988, Sakuma et al., 1988, Marletta et al., 1988). Thus, by the late 1980s researchers now had a substrate but they lacked knowledge of the enzyme that catalysed L-arginine's conversion to NO. Soon after, two key co-factors of this elusive enzyme were inferred: Ca^{2+} ions (Knowles et al., 1989) and nicotinamide adenine dinucleotide phosphate (NADPH; Forstermann et al., 1990) were essential for biological NO synthesis. By the beginning of the 1990s Bredt and Snyder isolated and purified an NADPH, Ca^{2+}-calmodulin dependent enzyme that synthesised NO from L-arginine which they termed NO synthase (NOS, Bredt and Snyder, 1990). This provided the first direct evidence that biological tissues contained synthetic machinery for NO.

The first NOS gene was cloned in 1991 (Bredt et al., 1991b) and expression analysis indicated that it encoded a central nervous system (CNS) specific NOS isoform which was termed "brain NOS", although it has since been renamed to "neuronal NOS" (nNOS, NOS1). This was shortly followed by the cloning of two other NOS isozymes: inducible NOS (iNOS, NOS2; Lyons et al., 1992; Xie et al., 1992) and endothelial NOS (eNOS, NOS3; Lamas et al., 1992), the latter being the isoform expressed in vascular endothelial tissue.

In as little as 15 years, NO had grown from being known as a mere environmental pollutant to a fundamentally important signalling molecule, so much so that in 1998 Ferid Murad, Robert Furchgott and Louis Ignarro were awarded the Nobel Prize in Physiology or Medicine for their pioneering work, whilst NO itself was awarded

the prestigious “molecule of the year” award by Science magazine in 1992. The impact this small molecule has had on the scientific community is reflected by the sheer volume of research articles on its biological function. A PubMed search reveals that, at the time of writing, over 108,000 articles have been published on NO (http://www.ncbi.nlm.nih.gov/pubmed; accessed 15/03/2011), with 107,000 of these subsequent to the 1986 Rochester meeting at which NO was first proposed to be a biological messenger.

Since these pioneering discoveries, NO has become a major focus of bioscience research and this small signalling molecule has now been implicated in a wide range of physiological processes that include vasodilation (Chen et al., 2008b, Gao, 2010); immunity (Tripathi et al., 2007, Coleman, 2001, Guzik et al., 2003, Moilanen and Vapaatalo, 1995); inflammation (Coleman, 2001, Tripathi et al., 2007, Swindle and Metcalfe, 2007, Wallace, 2005); nociception (Miclescu and Gordh, 2009, Luo and Cizkova, 2000, Schmidtko et al., 2009); apoptosis (Brown, Leon et al., 2008, Mannick, 2007); neuroprotection (Calabrese et al., 2007, Contestabile et al., 2003, Contestabile and Ciani, 2004); neurotransmission (Garthwaite, 2008, Vincent, 2010); nervous system development (Bicker, 2007, Contestabile, 2000); neurodegeneration (Chung, 2006, Boje, 2004, Molina et al., 1998) and ageing (Domek-Lopacinska and Strosznajder, 2010, Afanas'ev, 2009).

1.1.2 Isoforms of NOS

In mammals there are three members of the NOS family: neuronal NOS (nNOS; NOS1), endothelial NOS (eNOS; NOS3) and inducible NOS (iNOS; NOS2). NOS1 and NOS3 are Ca^{2+}-calmodulin (CaM) dependent enzymes constitutively expressed in a

variety of tissues and generate relatively small (nM) amounts of NO, whereas NOS2 is Ca^{2+} independent and generates high (μM) concentrations of NO in response to inflammatory factors and other physiological stressors (for review, see Guix et al., 2005). The following sections outline the general structural elements and catalytic activity of this family of enzymes.

1.1.2.1 NO synthesis from NOS isozymes

As a free radical with a short half-life (< 5s), NO cannot be stored in its free form but is rather synthesised on demand by the NOS enzymes. These catalyse the conversion of L-arginine to NO and L-citrulline in a five electron oxidation process requiring NADPH and O_2 (for reviews, see Daff, 2010, Alderton et al., 2001, Dudzinski et al., 2006). The synthesis of NO requires two successive monooxygenase reactions: in the first, one molecule of NADPH donates electrons to molecular oxygen which then oxidises the guanidine nitrogen terminal of bound L-arginine to release one molecule of water, thus converting L-arginine to N^{ω}-OH-L-arginine. In the second reaction, a further electron is contributed by NADPH and N^{ω}-OH-L-arginine undergoes a three electron oxidation to result in the formation of both NO and L-citrulline (see figure 1.1; for review, see Griffith and Stuehr, 1995). In addition all NOS isoforms can generate the free radical superoxide anion ($O_{2}^{\cdot -}$) from the reductase domain, especially in the absence of (6R)-5, 6, 7, 8-tetrahydrobiopterin (BH_4) and under low L-arginine concentrations, as this increases O_2 reduction to O_2^- (Xia et al., 1996, Bruckdorfer, 2005, Pou et al., 1999, Vasquez-Vivar et al., 1998).

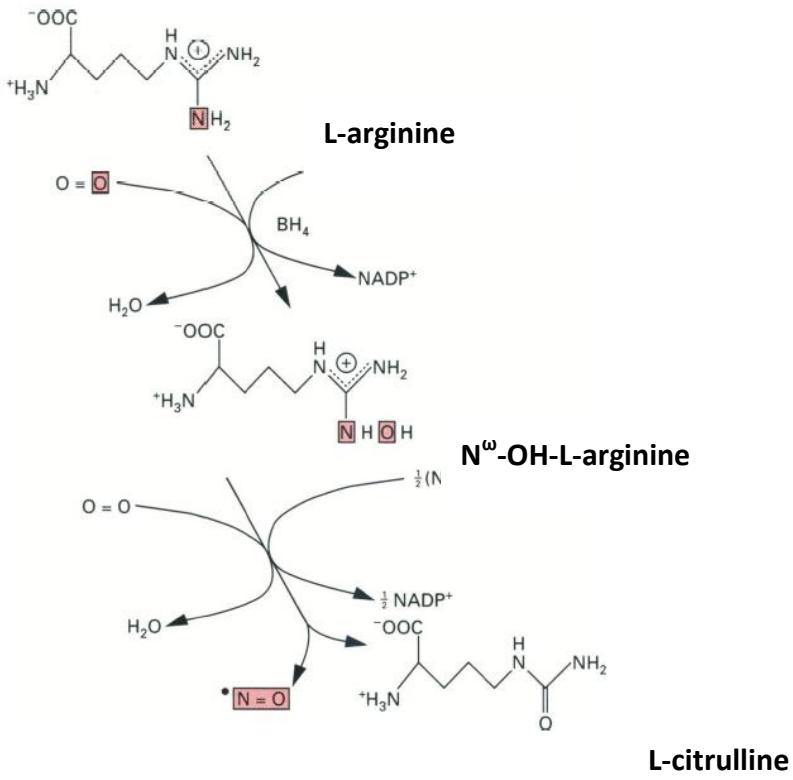

Figure 1.1: The synthesis of NO
A flow diagram outlining the reaction steps during the conversion of L-arginine to NO and L-citrulline. Red boxes indicate nitrogen and oxygen atoms used during formation of NO. (Knowles and Moncada, 1994).

1.1.2.2 Common structural features of NOS

The three mammalian NOS enzymes contain several common elements (summarised in figure 1.2). Each NOS enzyme is homodimeric, containing an N-terminal oxygenase domain and a C-terminal reductase domain. The oxygenase domain, which binds the substrate L-arginine, contains a BH_4 binding site and a cytochrome P-450-type haem active site. Common to their structure is also a CaM binding site that is linked to the

reductase-carboxyl domain. Ca^{2+}-CaM binding to this domain is essential for election transport across to L-arginine (for review, see Ghosh and Salerno, 2003, Alderton et al., 2001). In constitutively active NOS isoforms (NOS1, NOS3), elevated intracellular Ca^{2+} levels are required for CaM binding whereas, in the inducible isoform (NOS2), basal levels of Ca^{2+} facilitate tight binding of CaM. The reductase domain binds NADPH and also possesses binding sites for flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). Electrons pass from NADPH through FAD and FMN to reach the haem containing oxygenase domain where they can then convert L-arginine to L-citrulline and NO (see figure 1.3; for review, see Zhou and Zhu, 2009).

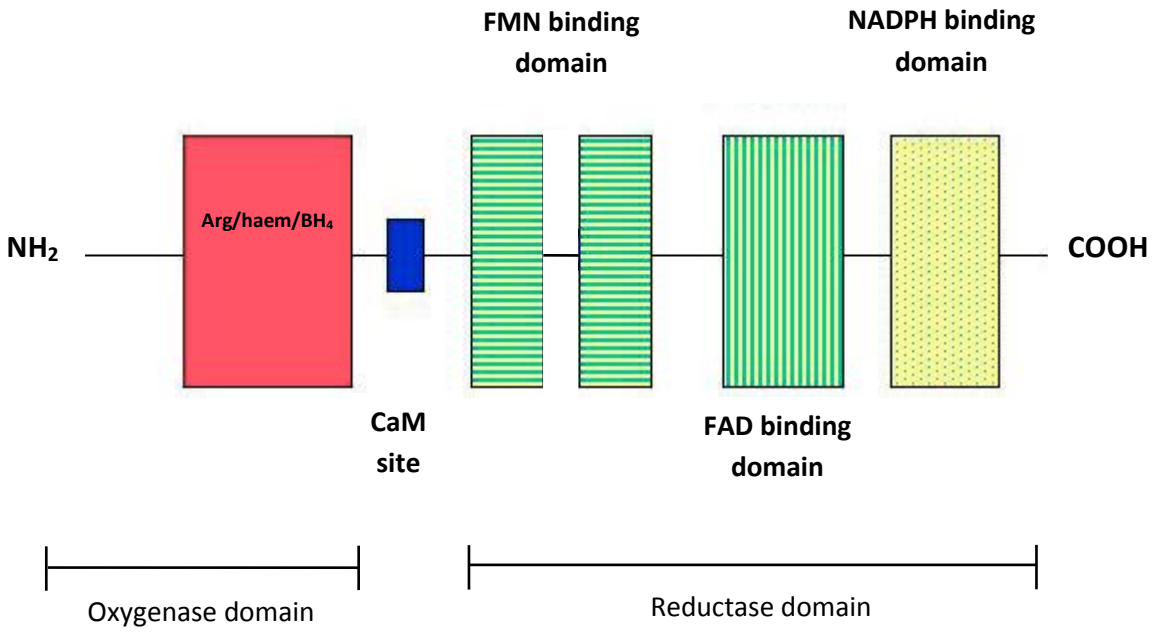

Figure 1.2: General structure of NOS
Schematic diagram of the common structural features of NOS isozymes. NOS monomers comprise an oxygenase and reductase domain. The oxygenase domain possesses binding sites for L-arginine (Arg), haem and (6R)-5, 6, 7, 8-tetrahydrobiopterin (BH_4). The reductase domain has binding domains for flavin mononucleotide (FMN); flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide phosphate (NADPH). The two domains are bridged by a calmodulin (CaM) binding site. (Ghosh and Salerno, 2003).

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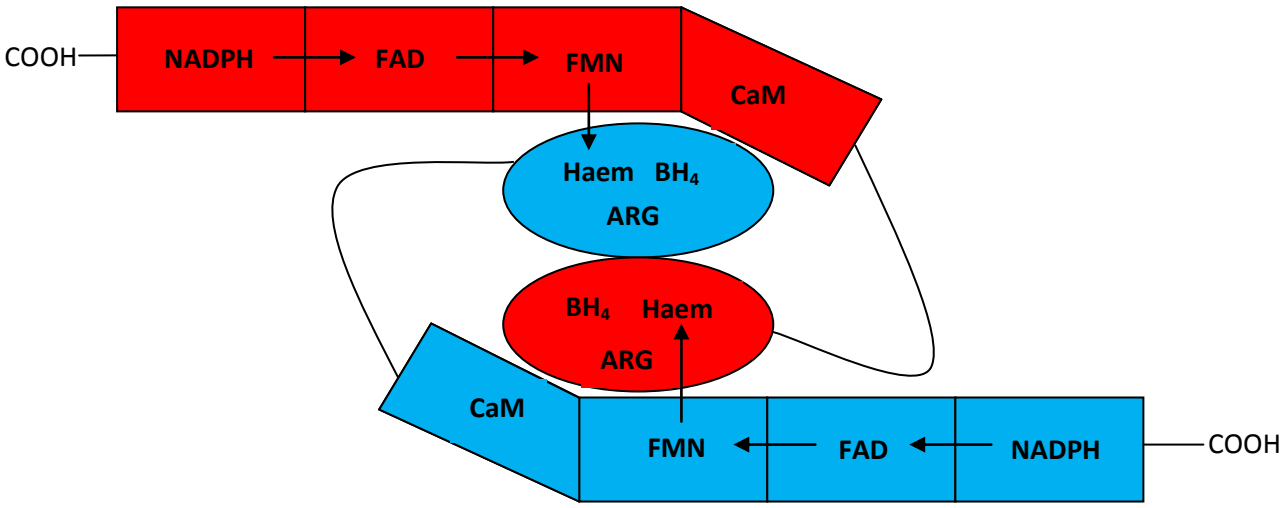

Figure 1.3: Electron flow through the NOS dimer during NO synthesis
During NO synthesis electrons donated from nicotinamide adenine dinucleotide phosphate (NADPH) flow through the flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) domains to the haem-containing oxygenase domain of the other monomer to synthesise NO from L-arginine (Arg). (Siddhanta et al., 1998).

1.1.2.3 NOS dimerisation

NOS activity requires homodimeric interaction between the two oxygenase domains to form the stable binding sites for BH_4, haem and L-arginine and for facilitating electron flow for NO synthesis (for review, see Zhou and Zhu, 2009, Alderton et al., 2001). In brief, NOS dimerisation is achieved through either formation of disulphide bridges or ligation of zinc ions between cysteine residues within the monomers (Alderton et al., 2001). Dimers are further stabilised by 'N-terminal hook' domains which interact with one another (Crane et al., 1999). The reductase domains

are also believed to contribute to dimer stabilisation though the precise role in this process is not yet known (Garcin et al., 2004).

1.1.3 A brief overview of the NOS isozymes

Whilst the three NOS isozymes contain several common elements that facilitate catalysis of NO, they also have key structural and functional differences which give them unique properties essential to their physiological function. For each enzyme, their features, and functional importance, are outlined below.

1.1.3.1 Neuronal NOS (nNOS) or NOS1

In vertebrates, although NOS1 is the predominant isoform expressed in the CNS (Bredt et al., 1991a, Bredt and Snyder, 1992, Dawson et al., 1992, Lowenstein and Snyder, 1992, Snyder, 1992), it is also found in cells of the peripheral nervous system (PNS; Dawson et al., 1991, Grozdanovic et al., 1992, Ceccatelli et al., 1994, Kirchgessner et al., 1994, Olsson et al., 2008, Holmqvist et al., 2004, Rao et al., 2008, Beck et al., 2009) and in skeletal muscle (Stamler and Meissner, 2001, Grozdanovic and Baumgarten, 1999). NOS1 is constitutively present, although its activity is dependent upon elevated levels of intracellular Ca^{2+} which stimulate the binding of the cofactor CaM to NOS1, thus linking NO synthesis to neuronal activity (Garthwaite et al., 1989, Garthwaite, 1991).

In mammals, four splice variants, NOS1α, NOS1β, NOS1γ and NOS1μ (see figure 1.4; Brenman et al., 1997) exist. In the CNS, NOS1α is by far the most predominant isoform whereas NOS1μ is expressed in fast skeletal muscle fibers. These isoforms possess an amino terminal post synaptic density protein-95, discs large, ZO-1 homology domain (PDZ domain) that anchors them to postsynaptic (NOS1α) or muscle dystrophin (NOS1μ) complexes. In contrast NOSβ and NOSγ lack this structure and are therefore localised to the cytoplasm in discrete regions of the CNS (Brenman et al., 1996a). These enzymes constitute only a small fraction of CNS NOS1 expression. Additionally NOSγ has only 3% of the catalytic activity that other NOS1 isoforms have (Brenman et al., 1996a). The physiological functions of NOS1 will be discussed in detail in section 1.1.6 below.

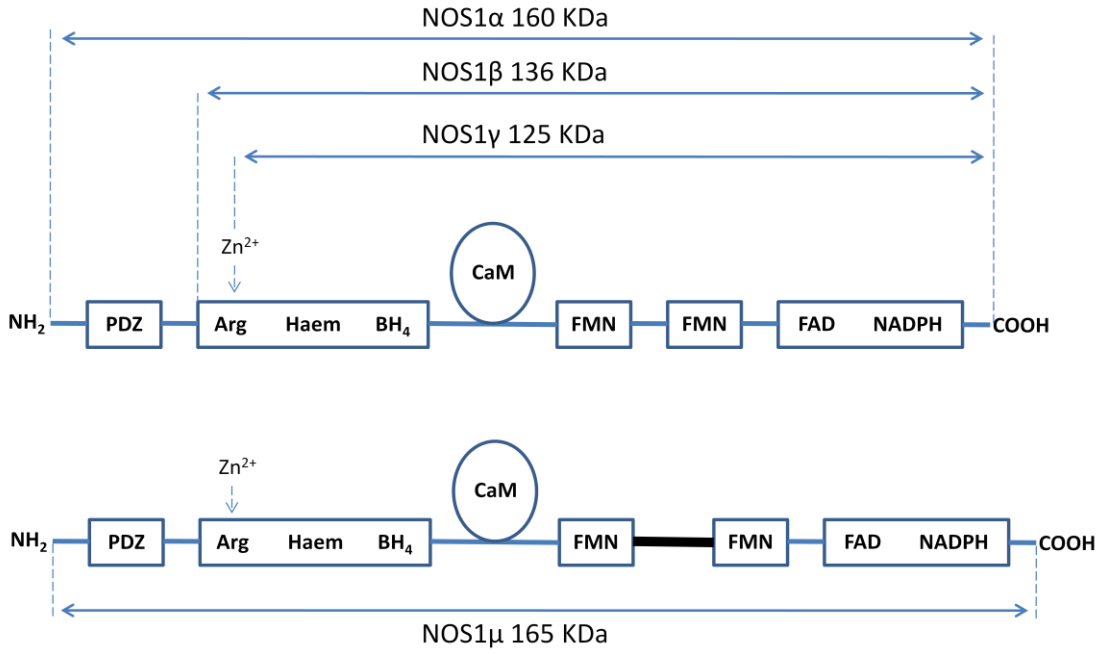

Figure 1.4 Splice variants of NOS1
Schematic of the four splice isoforms of mammalian NOS1: NOS1β: NOS1γ: NOS1μ and NOS1α. NOS1α is the common CNS isoform. NOS1α is identical but contains a 102bp insert (think line) between exons 16 and 17 whilst NOS1β and NOS1γ lack PDZ domain. (Guix et al., 2005).

1.1.3.2 Inducible NOS (iNOS) or NOS2

Mammalian NOS2 has a molecular mass of approximately 130kDa and shares around a 50% homology with the other NOS isoforms (Knowles and Moncada, 1994). NOS2 is unique among the NO synthesising enzymes in that it is the only isoform that is not constitutively expressed. Rather NOS2 expression is induced in response to physiological stressors such as inflammation (see below; Aktan, 2004). This enzyme further differs from constitutive NOS isoforms in that it binds CaM with a remarkably high affinity, which results in permanent association of these two molecules (Stevens-Truss and Marletta, 1995). Thus, CaM is essentially permanently bound to NOS2 which, upon induction, allows it to continuously generate NO independent of cellular Ca^{2+} concentrations.

NOS2 expression is principally induced by microbial products, such as lipopolysaccharide and inflammatory cytokines (e.g. interleukin-1, tumour necrosis factor-α and interferon-γ; for review, see Korhonen et al., 2005). Once synthesised, this enzyme produces high, steady state concentrations of NO until it is degraded (Coleman, 2001). This differs markedly from other NOS isozymes (NOS1 and NOS3) that rapidly produce NO in a "pulsatile" manner in response to Ca^{2+}/CaM binding (Guzik et al., 2003).

At least five splice isoforms of NOS2 have been detected in mammals (Eissa et al., 1996, Tiscornia et al., 2004). Although the functional consequences of this splicing have not yet been fully determined, it has been speculated at least one of the splice isoforms may be implicated in the downregulation of NO levels (Tiscornia et al., 2004).

Although NOS2 was first shown to be present in macrophages, it has since been identified in many activated cell types, including monocytes, eosinophils, hepatocytes, vascular smooth muscle cells, myocytes, fibroblasts and endothelial cells (for review, see Tripathi et al., 2007). Functionally NOS2 is believed to assist with host defence against infectious organisms such as; bacteria, viruses, parasites and fungi (Bogdan, 2001, Gao, 2010, Coleman, 2001) and also serves protective roles in pathophysiological conditions, such as cancer, where NOS2 can inhibit tumour cell proliferation, differentiation and metastatic spread as well as having cytotoxic effects on tumour cells (Lechner et al., 2005, Rieder et al., 2001). However, during chronic inflammation, prolonged activation of NOS2 contributes to pathogenesis of inflammatory conditions such as atherosclerosis (Lee et al., 2009, Upmacis, 2008), cancer (Muntane and la Mata, 2010, Vickers et al., 1999), sepsis (Fukuyama et al., 1997, Strand et al., 2000), myocardial infarction (Mihm et al., 2001, Chen et al., 2008a), diabetes (Holstad et al., 1997), Crohn's disease (Rachmilewitz et al., 1995, Guslandi, 1998), ulcerative colitis (Singer et al., 1996, Kimura et al., 1998), amyotrophic lateral sclerosis (ALS; Casoni et al., 2005, Sasaki et al., 2000), Parkinson's disease (Chinta and Andersen, 2010, Danielson et al., 2009) and Alzheimer's disease (Horiguchi et al., 2003, Williamson et al., 2002). In these conditions, persistently high concentrations of NO lead to generation of superoxide which give rise to reactive oxygen species that damage proteins, lipids, DNA and mitochondria. In addition, excessive S-nitrosylation of proteins has been shown to cause pathological changes in cellular respiration and survival (for review, see Tripathi et al., 2007).

1.1.3.3 Endothelial NOS (eNOS) or NOS3

The NOS3 isozyme is a constitutively expressed 135kD membrane-bound protein (Lamas et al., 1992) that, like NOS1, requires association with the Ca^{2+}/CaM complex to function. In mammals, there are at least three splice isoforms, eNOS13A, eNOS13B and eNOS13C (Lorenz et al., 2007) which are capable of forming heterodimers with the full length NOS3 to reduce its catalytic activity (Lorenz et al., 2007). NOS3 is predominantly expressed in vascular endothelial cells (Wu, 2002) where it localises to specific subcellular domains known as caveolae through two post-translational modifications: myristoylation of the N-terminal glycine and palmytoylation of cysteine residues 15 and 26 (for review, see Dudzinski and Michel, 2007, Shaul, 2002). These modifications construct three acyl groups that help anchor NOS3 to caveolin. This interaction leads to the tonic inhibition of NOS3 as caveolin occupies the CaM binding domain, therefore attenuating enzyme activity at basal Ca^{2+} levels (Dudzinski et al., 2006, Michel, 1999). However when intracellular Ca^{2+} levels rise, the Ca^{2+}CaM complex displaces caveolin and NOS3 becomes activated to generate small (nM) quantities of NO for the duration of the Ca^{2+} signal (Michel, 1999).

NOS3 has key roles in regulating cardiovascular physiology (for review, see Rubio and Morales-Segura, 2004), where it controls smooth muscle tone (Locals and Welch, 1995, Moncada and Higgs, 2006) as well as platelet aggregation and adhesion (Radomski et al., 1990b, Radomski et al., 1990a, Radomski et al., 1987, Bodzenta-Lukaszyk et al., 1994). Furthermore it is also known to control proliferation of vascular smooth muscle cells (Jeremy et al., 1999) and apoptosis of cardiomyocytes (Razavi et al., 2005).

1.1.4 The structure and function of the neuronal NOS isoform, NOS1

NOS1α constitutes the vast majority of NOS expressed in the CNS and its structure ensures that it localises close to N-Methyl-D-Aspartate receptors (NMDARs), a major family of Ca^{2+} conducting ligand gated glutamate receptors. Through this association, NOS1 activity is directly coupled to glutamate signalling, allowing NO to modify cellular and synaptic physiology in response to excitatory neurotransmission. This section will discuss NOS1 localisation at the postsynaptic site, its regulation by neurotransmission and some of the physiological functions that NOS1 has been shown to regulate.

1.1.4.1 NOS1 localisation

In mammals, the NOS1α transcript encodes a 1434 amino acid protein with a predicted molecular weight of 160.8 kDa (Hall et al., 1994). This enzyme possesses a 250 amino acid PDZ domain (Brenman et al., 1996a) at its N-terminus which permits interaction with membrane associated guanylate kinase (MAGUK)-family proteins such as post synaptic density proteins (PSD)-95 and PSD-93 (Brenman et al., 1996a, Brenman et al., 1996b). The PSD proteins also interact with the C-termini of NMDARs, anchoring these receptors at the synapse and bringing them into close association with NOS1 (Sattler et al., 1999, Brenman et al., 1996b, Christopherson et al., 1999). Thus, local Ca^{2+} entry through stimulated NMDARs results in Ca^{2+}-CaM association and activation of NOS1 proteins that are tethered under the synapse. This allows NOS1 to modulate cellular physiology in response to excitatory glutamatergic signalling (for review, see Contestabile, 2000).

NOS1 association with the postsynaptic scaffold is regulated by several adaptor proteins such as the transmembrane protein "NOS1-interacting DHHC domain-containing protein with dendritic mRNA" (NIDD) which targets NOS1 to the postsynaptic domain, through binding of the NOS1 PDZ domain (Saitoh et al., 2004). Alternatively, proteins such as carboxy-terminal PDZ ligand of NOS1 (CAPON; Jaffrey et al., 1998) can induce dissociation of NOS1 from the postsynaptic domain by competing for the NOS1 PDZ binding site. In this way CAPON proteins can uncouple NOS1 from NMDAR-mediated activation (Jaffrey et al., 1998) and this may act to prevent NOS1 overactivation in pathophysiological conditions. In support of this premise, elevated levels of CAPON have been demonstrated after spinal injury (Cui et al. 2011, Chen et al., 2008c, Shen et al., 2008, Cheng et al., 2008) which presumably helps prevent synaptically driven NOS1 neurotoxicity and aids the recovery process (Cheng et al., 2008).

CAPON also interacts with Dexras1, a brain-enriched G protein of the Ras family that plays important roles in controlling the circadian rhythm (Cheng et al., 2006). Dexras1 binds to the N terminus of the CAPON/NOS1 complex, thus providing Dextras1 with a direct delivery of NO which is used to activate this enzyme through S-nitrosylation (see section 1.1.5.1.i; Fang et al., 2000), although recent evidence suggests that CAPON/NOS1 complexing is not critical for Dexras1 activity (Cheah et al., 2006).

Through interactions with adaptor proteins, NOS1 can be found at either the postsynaptic domain or distributed within the cytosol. Localisation depends on physiological conditions and cell type studied (Forstermann et al., 1998). Differences in

NOS1 localisation are often seen during development (Matsumoto et al., 1993b). For example, in the developing rat cerebellum particulate NOS1 activity increases during the first week of development and then decreases with maturity, whereas cytosolic NOS1 activity is low in the first week in development and increases in activity through maturity, suggestive of a shift of protein location throughout development (Matsumoto et al., 1993b).

1.1.4.2 Activation of NOS1 within the nervous system

NOS1 contains several phosphorylation sites that are important for regulating its catalytic activity. Phosphorylation is conducted by a range of kinases and phosphatases, such as, protein kinase A (PKA), Ca^{2+}/CaM-dependent protein kinase (CaMK) II, PKC and phosphatase 1 (for review, see Garthwaite, 2008). These can differentially affect NOS1 activity depending on the site of phosphorylation. One such example is Ser847 phosphorylation by both CaMKII and phosphatase 1 (Komeima et al., 2000, Watanabe et al., 2003, Hayashi et al., 1999). In cultured cortical and hippocampal neurons, NMDAR activation regulates NOS1 phosphorylation at Ser847 in a complex and bidirectional manner (Rameau et al., 2004). Normal physiological NMDAR activation leads to NOS1 phosphorylation and reduces its activity by inhibiting Ca^{2+}/CaM binding, therefore limiting the extent of NOS1 activation (Rameau et al., 2004). In contrast, pathological concentrations of glutamate induce phosphatase 1-dependent dephosphorylation of Ser847 which increases NOS1 activity, which may account for the overproduction of NO and excitotoxic neuronal damage observed during a wide range of acute and chronic neurological disorders (Rameau et al., 2004).

Recent studies have also shown that the Akt enzymes, a family of serine/threonine protein kinases, phosphorylate NOS1 at Ser1412 and induce NO synthesis, possibly by increasing its affinity for Ca^{2+}/CaM (Rameau et al., 2007). However this is attenuated by subsequent CAMKII dependent phosphorylation of Ser847 (as outlined above). This suggests that, under physiological glutamate concentrations, phosphorylation of Ser1412 is necessary for the initial activation of NOS1 but the subsequent accumulation of NO is likely limited by the phosphorylation state of Ser847 (Rameau et al., 2007, Rameau et al., 2004).

1.1.5 Targets of NOS1 and mechanisms of NO action

NO has many neurophysiological functions. Its highly diffusible nature allows it to rapidly traverse biological membranes to influence cellular physiology in a number of ways. However, in order to do this NO must react with several target molecules. Whilst NO contains an unpaired electron that will react with almost any other molecule containing exposed unpaired electrons (Beckman and Koppenol, 1996), the physiological actions of this molecule can be broadly segregated into two pathways: NO-receptor dependent and NO-receptor independent pathways (Krumenacker et al., 2004, Bryan et al., 2009).

1.1.5.1 NO receptor-independent signalling pathways

1.1.5.1. i Nitrosation/Nitrosylation

NO readily reacts with molecular oxygen to form nitrogen dioxide (NO_2; Beckman and Koppenol, 1996) and when present in large amounts, NO_2 and NO react to form dinitrogen trioxide (N_2O_3). This unstable molecule rapidly decomposes into nitrosonium (NO^+) and nitrate (NO_2^-) ions (see figure 1.5), the former of which can induce nitrosylation of thiols, secondary amines and phenolics (for review, see Korhonen et al., 2005). The most common form of protein nitrosylation is S-nitrosylation, where NO^+ forms relatively stable covalent interactions with the sulphur-containing thiol of cysteine residues, resulting in the formation of S-NO bonds (For review, see Martinez-Ruiz and Lamas, 2004). This ultimately leads to changes in protein structure and function (Hess et al., 2005, Duan and Chen, 2007, Jaffrey et al., 2001, Bryan et al., 2009).

A growing body of evidence suggests that S-nitrosylation can markedly alter neuronal physiology and function (Choi et al., 2000). For example, S-nitrosylation of the NR2A subunit at Csy399 causes functional changes in NMDARs that contain this subunit by reducing their Ca^{2+} permeability (Lipton et al., 1998, Choi et al., 2000), thus helping to attenuate Ca^{2+} induced neurotoxicity caused by prolonged NMDAR activation (Lipton et al., 1993). In contrast, this mode of signalling has been shown to regulate axonal development: NO-induced growth cone collapse of mouse dorsal root ganglion (DRG) neurons is thought to be mediated through S-nitrosylation of microtubule-associated protein (MAP) 1B, leading to a destabilisation of the axonal cytoskeleton and growth cone collapse (Stroissnigg et al., 2007).

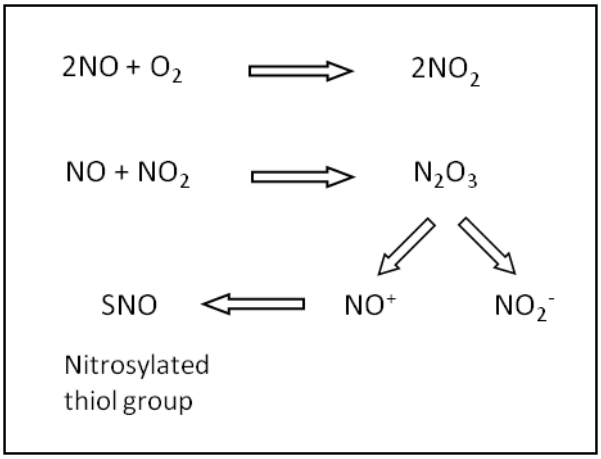

Figure 1.5 S-nitrosylation
A flow diagram showing the successive reaction steps during the process of S-nitrosylation. In the first reaction NO reacts with molecular oxygen (O_2) to form nitrogen dioxide (NO_2). NO_2 further reacts with NO to produce dinitrogen trioxide (N_2O_3) which decomposes in to nitrosonium (NO^+) and nitrite (NO_2^-) ions. NO^+ then S-nitrosylates thiols of secondary amines and phenols (Hanafy et al., 2001).

1.1.5.1. ii Nitration

As a consequence of aerobic metabolism, reactive oxygen species are continually generated in eukaryotic cells (Bryan et al., 2009). In order to protect cells from free-radical induced damage, enzymes metabolise free radical species to generate non-reactive products. One such enzyme is superoxide dismutase which reacts with superoxides to form hydrogen peroxide (H_2O_2) which is then converted to H_2O and O_2 (Tsang and Chung, 2009). However, at high (µM) concentrations NO can scavenge superoxide ions to form the highly reactive product peroxynitrite ($ONOO^-$) through a process known as nitration. In physiological environments peroxynitrite forms peroxynitrous acid which is a highly unstable and reactive oxidising species that readily decomposes to form several products including free radicals nitrogen dioxide ($NO^{\cdot}_2$) and hydroxyl radicals ($OH^{\cdot}$). It is believed that $ONOO^-$ facilitates the majority of its effects through this free radical production and one major physiological function of

this molecule is to nitrate tyrosine residues to form 3-nitrotyrosine (figure 1.6; for review, see Hanafy et al., 2001, Souza et al., 2008), a reaction that can impair the function of target proteins.

Tyrosine nitration has been widely documented to contribute to the pathogenesis of inflammatory maladies such as atherosclerosis (Lee et al., 2009, Upmacis, 2008), cancer (Muntane and la Mata, 2010, Vickers et al., 1999), sepsis (Fukuyama et al., 1997, Strand et al., 2000) and myocardial infarction (Mihm et al., 2001, Chen et al., 2008a). In the nervous system it also has a prominent role in neurodegenerative diseases such as ALS (Casoni et al., 2005, Sasaki et al., 2000), Parkinson's disease (Chinta and Andersen, 2011, Danielson et al., 2009) and Alzheimer's disease (Horiguchi et al., 2003, Williamson et al., 2002) in which elevated 3-nitrotyrosine levels are often observed causing irreversible protein damage.

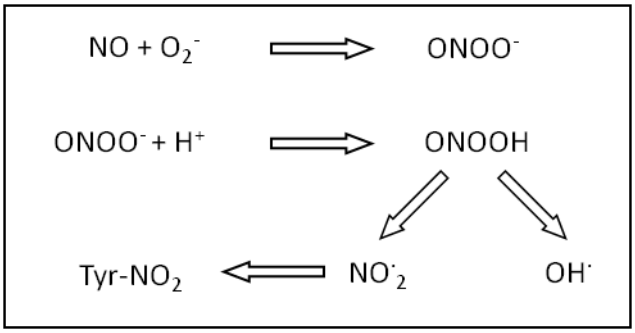

Figure 1.6 Nitration
A schematic diagram depicting the reactive steps during nitration. High levels of NO (μM) react readily with superoxide anions (O_2^-) to produce peroxynitrite ($ONOO^-$). This then reacts with hydrogen ions (H^+) to yield peroxynitrous acid (ONOOH). This unstable molecule rapidly decomposes to nitrogen dioxide ($NO^{\cdot}_2$) and hydroxyl radicals ($OH^{\cdot}$). Production of these free radicals then results in the nitration of tyrosine residues to form 3-nitrotyrosine. (Hanafy et al., 2001).

1.1.5.2 NO receptor-dependent signalling pathways

1.1.5.2. i cGMP-dependent signalling pathways

The enzyme soluble guanylyl cyclase (sGC) was the first physiological effector of NO to be discovered (Forstermann et al., 1986, Ignarro et al., 1986, Ignarro et al., 1982) and this enzyme remains the only known physiological receptor for this signalling molecule. When activated, sGC catalyses the conversion of guanosine-5'-triphosphate (GTP) to cGMP, a cyclic nucleotide second messenger molecule that regulates activity of target kinases and ion channels (for review, see Krumenacker et al., 2004). The catalytic activity of sGC is increased 5000-fold by binding of NO to the ferrous haem group of sGC (Garthwaite, 2010, Roy et al., 2008). Once bound NO forms a nitrosyl-iron complex with sGC, which induces a conformational change in the enzyme to increase its catalytic activity. Consequently modest amounts of NO can be amplified through this second messenger signalling pathway.

The sGCs are heterodimers that comprise an α and a β subunit (Humbert et al., 1990, Kamisaki et al., 1986). Each subunit contains three common domains: an N-terminal haem-binding domain, which binds NO (Ignarro et al., 1982), a dimerisation domain that is essential for assembly of the two subunits (Wilson and Chinkers, 1995) and a C-terminal catalytic domain which converts GTP to cGMP (Pyriochou and Papapetropoulos, 2005). To date, at least two isoforms of each sGC subunit have been identified: α1, α2, β1 β2 (for review, see Pyriochou and Papapetropoulos, 2005, Derbyshire and Marletta, 2009). Only heterodimeric enzymes harbour catalytic activity, with homodimeric sGCs showing little to no functionality (Harteneck et al., 1990, Buechler et al., 1991). Within the nervous system the heterodimer α_2/β_1 is the

most prevalent isoform and it anchors close to NOS1 at postsynaptic domains through α_2 subunit interactions with PSD-95 (Russwurm et al., 2001), as such, it has been termed "neuronal isoform of sGC" (Koesling et al., 2004). In contrast the α_1/β_1 dimer is found in the cardiovascular system and as such is termed the "vascular isoform of sGC" (Koesling et al., 2004).

1.1.5.2. ii cGMP mediated activation of protein kinases

The most common target of cGMP is PKG, a serine/threonine kinase that regulates function of a range of target proteins (for review, see Hanafy et al., 2001). PKG is activated by binding of cGMP to an allosteric site in the regulatory domain. This then enables PKG to catalyse the transfer of γ-phosphate from ATP to serine/threonine residues on target proteins (for review, see Francis et al. 2010). In mammals two families of PKG exist, PKGI and PKGII. The PKGI family is most commonly involved in NO mediated cGMP signalling and consists of two isoforms: PKG1α and PKG1β. Unlike PKGI, PKGII is membrane bound and was first identified in the rat intestinal mucosa, brain and kidney (Jarchau et al., 1994). In the intestine it has been largely shown to regulate ion transport in the epithelium (Markert et al., 1995, Vaandrager et al., 2000), whereas other physiological roles of PKGII include bone growth (Pfeifer et al., 1996), rennin secretion in juxtaglomerular cells (Wagner et al., 1998, Gambaryan et al., 1996) and regulation of the circadian clock (Tischkau et al., 2004). However, whilst both have cGMP binding sites PKGIα has a 10-fold greater affinity for cGMP than PKGIβ (Francis et al. 2010, Sekhar et al., 1992). The PKG isoforms also differ in their spatial distribution with PKGIα concentrated in the lung, heart, cerebellum and dorsal root

ganglion whereas PKG1β is concentrated in the hippocampus, olfactory bulb, smooth muscle and platelets (Hofmann et al., 2006, Hanafy et al., 2001).

PKG can phosphorylate a myriad of different proteins (for review, see Francis et al. 2010), which in the CNS include Kv3 potassium channels (Steinert et al., 2008, Moreno et al., 2001), brain specific G-septin (Xue et al., 2004, Xue et al., 2000), G-substrate in cerebellar Purkinje cells (Endo et al., 2003, Endo et al., 1999), GABA-A receptors (Nugent et al., 2009), ryanodine receptors (Takasago et al., 1991, Suko et al., 1993), leak K^+ channels (Kang et al., 2007), serotonin transporters (Ramamoorthy et al., 2007, Zhang et al., 2007) and tyrosine hydroxylase (Rodriguez-Pascual et al., 1999). In addition, PKG can also regulate its own catalytic activity through phosphorylation of sGC (Murthy, 2001, Zhou et al., 2008, Ferrero et al., 2000) which renders it inactive and through phosphodiesterase 5-depedent phosphorylation which promotes cGMP degradation (Murthy, 2001, Corbin et al., 2000, Rybalkin et al., 2002) thus creating negative regulatory feedback loops for the NO/sGC/cGMP/PKG pathway. NO mediated PKG activity will be covered in greater detail in chapter 5.

The cGMP signalling pathway can also activate PKA (Matsumoto et al., 2009, Muller, 2000, Muller and Hildebrandt, 2002), whilst the canonical ligand for this kinase is the second messenger 3',5'- cyclic adenosine monophosphate (cAMP) its cyclic nucleotide binding domain will also bind cGMP, albeit with 50-fold lower affinity (Francis et al. 2010). Despite its relatively low affinity cGMP-dependent PKA signalling has been shown to exert physiological functions in the CNS, for example, during insect associative olfactory learning (Matsumoto et al., 2009, Muller, 2000).

1.1.5.2. iii Cyclic nucleotide-gated channels

Both cGMP and cAMP are physiological ligands of cyclic nucleotide-gated (CNG) channels, of which there are two types: ligand gated CNG channels and voltage gated, hyperpolarisation-activated CNG channels (for review, see Biel, 2009). Hyperpolarisation-activated channels are cation channels that are activated by changes in membrane voltage (hyperpolarisation) and by cyclic nucleotides which act as ligands for these channels (DiFrancesco and Tortora, 1991). Within the nervous system CNG channels have been shown to have key roles in visual and olfactory function (Bradley et al., 2005, Broillet and Firestein, 1996, Kaupp and Seifert, 2002, Biel, 2009, Nakamura and Gold, 1987) as well as regulating neurite outgrowth (Togashi et al., 2008, Kafitz et al., 2000, Goldberg, 1998), chronic pain (Takasu et al. 2010, Jiang et al., 2008), LTP (Parent et al., 1998) and neurotransmission (Murphy and Isaacson, 2003, Savchenko et al., 1997, Carr et al., 2007, Papp et al., 2006).

1.1.6 Physiological roles of NOS1 in the embryonic nervous system

NO signalling has been show to regulate many aspects of nervous system development, from neurogenesis to synapse formation (for review, see Contestabile, 2000, Bicker, 2005, Contestabile and Ciani, 2004, Godfrey and Schwarte, 2003). Nervous system development begins with neurogenesis, and a range of studies show that NOS is expressed in neurogenic regions of the developing brain and spinal cord (Foster and Phelps, 2000, Holmqvist et al., 2000, Peunova et al., 2001, Islam et al., 2003, Moreno-Lopez et al., 2000, Romero-Grimaldi et al., 2008, Chen et al., 2004).

Subsequent functional studies have demonstrated that NO can regulate the proliferation and differentiation of neuronal precursor cells. In this context exogenous application of NO leads to decreases in cell proliferation in progenitor cells of the rodent subventricular zone (Matarredona et al., 2004), chick retina (Magalhaes et al., 2006) and *Drosophilla* imaginal disc (Kuzin et al., 1996), whereas inhibition of endogenous NO increases cell proliferation in the developing rat cerebellum (Ciani et al., 2006, Tanaka et al., 1994), and *Xenopus* optic tectum and neuroectoderm (Peunova et al., 2001, Peunova et al., 2007). Migration of postmitotic neurons from their area of birth to their final destination has also been shown to be regulated by NO. For example, NOS inhibition significantly reduces migration of cultured human neuronal progenitor cells (Tegenge and Bicker, 2009, Tegenge et al. 2010). Similar effects on migration are also observed in a number of other neuronal cells such as granule cells (Tanaka et al., 1994), Cajal-Retzius cells of the marginal zone (Santacana et al., 1998), sympathetic preganglionic neurons (Foster and Phelps, 2000) and invertebrate midgut neurons (Haase and Bicker, 2003).

After terminal differentiation neurons subsequently undergo neuritogenesis, and tissue and cell culture studies have implicated a role for NO in regulating this process (Van Wagenen and Rehder, 1999, Van Wagenen and Rehder, 2001, Welshhans and Rehder, 2005, Welshhans and Rehder, 2007, Trimm and Rehder, 2004, Ditlevsen et al., 2007, Yamazaki et al., 2005, Rialas et al., 2000, Hess et al., 1993, He et al., 2002a, Yamada et al., 2006). These studies will be discussed in detail in chapter 4.

Once axons reach their targets synaptic contacts are established and NO can also influence this process. Here NO can have very different roles which encompass

elimination/refinement of synaptic connections (Cramer and Sur, 1999, Cramer et al., 1996, Campello-Costa et al., 2000, Wu et al., 1994, Ernst et al., 1999, Wu et al., 2001) through to postsynaptic differentiation (Yang et al., 1997, Chao et al., 1997, Ribera et al., 1998, Kusner and Kaminski, 1996, Schwarte and Godfrey, 2004, Wildemann and Bicker, 1999, Jones and Werle, 2000, Godfrey et al., 2007). The specific roles of NO during synaptogenesis will be covered in detail in chapter 5.

1.1.7 Physiological roles of NOS1 in the adult central nervous system

In the adult nervous system, one of the most prominent roles for NOS1 is retrograde signalling during long term depression (LTD) and long term potentiation (LTP), which are believed to be the neural representations of learning and memory (for review, see Massey and Bashir, 2007, Raymond, 2007, Cooke and Bliss, 2006).

LTP has been widely studied in the hippocampal formation, a structure critical for short term and spatial memory. Here, coincident pre- and post-synaptic activity over prolonged periods induces LTP, a long-lasting strengthening of the synaptic connection. As LTP requires coincident activity, it is believed that a postsynaptic signal capable of diffusing retrogradely to the presynaptic terminal is required to induce long terms changes in synaptic strength (O'Dell et al., 1991). The highly diffusible and activity dependent nature of NO has made it an attractive candidate retrograde signal during LTP (Huang, 1997, Arancio et al., 1996a, Arancio et al., 1996b, O'Dell et al., 1991, Maffei et al., 2003). In support of this premise, LTP has been shown to be attenuated by NOS inhibition (O'Dell et al., 1991, Doyle et al., 1996, Bon and

Garthwaite, 2003, Bon and Garthwaite, 2001, Malen and Chapman, 1997, Jacoby et al., 2001, Maffei et al., 2003).

In a similar manner NO has also been shown to promote motor memory and learning by depressing inhibitory synaptic connections in the cerebellum through LTD, a weakening of synaptic strength induced by simultaneous activation of excitatory pathways in the cerebellum (Shibuki and Okada, 1991, Lev-Ram et al., 1995, Lev-Ram et al., 1997b, Lev-Ram et al., 1997a, Hartell, 1994, Reynolds and Hartell, 2001). In the cerebellar circuit, inhibitory Purkinje cells receive excitatory synaptic input from both parallel and climbing fibers. Coincident stimulation of these inputs reduces the strength of the parallel fiber-Purkinje cell synapses which causes disinhibition of deep cerebellar nuclei and promotes motor learning (for review, see Massey and Bashir, 2007). This effect depends upon elevated Ca^{2+} in climbing fibers coupled with NO synthesis in parallel fibers. When these events coincide, cGMP/PKG signalling is activated in Purkinje cell dendrites and alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptors are phosphorylated, hence facilitating LTD through increased desensitisation (for review, see Feil and Kleppisch, 2008).

Aside from its role in learning and memory, NO has also been implicated in many neuropathalogical disease states (for review, see Zhou and Zhu, 2009). Whilst this is principally caused by NOS2 activation (Pannu and Singh, 2006), NOS1 has also been shown to play fundamental roles. Evidence for this comes from the observation that NOS1 knockout mice treated with MPTP, a pharmacological tool that causes Parkinson's-like destruction of dopaminergic neurons in the substantia nigra, are resistant to neuronal damage (Przedborski et al., 1996) as are wild type mice treated

with NOS1 inhibitors (Schulz et al., 1995, Di Matteo et al., 2006, Muramatsu et al., 2002, Kurosaki et al., 2002, Przedborski et al., 1996). These observations may have clinical relevance as elevated NOS1 levels have been detected in patients with Parkinson's disease (Gatto et al., 2000, Eve et al., 1998).

Alzheimer's disease is a neurodegenerative disease that is characterised by synaptic degeneration, formation of β-amyloid peptide plaques and the presence of neurofibrillary tangles consisting of tau proteins that have been hyperphosphorylated. The numbers of NOS1 immunoreactive neurons are reduced in the brains of organisms with Alzheimer's disease, suggesting that NOS1 neurons are more vulnerable to neurodegeneration (Thorns et al., 1998, Norris et al., 1996). Furthermore, NOS1 localises in β-amyloid plaques and neurofibrillary tangles, thus further implicating a potential role for NOS1 in the progression of Alzheimer's disease (Thorns et al., 1998).

Finally, in ALS, an atrophic muscle weakening disease, spinal NOS1 expression is elevated (Sasaki et al., 2000). Additionally, treatment of a NOS1 specific inhibitor reduced progression of motoneuron degeneration in the wobbler mouse model of Motoneuron Disease (Ikeda et al., 1998). Taken together these findings suggest that NOS1 levels can change in a range of neurodegenerative disease states, although it is not clear whether this contributes to, or is a consequence of, disease pathology.

1.2 Zebrafish

1.2.1 Introduction to zebrafish as vertebrate model for study

The zebrafish (*Danio rerio)* is a small cyprinid fish that inhabits slow flowing, turbid rivers and pools, of northern India and Bangladesh (for review, see Engeszer et al., 2007). Over the last two decades this organism has become a premiere vertebrate model for developmental biology studies. Its emergence as a model for bioscience research stems from the pioneering work of George Streisinger (reviewed in Grunwald and Eisen, 2002) who, after spending two decades studying the molecular biology of bacteriophages (Streisinger et al., 1966, Okada et al., 1968) became interested in applying genetic analysis techniques to more complex model systems. Streisinger was a keen aquarist and brought many teleost species into the laboratory but settled on the zebrafish as they had several key characteristics that made them ideal for the genetic analysis of vertebrate development (see section 1.2.2). Streisinger began working alone on developing this model and pioneered several key genetic methods for use in this organism including the generation of clonal homozygous fish (Streisinger et al., 1981) and the identification of the first mutant lines: *golden, albino* and *sparse* (Streisinger et al., 1986).

During these formative years, several of Streisinger's colleagues (notably Charles Kimmel, Monte Westerfield and Judith Eisen) at the University of Oregon became increasingly interested in the power of Streisinger's new model and they themselves began to adopt the zebrafish as a tool for their own research. As such, following Streisinger's untimely death in 1984, Kimmel and Westerfield established workshops dedicated to the promotion of zebrafish as an internationally competitive

research model. Interest spread rapidly throughout Europe and the United States, culminating in the development of large-scale zebrafish mutagenic screening programmes by Christiane Nüsslein-Volhard and Wolfgang Driever in Germany (for review, see Granato and Nusslein-Volhard, 1996) which led to the generation of some 4,000 mutant lines to which an entire volume of the journal Development was dedicated in 1996 (volume 123; 1996). This was a major landmark, helping to place the zebrafish at the forefront of vertebrate genetics and leading to the initiation of the zebrafish genome sequencing project (in 2000). Over the following decade, zebrafish research across the world proliferated and to date the zebrafish public database Zfin catalogues some 13,990 publications using the zebrafish model.

1.2.2 The advantages of zebrafish as a vertebrate model for study

Zebrafish have many attributes that lend themselves to developmental studies. They are small, hardy and inexpensive to maintain and as such can be kept in a laboratory setting in large numbers with minimal maintenance. Moreover, zebrafish breed all year round and are highly fecund, with a pair of zebrafish able to generate several hundred eggs per day, thus providing ample embryos for daily experimentation. The zebrafish genome has been sequenced and a range of forward and reverse genetic tools are available for manipulating gene expression (Dahm and Geisler, 2006, Teh et al., 2005, Driever et al., 1994). Moreover, powerful imaging, physiological and behavioural methods have been adapted for use in zebrafish, thus enabling the study of molecular, cellular and behavioural aspects of biology (for review, see Fetcho et al., 2008, Lewis and Eisen, 2003, Drapeau et al., 2002, Peal et al.,

Key and Devine, 2003). However, the principal advantage of working with zebrafish stems from key characteristics that facilitate *in vivo* developmental studies: notably, as zebrafish embryos are optically transparent and externally fertilised the growth of individual cells, tissues and organs can be studied from the onset of development in intact living preparations. These advantages have allowed researchers to conduct detailed *in vivo* studies of developmental processes that are difficult, if not impossible, to undertake in mammals.

1.2.3 The zebrafish spinal cord as a model for neurodevelopmental studies

The zebrafish spinal cord has become a leading model for neurodevelopmental studies. Like that of other vertebrate species, this region contains a series of small neural circuits that are dedicated to the generation of locomotor behaviour. However, in comparison to mammalian embryos the zebrafish spinal cord is remarkably simple, containing few neurons each with stereotyped axonal trajectories and well characterised defined connectivity (Kuwada et al., 1990, Bernhardt et al., 1990, Higashijima et al., 2004b). This makes it a tractable model for study and, like that of other lower vertebrates such as the lamprey and *Xenopus* tadpole (for review, see Grillner, 2003, Roberts et al.), the zebrafish spinal cord has become a powerful tool for studying the neural basis of locomotor activity and neural circuit development in general (for review, see Drapeau et al., 2002, Lewis and Eisen, 2003; Saint-Amant, 2006, Brustein et al., 2003).

1.2.4 Zebrafish spinal cord development

In zebrafish, as with all anamniotes, spinal neurons develop in two discrete waves (Myers et al., 1986, Myers, 1985, Beattie, 2000, Lewis and Eisen, 2003). The first generates a very small number of so called “primary” neurons that pioneer a series of simple axonal scaffolds in the spinal cord between 10 and 18 hours post fertilisation (hpf). Subsequently, an additional wave of “secondary” neurons develop (16hpf), which assemble onto the primary scaffolds and comprise the bulk of the spinal cord tissue (for review, see Lewis and Eisen, 2003). The primary and secondary neuron populations have been characterised in detail and much is known of their anatomy, connectivity and function (see below).

1.2.4.1 Neuron classes of the early spinal cord

1.2.4.1. i Primary neurogenesis

The first primary neurons to form in the spinal cord are the primary motoneurons. These emerge during later stages of gastrulation, at around 9-10 hpf (Myers et al., 1986) and are ventrally located. These begin to extend their axons in to the periphery around 17hpf (see section 1.2.6 for more detail; Myers et al., 1986). The second neuronal population to exit the cell cycle are the Rohon-Beard sensory neurons (Korzh et al., 1993) which are first observed at around 10hpf. These have extended axons by 15hpf (Kuwada et al., 1990), in both rostral and caudal directions, to pioneer the dorsal longitudinal fasciculus (DLF), the major dorsal fascicle of the spinal cord (Kuwada et al., 1990, Bernhardt et al., 1990).

By 18hpf, the primary motoneurons and Rohon-Beard sensory neurons are joined by six interneuron classes (Drapeau et al., 2002): GABAergic dorsal longitudinal ascending (DoLA; Higashijima et al., 2004b), GABAergic ventral longitudinal descending (VeLD; Bernhardt et al., 1992, Batista et al., 2008), glutamatergic commissural primary ascending (CoPA; Higashijima et al., 2004b), glycinergic/glutamatergic commissural secondary ascending (CoSA; Higashijima et al., 2004b); GABAergic Kolmer-Agduhr (KA; Dale et al., 1987, Martin et al., 1998, Higashijima et al., 2004b) and ipsilateral caudal (IC) neurons (Mendelson, 1986). By 18hpf, the DoLA, have extended ipsilaterally and the CoPA and CoSA extended contralateral axons that project rostrally into the DLF (Bernhardt et al., 1990), these are accompanied by the IC and VeLD interneurons which have descending ipsilateral axons (Bernhardt et al., 1990, Mendelson, 1986, Drapeau et al., 2002). The IC cells which are located only in the rostral spinal cord, form a simple series of repeating neuronal circuits that extend through the rostrocaudal aspect of the spinal cord. Finally the KAs are positioned in the ventral spinal cord and have short ipsilateral ascending axons and central canal contacting cilia (Dale et al., 1987, Martin et al., 1998, Wyart et al., 2009). Furthermore a recent study has shown that KAs are important for the initiation of swimming and provide a positive drive to the CPG (Wyart et al., 2009).

1.2.4.1. ii Secondary neurogenesis

The second wave of neurogenesis begins at 16hpf with the birth of secondary motoneurons which subsequently extend into the periphery between 26-34hpf (Myers et al, 1986). A further eight classes of interneurons develop during the second wave of

neurogenesis. These are the glutamatergic circumferential descending (CiD; Higashijima et al., 2004b), glycinergic circumferential ascending (CiA; Higashijima et al., 2004b), glycinergic commissural bifurcating longitudinal (CoBL; Higashijima et al., 2004b), glutamatergic unipolar commissural descending (UCoD; Higashijima et al., 2004b), glutamatergic multipolar, commissural descending (MCoD; Higashijima et al., 2004b); glutamatergic ventral medial (VeMe; Higashijima et al., 2004b), ventral serotonergic (VeSe; McLean and Fetcho, 2004) and the glycinergic commissural local (CoLo; Satou et al., 2009). CiD, CiA and CoBLs are first detected at around 22hpf (Bernhardt et al., 1990): CoBLs are located dorsolaterally in the spinal cord and have commissural axons that bifurcate to project both rostral and caudal axons through the ventrolateral cord (Bernhardt et al., 1990). CiA and CiD neurons are dorsally located and have circumferential axons (Bernhardt et al., 1990), however CiAs have axons that project rostrally on the ipsilateral side to join the ventral DLF, whereas CiDs have descending axons that project to the ventral aspect of the DLF (Bernhardt et al., 1990).

UCoD and MCoD interneurons both have contralateral descending axons in the ventral spinal cord (Hale et al., 2001, Higashijima et al., 2004b). However UCoD cell bodies are positioned near the lateral margin (Hale et al., 2001) whereas MCoDs are positioned ventrally in the spinal cord (Higashijima et al., 2004b). Furthermore VeMe and VeSe interneurons are both located ventrally in the spinal cord and have descending axons (McLean and Fetcho, 2004, Hale et al., 2001, Higashijima et al., 2004b). Finally, CoLos are not observed in the spinal cord until around 40hpf and are located ventromedially with a largely descending commissural axon that runs along the

medial longitudinal fascicle (MLF), however a short ascending axon is also often detected (Satou et al., 2009).

1.2.5 Temporal onset of motor behaviours during development

The earliest spinally driven motor activity occurs at 17hpf, which is concomitant with the time in which primary motoneurons form synaptic connections with the muscle (Saint-Amant and Drapeau, 1998). This activity is known as "spontaneous coiling" as it is characterised by low frequency (0.1-1Hz) alternating contractions, or coils, of the trunk (Saint-Amant and Drapeau, 1998). Coiling is a unique form of activity seen only during a brief developmental window between 17 and 30hpf (for review, see Saint-Amant, 2006, Drapeau et al., 2002). At the onset of spontaneous coiling the spinal cord contains only a simple scaffold of primary neurons (Saint-Amant, 2006) that have yet to establish chemical synaptic connections. Patch clamp studies have shown that the electrical drive for coiling is generated by voltage-dependent conductances of immature spinal neurons that propagate activity via electrical synapses (Saint-Amant and Drapeau, 2001, Saint-Amant and Drapeau, 2000).

As the spinal cord matures, so does the locomotor behaviour, such that by 21hpf embryos first develop sensory responses to touch. At this stage tactile stimulation of the head or tail results in thrashing of their tail (Saint-Amant and Drapeau, 1998), a behaviour that involves the earliest chemical synapses of the spinal cord (Saint-Amant and Drapeau, 2001). Shortly thereafter, at 26hpf, the earliest forms of swimming behaviour emerge (Saint-Amant and Drapeau, 1998). Initially, bouts of

swimming are short, low frequency (ca. 10Hz) and poorly coordinated. However, as the fish develops and secondary neurons become incorporated into the circuit, swimming matures to generate high frequency (typically 30-50Hz) 'burst' swimming by 48hpf and to more mature 'beat-and-glide' swimming by 96hpf (for review, see Brustein et al., 2003).

1.2.6 The development zebrafish motoneurons

1.2.6.1 Motoneuron specification

Evidence to date suggests that zebrafish motoneuron specification involves a range of genes that are homologous to those that specify mammalian motoneurons (for review, see Lewis and Eisen, 2003). Morphogenic gradients established by the Hedgehog family of proteins are thought to be crucial for specifying positional fate of zebrafish motoneurons. The Hedgehogs are secreted from floor plate and notochord cells to regulate neuron specification in a concentration-dependent manner (for review, see Poh et al., 2002). Clear evidence that these proteins have a role in zebrafish spinal development comes from studies showing that mutation of the *smoothened* gene, which encodes a g-protein required for Hedgehog signal transduction, perturbs primary motoneuron differentiation (Chen et al., 2001). Hedgehog signalling is likely to regulate expression of many downstream target genes (such as *olig2, nkx6.1* and *nkx6*) that are involved in motoneuron specification (Park et al., 2002, Cheesman et al., 2004, Hutchinson et al., 2007).

At least three members of the LIM homeodomain gene family, *islet1*, *islet2* and *lim3,* work downstream of Hedgehog to regulate specification of zebrafish motoneurons (Lewis and Eisen, 2003, Appel et al., 1995, Inoue et al., 1994, Tokumoto et al., 1995). All primary motoneurons express *lim3* throughout early development and all also express *Islet1* from around 11hpf (Appel et al., 1995). However in both CaP and VaP motoneurons, *Islet1* expression is transient, being downregulated by 13hpf. As such, from 13hpf the CaP and VaP motoneurons express *islet2* (Appel et al., 1995). Temporal expression of *islet* genes are crucial for determination of primary motoneuron identity: when presumptive MiPs are translocated to the CaP domain of the spinal cord they begin to express *islet2* and adopt a CaP fate. This indicates that LIM gene expression determines motoneuron subtype (Appel et al., 1995). In further support of this, when islet1 is knocked down using antisense morpholino oligonucleotide (AMO) injection, motoneurons acquire an interneuron-like identity, extending axons through the spinal cord rather than into the somitic tissue (Hutchinson and Eisen, 2006). In a similar manner, when expression of the Met receptor tyrosine kinase, which is expressed in all primary motoneurons is inhibited with antisense methods, motoneurons also develop an interneuron phenotype and aberrantly express the inhibitory neurotransmitter GABA as well as ACh, suggesting that Met is required to prevent motoneurons from expressing interneuron-like identities and aids in their specification (Tallafuss and Eisen, 2008).

More recently, another LIM homeodomain gene *Hb9* was demonstrated to potentially regulate motoneuron specificity (Nakano et al., 2005). In mice, the 125bp *Hb9* enhancer region is shown to drive motoneuron specific gene expression of *Hb9*

and an identical copy is found in zebrafish, suggesting that *Hb9* is expressed also in specific motoneuron populations (Nakano et al., 2005). Taking these studies together it seems as though Hedgehog signalling from the floor plate induces *olig2, nkx6* and *nkx6.1* expression in progenitor cells from which motoneurons develop, this then initiates the expression of different patterns of LIM homeodomain genes that specify motoneuron fate (Lewis and Eisen, 2003).

1.2.6.2 Primary motoneuron birth and axon outgrowth

Primary motoneurons are larger and fewer in number than secondary motoneurons (11.3µm *cf.* 6.7µm; Myers et al., 1986) and although they are born during gastrulation, they do not undergo axonogenesis until around 17hpf (Eisen, 1991). By this stage, each hemisegment of the spinal cord contains three to four primary motoneurons: the caudal primary motoneuron (CaP), middle primary motoneuron (MiP) and rostral primary motoneuron (RoP; Figure 1.7; Myers et al., 1986). Additionally, a variable primary (VaP) can be observed transiently during development in roughly half of the spinal hemisegments (Eisen et al., 1990). During axonogenesis all motoneurons exit the spinal cord through a common exit point, initially following a common pathway pioneered by the CaP axon to the horizontal myoseptum (Myers et al., 1986). Here motoneuron axons contact the "muscle pioneers", a unique population of early differentiation muscle cells that form along the dorsovental midline near the future horizontal myoseptum (Eisen et al., 1989, Myers et al., 1986). These cells express unique markers such as *Engrailed* (Ekker et al., 1992, Fjose et al., 1992, Hatta et al., 1991) and act as choice points for the three primary

motoneuron axons (Melancon et al., 1997) which pause upon contact with the muscle pioneers before taking separate, stereotyped trajectories to pioneer tracts in the ventral (CaP), dorsal (MiP) and lateral (RoP) regions of the myotome (for reviews, see Beattie, 2000, Hjorth and Key, 2002, Drapeau et al., 2002; see figure 1.7). These primary motor axons never diverge from their stereotypical innervation domains and as such, the outgrowth of primary motoneurons is likely to be tightly regulated by intrinsic and extrinsic molecular cues (Beattie, 2000).

1.2.6.3 Secondary motoneuron birth and axon outgrowth

In comparison to primary motoneurons, secondary motoneurons have received relatively little attention. However, it is known that they are born around 6 hours after primary motoneurons and continue neurogenesis until around 25 hours after (Myers et al., 1986). There are around 20 secondary motoneurons per hemisegment that have smaller somata and finer axons than that of primary motoneurons. Their axons begin to extend at around 22hpf (Pike et al., 1992), exiting the spinal cord to innervate the musculature by following one of the three pathways pioneered by the primary motoneurons (Myers, 1985, Myers et al., 1986).

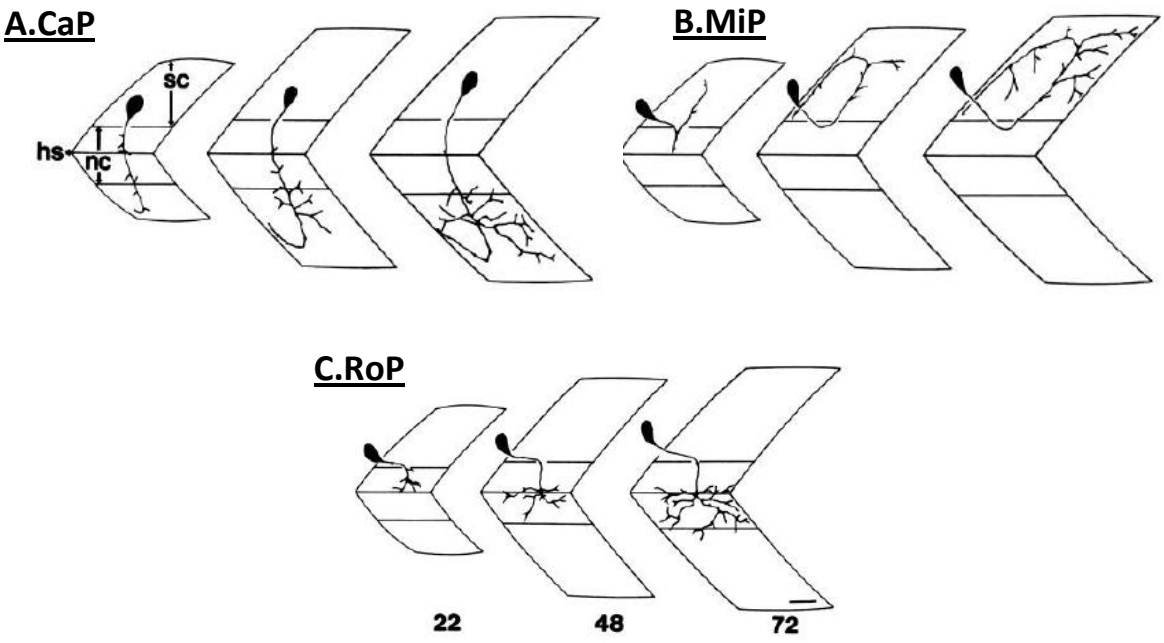

Figure 1.7 Primary motoneuron outgrowth
The stereotypical outgrowth of CaP **(A)**, MiP **(B)** and RoP **(C)** motoneurons across the first three days of development (22-72 hours post fertilisation (hpf)). ***A,*** By 22hpf the CaP axon has exited the spinal cord (sc) and pioneered the common pathway to the horizontal myoseptum (hs) before extending into the ventral myotome. Over the next 48 hours the CaP axon arborises extensively within its ventral territory. nc, notochord. ***B,*** At 22hpf the MiP axon projects along the common pathway to the hs then turns and projects in to the dorsal myotome. At around 24hpf the MiP retracts its ventral process and by 72hpf has innervated large regions of the dorsal myotome. ***C,*** The RoP axon follows the same pathway as the CaP and MiP to the hs at 22hpf, the axon then arborises laterally over the following 48 hours to innervate the surrounding myotomal muscle. Scale bar: ***A-C*** (in ***C***), 20µm. (Pike and Eisen, 1990).

1.2.7. Signalling molecules that regulate motor axon outgrowth

1.2.7.1 Interaction between motoneurons is not required for axon outgrowth

Eisen and colleagues have used a series of laser ablation studies to define how interactions between primary motoneurons affect axon growth. Here ablation of any single primary motoneuron does not impact the outgrowth of neighbouring primary motoneurons (Eisen et al., 1989, Pike and Eisen, 1990), suggesting that although primary motoneurons share a common path, they do not rely on interactions with one another to extend axons with the appropriate trajectory (Pike and Eisen, 1990, Eisen et al., 1989). In further support of this proposal, when CaPs are transplanted onto the horizontal myoseptum of a different somite where the native primary motoneurons have been removed, the transplanted cell extends axons with a trajectory appropriate to its class (Gatchalian and Eisen, 1992).

Similarly, whilst secondary motoneurons extend axons along tracts carved by the primary motoneurons, their outgrowth is not dependent on the presence of these pioneering axons. This has been demonstrated in an elegant series of laser ablation studies where removal of CaP axons does not inhibit secondary motoneuron outgrowth and secondary axons still extended ventrally along their normal pathway, albeit at a slower rate (Pike et al., 1992). Collectively these studies suggest that primary-primary and primary-secondary motoneuron interactions are not required for normal axon outgrowth. Rather, extracellular cues in the developing muscle likely guide motor axons to their destinations independently.

1.2.7.2 Myotomal factors affecting motor axon pathfinding

At the onset of motoneuron outgrowth, the myotomal tissue is immature and comprises only two cell types: lateral presomitic cells and adaxial cells, which give rise to embryonic white (EW) and embryonic red (ER) muscle fibers respectively (Devoto et al., 1996). The majority of adaxial cells exhibit a unique migratory pattern during development, forming adjacent to the notochord before elongating across the somite and migrating radially to occupy a superficial position at the lateral-most margins of the trunk whilst the remaining adaxial cells remain at the future horizontal myoseptum to become the adaxial muscle pioneer cells (Devoto et al., 1996). Lateral presomitic cells, which are initially positioned lateral to adaxial cells, invade the space left by migrating adaxial muscle, becoming located medially within the myotome (Devoto et al., 1996).

As motoneuron axons extend along their pathways into the myotomal territory, they are believed to encounter various attractant and repellent cues that are laid down by the nascent somitic tissue. These cues ensure that motor axons maintain a tight, stereotyped trajectory as they grow towards their targets. The following sections detail the evidence to support the premise that myotomal signals generated by the developing myotomal cell populations influence various aspects of motor axon growth.

1.2.7.2. i Adaxial muscle cells are critical for motor axon pathfinding

Before diverging to dorsal, ventral and medial regions of the myotome all motoneurons of the developing zebrafish trunk extend axons along a common

pathway to contact the muscle pioneers at the dorsovental midline. Analysis of zebrafish mutant lines that exhibit defects in motor axon development have demonstrated that the muscle pioneers could be critical choice points for motoneurons, sending instructive cues that control the trajectory of motoneuron axons (Eisen and Pike, 1991). Evidence in support of this argument was first provided through analysis of the mutant *spadetail* (Eisen and Pike, 1991). *Spadetail* encodes a T-box transcription factor that regulates many aspects of neuronal development (Griffin et al., 1998). *Spadetail* mutants lack muscle pioneers and have primary motoneuron axons which grow aberrantly, extending outside of the common pathway (Eisen and Pike, 1991). The motoneuron phenotype is not caused by disrupted autonomous development as when wild type primary motoneurons are transplanted in to *spadetail* background, similar defects in axonal outgrowth are observed (Eisen and Pike, 1991). Thus it was hypothesised that muscle pioneers are critical for controlling early stages of primary motoneuron outgrowth (Eisen and Pike, 1991).

Subsequent analysis of another mutant line, *stumpy* (Beattie et al., 2000, Hilario et al., 2010) further suggests that adaxial muscle pioneers are required for normal motoneuron outgrowth. *Stumpy* is demonstrated to affect primary motoneuron outgrowth, as these axons extend along their common pathway but stall at the horizontal myoseptum (Beattie et al., 2000). Further work has demonstrated that *stumpy* encodes CollagenXIXa1 which is also expressed at specific choice points (such as the muscle pioneers) where growing axons pause before changing trajectory in the muscle (Hilario et al., 2010). However, in a contrasting study, when muscle pioneers are ablated, it does not affect outgrowth of primary motoneurons (Melancon

et al., 1997). As such it is presently unclear whether expression of cues at the horizontal myoseptum regulates motoneuron outgrowth.

Studies of two other axon guidance mutants *diwanka* (Zeller and Granato, 1999, Schneider and Granato, 2006) and *unplugged* (Zhang et al., 2004a, Zhang and Granato, 2000, Jing et al., 2010) have provided conclusive evidence that the migrating adaxial muscle cells are critical for guiding pioneering motor axons through the somitic tissue. The *diwanka* mutant displays an array of abnormalities in primary motor axon outgrowth, from an absence of motor axons on common pathways to growth cones projecting beyond common pathways (Zeller and Granato, 1999). By performing a detailed series of transplantation studies, in which various wild type cells were transplanted to *diwanka* host embryos, it was found that only wild type adaxial muscle transplantation rescues the *diwanka* motoneuron phenotype. This suggests that *diwanka* expression in adaxial fibers is required for motor axon guidance (Zeller and Granato, 1999). Subsequent positional cloning has determined that the *diwanka* gene encodes the LH3 enzyme, which through its lysyl hydroxylase and glycosyltransferase activity, modifies extracellular proteins (Schneider and Granato, 2006).

The *unplugged* gene, which encodes a homolog of muscle-specific kinase (MuSK; Zhang et al., 2004a) expressed in adaxial muscle cells, also regulates primary motor axon outgrowth. In mammals MuSK is the canonical receptor for agrin, a secreted trophic molecule that is thought to trap and stabilise AChRs under nascent neuromuscular synapses. However, the earliest AChR clusters which assemble long before growth cones have made contact with them form in a MuSK-dependent but agrin-independent manner. Studies of the *unplugged* mutant have shed new light on

how early stages of AChR clustering arise. In wild type embryos, AChRs pre-pattern into tight clusters at the central zone of adaxial fibers before motor axon begin to extend (Panzer et al., 2005, Panzer et al., 2006). Disruption of *unplugged*/MuSK causes loss of AChR clustering, resulting in their dispersion across the surface of adaxial fibers. This consequently causes motor axons to stray off path towards dispersed receptors (Jing et al., 2009). The subsequent finding that blocking *dishevelled,* a component of the non-canonical Wnt signalling pathway, results in identical phenotypes to unplugged/MuSK mutants implicates a role for Wnt in controlling the initial stages of AChR patterning (Jing et al., 2009). The current hypothesis is that the Wnt ligand Wnt11r, rather than agrin, binds to MuSK and triggers pre-patterning of AChRs in a focal region in the centre of the myotome. Primary motor axons subsequently extend under these clusters, maintaining a tight trajectory above them as the muscle pioneers migrate throughout the somitic tissue (Jing et al., 2009).

Although much less is known of how migrating adaxial cells control secondary motoneuron outgrowth, both *unplugged* (Zhang et al., 2001) and *diwanka* (Zeller et al., 2002) mutants have been shown to display disruptions in secondary motoneuron outgrowth that are similar to those observed during primary motor development, suggesting that at least some guidance cues are conserved between both motoneuron populations (Zeller et al., 2002, Zhang et al., 2001). In addition, as the *you-too/gli2* mutant, which lacks adaxial cells, causes disruptions in secondary motoneuron outgrowth (Zeller et al., 2002), it is likely that adaxial cells are critical for guiding secondary motor axons to their targets.

1.2.7.3 Non-adaxial cues that influence motor axon pathfinding

Although adaxial cells are critical for guiding developing motor axons, other regions of the somite are of equal importance. For instance, the *topped* gene, which codes an as yet unidentified protein expressed in ventromedial white muscle cells regulates motor axon growth. Mutation of this gene causes CaP axons to stall at, or close to, the horizontal myoseptum before eventually passing over the horizontal myoseptum into the ventral myotome (Rodino-Klapac and Beattie, 2004). Through generation of genetic mosaic embryos, it has been found that *topped* expression in ventromedial fast muscle cells is required to rescue this phenotype (Rodino-Klapac and Beattie, 2004). The *topped* mutant also displays a significant delay in secondary motor axon outgrowth (Rodino-Klapac and Beattie, 2004).

Perhaps the most comprehensively studied zebrafish motor axon guidance molecules are the semaphorins (He et al., 2002b, Halloran et al., 1999, Sato-Maeda et al., 2006, Hilario et al., 2009, Roos et al., 1999), a large family of secreted and membrane bound proteins that act as ligands for the plexin, neuropilin and integrin receptors expressed on the surface of developing axons (Feldner et al., 2005, Feldner et al., 2007, Palaisa and Granato, 2007, Schlomann et al., 2009). In vertebrates, semaphorin receptor signalling typically causes growth cone repulsion through activation of Ras and Rho GTPases that induce cytoskeletal changes in the developing axon (for review, see Puschel, 2007) and a range of studies, outlined below, demonstrate that these molecules exert similar effects at the developing zebrafish neuromuscular junction.

In zebrafish, semaphorin3A1 is expressed in both dorsal and ventral domains of the developing myotome but is not present in the horizontal myoseptum (Halloran et al., 2000). Mis-expression of semaphorin3A1 in the horizontal myoseptum causes motor axons to stall just outside of the spinal cord, suggesting that this molecule repels developing growth cones (Halloran et al., 2000). Despite this, it should be noted that during normal development CaP axons extend ventrally past the horizontal myoseptum into semaphorin3A1 expressing domains, so whilst this molecule can repel nascent growth cones that have recently emerged from the spinal cord, axons presumably become less sensitive to this repulsive cue as they navigate beyond the horizontal myoseptum (Sato-Maeda et al., 2006).

A second semaphorin implicated in zebrafish motor axon outgrowth is semaphorin3A2. This signalling molecule is expressed in posterior regions of somites whilst anterior regions are devoid of expression (Roos et al., 1999). Over-expression of semaphorin3A2 results in ventral axons that are either truncated or fail to exit the spinal cord, however in adjacent somites ventral axon growth is unperturbed (Roos et al., 1999). This suggests that semaphorin 3A2 guides outgrowth of ventral motor axons by preventing motor axons from innervating the posterior half of the somite during initial axonogenesis (Roos et al., 1999).

One final semaphorin, 5A, has been shown to regulate primary motor axon guidance. Here antisense knockdown of semaphorin5A leads to stalling and aberrant branching of CaP axons at intermediate targets, which can be rescued by injection of full-length *semaphorin5A* RNA (Hilario et al., 2009). Semaphorin5A consists of two domains: the thrombospondin repeat domain and the sema domain, which are shown

to work together to guide motor axons in the myotome. In this context, the thrombospondin repeat domain is suggested to act as a positive cue to motor axons by guiding them to the ventral myotome, whereas the sema domain acts to prevent axon branching and keeps axons on the correct pathway (Hilario et al., 2009).

Semaphorin receptor studies have also provided compelling evidence that semaphorins regulate motor axon guidance. *Sidetracked* is a zebrafish gene that encodes the semaphorin receptor plexinA3 that is expressed in primary motoneurons during early periods of axonogenesis (Palaisa and Granato, 2007, Feldner et al., 2007). Antisense knockdown of plexinA3 in wild type embryos results in aberrant primary motor axon growth characterised by either additional nerves exiting the spinal cord or by aberrant branching, a phenotype mimicked in *sidetracked* mutants (Feldner et al., 2007, Birely et al., 2005). A second semaphorin receptor, neuropilin1a, has also been implicated in regulating motor axon guidance. Neuropilin transcript is detected in primary motoneurons as they undergo axonogenesis and antisense knockdown of neuropilin results in abnormalities in outgrowth of dorsal and ventral projecting primary motoneurons (Feldner et al., 2005).

The integrin family of semaphorin receptors also control motor axon growth. Evidence for this comes from studies showing that peptide integrin receptor antagonists cause either truncation of motor nerves or aberrant motor axon branching (Becker et al., 2003). Additionally, two integrin receptors, tenascin-C (Schweitzer et al., 2005) and laminin-α1 (Paulus and Halloran, 2006) have been shown to specifically regulate zebrafish motor axon development. Tenascin-C is expressed at the horizontal myoseptum and over-expression of tenascin-C results in a halt in outgrowth of ventral

motor axons at 24hpf, but not 33hpf (Schweitzer et al., 2005). Furthermore, a reduction in tenascin-C expression, through AMO knockdown, induces aberrant branching of ventral motor nerves at 33hpf but not 24hpf (33hpf; Schweitzer et al., 2005). Similarly, the *bashful/laminin-α1* mutant exhibits a multitude of primary motoneuron pathfinding errors and aberrant branching in the myotome (Paulus and Halloran, 2006). All together this suggests that integrins in the ECM could act as surfaces for growing axons to adhere to along the common pathway thus promoting axon outgrowth.

Finally, agrin, the heparin sulphate proteoglycan ligand for MuSK receptors also regulates zebrafish motor axon outgrowth (Magill-Solc and McMahan, 1988). Agrin knockdown zebrafish are characterised by truncated CaP axons that branch aberrantly during early axonogenesis (Kim et al., 2007), an effect that appears to be mediated through fibroblast growth factor interactions, rather than canonical MuSK interactions. Nonetheless, it is likely that agrin has important independent roles during zebrafish motor axon extension.

Finally, both primary and secondary motoneurons themselves can express receptors on their axons in order to receive cues from the surrounding environment, such as receptors for the glial cell line-derived neurotrophic factor (GDNF; Shepherd et al., 2001) and neurolin (DM-GRASP; Ott et al., 2001, Fashena and Westerfield, 1999). In primary motoneurons the GDNF receptor subunit GFRα is expressed on CaP motoneurons whilst GDNF is expressed on ventral somites during axonogenesis, suggesting that GDNF in the ventral myotome might act as an attractant cue for CaP axons during outgrowth. This was indeed the case as focal overexpression of GDNF in

the somite led to excessive branching towards somitic cells that overexpressed GDNF (Shepherd et al., 2001). Secondary motoneurons express the axonal cell adhesion glycoprotein DM-GRASP and injection with an inhibitory antibody against DM-GRASP results in defasiculation of motor axons, aberrant branching and pathfinding errors (Ott et al., 2001). As such, DM-GRASP has been suggested to regulate axon outgrowth through the adhesion of secondary motoneurons to the pioneered tracts of the primary motoneurons (Fashena and Westerfield, 1999).

1.2.8 Primary and secondary motoneurons innervate different muscle populations and are involved in different aspects of motor activity

By late embryonic and early larval stages, both lateral presomitic and adaxial cells have migrated and given rise to the EW and ER muscle fiber populations respectively (Devoto et al., 1996, Buss and Drapeau, 2000, Buss and Drapeau, 2002, van Raamsdonk et al., 1982). In stark contrast to mammals, where muscle fibers are initially polyneuronally innervated before being pruned back to form single motor units (for review, see Sanes and Lichtman, 1999), zebrafish muscle fibers become polyneuronally innervated during development and remain so throughout life and undergo little to no synaptic pruning (Westerfield et al., 1986). The ER and EW populations are differentially innervated by primary and secondary motoneuron pools. The primary motoneurons appear to selectively innervate WM fibers, and processes arising from these cells branch extensively to make multiple synaptic contacts with all EW muscle fibers within either the ventral (CaP), medial (RoP) or dorsal (MiP) musculature (Figure 1.8; Westerfield et al., 1986). In contrast secondary motoneuron

axons branch much less, with each neuron terminating on a small subset of either ER or EW muscle fibers (Figure 1.8; Westerfield et al., 1986).

As the fish matures, EW and ER muscle becomes functionally distinct so that, as in mammals, EW populations are recruited during bursts of powerful and fast activity whereas ER muscles are recruited during sustained, slower activity (Liu and Westerfield, 1988, Westerfield et al., 1986). This is coordinated by a gradient of spinal neuron recruitment that is governed by neuron age and dorsovental position, with earliest born dorsally situated neurons (the primary neurons) recruited only during fast, powerful swimming, whereas medial neurons of intermediate age are recruited during normal frequency swimming and ventral, immature neurons are recruited during slow, bouts of locomotion (McLean and Fetcho, 2009).

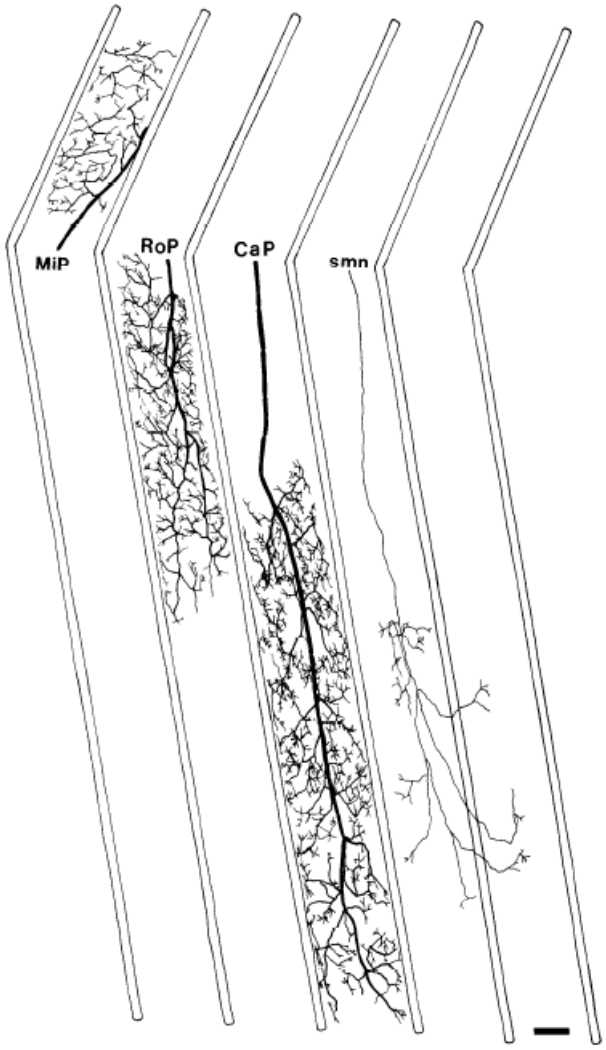

Figure 1.8 Myotomal innervation patterns of primary and secondary motoneuron axons
The first, second and third segments depict the innervation domains of the MiP, RoP and CaP axons in the dorsal, lateral and ventral musculature of adult zebrafish. The fourth segment shows the smaller terminal field of innervation of the secondary motoneurons (smn) in comparison to the primary motoneurons. Scale bar: 300µm. (Westerfield et al., 1986).

1.3 Aims and Objectives

The main aim of this thesis is to delineate the roles of NO during zebrafish spinal cord development. This will be achieved through 3 principal objectives which will form the main chapters of this thesis:

1. To determine the spatial and temporal distribution of NOS1 during early zebrafish ontogeny (Chapter 3).
2. To determine the roles of NO during development of zebrafish motor axons (Chapter 4).
3. To determine whether cGMP dependent pathways mediate the effects of NO and the role of nitrergic signalling in regulating neuromuscular maturation (Chapter 5).

Chapters 3 to 5 comprise data from the published article:

Bradley, S., Tossell, K., Lockley, R. & McDearmid, J. R. (2010) Nitric oxide synthase regulates morphogenesis of zebrafish spinal cord motoneurons. *The Journal of Neuroscience*, 30, 16818-31.

S. Bradley gathered all presented data within this published article. K. Tossell conducted preliminary NOS1 *in situ* hybridisation experiments and provided advice and expertise on cloning of NOS1 and *in situ* hybridisation techniques. R. Lockley assisted with some L.NAME experiments and J. McDearmid oversaw the project and provided funding.

2. Materials and Methods

2.1 Maintenance of zebrafish

Adult zebrafish were maintained according to established procedures (Westerfield, 1994) and in compliance with the Animals (Scientific Procedures) Act 1986. Embryos were harvested and raised in egg water at 28.5°C until the required developmental stage. Staging was performed in accordance with Kimmel et al., (1995).

2.2 Solutions and Buffers

All solutions were sterilised by autoclaving where appropriate.

Table 2.1. Table of solutions and buffers used

AP buffer:	0.1M Tris-HCl (pH 8.0 or 9.5); 0.1M NaCl; 50mM $MgCl_2$; 0.1% Tween20 made up to 50ml with double-distilled, deionised water (ddH_2O).
Block solution:	3% milk powder; 1% DMSO; 0.1% Triton-X 100 in 1x phosphate buffered saline (PBS).
Egg water:	Mix 1.5ml stock salt solution with 1L dH_2O.
Evans extracellular solution:	134mM NaCl; 2.9mM KCl; 2.1mM $CaCl_2$; 1.2mM $MgCl_2$; 10mM HEPES dissolved in ddH_2O; pH 7.8.
Hybridisation buffer:	50% formamide; 5xSSC; 5mM EDTA; 0.1% CHAPS; 0.1% Tween20; 50µg/ml Heparin; 1mg/ml Torula RNA made up to 50ml with ddH_2O.

LB broth:	10g tryptone; 5g yeast extract; 10g NaCl dissolved in 1L ddH_2O.
LB Agar:	LB broth containing 15g microagar per litre.
LB Agar plates	35g of LB agar dissolved in 1L of ddH_2O. Once cooled ampicillin was added to result in a final concentration of 50µg/ml. The mixture was the poured into 90mm petri dishes and left to set.
MS-222:	0.2-0.4% Ethyl 3-aminobenzoate methanesulfonic acid (MS-222) dissolved in Evans extracellular solution.
Stock Salts:	Mix 40g of Instant Ocean® sea salt with 1L dH_2O.
4% Paraformaldehyde (PFA):	4% paraformaldehyde dissolved in 1x PBS (stored in aliquots at -80°C).
1x Phosphate buffered saline (PBS):	4g NaCl; 0.1g KCl; 2.84 mM sodium phosphate dibasic; 0.6 mM sodium phosphate monobasic in 500ml dH_2O.
1x PBSTX:	PBS containing 0.1% Triton-X 100.
1x PBSTw:	PBS containing 0.1% Tween20.
20x SSC:	3M NaCl; 0.3M sodium citrate.

50x TAE:	2M Tris-base; 2M acetic acid; 50mM EDTA.

2.3 RNA extraction from whole embryos

For RNA extraction approximately 100 24hpf and 48hpf embryos were collected and de-chorionated. These were then transferred to separate 1.5ml eppendorf tubes and excess water was removed with a pipette. The embryos were then frozen by placing eppendorfs on dry ice. Total RNA was isolated from embryos using the NucleoSpin® RNA II kit (Macherey Nagel). To do this, embryos were transferred to a solution containing 350µl RA1 buffer (Macherey Nagel) and 3.5µl β-mercaptoethanol (Sigma), homogenised and vortexed vigorously. The lysate was then filtered through filter columns (NucleoSpin®) by centrifuging for 1minute (min) at 11,000 revolutions per min (rpm). Next 70% ethanol (350µl) was added to the lysate and mixed by repeatedly agitating the solution with a pipette. The lysate was transferred to an RNA II column (NucleoSpin®) and centrifuged for 30seconds (s) at 11,000rpm. 350µl of membrane desalting buffer was then added to the column before centrifuging for 1min at 11,000rpm. DNA digestion was completed by pipetting DNase reaction mixture onto the column followed by incubation at room temperature for 15min. Subsequently the column was washed three times, with brief centrifugation (30s at 11,000rpm after first two washes, 2min at 11,000rpm after final wash). Finally the RNA was eluted in 60µl RNase free water and centrifuged for 1min at 11,000rpm. The concentration of RNA was determined by UV absorption and stored as stock at -80°C.

2.4 Primer sequences

Table 2.2 Primer sequences

Gene	Primer sequences	Accession Number
nos1	Forward 5'-AGCGCTGACGCACAGTAAGACACA-3' Forward internal_XhoI 5'-GCCCTCGAGGACACAATGCAGGAATCAGAG-3' Reverse 5'-TAGCCGGTCAACATCGGCGTTTGA-3' Reverse internal_EcoR1 5'-GCCGAATTCGCAACATCTTTTGATTGATGT-3'	NP_571735

2.5 cDNA synthesis

cDNA templates were synthesised by reverse transcription of RNA isolated from 24 and 48hr embryos (as described in 2.3). Briefly ~4µg of total RNA, 1µl of oligo dt primer (Roche) and 2µl RNase free water (Fisher Scientific) were heated to 65°C for 10min and quickly cooled down to 4°C. Samples were made up to a final volume of 20µl with 4µl of 5x RT buffer, 0.5µl of RNase inhibitor (40units/µl), 2µl of dNTP mixture (10mM) and 0.5µl of RTase (Roche). Reactions were incubated at 55°C for 30min and then heated to 85°C for 5min to inactivate the RTase. The resulting cDNA produced was stored at -20°C until required.

2.6 Agarose Gel Electrophoresis

Validation and separation of DNA was conducted using agarose gel electrophoresis. Agarose gel was prepared at a concentration of 1.2% in TAE buffer. 2µl of edithium bromide (Fisher Scientific) was added to 50ml of gel solution prior to pouring into the casting tray. 6x gel loading dye (New England Biolabs (NEB)) were added to the DNA sample before loading onto the gel to aid visualisation. Once set the gel was submerged in 1 x TAE buffer and DNA samples loaded into lanes. An appropriate ladder (either 1kb or 100bp) was loaded alongside the DNA samples in order to determine the size of the samples. Electrophoresis was then conducted at 100V and 5W for around 50min. Edithium bromide labelled DNA was visualised under UV light.

2.7 Cloning of NOS1

Gene specific primers (see section 2.4) were designed to amplify a 1.2kb fragment of the *NOS1* cDNA sequence. Template cDNA (above) was mixed with 25µl of Taq master mix (25units/µl Taq polymerase: 200µM dNTP; Qiagen), *NOS1* forward and reverse primer (1µl each) and 20.5µl of water. PCR was conducted using a 2min hot start (94°C) before being held at 94°C for 3min (initial denature). Subsequently 30 PCR cycles, each comprising a 30s, 94°C denature, a 58°C, 30s annealing and a 72°C, 1min extension step to amplify the cDNA. The PCR reaction was then held at 72°C for 10min for the final extension before being rapidly cooled to 4°C. The reaction product was run on a 1.2% agarose/TAE gel to validate amplification of *NOS1* fragment.

2.7.1 Nested PCR

5µl of single stranded DNA from the first PCR was mixed with Taq mix (25µl), *NOS1* forward and reverse internal primers (1µl each) and 18µl of water. For nested PCR, an identical cycle to that outlined in section 2.7 was used, except the annealing temperature was set to 65°C. The nested PCR product was run on a gel to confirm successful amplification of the 1.2kb *NOS1* fragment.

2.7.2 DNA purification and ligation

DNA purification was conducted using E.Z.N.A.™ Cycle-Pure Kit (Omega). Using the protocol provided; DNA was purified and eluted then validated by running on a gel. For ligation *NOS1* insert and pGEM®-T easy vector (Promega) were mixed together at a ratio of approximately 3:1. T4 DNA ligase and 1x ligase buffer (Promega) were added. The reaction was made up to a final volume of 10µl with water. The reaction was gently mixed and incubated for 1hour (hr) at room temperature (RT) to ensure ligation of the *NOS*1 insert into the vector.

2.7.3 Transformation

Plasmid DNA was transformed into *E.Coli* competent cells (JM109, Promega). Transformation was initiated by pipetting 2µl of plasmid DNA into 20µl of competent cells. The mixture was placed on ice (20min) before being heat shocked at 42°C for 30s and then rapidly cooled on ice for 2min before the addition of 250µl of LB broth. After 1hr incubation at 37°C, the mixture was then plated on LB agar plates. The plates were subsequently incubated at 37°C for 14-16hrs. Resulting colonies were picked under

sterile conditions and validation of *NOS1* DNA inserts was conducted with colony PCR. Positive colonies were then picked from the plate and placed in LB broth for multiplication (containing 5µg/ml Ampicillin; Melford) overnight at 37 °C on an agitator at 200rpm.

2.7.4 Preparation of Plasmid DNA and sequencing

To extract and purify plasmid the E.Z.N.A.™ Plasmid Miniprep Kit I (Omega Bio-Tek) was used. The concentration of the eluted plasmid DNA was determined by spectroscopy using the following conversion factor: 1 OD_{260} = 50µg/ml DNA. Subsequently 10µl of eluted plasmid DNA was sent for sequencing at Genome Enterprise Limited, Norwich Biosciences Institutes.

2.8 Whole mount *in situ* hybridisation

2.8.1 Probe synthesis and linearisation

NOS1 probe was synthesised as outlined below. *Islet* probes were a kind gift from Professor Steve Wilson at UCL. For linearisation, plasmid DNA was treated with the appropriate enzyme (SAL1) for 3hrs at 37°C.

2.8.2 Phenol/Chloroform extraction and ethanol precipitation of DNA

Linearised DNA was purified with phenol/chloroform extraction. An equal volume of phenol/chloroform solution (150µl, Fisher Scientific) was added to the linearised DNA mixture (150µl) and vortexed vigorously before centrifugation at 13,000rpm for 2min. The upper aqueous layer was extracted and added to a 1/10

NaOAC and 2.5 x EtOH solution. This was inverted twice and incubated at -20°C for 30min. Subsequently the mixture was centrifuged at 13,000rpm for 15min and the resulting DNA pellet washed in 70% EtOH. After a final 5min spin at 13,000rpm, the EtOH was removed and the pellet air dried for 10min. The pellet was re-suspended in 15µl of ddH_2O.

2.8.3 Transcription

Linearised DNA was transcribed with T7 RNA polymerase. 1.5µg linearised DNA was incubated with 1x reaction buffer (NEB), 10% DIG mix (or FITC mix; Roche, for a FITC labelled probe), 5% RNase inhibitor (NEB), 5% of RNA polymerase (NEB) and made up to a final volume of 20µl with ddH_2O. DNA was transcribed at 37°C for 2-5hrs. Following transcription the RNA was treated with 2µl of DNase for 1hr at 37°C. Then 300µl of 100% EtOH, 10µl 4M LiCl and 80µl of ddH_2O were added to the mixture and centrifuged for 20min at 14,000rpm. The RNA pellet formed was washed in 70% EtOH and centrifuged again at 14,000rpm for 5min. All alcohol was removed, and the pellet was left to air dry before re-suspending in 20µl of ddH_2O and 20µl of hybridisation buffer. The probe was then run on a 1.2% agarose gel to check quality before being stored at -20°C.

2.8.4 One colour whole mount in situ hybridisation

Embryos and larvae (1d,2d and 3d) were anaesthetised in 0.02% MS-222 and subsequently fixed in 4% PFA for 4hr at RT. After several washes in PBS samples were immersed in 100% methanol at -20°C for 30min. Fish were then rehydrated in a methanol/PBS gradient (75%, 50% and 25% for 10min each) before washing in PBSTw

for 10min. Samples were then incubated in 10µg/ml of proteinase K (Roche) in PBSTw for 5min per day of development and then rinsed in PBSTw before re-fixation with 4% PFA (1hr at RT°). After washing in PBSTw, specimens were incubated in pre-warmed hybridisation buffer (65°C > 1hr) and then mRNA digoxygenin (DIG) labelled probe/hybridisation buffer (1:40) added for overnight hybridisation (at 65-70°C). The following day, fish were rinsed and incubated twice in pre-warmed 50% formamide/2xSSC/0.1% Tween20 at 65°C for 30min, followed by two incubations in pre-warmed 2xSSC/0.1% Tween20 for 30min each and two final incubations in 0.2xSSC/0.1% Tween 20, each for 30min at 65°C. After rinsing with PBSTw and addition of blocking solution (Roche) containing 5% sheep serum (Sigma) fish were exposed to anti-DIG alkaline phosphatase (AP) conjugated antibody (Roche) (overnight at 4°C). After subsequent washing with PBSTw 6 times, AP buffer (pH 9.5) was added for 10min and then exchanged for BM-purple AP substrate (Roche). Specimens were kept in dark conditions but were frequently monitored until precipitate could be observed. The reaction was stopped by washing with PBSTw before fixing with 4% PFA for 1hr. Embryos and larvae were then processed for whole mount and sectioned imaging (see section 2.18).

2.8.5 Two colour whole mount in situ hybridisation

Two colour hybridisation was performed as described above (section 2.8.4) with the following modifications. DIG labelled and a fluorescein (FITC) labelled probes were co-applied during the hybridisation step. After development of the DIG staining

specimens were washed extensively in PBSTw and then incubated at 70°C for 35min in order to deactivate the AP. Fish were then placed in block solution containing 5% sheep serum for 1hr at RT° before addition of anti-FITC -AP conjugated antibody (Roche) for overnight incubation at 4°C. After thorough washing AP buffer (pH 8.0) was added for 10min and subsequently exchanged for FAST RED solution for colouration, which was prepared using SIGMA*FAST*™ Fast Red TR/Naphthol AS-MX Tablets (Sigma). The red colour reaction was terminated by washing in PBSTw.

2.9 Morpholino injection of zebrafish embryos

Injection dishes were constructed using Sylgard®184 silicone elastomer kit (Dow Corning) in which small, egg sized wells were cut to provide a means of securing embryos during injection. AMOs were obtained from Gene Tools LLC. The *NOS1* AMO (sequence: 5'-ACGCTGGGCTCTGATTCCTGCATTG-3') was designed to target the translational initiation site of the *NOS1* mRNA. The control 'mismatch' AMO (5'-ACGCTcGcCTCTcATTCgTcCATTG-3') had 5 nucleotides altered along its sequence. AMO injections were performed as previously described (Nasevicius and Ekker, 2000). AMOs were dissolved in Evans physiological saline (Drapeau et al., 1999) to achieve a final concentration of 10-100µM. Injection needles (Harvard apparatus; borosilicate filamented glass; tip diametre *ca.* 10µm) filled with AMO solution were inserted into the blastomeres of 1-4 cell stage embryos and a brief 30ms pulse of positive pressure was used to dispense *ca.* 3 nl of AMO into the embryo using a Picosprizer III (Parker) microinjector. The pressure for injection was determined through trial and error, with 30ms being the optimum. Subsequently, eggs were allowed to develop at 28.5°C to the required developmental stage.

2.10 HuC GFP plasmid injections of zebrafish embryos

A DNA construct that contained GFP fused with the neural specific HuC promoter was kindly donated by Dr T. Hawkins (UCL). 50ng/µl HuC GFP plasmid was injected in embryos as outlined for the AMO injection protocol (above). Embryos were left to develop at 28°C until 1dpf at which point they observed under fluorescent light for stochastic GFP labelling. Labelled embryos were either injected with 1mM N_{ω}-Nitro-L-arginine methyl ester hydrochloride (L.NAME; see section 2.11) or left as untreated controls.

2.11 Drug administration via injection

For drugs that do not readily penetrate the skin we injected of the drug directly into the yolk sac. Drugs were dissolved in Evans physiological saline containing fast green (0.2%; Sigma). For injection, glass microinjection needles (tip diametre: 10-20µm) filled with drug solution were inserted into the yolk of 24hpf embryos. Around 50 nl of drug was pressure-ejected into the yolk using a Picosprizer III microinjector. Injections were repeated every 24hr until the required developmental stage was reached. Between injections, embryos were incubated at 28.5°C.

2.12 Drug administration via incubation

Drugs were diluted to the desired concentration using egg water (see table 2.3 for concentrations). 24hpf embryos were placed in the drug/egg water solution and incubated at 28.5°C until the desired developmental stage was reached. To diminish

the effects of drug depletion, incubation solutions were exchanged with freshly made media every 24hrs.

2.13 Pharmacological reagents

Table 2.3 Drugs used in this study

Drug	Concentration	Function	Injected/ incubated	Supplier
Diethylenetriamine/nitric oxide adduct (DETA NO)	500µM	NO donor	Incubated	Sigma
N_{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME)	1mM	Pan-specific NOS inhibitor	Injected	Sigma
Carboxy-PTIO, potassium salt (c-PTIO)	900µM	NO scavenger	Injected	Tocris bioscience
8-(4-Chlorophenylthio)-guanosine 3', 5'-cyclic monophosphate sodium salt (cGMP)	250µM-500µM	cGMP analogue	Incubated	Sigma
1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ)	50-100µM	sGC inhibitor	Injected	Ascent Scientific
Methylene blue	$3x10^{-3}$-$3x10^{-6}$ %	sGC inhibitor	Incubated	Acros Organics
PKG inhibitor peptide	250µM	PKG inhibitor	Injected	Tocris
8-bromo-cyclic adenosine diphosphate ribose (8-Bromo-cADPR)	1mM	cyclic adenosine diphosphate ribose (cADPR) antagonist	Injected	Sigma

Drug concentrations were determined by conducting a dose response curve, however the concentration range for the initial dose response curve was established from previous published literature regarding each drug type.

2.14 Labelling of putative neuromuscular synapses

Fish were anaesthetised in 0.2% MS-222 and subsequently fixed in 4% PFA for 90min at room temperature. Following thorough rinsing in PBSTX, 1mg/ml collagenase (Sigma) in PBS was added for 8min per 24hrs of age. After rinsing to remove collagenase, fish were placed in block solution (see table 2.1) containing 10µg/ml of rhodamine-conjugated α-bungarotoxin (Rh-α-BTX; Sigma) for 30min. The block/Rh-α-BTX solution was then rinsed with PBSTX (repeated 4 times) and subsequent SV2 immunohistochemistry was performed (section 2.16) to label pre-synaptic sites.

2.15 Nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase histochemistry

Embryos and larvae were anaesthetised in 0.02% MS-222 and fixed at RT° in 4% PFA for 90min. The PFA was rinsed off using four 10min PBSTX washes before being washed three times in 30% sucrose solution for 10min. Diaphorase staining was then conducted by incubating fish in 1ml of PBS containing 0.5mg NADPH (Melford) and 0.1mg of nitro blue tetrazolium (NBT; Melford) for 1-2hrs at 4°C. The reaction was terminated by rinsing in PBSTX.

2.16 Immunohistochemistry

Embryos and larvae were anaesthetised in 0.02% MS-222 and fixed in 4% PFA for 90min at RT°, then the PFA was thoroughly rinsed off with PBSTX solution. For older fish (3dpf) an optional 5min dH_2O permabilisation step was then conducted. Next, specimens were incubated in 500µl of blocking solution for 1hr before incubation

in primary antibody solution and either for 3-4hrs at RT° or overnight at 4°C. The primary antibody solution was subsequently removed and thoroughly rinsed with PBST before addition of block solution and incubation for 40min. This was then replaced with block solution containing secondary antibody in which fish were incubated for 3-4hrs at RT° or overnight at 4°C. After washing in PBST specimens were cleared in a gradient of glycerol (25%,50% and 75%) and mounted on a microscope slide for confocal microscopy imaging.

2.17 Antibodies used during this study

Table 2.4a Primary antibodies

Primary Antibody	Origin	Concentration	Supplier
Anti-Znp1	Mouse monoclonal	1:200	Developmental Studies Hybridoma Bank (DSHB)
Anti-Islet1/2	Mouse monoclonal	1:200	DSHB
Anti-Zn8	Mouse monoclonal	1:200	DSHB
Anti-3A10	Mouse monoclonal	1:200	DSHB
Anti-SV2	Mouse monoclonal	1:200	DSHB
Anti-NOS1	Rabbit polyclonal	1:200	Abcam
Anti-Serotonin	Mouse monoclonal	1:100	Abcam
Anti-Glutamate	Mouse monoclonal	1:400	Abcam
Anti-GABA	Rabbit polyclonal	1:200	Sigma

Table 2.4b Secondary antibodies

Secondary Antibody	Origin	Concentration	Supplier
Alexa Fluor® 488	Goat anti-mouse	1:500	Invitrogen
Alexa Fluor® 568	Rabbit anti-goat	1:500	Invitrogen

2.18 Cryosectioning

Fixed embryos and larvae were cryoprotected with a sucrose gradient (25%, 50%, and 75 %) at RT ° before being stored overnight in fresh 30% sucrose/PBS solution (4°C). Specimens were then transferred to cryomoulds containing O.C.T (Fisher Scientific) and orientated in the necessary direction for sectioning. The mould was rapidly frozen on dry ice and frozen specimens were sectioned with a cryostat (Bright Instruments) using a cutting thickness of 15-30µm. Individual sections were picked up onto polylysine pre-coated slides (Fisher Scientific) and left to air dry.

2.19 Confocal microscopy and image analysis

Image acquisition was performed using an Olympus FV1000 confocal microscope and Olympus Fluoview FV1000 imaging software. Images were captured in z-stacks at 1-3µm increments. Motor axon branch analysis was performed offline using the ImageJ plugin NeuronJ. Here motor axons were traced and quantified, however tracing of motor axons was restricted to fascicles ventral to the spinal cord as labelling of spinal neuropil precluded accurate examination of dorsal processes. For fish at 48hpf, branch patterns of three consecutive somites were analysed per fish, whilst at 72hpf, due to the density of branches present, analysis was limited to one somite.

2.20 Co-localisation analysis

Co-localisation of Rh-α-BTX and SV2 labelled sites was determined with ImarisColoc software (Bitplane). For analysis at 2dpf, one 73 x 75 pixel area was selected and positioned so that the bottom left hand corner of the selected area

contacted the distal tip of the main axonal fascicle ensuring co-localisation of the CaP axon alone. At 2dpf this analysis was performed on 3 consecutive somites. At 3dpf one somitic segment was analysed, in which three 73 x 75 pixel areas were selected that were evenly distributed from the ventral midline. At both ages the images in the pixel areas were thresholded and used for automated voxel colocalisation analysis.

2.21 Kinematic Analysis

Swimming activity was filmed with a Basler Pilot video at a 200Hz capture rate. Captured sequences of activity were analysed offline using ImageJ. Tail beat frequency, velocity and maximal bend amplitude of the first five cycles of each swimming episode were calculated as previously described (Budick and O'Malley, 2000).

2.22 Statistical Analysis

Results were calculated as means ± standard error of the mean (S.E.M). Statistical analyses were conducted using unpaired Student's *t*-tests.

3. The spatial and temporal distribution of NOS1 during early zebrafish ontogeny

3.1 Introduction

Over recent years it has become widely accepted that NO plays fundamental roles in regulating neuronal physiology in many regions of the adult CNS (for review, see Calabrese et al., 2007) and recent studies suggest that NO also has important roles during nervous system development (for review, see Vincent, 2010, Contestabile and Ciani, 2004, Godfrey and Schwarte, 2003, Contestabile, 2000). As such, a detailed knowledge of the developmental distribution of NOS1 isozymes is fundamental to the identification of NO's developmental roles in immature nervous tissue. However, despite this, there remains limited knowledge of the developmental expression of NOS1 enzymes in the nascent vertebrate CNS.

3.1.1 Methods for NOS1 detection

The most common methods of visualising nitrergic neurons are through *NOS1* transcript labelling with *in-situ* hybridisation, NOS1 protein staining with immunohistochemistry and detection of NOS1 catalytic activity with NADPH diaphorase (NADPH-d) histochemistry. Each of these techniques has merits and caveats, and a brief summary of these is detailed below.

In-situ hybridisation facilitates the detection of RNA molecules through hybridisation of a radioisotope, chromagen or florescent labelled antisense probe to a specific target RNA *in situ* (for review, see Jin and Lloyd, 1997). This method can be highly specific, allowing precise detection of individual RNA transcripts within a tissue. However, knowledge of the RNA sequence of interest is required for probe

manufacture. Additionally, as RNA labelling depends upon probe complementary, the presence of alternatively spliced transcripts should be considered when designing probes against RNAs that are known to be spliced. One final consideration is that because gene expression is tightly regulated at the post-transcriptional level, it cannot be assumed that the presence of mRNA signifies the presence of its protein product (for review, see Djuranovic et al., 2011 , Fabian et al., 2010).

Direct detection of NOS1 protein can be achieved through immunohistochemical methods. This approach can provide information on inter- and intracellular distribution of proteins. Immunolabelling essentially involves the reaction of biological samples with primary antibodies targeted against the protein of interest before application of probe-labelled (e.g. with chromagen, fluorophore, radioisotope) secondary antibodies that permit visualisation of primary localisation. Primary antibodies are synthesised via one of two methods: “monoclonal” antibodies are generated through immunisation of a single clonal cell line and thus are highly specific, recognising a single epitope of the target antigen. In contrast “polyclonal” antibodies are generated by animal immunisation and thus comprise a population of antibodies that recognise a range of epitopes. Their ability to recognise several epitopes can render them less specific but also makes them useful for amplification of weak signals and for reliable detection of protein polymorphisms. A wide range of commercial antibodies are now available for purchase, which greatly facilitates the application of this method. However, in situations where antibodies of interest are not commercially available, they must be manufactured by the end user, which is expensive both in terms of cost and time.

The final method for detecting NOS1 activity relies on the diaphorase activity of this enzyme. In the presence of NADPH, NOS1 reduces the chromogenic dye nitro blue tetrazolium (NBT) to generate a dark blue formazan precipitate (Hope et al., 1991). Pioneering work by (Dawson et al., 1991) and (Hope et al., 1991) showed that NOS1 diaphorase activity was selectively retained after gentle fixation and thus NADPH-d could be used to detect presence of NOS1 in fixed biological tissue (Hope et al., 1991, Dawson et al., 1991, Lopez and Gonzalez, 2002, Villani et al., 2001, Ramanathan et al., 2006, Devadas et al., 2001, Freire et al., 2008). As this method does not require the manufacture of probes targeted against a specific nucleotide or amino acid sequence, it offers a cost-effective and rapid method for studying NOS1 expression in organisms that lack sequencing information or commercially available antibodies. However, as other enzymes, such as cytochrome P-450 oxidoreductase (Ott and Burrows, 1999, Kishimoto et al., 1993) can also reduce NBT, care must be taken to ensure that detected signals are specifically attributable to NOS activity (Matsumoto et al., 1993a, Tracey et al., 1993).

3.1.2 NOS1 in the developing brain

3.1.2.1 Mammals

Whilst all four NOS1 splice variants are expressed in the adult mammalian CNS, NOS1α is by far the most prevalent isoform and the only one to couple synaptic NMDA receptor activity to NO synthesis. In contrast, the cytoplasmic NOS1β isoform is expressed in sparse, discrete CNS regions such as the brainstem and NOS1μ is found localised in skeletal muscle (Silvagno et al., 1996, Brenman et al., 1997). Finally,

although NOS1γ is expressed in the CNS, it has little catalytic activity. Whilst attempts have been made to characterise the distribution of NOS1 splice variants in the adult nervous system, little is known of their regional expression during embryonic development. This is principally because staining approaches which do not discriminate between splice isoforms are typically used to map NOS1 distribution in the embryonic CNS. As such, very little is known of the expression patterns of individual NOS1 isoforms during development and as such, no attempt will be made to discriminate between them here.

The neurodevelopmental expression of NOS1 has been examined in a wide range of mammalian species including humans (Judas et al., 1999, Yan and Ribak, 1997), cats (Scheiner et al., 2001, Riche et al., 1995), sheep (Northington et al., 1996, Wood et al., 2005), hamsters (Zhang et al., 2002, Zhang et al., 2004b), mice (Gotti et al., 2005, Oermann et al., 1999) and rats (Chung et al., 2004, Keilhoff et al., 1996, Samama et al., 1995, Terada et al., 1996, Terada et al., 2001, Iwase et al., 1998). However, in the majority of these organisms only specific developmental stages and/or CNS regions were examined. At present, comprehensive studies of NOS1 expression have only been conducted in murine species and a brief summary of these findings is outlined below.

Immunohistochemical methods have revealed that NOS1 is first expressed in murine brain at around embryonic day 15 (E15), shortly after pioneering axons have begun to extend towards their target fields. At this stage, NOS1 immunoreactive and diaphorase positive cell clusters can be observed in the hypothalamus (Terada et al., 1996, Terada et al., 2001, McClellan et al.), amygdala (Guirado et al., 2008, Olmos et

al., 2005, Keilhoff et al., 1996), pons (Terada et al., 1996, Terada et al., 2001), striatum (Samama et al., 1995), neocortical subplate (Derer and Derer, 1993), pallium (Guirado et al., 2003), inferior colliculus (Iwase et al., 1998) and ventral thalamus (Terada et al., 1996). As embryonic development progresses NOS1 immunopositive cells expand in number such that by around E19, they are widely distributed throughout the hypothalamus, striatum, amygdala, thalamus and pons (Murata and Masuko, 2003, Terada et al., 1996, McClellan et al., Olmos et al., 2005, Giuili et al., 1994). In addition, at this stage the first NOS1 immunoreactive and NADPH-d positive cells can be detected in the superior colliculus, dorsal raphe nucleus, tegmentum and cerebellum (Terada et al., 1996, Terada et al., 2001, Keilhoff et al., 1996).

At the time of birth (P0), cells of the hippocampus begin to acquire a nitrergic identity. This process is gradual, with a steady increase in NOS1 immunoreactive cells observed between P0 and P14 (Iwase et al., 1998). By P14, NOS1 is observed in cells of the granule cell layer, pyramidal layers within CA1-3, strata oriens and radiatum (Giuili et al., 1994, Chung et al., 2004). In addition, olfactory bulb neurons express *NOS1* mRNA shortly after birth (P3), with transcript localising in cells of the glomerular layer and the inner granular layer (Giuili et al., 1994, Iwase et al., 1998, Samama et al., 1995). Furthermore NOS1 also becomes widespread throughout the cortex, localising to cortical layers II,III, V and VI, as well as throughout the striatum and in the amygdaloid complex (Giuili et al., 1994, Derer and Derer, 1993, Terada et al., 2001, Iwase et al., 1998, Chung et al., 2004). Finally, the regions of NOS1 expression remain largely similar from P14 into adulthood (Terada et al., 2001, Giuili et al., 1994) with the

regions of highest NOS1 expression in the adult being the cerebellum and olfactory bulb (Gotti et al., 2005, Bredt et al., 1991a).

3.1.2.2 Lower vertebrates

The developmental expression of NOS1 in the brain has been investigated in teleost (Ando et al., 2004, Holmqvist et al., 2004, Poon et al., 2003, Villani et al., 2001, Villani, 1999), avian (Dermon and Stamatakis, 1994, Real et al., 2008, Suarez et al., 2005, Wu et al., 2001, Williams et al., 1994) and amphibian (Lopez and Gonzalez, 2002, Moreno et al., 2002, McLean and Sillar, 2001, Renteria and Constantine-Paton, 1996, Guglielmotti and Fiorino, 1999) species. Here, in contrast to mammals, there little evidence to suggest that NOS1 undergoes complex alternative splicing, with bioinformatic analysis to date suggesting the presence of a single NOS1 isoform (accession #: AF380137.1 (Fugu); AF053935 (*Xenopus*)). In these animals, discrete NOS1 expressing regions can be observed during embryonic stages and although the CNS bauplan of lower vertebrates diverges from mammals, broadly comparable NOS1 expression domains can be observed. To date, detailed accounts of spatiotemporal distribution of nitrergic cells of the CNS have been conducted in *Xenopus,* lamprey and zebrafish and a brief account of each is outlined in the following sections.

3.1.2.2.i NOS expression during Xenopus development

In *Xenopus,* the early developmental distribution of NOS1 protein is largely unknown. However, detailed transcript and diaphorase studies have characterised the

distribution of nitrergic cells from embryonic stages through to metamorphosis. In *Xenopus* the earliest signs of NADPH-d reactivity are observed in the caudal hindbrain, where a single cluster of nitrergic cells emerges at stage 29/30 (McLean and Sillar, 2001). Less than 10 hours later, a second hindbrain cluster exhibits diaphorase reactivity, as do receptor cells of the olfactory placode (McLean and Sillar, 2001, Lopez and Gonzalez, 2002). At around the hatching period, (stages 35-39) the number of NADPH-d labelled cells in the caudal hindbrain increases and a third subgroup differentiates in this region (Lopez and Gonzalez, 2002, McLean and Sillar, 2000, McLean and Sillar, 2001). Furthermore, during this period, additional weakly-diaphorase reactive neurons can be observed in the ventral and medial aspects of the forebrain, as well as in the caudal midbrain (McLean and Sillar, 2001).

During larval stages (stages 40-44), new nitrergic cell clusters emerge in the rostral rhombencephalic layer within the dorsolateral tegmentum, a cell group that comprises the most intensely diaphorase labelled neurons in the juvenile and adult brain (Lopez and Gonzalez, 2002). By stage 44 diaphorase positive cells are also detected in the developing tectum, telencephalon and diencephalon (McLean and Sillar, 2001). At stages 44-46, NOS1 expression increases substantially with abundant NADPH-d positive cells identified in olfactory sense organs, vestibuloacoustic organs and in both amacrine cells and the inner nuclear layer of the retina (Lopez and Gonzalez, 2002). In addition, by stage 47, transcript for *XNOS* (the neuronal isoform of *Xenopus* NOS) can be detected in the telencephalon, retina, optic tectum, midbrain and hindbrain (Peunova et al., 2001). Furthermore these *XNOS* expressing cells are shown to lie adjacent to proliferative zones in the optic tectum, telencephalon and

hindbrain, suggesting roles for NO in neuronal proliferation during early brain development (Peunova et al., 2001). Detailed mapping with diaphorase histochemistry at this stage (47) suggests that cell groups undergo progressive morphological maturation and additionally, new populations emerge in the pallium, the amygdaloid complex, ventral hypothalamus and pre-optic region (Lopez and Gonzalez, 2002, McLean and Sillar, 2001, Cogen and Cohen-Cory, 2000)

At stage 48, the nitrergic populations outlined above are all still present, but additional diaphorase positive neurons appear as a fourth small, caudal hindbrain subgroup (McLean and Sillar, 2001). At the onset of metamorphosis (stage 55) new nitrergic cell clusters emerge in the thalamus and the trigeminal sensory complex and by stage 60 diaphorase positive cells are apparent and become more widespread in the striatum, thalamus, trigeminal sensory complex, raphe and amygdaloid complex (Lopez and Gonzalez, 2002). By metamorphic climax the distribution of nitrergic cells observed in the brain is similar to that present in the adult brain (Lopez and Gonzalez, 2002, Bruning and Mayer, 1996a).

3.1.2.2.ii NOS1 expression during lamprey development

In the Lamprey, NOS expression during early development has been examined using NADPH-diaphorase histochemsitry. In the larval lamprey diaphorase positive cells are detected extensively in the olfactory epithelium and within the olfactory bulb (Schober et al., 1994; Zielinski et al., 1996). High levels of NOS are also detectable in fibers throughout the telencephalon, aswell as in the mesencephalon, metencephalon, pineal organ, habenular region, adenohypophysis and in the subcommissural organ (Schober et al, 1994).

3.1.2.2.iii NOS1 expression during zebrafish development

Whilst no studies have examined the developmental distribution of NOS1 protein, transcript studies have characterised the localisation of *NOS1* mRNA throughout embryonic and early larval stages. In zebrafish, *NOS1* transcript is first detected at the onset of forebrain neurogenesis in the ventrorostral cell cluster (VRC) at around 16-19hpf (Poon et al., 2003, Holmqvist et al., 2004). As development proceeds transcript becomes detectable in the pioneering forebrain ventrocaudal cell cluster (VCC) at 26-30hpf and dorsorostral cell cluster (DRC) at 35hpf (Holmqvist et al., 2004). In addition, hindbrain cell clusters emerge at around this time (Holmqvist et al., 2004). *NOS1* transcript expression continues to increase throughout late embryonic stages such that by 48hpf it can be observed in the telencephalic subventricular zone, dorsal hypothalamus, midbrain and hindbrain (Poon et al., 2003). At posthatching stages, *NOS1* transcript can be detected in a wide range of brain structures such as the

olfactory bulb, pre-optic area, retina, thalamus, pre-tectum, hypothalamus, posterior tuberculum and cerebellum (Poon et al., 2003, Holmqvist et al., 2004).

3.1.3 NOS1 expression in the developing spinal cord

3.1.3.1 Murine species

In murines, the first population of NADPH-d positive neurons can be observed at E12-E14 in the dorsolateral region of the ventral horn and due to their spatial distribution were presumed to be developing autonomic motoneurons (Bruning and Mayer, 1996b, Wetts et al., 1995, Huber et al., 1995). These spinal cells remain NADPH-d positive as they migrate to their terminal domain suggesting that NO may be involved in regulating early neuronal migration (Bruning and Mayer, 1996b). By E15 several sparse clusters of NADPH-d reactive cells are observed in the ventral horn in caudal regions of both cervical and lumbar murine spinal cord domains (Bruning and Mayer, 1996b, Wetts et al., 1995). These cells have been proposed to be somatic motoneurons but if this is the case, expression is transient as by birth somatic motoneurons do not express NOS1 (Wetts et al., 1995). A further group of cells that transiently express NOS are first detected at E16 adjacent to the autonomic motoneuron populations. Here diaphorase reactivity persists during all embryonic stages but decreases after birth with an absence of staining by P8 (Wetts et al., 1995). At E17 modest NADPH-d reactivity is detected in the neck of the dorsal horn (laminae V/VI), dorsal regions of the intermediate zone (lamina VII) and around the central canal (lamina X; Takemura et al., 1996, Wetts et al., 1995). Finally by E19 diaphorase positive

cells are more numerous in the dorsal horn with the additional presence of NADPH-d reactive cells in laminae III (Wetts et al., 1995).

After birth, intense NADPH-d reactivity is readily observed in laminae II/III/IV, although this labelling decreases to a moderate level by P10. By P15 diaphorase reactivity is scattered evenly across the entire width of the dorsal horn laminae at all spinal cord levels (Wetts et al., 1995). Furthermore diaphorase labelling of cells in the lamina II-VII and X, as well as diaphorase reactivity in autonomic motoneurons remained largely the same through postnatal life and in to adulthood (Dun et al., 1993, Saito et al., 1994, Spike et al., 1993).

3.1.3.2 NOS1 expression in the developing *Xenopus* spinal cord

In comparison to mammals, spinal NOS1 expression occurs relatively late during development, at pre-metamorphic stages (stage 47-48; Ramanathan et al., 2006, McLean and Sillar, 2001). Here, NOS immunohistochemistry and diaphorase staining show that nitrergic cells are restricted to a small isolated neuron cluster of the rostral spinal cord (McLean and Sillar, 2001, Ramanathan et al., 2006). Shortly after, just prior to the emergence of the limb buds (stage 51), two spatially separated NOS-positive neuronal clusters are observed in the spinal cord (Ramanathan et al., 2006). These can be further divided in to three topographic subgroups: the first are dorsally located, second are medially located with ventrally projecting neurites and third are located ventrally with axons that cross the ventral midline (Ramanathan et al., 2006, McLean and Sillar, 2001). During early metamorphosis (stage 53-56), weak diaphorase

reactivity is detected in the previously unstained region separating the two nitrergic clusters and diaphorase labelling is increased in the three spinal cell groups. By stage 58, NADPH-d reactivity is apparent in cells and fibers along the entire spinal cord but at metamorphic climax NOS expression declines (Ramanathan et al., 2006). As the onset and peak of NOS expression coincides with the emergence of forelimbs and declines thereafter, it has been proposed that NO regulates this transitional period of development. This is further supported as in *Rana esculenta* NOS expression in DRGs and dorsal horn neurons also coincides with periods of metamorphosis (Cristino et al., 2004).

3.1.3.3 NOS1 expression in the lamprey spinal cord

There is a paucity of data describing the expression of NOS1 within the developing lamprey spinal cord, however Schober et al., (1994) show that NADPH-d is detected in the axons of large Müller cells in the cervical spinal cord. In the adult lamprey the distribution of NOS1 in the spinal cord has been studied in greater detail. Here using both NADPH-d and NOS1 immunohistochemistry, NOS is detected in stretch-sensitive edge cells that span the length of the spinal cord at irregular intervals. Additionally NOS is also expressed in interneurons, dorsal cutaneous sensory neurons and motoneurons (Kyriakatos et al., 2009).

3.1.3.4 NOS1 expression in the developing zebrafish spinal cord

In the developing zebrafish, spinal cord *NOS1* mRNA has been reported to appear close to hatching period (55hpf) and is characterised by an intensely labelled band of staining spanning the ventral-most aspect of the spinal cord (Holmqvist et al., 2004). These cells have been shown to persist through to at least the third day of development, although their fate at later stages is currently unknown. Whilst cells have not been formally classified it has been postulated by Poon et al., (2003) that, according to their spatial distribution, they comprise secondary motoneuron populations.

3.1.4 Aims of this study

The principal aim of this study is to map the distribution of *NOS1* mRNA and protein during embryonic and early larval stages of zebrafish development, with a particular focus on the ontogeny of spinal nitrergic systems. Although previous studies (Holmqvist et al., 2004, Poon et al., 2003) have examined the spatial distribution of *NOS1* RNA during specific time points in development, the temporal resolution of these studies is limited and nothing is currently known of NOS1 protein distribution patterns in the zebrafish CNS. This is particularly relevant to spinal nitrergic systems where little is known of the timing of NOS1 expression in this region or the identity of nitrergic cells of the spinal cord.

This study demonstrates that during early life, *NOS1* RNA and protein expression patterns broadly overlap in the developing brain and spinal cord. Moreover,

it characterises spinal nitrergic populations in detail, showing that two spinal populations exist: a transient dorsal cell group and a persistent ventral cell group, each emerging within the first 30-35 hours of life. Morphological and histochemical analyses of these populations suggest that they are interneurons and that the ventral population comprises a novel, previously undescribed cell class.

3.2 Results

3.2.1 NOS1 expression in the 24-30hpf zebrafish CNS

3.2.1.1 NOS1 expression in the brain:

Consistent with previous reports (Poon et al., 2003, Holmqvist et al., 2004), *NOS1* riboprobe labelling revealed that *NOS1* transcript was first observed in discrete forebrain regions at 24hpf. At this stage, mRNA was restricted to VRC cell clusters (Figure 3.1B-D; n=5). Expression in this region persisted at 30hpf, with additional faint labelling observed in the POC at this stage (Figure 3.2C-D). During this developmental window, no transcript was observed in midbrain or hindbrain regions.

In contrast to riboprobe studies, NOS1 immunohistochemistry did not yield positive results at stages ranging from 24-30hpf (not shown). This is likely due to either low levels of protein expression that preclude immunohistochemical detection or a delay between transcription and translation of NOS1.

3.2.1.2 NOS1 expression in the spinal cord:

Whilst at 24hpf *NOS1* mRNA was not detectable in spinal tissue (not shown), by 30hpf the first signs of transcript could be observed (Figure 3.2E-F). At this stage, staining restricted to large cell bodies located in the dorsolateral spinal cord. Labelled cells were remarkably infrequent with no more than three cells labelled throughout the entire length of the spinal cord (n=5 of 5 embryos).

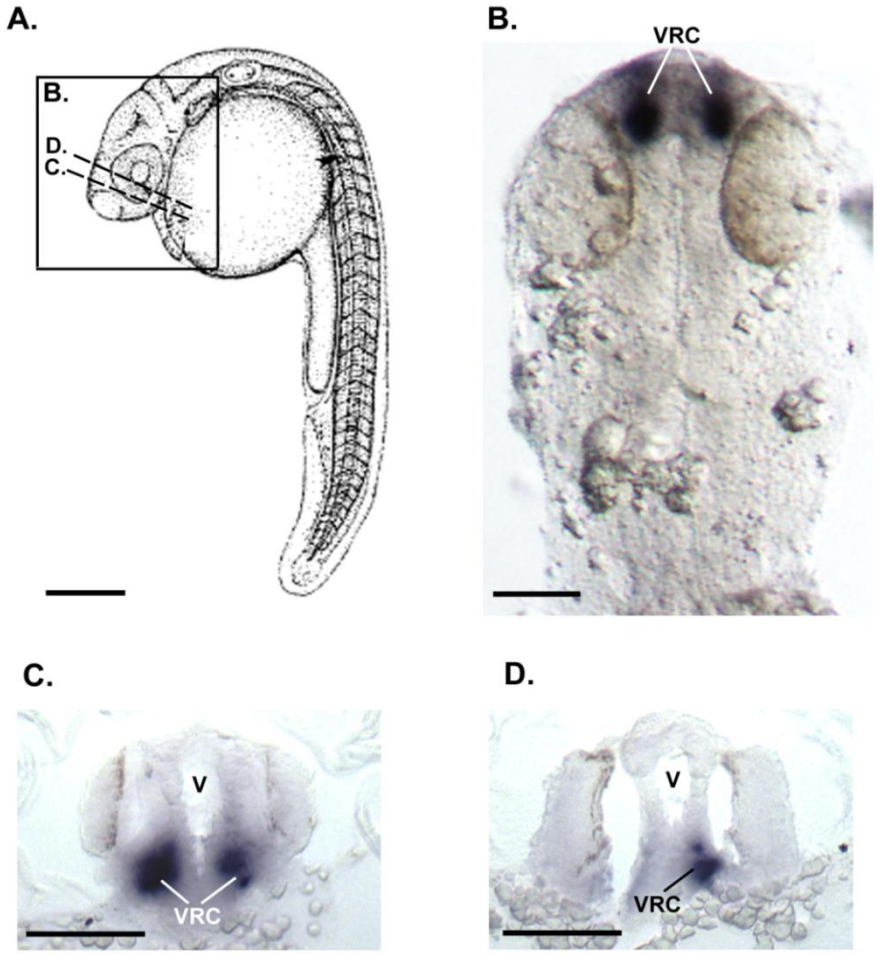

Figure 3.1 *NOS*1 mRNA expression at 24hpf

A, Schematic diagram of a 24hpf zebrafish embryo (from Kimmel et al, 1995) illustrating the regions depicted in *B-D*. ***B.*** Whole mount dorsal view of region boxed in ***A*** displaying bilateral *NOS1* mRNA expression in the ventrorostral cell clusters (VRC). ***C, D,*** Brain cross sections displaying *NOS1* transcript in the VRCs at 24hpf. V, ventricle. Scale bars: ***A***, 250µm; ***B-D***, 100µm.

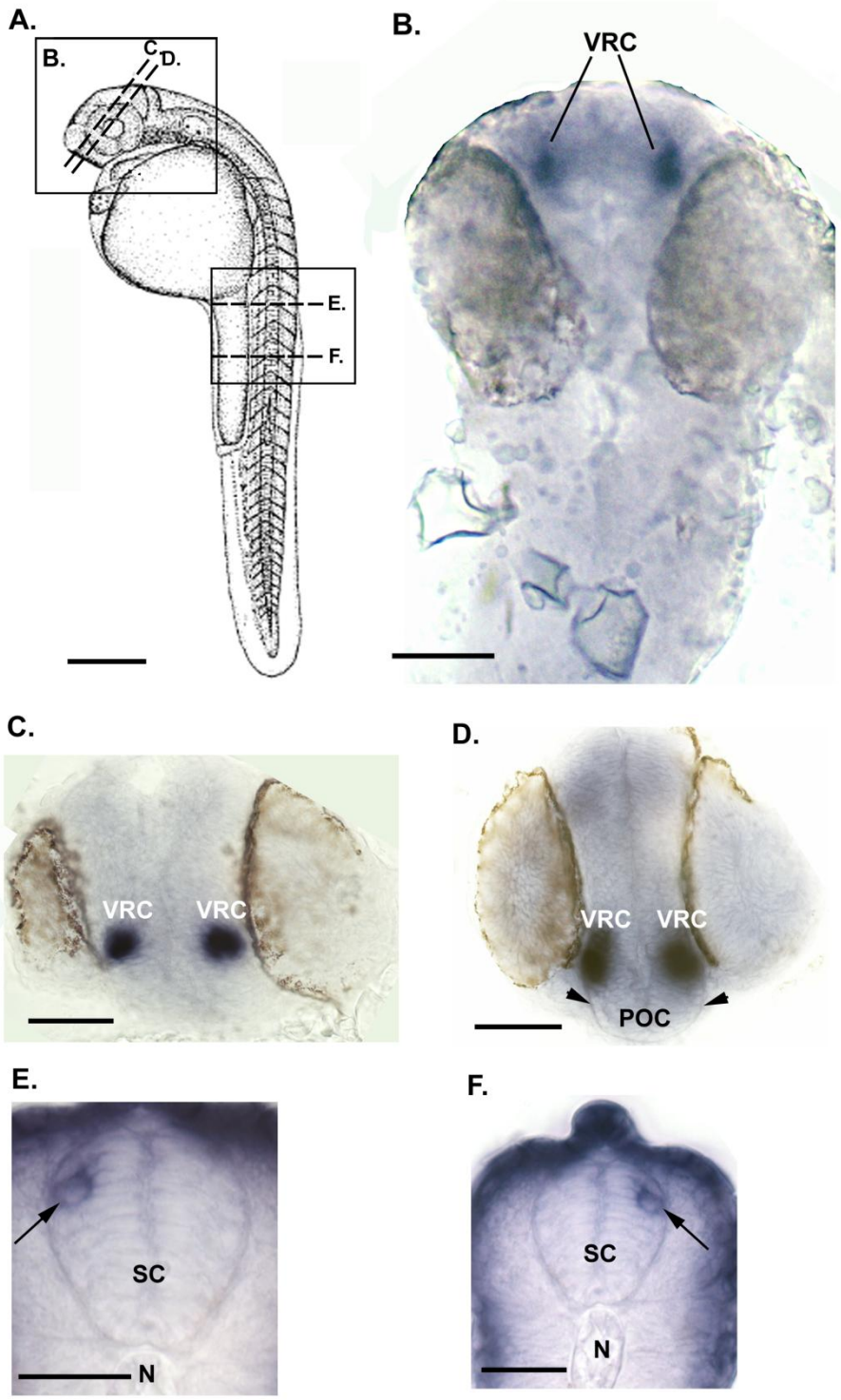

Figure 3.2 *NOS1* mRNA expression at 30hpf

A. Schematic diagram of a 30hpf embryo (from Kimmel et al, 1995), dashed lines mark the regions of interest in *B-F*. ***B,*** Dorsal whole mount view of *NOS1* transcript expression in the ventrorostral cell cluster (VRC) at 30hpf. ***C-D,*** *NOS1* transcript localises in the VRCs in sectioned brain tissue and also at the post-optic commissure (POC; arrowheads) in ***D***. ***E-F,*** Spinal cross sections reveal that *NOS1* mRNA localises solely in dorsal spinal cells at 30hpf (arrows). SC, spinal cord; N, notochord. Scale bars: ***A***, 250µm; ***B-D***, 100µm; ***E-F***, 50µm.

3.2.2 NOS1 expression in the 35-40hpf zebrafish CNS

3.2.2.1 NOS1 expression in the brain

Between 35-40hpf *NOS1* transcript expression was restricted to forebrain regions with no staining observed in midbrain/hindbrain structures. At 35hpf *NOS1* transcript persisted in the VRC and, additionally, two new *NOS1* expressing clusters were observed in the DRC and the VCC (Figure 3.3B-C; 3.4B-E; n=4). An identical distribution of transcript was also observed at 40hpf (Figure 3.6B-E; n=4).

Immunohistochemical analysis revealed that at 35hpf NOS1 protein was first detected in the CNS. At this stage, expression patterns broadly correlated with transcript, with staining in the VRC, DRC, POC (Figure 3.5D-F; n=5), although in contrast to transcript studies protein staining was not detected in the VCC but could be detected in the olfactory placode (Figure 3.5B, C, and E) and hypothalamic regions (Figure 3.5G (arrow)).

3.2.2.2 NOS1 expression the spinal cord

By 35hpf a ventral population of NOS1 expressing cells began to accompany those of the dorsal spinal cord (Figure 3.3D; 3.4F-G; n=4). These were more abundant than the dorsal cells, with expression present in each somite of the rostral spinal cord (somites 1-7)**.** By 40hpf and onwards *NOS1* transcript was no longer detected in dorsal spinal cells but remained prevalent in ventral spinal domains with *NOS1* riboprobe labelling through to somite 12 (Figure 3.6E-G; n=4).

At 35hpf corresponding patterns of expression in the spinal cord were also observed with immunohistochemical methods. Here protein labelling identified a

similar pattern of NOS1 expression, with weak NOS1 protein labelling in both dorsal and ventral spinal domains (Figure 3.5H-I; n=4).

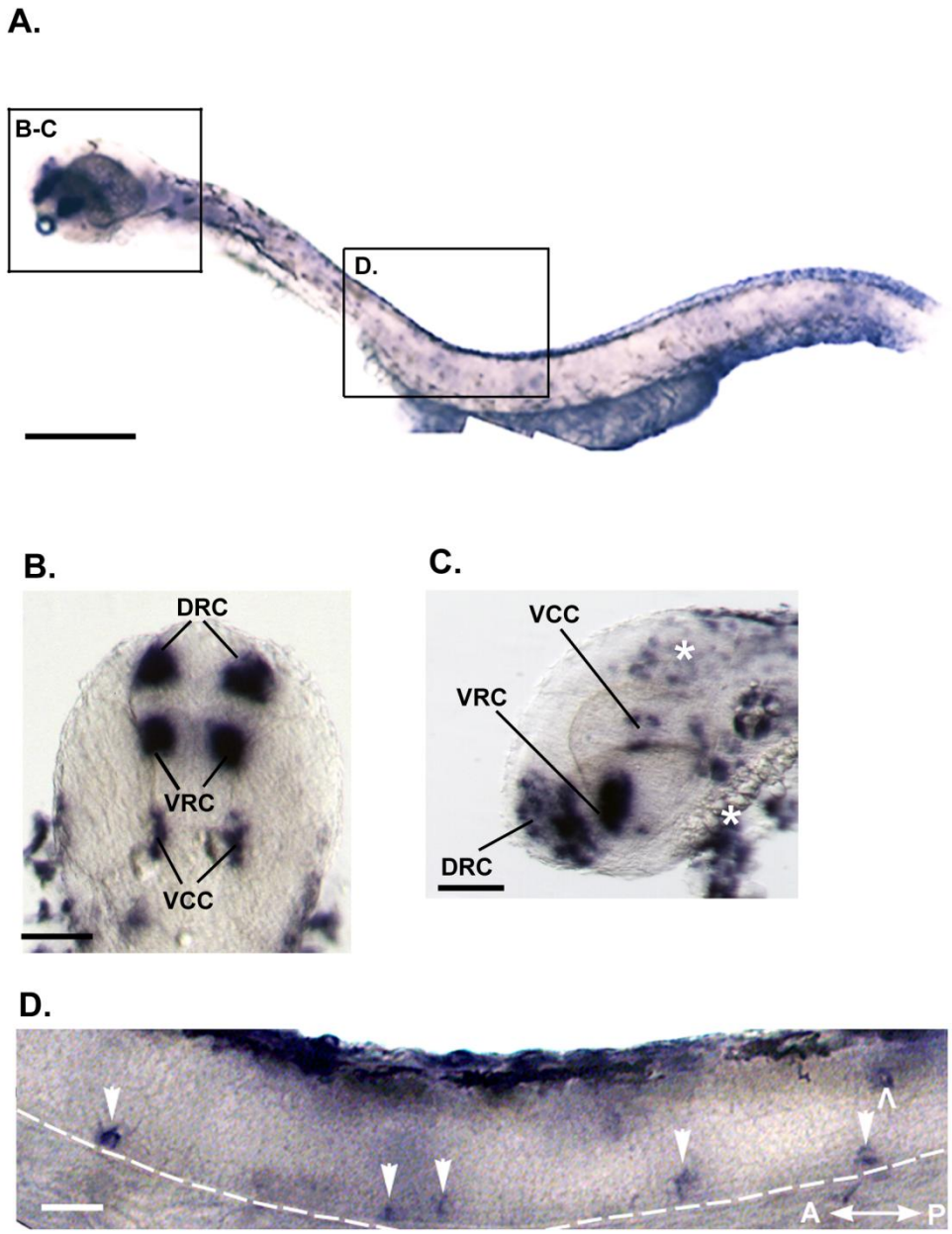

Figure 3.3 Whole mount *NOS1* mRNA expression at 35hpf
A, Whole embryo labelled with *NOS1* riboprobe displaying the regions depicted in *B-C*. ***B,*** Dorsal whole mount view of *NOS1* expressing areas in the embryonic brain. *NOS1* localises in three specific clusters the dorsorostral cluster (DRC); ventrorostral cluster (VRC) and the ventrocaudal cluster (VCC). ***C,*** Lateral view of *NOS1* expressing regions in the brain at 35hpf, asterisks denote *NOS1* transcript in the skin. ***D,*** Longitudinal image of spinal cord tissue labelled with *NOS1* riboprobe. Closed arrowheads indicate *NOS1* expression in the ventral spinal cord and the open arrowhead demonstrates *NOS1* labelling in the dorsal spinal cord. Dotted line in ***D*** delimits the ventral border of the spinal cord. A, anterior; P, posterior. Scale bars: ***A,*** 100µm; ***B-C,*** 50µm; ***D,*** 15µm.

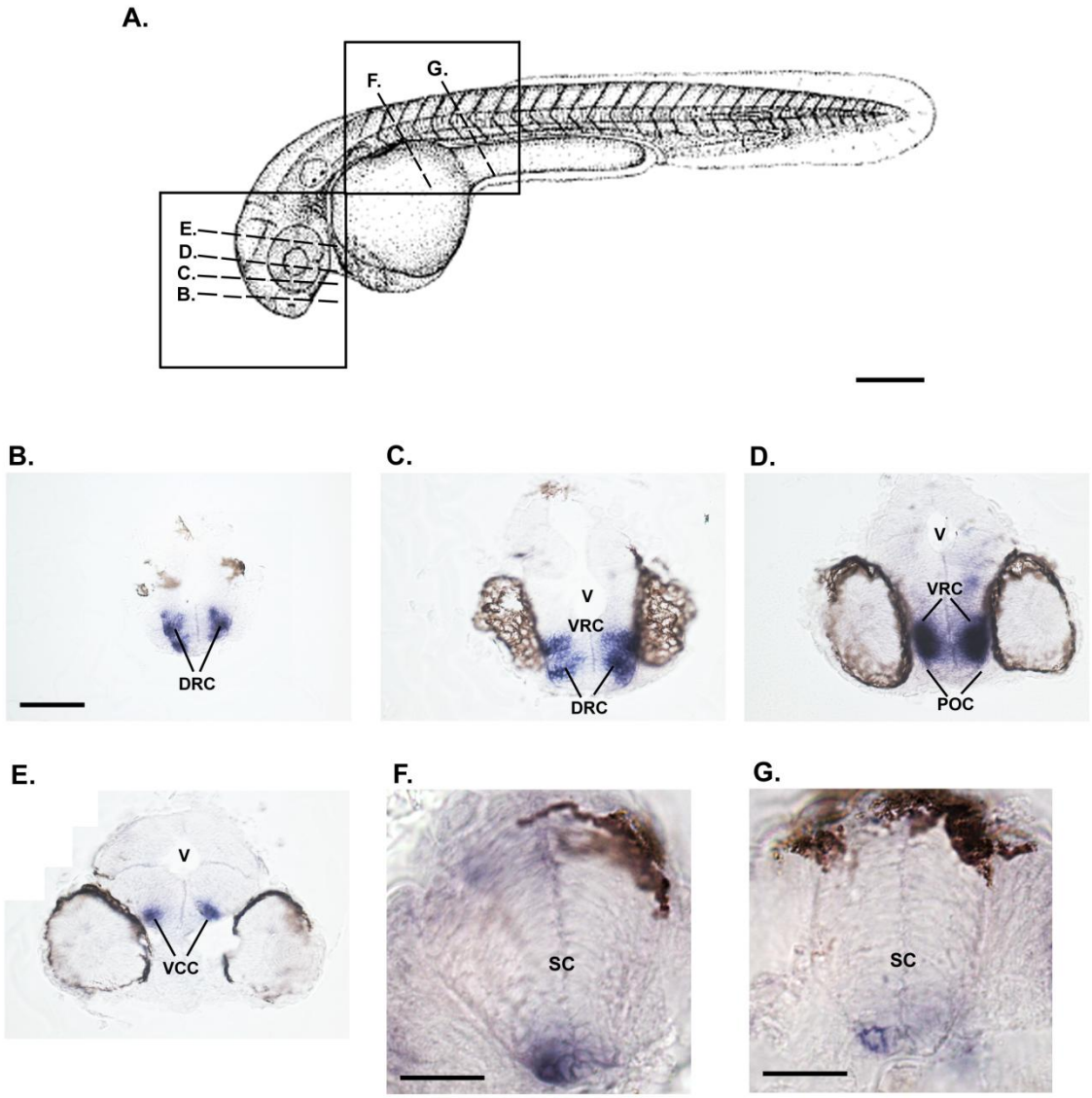

Figure 3.4 *NOS1* mRNA expression in sectioned tissue at 35hpf

A, Schematic diagram of a 35hpf embryo (from Kimmel et al, 1995) illustrating the approximate locations of the sections in *B-G.* ***B-E,*** *NOS1* mRNA expression in brain cross sections, transcript localises in the dorsorostral cluster (DRC; ***B-C***), the ventrorostral cluster (VRC; ***D***), the ventrocaudal cluster (VCC; ***E***) and faint labelling is observed in the post-optic commissure (POC; ***D***). ***F-G,*** Spinal cross sections labelled with *NOS1* riboprobe at 35hpf. *NOS1* transcript is detected in ventrally located spinal neurons. V, ventricle. Scale bars: ***A***, 250µm; ***B-E*** (in ***B***), 50µm; ***F-G***, 25µm.

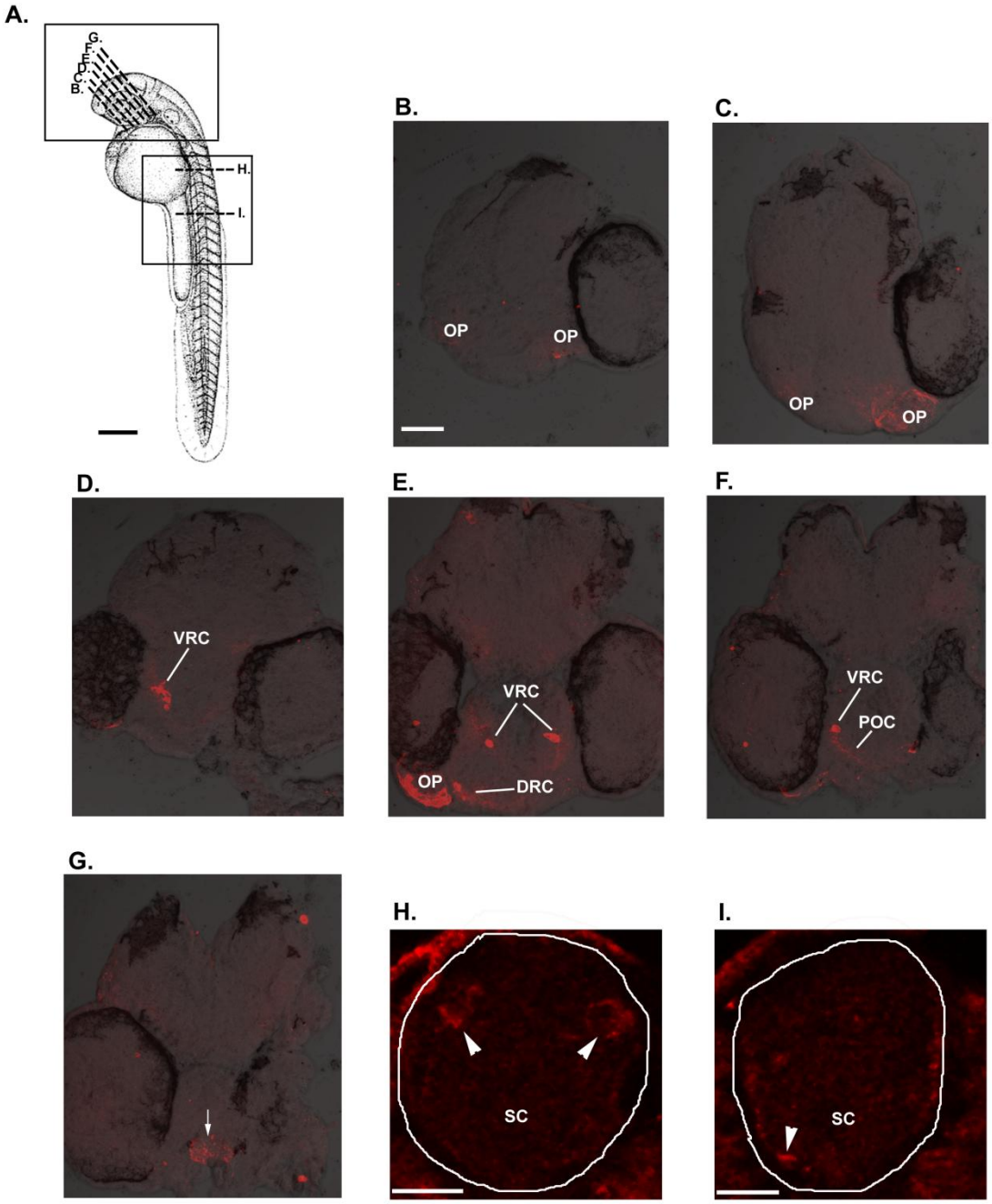

Figure 3.5 NOS1 protein expression in the brain and spinal cord at 35hpf

A, Schematic diagram of a 35hpf zebrafish embryo (from Kimmel et al, 1995) illustrating approximate locations of panels *B-I*. ***B-G,*** NOS1-immunolabeled cells in sectioned brain tissue at 35hpf. NOS1-immunopositive cells are identified in the olfactory placode (OP; ***B-C***), ventrorostral cluster (VRC; ***D-F***), dorsorostral cluster (DRC; ***E***) and post-optic commissure (POC; ***F***). Arrow in ***G*** depicts possible NOS1 immunopositive cells in the hypothalamic region. ***H-I,*** NOS1 immunolabelled cells in the dorsal (***H***) and ventral (***I***) spinal cord (SC) at 35hpf. Scale bars: ***A***, 250µm; ***B-G*** (in ***B***), 50µm; ***H-I***, 10µm.

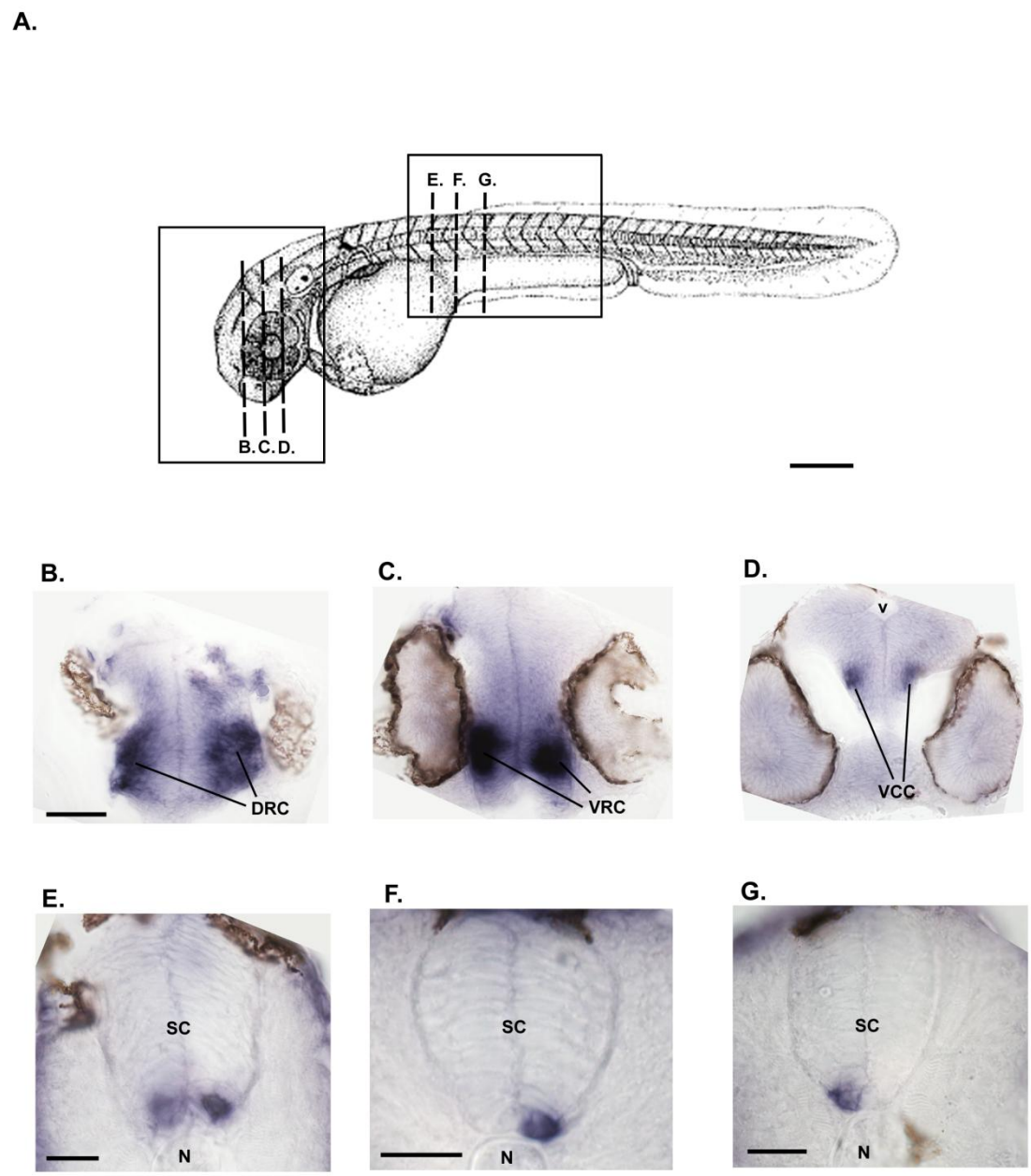

Figure 3.6 *NOS1* mRNA expression in the brain and spinal cord at 40hpf
A, Schematic diagram of a 40hpf zebrafish (from Kimmel et al, 1995), hashed lines illustrate the approximate regions depicted in panels *B-G*. ***B-D*** *NOS1* transcript localises in the dorsorostral cluster (DRC; ***B***), ventrorostral cluster (VRC; ***C***) and ventrocaudal cluster (VCC; ***D***) in sectioned brain tissue. ***E-G,*** Spinal cross sections displaying *NOS1* mRNA in ventral spinal cells at 40hpf. N, notochord; SC, spinal cord. Scale bars: ***A***, 250µm; ***B-D*** (in ***B***), 50µm; ***E-G***, 25µm.

3.2.3 NOS1 expression in the 48hpf zebrafish embryo

3.2.3.1 NOS1 expression in the brain

By the second day of development (48hpf) *NOS1* riboprobe labelling became much more widespread throughout the CNS, with mRNA localising in the olfactory bulb, pre-optic area, thalamic region and tegmentum (Figure 3.7A-C; n=8). As expression domains were relatively large, it became difficult to definitively define NOS1 expressing brain regions in whole mount preparations. However, examination of sectioned tissue facilitated the identification of additional specific NOS1 reactive brain regions, such as the sub-pallium, pallium and hypothalamic domains (Figure 3.8 C-D, G).

Immunohistochemical staining of 48hpf sectioned brain tissue revealed that many *NOS1* mRNA expressing regions also labelled positively for NOS1 protein, these included the pre-optic area, hypothalamic domain, thalamic region and tegmentum (Figure 3.9B-F). However, unlike transcript studies, protein expression was not observed in the sub-pallium, pallium, olfactory bulb or medulla oblongata. The final discrepancy is the persistence of NOS1 protein expression in the olfactory placode, which was primarily observed at 35hpf (Figure 3.9A-B *cf*. Figure 3.5B, C, E). In conclusion, whilst it is clear that mRNA and protein expression domains broadly overlap, there are some regions, specifically those that display weaker transcript expression, such as the medulla oblongata, in which protein cannot be detected.

3.2.3.2 NOS1 expression in the spinal cord

At this stage *NOS1* mRNA ventral spinal expression was present along the length of the ventral spinal cord with nitrergic spinal cells present in all but the most caudal regions (Figure 3.7A, D; n=8). Furthermore, transverse sections also reveal *NOS1* riboprobe (Figure 3.8K-M; n=8) and protein labelling in the same region of the ventral spinal cord (Figure 3.9G-I; n=10).

3.2.3.3 NADPH-d distribution in the spinal cord

NADPH-d histochemistry was first used to examine NOS1 expression in the brain and spinal cord of wholemount tissue. In these preparations, intense staining made it difficult to detect a specific diaphorase signal. As such, sectioned tissue was used. In contrast to *NOS1* transcript and protein expression, diaphorase staining was not found to localise in cell bodies of the brain (not shown) or spinal cord (Figure 3.10 A,D; n=3). Rather it accumulated in the neuropil. As NADPH-d did not correlate with expression of *NOS1* transcript or protein (Figure 3.10 A,D *cf.* B-C; E-F) it was not used as a marker of nitrergic cell populations during the remainder of the study.

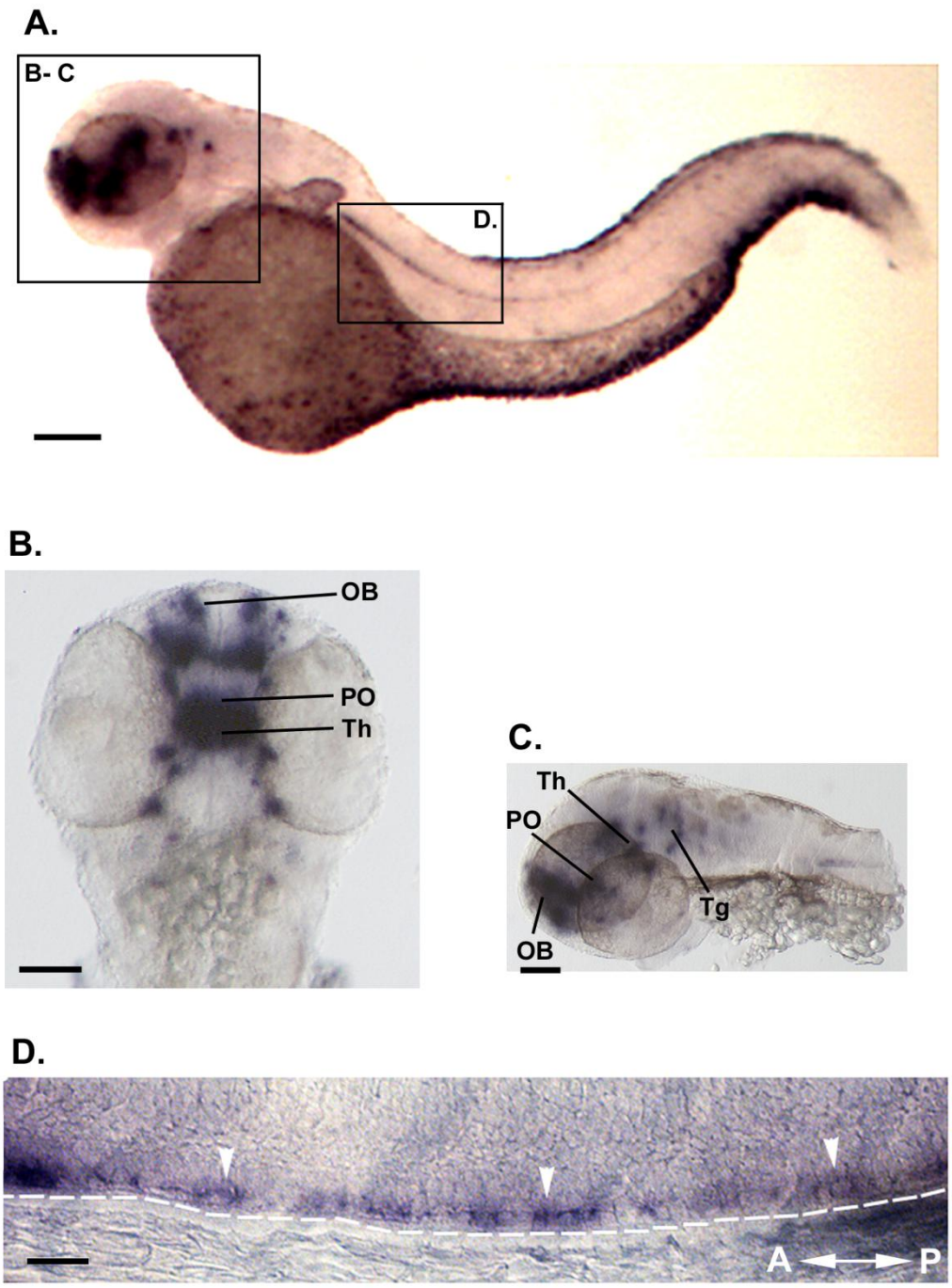

Figure 3.7 Whole mount *NOS1* transcript expression at 48hpf
A, Whole NOS1-riboprobe labelled embryo at 48hpf. Boxes demark regions outlined in panels ***B-D***. ***B,*** Dorsal view of *NOS1* mRNA expressing brain regions. *NOS1* transcript localises in the olfactory bulb (OB), pre-optic (PO) region and thalamic (Th) region. ***C,*** Lateral whole mount view illustrating *NOS1* mRNA in the OB, PO, Th, and also in the tegmentum (Tg). ***D,*** Longitudinal image of the spinal cord at 48hpf stained with *NOS1* riboprobes. Arrowheads indicate *NOS1* expressing spinal cells spanning the ventral spinal cord. Dotted line in ***D*** delimits the ventral border of the spinal cord. A, anterior; P, posterior. Scale bars: ***A-C,*** 100µm; ***D,*** 15µm.

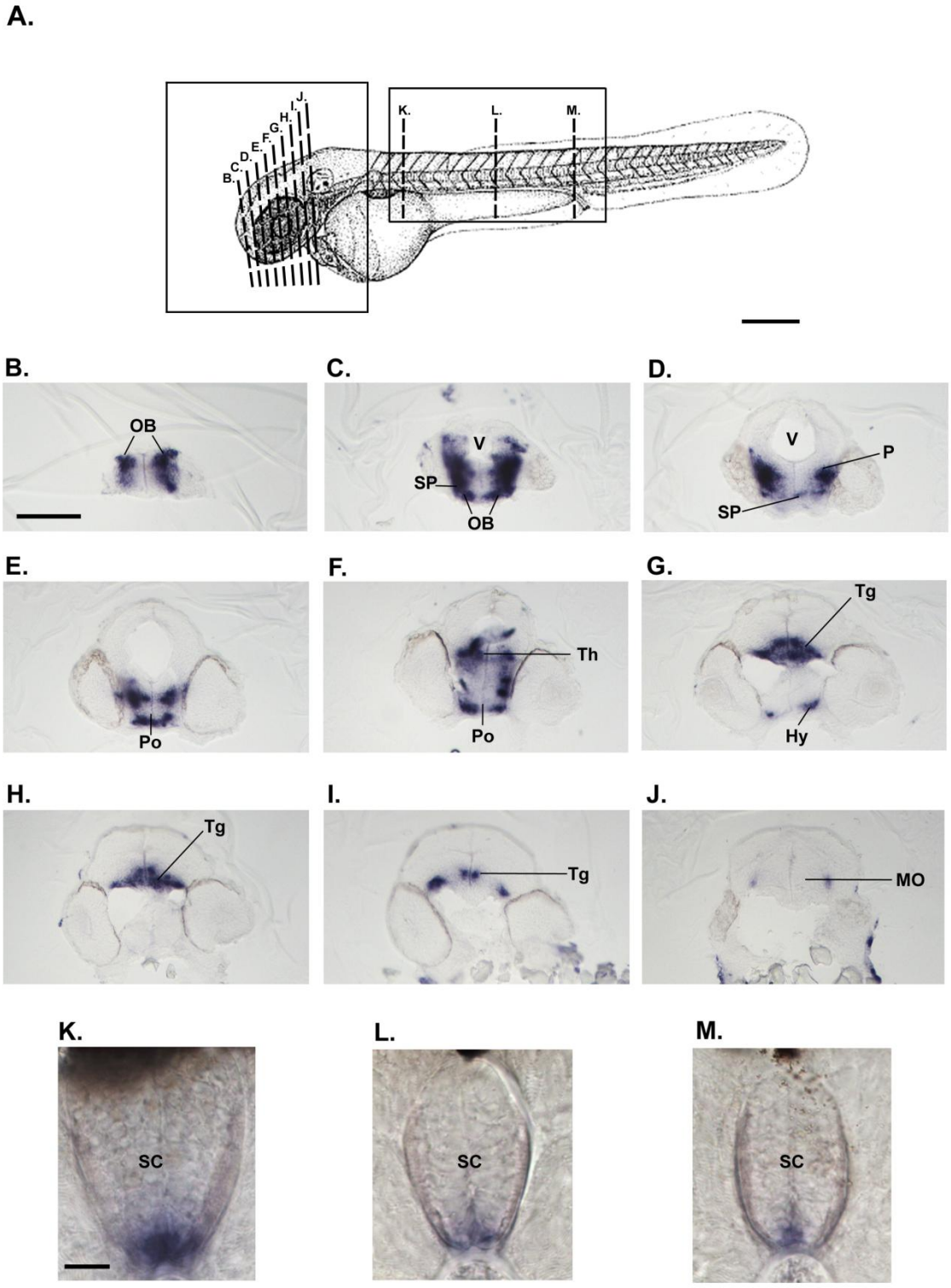

Figure 3.8 *NOS1* mRNA expression in sectioned tissue at 48hpf

A. Schematic diagram of a 48hpf embryo (from Kimmel et al, 1995). dashed lines illustrate the approximate region depicted in subsequent panels. ***B-J,*** Cross sections of brain tissue labelled with *NOS1* riboprobes. *NOS1* mRNA expression is detected in the olfactory bulb (OB; ***B, C***), subpallium (SP; ***C, D***), pallium (P; ***D***), pre-optic region (Po; ***E, F***), thalamic region (Th; ***F***), tegmentum (Tg; ***G-I***), hypothalamus (Hy; ***G***) and medulla oblongata (MO; ***J***). ***K-M,*** *NOS1* transcript localises in the ventral spinal cord (SC) in sectioned tissue. Scale bars: ***A***, 250µm; ***B-J*** (in ***B***), 50µm; ***K-M***, 25µm.

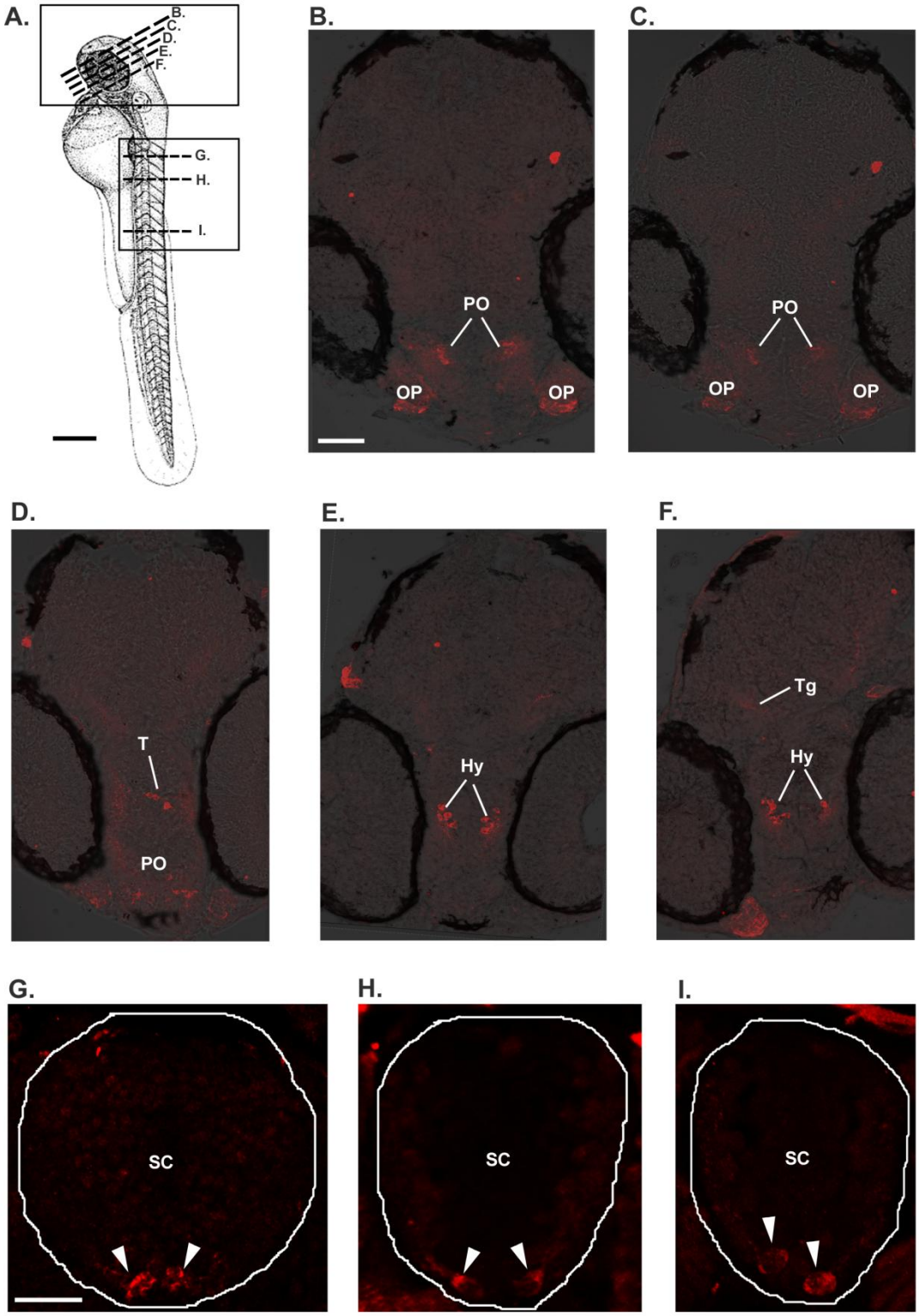

Figure 3.9. NOS1 protein expression in the brain and spinal cord at 48hpf
A, Schematic diagram of a 48hpf zebrafish embryo (taken from Kimmel et al, 1995), demonstrating the regions of interest for the subsequent panels. ***B-F,*** NOS1 immunopositive cells are detected in the olfactory placode (OP; ***B, C***), pre-optic region (PO; ***B-D***), thalamic region (T; ***D***), hypothalamic domains (Hy; ***E, F***) and tegmentum (Tg; ***F***). ***G-I,*** NOS1 protein is expressed in ventral spinal cells in sectioned spinal cord (sc) tissue. Scale bars: ***A***, 250µm; ***B-F***, 50µm; ***G-I***, 20µm.

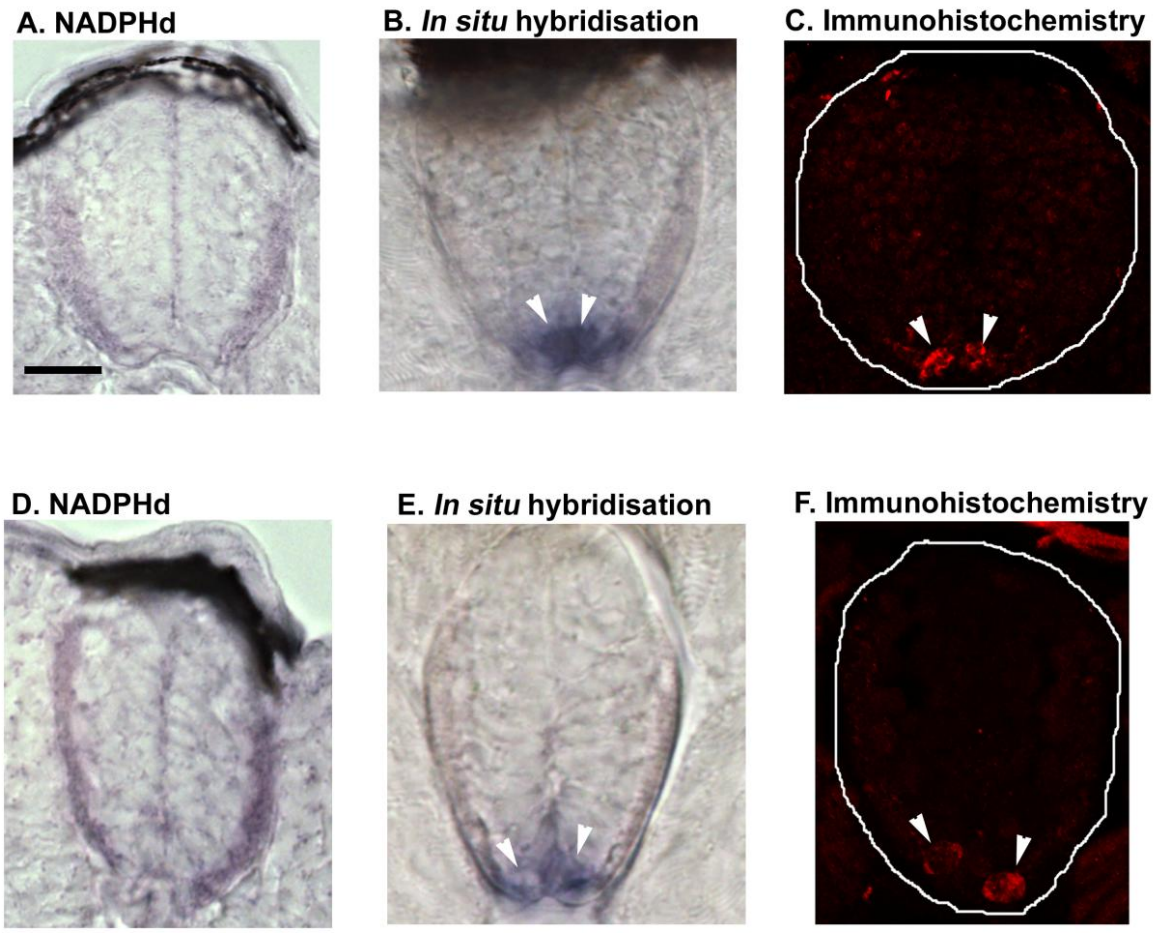

Figure 3.10 NADPH diaphorase histochemistry in sectioned spinal tissue at 48hpf
A-F, Transverse spinal sections demonstrating NADPHd histochemical staining (***A,D***), *in situ* hybridisation (***B,E***) and NOS1 immunohistochemical staining (***C,F***) in 48hpf embryos. NADPH diaphorase staining does not localise in ventral spinal cells (***A, D***) and does not correlate with *NOS1* mRNA (***B, E***) or NOS1 protein (***C, F***) expression patterns in the ventral spinal cord. Scale bar: ***A-F*** (in ***A***), 25µm.

3.2.4 Spatial distribution of NOS1 throughout the CNS in larval zebrafish (72hpf)

3.2.4.1 NOS1 expression in the brain

By the third day of development, *NOS1* mRNA expression was widespread throughout the brain (Figure 3.11A-B). Due to the dense accumulation of *NOS1* transcript throughout the brain, the specific *NOS1* expressing regions could only be determined in sectioned tissue. Transverse sections demonstrated *NOS1* riboprobe labelling within the olfactory bulb, pallium, sub-pallium, thalamic region, pre optic region, post optic commissure, posterior tuberculum, hypothalamic region, optic tectum and medulla oblongata (Figure 3.12B-J). Additionally *NOS1* mRNA was detected for the first time within the eye, in presumptive amacrine cells and cells of the inner plexiform layer (Figure 3.12E-F). The expression pattern of *NOS1* mRNA at 72hpf was similar to that observed at 48hpf, however during this 24hr period some brain regions such as the thalamic region, hypothalamic region and medulla oblongata exhibited progressively more intense, spatially expanded expression (Figure 3.12D-J *cf*. Figure 3.8F-J) whereas staining in the olfactory bulb and pre optic region became less intense and more spatially restricted by 72hpf (Figure 3.12B-D *cf*. Figure 3.8B-F). This might reflect spatial and temporal differences in the developmental functions of the NOS1 gene.

By 72hpf NOS1 immunoreactive cells were identified within the majority of brain regions that expressed NOS1 transcript (Figure 3.13B-J). However, in comparison to mRNA, protein labelling was more restricted in these domains (Figure 3.13B-J *cf*. Figure 3.12B-J). Furthermore, no protein was detected in the amacrine cells, pallium or

sub-pallium. At this stage populations of NOS1 immunoreactive fibers could also be detected in regions outside of the brain that corresponded to the branchial arch (Figure 3.13G ;arrows) and the enteric nervous system (Figure 3.13I-J; arrows).

3.2.4.2 NOS1 expression in the spinal cord

By 72hpf *NOS1* riboprobe labelling spanned the ventral aspect of the spinal cord (Figure 3.11A, C). Examination of sectioned tissue at this stage revealed that NOS1 mRNA was present in caudal regions as well as more rostral regions (Figure 3.12M *cf.* Figure 3.12K-L). In addition, NOS1 immunohistochemistry identified correlated regions of protein expression along the ventral spinal cord in a similar manner to *NOS1* mRNA expressing domains (Figure 3.13K-M).

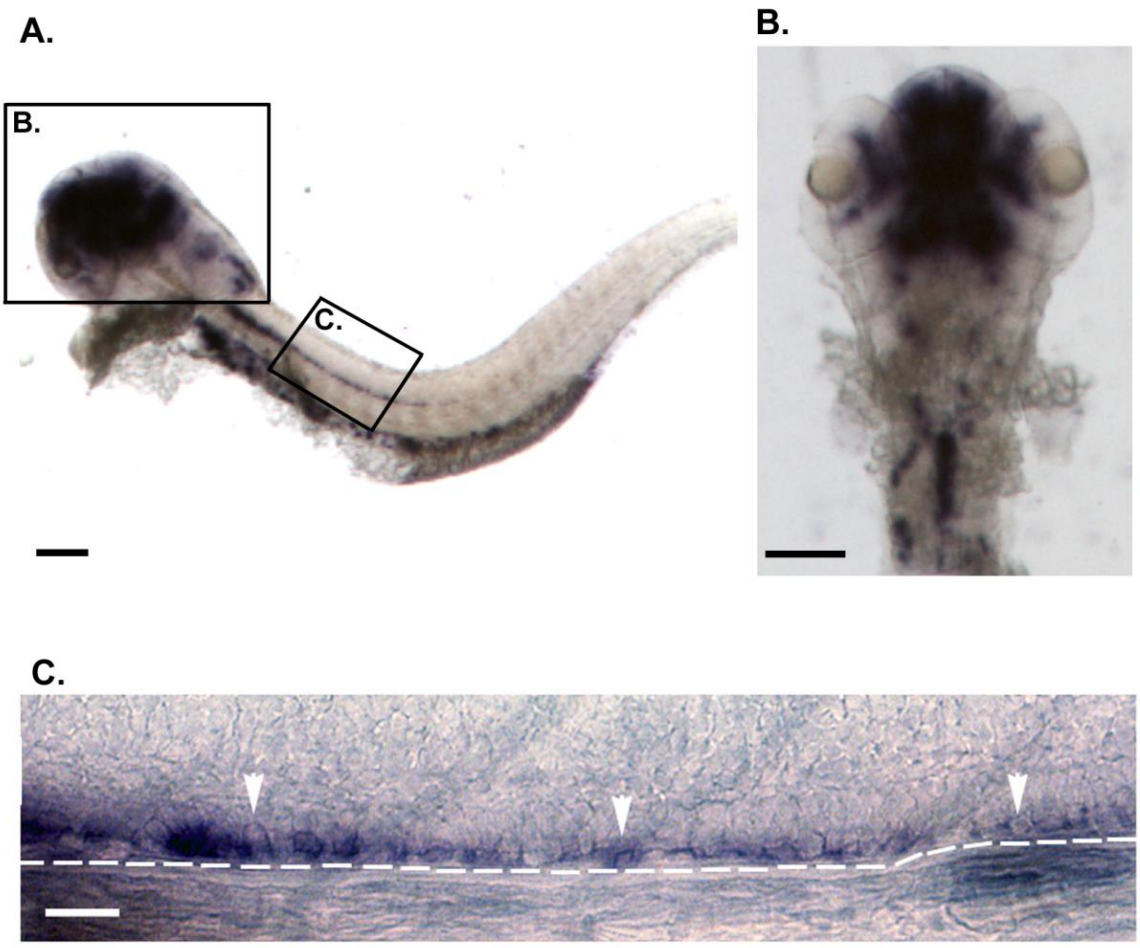

Figure 3.11 Distribution of *NOS1* transcript in whole mount larvae at 72hpf
A, Whole NOS1 riboprobe labelled 72hpf zebrafish. *NOS1* is expressed heavily throughout the brain by 72hpf, as such specific domains could not be determined. Boxes illustrate region of interest in the subsequent panels. ***B,*** Dorsal view of *NOS1* expression within the brain in whole mount 72hpf zebrafish larvae. ***C,*** Longitudinal image of the spinal cord depicting *NOS1* mRNA expression spanning the ventral spinal cord. Arrowheads indicate regions of *NOS1* expression. Dotted line in ***C*** delimits the ventral border of the spinal cord. Scale bars: ***A-B,*** 100µm; ***C,*** 15µm.

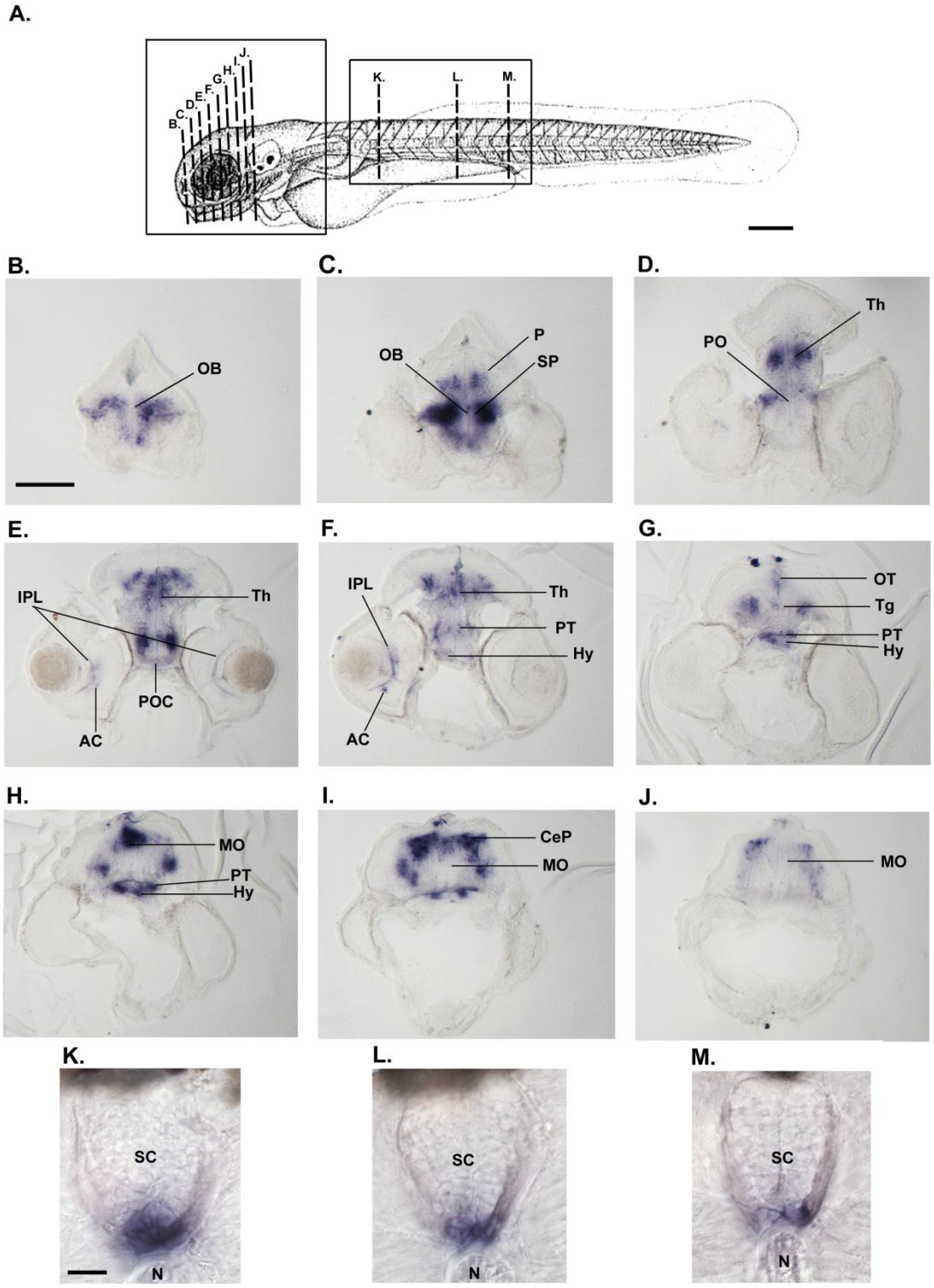

Figure 3.12 *NOS1* mRNA distribution in sectioned brain and spinal cord tissue at 72hpf
A, Schematic diagram of a 72hpf zebrafish (taken from Kimmel et al, 1995), dashed lines illustrate the approximate locations depicted in the subsequent panels. ***B-J,*** *NOS1* transcript localises in the olfactory bulb (OB; ***B-C***), pallium (P; ***B***), subpallium (SP; ***B***), pre-optic region (PO; ***D***), thalamic region (Th; ***D-F***), post-optic commissure (POC; ***E***), amacrine cells (AC; ***E-F***), inner plexiform layer (IPL; ***E-F***), posterior tuberculum (PT; ***F-H***), hypothalamus (Hy; ***F-H***), tegmentum (Tg; ***G***), optic tectum (OT; ***G***), medulla oblongata (MO; ***H-J***) and cerebellar plate (CeP; ***I***) in sectioned brain tissue at 72hpf. ***K-L,*** *NOS1* mRNA is expressed in the ventral aspect of the spinal cord (SC) at 72hpf in cross sectioned spinal tissue. N, notochord. Scale bars: ***A***, 250µm; ***B-J*** (in ***B***), 50µm; ***K-L*** (in ***K***), 10µm.

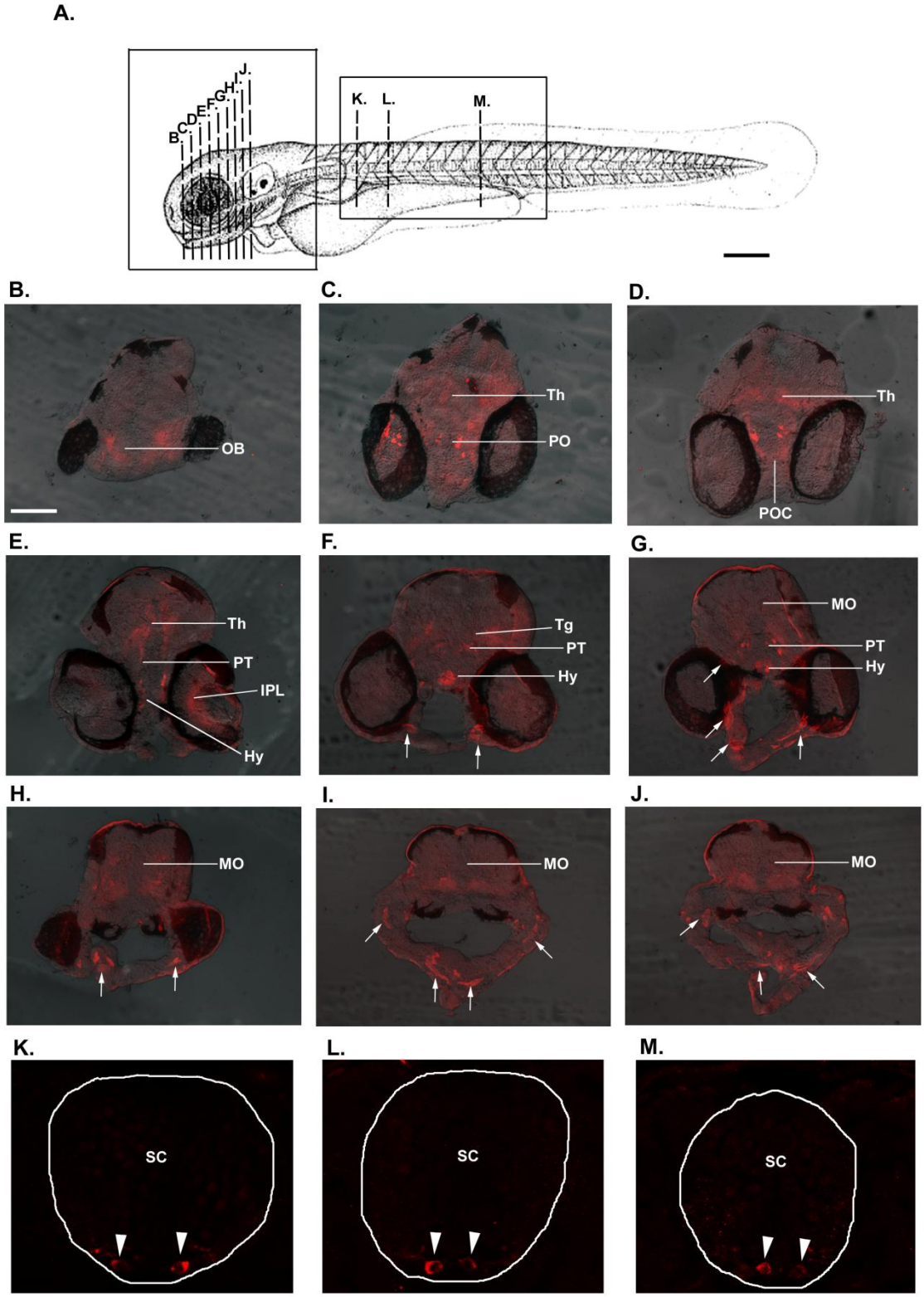

Figure 3.13 NOS1 protein expression in sectioned brain and spinal tissue at 72hpf.
A, Schematic diagram of a 72hpf zebrafish (taken from Kimmel et al, 1995), demonstrating the regions of interest for the subsequent panels. ***B-J*** NOS1 protein is distributed in the regions of the olfactory bulb (OB; ***B***), pre-optic area (PO; ***C***), thalamus (Th; ***C-E***), post-optic commissure (POC; ***D***), hypothalamus (Hy; ***E-G***), posterior tuberculum (PT; ***E-G***), inner plexiform layer (IPL; ***E***), tegmentum (Tg; ***F***), and medulla oblongata (MO; ***G-J***). Arrows indicate NOS1 immunopositive fibers outside of the brain, in brachial arches (***F-H***) and in the gut (***I-J***). ***K-M,*** NOS1 protein localises in ventral spinal cells in spinal cord (SC) cross sections. Scale bars: ***A***, 250µm; ***B-J*** (in ***B***) 100µm; ***K-M*** (in ***K***) 10µm.

3.2.5 Characteristics of NOS1 expressing cells in the developing spinal cord

Initial studies demonstrated that at distinct points during zebrafish development, NOS1 is expressed in dorsolateral (Figures 3.2,3.3;3.5) and ventromedial (Figures 3.3-3.9) domains within the spinal cord. Expression in these regions differed temporally, with dorsovental cells transiently expressing NOS1 between 30 and 40hpf whilst ventromedial cells expressed NOS1 from 35hpf onwards. As expression in dorsal populations was transient and sparse, the persistent ventral population was chosen as the principal focus of study.

Ventral nitrergic spinal neurons were examined between 48hpf and 72hpf as by these stages they were prevalent and likely to exhibit clear morphological characteristics. NOS1 immunostaining of nitrergic cells at these stages revealed that they had characteristics of spinal interneurons, with processes projecting to form a loose fascicle immediately dorsal to their somata (Figure 3.14A-B). The majority of NOS1 cells had processes that projected caudally before joining the fascicle (Figure 3.14D), although a smaller proportion were shown to have short bipolar processes (Figure 3.14D, lower panel).

To more accurately gauge the dorsovental location of nitrergic cells, isolated spinal tissue was co-stained with anti-NOS1 and anti-3A10, the latter of which recognises TAG-1 (Furley et al., 1990) expressed by a subset of hindbrain neurons that send axons through the MLF of the spinal cord. This revealed that nitrergic spinal neurons lie directly below the MLF (Figure 3.14C) with axons that appeared to either join or run in close proximity to the MLF (Figure 3.14C).

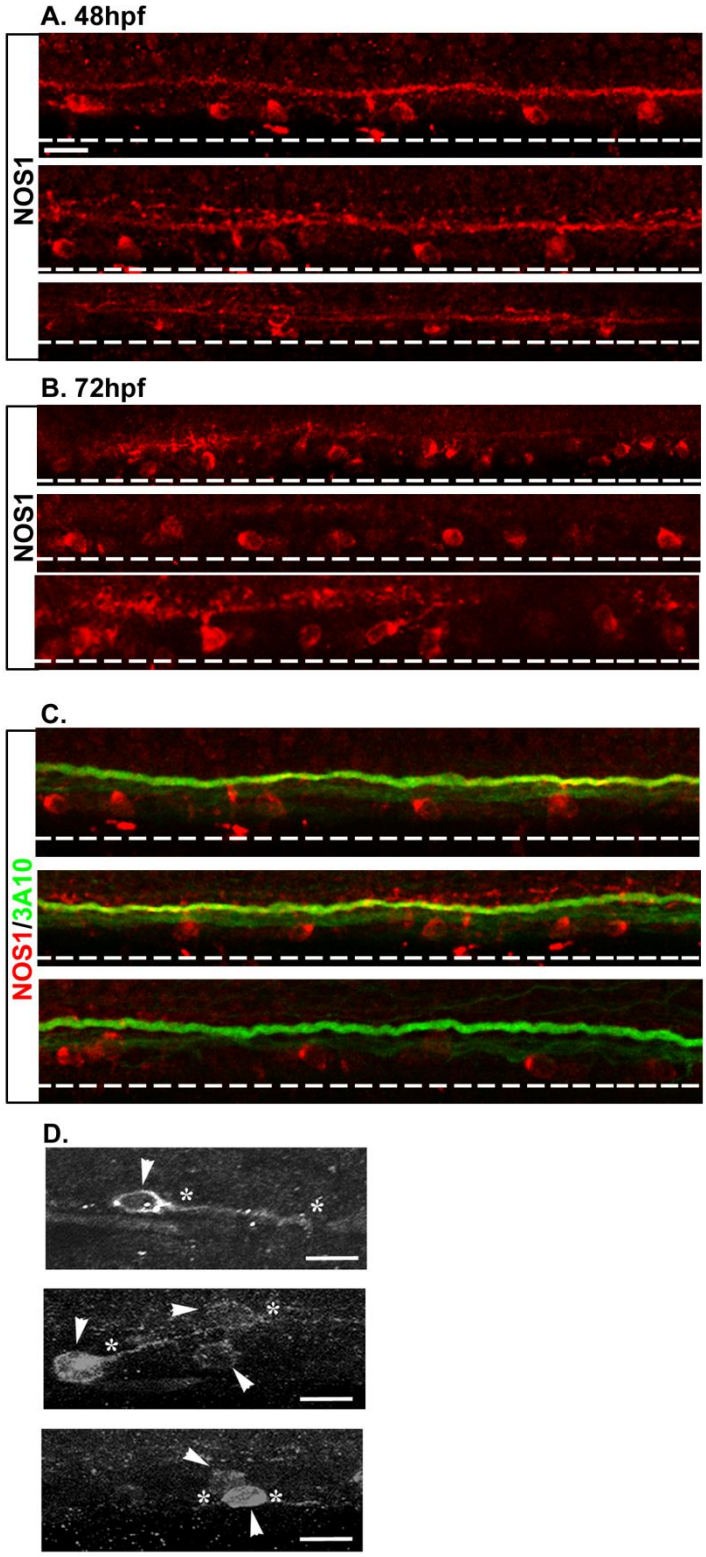

Figure 3.14. NOS1 expressing cells in the ventral spinal cord
A-B, Longitudinal sections of spinal tissue showing NOS1 immunopositive spinal cells spanning the ventral aspect of the spinal cord at 48hpf (***A***) and 72hpf (***B***). ***C,*** NOS1 immunopositive cells (red) lie directly beneath the Mauthner axon (immunolabelled with anti-3A10 antibodies, green) in the ventral spinal cord. ***D,*** High magnification images of individual NOS1 immunopositive cells. Asterisks illustrate processes extending from the soma (arrowhead). Dotted lines demark the ventral border of the spinal cord. Scale bars: ***A-C*** (in ***C***), 10µm; ***D***, 8µm.

3.2.6 Classification of NOS1 expressing spinal cells

Previous studies have postulated that, based on their ventromedial location, ventral nitrergic cells of the spinal cord are in fact secondary motoneurons (Poon et al., 2003). Whilst their extreme ventral location argued against this, the possibility that motoneurons expressed NOS1 was investigated with dual-riboprobe labelling using anti-NOS1 and the motoneuron-specific marker islet1/2. *NOS1* expression domains were found to be spatially distinct from those of motoneurons, with nitrergic cells lying directly ventral to *islet* expressing motoneuron pools (Figure 3.15A-B). This finding was confirmed with immunohistochemical staining of NOS1 and islet1/2 protein, which exhibited a pattern of staining indistinguishable from that seen in transcript studies (Figure 3.15C). To examine the spatial relationship between motoneurons and nitrergic cells in detail, GFP was expressed in individual motoneurons prior to immunostaining with NOS1 antibodies. This demonstrated that motor axons coursed close to nitrergic neurons as they extended ventrally towards their spinal exit points (Figure 3.15D-E).

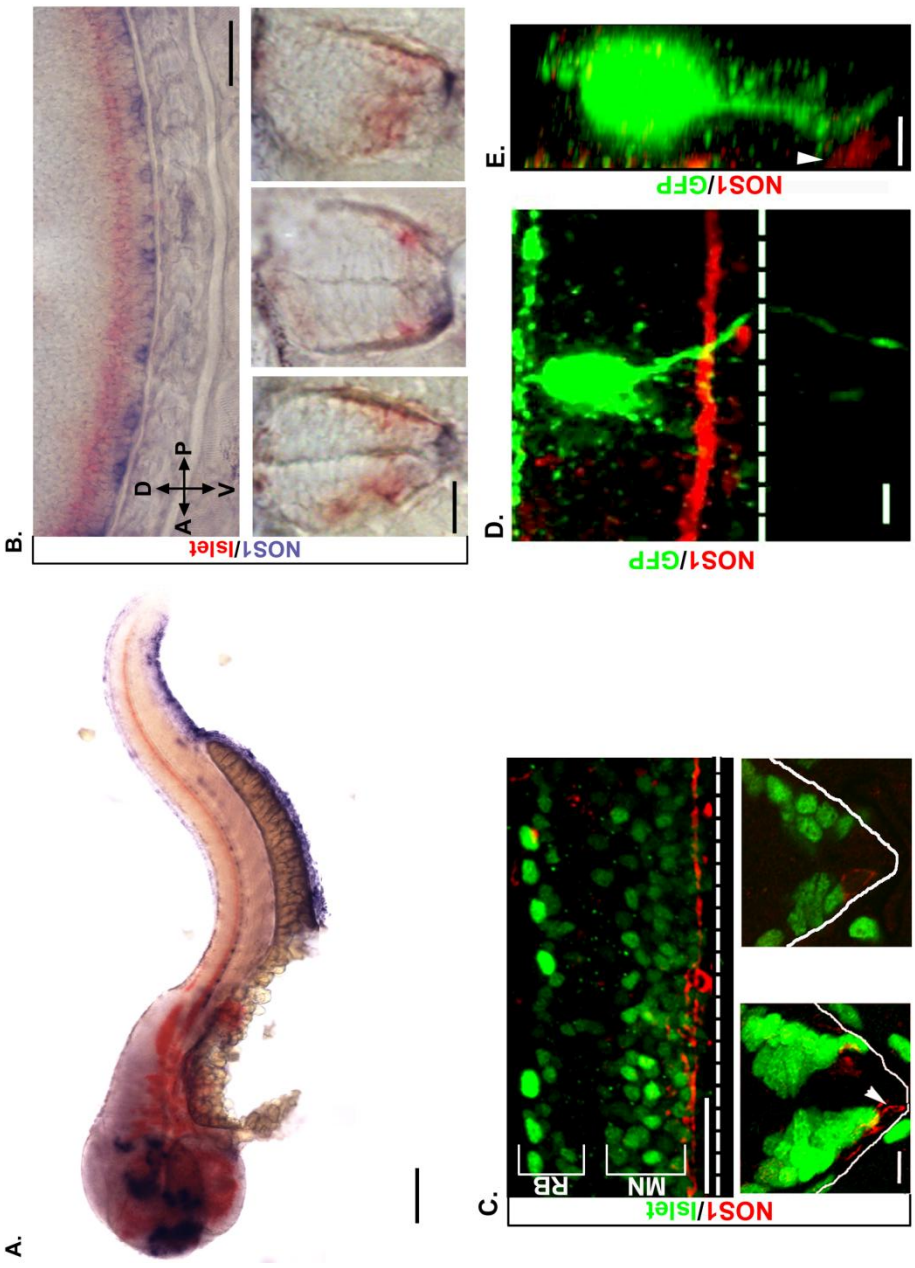

Figure 3.15 Spatial relationship between nitrergic cells and motoneurons
A, Wholemount *NOS1* (blue) and *islet 1/2* (red) dual riboprobe-labelled 48hpf embryo demonstrates that nitrergic spinal cells are located ventrally to motoneurons. ***B,*** Longitudinal section (top panel) and cross sections (lower panel) of spinal probe labelled spinal tissue reveals that *NOS1* labelled cells lie ventrally to *Islet* labelled cells. ***C,*** Top panel: longitudinal image of a 48hpf spinal cord immunolabelled with NOS1 (red) and islet 1/2 (green). Lower panels: transverse spinal cord sections labelled with NOS1 and islet 1/2. ***D,*** Longitudinal image of GFP-labelled primary motoneuron (green) contacting NOS1-immunoreactive processes (red). ***E,*** Z-stack image of panel ***D*** rotated 90° to reveal the close spatial relationship of the motoneuron axon to a nitrergic spinal cell as the axon exit the spinal cord. Dashed lines delimit the ventral border of the spinal cord. Solid lines mark the lateral margins of the ventral border of the spinal cord. D, dorsal; V, ventral; A, anterior; P, posterior. Scale bars: ***A***, 100µm; ***B***, 25µm; ***C-E***, 8µm.

3.2.7 Spatial relationship between nitrergic interneurons and known ventral spinal cell classes

The next aim was to determine which interneuron class expressed NOS1. Previous studies have shown that only three classes of neuron; KA; VeMe and VeSe cells (Figure 3.16A) occupy a position directly ventral to the MLF (Higashijima et al., 2004b, Hale et al., 2001, McLean and Fetcho, 2004, Martin et al., 1998). These interneuron classes differ in their neurotransmitter phenotype and axon trajectory: VeMe and VeSe have descending axons and express glutamate and serotonin respectively (Higashijima et al., 2004a, Higashijima et al., 2004b, Hale et al., 2001, McLean and Fetcho, 2004) whilst KA neurons have ascending axons and express GABA (Higashijima et al., 2004b, Martin et al., 1998).

To ascertain whether any of the aforementioned interneuron populations are nitrergic, spinal cord sections were immunolabelled with NOS1 and either anti-GABA (Figure 3.16B), anti-glutamate (Figure 3.16C) or anti-serotonin (Figure 3.16D-E) antibodies. NOS1 did not co-localise with GABA suggesting that nitrergic neurons were not KA interneurons (Figure 3.16B). Similarly, no co-localisation was observed with glutamate, indicating that nitrergic neurons were not VeMe cells (Figure 3.16C). Finally, NOS1 immunoreactive cells were also found to be discrete from VeSe cells, as NOS1 and serotonin antibody staining did not overlap (Figure 3.16D-E). This suggests that nitrergic neurons comprise a novel class of spinal interneuron.

To compare the number of nitrergic cells to known interneuron classes of the ventral spinal cord, the spatial distribution of VeMes, VeSes and KAs was compared to the distribution of nitrergic spinal cells over a 30μm section of the spinal cord. In doing

so, it was observed that NOS1 expressing spinal cells display a similar density to the VeMe cell class (Figure 3.16F). In conclusion, the above data support the proposal that although NOS1 expressing cells share a similar spatio-temporal distribution to VeMe and VeSe cells, they appear to belong to a novel nitrergic cell class of the ventral spinal cord.

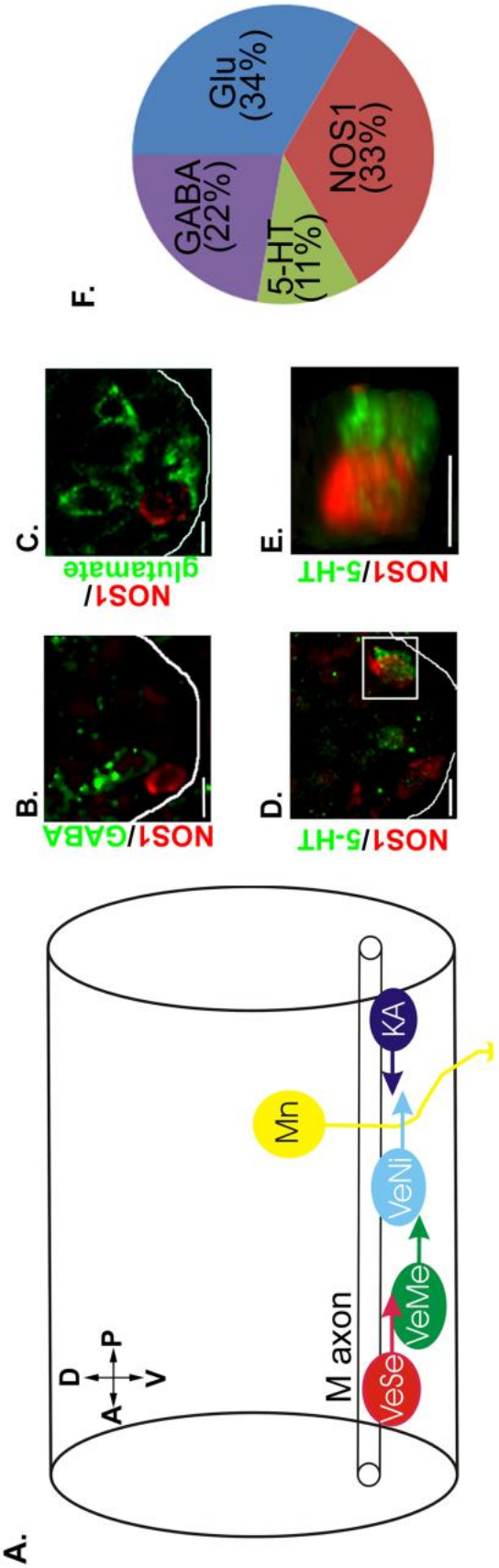

Figure 3.16 Spatial relationship between nitrergic cells and known cell classes of the ventral spinal cord.
A, A schematic diagram illustrating known cell classes of the ventral spinal cord. Serotonergic VeSe (red), glutamatergic VeMe (green), GABAergic KA (dark blue) and ventral nitrergic (VeNi; light blue) all lie beneath the M axon. ***B-D,*** Cross sections of the ventral spinal cord at 72hpf labelled with NOS1 (red) and GABA (green; ***B***), glutamate (***C***) and serotonin (***D***). ***E,*** Region highlighted in ***D*** rotated through 90° to reveal the spatial separation between NOS1 and serotonergic immunopositive cells. ***F,*** Relative proportion of glutamate (2 cells/30µm), GABA (3 cells/30µm), serotonin (1 cell/30µm) and NOS1 (3 cells/30µm) immunopositive cells in the spinal cord. A, anterior; P, posterior; D, dorsal; V, ventral. Solid lines in ***B-D*** demark the lateral margins of the ventral spinal cord. Scale bars: ***B-E,*** 8µm.

3.3 Discussion

This study has examined the developmental distribution of NOS1 with combined *in situ* hybridisation and immunohistochemical techniques. In agreement with previous studies, NOS1 was observed in several discrete CNS regions from early stages of life. Moreover, the close correlation between *NOS1* mRNA and protein expression demonstrates that this enzyme is translated in major NOS1 expressing regions of the zebrafish embryo. Finally, detailed analysis of spinal nitrergic populations suggests that they comprise a novel population of interneurons that occupy ventral positions, just below the motoneuron populations. As these cells do not co-express any known neurotransmitters of the ventral spinal cord, they likely represent a novel, previously unknown class of spinal cord interneuron.

3.3.1 Comparison of NOS detection methods in early stage zebrafish

Of the three methods used to detect NOS1 expression in this study antibody staining and riboprobe labelling generated broadly comparable results whilst NADPHd histochemistry did not. This suggests that although the diaphorase approach is a reliable method for labelling nitrergic cells in a wide range of species (Foster and Phelps, 2000, Dawson et al., 1991, Virgili et al., 2001, Bruning and Mayer, 1996a, Smeets et al., 1997, Campese et al., 2007, Lopez and Gonzalez, 2002, Hope et al., 1991) it is of little utility in early stage zebrafish. There are two explanations why the diaphorase method might not have generated meaningful results. First, this approach depends upon the ability of NOS1 to resist fixation and it is possible that in zebrafish,

this enzyme does not retain catalytic activity under these conditions. Second, as NOS only represents a fraction of the total NADPHd activity present in cells (Tracey et al., 1993) it is possible that strong, non-specific oxidoreductase reactions precluded accurate detection of NOS1 (Kishimoto et al., 1993). In support of the latter argument, several studies have shown that diaphorase staining can be detected in neurons that do not express NOS1 (Kluchova et al., 2002, Dawson et al., 1991, Virgili et al., 2001, Smeets et al., 1997, Lopez and Gonzalez, 2002, Huang et al., 1997, Giraldez-Perez et al., 2008, Pullen and Humphreys, 1995).

In contrast to the diaphorase staining method, there was broad correlation between *NOS1* transcript and protein expression domains. As these independent approaches generated comparable findings, it is highly likely that expression patterns observed with these methods were due the presence NOS1 transcript/protein. However, two issues merit consideration. First, the probe used in this study was targeted against the first six exons (1200bp) of the *NOS1* mRNA sequence and as such this probe may not have detected alternatively spliced NOS1 transcripts that involve exon addition/removal within this region. However, this is unlikely as there is currently little evidence that NOS1 is alternately spliced in lower vertebrates and bioinformatic analysis of zebrafish NOS1 (accession #: NP_571735) shows that whilst 3 isoforms of *NOS1* mRNA exist, they differ only in their non-coding regions and therefore generate an identical protein product. Moreover, prior transcript studies that used probes targeted to different exons of zebrafish NOS1 RNA revealed essentially identical staining patterns to those described here (Holmqvist et al., 2004, Poon et al., 2003).

The second issue relates to antibody specificity. As there are currently no commercially available antibodies designed to target zebrafish NOS1, this study employed a polyclonal antibody targeted against amino acids 1411-1425 of human NOS1. Bioinformatic comparison revealed 93% identity between human and zebrafish NOS1 in the region targeted by this antibody, suggesting that it is likely to cross-react with zebrafish NOS1. In support of this, NOS1 antibody staining patterns closely resembled those observed with NOS1 riboprobe labelling. However, it is possible that this antibody may have recognised additional epitopes within the zebrafish proteome that are not present on the NOS1 protein, which could account for the occasional discrepancy between antibody and transcript labelling.

3.3.2 Spatio-temporal distribution of NOS1 within the zebrafish brain during early development

3.3.2.1 NOS1 expression in the 24-40hpf zebrafish brain

In agreement with previous studies (Poon et al., 2003, Holmqvist et al., 2004), the current findings demonstrate that *NOS1* mRNA is first detected in the embryonic VRC. However, the precise onset of NOS1 expression remains open to debate: Poon et al. (2003) first detected *NOS1* mRNA in the VRCs at 16hpf whilst Holmqvist et al. (2004) observed expression at 19hpf. The current study did not attempt to detect NOS1 expression until 24hpf. Irrespective of the precise timing of expression, the precocious emergence of NOS1 in this region, which broadly corresponds to the extension of pioneering axons from the forebrain, suggests that NO signalling may regulate early stages of neuronal development (Ross et al., 1992). By 40hpf NOS1 expression is

restricted to forebrain regions with additional transcript detected in the VCC and DRC. However, at this stage differences were observed between protein and mRNA expression. Specifically, NOS1 protein was not detected in the VCC, a region where transcript could be seen. The most likely explanation for this is that there is a delay between transcription and translation in these cells. In contrast, protein was observed in the olfactory placodes which was consistently devoid of transcript. One possible explanation for this is that the antibody was detecting additional epitopes other than NOS1 as expression between mRNA and transcript in other brain regions was largely consistent. Despite these differences, the progression of NOS1 expression in the VCC and DRC is in general harmony with previous teleost studies (Poon et al., 2003, Holmqvist et al., 2004).

3.3.2.2 NOS1 expression in the 48-72hpf zebrafish brain

At the second day of development NOS1 transcript expression had increased greatly with regions of the olfactory bulb, pre-optic area, thalamic region, tegmentum, medulla oblongata sub-pallium, pallium, post optic commissure and hypothalamic domains expressing *NOS1* mRNA. Although protein expression correlated largely with *NOS1* transcript distribution, some areas such as the pallium, sub-pallium, olfactory bulb and medulla oblongata demonstrated no protein labelling. Again, the most likely explanation for this is that there is a delay between the onset of mRNA expression and protein translation. Although there are obvious temporal differences in development, NOS1 is expressed in broadly homologous brain regions of late embryonic/early postnatal murine species (Terada et al., 1996, Terada et al., 2001, Giuili et al., 1994,

Keilhoff et al., 1996) and embryonic to metamorphosising amphibian species (Lopez and Gonzalez, 2002, McLean and Sillar, 2001).

By the third day of development *NOS1* transcript persists in the same regions observed at 48hpf, with the addition of new NOS1 expressing cells in the posterior tuberculum, optic tectum and retinal cells. These domains share a similar temporal homology with *Xenopus* as NADPH reactivity does not appear in retinal cells or the tectum until pre-metamorphic stages (McLean and Sillar, 2001, Lopez and Gonzalez, 2002, Peunova et al., 2001, Cogen and Cohen-Cory, 2000) which roughly correlates to day three in zebrafish development.

3.3.3 Expression of NOS1 within the zebrafish spinal cord during early development

Although NOS1 expressing spinal cells have been documented to first appear at *ca.* 55hpf in the ventral aspect of the spinal cord (Holmqvist et al., 2004), the current study demonstrates that it is expressed much earlier and is segregated into two distinct populations. First, from as early as 30hpf, a small number of dorsolateral cells transiently express NOS1 for no more than 10h. Second from 35hpf onwards, a persistent population of nitrergic cells express NOS1 in the ventral spinal cord.

The most prevalent region of NOS1 expression was ventral to the MLF. In agreement with this finding, NOS1 expressing cells have also been detected in the adult ventral spinal cord of goldfish (Giraldez-Perez et al., 2008), frogs (Munoz et al., 2000, Bruning and Mayer, 2001), rats (Dun et al., 1993, Saito et al., 1994, Terenghi et

al., 1993), pigeons (Necker, 2004), humans (Terenghi et al., 1993) and mice (Bruning and Mayer, 1996b, Dun et al., 1993) as well as in developing mammals (Foster and Phelps, 2000, Wetts et al., 1995) avians (Necker, 2005), teleosts (Holmqvist et al., 2004, Poon et al., 2003, Villani, 1999) and amphibians (Ramanathan et al., 2006, McLean and Sillar, 2001, Crowe et al., 1995). This therefore suggests that NOS1 expressing ventral neurons serve an evolutionarily important function.

The spinal dorsal population comprised only a small number of cells during a transient developmental period (between 30hpf and 40hpf). Although this study did not attempt to define the transient NOS1 expressing cell population, based on their size and location they are likely to comprise the DoLA population (Kuwada et al., 1990, Higashijima et al., 2004b, McLean and Fetcho, 2008). Transient expression in this region is suggestive of discrete developmental functions for NOS1 in a small subset of dorsal interneurons. In mammals, NOS1 is similarly expressed in cells of the dorsal spinal cord (Saito et al., 1994, Dun et al., 1993, Bruning and Mayer, 2001, Necker, 2004, Giraldez-Perez et al., 2008). This region is involved in sensory processing and functional studies have demonstrated that NO regulates sensory transduction in developing and adult spinal tissue (Chu et al., 2005, Guan et al., 2007, Schmidtko et al., 2009). In zebrafish, stable NOS1 expression was not observed during early development which on first glance suggests that NO is not involved in sensory processing. However, at the stages studied, spinal sensory transduction is immature, being mediated exclusively by the “Rohon-Beard” mechanosensory neurons. These cells act as a transient and primitive mechanosensory system in the embryo but subsequently undergo programmed cell death at around day 5 (Reyes et al., 2004) to

be replaced by peripheral dorsal root ganglion (DRG) neurons that adopt mature sensory functions (An et al., 2002). As this study did not cover the stage by which zebrafish sensory transduction is mediated by DRGs, the possibility remains that by later stages, when dorsal sensory circuits are assembled, NOS1 is involved in sensory transduction. In support of this hypothesis, other anamniotes such as *Rana esculenta* develop NOS expression in DRGs during early metamorphosis (stage 43; Cristino et al., 2004) and by adulthood all DRGs are NADPH-d reactive (Crowe et al., 1995).

3.3.4 Classification of NOS1 expressing cells within the ventral spinal cord

Previous studies have suggested that, based on their dorsoventral location, ventral nitrergic neurons comprise a subset of spinal motoneurons (Poon et al., 2003). However, the present study demonstrates that this is not the case. Thus zebrafish motoneurons, like those of other vertebrates (Necker, 2004, Ramanathan et al., 2006, Bruning and Mayer, 2001,Kluchova et al., 2002, Dun et al., 1993, Saito et al., 1994, Wetts and Vaughn, 1994, Spike et al., 1993) do not express NOS1, at least under normal physiological conditions. Rather, ventral nitrergic cells appear to comprise a distinct population of spinal interneuron. These cells lie directly beneath the MLF in a domain occupied by three known interneuron classes; KAs, VeMes and VeSes (Bernhardt et al., 1990, McLean and Fetcho, 2004, Hale et al., 2001). However, immunohistochemistry demonstrated that none of the aforementioned interneuron cell classes express NOS1, therefore suggesting that ventral nitrergic cells comprise a novel class of spinal interneuron that reside in close apposition to motoneurons as well as other ventral interneuron classes.

Nitrergic interneurons have been identified in the ventral spinal cord of the chick by embryonic day 5. These also reside in ventral domains close to motoneuron populations and are shown to modulate the elaboration of motoneuron dendrites (Xiong et al., 2007). The early development of zebrafish nitrergic neurons and their close proximity to nascent motoneurons suggests that they may act to regulate aspects of development of these cells. In further support of this proposal, NO has previously been demonstrated to modulate neurite development in a range of both vertebrate and invertebrate neuronal cell types (Trimm and Rehder, 2004, Van Wagenen and Rehder, 1999, Van Wagenen and Rehder, 2001, Welshhans and Rehder, 2005, Welshhans and Rehder, 2007, Ditlevsen et al., 2007, Hindley et al., 1997, Yamazaki et al., 2006, Seidel and Bicker, 2000, Cogen and Cohen-Cory, 2000, Campello-Costa et al., 2000). This possibility is addressed in detail in Chapter 4.

4. Roles of NO during development of zebrafish motor axons

4.1 Introduction

The development of functional synaptic contacts is a key event during neural network assembly that that involves the addition, stabilisation and elimination of neurites (Luo, 2002). Plasticity during axonodendritic development helps ensure that neurons assemble appropriate numbers of contacts with the correct targets, and a major goal of neuroscience research is to understand the signalling pathways that regulate this process. Although recent research has contributed substantial insights in to potential factors that exert control over neurite outgrowth (for review, see Sanes and Yamagata, 2009), the precise mechanisms remain to be established.

A mounting body of evidence suggests that NO has key regulatory functions during nervous system ontogeny (Magalhaes et al., 2006, Peunova et al., 2001, Peunova and Enikolopov, 1995, Villalobo, 2006) and recent studies have demonstrated that NO can influence neurite growth and synaptogenesis in a range of cell and tissue culture systems. However, the functional role of NO during *in vivo* development remains poorly described.

4.1.1 Nitrergic regulation of axon development and synapse elimination in cell and tissue culture models

4.1.1.1 Invertebrate models

Cultured invertebrate neurons have proven to be an invaluable tool for studying the regulation of growth cone behaviour and synapse formation. Their large size and amenability to culture methods has facilitated detailed study of growth cone

dynamics at a resolution not afforded by mammalian models. In this context, studies of cultured *Helisoma trivolvis* B5 neurons have generated a comprehensive and detailed account of the role NO signalling plays during growth cone navigation. Here, NO is believed to act as a "stop and search" signal which ensures that growing axons halt to sample for instructive cues within their environment. Initial evidence for this came from local application of NO donors (3-morpholinosydnonimine (SIN-1) or 3-(2-hydroxy-1-methyl-2-nitrosohydazino)-*N*-methyl-1-propanamine (NOC-7) to developing *Helisoma* B5 growth cones. Exposure to these drugs effectively slows neurite outgrowth although the dynamics of this response depends on the specific concentration of donor applied: lower (100µM) concentrations slow neurite outgrowth, intermediate concentrations (250µM) halt neurite outgrowth and high concentrations (1mM) result in neurite collapse and retraction (Trimm and Rehder, 2004). In addition, low and intermediate NO concentrations increase the sensory radius of the growth cone by increasing filopodia length and decreasing filopodia number (Van Wagenen and Rehder, 1999, Van Wagenen and Rehder, 2001, Trimm and Rehder, 2004, Welshhans and Rehder, 2005, Welshhans and Rehder, 2007). This combined effect is thought to slow down the rate of growth cone advance ("stopping") whilst at the same time allowing it to interrogate the local environment for instructive signals ("searching"). However, a basal level of NO signalling is also likely to be critical for facilitating normal neurite growth as pharmacological NOS blockade (with L-NAME) also slows neurite outgrowth (Trimm and Rehder, 2004).

The physiological relevance of the aforementioned responses has been confirmed by using a "sender-receiver" paradigm where NOS-expressing B5 cells (the

"senders") are positioned near to isolated NOS-negative "receiver" cells. Stimulation of the sender promotes NO synthesis, allowing the consequences to receiver growth cones activity to be monitored. Using this paradigm, it has been shown that the sender can elicit changes in receiver growth cone behaviour that mimic those seen with NO donors. Moreover, by placing the sender at incremental distances from the responder, it has been shown that a single physiological source of NO can evoke changes in growth cones that lie some 100-150µm away, although maximal effects were observed between 20 and 60µm (Tornieri and Rehder, 2007). This demonstrates that a single physiological source of NO can have potent effects on the growth of cells that reside at some distance from it.

Cell and tissue culture models have also provided evidence that NO regulates maturation of invertebrate visual circuits. In *Drosophila*, NOS is expressed in the optic lobe at a time when photoreceptors are innervating this region and establishing an ordered topographic arrangement of synaptic connections (Gibbs and Truman, 1998). Inhibition of NOS with L-NAME (100µM) or application of the NO scavenger 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO) perturbs patterning of optic lobe connections, causing retinal axons to project beyond their targets.

4.1.1.2 Vertebrate models

Nitrergic regulation of axon growth has also been comprehensively studied in vertebrate cell and tissue culture models with studies often generating conflicting reports. First, several studies have shown that NO enhances the growth of nascent

neurites, an effect which often depends upon the presence of cues in the ECM that trigger NO-dependent changes in neurite extension. For example, the ability of neural cell adhesion molecule (NCAM) to promote neurite extension in isolated rat hippocampal neurons is inhibited by the NOS inhibitor NG-Monomethyl-L-arginine acetate (L-NMMA; Ditlevsen et al., 2007), suggesting that NCAM-dependent neurite growth requires functional NOS activity.

NO has also been shown to promote neurite outgrowth in rat pheochromocytoma PC12 cells. This is a carcinoma cell line derived from the adrenal medulla which, upon treatment with nerve growth factor (NGF), adopts a neuronal fate and begins to extend neuritic processes (Fujita et al., 1989). Studies show that the effects of NGF are, at least in part, mediated by NO signalling: NGF treatment induces NOS catalysis (Peunova and Enikolopov, 1995) and pre-incubation with L-NAME (Yamazaki et al., 2006) disrupts neurite outgrowth. Similarly, the ECM component laminin also promotes NO synthesis and, like NGF, NOS inhibition disrupts this effect (Rialas et al., 2000).

In contrast, a number of reports also show that NO inhibits outgrowth of cultured neurites. In rat dentate granule cells, NOS1 localises at sites of neurite initiation (Gotti et al., 2005) and exogenous application of the NO donors $(CH_3N[N(O)NO]^-(CH_2)_3NH_3^+$ (NOC13) and S-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) induces growth cone collapse, an effect which is attenuated by prior application of the sGC inhibitor 6-anilino-5,8-quinolinequinone (LY83583; Yamada et al., 2006). Likewise, in rat DRG neurons, exposure to the NO donor SIN-1 results in rapid growth cone collapse (Hess et al., 1993) whilst NOC-7 induces a similar effect in chick sensory

neurons (He et al., 2002a). NO has also been shown to repress neuritogenesis in cultured vertebrate visual neurons: growth cones of *Xenopus* (Renteria and Constantine-Paton, 1996) and chick retinal ganglion cells collapse following exposure to NO donors (Ernst et al., 1999, Gallo et al., 2002). However, this effect can be are occluded by pre-treatment with brain derived neurotrophic factor (BDNF), which stabilises filamentous actin (F-actin) and prevents NO-induced depolymerisation of the growth cone cytoskeleton (Ernst et al., 2000). This suggests that interaction between presynaptic BDNF secretion and postsynaptic NO synthesis may regulate elimination and retention of retinal axons during development.

4.1.2 Nitrergic regulation of axon development and synapse elimination in vivo

4.1.2.1 Pharmacological studies in invertebrate models

A growing body of invertebrate studies demonstrates that NO can also regulate axon growth during *in vivo* development. For example, in the grasshopper embryo the NO signalling pathway regulates axonogenesis of the developing antenna pioneer neurons. Developmental inhibition of NOS results in perturbation of antennal axonogenesis causing retardation of outgrowth and thus preventing growth cones from reaching the CNS (Seidel and Bicker, 2000).

4.1.2.2 Pharmacological studies in vertebrate models

Whilst cell and tissue culture studies suggest that axonal modelling in a wide range of vertebrate brain regions is regulated by NO, supportive *in vivo* evidence is

principally limited to nascent visual circuits. During early stages of mammalian development, axons of retinal ganglion cells grow through the optic nerve, either cross to the contralateral side or remain ipsilateral at the optic chiasm, then continue growth through the optic tract where they terminate at either the lateral geniculate nucleus (LGN) or the visual cortex. Here axons segregate into a number of regular feature maps which include retinotopic maps, eye-specific layer, ocular dominance columns, orientation maps and direction preference maps (for review, see Huberman et al., 2008). In the adult visual system retinal ganglion axons from both eyes terminate in adjacent but non-overlapping layers, however during development axonal inputs from the two eyes are intermixed. In order to achieve synaptic specificity maps undergo a process of refinement which involves weakening and elimination of imprecise connections whereas correctly targeted projections are strengthened and maintained (for review, see Shatz, 1996). This process is activity-dependent with correlated activity of input neurons causing synaptic strengthening.

Previous studies have shown that activity-dependent visual synapse remodelling requires postsynaptic NMDA receptor activation (Hahm et al., 1991, Ernst et al., 1999, Cline et al., 1987, Kleinschmidt et al., 1987), leading to the proposal that a retrograde signal drives concomitant presynaptic changes in axon structure. The activity-dependence and diffusible nature of NO made it an attractive candidate retrograde signal during this process. The first direct demonstration that NO regulates activity-dependent refinement of visual circuits was derived from studies of On/Off sublaminae in the ferret LGN. Here NOS1 is transiently expressed in postsynaptic domains of the LGN concomitant with periods of axon refinement and synaptic pruning

(Cramer et al., 1996, Cramer and Sur, 1999). Inhibition of NOS with daily injections of 7-NI significantly reduces sublamination and disrupts the formation of On/Off sublaminae (Cramer and Sur, 1999). Circumstantial evidence that this effect is coupled to NMDAR signalling comes from studies showing that blockade of LGN NMDARs with MK801 mimics that of NOS disruption (Hahm et al., 1991). Subsequently, a similar role for NO in the refinement of connections in the rat superior colliculus, a midbrain structure involved in generation of visuomotor reflexes, was identified. Here, high levels of diaphorase activity can be observed during periods of refinement of superior colliculus connections (Tenorio et al., 1996, Campello-Costa et al., 2000) and developmental treatment with NOS inhibitor N_{ω}-nitro-L-arginine (L-NOARG) inhibits pruning of retinal projections up to the second postnatal week, which is past the point that these are normally refined (Campello-Costa et al., 2000).

NO has also been shown to play a role in wiring of lower vertebrate retinotectal pathways. The observations that chick postsynaptic tectal neurons become NADPH-diaphorase positive upon innervation by retinal axons, and that regions that fail to become innervated by axons never exhibit diaphorase reactivity (Williams et al., 1994), suggest that NO signalling may be important for refinement of synaptic connections in this region. Subsequent pharmacological studies demonstrated that L-NAME and L-NOARG prevent the elimination of transient retinotectal projections (Wu et al., 1994) and lead to a loss of topographic precision during innervation (Wu et al., 2001). The observation that MK-801 can cause a dose-dependent decrease in NOS activity (Ernst et al., 1999) and also delay pruning of misdirected ipsilateral retinotectal projections,

suggests that NO acts as a retrograde synaptic signal during activity-dependent refinement of chick visual circuits (Ernst et al., 1999).

Time-lapse imaging studies of the *Xenopus* tadpole optical tectum have demonstrated that the NO-dependent axonal remodelling has complex temporal dynamics. Here, tectal injection of NO donors (200µM SNAP or 1mM (*Z*)-1-[2-(2-aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA-NONOate)) increases the addition of new branches but not their long term stabilisation, whereas inhibition of NOS (1mM 3-bromo-7-nitroindazole (3B-7-NI)) results in a more dynamic remodelling characterised by increases in both addition and elimination of individual branches (Cogen and Cohen-Cory, 2000). As NOS inhibition promotes branch addition and elimination, NO may have a role in promoting the transient stabilisation of co-active synaptic connections (Cogen and Cohen-Cory, 2000).

Whilst NO and glutamate signalling clearly have the capacity to remodel visual circuits, there is little direct evidence to suggest these systems work co-operatively. Indeed, notwithstanding evidence that segregation of ferret LGN on/off sublaminae and chick ipsilateral retinotectal projections requires interaction between NMDA and NO signalling (Hahm et al., 1991, Cramer et al., 1996, Ernst et al., 1999), there is a significant body of evidence demonstrating that these pathways do not interact. For example, the formation of artificially induced ocular dominance columns in 3-eyed *Rana pipiens* tadpoles is regulated by NMDA but not NO signalling (Cline et al., 1987). Similarly, inhibition of NOS in the feline visual cortex does not have a significant effect on ocular dominance shifts induced by monocular deprivation (Ruthazer et al., 1996, Reid et al., 1996) whereas NMDAR blockade renders this region resistant to sensory

deprivation (Kleinschmidt et al., 1987, Gu et al., 1989). Whilst these studies suggest that NO does not act as a retrograde signal during activity-dependent remodelling, it is important to note that, due to multiple NOS isozymes and drug efficacy limitations, residual NOS activity was most likely present during the aforementioned studies (Reid et al., 1996). Thus, the precise functional roles of NOS/NMDAR during *in vivo* visual circuit formation remain poorly understood.

4.1.2.3 NOS knockout studies in mice

As pharmacological studies suggest that NO has key roles during visual system development, it was assumed that genetic NOS1 ablation would yield similar findings. However, in this context, murine models have failed to provide strong evidence that NO regulates synaptogenesis. For example, the refinement of mouse ipsilateral retinocollicular and superior colliculular pathways are unaltered by NOS1 knockout (Mize et al., 1998, Scheiner et al., 1999) and similarly axon guidance is unperturbed in DRG neurons of these animals (Tojima et al., 2009). Outside the visual system, one study has shown that NOS1 knockout reduces the size of dendritic trees in developing spinal motoneurons, an effect which relies on activity-dependent NMDA receptor signalling (Inglis et al., 1998). However, notwithstanding this study, other mouse knockout studies have failed to provide compelling evidence that NOS1 abrogation plays widespread roles in the refinement of neuronal circuits. However, two caveats must be considered before discounting the involvement of NOS1 in this process. First, other NOS isozymes may be able to functionally compensate for the loss of NOS1 (Tao et al., 2004, Hervera et al., 2010). Indeed, this appears to be the case in at least some

parts of the developing visual system. During development, synapses from the retinocollicular pathway undergo dramatic refinement across the medio-lateral axis of the superior colliculus and whilst onset of refinement is unperturbed in NOS1 knockout mice (Mize et al., 1998), it is markedly delayed in NOS1/NOS3 double knockouts (Wu et al., 2000a, Wu et al., 2000b), although it is important to note that refinement still occurs in these mice at later stages of development (Wu et al., 2000a, Wu et al., 2000b). Second, the lines used for the majority of knockout studies harbour a deletion within the second exon of NOS1. However, as NOS1β and NOS1γ splice isoforms use alternate translation start sites that exclude exon two, some residual NOS1 activity persists in knockout animals (Brenman et al., 1997) and this may be sufficient to mask the effects of NOS1 disruption.

4.1.3 Aims of this study

Although there is strong evidence to suggest that NO regulates the growth of cultured vertebrate neurons, there remains little evidence to support the premise that this molecule has functional roles during *in vivo* development. Moreover, the currently available evidence is limited to visual pathways and little is known of how other regions rich in NOS1 expression (such as the spinal cord) are affected by NO signalling. This chapter aims to directly address these problems by studying the functional role of NO signalling during *in vivo* zebrafish spinal cord development. This region contains a subset of nitrergic interneurons that form in the ventral spinal cord mid-way through embryonic life (see chapter 3) and using combined molecular and pharmacological

approaches spinal NO signalling is disrupted and the morphological changes in spinal neuron development assessed. Using this approach, a novel role for NO signalling during motoneuron development is identified: disruption of nitrergic signalling impacts motor axon arborisation by controlling the rate of neuromuscular arbour addition over the first three days of development. Further analysis reveals that NO signalling acts by influencing the branching of primary motoneurons, without affecting the growth of secondary motoneuron or spinal interneuron axons. Taken together, these findings show for the first time that endogenous NO signalling is a potent endogenous regulator of vertebrate motor axon development.

4.2 Results

4.2.1 Antisense knockdown of zebrafish NOS1 dramatically increases motor axon branching

As a first step towards investigating the functional role of NOS1 in spinal tissue, antisense morpholino oligonucleotides (AMOs) were used to disrupt NOS1 translation from the onset of development. To do this an AMO was designed to recognise and bind the 5' UTR region of zebrafish *NOS1* mRNA. Injection of this construct (10µM) into early stage (1-4 cell) zebrafish blastulae (see Methods section 2.9) would be expected to specifically inhibit ribosomal access to *NOS1* mRNA, resulting in "knockdown" of NOS1 translation.

To confirm that AMO-injected fish exhibited reduced spinal NOS1 protein expression, embryos were raised until the appropriate stage and processed for NOS1

immunoreactivity. AMO injected fish revealed a complete loss of NOS1-specific staining at 48hpf (Figure 4.1B *cf.* 4.1A; n=12) and a marked reduction of NOS1 staining at 72hpf (Figure 4.1D *cf.* 4.1C; n=12). Thus the AMO abolished NOS1 protein expression during the first two days of life and resulted in diminished expression on the third, with residual immunoreactivity likely a consequence of reduced AMO efficacy as the embryo increased in volume (Nasevicius and Ekker, 2000, Ekker, 2000). To validate the specificity of the NOS1 AMO, embryos were injected with a control 'mismatch' AMO (10µM), in which 5 nucleotides were switched along its sequence, thereby rendering it unable to bind to NOS1 mRNA. As expected, injection of this AMO failed to disrupt NOS1 antibody staining at 48hpf (Figure 4.1F *cf.* 4.1E; n=7).

In order to confirm that NOS1 AMOs did not cause gross developmental defects (Scherer and Rossi, 2003, Branch, 1998, Gerety and Wilkinson), the morphology of AMO injected fish at 72hpf was compared to uninjected controls. Figure 4.1G shows that, upon visual comparison, NOS1 knockdown larvae were morphologically similar to control larvae of the same age. Taken together these findings show that AMO-mediated knockdown methods reliably abrogate NOS1 protein expression without causing gross developmental perturbations.

During the first three days of embryonic life studied here, primary and secondary motoneurons undergo axonogenesis at around 17hpf (Eisen, 1991) and 22hpf (Pike et al., 1992) respectively. Once they have extended their axons in to the musculature, primary motor axons branch extensively throughout development to make multiple synaptic contacts with all EW muscle fibers within their reach (Westerfield et al., 1986). Secondary motoneuron axons branch much less and

terminate on a small subset of both ER and EW muscle fibers within their region (Westerfield et al., 1986). To determine the developmental consequence of NOS1 knockdown on spinal motoneuron development, znp-1 antibodies were used (Melancon et al., 1997) which label synaptotagmin 2 proteins that are concentrated in primary and secondary motor axons, as well as spinal interneuron axons, of the zebrafish trunk. As such, immunostaining of control embryos at 35hpf revealed that motor axon fascicles had extended into the musculature but formed only a small number of arbours. Similarly, motor axons of 35hpf NOS1 AMO-injected fish had exited the spinal cord and appeared to take appropriate trajectories within the muscle in a manner indistinguishable from control fish (Figure 4.2D *cf.* 4.2A; figure 4.2G). In contrast, 48hpf NOS1 AMO injected fish exhibited a 57% increase in motor axon branches when compared to age matched controls (Figure 4.2E *cf.* 4.2B; Figure 4.2H; control: 23 ± 1.5 branches per fascicle (bpf) *cf.* AMO: 36 ± 1.5bpf, $p=1x10^{-4}$). This phenotype persisted into the third day in development with NOS1 AMO fish having 40% more motor axon collaterals than controls (Figure 4.2F *cf.* 4.2C; Figure 4.2I; control: 70 ± 3.9bpf *cf*. AMO: 98 ± 4.2bpf, $p=4x10^{-4}$). Conversely, mismatch AMO injected fish displayed a phenotype indistinguishable from controls at any of the developmental stages studied (Figure 4.2G-I; 35hpf: 3.9 ± 0.4bpf, p=0.20; 48hpf: 22.6 ± 1.1bpf, p=0.60; 72hpf: 75 ± 3.9bpf, p=0.40).

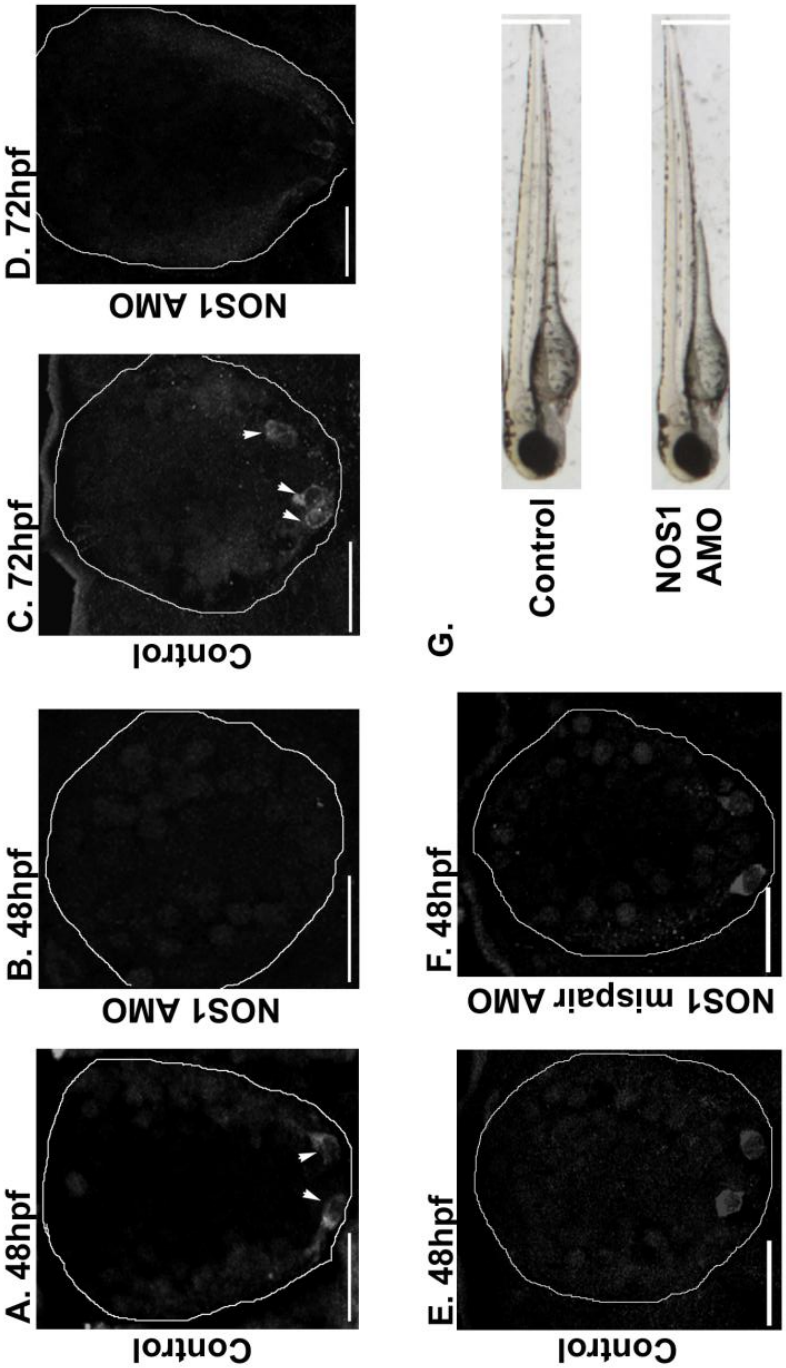

Figure 4.1 Validation of NOS1 knockdown via AMO injection

A, Transverse spinal section immunostained with NOS1 at 48hpf. ***B,*** NOS1 antibody staining is abolished in NOS1 AMO-injected embryos at 48hpf. ***C,*** 72hpf transverse spinal section immunostained for NOS1. ***D,*** NOS1 antibody staining is largely reduced in NOS1 AMO-injected larvae at 72hpf. ***E-F,*** NOS1 immunostained transverse spinal sections at 48hpf. NOS1 mispair AMO failed to disrupt NOS1 immunoreactivity (***F***). ***G,*** NOS1 AMO larvae are morphologically indistinguishable from control larvae of the same age. Scale bars: ***A-F,*** 20µm; ***G***, 600µm.

4.2.2 Antisense knockdown of endogenous NOS1 via AMO injection leads to increases in branch number but not branch length

To further characterise how NOS1 knockdown impacts motor axon growth, the number and length of neuromuscular arbours was quantified in control and NOS1 AMO fish at 48hpf (Table 4.1). To do this, each branch was assigned an order which was defined as follows: first order branches were classed as those arising from the primary axon tract; second order branches were those branches that originated from a first order branch; third order from a second order branch and so forth. Quantification revealed that for each branch order, length was not significantly affected by either NOS1 AMO injection or DETA NO (500μM) treatment. However, the total number of branches was significantly altered in both conditions, with the first to fourth order branch number significantly increased in NOS1 knockdown and significantly decreased in DETA-NO treated fish (Table 4.1). In contrast, the total number of fifth order branches was not significantly affected by either treatment, an effect that is likely due to the small number of quinary branches formed by this stage of development. Nonetheless, these findings suggest that during early development, loss of endogenous NO signalling accelerates the rate of motor axon arbour addition without altering the length of individual arbours.

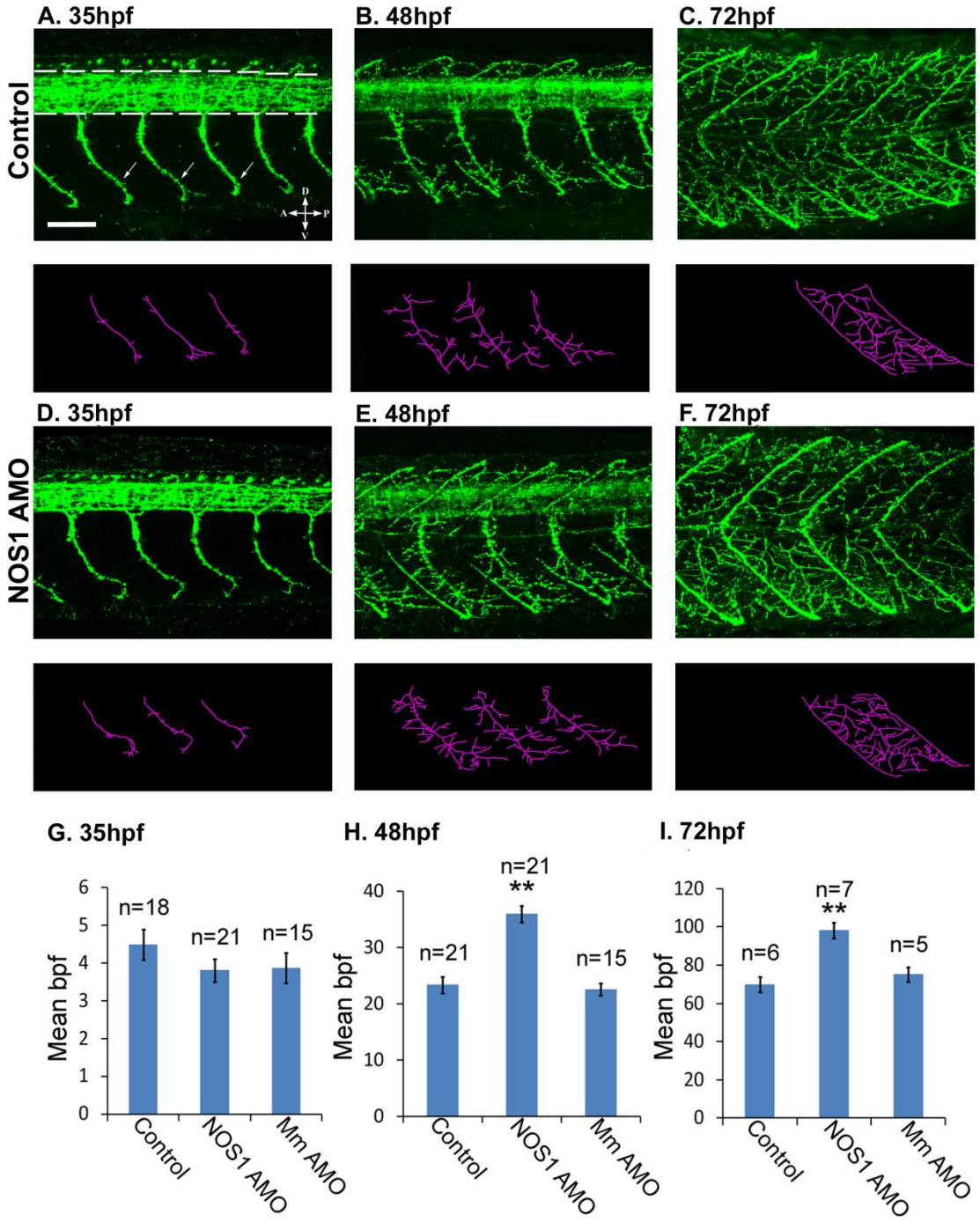

Figure 4.2 NOS1 knockdown increases motor axon branch formation

A-F, Top panels: lateral views of znp1 trunk staining in control (***A-C***) and NOS1 AMO-injected fish (***D-F***) at 35, 48 and 72hpf. Znp1 labels the neuropil (outline by hashed lines in ***A***) and motoneuron axons (arrows in ***F***). Lower panel: Neuron J tracings of motor axons ventral to the spinal cord derived directly from the panel above. ***G-N,*** Bar charts depicting the mean number (± SEM) of motor axon branches per fascicle (bpf) for control, NOS1 and mismatch (Mm) AMO at 35 (***G***), 48 (***H***) and 72hpf (***I***). Inset in ***A*** indicates the orientation of current and all subsequent figures, A, anterior; P, posterior; D, dorsal; V, ventral. Scale bars: ***A-F*** (in ***A***), 50µm. **p ≤ 0.0005.

Table 4.1 Perturbation of NOS1 levels affects branch addition but not length.

Branch order	Branch number			Branch length (μm)		
	Control	DETA NO	NOS1 AMO	Control	DETA NO	NOS1 AMO
Primary	10.8 ± 0.6	5.8 ± 0.4**	14.9 ± 0.8**	14.3 ± 0.7	11.8 ± 0.9	12.7 ± 0.6
Secondary	8.3 ± 0.8	3 ± 0.6**	11.7 ± 0.7*	9.3 ± 0.5	10.2 ± 0.7	9.5 ± 0.6
Tertiary	3.5 ± 0.5	1.3 ± 0.5*	6.3 ± 0.5**	8.3 ± 0.5	8.5 ± 0.9	8.4 ± 0.7
Quartary	0.8 ± 0.1	0.2 ± 0.2*	2.5 ± 0.5*	8.8 ± 1.3	7.9 ± 1.0	10 ± 1.2
Quinary	1.5 ± 0.5	0.0	1.6 ± 0.2	6.3 ± 1.6	0.0	4.8 ± 1.0

Table indicating the number and length of first order to fifth order branches per fascicle in 48hpf control, DETA treated and NOS1 AMO injected fish. All values are shown as mean ± SEM; n=21 for each condition; **p≤0.0005, *p≤0.005.

4.2.3 Pharmacological perturbation of NO signalling mimics the effects of NOS1 abrogation

The aforementioned results suggest that loss of endogenous NOS1 increases motor axon arborisation during early development. To confirm that this was due to attenuation of nitrergic transmission, fish were exposed to pharmacological agents deigned to disrupt NO signalling during development. First, NOS activity was inhibited with the pan-specific NOS inhibitor L-NAME (1mM). This drug was injected into the yolk sac of 24hpf fish and the consequence to motor axon branching examined over the following 48 hrs with znp1 immunohistochemistry. Similar to the NOS1 AMO phenotype, L-NAME treatment did not perturb outgrowth of the motoneuron fascicles at 35hpf (Figure 4.3A *cf.* Figure 4.3D) but caused a dramatic increase in motor axon branching at later stages such that L-NAME injected fish had 39% and 40% more motor

axon branches at 48hpf and 72hpf respectively (Figure 4.3E *cf.* Figure 4.3B; Figure 4.3H; control: 23 ± 1.9bpf *cf.* L-NAME: 32 ± 0.9bpf, $p=1x10^{-4}$; Figure 4.3F *cf.* Figure 4.3C; Figure 4.3I; control: 67 ± 2.5bpf *cf.* L-NAME: 94 ± 4.6bpf, $p=1x10^{-4}$).

To further assess the consequences of disrupting NO signalling, fish were exposed to the NO scavenger c-PTIO (900µM), a drug that rapidly scavenges free NO molecules to yield imidazolineoxyl and free NO_2 (Pfeiffer et al, 1997). When 24hpf embryos were injected with c-PTIO and their motor axons examined over the following 48hpf, a dramatic increase in motor axon branch numbers was observed from the onset of the second day of development. Specifically, c-PTIO treatment did not affect branch numbers at 35hpf but caused a 61% and 40% increase in motor axon branches at 48hpf (Figure 4.4E *cf.* Figure 4.4B; Figure 4.4H; control: 23 ± 1.9bpf *cf.* c-PTIO: 37 ± 1.2bpf, $p=1x10^{-4}$) and 72hpf (Figure 4.4F *cf.* Figure 4.4C; Figure 4.4I; control: 67 ± 2.5bpf *cf.* c-PTIO: 94 ± 3.3bpf, $p=1x10^{-4}$) respectively. Taken together these results strongly suggest that disruption of NO, either through abrogation of NOS1 function or scavenging of free NO causes marked increases in motor axon branching.

4.2.4 The NO donor, DETA-NO inhibits motor axon branch addition

Since the above findings show that loss of NO signalling increases motor axon branching, the next aim was to determine whether elevation of NO had the converse effect. In order to address this issue, 1dpf embryos were incubated in the NO donor DETA-NO (500µM). Subsequent znp-1 immunostaining revealed that this drug significantly decreased motor axon branch numbers by 27% (Figure 4.5D *cf.* Figure

4.5A; control: 5 ± 0.4bpf *cf.* DETA NO: 3.75 ± 0.3bpf, p=0.01) at 35hpf, 59% at 48hpf (Figure 4.5E *cf.* Figure 4.5B; Figure 4.5H; control: 23.4 ± 1.5bpf *cf.* DETA-NO: 9.7 ± 0.9bpf, $p=1x10^{-4}$) and 61% at 72hpf (Figure 4.5F *cf.* Figure 4.5C; Figure 4.5I; control: 70 ± 3.9bpf *cf.* DETA-NO: 27.4 ± 1.7bpf, $p=1x10^{-4}$). In order to establish whether the observed effect was due to liberation of NO gas rather than non-specific actions of DETA breakdown products, embryos were incubated in NO depleted DETA. DETA NO has an approximate half life of 20hr therefore the drug was left in solution for 1 week to deplete NO stores. As predicted these fish did not exhibit altered numbers of motor axon branches when compared to age matched controls (Figure 4.5H; control: 23.4 ± 1.5bpf *cf.* DETA: 26.9 ± 1.2bpf, p= 0.06).

To determine whether NO slowed the rate of motoneuron branch addition or alternately, induced collapse and retraction of pre-existing branches, late stage embryos (48hpf) were exposed to a 24h DETA-NO treatment. Here it was posited that if DETA-NO simply slowed branch addition then the number of branches formed after the 24h treatment period would be greater than that of 48hpf, but less than that of 72hpf control fish. Conversely, if NO was causing retraction and collapse of established branches, they would resemble much younger embryos because pre-existing branches would be stripped by DETA-NO treatment. Subsequent analysis suggests that NO slows the rate of branch addition rather than stripping established branches, as 72hpf larvae that were transiently exposed to DETA-NO revealed 41% fewer motor axon branches than untreated controls of the same age (Figure 4.6A-C; control: 69.2 ± 2.9bpf *cf.* DETA-NO: 40.7 ± 3.4bpf, $p=1x10^{-4}$) but 74% more collaterals than 48hpf controls.

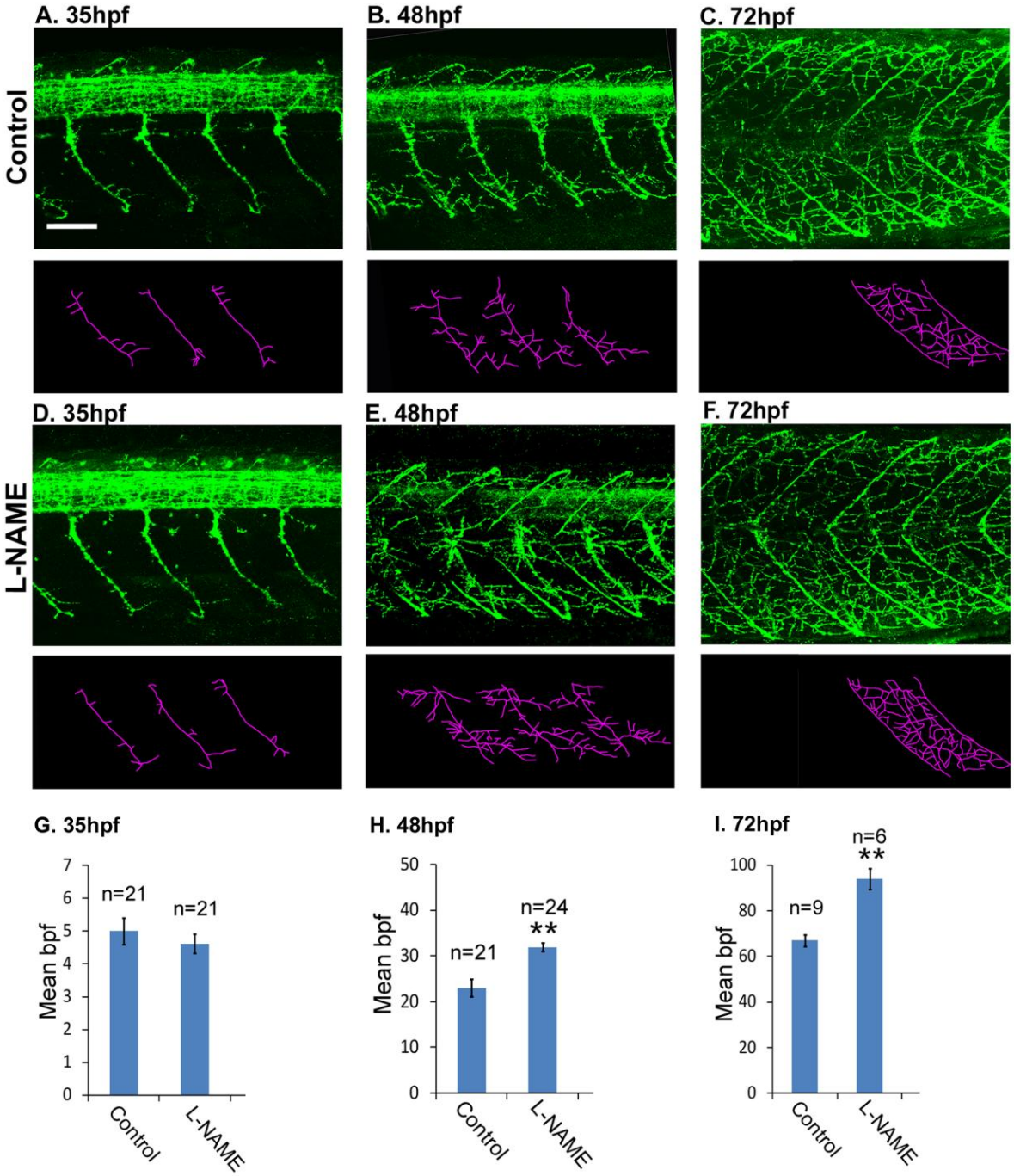

Figure 4.3 Exposure to the NOS inhibitor L-NAME increases motor axon branching
A-F, Top panels: znp1 antibody stained trunk tissue in control (***A-C***) and L-NAME-injected (***D-F***) fish at 35, 48 and 72hpf. Lower panels: Neuron J tracings of motor axons derived from the panel directly above. ***G-I,*** Bar charts depicting the mean (± SEM) number of axon branches per fascicle (bpf) for each experimental condition at 35 (***G***), 48 (***H***) and 72 (***I***) hpf. Scale bars: ***A-F*** (in ***A***), 50µm. **p ≤ 0.0005. Some data in 4.3H was contributed by Rachel Lockley.

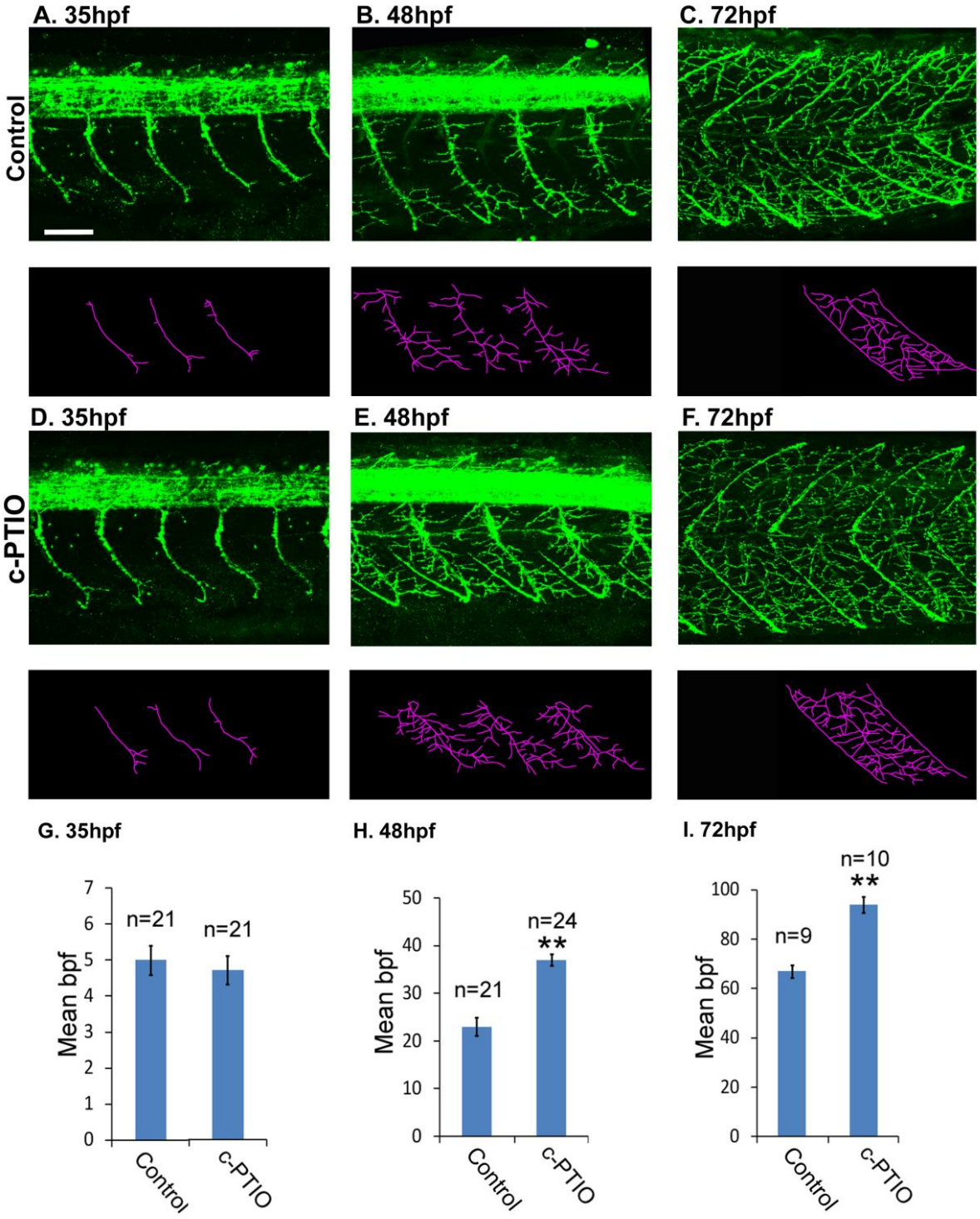

Figure 4.4 Injection of the NO scavenger c-PTIO leads to increased motor axon branching

A-F, Upper panels: lateral images of znp1 labelled trunk tissue of control (***A-C***) and c-PTIO-injected (***D-F***) fish at 35, 48 and 72hpf. Lower panels: Neuron J tracings derived from the image directly above of ventral motor axon branches. ***G-I,*** Bar charts showing the mean (± SEM) number of motor axon branches per fascicle (bpf) for both control and c-PTIO fish at 35 (***G***), 48 (***H***) and 72 (***I***) hpf Scale bars: ***A-F*** (in ***A***), 50µm. **p ≤ 0.0005.

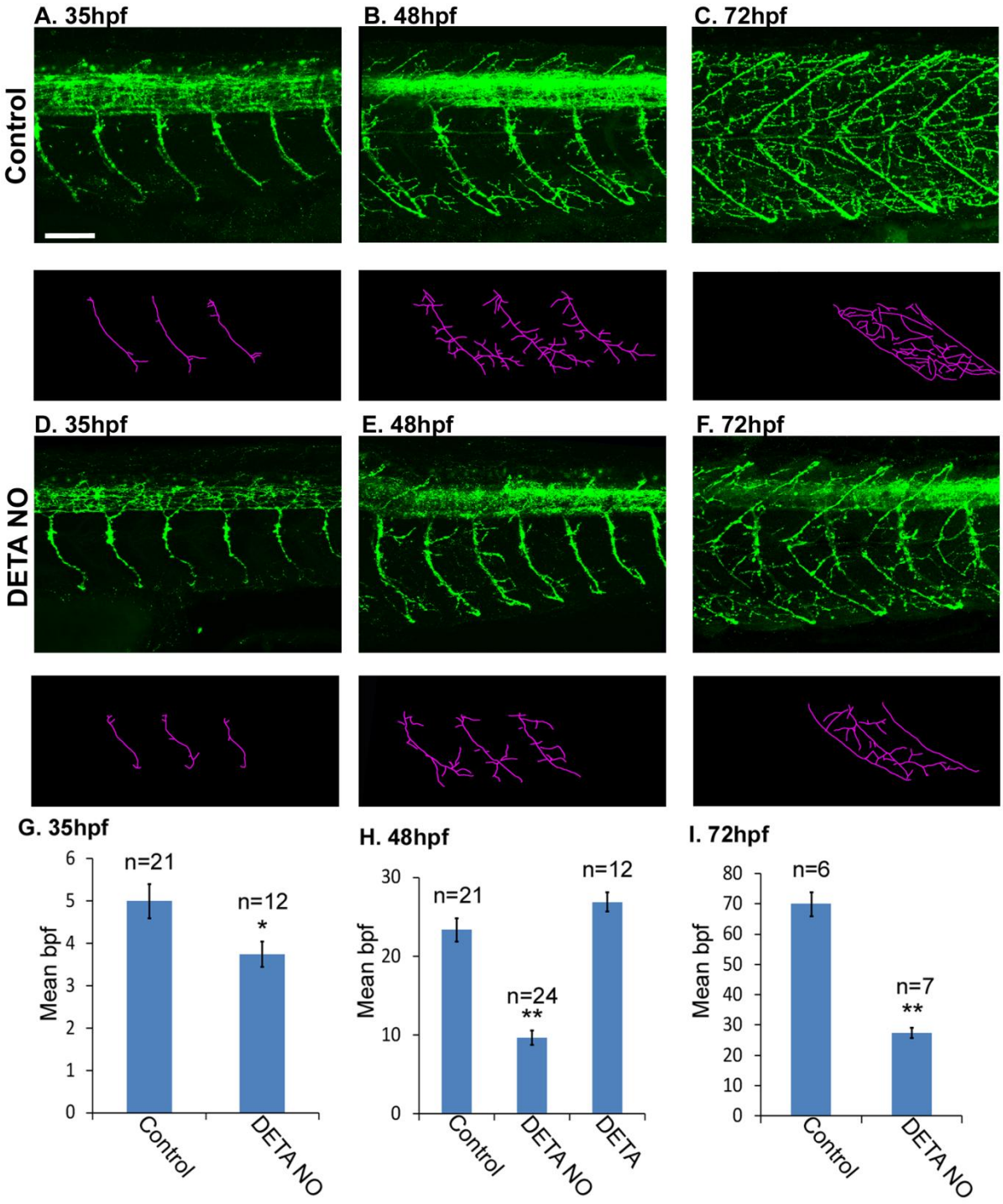

Figure 4.5 Developmental elevation of NO decreases motor axon branch numbers
A-F, Upper panels: znp1 immunostained trunk tissue of control (***A-C***) and DETA NO (***D-F***) treated fish at 35, 48 and 72hpf. Lower panels: Neuron J tracings of motor axon branches derived from image above. ***G-I,*** Bar charts displaying the mean (± SEM) branches per fascicle (bpf) for control, DETA NO and DETA depleted (DETA) at 35 (***G***), 48 (***H***) and 72(***I***) hpf. Scale bar (in ***A***), 50µm. **p ≤ 0.0005.

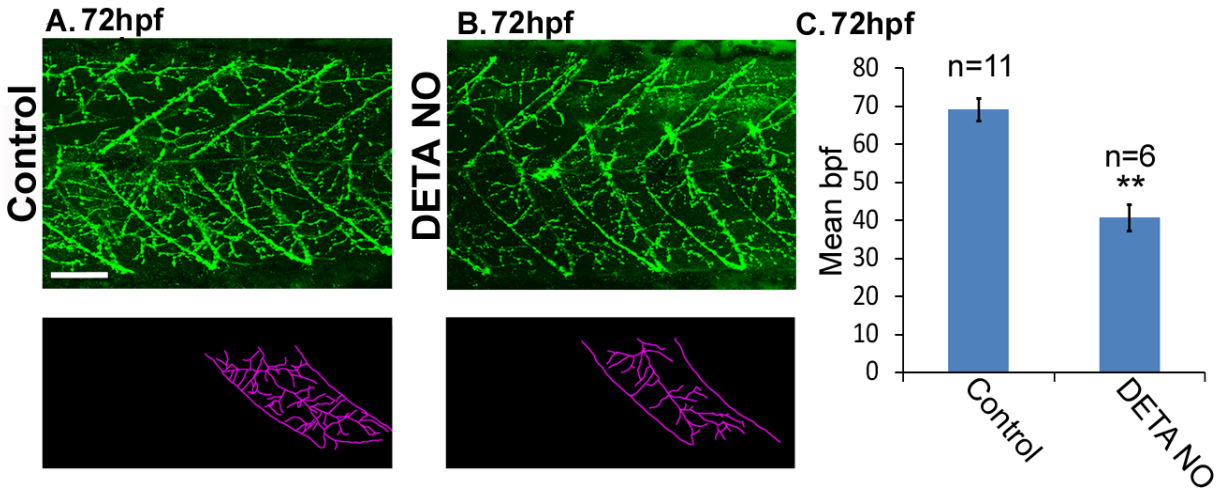

Figure 4.6 Elevation of NO between 48 and 72hpf decreases motor axon branch numbers
A-B, Upper panels: Lateral images of znp1 labelled trunk of a 72hpf control (***A***) and a 72hpf larvae subjected to a 24h DETA NO treatment at 48hpf (***B***) immunolabelled with znp1 antibodies. Lower panels: Neuron J tracings of motor axon branches from the panel directly above. ***C,*** Bar chart depicting the mean (± SEM) number of branches per fascicle (bpf) in both control and DETA NO treated fish. Scale bar: ***A-B*** (in ***A***), 50µm. **p ≤ 0.005.

4.2.5 Disruption of NO signalling does not affect motoneuron differentiation

A range of studies show that NO can regulate neuronal differentiation (Nisoli et al., 1998, Contestabile and Ciani, 2004, Phung et al., 1999, Peunova and Enikolopov, 1995) raising the possibility that NO regulates zebrafish motor axon development by modulating motoneuron genesis. To address this issue, motoneuron numbers were quantified with islet1/2 antibodies, which label nuclei of ventrally located primary and secondary motoneurons as well as those of the dorsally located Rohon-Beard sensory neurons (Inoue et al., 1994). As such, this enabled motoneuron numbers to be counted visually from confocal Z stack images. Embryos injected with NOS1 AMO (10µM) or exposed to DETA-NO (500µM) displayed a small yet significant decrease in

motoneuron numbers whilst no difference was detected in L-NAME (1mM) or c- PTIO (900μM) injected embryos (Figure 4.7 A-F; control: 82.1 ± 2.9*;* AMO: 71.8 ± 2.3, p=0.01; C-PTIO: 87.2 ± 3.2, p=0.26; L-NAME: 79.0 ± 2.2, p= 0.44; DETA-NO: 72.5 ± 2.9, p=0.05;). The foregoing observations suggest that excess motor axon branches formed in NO-depleted conditions do not arise from the genesis of supernumerary motoneurons.

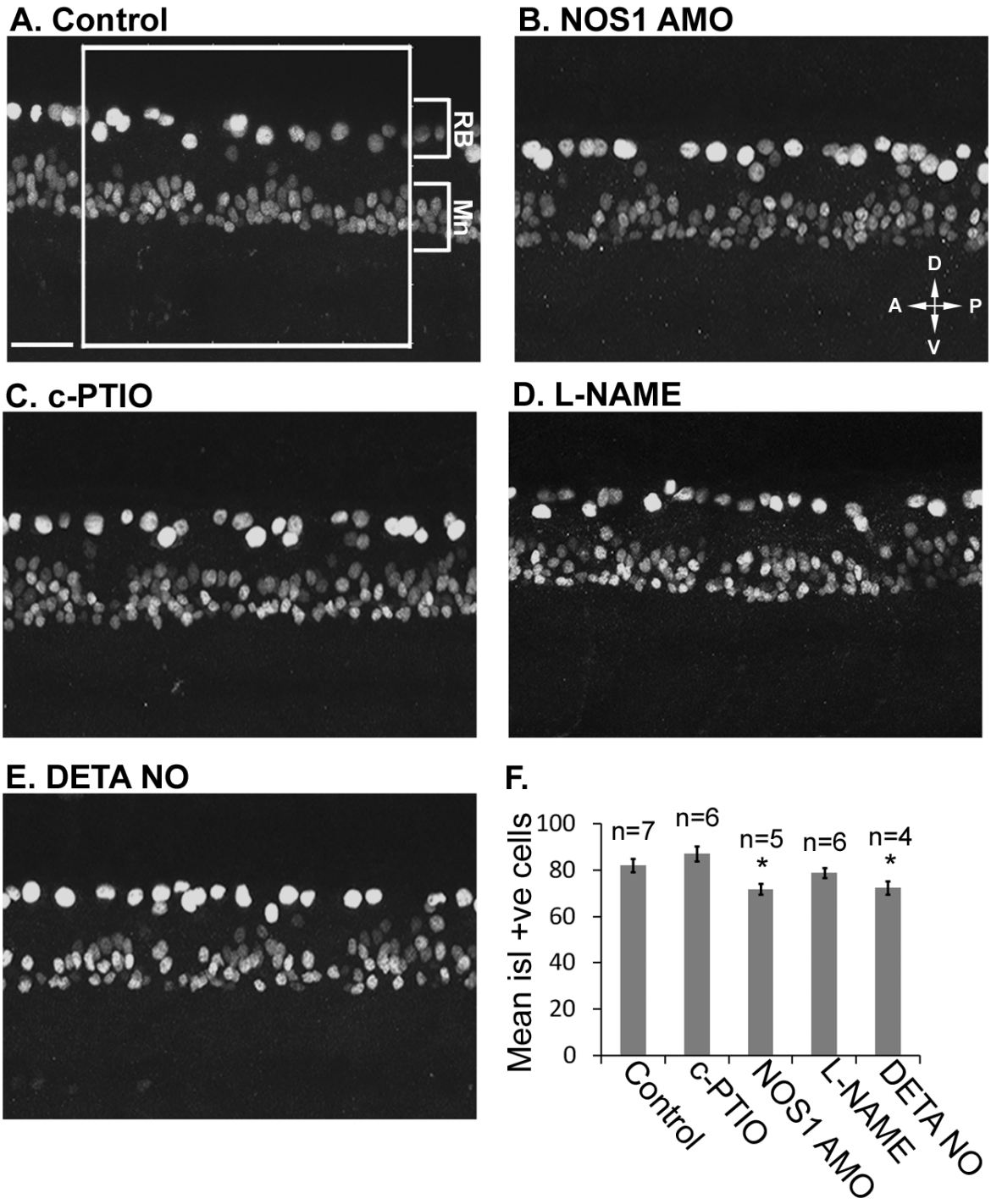

Figure 4.7 Perturbation of NO signalling does not increase motoneuron numbers

A-F, Lateral views of anti-islet1/2 immunostained spinal cords of control (***A***), NOS1 AMO (***B***), c-PTIO (***C***), L-NAME (***D***) and DETA NO (***E***) treated fish at 35hpf. Islet 1/2 labels both motoneuron (Mn) and Rohon-Beard (RB) nuclei in the ventral and dorsal spinal cord respectively. ***F,*** Bar chart depicting the mean number (± SEM) of islet positive motoneuron nuclei for each experimental condition at 35hpf. Inset in ***B*** indicates the orientation of current figure, A, anterior; P, posterior; D, dorsal; V, ventral Scale bar: ***A-E,*** (in ***A***), 50µm. * $p \leq 0.05$.

4.2.6 Primary, but not secondary motoneurons are responsive to NO signalling

To distinguish between effects of NO on primary and secondary motoneuron populations, two approaches were taken. First, zn8 antibodies were used to label DM-GRASP proteins expressed on axons of secondary, but not primary, motoneurons (Menelaou et al., 2008). As expected, immunohistochemical analysis of secondary motor axons in 72hpf control fish revealed the presence of very few branches (Westerfield et al., 1986). Developmental exposure to c-PTIO (900µM) or NOS1 AMOs (10µM) did not induce significant changes in secondary motor axon branching (Figure 4.8A-C), suggesting that perturbation of NO levels does not cause premature arborisation of secondary motoneuron axons.

The second approach utilised transgenic techniques to selectively label individual primary and secondary motoneurons. In order to do this, a DNA construct containing GFP fused with the neural-specific HuC promoter (Park et al., 2000) was injected into 1-4 cell stage blastulae. Subsequently, fish were screened for GFP fluorescence at 24hpf and those containing individually labelled motoneurons, of either type, were selected and either injected with the NOS antagonist L-NAME (1mM) or used as untreated controls. Subsequent analysis of GFP-labelled motoneurons at 48hpf revealed that L-NAME caused a 130% increase in primary motoneuron branches when compared to age matched controls (Figure 4.8D,F; control: 11.0 ± 0.5 branches *cf.* L-NAME: 25.3 ± 0.7 branches, $p=1x10^{-4}$). However, L-NAME treatment failed to significantly affect branching of secondary motoneuron axons (Figure 4.8E-F; control: 3.7 ± 0.4 branches *cf.* L-NAME: 4.3 ± 0.4 branches, p=0.37). These findings strongly

suggest that, at the developmental stages used in this study, NO principally regulates primary motoneuron morphogenesis.

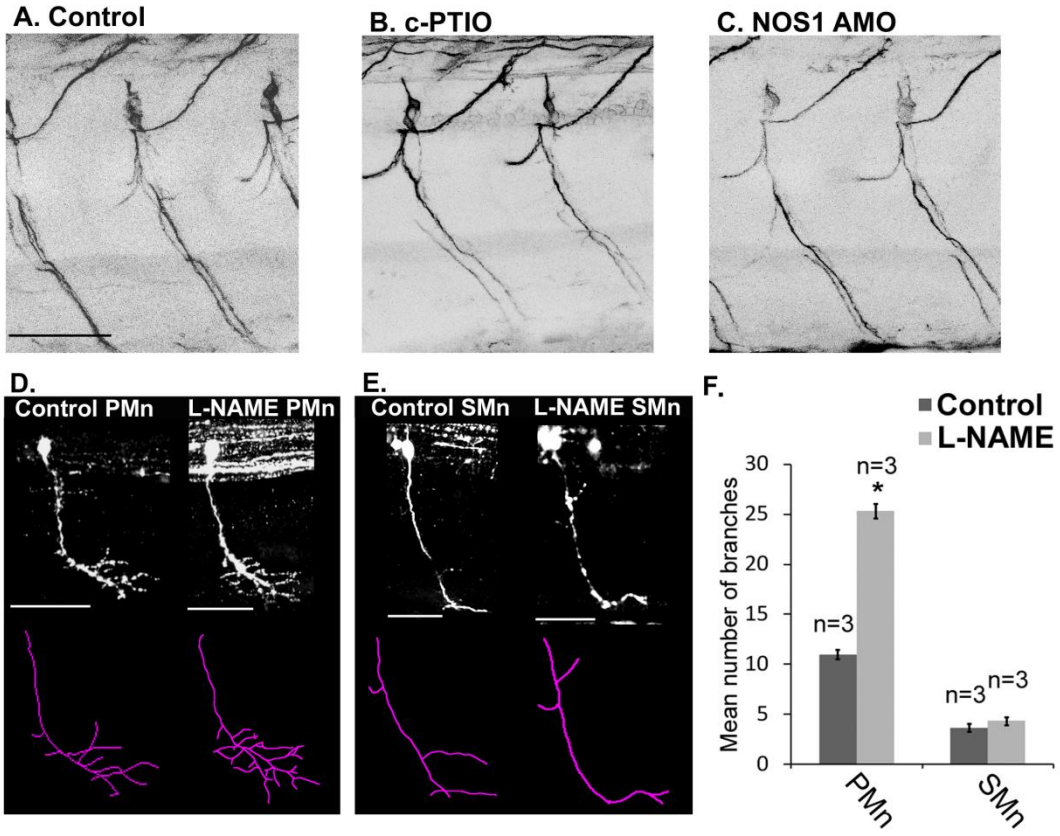

Figure 4.8 Secondary motoneuron branches are not affected by disruption of NO signalling

A-C, LUT inverted images of secondary motoneuron branches at 72hpf immunolabelled with zn8 in control (***A***), c-PTIO (***B***) and NOS1 AMO (***C***) fish. Images are lookup table (LUT) inverted to increase the clarity of axonal processes. ***D,*** Top panels: GFP–labelled primary motoneuron (PMn) of a control and L-NAME treated fish at 48hpf. Bottom panels: Neuron J tracings of the processes emanating from the motoneuron axon in the panels directly above. ***E,*** Top panels: GFP-labelled secondary motoneuron (SMn) in a control and L-NAME treated fish at 48hpf. Bottom panels: Neuron J tracings derived from the panels directly above. ***F,*** Bar chart displaying the mean (± SEM) number of branches per PMn and SMn fascicle in both control and L-NAME treated fish. Scale bar: ***A-C*** (in ***A***) and ***D-E***, 50µm. * $p \leq 0.005$.

4.2.7 Effects of NOS inhibition on axons of early developing interneurons

During early periods of motoneuron growth, interneurons are also extending axons and forming synaptic connections. In order to determine whether NO also regulates development of these cells (Figure 4.9), interneurons were stochastically labelled by injecting early stage blastulae with the GFP-HuC plasmid. At 24hpf embryos were screened for GFP expression and those containing labelled interneurons were either injected with L-NAME or raised as untreated controls. Subsequently, axonal trajectories and branch morphologies of labelled interneurons were examined at 48hpf. Upon visual comparison, in contrast to the effects observed in motoneuron labelling studies, L-NAME did not have a demonstrable impact on branching patterns of either primary commissural or ipsilateral interneurons (Figure 4.10-4.11). In addition, upon visual comparison, L-NAME did not disrupt morphology of NOS1 immunoreactive cells (Figure 4.11D). These findings suggest that NO signalling does not regulate axonal architecture of early developing interneuron classes.

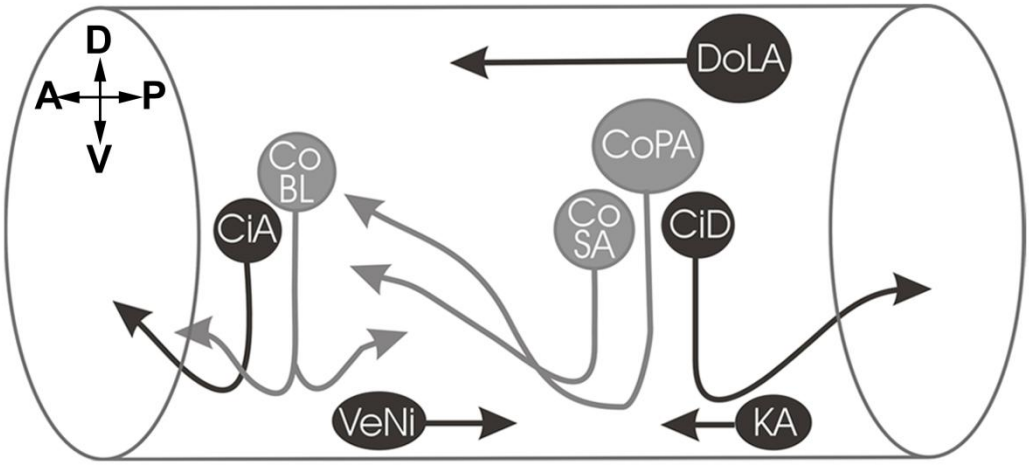

Figure 4.9 Schematic diagram depicting early developing commissural and ipsilateral interneuron classes.

Early born commissural interneurons are shaded light grey and comprise the commissural bifurcating longitudinal (CoBL); commissural primary ascending (CoPA) and commissural secondary ascending (CoSA) interneurons. Early born ipsilateral projecting interneurons are labelled in dark grey and comprise the circumferential descending (CiD); circumferential ascending (CiA); Kolmer-Agduhr (KA), dorsal longitudinal ascending (DoLA) and ventral nitrergic (VeNi) interneuron cell classes. D, dorsal; V, ventral; A, anterior; P, posterior.

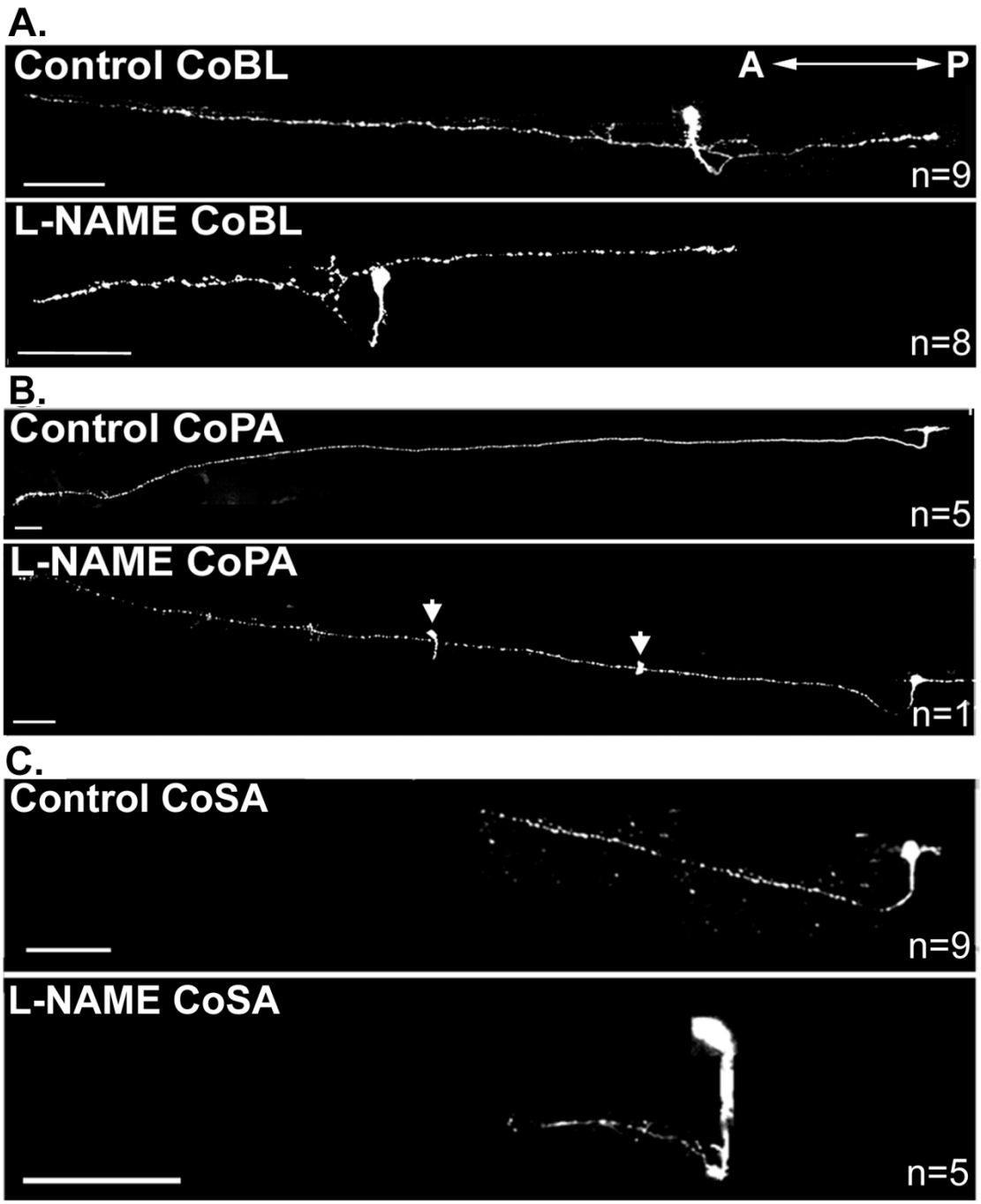

Figure 4.10 NOS inhibition does not regulate commissural interneurons growth
A-C, Optical images of GFP-immunostained commissural interneurons; commissural bifurcating longitudinal (CoBL; ***A***), commissural primary ascending (CoPA; ***B***) and commissural secondary ascending (CoSA; ***C***) interneurons in both control and L-NAME fish at 48hpf. Arrowheads indicate additional GFP-labelled neurons that lie close to the imaged axons. A, anterior; P, posterior. Scale bars: ***A-C***, 50µm.

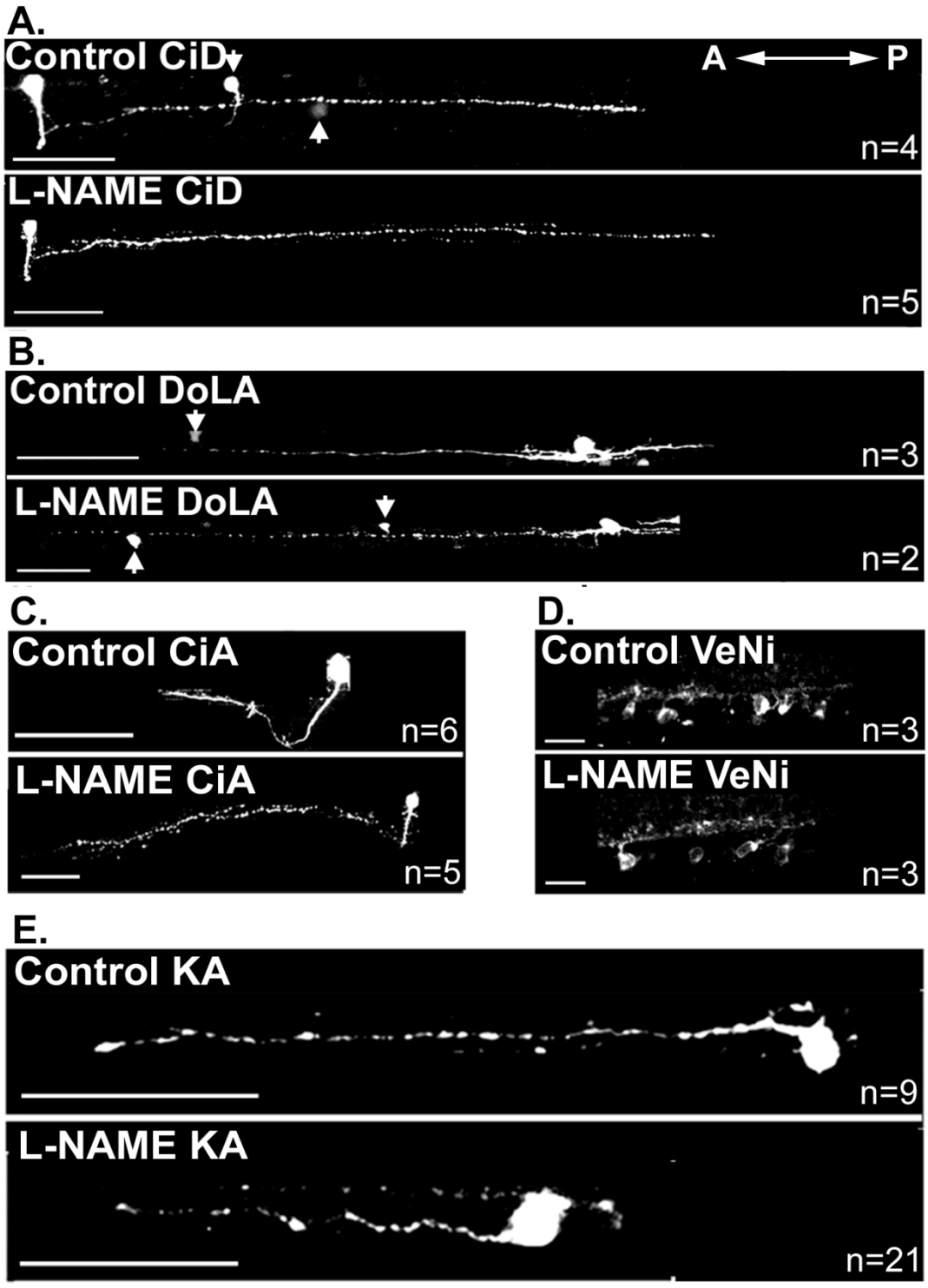

Figure 4.11 Perturbation of NOS signalling does not alter axon branching patterns of early born ipsilateral interneurons

A-E, GFP-immunolabelled ipsilateral interneurons in both control and L-NAME treated fish at 48hpf. Ipsilateral interneurons consisted of circumferential descending (CiD; ***A***); dorsal longitudinal ascending (DoLA; ***B***); circumferential ascending (CiA); ventral nitrergic (VeNi) and Kolmer-Agduhr (KA). Arrowheads indicate additional GFP-labelled neurons that lie close to the imaged axons. A, anterior; P, posterior. Scale bars: ***A-C***, ***E***, 50µM; ***D***, 10µm.

4.3 Discussion

The principal finding of this study is that the NOS1 enzyme is endogenously active during zebrafish spinal cord ontogeny and functions to regulate spinal motoneuron development through NO biosynthesis. This is supported by the finding that reduction of endogenous NOS1 signalling, via antisense knockdown or pharmacological inhibition, causes supernumerary motor axon branch formation. Evidence that NO synthesis underpins this effect comes from the observation that the NO scavenger c-PTIO mimics these effects whilst exogenous elevation of NO with DETA-NO inhibits branch development. Detailed analysis of individual neuronal populations demonstrates that this effect of NO is restricted to primary motor axons, with secondary motoneurons and interneurons unaffected by altered NO levels. These findings demonstrate that nitrergic signalling has important roles during morphogenesis of zebrafish motor axons.

4.3.1 Methodological considerations

In contrast to mammals, zebrafish have only one constitutively active NOS gene (NOS1) (Holmqvist et al., 2000) but two inducible NOS2 genes (NOS2a and NOS2b) (Lepiller et al., 2009, Poon et al., 2008). Bioinformatic analysis reveals that the zebrafish NOS1 gene codes for 3 mRNAs (accession #: NP_571735) which differ solely in the length of their non-coding regions. As each of these shares an identical start codon sequence, the translational blocking AMO used in this study will recognise and bind all known constitutively active forms of zebrafish NOS1. In addition, the presence

of a single constitutively active NOS gene in zebrafish suggests that that all constitutive NOS function would be abolished by NOS1 AMO treatment. Thus, notwithstanding the presence of hitherto unidentified NOS1 splice isoforms with divergent start codon sequences, it likely that in the current study all constitutive NOS activity is abolished by NOS1 AMO treatment.

4.3.2 Nitrergic regulation of motoneuron axonal arborisation

This finding shows that pharmacological and molecular perturbation of NO signalling induces formation of supernumerary motor axon branches whilst exogenous application of NO leads to a reduction in branch formation. This suggests that NO has suppressive actions during zebrafish motor axon development. Similar inhibitory actions of NO have been reported in the outgrowth of B5 neuron of the *Helisoma* buccal ganglia (Trimm and Rehder, 2004, Van Wagenen and Rehder, 1999, Van Wagenen and Rehder, 2001, Welshhans and Rehder, 2005, Welshhans and Rehder, 2007), rat DRG, (Hess et al., 1993) and the rat dentate granule neurons (Yamada et al., 2006). Additionally within the visual system NO induces collapse of retinal ganglion cells in both *Xenopus* (Renteria and Constantine-Paton, 1996) and the chick (Ernst et al., 2000, Gallo et al., 2002) as well as modulating retraction of inappropriate synaptic connections in vertebrates (Campello-Costa et al., 2000, Wu et al., 2001, Cramer et al., 1996) and invertebrates alike (Gibbs and Truman, 1998). However, these findings conflict with reports demonstrating that NO promotes outgrowth of grasshopper antennal pioneer neurons *in vivo* (Seidel and Bicker, 2000), as well as in a range of cell culture studies (Hindley et al., 1997, Rialas et al., 2000, Yamazaki et al., 2001, Yamazaki

et al., 2006, Yamazaki et al., 2004). These disparate actions of NO are difficult to reconcile but several factors may influence their outcome. First, diverse pharmacological tools used at different concentrations could generate responses with varying specificity and efficacy. Second, responses observed in cell and tissue culture experiments may diverge from those observed during *in vivo* studies as they cannot accurately mimic the complex physiological environment of whole animal systems. This is particularly relevant to NO signalling as its effects are often likely to rely on upon the presence of multifactorial ECM cues that interact in a complex manner to instruct axon guidance and synaptogenesis (Ditlevsen et al., 2007, Peunova and Enikolopov, 1995, Yamazaki et al., 2006, Rialas et al., 2000, Hindley et al., 1997). Third, responses to NO may reflect functional differences in NO signalling that are governed by neuron type and developmental stage.

Notwithstanding the aforementioned problems, the current study used three independent methods; molecular NOS1 abrogation, pharmacological NOS inhibition and NO scavenging; to depress NO signalling during development. All three approaches generated comparable phenotypes: enhanced motor axon branching. Moreover, as DETA-NO exposure had the opposite effect, notably inhibition of axon arbour formation, these findings collectively demonstrate that NO acts to suppress motor axon arborisation in the zebrafish trunk. However, the possibility remains that NO could generate different responses that are dependent on frequency of exposure, concentration and developmental stage. Future studies will help determine if this is indeed the case.

4.3.3 Dynamics of axon branch regulation by nitric oxide

Axon development is a highly dynamic process, involving rapid addition and elimination of arbours, and precisely how NO influences the dynamism of zebrafish motor axon development remains to be determined. However, two inferences can be drawn from the present study. First, NO signalling has a potent effect on axon arbour number without impacting arbour length. Thus, this signalling molecule does not have a direct influence over growth rate of established axon arbours. Second, exposing late stage embryos (48hpf) to exogenous NO does not induce axon stripping, nor does it prevent addition of new axons but rather reduces the rate of arbour addition. These findings strongly suggest that NO's principal function is to reduce the frequency of arbour initiation events, rather than to prune existing synaptic contacts.

Real-time imaging of labelled neurons in the *Xenopus* visual system have permitted detailed study of NOs effects on axon arbour dynamics *in vivo* (Cogen and Cohen-Cory, 2000). Here, NOS inhibitors increase both axonal branch addition and elimination, as well as causing lengthening of pre-existing branches, whereas application of an NO donor results solely in increased arbour addition, suggesting that growth cone responses to NO have a complex temporal component (Cogen and Cohen-Cory, 2000). Whilst it is possible that similar mechanisms drive changes at the zebrafish neuromuscular junction, the finding that modulation of NO does not affect axon branch length suggests a divergent mechanism of action. Future time-lapse studies of motor axon morphogenesis throughout early development will help determine if this is the case.

4.3.4 Does NO regulate arbour growth or synaptic pruning?

A widely held view is that NO acts as a retrograde signal during activity-dependent pruning of developing synaptic connections. For example, in visual circuits, disruption of NO often results in the maintenance of excess, misdirected and inappropriate synaptic connections (Wu et al., 2000a, Wu et al., 2000b, Ernst et al., 2000, Gibbs and Truman, 1998, Wu et al., 1994, Cramer et al., 1996, Ernst et al., 1999). Yet, parallels cannot be easily drawn between these studies and the data presented here for two reasons. First, there is no evidence that NO is synthesised at the neuromuscular junction, thus precluding its involvement in retrograde signalling processes and; second, there is no evidence to suggest that pruning occurs at zebrafish neuromuscular synapses (Liu and Westerfield, 1990). In contrast to their mammalian counterparts (Jansen and Fladby, 1990, Colman and Lichtman, 1993), zebrafish neuromuscular synaptogenesis is extraordinarily economical, with axons making precise, stable contacts with their targets. Instead, these data point towards a role for spinally secreted NO in repression of branch initiation at distal compartments of motoneuron axons, rather than retrograde neuromuscular signalling modulating local synapse stability.

4.3.5 Nitric oxide does not regulate motoneuron differentiation

A range of studies show that NO modulates differentiation by triggering growth arrest. In PC12 cells, inhibition of NOS reverses the cytostatic action of NGF and cells continue to proliferate instead (Peunova and Enikolopov, 1995, Phung et al., 1999).

Similarly, NOS inhibitors increase proliferation of *Drosophila* imaginal disc cells (Kuzin et al., 1996) whilst NO exposure decreases proliferation in the *Xenopus* tadpole CNS (Peunova et al., 2001). In addition, a previous study has provided evidence that NO regulates proliferation and differentiation of zebrafish spinal cord neurons (Gibbs, 2003, Gibbs et al., 2001): this study suggests that prior to its closure the ventral neural tube as well as the notochord are diaphorase reactive. Moreover, pharmacological inhibition of NOS during this period causes floor plate expansion and increased cell division (Gibbs et al., 2001, Gibbs, 2003). However, as subsequent studies have failed to detect either NOS1 RNA or protein expression in the early neural tube, it is unlikely that diaphorase staining reflects the presence of *bona fide* nitrergic cells (Holmqvist et al., 2004, Poon et al., 2003, Bradley et al., 2010; also see chapter 3) Indeed, existing protein and RNA evidence strongly suggests that NOS1 is expressed only after the vast majority of spinal cord motoneurons have terminally differentiated (by 16hpf; Myers et al., 1986). In support of this argument, the present study demonstrates that AMO-directed perturbation of NOS1 from the onset of development does not cause a significant increase in the number of Islet1/2 expressing motoneuron nuclei. Thus, the observed changes in motor axon arborisation reported in the current study are likely to arise from changes in motor axon branch dynamics, rather than changes in motoneuron number. Whether NO influences differentiation of neurons in other brain regions remains to be determined.

4.3.6 Primary motoneurons are selective targets of nitric oxide

Analysis of the effects of NO on axons of primary and secondary motoneuron populations suggests that this signalling molecule selectively regulates primary motoneuron arborisation. Evidence for this comes from the observation that zn8 labelled secondary motor axons do not exhibit increased branching when NOS1 is perturbed. Moreover, analysis of individually GFP labelled motoneurons demonstrated that axon branch numbers of primary motoneurons dramatically increases in NO depleted conditions whereas those of secondary motoneurons are not affected. Therefore, at the developmental stages examined, NO principally regulates primary motor axon development. The most plausible explanation for this is that secondary motoneurons lack the necessary signal transduction elements (e.g. sGC,cGMP,PKG) required for NO signalling. Similar specificity of neuronal cell types to NO signalling is shown in *Helisoma trivolvis*. Here B5 neurons show strong sGC immunoreactivity and exhibit altered growth cone behaviour upon NO exposure whilst B19 neurons have little sGC immunoreactivity and do not respond to NO (Van Wagenen and Rehder, 2001). Nonetheless, it remains to be established whether secondary motoneurons become competent to nitrergic signals during later periods of development. Further studies focusing on how secondary motoneuron populations of later-stage larvae respond to manipulated NO levels will help identify if this is the case.

Similarly, comparison of stochastically GFP-labelled interneuron morphology in wild type and L-NAME treated fish suggests that the growth of early born interneuron axons is unaffected by NO signalling. However, it remains to be determined whether any of these interneuron classes become responsive to NO as development proceeds,

or indeed whether later born secondary interneurons, which were not examined in this study, are responsive to NO signalling. Additionally, no attempt was made during the current study to determine whether other neuronal compartments, such as dendritic processes, are altered in response to perturbations of NO signalling, as has been shown in mouse knockout models (Inglis et al., 1998). Future studies are needed to explore the role of NO during dendrite development.

4.3.7 Ventral nitrergic interneurons as a source of NO emission

This study suggests that during nitrergic regulation of motoneuron growth, the most likely source of NO arises from a discrete population of ventral spinal cord cells. These cells lie in close proximity to developing motoneurons and express NOS1 at around 35hpf (Chapter 3, section 3.2.3.2), close to the onset of motor axon branching. Thus ventral nitrergic interneurons are likely to regulate primary motoneuron development in an NO dependent manner.

4.3.8 Possible modes of nitrergic signalling

Assuming that the source of NO is spinal, there are two possible modes of NO signalling. First, local anterograde signalling of NO at *en passant* motoneuron-nitrergic interneuron synapses could activate signalling cascades that regulate motoneuron branching. Here transynaptic signalling could induce long-range changes in developing axons through either translocation of elements of the NO signalling pathway (Shelly et

al.), long range Ca^{2+} sweeps (Yamada et al., 2008) or by affecting postsynaptic changes in excitability that induces plastic changes at the developing motor axons (Hua et al., 2005). Several studies have shown that NO can act as an anterograde signalling molecule (Park et al., 1998, Lev-Ram et al., 1995, de Vente et al., 2000) to mediate motoneuron excitability which during early development can affect properties of motoneuron development such as axonal outgrowth, pathfinding and synapse formation (Moreno and Ribera, 2010, Hanson et al., 2008, Zhang and Poo, 2001).

The second possible mode of NO action is via paracrine signalling. Here, rather than diffusing across local synapses, NO could regulate motor axon branching through either local diffusion to multiple motoneuron somata, as has been shown to occur in the chick and mouse spinal cord (Xiong et al., 2007, Kalb and Agostini, 1993, Inglis et al., 1998), or by long range diffusion to developing neurites in the periphery. In the latter scenario, downstream components of the NO signalling cascade would need to reside within the growth cone (Van Wagenen and Rehder, 2001) and the effects of NO would be dependent upon its ability to diffuse long distances. However, previous studies have shown that NO is capable of diffusing up to 200µm from its point of synthesis (Lancaster, 1994, Wood and Garthwaite, 1994) and since zebrafish motoneuron axons extend no more than ca. 100µm (at 48hpf) to 150µm (at 72pf) from the ventral spinal cord during the stages studied, the majority of motor axon branches could be within range of paracrine nitrergic signals.

5. cGMP dependent pathways that mediate the effects of NO and the role of nitrergic signalling in regulating neuromuscular maturation

5.1 Introduction

In order for NO to exert its effects it must react with other molecules. The canonical target of NO is the enzyme sGC, which leads to the formation of cGMP in target cells. cGMP is an key second messenger present in almost every cell type (Hardman and Sutherland, 1969), regulating a wide range of physiological processes including vasodilation (Rubio and Morales-Segura, 2004, Munzel et al., 2003), platelet aggregation (Pigazzi et al., 1999), apoptosis (Brown, 2010, Blaise et al., 2005), ageing (Domek-Lopacinska and Strosznajder, 2010), learning and memory (Taqatqeh et al., 2009, Bon and Garthwaite, 2003, Hawkins et al., 1994), nociception (Schmidtko et al., 2009, Meller and Gebhart, 1993), neurotransmission (Garthwaite, 2008), neurite outgrowth (Bradley et al., 2010, Welshhans and Rehder, 2005, Welshhans and Rehder, 2007) and axon guidance (Tojima et al., 2009, Dickson, 2002, Song et al., 1998). Indeed, this small signalling molecule, which acts principally on protein kinases and cyclic nucleotide gated ion channels (see Chapter 1; for review, see Francis et al. 2010, Bryan et al., 2009, Friebe and Koesling, 2003), has been shown to control several aspects of axon development and evidence in support of this is detailed in the following sections.

5.1.1 cGMP dependent regulation of neurite development in cell and tissue culture models

5.1.1.1 Invertebrate models

A large body of evidence demonstrates that the cGMP signalling pathway regulates development of invertebrate neurites and that these effects are often dependent on nitrergic activation of sGC. In cultured *Drosophila* nervous tissue application of the membrane permeable cGMP analogue 8-bromo-cGMP attenuates

the effects of NOS inhibition on retinal axons overgrowth (Gibbs and Truman, 1998), suggesting that NO signals through cGMP to stabilise retinal growth cones in the developing invertebrate visual system. Similarly, in cultured *Helisoma* B5 neurons cGMP analogues mimic the effects of NO exposure, causing a dose-dependent effect on neurite outgrowth with low concentrations (250µM) slowing neurite outgrowth and high concentrations (1mM) causing collapse and retraction of neurites (Trimm and Rehder, 2004).

Detailed analysis of the downstream targets of B5 responses to cGMP suggests that it regulates axon development through modification of calcium dynamics at the growth cone (Trimm and Rehder, 2004, Welshhans and Rehder, 2005, Welshhans and Rehder, 2007). This occurs through a complex cascade that involves protein kinases and additional second messenger systems (Welshhans and Rehder, 2007). The first step in this process involves activation of PKG, a canonical target of cGMP that phosphorylates serine and threonine residues of proteins, thereby modifying their actions (for review, see Francis et al. 2010). Immunohistochemical detection of PKG protein demonstrates that it is distributed within the central domain of the growth cone (Welshhans and Rehder, 2007) and preincubation with the PKG inhibitor KT5823 inhibits NO-mediated changes in filopodia length and number, suggesting that this enzyme acts downstream of NO (Welshhans and Rehder, 2005). The role of PKG appears to be to activate the second messenger, and ryanodine receptor ligand, cyclic adenosine diphosphate ribose (cADPR; Willmott et al., 1996, Lee et al., 1998, Galione et al., 1993). Evidence for this comes from studies showing that block of adenosine diphosphate ribosyl cyclase (ADP-ribosyl cyclase) with nicotinamide or 8-Bromo-cADPR

attenuates the ability of NO to alter growth cone behaviour (Welshhans and Rehder, 2005). Subsequently, cADPR induces Ca^{2+} release from ryanodine sensitive stores, through either direct binding to the ryanodine receptor (Meszaros et al., 1993) or interaction with immunophilin protein FKBP12.6 (Higashida et al., 2007, Guse, 2005, Noguchi et al., 1997, Wang et al., 2004). Thus, when ryanodine receptors are blocked with ryanodine, 9,21-dehydro, NO can no longer increase growth cone Ca^{2+} or alter filopodial growth (Welshhans and Rehder, 2007). Overall these studies provide evidence that NO modulates growth cone navigation and neurite outgrowth through a signalling pathway comprising sGC, cGMP, PKG, cADPR and ryanodine receptors.

5.1.1.2 Vertebrate models

The role of cGMP signalling in mediating the actions of NO have also been characterised using cell and tissue culture systems in vertebrate models and as outlined in chapter 4, these often depend upon the presence of components of the ECM. For example, NO/cGMP has been shown to play a role in regulating NCAM-mediated outgrowth of hippocampal neurites. Application of the sGC antagonist ODQ mimics the effects of NOS inhibition by inhibiting NCAM-mediated increases in neurite growth, whereas addition of the cGMP analogue 8-bromo-cGMP has the opposite effect (Ditlevsen et al., 2007). PKG is likely to be a principal downstream target of this response as the PKG inhibitor KT5823 also causes inhibition of NCAM-stimulated neurite extension (Ditlevsen et al., 2007).

In cultured PC12 cells, NGF promotes neurite outgrowth in a NO-dependent manner and sGC/cGMP appear to be key elements of this response. Here application of a sGC inhibitor methylene blue (Gruetter et al., 1979, Gruetter et al., 1981) attenuates the ability of the NO donor SNP to enhance NGF-mediated neurite outgrowth (Hindley et al., 1997), whilst cGMP analogues potentiate the ability of NGF to induce neurite formation (Hindley et al., 1997, Yamazaki et al., 2005). PKG also appears to be a downstream effector of these responses as NO/cGMP induced increases in neurite outgrowth can be occluded by PKG inhibition (Yamazaki et al., 2005).

In addition to regulating neurite outgrowth, ECM-induced growth cone turning responses are also modulated by the NO/sGC/cGMP pathway. In chick DRG neurons cultured on laminin substrate, growth cone repulsion can be induced by release of caged Ca^{2+} (with laser induced photolysis) at the growth cone (Tojima et al., 2009). In contrast, when cells are cultured on an L1 substrate, the same paradigm induces growth cone attraction. The differential growth responses induced by these substrates is likely mediated by NO/sGC/cGMP signalling as manipulation of this pathway can switch growth behaviour such that in the presence of laminin, disruption of NO/cGMP synthesis causes growth cone attraction whereas in the presence of L1, elevated NO/cGMP causes growth cone repulsion. These responses depend upon PKG and ryanodine-dependent increases in growth cone Ca^{2+} (Tojima et al., 2009). This suggests that during growth cone navigation, NO/sGC/cGMP signalling can induce complex behaviours at the growth cone that depend upon interplay between extracellular cues and local Ca^{2+} signalling.

The NO/sGC/cGMP signalling cascade has also been demonstrated to regulate axon navigation in cultured *Xenopus* spinal neurons. Here, netrin acts as an attractive cue. However, this response can be converted to repulsion through bath application of either SNAP or 8-bromo-cGMP (Nishiyama et al., 2003). In contrast, repulsion induced by semaphorin 3 can be switched to attraction through bath application of 8-bromo-cGMP (Song et al., 1998). In addition, whilst the NO donor SNAP does not induce growth cone attraction, it does suppress the repulsive actions of semaphorin 3 (Song et al., 1998).

Whilst PKG often mediates neurite responses to cGMP, it is not the only downstream target of this second messenger. In cultures of *Xenopus* spinal neurons, studies have demonstrated that cGMP directly influences semaphorin 3A-induced repulsion by triggering Ca^{2+} influx through cyclic nucleotide-gated channels (Togashi et al., 2008), although this response does not involve NO. Similarly, 8-bromo-cGMP inhibits extension of rat olfactory receptor growth cones in a cyclic nucleotide-gated channel-dependent manner, although whether NO plays a role in this response is currently unknown (Kafitz et al., 2000).

5.1.2 cGMP dependent regulation of neurite development in vivo

5.1.2.1 Pharmacological studies in invertebrate models

To date, only a small number of studies have shown that sGC/cGMP signalling influences invertebrate development *in vivo*. In grasshopper embryos, cGMP is detected in outgrowing pioneer neurons after stimulation with the NO donor SNP

(Seidel and Bicker, 2000). Inhibition of sGC with ODQ inhibits axon outgrowth, an effect which can be rescued upon 8-bromo-cGMP application (Seidel and Bicker, 2000).

In a similar manner, cGMP has also been implicated in growth cone navigation in *Drosophila*. Here semaphorin 1A/plexin A mediates defasiculation of motor axon bundles. This response requires guanylyl cyclase Gyc76C, as mutations of this gene abolish this effect and axons fail to innervate their synaptic targets (Ayoob et al., 2004). Furthermore the sGC/cGMP signalling cascade also regulates refinement of *Drosophila* visual synapses (Gibbs et al., 2001): mutants for the sGC α subunit gene Gcα1, display minor defects in visual system organisation with the extension of retinal axons slightly beyond their targets (Gibbs et al., 2001).

5.1.2.2 Pharmacological studies in vertebrate models

Relatively few studies have examined possible roles of cGMP during *in vivo* vertebrate development. However one key study demonstrates a role for cGMP in motoneuron dendrite elaboration. Here inhibition of sGC, through ODQ application, culminates in a *ca*. 45% reduction in dendritic tree size, a 40% reduction in branching and a 30% reduction in motoneuron cell body size, effects that can be mimicked by NOS disruption (Xiong et al., 2007). Similar reductions are also observed upon PKG inhibition, supportive of the notion that the NO/cGMP/PKG pathway mediates motoneuron dendritic growth (Xiong et al., 2007). Additionally, in the developing visual system of the ferret, the sGC/cGMP/PKG signalling cascade mediates the effects of NO during refinement of retinogeniculate connections (Leamey et al., 2001). Here cGMP is

expressed during periods of sublamination in the LGN and inhibition of sGC or PKG disrupts this process (Leamey et al., 2001).

5.2 NO mediated effects on neuromuscular development

Once motor axons have undergone axonogenesis, they innervate the surrounding musculature (see chapter 1, section 1.2.8) to form synapses with their respective targets. Over recent years NO has been implicated to mediate neuromuscular synaptogenesis and the subsequent maturation of locomotor behaviours. A brief summary of the literature regarding the role of NO in each one of these processes is outlined below.

5.2.1 NO mediated effects on neuromuscular synaptogenesis

In mammals, the NOS1μ isoform localises within developing muscle fibres (for review, see Blottner and Luck, 2001) and although there is little evidence to suggest that this enzyme is also expressed in the muscle of lower vertebrates, there is some evidence that NO can influence embryonic chick and tadpole neuromuscular development (Schwarte and Godfrey, 2004, Jones and Werle, 2000). In *Xenopus* and avian muscle cells, application of NO donors causes an increase in aggregation of AChRs at the embryonic NMJ (Schwarte and Godfrey, 2004, Jones and Werle, 2000) whilst NOS inhibition suppresses AChR aggregation. Furthermore, elevation of downstream NO targets (sGC, cGMP and PKG), culminates in increases in AChR

aggregation by as much as 100-200% (Godfrey and Schwarte, 2003, Godfrey et al., 2007). In addition, further studies have demonstrated additional roles for NO at the NMJ, indicating that it acts as a neuromodulator, enhancing (Wildemann and Bicker, 1999) or decreasing (Ribera et al., 1998, Wang et al., 1995, Thomas and Robitaille, 2001) ACh release.

5.2.2 NO signalling during locomotor maturation and function

Previous work on both invertebrates and vertebrates has demonstrated that NO exposure can also modify parametres of locomotor networks. Here, exogenous elevation of NO can stimulate both non-rhythmic and rhythmic motor activity. For example, in *Rana temporaria*, bath application of NO donors, SNAP or DEANO, induces non-rhythmic motor bursts, however this treatment failed to induce or attenuate any parametres of rhythmic swimming (McLean et al., 2001). In contrast, bath application of NO to either semi-intact or free swimming jellyfish (*Aglantha digitale*) induces slow rhythmic swimming behaviours that are largely associated with prey capture (Moroz et al., 2004). Furthermore, the NO-induced rhythmic swimming behaviour is mimicked upon bath application of cGMP analogue, 8-Br-cGMP and attenuated after application of either a NOS inhibitor or NO scavenger, thus suggesting rhythmic swimming behaviours in jellyfish are modulated through the NO/cGMP pathway (Moroz et al., 2004).

Studies of *Xenopus* and lamprey spinal networks suggest that NO regulates the strength of synaptic connections in locomotor circuits. In *Xenopus* tadpoles NO

decreases swim episode duration and swimming frequency through facilitation of both GABAergic and glycinergic inhibitory synapses (McLean and Sillar, 2000, McLean and Sillar, 2002, McLean and Sillar, 2004). These effects are mediated by two distinct pathways. First, NO reduces swim frequency by acting indirectly as a "metamodulator" to elicit noradrenaline dependent presynaptic potentiation of glycinergic synapses (McDearmid et al., 1997). This causes a potentiation of the mid-cycle inhibitory phase of each cycle, prolonging its duration and delaying the onset of each subsequent excitatory phase. Second, NO reduces swim episodes by directly promoting GABA release from reticulospinal inhibitory neurons, which prematurely terminates swim episodes (McLean and Sillar, 2004).

In contrast, in the lamprey spinal cord, NO enhances locomotor frequency, an effect achieved by modulation of both inhibitory and excitatory synapses. Here NO donors cause presynaptic suppression of glycine release, leading to a weakening of mid-cycle inhibition during locomotor episodes. In concert with this effect, NO also potentiates the strength of glutamatergic synapses through both pre and postsynaptic mechanisms. The shift in excitatory drive culminates in an increase in locomotor frequency (Kyriakatos et al., 2009).

In higher vertebrates, acute application of NO also affects motor control. Here locomotor behaviour and fine motor control are disrupted through injection of a variety of NOS inhibitors (Dzoljic et al., 1997, Araki et al., 2001, Uzbay, 2001, Del Bel et al., 2002). However, exogenous application of the NO donor SNP can also cause a reduction in motor behaviour (Dall'Igna et al., 2001), thus suggesting that a basal level of NO may be required for efficient motor output in mice.

Whilst acute effects of NO on spinal rhythm generation have been explored in some detail, the effects of chronic developmental disruption of this signalling molecule are less clear. In *Drosophila* sGC knockout models, larval locomotion is increased during foraging (Riedl et al., 2005). Moreover as decreases are observed in NO-stimulated GC activity in these mutants, this supports the functional role of NO/sGC signalling in locomotor behaviours during development (Riedl et al., 2005). NOS1 knockout can also affect adult motor performance, with NOS1 knockout mice demonstrating increases in locomotion and touch escape reactions (Weitzdoerfer et al., 2004) as well as an array of other phenotypic behaviours such as: chronic aggressive behaviour; excessive and inappropriate mounting behaviour and impaired learning and memory (Kriegsfeld et al., 1997). However, whether these effects arise from developmental perturbations in nervous system structure is not yet known.

5.3 Aims of this study

The aims of this chapter are twofold: first, it seeks to elucidate the downstream signal transduction pathway through which NO signalling regulates axon branching of zebrafish spinal cord motoneurons and second, it seeks to define the functional consequences of perturbed NO signalling by assessing how disrupted NO signalling affects neuromuscular synaptogenesis and locomotor behaviour. The evidence presented in this study demonstrates that NO regulates motor axon branching through the canonical sGC/cGMP signalling pathway. Moreover, it demonstrates that perturbed NO signalling dramatically changes functional output of the developing

spinal network by modulating neuromuscular synapse density and maturation of motor control.

5.4 Results

5.4.1 Inhibition of soluble guanylyl cyclase increases motor axon branching

In order to gain an insight into how chronic disruption of the sGC/cGMP pathway regulates zebrafish motor axon development, ODQ (50µM), a pharmacological inhibitor sGC was injected into 24hpf zebrafish embryos and the effects on motor axon growth assessed on the following day with znp1 immunohistochemistry. ODQ treatment was found to cause a dramatic 72% increase in motor axon branch number at 48hpf (Figure 5.1 A-C; control: 19.7 ± 1.1bpf *cf.* ODQ: 33.9 ± 1bpf, $p=1x10^{-4}$), similar to the phenotype observed upon perturbation of NO signalling (see Figure 4.2-4.4).

In order to confirm that sGC inhibition affected motor axon growth, embryos were also raised in methylene blue (Gruetter et al., 1979, Gruetter et al., 1981), a classical inhibitor of this enzyme. Incubation of early stage zebrafish in methylene blue caused a significant increase in motor axon arborisation over a range of concentrations (Figure 5.2 A-F; control: 23.1 ± 1.8bpf; Mb ($3x10^{-6}$): 33.8 ± 4bpf, p=0.02; Mb ($3x10^{-5}$): 35.5 ± 1.9bpf, $p=1x10^{-4}$; Mb ($3x10^{-4}$): 31.1 ± 2.2bpf, p=0.04; Mb ($3x10^{-3}$): 35.7 ± 1.3bpf, $p=1x10^{-4}$), although the effects of this drug were not as dramatic as those observed following ODQ treatment. There is also a lack of dose dependence across the concentrations of methylene blue used demonstrating that this drug has a high

efficacy at a wide range of concentrations. Nonetheless, these findings strongly suggest that sGC inhibition mimics the effects of NOS1 perturbation by increasing motor axon branching.

5.4.2 Blockade of sGC occludes the effects of NO donors

The findings above suggest that blockade of sGC signalling mimics the effects NOS1 perturbation (Chapter 4; figure 4.2-4.4). To confirm that NO signalling modulates sGC activity, the ability of ODQ to rescue the DETA-NO phenotype was examined. To do this, 1dpf embryos were pre-injected with ODQ (50µM) before incubation in the NO donor DETA-NO (500µM) for 24hr. At 48hpf znp-1 labelled motor axon branching patterns of ODQ-injected fish, exposed to DETA-NO, were compared to those of fish exposed to DETA-NO alone. As expected, DETA-NO treatment caused a dramatic reduction in axon branch numbers (Figure 5.3A-D; control: 23.2 ± 1.2bpf *cf.* DETA-NO: 9.7 ± 0.9bpf *cf.* ODQ+DETA-NO: 23.3 ± 1.6bpf, $p=1x\ 10^{-4}$). However, pre-treatment with ODQ occluded this effect restoring branch numbers to those seen in control fish. These findings strongly suggest that the actions of NO are mediated through the sGC signalling pathway.

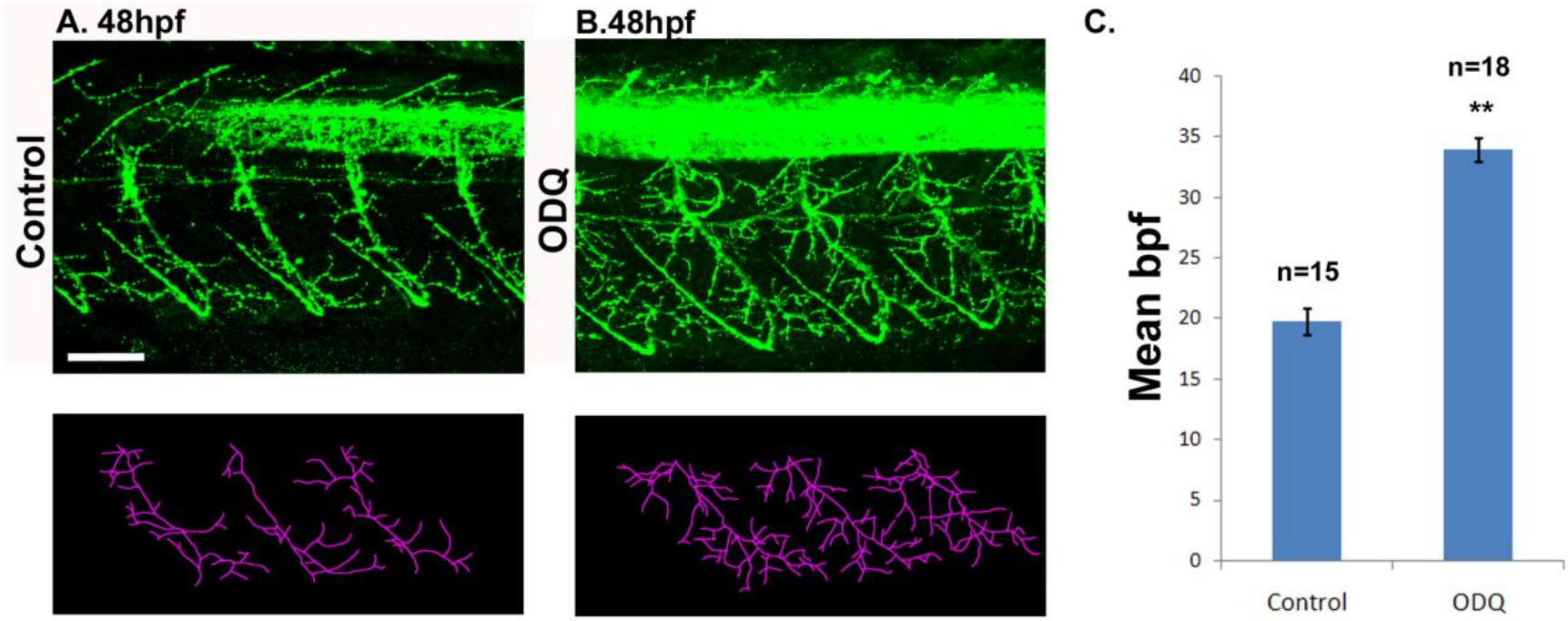

Figure 5.1 Developmental inhibition of sGC with ODQ increases motor axon branching

A-B, Upper panels: lateral views of znp1 labelled trunks (***A***) and ODQ-injected (***B***) fish at 48hpf. Lower panels: Neuron J tracings of motor axon collaterals derived from the image directly above. ***C,*** Bar char depicting the mean (± SEM) branches per fascicle (bpf) for both control and ODQ treated fish. Scale bar: ***A-B***, 50µm. ** $p \leq 0.0005$.

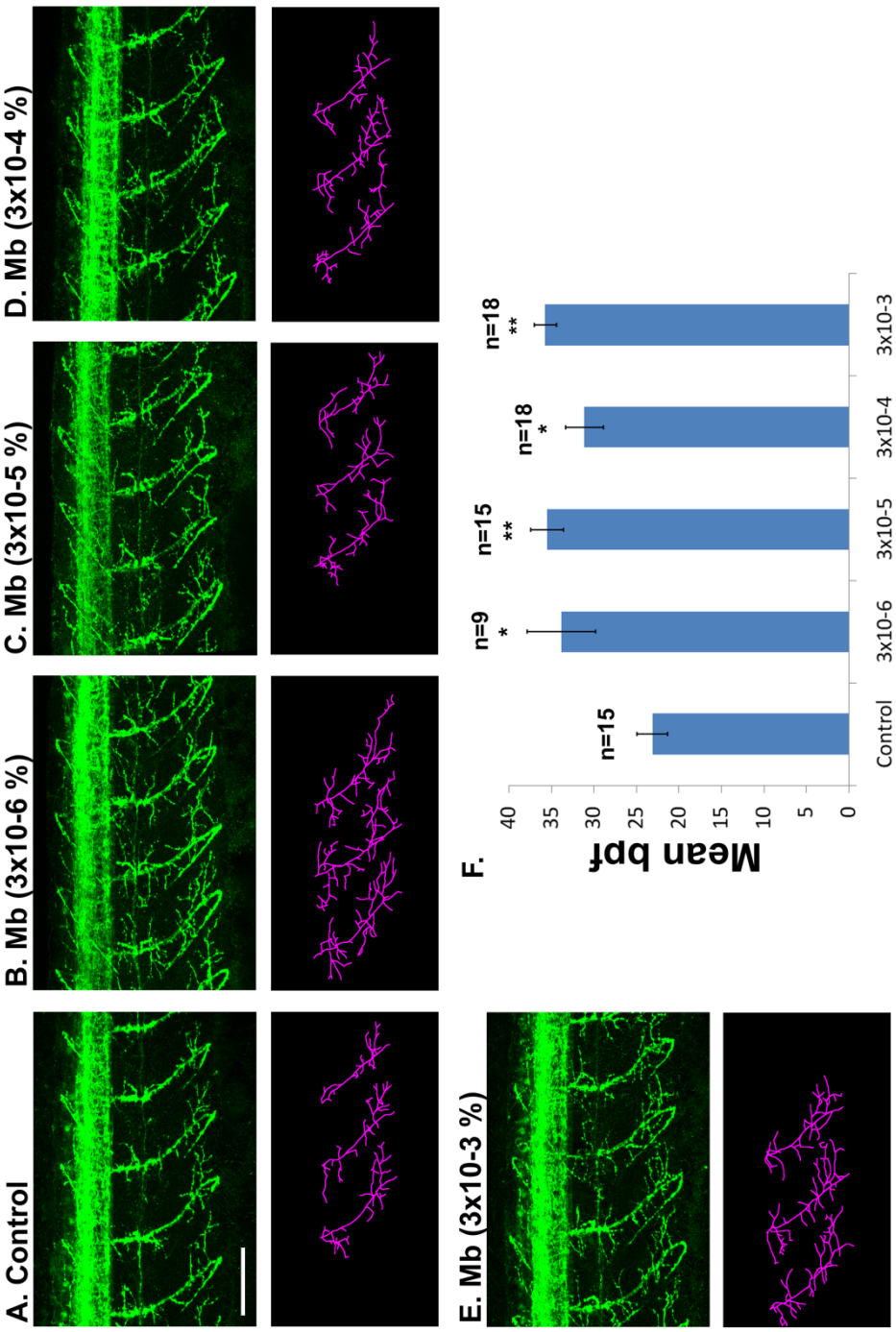

Figure 5.2 Pharmacological inhibition of sGC with methylene blue increases in motor axon arborisation

A-E, Upper panels: znp1 immunostained trunks (***A***) and methylene blue treated fish; $3x10^{-6}$% (***B***); $3x10^{-5}$% (***C***); $3x10^{-4}$% (***D***); $3x10^{-3}$% (***E***). Lower panels: Neuron J tracings of motoneuron branches derived directly from the image above. ***F,*** Bar chart to show the mean (± SEM) branches per fascicle (bpf) for each experimental condition. Scale bar: ***A-E*** (in ***A***), 50µm. * $p \leq 0.05$; ** $p \leq 0.0005$.

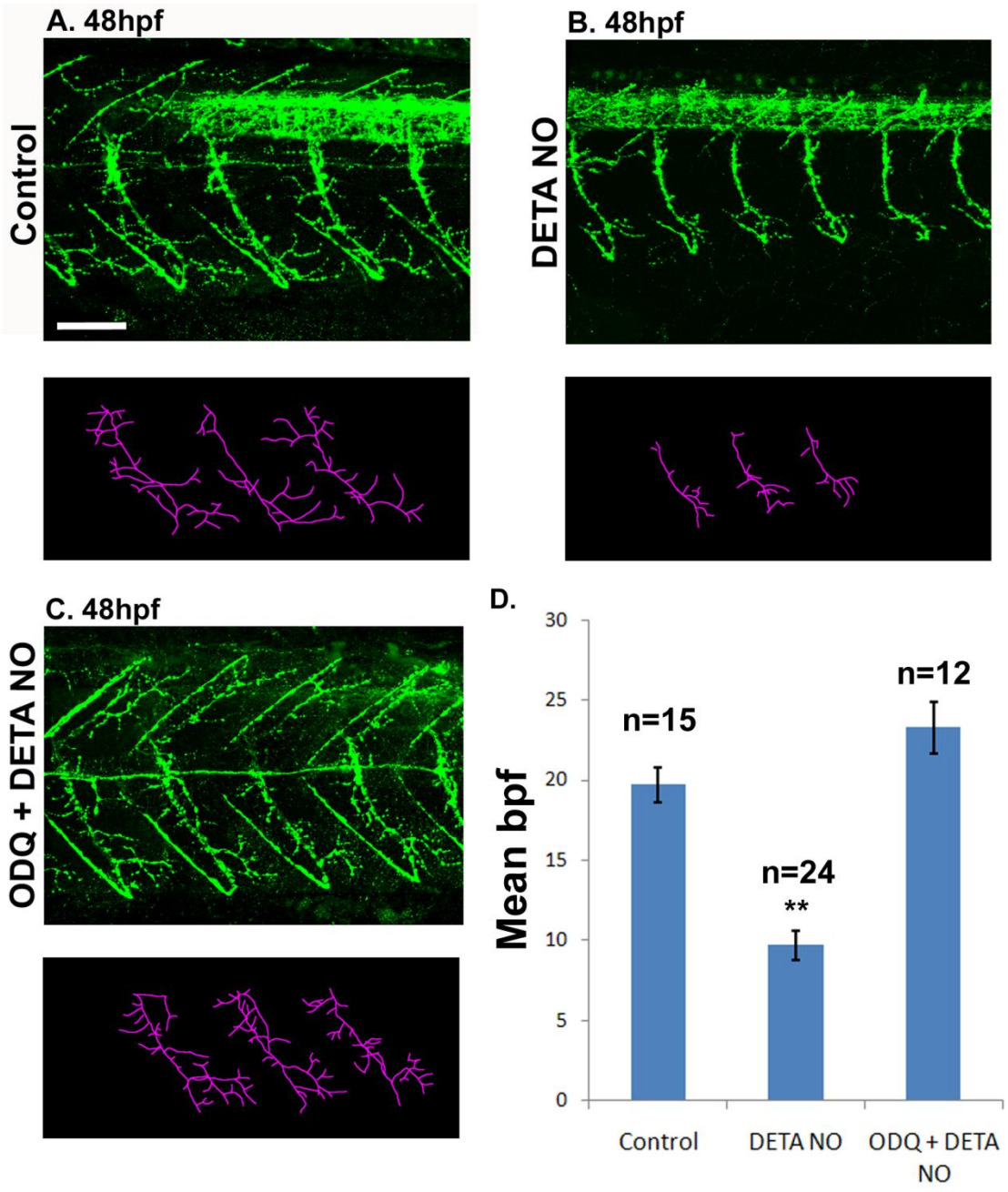

Figure 5.3 DETA NO-induced decreases in motor axon branch numbers is rescued by inhibition of sGC during DETA NO treatment

A-C, Upper panels: lateral views of znp1 labelled trunks of control (***A***), DETA NO (***B***) treated and ODQ+DETA NO (***C***) treated embryos at 48hpf. Lower panels: Neuron J tracings of motor axon branches derived from the panel directly above. ***D,*** Bar chart displaying the mean (± SEM) branches per fascicle (bpf) for each of the experimental conditions above. Scale bar: ***A-C*** (in ***A***) 50µm. ** $p \leq 0.0005$.

5.4.3 Exogenous cyclic guanosine monophosphate mimics the effects of NO donors

Next, the effects of exogenous cGMP application on motor axon branching were investigated using a membrane permeable analogue of cGMP. Incubation of the cGMP analogue (250µM-750µM) at 24hpf resulted in a significant 60% decrease in motor axon branching by 48hpf (Figure 5.4A-C; control: 25.6 ± 1.7bpf *cf.* cGMP: 10.2 ± 0.7bpf, $p=1x10^{-4}$), an effect that was strikingly similar to that caused by DETA-NO treatment (Figure 5.3B). This suggests that NOs effects on motor axon morphogenesis are mediated by the sGC-cGMP pathway.

5.4.4 PKG inhibition does not alter motor axon branching

To explore whether PKG is a downstream target of cGMP signalling, 1dpf embryos were injected with 250µM of a PKG inhibitor peptide and incubated for a 24hr period. At 48hpf, treatment with the PKG inhibitor had no effects on motor axon branching with resulting branch numbers identical to control (Figure 5.5A-C; control: 30.9 ± 1.1bpf *cf.* PKG inhibitor: 30.4 ± 2.5bpf, p=0.8). These findings suggest that PKG does not have a role in regulating motor axon growth.

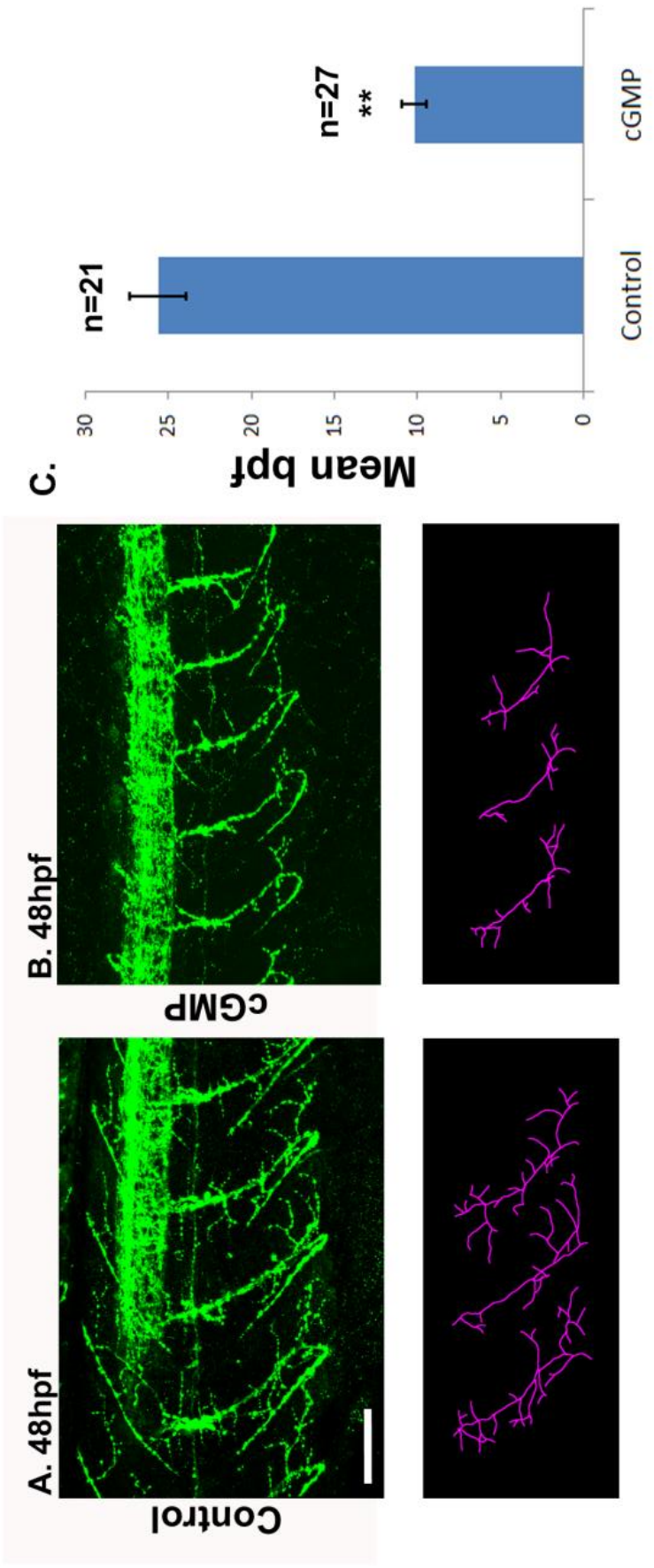

Figure 5.4 Exogenous elevation of cGMP levels decreases motor axon branching

A-B Upper panel: znp1 immunolabelled trunks of control (***A***) and cGMP (***B***) treated embryos at 48hpf. Lower panels: Neuron J tracings of the motor axon branches from the image directly above. ***C,*** Bar chart depicting the mean (± SEM) branches per fascicle (bpf) for both control and cGMP treated fish. Scale bar: ***A-B*** (in ***A***), 50µm. ** $p \leq 0.0005$.

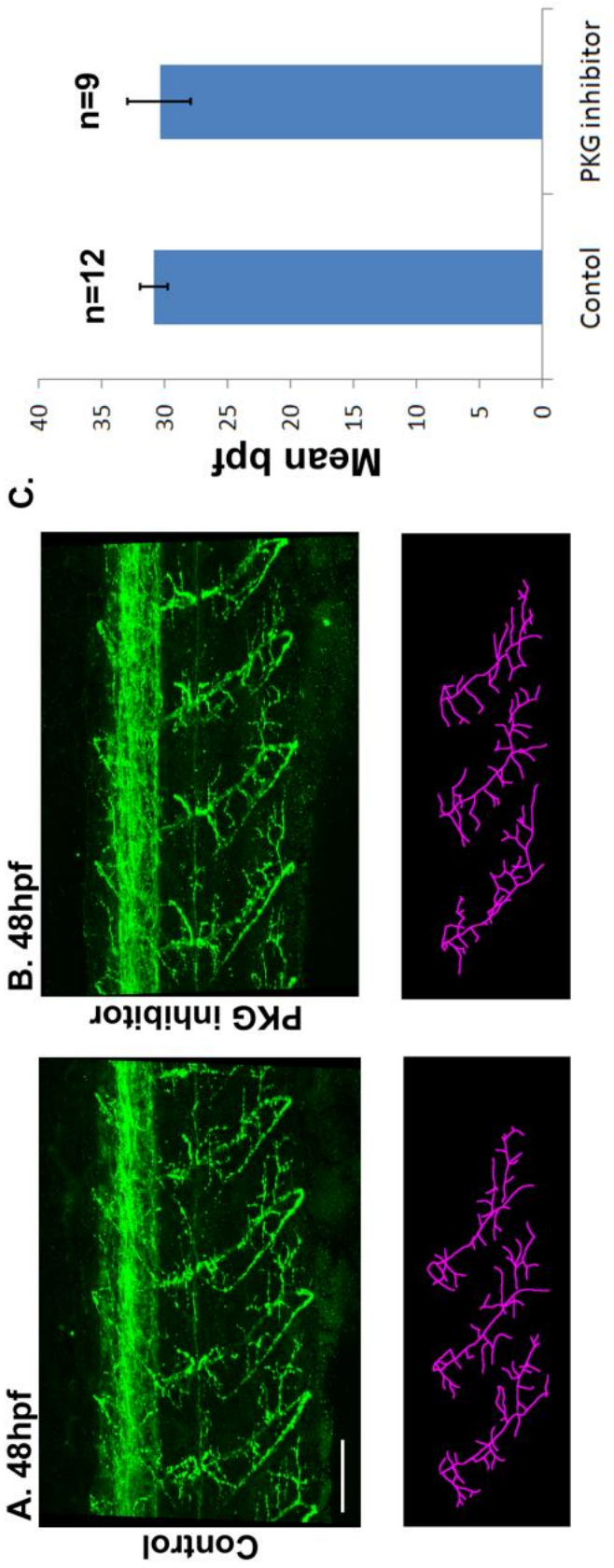

Figure 5.5 inhibition of PKG does not alter motor axon branch numbers

A-B, Upper panels: lateral views of znp1 immunolabelled trunks of control (***A***) and PKG inhibitor (***B***) treated fish at 48hpf. Lower panels: Neuron J tracings of motor axon branches derived from the panel directly above. ***C,*** Bar chart depicting the mean (± SEM) number of motor axon branches per fascicle (bpf) in both control and PKG inhibitor treated fish. Scale bar: ***A-C*** (in ***A***), 50µm.

5.4.5 Inhibition of cADPR signalling increases motor axon branch branching

As cADPR has been shown to be a component of the NO signalling response at *Helisoma* B5 growth cones (Welshhans and Rehder, 2005, Welshhans and Rehder, 2007), the possibility that this messenger influences zebrafish motor axon development was also investigated. To do this 24hpf embryos were injected with the cADPR antagonist, 8-Bromo-cADPR (1mM), and the effects on motor axon growth assessed with znp1 staining at 48hpf. Injection of 8-bromo-cADPR resulted in a 17% increase in motor axon branching by 48hpf (Figure 5.6A-C; control: 30.9 ± 1.1bpf *cf.* 8-bromo-cADPR: 36.3 ± 1bpf, $p=1x10^{-3}$). Although this is a modest increase compared to that induced by sGC or NOS1 perturbation (Figure 5.1-5.2; Chapter 4; figure 4.2-4.4), it nonetheless indicates that cADPR has the capacity to modify motor axon branching.

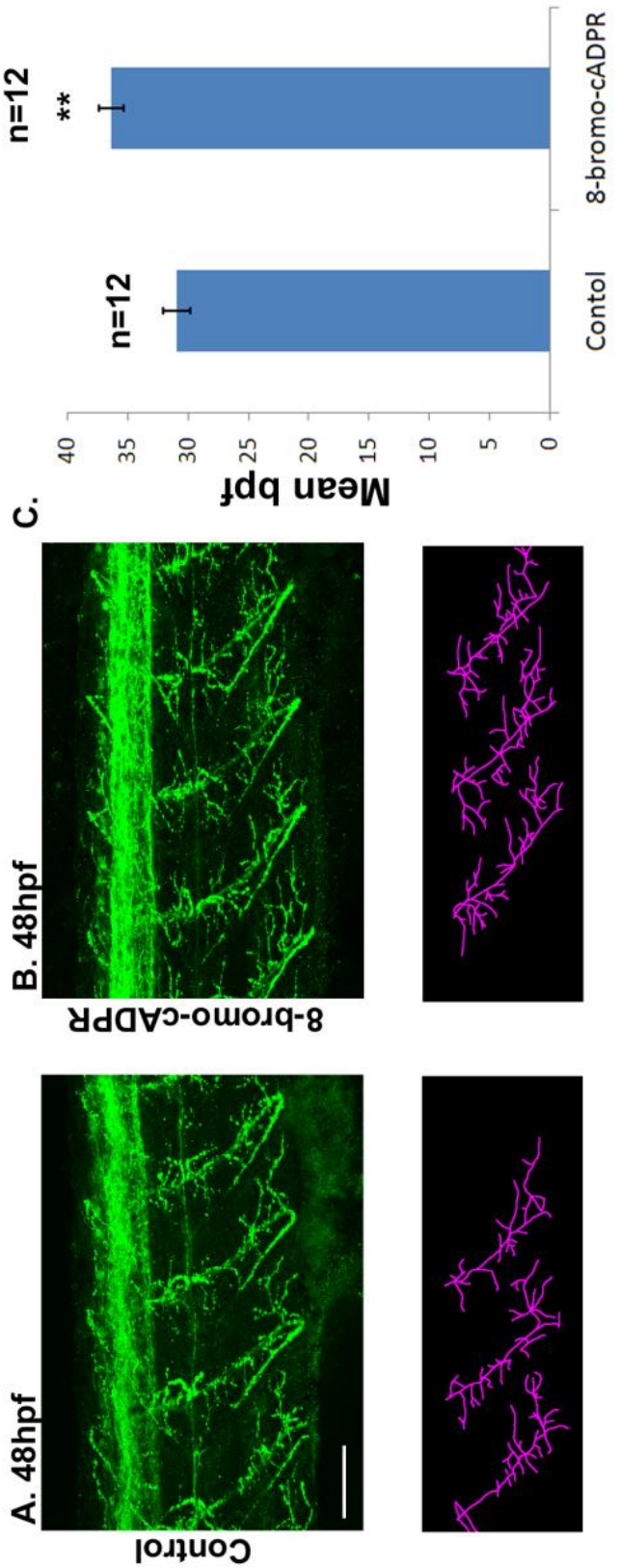

Figure 5.6 Inhibition of cADPR increases motor axon branching
A-B, Upper panels: znp1 immunostained trunks of control (***A***) and 8-bromo-cADPR (***B***) treated fish at 48hpf. Lower panels: Neuron J tracings of motor axon branching derived from the image directly above. ***C,*** Bar chart displaying the mean (± SEM) number of motor axon branches per fascicle (bpf) in both control and 8-bromo-cADPR treated fish. Scale bar: ***A-B*** (in ***A***), 50µm. ** $p \leq 0.005$.

5.4.6 NO signalling affects neuromuscular synaptogenesis

Whilst anatomical studies of motor axons provide an important insight into presynaptic changes in neuromuscular development, they do not provide information on accompanying postsynaptic changes and synapse formation. To gain an insight into whether NO can also influence neuromuscular synaptogenesis, NO signalling was disrupted in early stage embryos and the impact on synapse formation assessed with SV2 and Rh-α-bungarotoxin staining, which are markers for presynaptic and postsynaptic neuromuscular domains respectively (Panzer et al., 2005, Panzer et al., 2006). For the purposes of this study, areas where these markers co-localised were considered to represent putative neuromuscular synapses.

Initially, NOS1 function was perturbed during development with the NOS inhibitor L-NAME (1mM) and the consequences to synaptic markers assessed at 48 and 72hpf respectively. Fish injected with L-NAME were found to exhibit a dramatic increase in SV2/Rh-α-BTX co localisation in the muscle tissue at 48hpf (Figure 5.7A-E; control: 2.5 $x10^4$ ± 0.1 $x10^4$ *cf.* L-NAME: 4.6 $x10^4$ ± 0.2 $x10^4$ co-localised voxels, $p=1x10^{-4}$) and 72hpf (Figure 5.8A-E; control: 3.1 $x10^4$ ± 0.3 $x10^4$ *cf.* L-NAME: 6.1 $x10^4$ ± 0.8 $x10^4$ co-localised voxels, $p=1x10^{-4}$), which represents an 84% and 97% increase in co-localised domains at 2d and 3d respectively in comparison to age matched controls.

Subsequently the effects of exogenous NO application on neuromuscular markers was assessed by raising embryos in DETA NO (500μM). These fish had significantly reduced levels of SV2/Rh-α-BTX co-localisation at both 48hpf and 72hpf: at 48hpf DETA-NO treated fish demonstrated a 40% reduction in SV2/Rh-α-BTX co-localisation (Figure 5.9A-E; control: 1.5 $x10^4$ ± 0.2 $x10^4$ *cf.* DETA NO: 0.9 $x10^4$ ± 0.1 $x10^4$

co-localised voxels, p=0.05). Similarly by 72hpf, DETA NO treated fish displayed a 43% decrease in SV2/Rh-α-BTX co-localisation (Figure 5.10A-E; control 2.1 $x10^4 \pm 0.1$ $x10^4$ *cf.* DETA NO: 1.2 $x10^4 \pm 0.2$ $x10^4$ co-localised voxels, $p=3x10^{-3}$). Taken together these observations suggest that NO signalling potently regulates neuromuscular synaptogenesis during early stage zebrafish development.

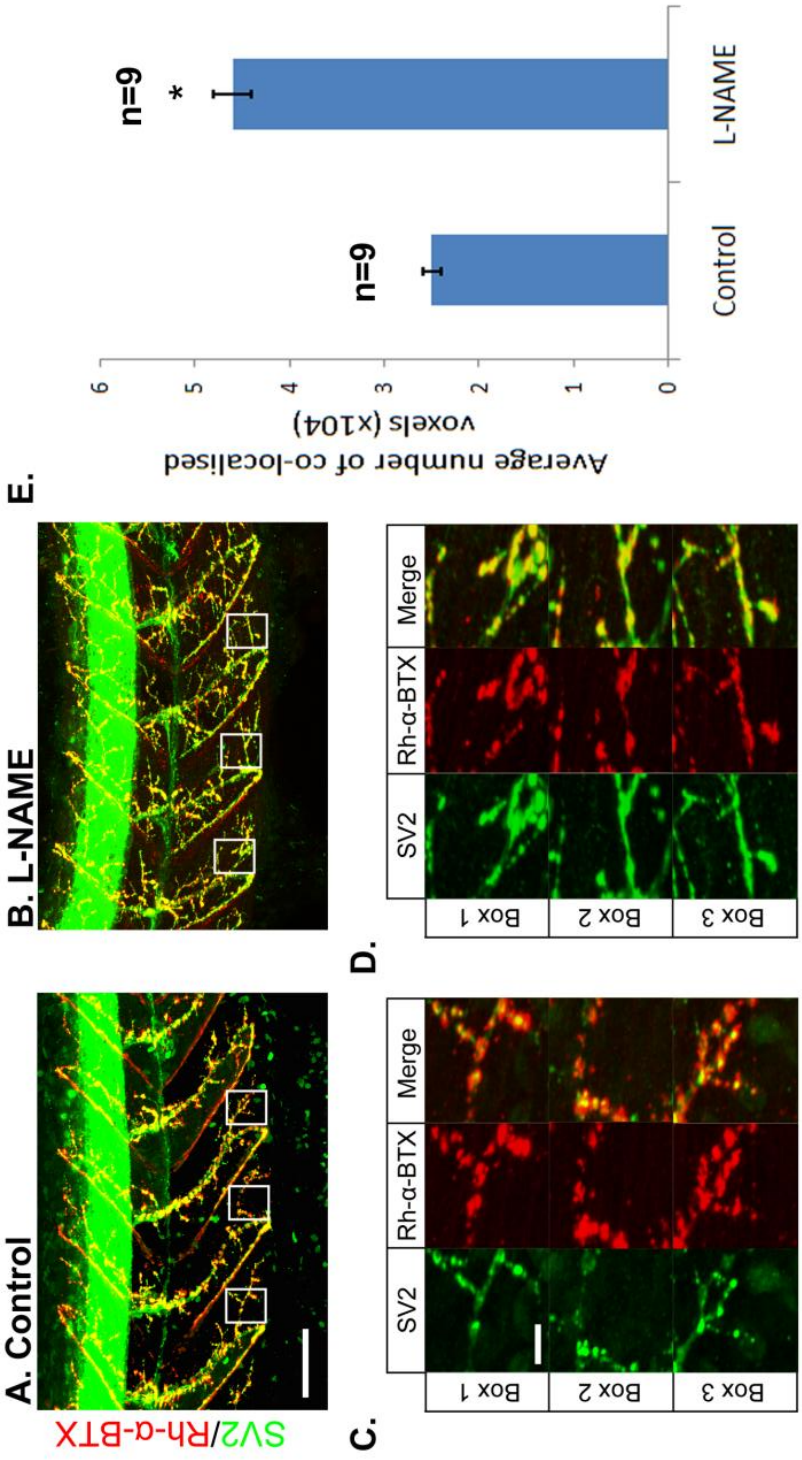

Figure 5.7 Developmental inhibition of NOS increases neuromuscular synapse numbers at 48hpf

A-B, lateral views of zebrafish trunks immunostained with SV2 (green)/Rh-α-BTX (red) and merged in control (***A***) and L-NAME (***B***) treated fish at 48hpf. Boxes in ***A*** and ***B*** represent the regions selected for co localisation analysis in ***C*** and ***D***.***C-D,*** High power images of single and merged SV2/Rh-α-BTX channels from the area demarked in ***A*** and ***B***. ***E,*** Bar chart to represent the average number (± SEM) of co-localised voxels in the selected regions of both control and L-NAME treated fish. Scale bar: ***A-B*** (in ***A***), 50µm; ***C-D*** (in ***C***), 8µm. *p ≤ 0.0005.

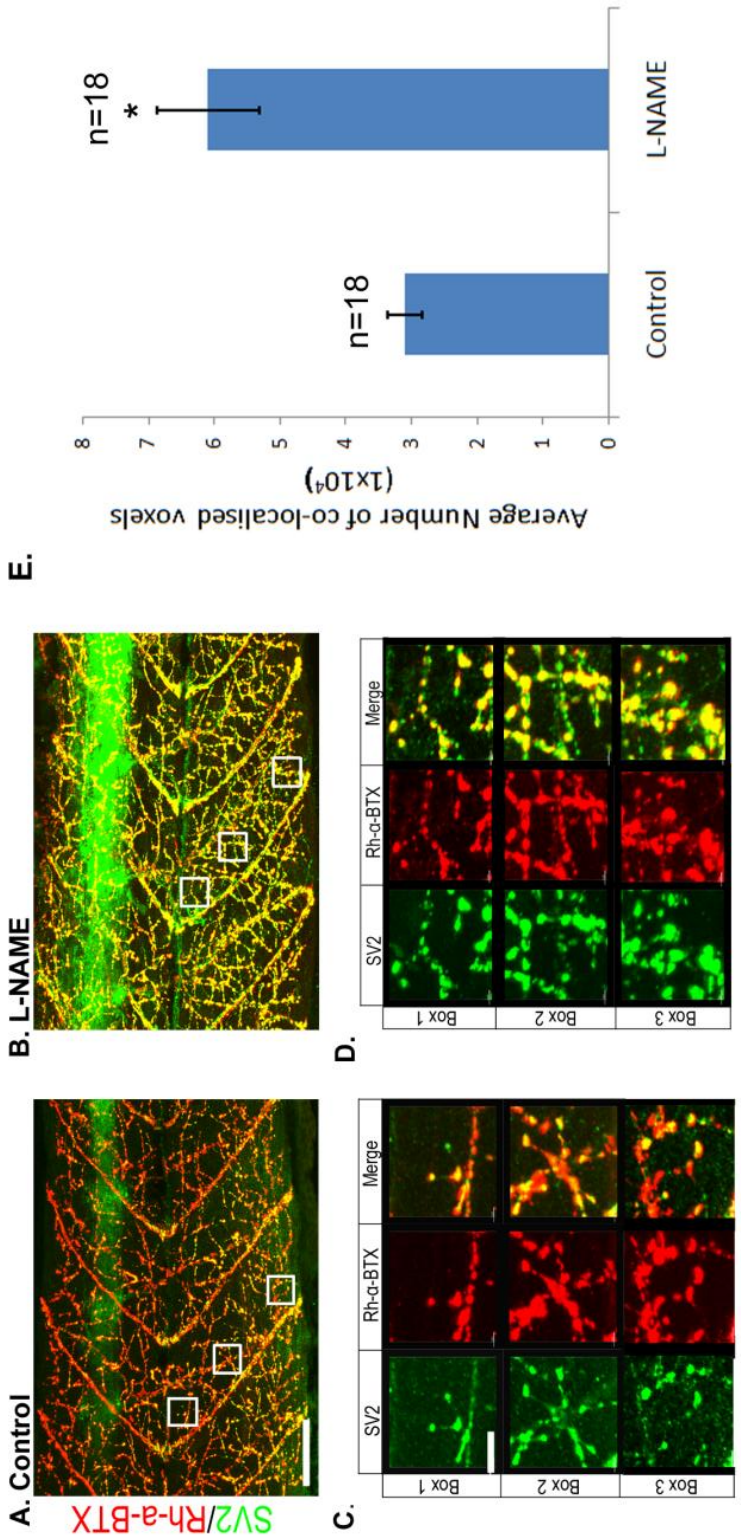

Figure 5.8 Developmental inhibition of NOS increases neuromuscular synapse numbers at 72hpf
A-B, Merged image of SV2 (green)/ Rh-α-BTX (red) immunostaining on control (***A***) and L-NAME (***B***) treated fish at 72hpf. Boxes in ***A*** and ***B*** outline the regions chosen for co-localisation analysis in ***C*** and ***D***. ***C-D,*** Magnified images of separate and merged SV2/Rh-α-BTX channels from the boxed outlines in ***A*** and ***B***. ***E,*** Bar chart depicting the average (± SEM) number of co-localised voxels in the outlined regions of interest for both control and L-NAME treated fish. Scale bars: ***A-B*** (in ***A***), 50µm; ***C-D*** (in ***C***), 8µm. * $p \leq 0.0005$.

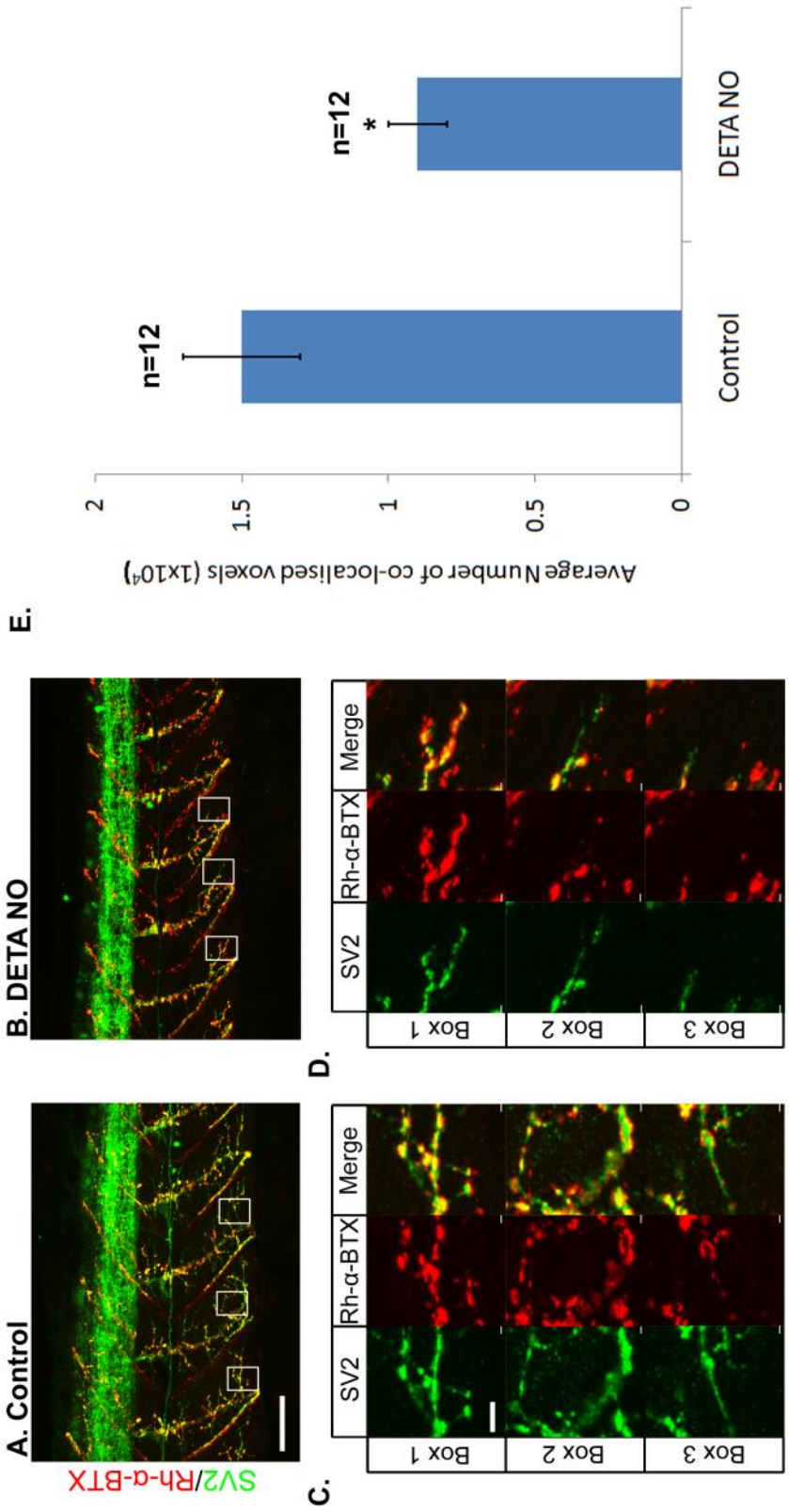

Figure 5.9 Elevation of NO signalling decreases neuromuscular synapse numbers at 48hpf

A-B, Lateral view of control (***A***) and L-NAME (***B***) treated zebrafish trunks dual labelled with SV2 (green) and Rh-α-BTX (red). Boxes outlined in ***A*** and ***B*** depict the regions for co-localisation analysis in ***C*** and ***D***. ***C-D,*** Single and merged SV2 and Rh-α-BTX channels taken from the regions outlined in ***A*** and ***B***. ***E,*** Bar chart showing the average (± SEM) number of co-localised voxels in both control and L-NAME treated fish from the boxes outlined in ***A*** and ***B***. Scale bars: ***A-B*** (in ***A***), 50µm; ***C-D*** (in ***C***), 8µm. * $p \leq 0.05$.

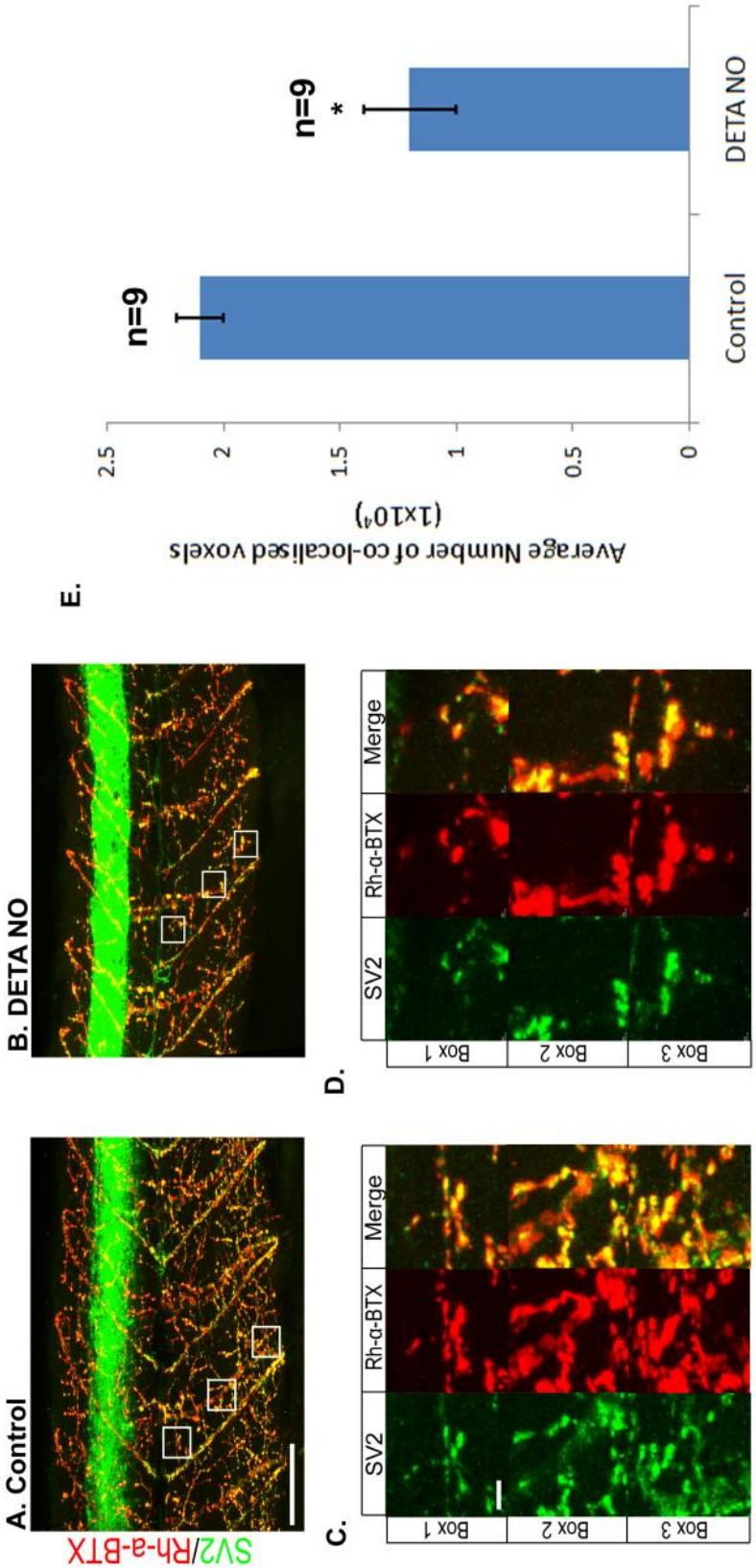

Figure 5.10 Elevation of NO signalling decreases neuromuscular synapse numbers at 72hpf

A-B, Lateral views of 72hpf zebrafish trunks immunolabelled with SV2 (green) and Rh-α-BTX (red) in control (***A***) and DETA NO (***B***) treated fish. Boxes outlined in ***A*** and ***B*** demark the regions selected for co-localisation analysis in ***C*** and ***D***. ***C-D*** single and merged channels of SV2/Rh-α-BTX staining taken from the boxes shown in panels ***A*** and ***B***. ***E,*** Bar chart depicting the average (± SEM) number of co-localised voxels in the boxes selected in ***A*** and ***B***. Scale bars: ***A-B*** (in ***A***), 50µm; ***C-D*** (in ***C***), 8µm. * $p \leq 0.005$

5.4.7 Developmental disruption of nitrergic signalling affects locomotor output

As NO signalling had a profound influence over neuromuscular synaptogenesis, the possibility that this messenger also regulates maturation of locomotor behaviour was investigated. To do this, NO signalling was perturbed between 24 and 72hpf and the consequences on locomotor performance studied thereafter through kinematic analysis of the following locomotor parametres: velocity, tail beat frequency and maximal tail bend amplitude (Budick and O'Malley, 2000).

Swimming velocity of 72hpf NOS1 knockdown larvae was found to be significantly higher than that of age-matched controls (Figure 5.11B; control: 75.3 ± 5.2mm/s *cf.* NOS1 AMO: 90.1 ± 4.6mm/s, p=0.03), whilst larvae raised in DETA NO throughout early development swam at dramatically reduced velocities (Figure 5.11B; control: 75.3 ± 5.2mm/s *cf.* DETA NO: 29.7 ± 2.1mm/s, $p=1x10^{-4}$). Likewise, tail beat frequencies were significantly higher in NOS1 AMO injected larvae (Figure 5.11C; control: 32.2 ± 1.5Hz *cf.* NOS1 AMO: 37.7 ± 1.1Hz, p=0.04) and significantly lower in DETA-NO exposed larvae (Figure 5.11C; control: 32.2 ±1.5Hz *cf.* DETA NO: 22.3 ± 1.1Hz, $p=1x10^{-4}$). Finally, NOS1 AMO injected fish were capable of generating larger maximal bend amplitudes compared to controls (Figure 5.11A,D; control: 98.3 ± 3.2° *cf.* NOS1 AMO: 108 ± 2°, p=0.01). In contrast, DETA NO treated fish produced maximal bend amplitudes that were dramatically lower than those of control fish of the same age (Figure 5.11A,D; control: 98.3 ± 3.2° *cf.* DETA NO: 59.9 ± 3.3°, $p=1x10^{-4}$). Taking the above findings into consideration, these data suggest that modulation of NO levels

during early motoneuron maturation leads to alterations in several parametres of locomotor output.

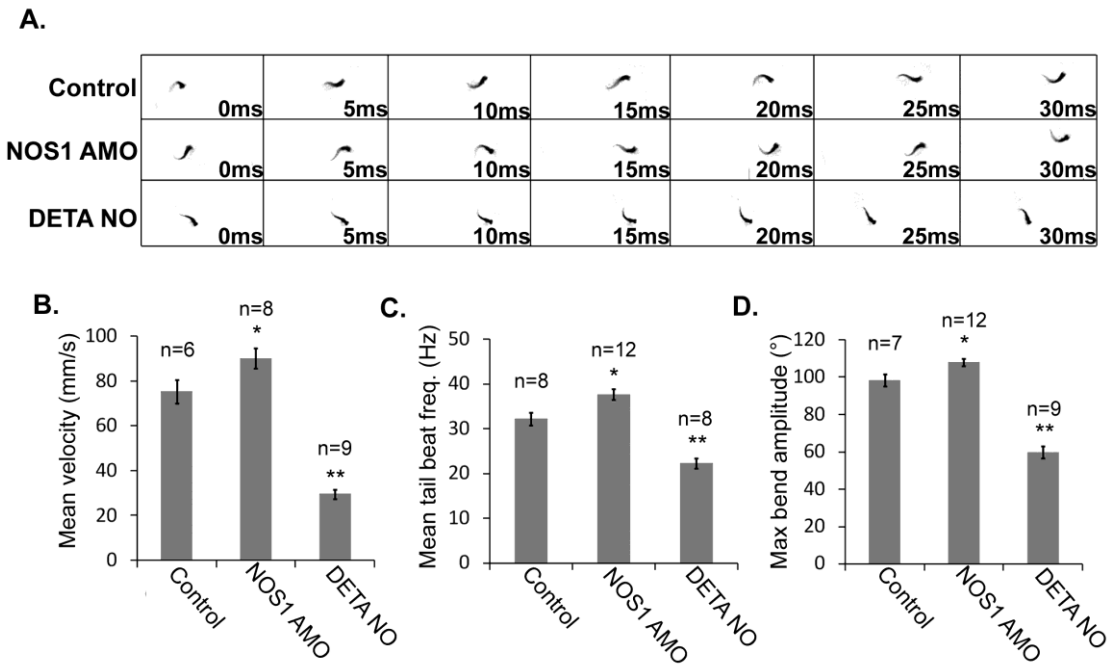

Figure 5.11 NO signalling affects locomotor behaviour
A, Locomotor behaviour captured at 200 frames·sec^{-1} in control, NOS1 AMO-injected and DETA NO-treated fish at 72hpf. ***B-D,*** Bar charts displaying mean (± SEM) velocity (***B***), tail beat frequency (***C***) and maximal tail bend amplitude (***D***) for control, NOS1 AMO-injected and DETA NO-treated fish at 72hpf. ** $p \leq 0.0005$; * $p \leq 0.05$.

5.5 Discussion

This chapter demonstrates that the sGC/cGMP signalling pathway potently regulates the growth of zebrafish motor axons in a manner similar to NO, with elevated cGMP levels inhibiting and reduced cGMP levels promoting axon branch development. Importantly, this study also shows that blockade of sGC activity occludes NOs ability to inhibit motor axon growth, providing strong evidence that the sGC/cGMP signalling is the principal transducer of NOs effects. Additional examination of downstream candidates failed to provide evidence for an involvement of PKG but did provide indirect evidence that cADPR signalling may play a role in mediating these actions.

In addition, this chapter has demonstrated that disrupting the NO/cGMP signalling pathway has clear functional consequences. It demonstrates that NO-induced changes in motor axon branching culminate in dramatic alterations in neuromuscular synaptogenesis, with high levels of NO suppressing synaptogenesis and low levels of NO promoting it. Analysis of motor behaviour shows that these changes can cause disruptions in the maturation of locomotor activity, especially under conditions where NO levels are high. Collectively, this work demonstrates for the first time the sGC/cGMP signalling pathway is a potent regulator of zebrafish motoneuron maturation during *in vivo* development.

5.5.1 The sGC/cGMP pathway is a downstream effector of NO

In a wide range of culture models, cGMP has been identified as the principal downstream effector of NOs actions on the developing neurite (Hindley et al., 1997, Gibbs et al., 2001, Gibbs and Truman, 1998, Tojima et al., 2009, Seidel and Bicker, 2000, Haase and Bicker, 2003, Xiong et al., 2007, Tornieri and Rehder, 2007, Welshhans and Rehder, 2005, Welshhans and Rehder, 2007, Trimm and Rehder, 2004, Van Wagenen and Rehder, 1999, Van Wagenen and Rehder, 2001). This study provides additional *in vivo* support to this proposal, showing that sGC/cGMP signalling is a critical target for nitrergic modulation of zebrafish motoneuron growth. Evidence for this comes from the finding that the sGC inhibitors, ODQ and methylene blue, mimic the effects of NOS1 perturbation, whilst the GMP analogue 8-(4-chlorophenylthio)-cGMP phenocopies that of NO donors. Moreover, the finding that ODQ occludes the effects of DETA NO provides direct evidence that sGC is the primary downstream effector of NO. Thus, these studies support the notion that canonical cGMP signalling cascades underpin NOs ability to regulates motor axon branching.

It is important to note that the sGC/cGMP is not the only pathway that mediates the effects of NO in physiological systems: nitrosylation and nitration are also key transducers of this molecule (see chapter 1 for overview). However, as manipulation of either sGC, cGMP or NO yielded near identical results, it was not considered necessary to explore these pathways during the course of this study. However, this does not preclude their involvement in other developmental processes and future studies will help determine if this is the case.

5.5.2 Possible downstream targets of cGMP signalling

cGMP is known to act on three major targets: cyclic nucleotide-gated ion channels, phosphodiesterases and PKG (Nakamura and Gold, 1987, Firestein et al., 1991, Francis et al. 2010, Hanafy et al., 2001, Bryan et al., 2009, Krumenacker et al., 2004, Garthwaite, 2008, Garthwaite, Bradley et al., 2005). Of these, PKG is possibly the most common and there is strong evidence to suggest that this protein is involved in regulating neurite growth (Welshhans and Rehder, 2005, Welshhans and Rehder, 2007, Ditlevsen et al., 2007, Yamazaki et al., 2005, Xiong et al., 2007, Leamey et al., 2001). During the current study, exposure to peptide inhibitors of PKG had no effect on motor arbour formation, suggesting that this enzyme does not have a role in nitrergic regulation of motor axon growth. However as the inhibitor used was large and lipophobic, it is possible that, in zerbrafish, accessibility problems may have diminished its pharmacological actions. Taking these caveats in to consideration it is not possible to determine whether PKG is functionally involved in nitrergic regulation of motor axon growth. As such, the possible role of PKG in control of zebrafish motor axon development merits further investigation.

Although this study did not determine a role for PKG in NO transduction, preliminary experiments demonstrate that inhibition of cADPR, a downstream effector of NO signalling in *Helisoma* B5 neurons (Welshhans and Rehder, 2005, Welshhans and Rehder, 2007), increases branching of zebrafish motor axons. As this effect was similar, to that elicited by NO/cGMP, it is tempting to speculate that cADPR may be a component of the NO signalling pathway. However before this can be validated, experiments to determine whether these pathways are coupled must be conducted.

Future experiments examining whether blocking cADPR signalling renders NO ineffective will help to determine whether this is indeed the case.

5.5.3 Implications for the use of methylene blue to prevent fungal growth during zebrafish culture

Methylene blue is a widely used reagent for preventing fungal blooms during the first few days of zebrafish development (Westerfield, 2007). When used at 3 $x10^{-5}$ %, this treatment dramatically improves survival and embryo yield without causing any obvious developmental defects. However, methylene blue is also an effective antagonist of sGC (Masaki and Kondo, 1999, Hwang et al., 1998, Gibbs and Truman, 1998, Dierks and Burstyn, 1998) and there is some evidence to suggest that it also inhibits NOS (Luo et al., 1995, Mayer et al., 1993b, Mayer et al., 1993a). Findings presented in this study suggest that, at concentrations rountinely used for zebrafish embryo culture, methylene blue can induce changes in motor axon growth that mirror those seen with NOS and sGC inhibitors. This finding, which provides evidence that methylene blue inhibits zebrafish cGMP signalling, therefore raises doubt over its utility as a zebrafish culture reagent: the widely used practice of methylene blue culture may diminish cGMP signalling and impact a whole range of developmental processes ranging from axon growth to cardiovascular development (Pelster et al., 2005).

5.5.4 NO signalling and neuromuscular synaptogenesis

The findings summarised in chapter 4 demonstrate that NO regulates branching of developing motoneuron axons, which suggests that it may also regulate neuromuscular synaptogenesis. In support of this premise, the density of pre- and postsynaptic neuromuscular markers can be dramatically altered by developmental manipulation of NO signalling. In this context, shifts in neuromuscular synapse density mirror those of motor axon branch density, with NOS inhibition causing an increase in synapses and NO depletion causing a decrease in synapses. This finding provides strong evidence that NO signalling tightly regulates neuromuscular synaptogenesis.

In other biological systems, NO has clearly demonstrable effects on synaptic plasticity (Cogen and Cohen-Cory, 2000, Truman et al., 1996, Campello-Costa et al., 2000, Roskams et al., 1994, Sunico et al., 2005, Wu et al., 1994). For example, in visual circuits NO modulates activity-dependent refinement of synaptic connections (Gibbs et al., 2001, Gibbs and Truman, 1998, Cramer et al., 1996, Cramer and Sur, 1999, Tenorio et al., 1996, Wu et al., 2001, Williams et al., 1994). Moreover, previous studies also support the proposal that NO regulates formation of the vertebrate NMJ: here NO signalling promotes AChR clustering in muscle cells of *Xenopus* (Schwarte and Godfrey, 2004) and chick (Jones and Werle, 2000). Whilst a similar effect could account for the findings described here, two observations argue against this. First, although NOS1 is localised in the skeletal muscle at the NMJ in mammals (Yang et al., 1997, Chao et al., 1997, Ribera et al., 1998, Kusner and Kaminski, 1996) there is no evidence to suggest its presence in muscle tissue of zebrafish embryos (Chapter 3; see also (Holmqvist et

al., 2004, Poon et al., 2003). Second, in zebrafish NOS1 is not expressed in the ventral spinal cord until 35hpf which is long after AChRs have formed clusters within in the developing myotome (Panzer et al., 2005, Panzer et al., 2006). Taken together, it is unlikely that the effects observed in the current study are a consequence of NO-dependent remodelling of postsynaptic domains.

Given the absence of NOS in muscle tissue, how might NO signalling influence NMJ development? One possibility is that spinal NO signalling modulates electrical or synaptic properties of developing motoneurons. Indeed, NO has been shown to regulate transmitter release at the developing NMJ (Thomas and Robitaille, 2001, Wang et al., 1995, Ribera et al., 1998) causing reduced end plate potential amplitudes (Thomas and Robitaille, 2001). Similarly in *Torpedo marmorata* synaptosomes, application of NO inhibits the depolarisation-induced release of ACh (Ribera et al., 1998). However, in contrast, NO has also been demonstrated to increase neurotransmission at the rat NMJ (Nickels et al., 2007). Thus, changes in neuromuscular development could be a homeostatic consequence of disrupted motoneuron function (for review, see Turrigiano and Nelson, 2004).

5.5.5 Nitric oxide modulates the maturation of motor behaviours

As NO has repercussions on neuromuscular synaptogenesis, it was important to determine whether locomotor performance was impacted by disrupting nitrergic pathways. Fish exposed to DETA NO throughout early development had greatly reduced swimming velocities and maximal tail bend amplitudes in comparison with

fish of the same age. In contrast, NOS1 AMO injected fish exhibited a small yet significant increase in both their swimming velocity and maximal bend amplitudes.

One possible explanation for these changes in motor output could be due to alterations in EW muscle innervation by the primary motoneurons. As previously shown, DETA NO treatment results in less motor axon collaterals therefore it seems feasible to suggest that this would lead to significantly reduced levels of EW muscle recruitment by the primary motoneurons; either by preventing innervations in a proportion of EW muscle fibers altogether or by reducing the number of terminal contacts made on each fiber. Either of the above scenarios would result in dramatically reduced levels of motor output. In contrast, perturbation of NO signalling results in increased motor axon branching which, in turn could lead to increased innervations of the EW muscle. However, as the neuromuscular contacts on the EW fibers are sufficient to generate optimal muscle contractions, the addition of extra synaptic contacts is unlikely to boost motor performance dramatically, but still significantly.

Additionally, it is demonstrated that NO also appears to modulate tail beat frequency; with DETA NO treated fish displaying reduced frequencies and NOS1 AMO injected fish demonstrating increased frequencies. These phenotypes are likely to arise from effects on pre-motor circuitry rather than changes in EW muscle innervations and therefore it is likely that electrical and/or synaptic properties of interneurons are modulated by this signalling molecule. Indeed, NO is known to regulate aspects of locomotor circuitry: this molecule facilitates glycinergic and GABAergic inhibition. In *Xenopus,* NO is shown to decrease both swim episode duration and swim frequency through facilitation of GABAergic and glycinergic synapses respectively (McLean and

Sillar, 2000, McLean and Sillar, 2002, McLean and Sillar, 2004). However in the lamprey NO has a contrasting effect. Here NO enhances locomotor frequency through suppression of glycinergic synapse and activation of excitatory glutamatergic synapses (Kyriakatos et al., 2009). Further research is required to gain a full understanding of whether NO modulates changes in electrical and synaptic properties of zebrafish spinal cord interneurons.

6. Final discussion

The work presented in this thesis details a novel developmental role of the small simple messenger molecule NO during zebrafish motoneuron maturation. The principal findings of this study are that endogenous NO signalling regulates primary motor axon branching in a cGMP-dependent manner. This pathway has the potential to cause dramatic restructuring of neuromuscular synapses which consequently impact the maturation of locomotor performance in postembryonic life.

Work in Chapter 3 uses for the first time a combined protein and transcript approach to define the spatial distribution of NOS1 during zebrafish spinal cord development, showing that NOS1 is expressed in a discrete population of spinal interneurons which lie in close proximity to developing motoneuron pools. These cells are the only cells of the early trunk to persistently express NOS1 and their spatial position suggests that they have the potential to influence the growth of motoneuron populations directly above them. In broad agreement with these observations, NO synthesising cells have been reported in the ventral spinal cord of a range of vertebrates (Wetts et al., 1995, Ramanathan et al., 2006, Giraldez-Perez et al., 2008, Munoz et al., 2000, Bruning and Mayer, 2001, Dun et al., 1993, Terenghi et al., 1993, Saito et al., 1994, Necker, 2004, Crowe et al., 1995). However, almost nothing is known of the functions these cells serve in developing or adult spinal networks (but see Inglis et al., 1998).

Subsequently, in Chapter Four, developmental roles of spinal NOS1 activity were investigated. Using a range of molecular and pharmacological methods, this work unearthed one novel and important function of NO signalling. Specifically, this molecule acts to temper the growth of nascent spinal cord motoneuron axons. The

effects of NO appear to be confined to primary motoneurons, with axons of interneurons and secondary motoneurons unaffected by NO disruption. Because spinal nitrergic neurons are the only NO generating cells that lie in proximity to primary motoneuron populations, the most parsimonious conclusion is that these cells regulate motor axon growth through NO biosynthesis. This finding is of considerable significance because previous *in vivo* studies have only demonstrated axonogenic roles of NO during in nascent visual circuits (Cramer et al., 1996, Cramer and Sur, 1999, Campello-Costa et al., 2000, Wu et al., 1994, Wu et al., 2001, Ernst et al., 1999, Cogen and Cohen-Cory, 2000). To date almost nothing is known of how this molecule influences development of neurons in other regions of the nervous system. As such, the work presented in this thesis represents one of the first studies to demonstrate that NO signalling regulates aspects of vertebrate motoneuron development in an intact, living embryo and suggests that this signalling molecule may have broader developmental functions than previously recognised.

The findings presented in Chapter 5 aimed to delineate the signalling pathway through which NO regulates primary motoneuron branching. By employing a series of pharmacological manipulations, it was determined that this molecule exerts its actions principally through the sGC/cGMP signalling pathway. Whilst other modes of action, such as S-nitrosylation, cannot be completely excluded at this stage the observation that sGC inhibition rescued the DETA-NO phenotype strongly suggests that cGMP is the principal mediator of NOs effects. There is already a wealth of evidence demonstrating that cGMP can affect axon outgrowth in other models (Hindley et al., 1997, Gibbs et al., 2001, Gibbs and Truman, 1998, Tojima et al., 2009, Xiong et al., 2007, Tornieri and

Rehder, 2007, Welshhans and Rehder, 2005, Welshhans and Rehder, 2007, Trimm and Rehder, 2004, Van Wagenen and Rehder, 2001). However, the findings presented in this thesis are the first to show that cGMP signalling regulates axon growth in zebrafish, and provide one of the first *in vivo* demonstrations that cGMP influences vertebrate motoneuron growth. The downstream targets of cGMP await further investigation, although the work presented here tentatively suggests that cADPR may be involved.

In addition to exploring the signalling pathways underpinning the NO response, experiments in Chapter 5 aimed to define the functional consequences of disrupting NO signalling. Immunohistochemistry revealed that NO/sGC/cGMP signalling regulates neuromuscular synaptogenesis. Thus, it is likely that this signalling pathway has key roles in sculpting neuromuscular development. Previous studies have demonstrated that modulating NO during development affects the maturation of motor behaviour: NOS1 knockout mice display increases in locomotor behaviour (Weitzdoerfer et al., 2004), as do sGC knockout flies. In corroboration with these studies, behavioural analysis confirmed that NO can indeed impact motor ontogeny. Kinematic studies of swimming in NO-perturbed fish showed that several parametres of locomotion were affected, especially when NO levels were elevated which markedly impeded neuromuscular synaptogenesis. This suggests that developmental imbalances of NO might have deleterious consequences on the subsequent acquisition and maturation of motor control, and could have a role in developmental neuromuscular diseases. The observation that parametres such as locomotor frequency were disrupted by NO signalling also suggests that this signalling molecule regulates aspects of premotor

interneuron development in the zebrafish spinal cord, although not by perturbing growth of their axons. However, investigations into additional effects of NO on dendritic architecture and excitability of premotor interneurons certainly merits future investigation.

Taken together, the work presented in this thesis provides, to the author's knowledge, the first account of the role nitrergic signalling plays at the developing vertebrate neuromuscular junction. These findings implicate NO in novel and important developmental processes that regulate the maturation of vertebrate motor behaviour.

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