Aminergic modulation of synaptic transmission during motor pattern generation in a vertebrate spinal cord

A thesis submitted to the University of St. Andrews for the degree of doctor of philosophy

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I, Simon David Merrywest, hereby certify that this thesis, which is approximately 37,000 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

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Abbreviations

5-HT: 5-hydroxytryptamine (serotonin)

BICUC: Bicuculline

CLON: clonidine

CNS: Central nervous system

CPG: Central pattern generator

CV: coefficient of variation

dla: Dorsolateral ascending

dlc: Dorsolateral commissural

EPSP: Excitatory post-synaptic potential

GABA: gamma-aminobutyric acid

IPSP: inhibitory post synaptic potential

KCl: Potassium chloride

mhr: mid-hindbrain reticulospinal

NA: noradrenaline

PHEE: phenylephrine

PHENT: phentolamine

PROP: propranolol

PROTRIP: Protriptyline

R-B: Rohon-Beard

RC-delay: Rostro-caudal delay

STRYC: Strychnine

TTX: Tetrodotoxin

VR: Ventral root

Abstract

1. I have examined the noradrenergic neuromodulation of sensory inputs and the motor output that this information triggers, in the intact nervous system of immobilised *Xenopus laevis* tadpoles.

2. Noradrenaline (NA) slowed swimming and reduced the rostro-caudal (RC-) delay in motor neuron firing. α_1- and α_2-adrenoreceptor activation mimicked the noradrenergic slowing of swimming, whilst α_1-receptors also reduced RC-delays, suggesting that the effects of NA are largely mediated via α-adrenoreceptors.

3. Strychnine counteracted the noradrenergic reduction of swimming frequency and RC-delays, whilst bicuculline only counteracted the slowing of swimming. Strychnine also reduced the effects of α_1-receptors on frequency and delays, whilst bicuculline had no effect. Blocking glycine or $GABA_A$ receptors counteracted the α_2-receptor-mediated slowing of swimming. α_1-receptors, therefore, utilise only glycinergic inhibition to modulate swimming frequency and RC-delays, whilst α_2-receptors slow swimming via glycinergic and GABAergic pathways.

4. NA and activation of α_1- and α_2-adrenoreceptors enhanced the GABAergic barrage produced by stimulation of the rostral cement gland in stage 37/38 embryos. In addition, NA pre-synaptically enhanced the spontaneous release of GABA in the quiescent periods between swimming episodes. α_1-receptor

activation similarly increased the probability of glycine release. Both NA and α_1-receptor activation enhanced the probability of rebound firing, presumably through a direct effect on cellular properties, which may contribute to the reduction in the RC-delay and the maintenance of longer cycle periods under NA.

5. Blocking α- and β-adrenoreceptors reduced the frequency of motor bursts and their caudo-rostral delay during struggling, but often the firing pattern switched to become rostro-caudal, a characteristic of swimming rather than struggling. Thus, NA appears to prime, and can activate, the switch between these two behaviours.

6. NA increased the swim-initiation threshold by revealing bicuculline-sensitive IPSPs in motor neurons. This suggests that NA unmasks a previously silent inhibitory pathway controlling the sensitivity of the Rohon-Beard pathway.

Chapter One

General Introduction

1.1 Summary

It is important to understand the anatomy and physiology underlying the integration of sensory cues and the generation of behaviour in the nervous system. Historically, such investigations have largely been limited to invertebrate model systems, because of the prohibitive complexity of the mammalian nervous system. However, the relatively simple nervous systems of 'lower' vertebrates such as the lamprey and the *Xenopus laevis* tadpole, which are homologous to those of mammals, have now yielded to more detailed multi-level examination and have proven invaluable model systems in which to study both the generation and modulation of behaviour.

Fictive swimming in immobilized *Xenopus* tadpoles involves ventral root impulses that alternate across the body and propagate from head to tail with a brief rostro-caudal (RC-) delay. Once initiated, this locomotor rhythm is self-sustaining, and unlike many other vertebrate preparations, does not require repetitive sensory stimulation or pharmacological intervention for its maintenance. At the time of hatching the *Xenopus* spinal cord contains only eight distinct neuron classes whose anatomy and synaptic interconnections have now been studied in some detail. More recent work has focused on the modulation of ongoing locomotion by classical neuromodulators such as the amines serotonin (5-HT) and noradrenaline (NA) and less conventional signaling molecules such as the gas nitric oxide (NO). The *Xenopus* preparation has also been utilised to investigate nervous system development and behavioural maturation, with 5-HT implicated in affording the animal a greater degree of behavioural flexibility.

In this chapter, I will introduce the rationale behind using the *Xenopus* tadpole preparation to investigate principles of neural network control, before describing in detail its neuroanatomy and how these neurons are organised into networks capable of initiating and maintaining motor activity. I then briefly discuss the role of neuromodulators in tuning the output of motor networks and review our knowledge of their role in *Xenopus*, with particular regard to 5-HT and NA.

1.2 Background.

Despite a detailed description of the gross anatomical structure of the vertebrate central nervous system (CNS), we are still far from achieving a complete picture of how its cellular and synaptic components operate in concert to produce such a plethora of behavioural outputs. *In vitro* studies of ion channels and receptors present on isolated neurons (for review see, Wicher *et al.*, 2001) have advanced our understanding of the nervous system at the cellular level, but the way in which these individual components are assembled and interact as neural networks are elucidated more clearly with the whole system intact. Early attempts to study intact nervous systems used invertebrates, where the simpler anatomical structure of the nervous system with large identifiable neurons yields more easily to physiological examination. As a result, many of these early model systems have been well described (for reviews see: *Aplysia*; Kupfermann *et al.*, 1991; lobster stomatogastric system, Selverston *et al.*, 1998; snail, Murphy, 2001; general, Kupfermann & Weiss, 2001; Marder & Calabrese, 1996). Whilst allowing us to understand several key principles of nervous system function, applicable knowledge accruing from these preparations is constrained by their limited physiological functions. In addition, in invertebrates general principles of neural network function may be rather different in detail from networks in their vertebrate counterparts. However, the use of relatively simple lower vertebrate preparations can provide more close approximations to the complex nervous systems of higher vertebrates. Organisms including the lamprey (e.g. Grillner *et al.*, 1995), the neonatal rat (e.g. Kiehn & Kjaerulff, 1998) and the *Xenopus laevis* tadpole (e.g. Roberts, 1990; Roberts *et al.*, 1998), whose nervous systems are relatively simple, are now beginning to broaden our knowledge of the

structure, function and modulation of the neural networks controlling motor behaviour.

1.3 The *Xenopus* tadpole preparation.

At the time of hatching from their egg membranes at around stage 37/38 (Nieuwkoop & Faber, 1956), tadpoles of *Xenopus laevis*, the South African clawed frog, possess a relatively simple nervous system, which contains well-described sensory receptors primarily for mechanoception and photoception. The neurobehavioural responses of hatchling *Xenopus* tadpoles to these stimuli can be studied by recording ventral root motor activity. Motor output in α-bungarotoxin paralysed animals, termed 'fictive' activity, can be elicited either by a brief electrical stimulus to the tail skin, or by dimming of the illumination. Following only a brief sensory stimulus, long episodes of fictive swimming can be elicited, which alternates between the two sides of the spinal cord and progresses from head to tail with a brief RC-delay, permitting swim frequencies at between 10-20Hz (Roberts *et al.*, 1998; Figure 1.3Ai). There is little variability in motor burst durations from one cycle to the next and cycle periods gradually decline during the course of an episode. Just 24 hours later at larval stage 42, swimming becomes far more variable, with burst durations and cycle periods fluctuating within each episode of swimming, thus affording the animal greater flexibility during swimming (Sillar, *et al.*, 1991; Sillar & Simmers, 1992; Sillar *et al.*, 1998; Figure 1.3Bi). In response to more prolonged or sustained stimuli an entirely different behaviour can be evoked. Termed struggling, it consists of rhythmic alternating bursts of activity, which progress along the body in a caudo-rostral direction (Kahn & Roberts, 1982c).

1.4 Neuroanatomy of the *Xenopus* tadpole.

Neuroanatomical techniques such as horseradish peroxidase (HRP) tracing and immunocytochemistry have revealed that, at the time of hatching, the spinal cord of the *Xenopus* tadpole contains only eight distinct classes of differentiated neurons (Figure 1.1C; Roberts & Clarke, 1982; Roberts, 2000). The Kölmer-Agduhr cells, located close to the neurocoel, are ciliated ependymal cells, which are immuno-positive for GABA (Dale *et al.*, 1987), but their function is, as yet, not known (Roberts & Clarke, 1982, Roberts, 1990). Three types of neurons, the Rohon-Beard (R-B) sensory neurons, the dorsolateral ascending (dla) and the dorsolateral commissural (dlc) sensory interneurons are the major constituents of the skin mechanosensory system. The remaining classes of interneuron, as well as the motor neurons themselves comprise the primary motor system of the spinal cord.

R-B cells are the primary mechanosensory receptors that innervate the skin of the tadpole, with free nerve endings and slowly conducting axons. Their cell bodies are located dorsally within the spinal cord and their central axons project ipsilaterally both rostrally, into the hindbrain and caudally (Roberts & Clarke 1982; Clarke *et al.*, 1984; Roberts *et al.*, 1983). R-B cells fire impulses in response to either electrical or mechanical stimulation of the skin and a single impulse in one R-B cell can trigger swimming activity, even in spinalised animals (Roberts & Clarke, 1983; Clarke *et al.*, 1984). R-B cells must therefore synapse with neurons in the spinal cord. Spinalisation also removes input from electrically-excitable skin cells whose sensory pathway is less well understood, but which requires processing in the trigeminal ganglion located at the mid-hindbrain boundary (Roberts, 1971). The initiation of swimming in animals spinalised below this level hence infers that the R-B pathway is important.

The dorsal spinal cord also contains two classes of sensory interneuron, which receive direct synaptic connections from R-B axonal projections in the dorsolateral part of the cord (Roberts & Clarke 1982; Roberts, 1990). The first, the dlc interneurons, are innervated by R-B cells on the same side as the initial source of cutaneous stimulation. Roberts & Sillar (1990) and Sillar & Roberts (1988a/b) provide further information about the structure and function of these dlc interneurons. They reported that the major excitatory input to dlc interneurons is via release of an excitatory amino acid from R-B synaptic terminals, which gives rise to excitatory post-synaptic potentials (EPSPs) within the dla interneurons. Dlc interneurons have been shown to make connections onto contralateral pre-motor interneurons; indeed the musculature on the opposite side of the animal to any stimulus is usually the first to contract and it is likely that dlc interneurons are responsible for transmission of this response across the cord (Sillar & Roberts, 1992).

Dorsolateral ascending (dla) interneurons are the second class of interneuron that receive direct synaptic excitation from the axons of R-B cells. These cells differ from dlc interneurons in that they receive innervation from R-B cells on both sides of the animal and their axons project ipsilaterally along the dorsolateral tract to the hindbrain. After an initial stimulus each R-B cell is capable of exciting many dorsolateral interneurons, which in turn synapse with many pre-motor interneurons, hence providing an overall amplification of the initial stimulus (Clarke & Roberts, 1984). Neither R-B nor dorsolateral interneurons are then involved in any further processing of this primary response. Indeed the interneurons are inhibited during swimming (Roberts & Sillar, 1990)

Three types of pre-motor interneurons: commissural, descending and ascending have been identified in the spinal cord of hatchling *Xenopus* (Clarke & Roberts, 1984). Commissural interneurons are unipolar and have an axon that crosses the cord and then ascends and descends in the marginal zone (Roberts & Clarke, 1982; Soffe *et al.*, 1984). Ascending interneurons are also unipolar and send an axonal projection ipsilaterally and rostrally. Descending interneurons are multipolar with dendrites extending into the dorsal and lateral tracts and an axon that descends along the ipsilateral tract. Finally, motor neurons have a ventral soma, with dendrites and axons in the lateral tract. The axons run caudally and ventrally before exiting the spinal cord to innervate the segmental myotomal musculature (Roberts *et al.*, 1983).

1.5 Physiology of motor activity in *Xenopus*.

Xenopus exhibit three principal motor behaviours following stimulation. A classical swimming response (in which motor neurons fire only once per cycle, alternating between the two sides and propagating from head to tail with a brief RC-delay; Kahn & Roberts, 1982a/b; Roberts, 1990; Figure 1.2A), a struggling response (discussed below and consisting of rhythmic bursts of motor activity propagating from tail to head; Kahn & Roberts, 1982c; Soffe, 1991, 1993; Figure 1.2A) and a simple avoidance reflex in which stimulation of the skin on one side causes the myotomes on the opposite side to contract, bending the body away from the source of stimulation (Zhao *et al.*, 1998).

During swimming, there are three principal components to the underlying synaptic drive. The first is a phasic EPSP that underlies the spike recorded in each cycle of swimming (Dale & Roberts, 1985). The second, related component of the

synaptic drive is a tonic excitation, seen as a sustained depolarisation throughout each episode of swimming. The origin of both of these components of the swimming pattern are still not fully understood, but both are known to result in part from the activation of excitatory amino acid receptors (Dale & Roberts, 1985). This excitatory amino acid transmitter (presumed to be glutamate) is thought to originate from descending pre-motor interneurons and interacts with two different receptor subtypes, to produce two EPSPs with different temporal characteristics. One form is NMDA receptor mediated and consists of EPSPs with a slow rise and fall time and long duration (~200ms) relative to the cycle period (~50-100ms). These slow EPSPs will thus summate from one cycle to the next and contribute to the tonic excitation seen during swimming.

The second type of excitation consists of fast rise (2-3ms) and fall (~50ms) excitation and is mediated partly via ionotropic AMPA receptors on the motor neurons and is thought to account for the phasic 'on-cycle' component of the synaptic drive (Dale & Roberts, 1985). Two further components of excitation have subsequently been shown to contribute to this on-cycle excitation. Roberts and Perrins (1995a,b, 1994) showed pharmacologically that motor neurons feedback onto themselves and pre-motor interneurons, using acetylcholine as a transmitter. By recording from neurons in which IPSPs were chemically blocked they were able to show that blocking nicotinic acetylcholine receptors led to a 20% drop in excitation. Blocking release of excitatory amino acids using cadmium produced a further 30% fall in excitation, which suggested that the remaining 50% was of electrical origin (Perrins & Roberts, 1995a,b,c). This electrical component is probably generated by electrotonic coupling between closely located motor neurons. Such coupling is thought to both enhance and synchronise the firing of neighbouring motor neurons.

Investigation of pre-motor interneurons showed that around 16% of their synaptic drive was cholinergic, implying that they too received positive feedback from motor neurons. Work on the related amphibian species *Rana* and *Bufo* (Perrins & Soffe, 1996) also revealed the presence of excitatory amino acid and cholinergic components of motor excitatory drive, suggesting a common role in amphibian species, although little or no electrical component was observed in *Rana* or *Bufo*.

The third component of the synaptic drive underlying the generation of the swimming rhythm occurs at mid-cycle and it is glycinergic and inhibitory. Glycine opens chloride ion channels on postsynaptic neurons, giving rise to IPSPs. These IPSPs are reversed in sign to become depolarising when recordings are made with KCl-filled microelectrodes indicating that they are chloride-dependent (Figure 1.4B). The glycinergic antagonist strychnine abolishes these potentials, indicating that they are glycinergic in origin (Figure 1.4D; Dale, 1985; Soffe, 1987). The inhibitory phase of the swimming cycle occurs in time with the motor activity on the opposite side of the cord and is thus thought to arise from the contralateral commissural interneurons that pass inhibition across the cord. The initial evidence for this contralateral control came from hemisection studies where the spinal cord was cut along the mid-line and then severed on one side to prevent any descending input. Mid-cycle inhibition was abolished on the opposite side of the cord to the cut, suggesting that the inhibitory neurons that generate the mid-cycle inhibition are on the opposite side of the cord to the motor neurons which they inhibit (Soffe & Roberts, 1982). Using paired recordings and intracellular HRP staining, the neurons which were responsible for conferring this mid-cycle inhibition were shown to be commissural interneurons (Dale, 1985). The pattern of reciprocal inhibition has been shown to occur not only in motor neurons, but also in commissural interneurons

(Dale, 1985) and excitatory interneurons during swimming (Dale & Roberts, 1985). Immunocytochemistry using anti-glycine antibodies has identified commissural interneurons as being the likely source of glycine (Dale *et al.*, 1986)

The physiological description of these eight spinal cell types permits an understanding about how they interact to produce a motor output in response to a sensory input. Stimulation of the primary afferent R-B cells leads to activation of dorsolateral sensory interneurons following release of an excitatory amino acid and activation of NMDA and non-NMDA receptors (Sillar & Roberts, 1988b). Dorsolateral commissural interneurons then carry the excitation across the cord and initiate firing of motor neurons and pre-motor interneurons. In this way, the initial response to a cutaneous sensory stimulation is for the animal to flex away from the stimulus by contracting its contralateral musculature. If, however, sufficient numbers of neurons are recruited by the initial stimulus, swimming will be initiated.

An important aspect of the *Xenopus* skin sensory system is that it allows for expression of a different form of rhythmic motor output, termed struggling. Both struggling and swimming are driven by essentially the same neural circuitry, use the same musculature and can both be initiated via stimulation of the R-B sensory pathway (Roberts *et al.*, 1983). Whilst a brief stimulation of R-B cells is capable of initiating swimming activity, more prolonged or repetitive stimulation of the skin is capable of inducing struggling, characterised by bursts of activity which propagate rostrally along the body. This observation has led to the hypothesis that a behavioural switch must occur in the neural circuitry to select struggling. Soffe (1991; 1993; 1996) has shown that up to 30% more motor neurons fire during struggling compared to swimming, although this does not account for the repetitive discharge so characteristic of struggling activity. However, R-B cells and

dorsolateral sensory interneurons do not fire during either swimming or struggling. Soffe also showed that levels of excitation are higher during struggling than swimming, whilst reduction of this excitation with kynurenic acid suggests the involvement of excitatory amino acid receptors. Local applications of excitatory amino acids to the spinal cord induce struggling by increasing levels of pre-motor and motor neuron activation. Pharmacological block of glycinergic inhibition has relatively little effect on the swimming rhythm, but does disrupt the generation of struggling behaviour (Soffe, 1996). It therefore seems likely that the trigger for switching from swimming to struggling activity derives from a component of the motor network other than the motor neurons. This indirectly suggests involvement of the sensory components such as R-B and dlc cells, although other effects such as the modulation of motor neuron membrane properties have also to be considered (Soffe, 1993). As a footnote, no distinct receptors for nociception have yet been identified in hatchling *Xenopus*. Studies have shown that R-B cells are substance P immunoreactive, although no role for it has yet been identified in the sensory pathway (Gallagher & Moody, 1987; Clarke *et al.*, 1984). It is conceivable that the repetitive firing of R-B cells is necessary to release substance P, which in turn is somehow responsible for triggering struggling. In relation to the present study, some limited work (Stevens & Brenner, 1996; Brenner *et al.*, 1994) has however shown that an analgesic response modulated by descending noradrenergic pathways and α_2-receptors is present in the grass frog *Rana pipens*.

1.6 Aminergic neuromodulation of sensory and motor processing.

Neural circuits have the ability to alter their activity according to the prevailing behavioural requirements and neuromodulators, molecules which alter

electrical properties and synaptic strengths, provide one important way of tuning the output of neural circuits. According to Pearson (1993), neuromodulators have three main roles: the ability to suppress or promote motor function, to initiate a new motor function when required and to modify neuronal and synaptic components of the network to allow them to generate different patterns of motor activity.

The results of studies on neuromodulation in motor circuits has caused the motor control field to abandon the traditional view that neuronal networks are hard-wired, static circuits (for a review see: Harris-Warrick & Marder, 1991, Pearson, 1993). Neuromodulation has been identified during a wide range of motor behaviours in both invertebrates and vertebrates, including feeding in *Aplysia* (Morgan *et al.*, 2000), digestion in the lobster stomatogastric system (Selverston *et al.*, 1998), control of respiration in the adult frog (Hedrick *et al.*, 1998) and locomotion in crayfish (e.g. Pearlstein *et al.*, 1998) and lamprey (Buchanan, 2001). Not only can neuromodulators affect ongoing motor activity, but they can also initiate new motor patterns [e.g. 5-HT can initiate swimming in leeches (Willard, 1981) and motor activity in the rat (Cazalets *et al.*, 1992; Kiehn & Kjaerulff, 1996), whilst NA can evoke hatching *in Rana temporaria* tadpoles (McDearmid, 1998) and walking in cats (Barbeau & Rossignol, 1991; Chau *et al.*, 1998)].

The primary role of the somatic sensory nervous system is to relay external tactile 'environmental' information to the CNS, be it, noci-, proprio- or mechanoreceptive, to not only elicit a behavioural response, but also to modify ongoing motor activity. Often this is to an organisms advantage in order that appropriate avoidance action can be taken where the stimuli may be potentially harmful. Vertebrate mechanoreceptive responses from primary cutaneous afferent terminals in the CNS usually involve release of an excitatory amino acid, (presumed

to be L-glutamate) that acts through AMPA receptors (e.g. Jahr & Jessell, 1985). This initial response is then relayed to motor neurons in the spinal cord (via interneuronal connections) so that a motor response can be generated (Jordan *et al.*, 1992; Sillar 1989). Nociceptive responses are relayed to the dorsal horn by Aδ- and C-fibres. Aδ-fibres conduct action potentials quickly as they are lightly myelinated, whereas C-fibres, which are unmyelinated and account for about 70% of nociceptors innervating the skin, conduct information more slowly and as such are responsible for the prolonged burning sensation experienced during pain. From here information is relayed rostrally to the thalamus via dorsal horn interneurons and subsequently to the somatosensory cortex for higher processing. The transmitters involved in this response are substance P and glutamate, which have been found to co-localise in the terminals of primary afferent neurons (Battaglia & Rustioni, 1988).

For pathways such as these to effectively control an animal's behaviour, they must be efficiently regulated in order that changing sensory cues may be rapidly converted into an appropriate behavioural response. Studies on many different organisms have converged to reveal many common features of sensory processing within the nervous system. Watson (1992), in reviewing pre-synaptic modulation of primary afferent input in both vertebrates and invertebrates, suggests that modulation of sensory inputs is very closely related to behaviour and provides information which can lead to behavioural modification. For example, in the stomatogastric ganglion of the spiny lobster *Panulirus interruptus*, amines such as NA, dopamine and 5-HT can change the strength of synaptic connections from primary sensory inputs onto second order interneurons and in doing so affect the resulting pattern of motor output (Johnson *et al.*, 1995). Such modulation is often complex, involving several different mechanisms acting on a single afferent terminal. Postsynaptically,

several distinct pathways can be engaged via activation by a single transmitter, hence initiating a further level of potential neuromodulation.

In the *Xenopus* spinal cord there are three principal levels at which modulation of both sensory and motor processing can occur; pre-synaptically via the synapses of the primary afferent R-B neurons, post-synaptically at the level of the sensory interneurons and thirdly via modulation of pre-motor interneurons and motor neurons by cyclic excitation and inhibition as described above. As in most vertebrates (e.g. cat, Barbeau *et al.*, 1987; Barbeau & Rossignol, 1991; rat, Cazalets *et al.*, 1992; Kiehn *et al.*, 1999; chick, Okado *et al.*, 1992), the biogenic amines NA and 5-HT exert modulatory effects on sensory and motor systems of the *Xenopus* spinal cord. In amphibians, as in other vertebrates, NA and 5-HT are localised in neurons associated with the isthmic region (the amphibian homologue of the locus coeruleus, Marin *et al.*, 1996) and the raphe nucleus (van Mier *et al.*, 1986; Sillar *et al.*, 1995a,b; Woolston *et al.*, 1994). These neurons have associated axonal processes that project into the spinal cord from around the time of hatching (Sanchez-Camacho *et al.*, 2002; van Mier *et al.*, 1996; Woolston *et al.*, 1994).

Both these amines have now been implicated in the pre-synaptic modulation of transmitter release from synaptic terminals. McDearmid *et al.*, (1997) studied the role of NA and 5-HT in the modulation of glycine release in the *Xenopus* spinal cord. As discussed above, commissural interneurons in the spinal circuitry use glycine as a reciprocal inhibitory transmitter, which causes opening of chloride ion channels, membrane hyperpolarisation and inhibition. In this study, NA was shown to strengthen the mid-cycle glycinergic IPSPs, and in doing so reduce the frequency of swimming (Figure 1.5A,B). 5-HT exerted the opposite effect and led to a weakening of glycinergic inhibition and to an increase in the intensity of motor

output (Figure 1.5A,B). 5-HT also modulates the *Xenopus* spinal circuitry at the sensory level. Sillar & Simmers (1994a) showed that 5-HT inhibits the initiation of fictive swimming in response to activation of R-B cells. Recordings from sensory dorsolateral interneurons revealed that 5-HT reversibly reduced the frequency of spontaneous EPSPs from R-B neurons, suggesting that 5-HT receptors are located on the terminals of these neurons, from where they pre-synaptically regulate release of transmitter onto dorsolateral neurons. Coupled with the evidence that 5-HT can enhance the motor pathway by reducing inhibition, it is suggested that the amine provides a mechanism by which *Xenopus* can initiate evasive swimming in response to a strong stimulus and in doing so inhibit any further input from the cutaneous sensory system, which could hinder its ability to escape.

5-HT has also been implicated in the maturation of the motor system in *Xenopus*. At stage 37/38, when serotonergic projections have only reached the rostral spinal cord (van Mier *et al.*, 1986), application of 5-HT mimics larval swimming activity in rostral, but not caudal ventral roots. At stages 40 and 42, however, when serotonergic projections have extended more caudally, 5-HT now increases burst durations along the length of the animal (Sillar *et al.*, 1992c; 1995b). If endogenous levels of 5-HT were raised, using the metabolic precursor, 5-hydroxytryptophan, similar effects were observed, indicating that the serotonergic system is functional and can influence swimming from around the time of hatching (Sillar *et al.*, 1992c). When the development of serotonergic projections from the raphe nucleus was interrupted with an aminergic neurotoxin, 5,7-dihydroxytryptamine, normal maturation of swimming at stage 42 could be retarded (Sillar *et al.*, 1995a), suggesting that the development of serotonergic projections into the spinal cord may be a prerequisite for the development of the more mature larval swimming pattern.

In the related amphibian, *Rana temporaria*, more extensive serotonergic projections are present in the spinal cord and 5-HT has a more dramatic effect on swimming, increasing the duration and intensity of motor bursts, whilst simultaneously slowing the swimming rhythm (Woolston *et al.*, 1994).

Motor output in vertebrates can be shaped by a range of voltage-dependent non-linear motor neuron membrane properties, which in response to appropriate inputs can confer long lasting neuronal effects. For example, in the spinal cord of the lamprey, activation of NMDA-type glutamate receptors triggers oscillations in membrane potential (Wallen & Grillner, 1987). The underlying mechanism involves the cyclical voltage-dependent blocking and un-blocking of the NMDA receptor ion channel by magnesium ions. This confers a region of negative slope resistance in the current/voltage relationship causing the membrane potential to oscillate between two quasi-stable levels. In the cat and the turtle another form of membrane bistability, long-lasting plateau potentials, relies upon 5-HT (Hounsgaard & Kiehn, 1989, Hounsgaard *et al.*, 1988). In *Xenopus* tadpoles, the presence of non-linear, voltage-dependent membrane potential oscillations relies upon the co-activation of 5-HT and NMDA receptors (Scrymgeour-Wedderburn *et al.*, 1997; Reith & Sillar, 1998; Figure 1.6). This property is conferred on motor neurons during the brief (ca. 24 hour) period between late embryonic (stage 37/38) and early larval (stage 42) development and parallels the in-growth of serotonergic projections into the cord (Sillar *et al.*, 1995a,b). Co-activation of NMDA and 5-HT receptors for the expression of voltage oscillations has also been reported in the related anuran species *Rana temporaria* (Sillar & Simmers, 1994b) and in the neonatal rat (Kiehn *et al.*, 1996; MacLean *et al.*, 1997, 1998).

1.7 Roles of GABA, glycine and nitric oxide in sensory and motor processing.

Although the monoamines NA and 5-HT are two of the principal neuromodulators acting within the *Xenopus* spinal cord, other neuroactive substances exist. Glycine has been mentioned above in its role as an inhibitory transmitter of motor pathways. GABA is another inhibitory amino acid which also plays a role in sensory modulation, where it has been shown to depolarise primary afferent R-B cells, and via $GABA_B$ receptors, pre-synaptically inhibits release of excitatory amino acids (Wall & Dale, 1993; Sillar, 1989). In lamprey too, $GABA_B$ receptor activation can modulate the flow of sensory information by inhibiting dorsal sensory cells and decreasing voltage-activated calcium currents (Batueva *et al.*, 1999). GABA also plays an important role in shaping motor output. When primary afferents of the rostral cement gland in *Xenopus* are activated, such as when the tadpole encounters an obstacle, mid-hindbrain (mhr) GABAergic neurons (Roberts *et al.*, 1987) fire and terminate swimming by activating $GABA_A$ receptors on spinal neurons (Boothby & Roberts, 1992a,b; Figure 1.3Aiii). As development proceeds, the cement gland degenerates and the reliability of the stopping response declines, so that by around stage 46, when the animal is free swimming, neither the cement gland nor the stopping response are present (Boothby & Roberts, 1992b). By larval stage 42, episodes of swimming often terminate with a barrage of apparently spontaneous GABAergic IPSPs which closely resemble those evoked by cement gland stimulation at stage 37/38 (Figure 1.3Bii), which suggests that during development the mhr neurons may have been retained and recruited into an intrinsic stopping pathway (Reith & Sillar, 1999).

More recent work on neuromodulation in the *Xenopus* system has focused on the gaseous signaling molecule nitric oxide. Using the NADPH-diaphorase staining

technique, three distinct brainstem populations of nitrergic neurons have been identified (McLean & Sillar, 2000, McLean *et al.*, 2000). Intriguingly, the location of these populations appears to correlate with the location of the serotonergic raphe nucleus, the isthmic region (the amphibian homologue of the locus coeruleus) and the GABAergic mid-hindbrain reticulospinal neurons (for a review see McLean *et al.*, 2000). Furthermore, application of nitric oxide donor compounds slows swimming and terminates swim episodes (McLean & Sillar, 2000), through net and parallel facilitation of glycinergic and GABAergic inhibition (McLean & Sillar, 2002).

1.8 Scope of the present study.

The many and varied aspects of nervous system function, modulation and development in the *Xenopus* tadpole discussed above are symptomatic of both its simplicity and its ability to yield to physiological and pharmacological investigation. In the results that follow I will concentrate my investigation on the noradrenergic modulation of the motor and sensory components of the *Xenopus* spinal pattern generator, together with the associated receptors that underlie this control pathway. The effects of specific classes of adrenoreceptors during swimming have been investigated, and their probable roles in mediating the modulatory effects of NA are discussed. The likely inhibitory pathways that are utilised during activation of these adrenoreceptor classes are then examined in some detail. Finally, the role of NA in desensitising the primary afferent skin sensory pathway is investigated and I suggest its possible role in motor pattern selection.

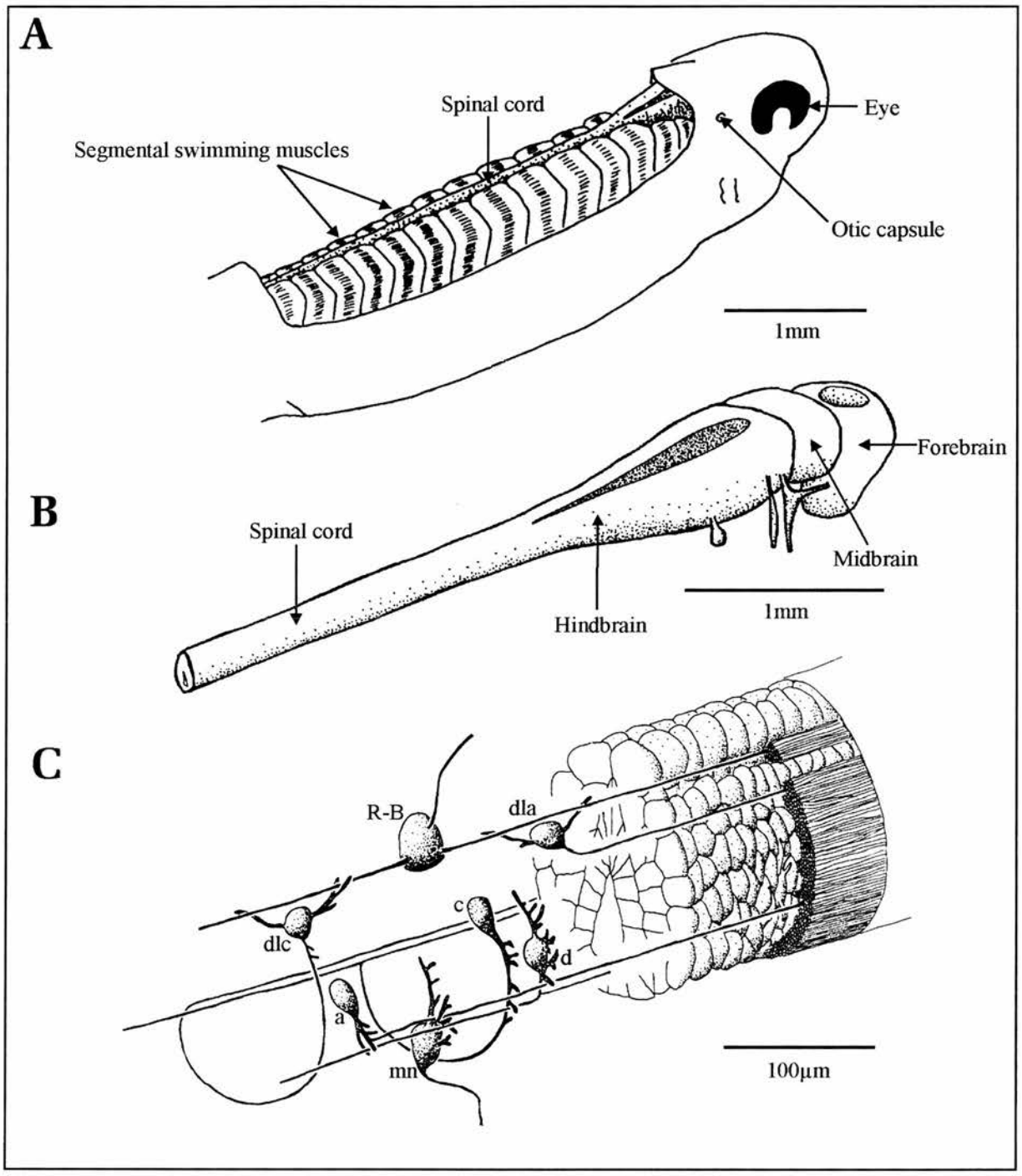

Figure 1.1: *Neuroanatomy of the Xenopus tadpole*. (**A,B**) Overview of the *Xenopus* central nervous system (at stage 37/38) illustrating the principal anatomical features. (**C**) Cutaway view through the spinal cord, showing the principal classes of neurons and their projections: Rohon-Beard (R-B); dorsolateral ascending (dla); dorsolateral commissural (dlc); descending interneurons (d); commissural interneurons (c); ascending interneurons (a) and motor neurons (mn). (Kölmer-Agduhr neurons not shown). Figure adapted from original drawing by S.R. Soffe, with permission.

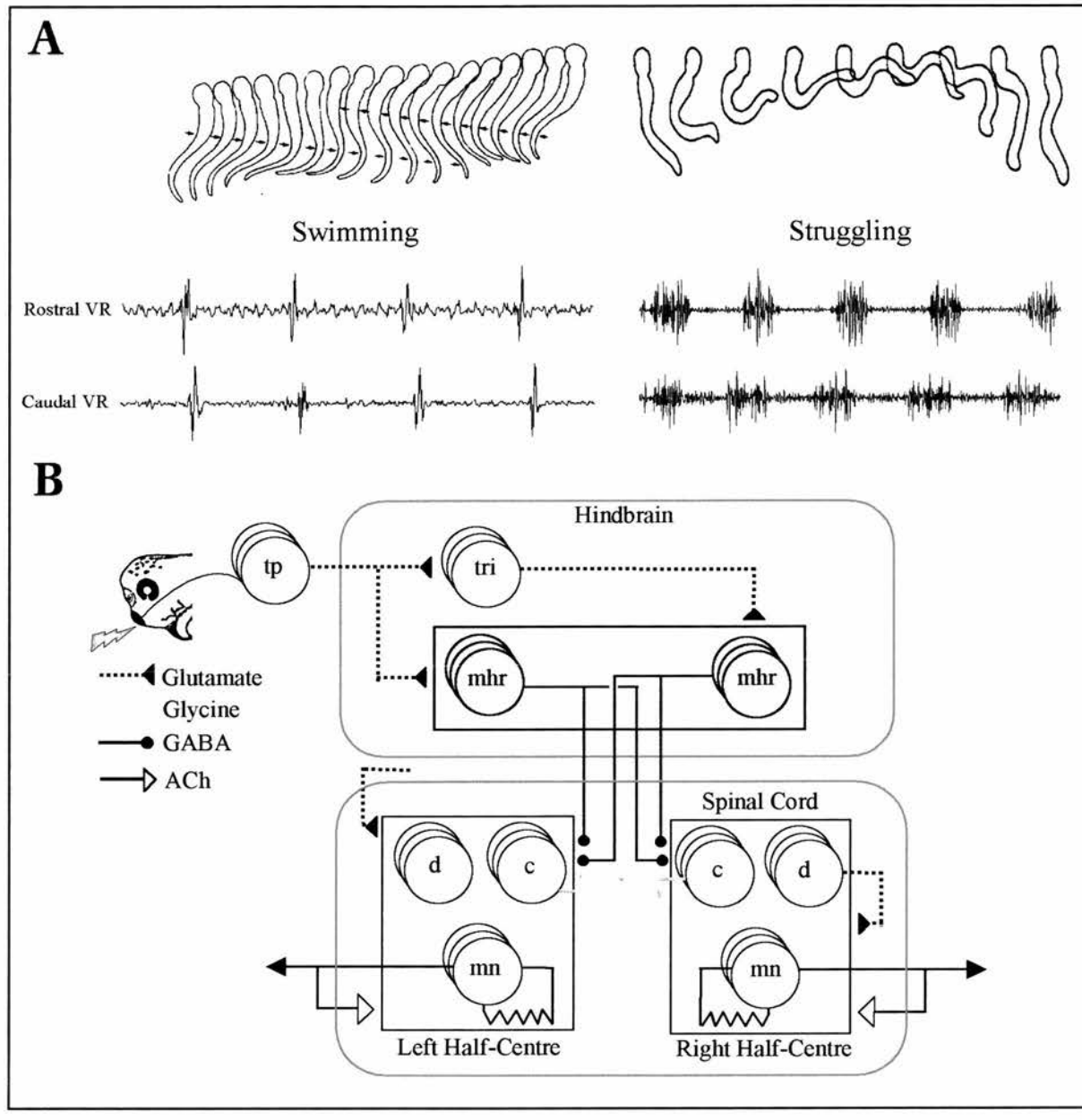

Figure 1.2: *Motor pattern generation in Xenopus tadpoles*. (**A**) *Xenopus* can generate two distinct behaviours -swimming (alternating rhythmic activity with a brief rostro-caudal delay) and struggling (alternating, more intense activity with a caudal-rostral activity). They are produced by the same neural circuitry, yet whilst struggling is elicited following a prolonged sensory stimulus, swimming can be initiated by only a brief sensory stimulus. Silhouettes in A modified from Kahn & Roberts 1982b,c. (**B**) The spinal cord is organised as half-centres consisting of motor neurons (mn), descending interneurons (d) and commissural interneurons (c), which inhibit the opposite half centre, allowing alternating activity across the spinal cord. The spinal cord also receives descending GABAergic inhibition from mid-hindbrain reticulospinal (mhr) neurons, which are activated following stimulation of trigeminal pressure (tp) receptors in the cement gland and the trigeminal ganglion (tri) in the hindbrain. Diagram in (B) adapted from Roberts *et al.*, 1998.

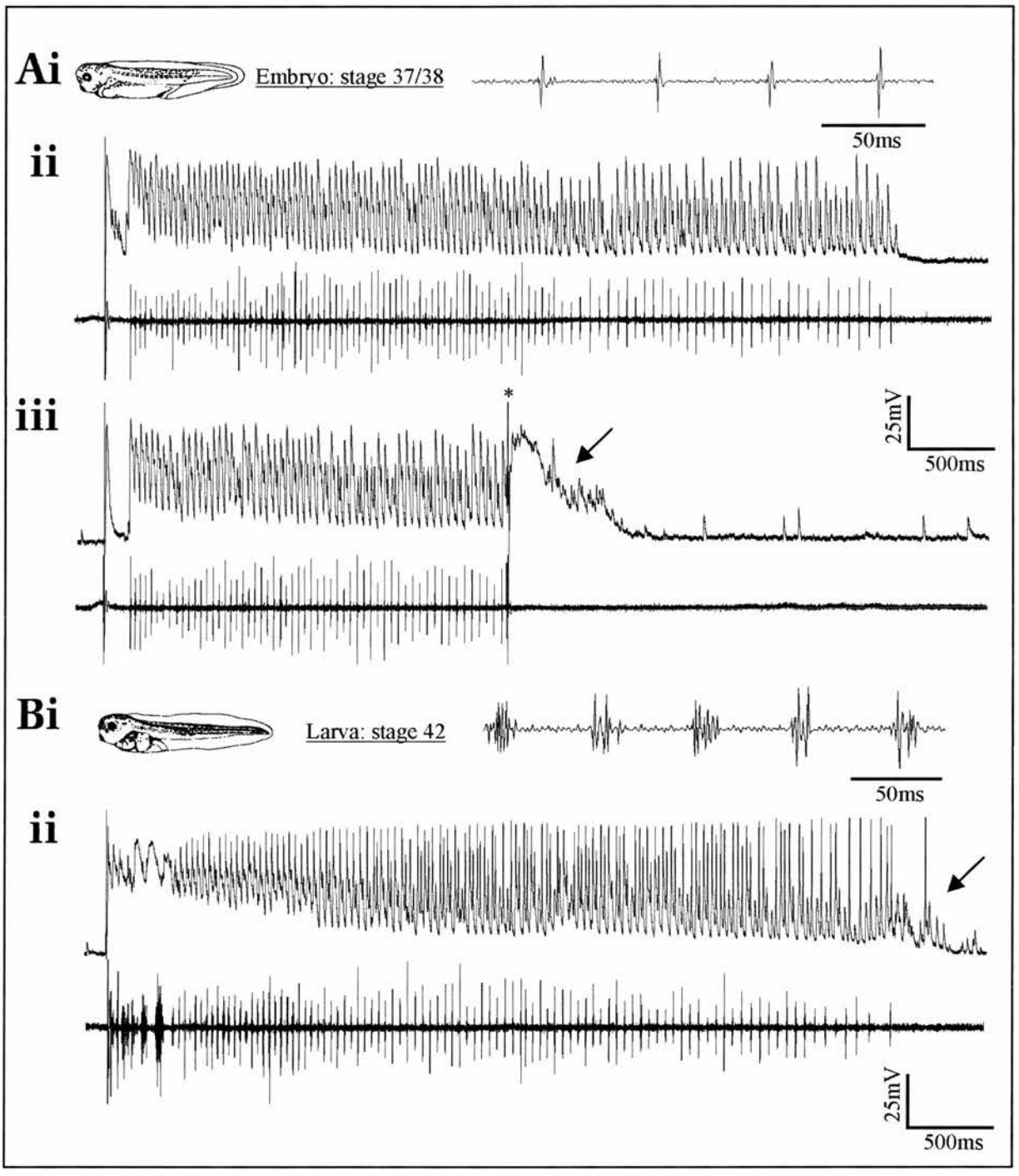

Figure 1.3: *Maturation of Xenopus swimming and the control of episode duration.* **(Ai)** Swimming in embryos is characterised by a single compound ventral root spike per cycle, and episodes of swimming **(Aii)** can be terminated prematurely by stimulation of the rostral cement gland (*). This activates mhr neurons which in turn release GABA onto the spinal CPG **(Aiii**; arrowed). **(Bi)** Twenty four hours later at larval stage 42, increased motor burst durations afford the animal greater flexibility during swimming, whilst episodes can now terminate spontaneously with a barrage of GABAergic inhibition **(Bii;** arrowed**)**. These potentials are depolarising when recording using KCl-filled electrodes- see chapter two and Figure 1.4 for an explanation.

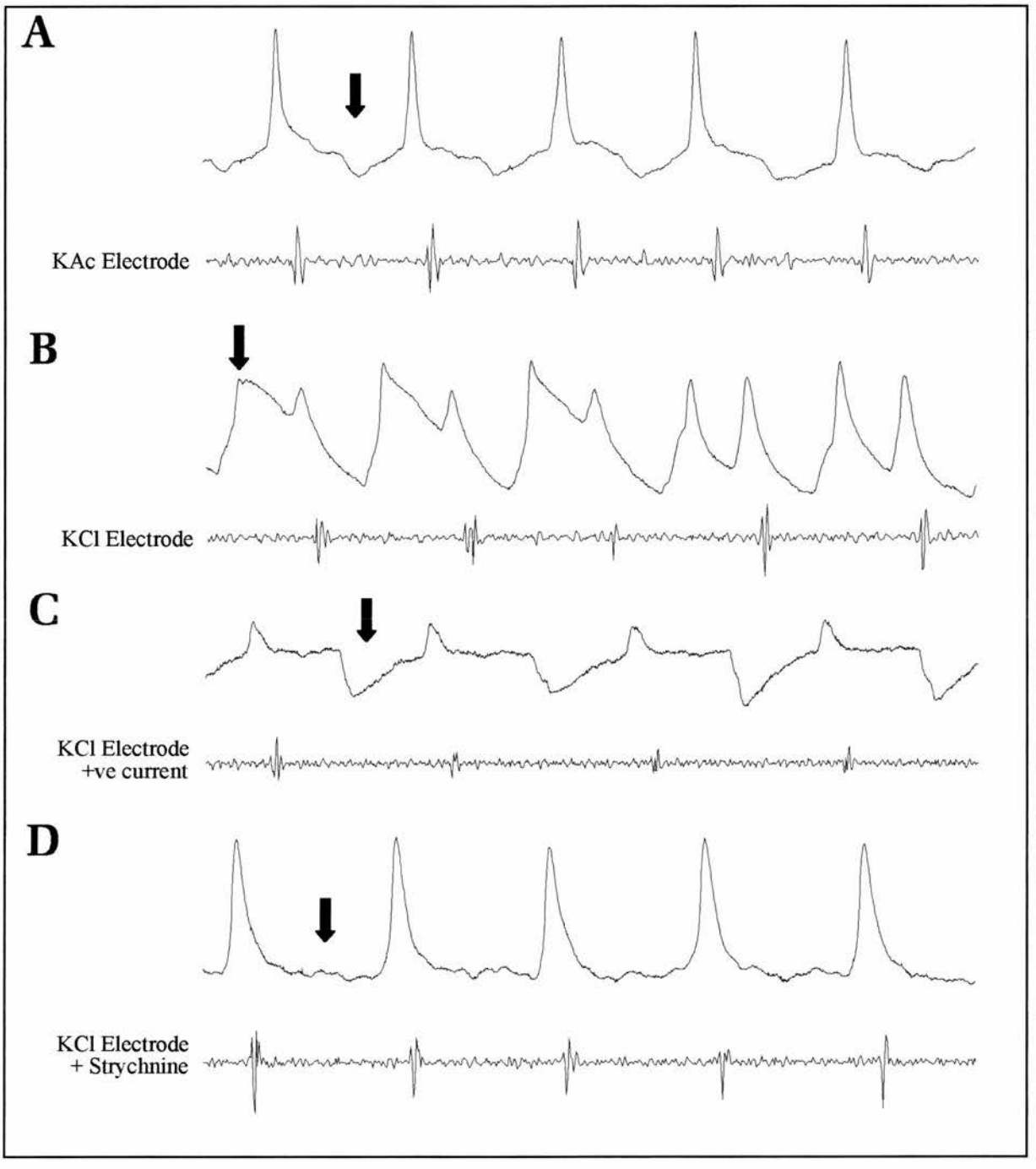

Figure 1.4: *Reciprocal inhibition in* <u>*Xenopus*</u> tadpoles. During episodes of swimming, sharp electrode recordings from presumed motor neurons in the ventral margin of the spinal cord reveal that the synaptic drive underlying ventral root motor bursts consists of alternating excitation and reciprocal mid-cycle inhibition (arrowed). (**A**) When recording using KAc-filled electrodes, mid-cycle IPSPs are hyperpolarising. (**B**) The inhibition is rendered depolarising when recording with KCl-filled electrodes due to chloride leakage into the neuron. (**C**) These depolarising IPSPs can, however, be reversed with the injection of depolarising current, allowing inhibition and excitation to be easily distinguished. (**D**) Mid-cycle inhibition is glycinergic and as such is blocked by the addition of strychnine.

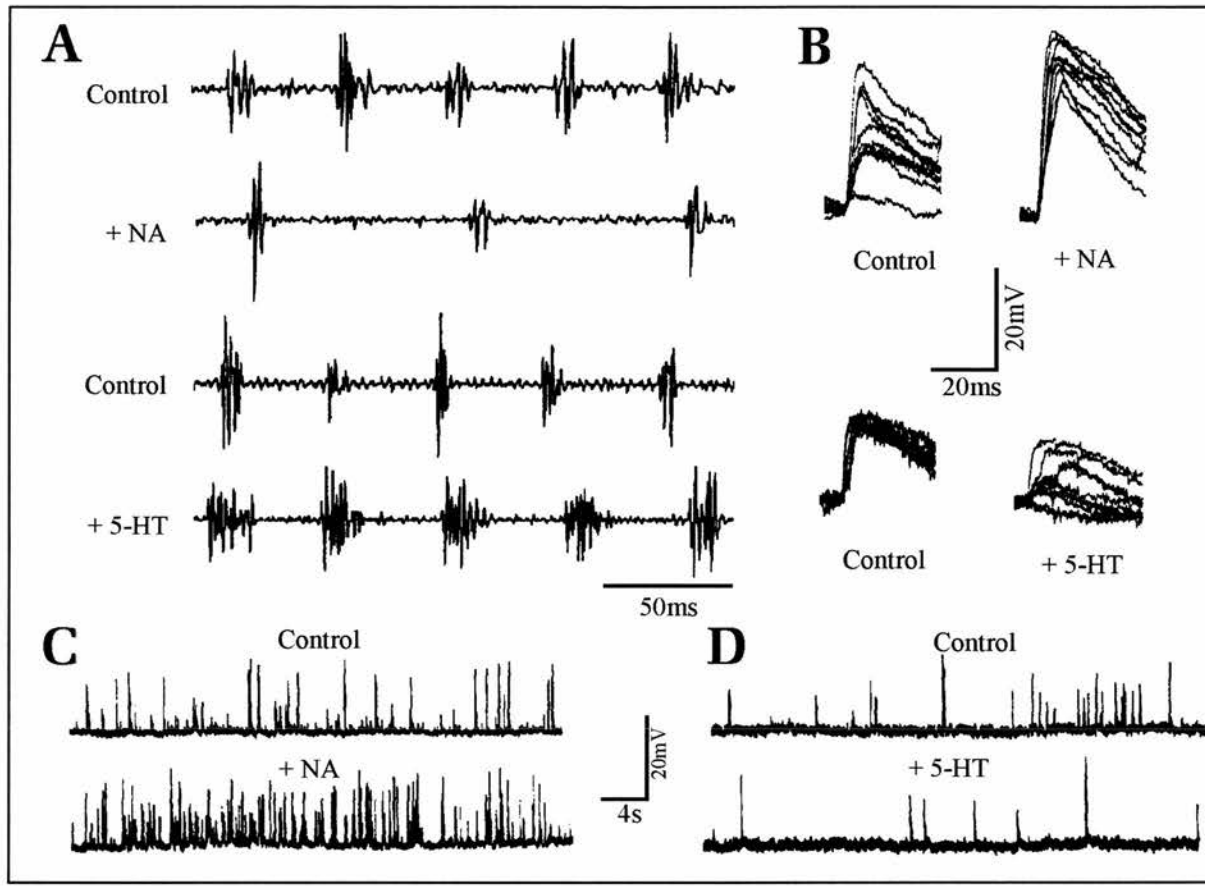

Figure 1.5: *Aminergic modulation of Xenopus swimming* (**A**) Bath application of NA slows swimming, whilst 5-HT increases its intensity. (**B**) NA increases and 5-HT decreases the amplitude of midcycle glycinergic IPSPs during swimming. NA also reduces the amplitude variability of the IPSPs. NA increases (**C**) and 5-HT (**D**) decreases the rate of spontaneous miniature glycinergic potentials, suggesting that both amines act pre-synaptically to affect the rate of transmitter release. Figure adapted from McDearmid *et al.*, 1997.

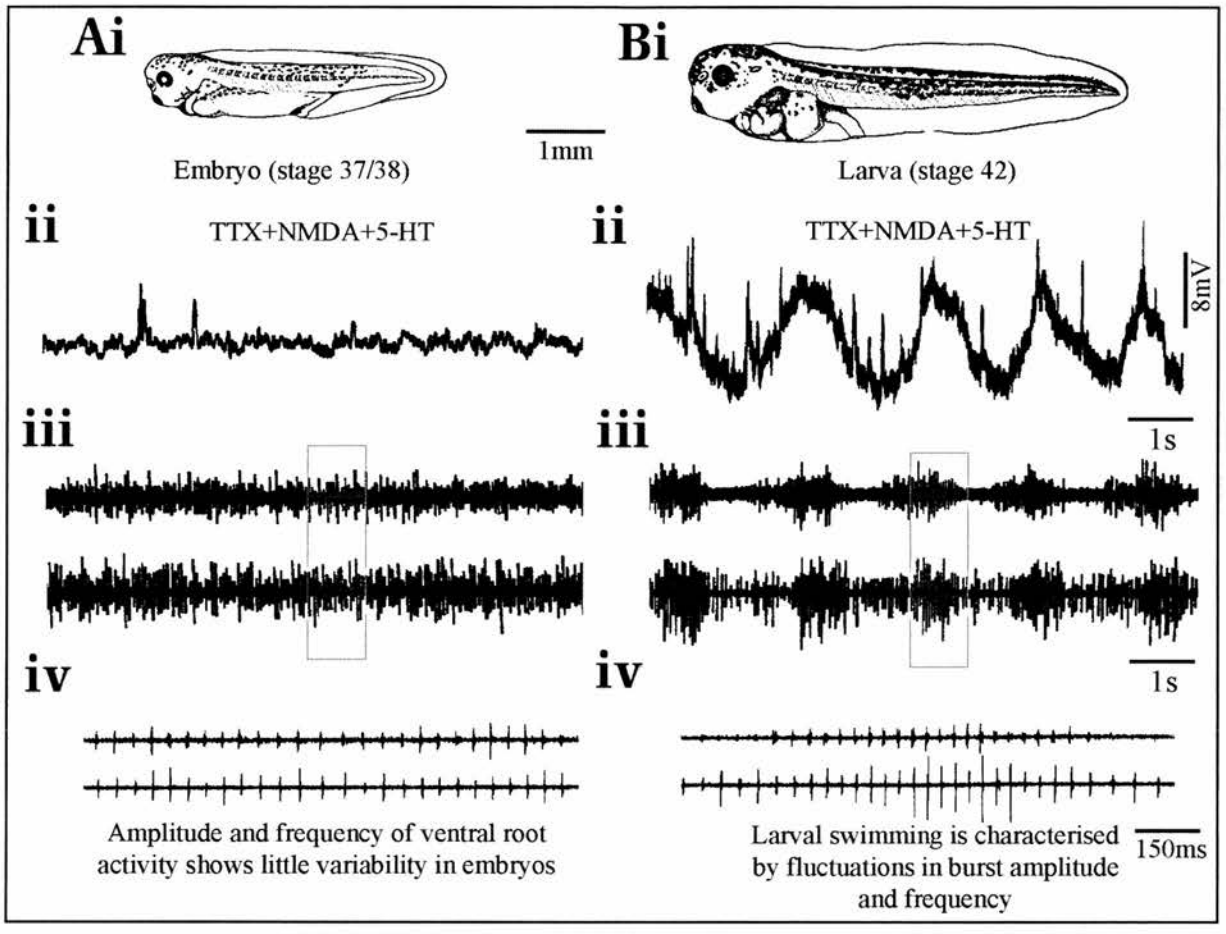

Figure 1.6: *5-HT-induced NMDA receptor-mediated voltage oscillations in Xenopus larvae*.(**A**) Intrinsic voltage oscillations are seldom present in late embryonic (stage 37/38; **Ai**) stage *Xenopus* tadpoles as shown (**Aii**) by the excerpt of activity from a spinal motor neuron in the presence of TTX, NMDA and 5-HT. (**Aiii-iv**) Recordings of ventral root activity reveal that in during embryonic swimming, motor bursts are of fairly constant frequency and amplitude. 24 hours later in early larvae (stage 42; **Bi**) voltage oscillations can be induced in the presence of TTX, NMDA and 5-HT (**Bii**). Ventral root recordings of larval swimming reveal corresponding fluctuations in burst amplitude and frequency (**Biii-iv**). Figure adapted from Reith & Sillar, 1998 and Scrymgeour-Wedderburn *et al.*, 1997.

Chapter Two

Materials and Methods

2.1 Animals.

All experiments were performed on pre-feeding, hatching stage embryos (stage 37/38) and post-hatching larvae (stage 42; staged according to Nieuwkoop & Faber, 1956; Figure 2.1A) of the South African clawed frog, *Xenopus laevis*. Experimental animals were obtained by induced breeding, following the injection of human chorionic gonadotropin (HCG, 1000 U/ml, Sigma, UK) into adult pairs from a laboratory colony. Eggs were kept in de-chlorinated tap water at 17-23°C, which allowed for differential rates of development and the provision of experimental animals over successive days.

2.2 The Preparation.

Following slitting of the tail fin to facilitate toxin penetration, the tadpoles were immobilized using the neuromuscular blocker α-Bungarotoxin (12.5μM, Sigma, UK) and transferred into a preparation bath (volume ~2ml) containing continuously re-circulating frog ringer solution (basic composition in mM: NaCl, 115; KCl, 2.5; $NaHCO_3$, 2.5; HEPES, 10; $MgCl_2$, 1; $CaCl_2$, 2 (extracellular), 4 (intracellular); pH 7.4). Animals were secured on their sides, with fine-etched tungsten pins through the notochord, onto a Sylgard-coated rotatable Perspex table within the preparation bath. After removal of the flank skin from around the level of the otic capsule to half way along the body, extracellular recordings of ventral root activity were made by placing glass suction electrodes (~40-70μm tip opening diameter) onto the exposed inter-myotomal clefts, wherein the motor axons are

located (Figure 2.1B). Recording sites were numbered as the position in clefts from the otic capsule and recordings were made from two ipsilateral muscle clefts.

For intracellular recordings, a block of myotomes overlying the rostral spinal cord was removed with tungsten dissecting needles. For ease of recording, following dissection, animals were re-pinned ventral side up onto the Sylgard platform and were rotated forwards, thus allowing better access to the ventral margin of the spinal cord (where motor neurons predominate; Roberts & Clarke, 1982). However, as a result recordings were often made from contralateral neurons (e.g Figure 2.1). Recordings were made with glass microelectrodes pulled on a Campden microelectrode puller (model 753). Microelectrodes were usually filled with 2M potassium chloride and had DC resistances of 100-150MΩ. Using KCl-filled microelectrodes reverses and enhances chloride dependent IPSPs. Thus, the mid-cycle glycinergic inhibition, which is chloride-dependent, can be reversed in sign to become depolarising when recording with KCl-filled electrodes, due to chloride leakage into the cell (Figure 1.4B; Soffe, 1987). Injecting depolarising current into the cell can however reverse these depolarising IPSPs, allowing excitation and inhibition to be easily distinguished (Figure 1.4C). Similarly, both GABAergic and glycinergic spontaneous IPSPs are chloride ion-dependent and are reversed in a similar way (Reith & Sillar, 1997).

Neurons were penetrated using capacity overcompensation and intracellular signals were amplified 10 times using a purpose built laboratory made amplifier (S.R. Soffe, University of Bristol). Episodes of fictive swimming activity (Figure 2.1C) were elicited either by dimming of the illumination or by applying a short current pulse (0.5-1ms) using a digitimer DS2 isolated stimulator (Digitimer, Welwyn Garden City, UK) via a separate glass suction electrode, located on the tail

skin (Figure 2.1B). In some experiments, swimming was terminated prematurely by applying a similar brief current pulse to the rostral cement gland via an additional suction electrode.

2.3 Pharmacological agents.

All drugs were purchased from Sigma (UK), except for propranolol (Tocris Cookson, Bristol, UK). Drugs were bath-applied to the perfusate by adding known concentrations to the stock bottle (100ml) to achieve the desired final concentration.

2.4 Parameters of fictive swimming.

The effects of NA and drugs acting on adrenoreceptors were investigated by analysing the following parameters of fictive swimming: (a) the episode duration (in seconds), measured from the first to the last motor burst observed during an episode; (b) the burst duration (in ms), measured as the duration of each discrete motor burst; (c) the burst amplitude (in mV), measured as the peak-to-peak interval, (d) the cycle period (in ms), measured as the time interval from the start of one burst to the start of the next; and (e) the rostro-caudal (RC-) delay between two ipsilateral ventral roots (in ms), measured as the time interval between the start of a rostral burst and the start of the corresponding caudal burst (Figure 2.1C). When investigating burst amplitudes, the coefficient of variation (CV) was used as an additional measure of the drug effects.

2.5 Data evaluation and statistical analysis.

Electrophysiological data were stored on a Vetter integrated videocassette recorder (Wintron Technologies, PA, USA). Data was digitised off-line with an A/D interface (Cambridge Electronic Design, Cambridge, UK; model 1401) and measured using the Spike 2 data analysis software (Cambridge Electronic Design;

version 3) and the DataView signal analysis program (W.J. Heitler, University of St. Andrews; Version 1.2h). Extracellular data analysis was performed on thirty consecutive cycles of motor activity, beginning 1s after the start of each of three different episodes (this minimises the possible contamination by effects from the initiating sensory stimulus). Data are presented as means and standard errors of the mean. Statistical procedures were computer aided (KaleidaGraph for Windows and MS Excel). r indicates the linear coefficient of correlation. Statistics were considered to be significant at $P<0.05$. N gives the number of animals; n indicates the number of samples pooled from one particular animal. Comparisons of two sets of data with equal variances were performed using the Student's *t*-test. Non-parametric comparisons were performed using the Mann-Whitney *U*-test.

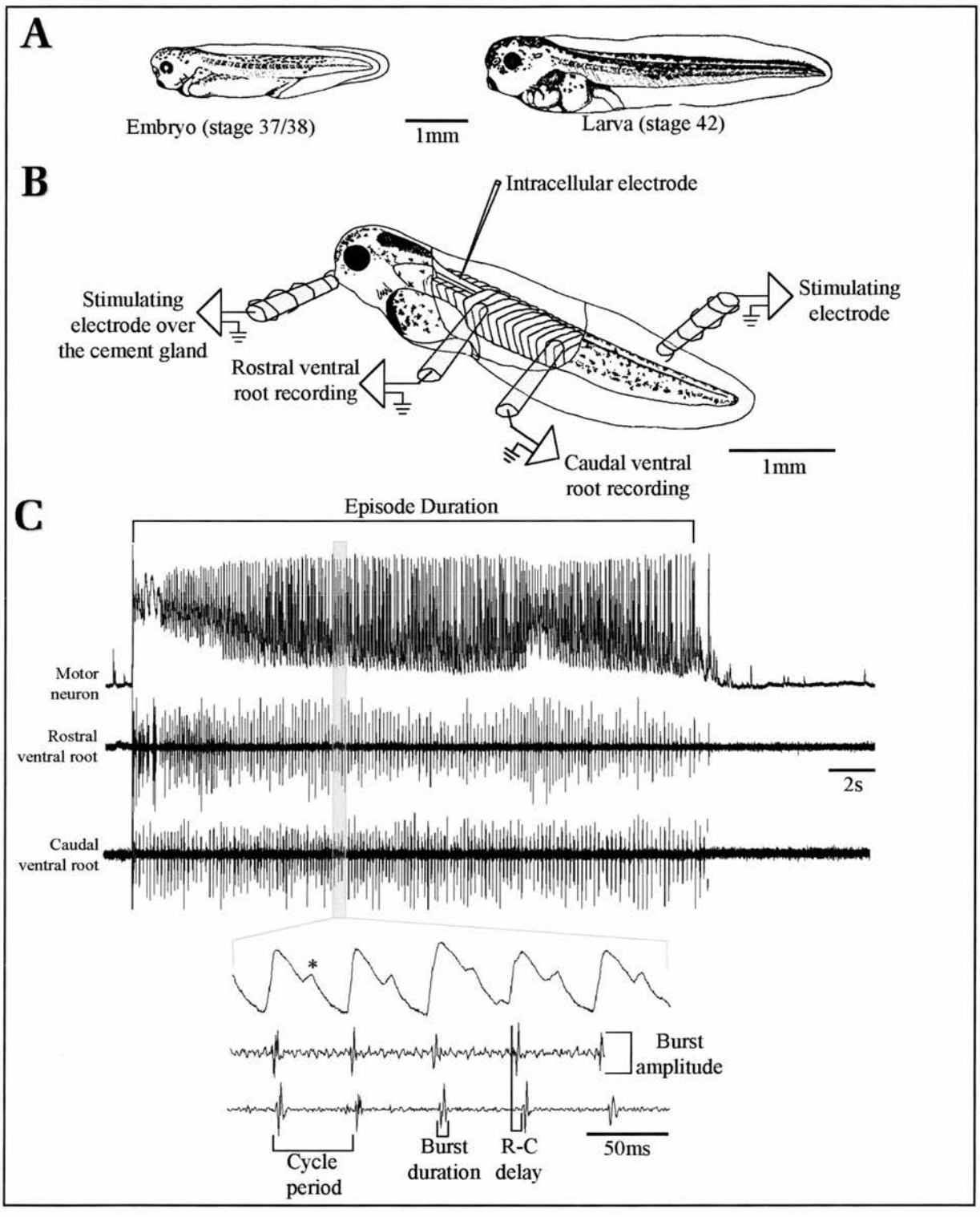

Figure 2.1: *The Xenopus tadpole preparation*: (**A**) An embryonic (stage 37/38) and a larval (stage 42) *Xenopus* tadpole. (**B**) Glass suction electrodes were used to make ipsilateral extracellular recordings from the inter-myotomal clefts, whilst intracellular recordings were made from presumed motor neurons along the ventral margin of the spinal cord using KCl-filled microelectrodes. (**C**) Excerpt of fictive swimming activity from two ipsilateral ventral roots and a motor neuron, illustrating the measured parameters of the fictive swimming rhythm (see text for details). Swimming is characterised by alternating excitation (marked *) and mid-cycle (on cycle in this example) glycinergic inhibition. Diagram in (B) courtesy of D.L.McLean.

Chapter Three

Adrenoreceptor-mediated modulation of the spinal cord locomotor – generating network during swimming

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Adrenoreceptor-mediated modulation of the spinal locomotor pattern during swimming in *Xenopus laevis* tadpoles.

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3.1 Summary.

This chapter investigates the possibility that the noradrenergic modulation of the spinal motor networks underlying swimming activity in *Xenopus* tadpoles may be mediated by different adrenoreceptor subtypes.

Bath application of NA reduced the frequency of swimming and the RC-delay in motor neuron activation, whilst subsequent application of the general α-receptor antagonist phentolamine reversed these effects. In embryos, activation of α_1-adrenoreceptors using the specific α_1-receptor agonist phenylephrine increased the duration of episodes of fictive swimming, whilst 24 hours later in larvae, they decreased. However, activation of α_2-adrenoreceptors using the agonist clonidine markedly reduced episode duration at both developmental stages. Cycle periods in both stages were increased by the activation of α_1- and/or α_2-receptor subclasses. Furthermore, α_1-receptor activation decreased the RC-delay in the head-to-tail propagation of swimming activity, while α_2-receptors apparently, do not influence these RC-delays. Activation of neither of the subclasses of α–receptor investigated had any consistent effect on the duration of ventral motor bursts, however α_2-receptors appear to reduce the amplitude of motor bursts.

These findings suggest that noradrenergic modulation of the swim-pattern generator in *Xenopus* tadpoles is mediated, at least in part, through the activation of α-adrenoreceptors. In addition, activation of particular receptor subclasses might enable the selective modulation of either the rhythm generating networks, the longitudinal co-ordination of those networks or control at both levels simultaneously.

3.2 Introduction.

The rhythmic motor patterns generated by neuronal networks during vertebrate locomotion are characterised by a high degree of intrinsic variability, which affords the animal the behavioural flexibility to respond to different environmental requirements (Kiehn *et al.*, 1997; Sillar *et al.*, 1997). Such variability can be achieved, for example, by direct behavioural selection in response to different afferent inputs, yet it can also be due to the intrinsic influences of centrally acting neuromodulators that affect the spinal motor circuitry itself (for reviews, see Pearson, 2000; Kiehn & Katz, 1999; Harris-Warrick, 1991).

In vertebrates, biogenic amines such as 5-HT and NA play important roles in initiating, sustaining and modulating motor output. There have been many studies which have focused on the role of 5-HT in a range of vertebrates (e.g. cat, Barbeau & Rossignol, 1991; rabbit, Viala & Buser, 1969; rat, Kiehn & Kjaerulff, 1996; Cazalets *et al.*, 1992; turtle, Hounsgaard & Kiehn, 1989; lamprey, Harris-Warrick & Cohen, 1985; tadpole, McDearmid *et al.*, 1997; Sillar *et al.*, 1992a). The effects of 5-HT on initiating and modulating vertebrate motor patterns are mediated at a range of 5-HT receptor sub-types (e.g. Wallis, 1994; Wedderburn & Sillar, 1994; Wikstroem *et al.*, 1995). The metabotropic α_1-, α_2-, β_1- and β_2-adrenoreceptor subclasses have been previously defined as putative receptors for catecholamines, based upon both their physiological actions and pharmacological specificity (e.g. Hirst & Neild. 1980; Lefkowitz & Caron 1985; Summers & McMartin, 1993). In vertebrates there is evidence that activation of adrenergic receptors can also modulate motor output (Forssberg & Grillner, 1973; Kiehn *et al.*, 1992; Barbeau & Rossignol, 1991; Sqalli-Houssaini & Cazalets, 2000).

NA plays a wide variety of roles in vertebrates, not only in the CNS. It has

for example been implicated in the control of apoptosis (e.g. Singh *et al.*, 2001), regulation of vascular flow and smooth muscle contraction (for a review see; Jackson & Cunnane, 2001) and plays a role in a number of pathological conditions such as depression (e.g. Leonard, 1997). Whilst its roles in a wide variety of peripheral nervous system functions is well reported, far less is known of its role in the CNS and in particular its involvement in the generation and modulation of behaviour. Noradrenergic neurons and projections have now been identified in several vertebrate central nervous systems (for a review see Jones, 1991; amphibians, e.g. Gonzalez & Smeets, 1995; Gonzalez *et al.*, 1995). In *Xenopus*, immunocytochemistry in the brain and spinal cord of adults has revealed tyrosine-hydroxylase-positive neurons in three locations- the isthmic region, the caudal brainstem and in the nucleus of the periventricular organ. (Gonzalez & Smeets, 1993). More recently, the presence of aminergic projections from the locus coeruleus into the spinal cord of embryos has been reported (Sanchez-Camacho *et al.*, 2002).

In higher vertebrates, such as the cat (Chau *et al.*, 1998; Kiehn *et al.*, 1992) and rat (Sqalli-Houssaini & Cazalets, 2000; Kiehn *et al.*, 1999), NA is able to initiate, sustain and modulate rhythmic motor activity in spinal cord preparations, yet in some lower vertebrate preparations NA appears not to play any significant role in neuromodulation (e.g. lamprey; Buchanan, 2001). In the spinal pattern generator of the *Xenopus* tadpole, however, NA has a strong effect, slowing swimming (McDearmid *et al.*, 1997), and simultaneously reducing the RC-delay in muscle activation (McDearmid, 1998).

Most previous work on NA modulation has been performed either on the spinal cord *in vitro* or on acutely spinalised preparations in which locomotor activity was triggered pharmacologically or by tonic electrical stimulation. In utilising the

relatively simple and essentially intact *Xenopus* preparation I have been able to study noradrenergic modulation in an animal whose spinal network generates self-sustaining rhythmic activity, without reliance on persistent drug application. This chapter therefore investigates the specific adrenoreceptor sub-types through which the modulatory effects of NA are most likely mediated. I have used both general and specific adrenoreceptor agonists and antagonists to investigate the role that pharmacologically distinct adrenoreceptors might play in the modulation of locomotion in *Xenopus* tadpoles.

3.3 The general α-adrenoreceptor antagonist phentolamine counteracts the effects of NA on cycle periods and RC- delays.

Unless stated otherwise, the data are from experiments on stage 42 larvae, but are equally valid for stage 37/38 embryos. Developmental comparisons between stages 37/38 and stage 42 are outlined only when a significant difference was present.

I first investigated the role of adrenoreceptors in modulating *Xenopus* swimming by bath applying the general α-receptor antagonist, phentolamine and general β-receptor antagonist propranolol after prior application of NA. Representative experiments for individual animals are shown in Figure 3.1. In the 12 preparations tested, bath application of 4-6μM NA significantly increased cycle periods by 36.7 ± 5.9% (N=12, P<0.001; Figure 3.1A-B, Figure 3.2A-B), reduced the RC-delay by 11.1 ± 7.7% (N=12, P<0.01; Figure 3.1C) and the swim episode duration by 23.6 ± 8.3% (N=12, P<0.05; Figure 3.1D). In addition, NA did not consistently influence the burst duration in either embryos or larvae (Figure 3.1E), nor did it have any consistent effects on the amplitude of the ventral root bursts (N=12, P>0.05; Figure 3.1F).

Subsequent application of phentolamine to 8 of the 12 animals decreased cycle periods by 17.6 ± 6.7% (N=8, P<0.001, Figure 3.1A,B) and increased RC-delays by 33.2 ± 11.6% (N=8, P<0.001, Figure 3.1C). However, after addition of phentolamine, episode durations were not significantly affected (N=8, P>0.05, Figure 3.1A).

In 4 further experiments, the β-receptor antagonist propranolol (40-45μM) was applied subsequent to NA. However, propranolol was unable to reverse the effects of NA on cycle periods (N=4, P>0.05, Figure 3.2A,B), RC-delays (N=4, P>0.05, Figure 3.2B,C) or episode durations (N=4, P>0.05, Figure 3.2D).

The ability of a general α-receptor antagonist to reverse the noradrenergic effects on swimming frequency and RC-delays suggests that NA might activate α-adrenoreceptors in order to modulate these parameters. My next strategy was therefore to look specifically at the pharmacology of this response using specific agonists to α_1- and α_2- adrenoreceptor classes to try and mimic the noradrenergic response.

3.4 The general α-receptor antagonist phentolamine counteracts the effects of selective α_1-receptor activation on the parameters of swimming.

In 3 embryos and 4 larvae, the specific α_1-agonist phenylephrine (50-100μM) was bath-applied. In embryos, episode duration under phenylephrine increased by 52 ± 12.3% (N=3, P<0.05, Figure 3.3A), whilst in larvae, episode durations decreased by 36.5 ± 3.4% (P<0.01; N=4, Figure 3.3A). In all 7 animals tested, cycle periods increased after α_1-receptor activation by 10.4 ± 3.2% (N=7, P<0.01; Figure 3.3B,C). Furthermore, the RC-delay was decreased after the application of phenylephrine by

15.3 ± 3.2% (N=7, P<0.01; Figure 3.3D). Like NA, phenylephrine also had no consistent effects on either the burst duration or the burst amplitude (N=7, P>0.05; data not shown). These results clearly show that activation of α_1-receptors can mimic the effects of NA on all the parameters of swimming. My next approach was therefore to counteract the effects of α_1-activation using phentolamine.

The effects of phenylephrine on the parameters of swimming could be reversed with subsequent application of the general α-receptor antagonist phentolamine. Cycle periods decreased by 8.5 ± 2.2% and RC-delays by 12.5 ± 3.2% after application of 50-70μM phentolamine (N=7, P<0.05; Figure 3.3B-D). The effects of phenylephrine on episode duration in both embryos and larvae were also counteracted after addition of phentolamine (N=7, P<0.05; Figure 3.3A). In 4 different preparations (all larvae) the effects of phenylephrine were investigated after prior application of phentolamine. In none of the experiments was a significant effect on cycle period or RC-delay observed after application of phenylephrine (N=4, P>0.05; Figure 3.3E,F). Phentolamine did not, however, prevent the reduction of episode duration by phenylephrine (N=4, P<0.05; data not shown). The finding that phentolamine could counteract the effects of α_1-receptor activation on all the parameters of swimming (except for episode duration), and could also occlude its effects when pre-applied, strongly suggests an intrinsic role for this receptor type in modulating swimming. Coupled with the earlier evidence that the effects of NA could be counteracted with phentolamine, it is likely that NA modulates, at least in part, the frequency and duration of tadpole swimming, together with its longitudinal co-ordination via activation of α_1-adrenoreceptors.

3.5 The general α-receptor antagonist phentolamine counteracts the effects of α_2-receptor activation on the parameters of swimming.

As phentolamine is a broad-spectrum α-adrenoreceptor antagonist, it was important not to overlook a possible role for α_2-adrenoreceptors in mediating the effects of NA. Therefore, in 9 preparations, the specific α_2-receptor agonist clonidine was bath-applied at concentrations of 35-200μM. After application of clonidine, episode durations were dramatically reduced by 75.8 ± 8.2% (N=9, P<0.05; Figure 3.4A). Cycle periods increased under clonidine by 15.5 ± 2.2% (N=9, P<0.001; Figure 3.4B,C,E,G). The response to clonidine depended on the applied concentration. The minimum concentration for eliciting a response was 35-50μM, although the effects peaked at 100-110μM, with even higher concentrations sometimes leading to bouts of struggling at the start of and during swimming episodes (Figure 3.4E; see also chapter 6 for a discussion of the effects of NA and α-adrenoreceptor agonists on struggling). Furthermore, there was no effect on RC-delays after application of clonidine (N=9, P>0.05; Figure 3.4D). For a given cycle period, the RC-delay did not differ under clonidine (intercepts not different, P>0.05; Figure 3.3G). Clonidine had no consistent effects on burst durations (N=9, P>0.05; data not shown), however, it reduced burst amplitudes by 14.1 ± 2.3% (N=9, P<0.001; Figure 3.4F).

Subsequent application of phentolamine (40-60μM) to 5 animals or returning to control saline (4 animals) counteracted the effects of α_2-receptor activation, reducing cycle periods by 10.6 ± 4.2% (N=9, P<0.05; Figure 3.4B,C). However, neither phentolamine, nor washing in control saline could counteract the effects on either episode duration (N=9, P>0.05; Figure 3.4A) or burst amplitudes (N=9,

P>0.05; Figure 3.4F). These results suggest that in addition to α_1-receptors, α_2-receptor activation appears also to play an intrinsic role in mediating the noradrenergic effects on both swimming frequency and duration. However, in contrast to α_1-receptors, α_2–receptor activation appears not play a role in the modulation of longitudinal co-ordination.

3.6 Discussion.

NA and activation of adrenoreceptors is known to have an important role in the initiation and modulation of spinal locomotor patterns in mammals (cats, e.g. Forssberg & Grillner, 1973: Barbeau *et al.*, 1987; Barbeau & Rossignol, 1991; Kiehn *et al.*, 1992; Rossignol *et al.*, 1998; Marcoux & Rossignol, 2000; rats, e.g. Kiehn *et al.*, 1999; Sqalli-Houssaini & Cazalets, 2000). Furthermore, the different receptor subtypes have been found to perform various specific functions in mediating these locomotor effects. For example, in the neonatal rat, α_1-receptor activation can increase the frequency of NMDA-induced activity (Sqalli-Houssaini & Cazalets, 2000; see also cats: Chau *et al.*, 1998). In this chapter I have investigated the potential involvement of adrenoreceptors in the modulation of swimming in the *Xenopus* tadpole. As discussed in detail above, this system has many advantages over other vertebrate preparations, particularly as it is an intact locomotor system that generates a self-sustaining swimming rhythm without reliance on persistent drug application.

In post-embryonic tadpoles (as in other aquatic animals, e.g. Lighthill, 1969; Grillner & Kashin, 1976), cycle periods and RC-delays are positively correlated (Tunstall & Sillar, 1993), thus introducing a constant phase-lag along the body and, presumably maintaining a uniform undulatory body shape, regardless of the speed of

locomotion (Von Seckendorf-Hoff & Wassersug, 1985). In embryos, however, co-ordination of swimming is characterised by a constant latency in the successive activation of the body segments irrespective of the actual swimming frequency (Tunstall & Sillar, 1993). Despite this, during swimming in embryos a uniform undulatory body shape is maintained even as swimming frequency changes. Although maintenance of this shape in the absence of a direct relationship with longitudinal co-ordination must presumably rely on some interaction with the environment, the mechanisms behind this are not known.

In larval tadpoles, two parallel aminergic neuromodulatory systems control locomotion in an antagonistic manner. NA strengthens mid-cycle glycinergic IPSPs by pre-synaptically enhancing the probability of transmitter release from known interneuron terminals, and in doing so reduces the frequency of swimming. 5-HT, however, has the opposite effect and reduces glycinergic inhibition, leading to an increase in the intensity of motor output (Figure 1.5; McDearmid *et al.*, 1997). Serotonergic raphe spinal neurons develop projections into the spinal cord during the transition from embryonic to the early larval stage (van Mier *et al.*, 1986, Woolston *et al.*, 1994). Neurons immuno-positive for dopamine and tyrosine hydroxylase are present in the brain stem and spinal cord of both embryos and larvae (Gonzalez *et al.*, 1994, reviewed in: Smeets & Gonzalez, 2000) and more recently, the presence of aminergic projections from the locus coeruleus into the spinal cord of embryos has been reported using antibodies against NA (Sanchez-Camacho *et al.*, 2002). Furthermore, as shown by these results, NA and adrenoreceptor agonists have comparable effects on the embryonic and larval locomotor network. Taken together, this anatomical and pharmacological evidence suggests that the noradrenergic neuromodulatory pathway is at least established and available for utilisation by the

late embryonic stage of development.

In this chapter I have shown that specific adrenoreceptor subclasses exert distinct effects on particular facets of the fictive swimming rhythm, which are largely independent of the stage of development. Firstly, α_1-receptor activation influences both swimming frequency and its longitudinal co-ordination (Figure 3.3), whilst α_2-receptors mainly modulate only swimming frequency (Figure 3.4) and are not involved in the longitudinal co-ordination of swimming. However, both receptor classes affect the duration of swimming episodes: α_2-receptor activation dramatically reduces the length of swimming bouts (Figure 3.4), as do α_1-receptors in larvae, but in embryos α_1-receptor activation increases episode duration (Figure 3.3). Furthermore, the effects of NA on cycle periods and RC-delays were reversed using a general α-receptor antagonist (Figure 3.1). Taken together, this suggests the participation of α-receptors in mediating the effects of NA on swimming frequency. In addition, in a recent study, β-receptor activation using specific pharmacological agonists was shown to modulate longitudinal co-ordination, yet seems not to affect cycle periods or episode durations (Fischer *et al.*, 2001; Figure 3.5). It is perhaps surprising therefore that the effects of NA on RC-delays were not counteracted by the β-receptor antagonist propranolol in this study. Neither of the receptor sub-classes I studied here was able to increase swimming intensity at any of the concentrations applied. Consistent with this finding, α_2-receptors are known to slow ongoing locomotion in other locomotor systems (e.g. cat: Chau *et al.*, 1998, rat: Sqalli-Houssaini & Cazalets, 2000). In tadpoles however, there is no evidence for either excitatory NA effects on the pattern mediated by α_1-receptors, or for a role for β-receptors in decreasing locomotor activity. Both of these effects have been

reported in rats (Sqalli-Houssaini & Cazalets, 2000). α_1-receptor activation did not have any effect on the motor burst duration, or the motor burst amplitude, which would suggest that adrenoreceptor activation might not have strong direct effects on motor neurons during the modulation of swimming. Indeed, previous findings have shown that the receptors mediating the effects of NA are presumed to be located presynaptically on the terminals of inhibitory neurons (McDearmid *et al.*, 1997). Burst amplitudes and durations appear to be strongly influenced by the excitatory drive onto motor neurons and its interaction with serotonin-dependent non-linear membrane properties (e.g. in *Rana*, Sillar & Simmers, 1994b; *Xenopus*, Scrymgeour-Wedderburn *et al.*, 1997; Reith & Sillar 1999; for other systems, Hounsgaard *et al.*, 1988; MacLean *et al.*, 1996). Activation of α_2-receptors by clonidine, however, decreased motor burst amplitudes during swimming (Figure 3.4F). However, since clonidine did not affect burst durations it seems possible that these adrenoreceptors are located distal to the spike-initiating zone. This is consistent with evidence from some higher vertebrates where α_2-receptors are presumed to be located post-synaptically (e.g. cat, Chau *et al.*, 1998). Furthermore, activation of α_2-receptors affects locomotor burst duration, and the application of NA modulates the electrical properties of motor neurons (Connell *et al.*, 1989; Elliot & Wallis, 1992; Sqalli-Houssaini & Cazalets, 2000; see also chapter five of this study). It remains to be determined, however, whether any subclasses of adrenoreceptors are present post-synaptically in *Xenopus* and contribute to the modulation of ongoing locomotion.

The only parameter of swimming in which there appears to be a developmental difference during adrenoreceptor modulation is the duration of swim episode, where significant differences in response to the activation of α_1-receptors were observed between embryos and larvae (Figure 3.3). It is relevant to consider

intrinsic mechanisms that regulate episode durations as possible targets for these noradrenergic effects. Swimming in embryos can be terminated prematurely by the activation of primary afferents innervating the rostral cement gland. This glutamatergic pathway activates mid-hindbrain reticulospinal (mhr) GABAergic neurons, which in turn release GABA from their spinal projections onto the CPG and hence terminate swimming (Boothby & Roberts, 1992a,b; for more detail, see chapter one; Figure 1.3Aiii). This pathway begins to functionally degenerate during the developmental transition between embryonic to free swimming larval stages, although the GABAergic mhr neurons appear to remain (Reith, 1995). Termination of swimming in larvae, however, often occurs spontaneously, coincident with a barrage of GABAergic IPSPs, presumably due to an intrinsic activation of an inhibitory pathway from the mhr neurons (Reith & Sillar, 1999; Figure 1.3Bii). A plausible explanation for the differences in episode duration in response to α_1-receptor activation at the two stages, therefore, is the developmental change in how this particular GABAergic inhibitory pathway, which does not markedly affect parts of the network controlling the other locomotor parameters, is engaged.

At present, only some of the cellular and synaptic mechanisms underlying the NA-mediated modulation of tadpole swimming have been investigated. It is known, that the NA-mediated increase in the mid-cycle inhibitory component of the swimming rhythm reduces swimming frequency via pre-synaptic enhancement of glycine release from the terminals of commissural interneurons (McDearmid *et al.*, 1997; Figure 1.5). Furthermore, as highlighted above, a known GABAergic pathway participates in the termination of swimming. The similar effects on episode duration after the application of either NA (Figure 3.1), or agonists at either $GABA_A$ receptors (Reith & Sillar, 1997, 1999) or subclasses of α-adrenoreceptors (this

chapter) raises the possibility that all of these pharmacological agonists share common targets, namely the fast inhibitory pathways that influence tadpole swimming. A more detailed examination of this hypothesis, focusing in particular upon the contributions of glycinergic and GABAergic inhibition to the generation, control and termination of swimming, should provide a clearer picture of the role of adrenoreceptors in mediating the noradrenergic modulation of swimming in the *Xenopus* tadpole.

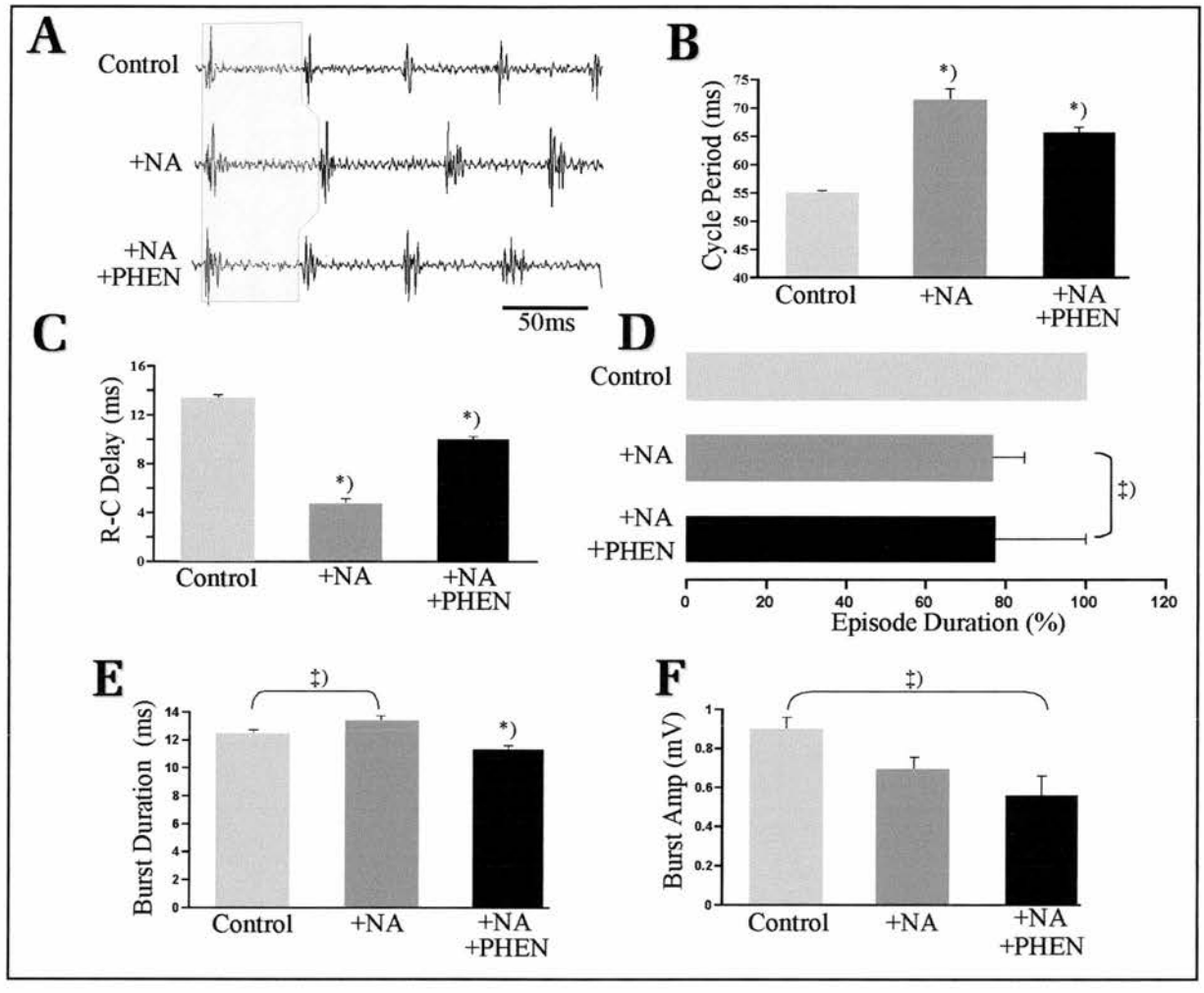

Figure 3.1: *Effects of phentolamine on the noradrenergic modulation of* swimming **(A)** Effects of NA and phentolamine (PHEN) on swimming frequency (as indicated by the grey shaded area). Changes in average cycle period **(B)** and RC-delay **(D)** under control, NA and phentolamine.**(D)** NA significantly reduces the duration of swimming episodes. Phentolamine does not reverse these effects. **(B)**. NA had no effect on the average duration of motor bursts **(E)** or their amplitude **(F)**. Data in (B-D) and (E-F) are from the same animal. Asterisks (*) indicate values different from the control ($P<0.01$) and from each other ($P<0.05$). ‡) Not significantly different ($P>0.05$).

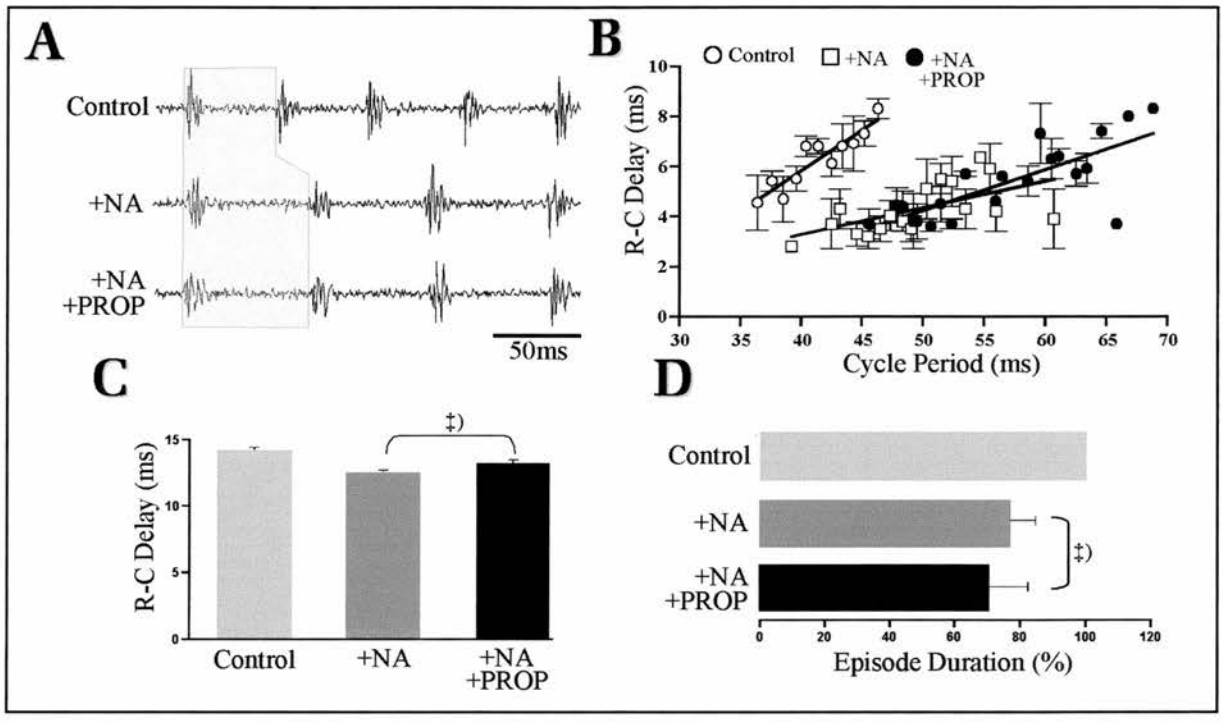

Figure 3.2: *Effects of propranolol on the noradrenergic modulation of swimming* (**A**) Propranolol (PROP) could not counteract the NA-mediated effects on cycle period, as indicated by the grey shaded area. (**B**) RC-delays are significantly correlated with the cycle period in all three conditions tested ($P<0.01$).Data were pooled from different observations at a given cycle period. Propranolol cannot counteract a reduction in RC-delays (**C**) or episode duration (**D**) by NA. Data in (A-B) and (C-D) from the same animal. Asterisks (*) indicate values different from the control ($P<0.01$) and from each other ($P<0.05$). ‡) Not significantly different ($P>0.05$).

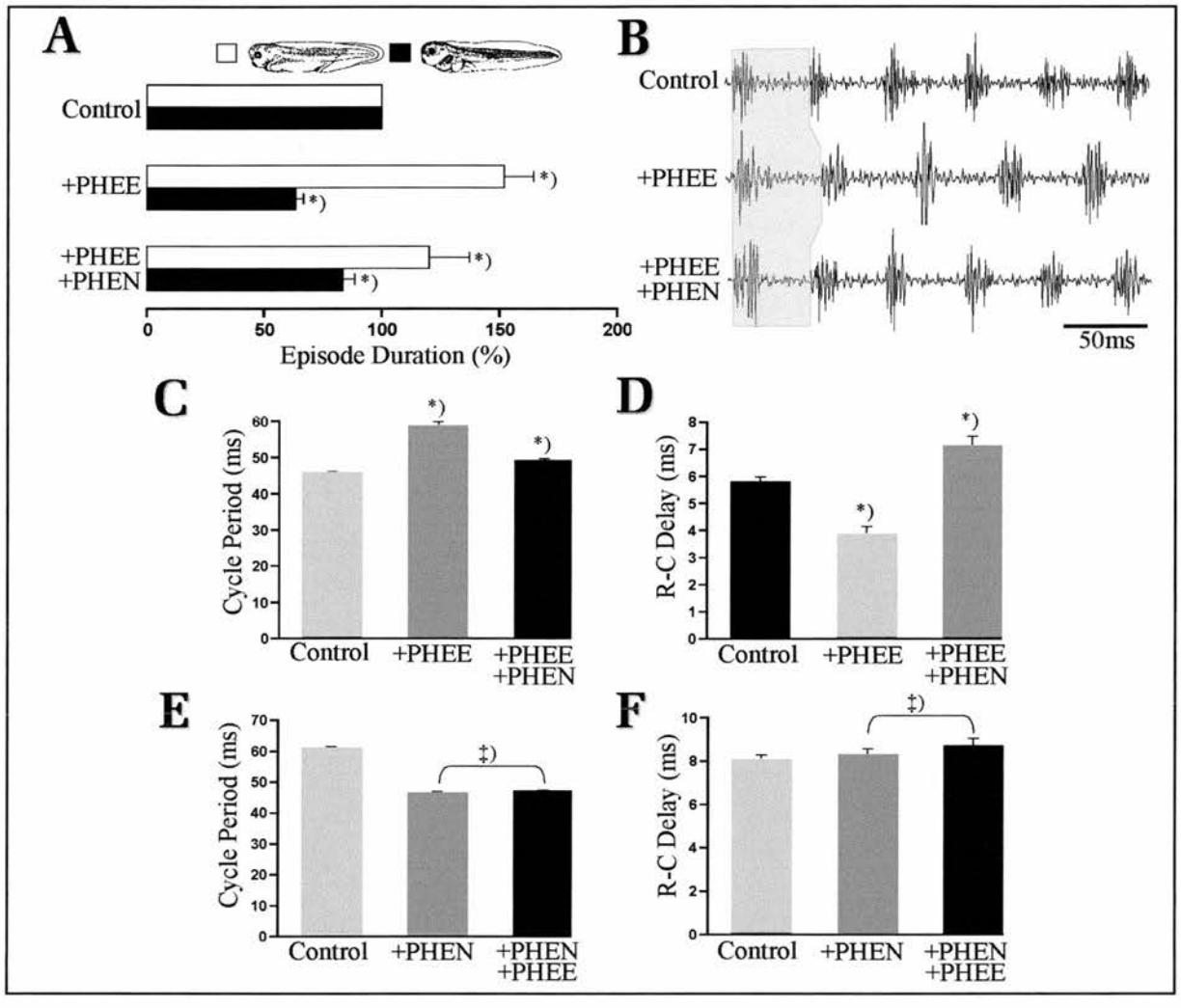

Figure 3.3: *Effects of phenylephrine and phentolamine on the parameters of swimming.* (**A**) Phenylephrine (PHEE) significantly increases embryonic episode duration, but shortens larval episodes. Subsequent addition of phentolamine reversed the effects of phenylephrine (**B**) Effects of phenylephrine and phentolamine on the swimming frequency (as indicated by the grey shaded area). Phentolamine could counteract an increase in cycle period (**C**) and decrease in RC-delay (**D**) after application of phenylephrine. Prior application of phentolamine could occlude the effects of phenylephrine on cycle periods (**E**) and RC-delays (**F**). Data in (B-D) and (E-F) are from the same animals. *) $P<0.01$ ($n=3$); ‡) Not significantly different ($P>0.05$, $n=3$).

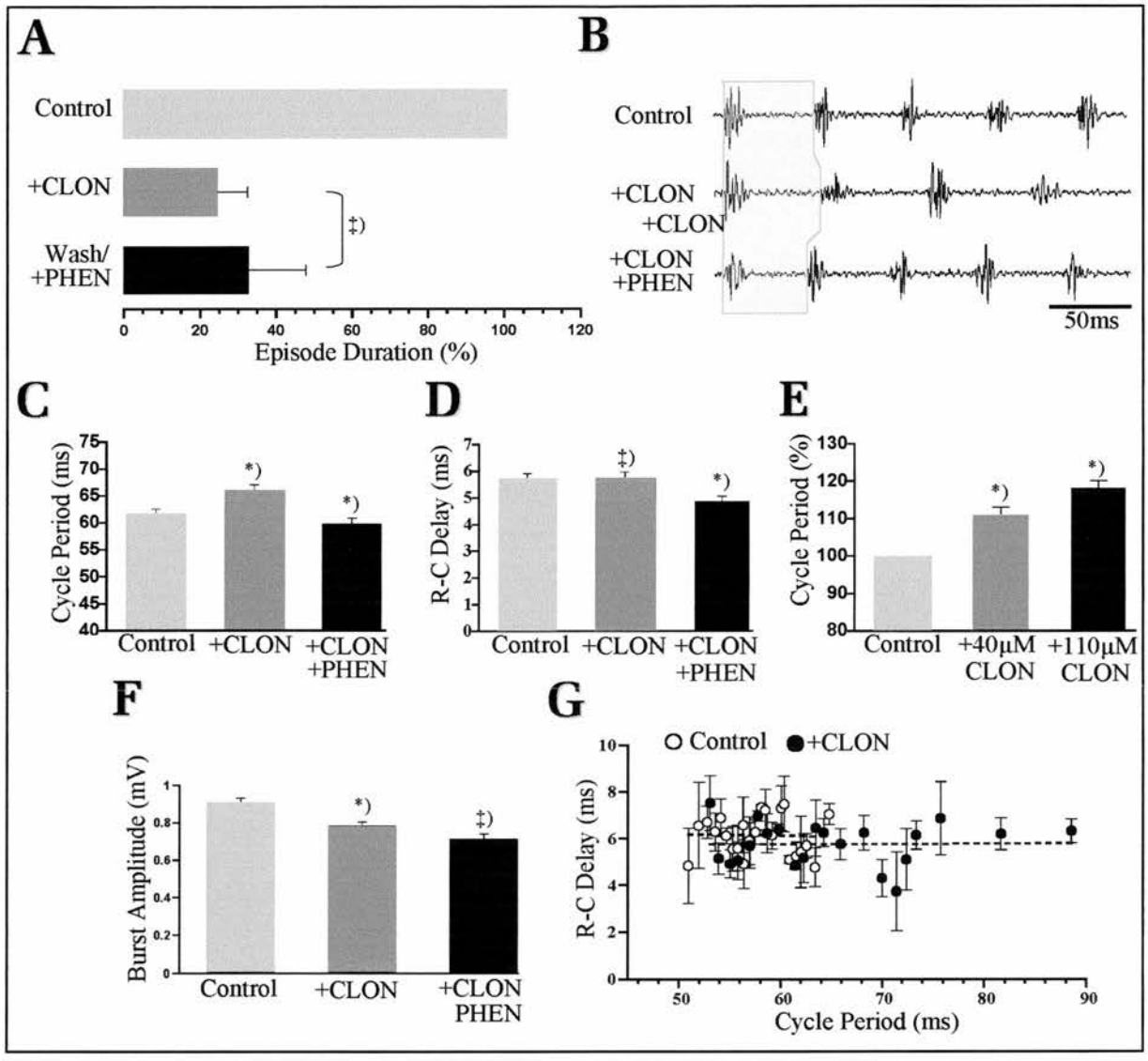

Figure 3.4: *Effects of clonidine and phentolamine on the parameters of fictive swimming.* (**A**) Clonidine (CLON) significantly reduces episode duration. No significant reversal was produced by washing or by application of phentolamine. (**B**) Effects of clonidine on swimming frequency (as indicated by grey shaded region). Clonidine reduced the swimming frequency, which was counteracted by phentolamine (**C**), but had no effect on RC-delay (**D**). (**E**) Dose dependent effects of clonidine on cycle period (data from 5 animals). (**F**) Clonidine significantly reduced the amplitude of motor bursts. Phentolamine could not reverse this effect. (**G**) The increase in cycle period under clonidine does not influence the RC-delay (dashed lines indicate no significant correlation, $P>0.05$). Data shown in (B-D) and are from the same animal. *) $P<0.01$ ($n=3$); ‡) Not significantly different ($P>0.05$, $n=3$).

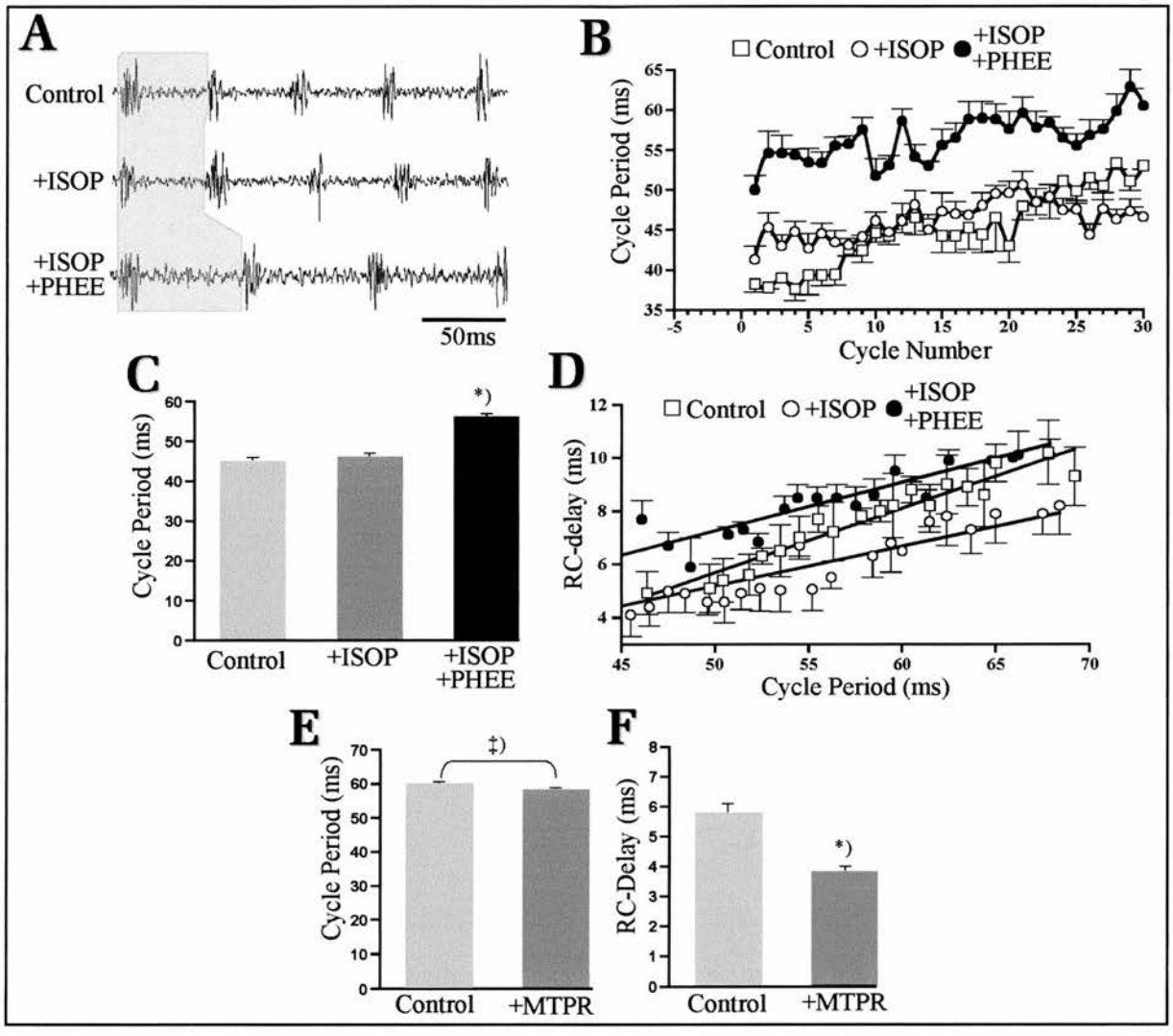

Figure 3.5: *Effects of isoproterenol and metaproterenol on the parameters of swimming.* (**A**) Effects on swimming frequency after application of isoproterenol *(general β-receoptor agonist; ISOP)* and phenylephrine (indicated by the grey shaded area). (**B**) Time course of cycle periods over 30 continuous cycles. Data points pooled from 3 episodes. (**C**) Isoproterenol had no effect on cycle periods, however, they were elongated after subsequent addition of phenylephrine. (**D**) Relationship between RC-delay and cycle period under control, isoproterenol and additionally, propranolol. Data points at a given cycle number pooled from 3 episodes. Data in (A)-(C) stem from the same larval preparation, (D) and (E)-(F) are from two further animals. RC-delays were significantly decreased under metaproterenol *(specific β_2-receptor agonist; MTPR;* $P<0.05$; **E**), however, it did not influence cycle period (**F**). *) $P<0.01$ ($n=3$); ‡) Not significantly different ($P>0.05$, $n=3$). Figure adapted from Fischer *et al.*, 2001

Chapter Four

Alpha-adrenoreceptor activation modulates swimming via glycinergic and $GABA_A$ergic inhibitory pathways

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Alpha-adrenoreceptor activation modulates swimming via glycinergic and $GABA_A$ergic inhibitory pathways in *Xenopus laevis* tadpoles

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4.1 Summary.

This chapter focuses on the possible neural pathways that underlie the adrenoreceptor-mediated modulation of fictive swimming in *Xenopus* tadpoles. As shown in chapter three, NA increases cycle periods whilst simultaneously reducing the duration of swimming episodes and the RC-delay in motor neuron activation. In addition, both swimming frequency and duration are reduced by selective pharmacological activation of α_1- and/or α_2-adrenoreceptors, whilst α_1-receptor activation also reduces RC-delays. These effects are consistent with facilitation of fast inhibitory pathways influencing the swimming circuitry.

Here I show that NA could still exert effects on fictive swimming after blocking either glycine or $GABA_A$ receptors with strychnine or bicuculline, respectively. However, after pre-application of NA, strychnine could counteract the noradrenergic effects on cycle periods and RC-delays, whilst bicuculline could only counteract effects on cycle periods, suggesting that both these inhibitory pathways are involved in the noradrenergic modulation of swimming. Furthermore, strychnine reduced the effects of α_1-receptors on cycle periods and delays, whilst bicuculline had no effect. However, blocking either glycine or $GABA_A$ receptors was able to reduce the increase in cycle period by α_2-receptors, whilst pre-application of bicuculline prevented a reduction in episode durations by NA, α_1- and α_2-receptors.

These findings suggest that the noradrenergic modulation of *Xenopus* swimming is mediated via α-adrenoreceptors, interacting with glycinergic and GABAergic inhibitory pathways. α_1-receptor activation utilises glycinergic pathways to modulate the swimming rhythm and its longitudinal co-ordination, whilst α_2-receptor activation affects swimming frequency via both glycinergic and GABAergic pathways. Activation of either α_1- or α_2-adrenoreceptors influences the GABAergic pathway controlling the duration of swimming episodes.

4.2 Introduction.

An inherent requirement of vertebrate locomotor systems is the ability to modulate their motor output in order to adapt to different environmental requirements. Neuromodulators provide one important way of achieving this by tuning the output of neural circuits. They have the ability to suppress or promote motor function, to initiate a new motor function when required and to modify neuronal and synaptic components of the network to allow them to generate different patterns of motor activity (Pearson, 1993). The pathways that mediate fast synaptic inhibition and which are known to be targets for neuromodulation utilise the amino acids glycine and GABA. Reciprocal glycinergic inhibition plays an important role in the co-ordination of alternating activity between the two sides of the body during undulatory locomotion (for an overview, see Roberts, 2000; Grillner *et al.*, 1995). GABA is involved in the modulation of sensory afferent transmission during locomotion (Dubuc *et al.*, 1988; Stuart & Redman, 1991) and may also affect network inter-segmental (lamprey; Tegner & Grillner, 2000) as well as intra-limb (rat; Cowley & Schmidt, 1995; Kremer & Lev-Tov, 1997) co-ordination. Furthermore, in *Xenopus*, brainstem GABA systems with descending projections are known to contribute to the termination of locomotor activity (Boothby & Roberts, 1992a,b; Reith & Sillar, 1999). Neither GABAergic nor glycinergic pathways are, however, necessary for basic rhythm generation because locomotor activity continues even after pharmacological blockade of post-synaptic receptors (e.g. rat, Tresch & Kiehn, 2000; lamprey, Cohen & Harris-Warrick, 1984; Alford & Williams, 1989; Hagevik & McClellan, 1994; *Xenopus* embryo, Roberts *et al.*, 1984, larva, Reith & Sillar, 1999; mudpuppy, Jovanovic *et al.*, 1999).

As discussed in chapter three, NA and 5-HT modulate swimming frequency

in *Xenopus* tadpoles by differentially affecting the glycinergic mid-cycle component of the synaptic drive underlying swimming (McDearmid *et al.*, 1997; Figure 1.5). Whilst NA enhances glycine release, increasing the opening of chloride ion channels, membrane hyperpolarisation and inhibition, thus slowing swimming and reducing the RC-delay along the body, 5-HT inhibits it, thus leading to relatively fast intense swimming. Furthermore, the noradrenergic modulation of the spinal swim-pattern generator in *Xenopus* appears to be mediated via activation of α- (see previous chapter) and β- (Fischer *et al.*, 2001) adrenoreceptors. Both α_1- and α_2-receptors modulate the frequency and duration of swimming, whilst α_1- and β-receptors modulate longitudinal co-ordination. In this chapter I have used a pharmacological approach to investigate the possibility that both GABA- and glycinergic inhibitory pathways are targets for neuromodulation by NA acting at the α- class of adrenoreceptors.

4.3 Blocking either glycine or $GABA_A$ receptors counteracts the noradrenergic effects on cycle periods and RC- delays.

The data presented here are derived from experiments on 72 animals of both embryonic and early larval stage *Xenopus* tadpoles. In the previous chapter (see also Fischer *et al.*, 2001), the only developmental difference found between these stages of development was the increase in the duration of embryonic swimming episodes (compared to a decrease in larvae) following α_1-receptor activation. I have therefore not otherwise discriminated between developmental stages.

My first approach was to bath apply NA before pharmacologically blocking either glycine or $GABA_A$ receptors and analysing any changes in the parameters of

swimming. Representative experiments for individual animals are shown in Figure 4.1. In all the animals tested, NA (4-6μM) increased cycle periods by 21.6 ± 3.5% (N=15, P<0.001; Figure 4.1A,B,D), reduced the RC-delay by 22.9 ± 4.9 % (N =15, P<0.001; Figure 4.1C, F) and the swim episode duration by 43.75 ± 10.7 (N =15, P<0.001; Figure 4.1E; see also chapter three).

The subsequent application of the glycine receptor antagonist strychnine (1μM, see Soffe, 1987) to 8 of these animals decreased cycle periods by 17.9 ± 6.6% (N =8, P<0.001; Figure 4.1A,B) and increased the RC-delay by 19.2 ± 12.7% (N =8, P<0.05; Figure 4.1C). However, there was no further effect on episode duration (N=8, P>0.05; Figure 4.1E). The application of the $GABA_A$ receptor antagonist bicuculline (30-40μM) to the remaining 7 animals significantly counteracted the effects of NA on cycle periods by 12.2 ± 5.2% (N =7, P<0.01; Figure 4.1D), but did not affect episode duration (N=7, P>0.05; Figure 4.1E). In addition, bicuculline did not counteract the noradrenergic effects on RC-delays, which were further reduced by 10.7 ± 5.1% (N=7, P<0.05; Figure 4.1F). This set of experiments indicates that the noradrenergic modulation of swimming frequency might target both GABAergic and glycinergic pathways, whereas the modulation of longitudinal co-ordination might utilise only glycinergic pathways. However, it should be noted that both strychnine and bicuculline can themselves reduce swimming frequency (e.g. Reith & Sillar, 1999). It is therefore important to investigate the effects of NA (and adrenoreceptor activation) in the presence of both of strychnine and bicuculline to discount that the effects in these experiments are due to the antagonists rather than to their reversal of NA's effects.

4.4 The effects of α_1-receptor activation on cycle periods and the longitudinal delay are counteracted by blocking glycine receptors, but not $GABA_A$ receptors.

The effects of NA on the locomotor rhythm are mediated by different sets of adrenoreceptors (see chapter three). My next approach was therefore to investigate the possible interaction between α_1-receptors and the glycinergic or $GABA_A$ergic inhibitory pathways. Representative examples of individual animals are given in Figures 4.2 & 4.3. The activation of α_1-receptors by bath application of the specific agonist phenylephrine (70-100μM) to 4 embryos and 4 larvae increased cycle periods by 15.1 ± 4.9 % (N=8, P<0.001; Figures 4.2A,B, 4.3A,B) and reduced the RC-delay by 9.38 ± 1.74% (N=8, P<0.001; Figure 4.2C, 4.3C). In embryos, episode duration increased under phenylephrine by 35.2 ± 15.1% (N=4, P<0.05), whereas, in larvae, episodes became shorter by 15.4 ± 5.6% (N=4, P<0.05; Figures 4.2D, 4.3D), confirming my findings in chapter three.

Subsequent application of strychnine (1μM) to 4 animals reduced cycle periods by 10.3 ± 2.3% (N=4, P<0.05; Figure 4.2A-B) and increased the RC-delay by 13.4 ± 4.4% (N=4, P<0.05; Figure 4.2C). However, in neither embryos nor larvae did the subsequent application of strychnine further affect episode duration (N=8, P>0.05; Figure 4.2D). In the remaining 4 animals, subsequent application of bicuculline (30-40μM) did not significantly counteract the effects of phenylephrine on cycle period (N=4, P>0.05; Figure 4.3A-B) or RC-delay (N=4, P>0.05; Figure 4.3C). In addition, there was no further effect on episode duration in either embryos or larvae (N=4, P>0.05; Figure 4.3D). These results are consistent with the conclusion that α_1-adrenoreceptor activation modulates swimming frequency and

longitudinal co-ordination by utilising glycinergic pathways, but apparently not GABAergic ones.

4.5 The effects of α_2-receptor activation on cycle periods are counteracted by blocking either glycine or $GABA_A$ receptors.

In the next set of experiments α_2-receptors were activated using clonidine, in order to investigate the interaction between α_2-receptors and the two inhibitory pathways (Figure 4.4). In 13 animals, clonidine (70-110μM) increased cycle periods by 9.8 ± 1.8% (N=13, P<0.05; Figure 4.4A-D), but did not affect the RC-delay (N=13, P>0.05; data not shown). Clonidine also reduced episode durations by 25.1 ± 6.6% (N=13; P<0.05; Figure 4.4E,F). Subsequent addition of strychnine to 9 animals reduced cycle periods by 14.4 ± 2.1% (N=9, P<0.05; Figure 4.4A,B), but had no effects on either the RC-delay (N=9, P>0.05; data not shown) or episode duration (N=9, P>0.05; Figure 4.4E). Under clonidine, addition of bicuculline to the remaining 4 animals reduced cycle periods by 18.5 ± 6.7% (N=4, P<0.001; Figure 4.4C,D) and RC-delays by 22.1 ± 9.1% (N=4, P<0.01; data not shown). However, episode duration was not affected (N=4, P>0.05; Figure 4.4F). These findings suggest that in contrast to α_1-receptor activation, α_2-receptors modulate swimming frequency by utilising both glycinergic and GABAergic pathways.

4.6 Blocking glycine receptors occludes the effects of α_1-receptor activation, but not of NA and α_2- receptor activation on cycle periods and the RC-delay.

To extend my study of the interaction between NA, α-receptors and the glycinergic inhibitory pathways, NA and α-receptor agonists were added to animals

in which glycine receptors had been pre-blocked by strychnine. Representative examples are shown in Figure 4.5. Application of strychnine (1μM) to 19 animals reduced cycle periods by 12.7 ± 1.6% (N=19, P<0.001; Figure 4.5A,B,D,F,H), increased the RC-delay by 14.8 ± 4.7% (N=19, P<0.05; Figure 4.5C,G) and reduced episode duration by 33.5 ± 6.0% (N=19, P<0.001; data not shown).

Subsequent application of NA (4-6μM) to 11 animals and clonidine (80-110μM) to 4 animals, increased cycle periods by 24.0 ± 5% (N=11, P<0.001; Figure 4.5A,B) and 9.4 ± 4.1% (N=4, P<0.001; Figure 4.5D) respectively. However, addition of phenylephrine to 4 animals, did not affect cycle periods in the presence of strychnine (N=4, P>0.05; Figure 4.5E,F). NA, clonidine and phenylephrine were not able to affect the RC-delay in the presence of strychnine (N=19, P>0.05; Figure 4.5G; data for NA and clonidine not shown). Under strychnine, clonidine further shortened episode durations by 31.1 ± 7.1% (N=4, P<0.05), whereas neither NA (N=11) nor phenylephrine (N=4) could affect episode durations (P>0.05; data not shown). In 5 animals, to which strychnine, then NA had been applied, bicuculline (30-40μM) was added. Bicuculline counteracted the noradrenergic effects on cycle periods, reducing them by 11.8 ± 2.9% (N=5, P<0.05; Figure 4.5H), but did not affect the RC delay or episode duration (N=5, P>0.05; data not shown). Consistent with the data described in the sections above, these experiments indicate that NA, acting at α_1-receptors, influences glycinergic inhibition to modulate swimming frequency and longitudinal co-ordination. However, acting via α_2 receptors, NA regulates both glycinergic and GABAergic pathways to modulate swimming frequency.

4.7 Blocking $GABA_A$ receptors does not prevent the effects of NA and α-receptors on cycle periods and RC-delays, but can occlude effects on episode duration.

To investigate the interaction between NA, α-receptors and $GABA_A$ergic inhibitory transmission, the effects of NA and α-receptor activation were studied whilst GABAergic inhibition was blocked by bicuculline (Figure 4.6). Application of bicuculline (30-40μM) to 17 animals reduced cycle periods by 10.8 ± 3.5% (N=17, P<0.001; Figure 4.6A-D,F) and the RC-delay by 15 ± 3.2% (N=17, P<0.001; Figure 4.6E). Furthermore, episode duration under bicuculline decreased by 25.9 ± 15.1% (N=22, P<0.01; data not shown).

After subsequent addition of phenylephrine to 7 animals and NA to 6 animals, cycle periods increased by 18.6 ± 5.6% (N=7, P<0.001; Figure 4.6A,B) and 12.4 ± 17.8% (N=6, P<0.001; Figure 4.6C,D) respectively. However, neither NA nor phenylephrine had any effect on the RC-delay (Figure 4.5E) or episode duration (N=13, P>0.05; data not shown). The addition of clonidine to 4 animals increased cycle periods by 8.8 ± 1.5% (N=4, P<0.05; Figure 4.6F), but did not affect the RC-delay or episode duration (N=7, P>0.05; data not shown). This suggests that the modulation of swimming frequency by NA, acting via α_1- and α_2-receptor activation, does exclusively involve the $GABA_A$ergic pathways.

4.8 Discussion.

NA modulates locomotor networks in a wide range of vertebrates (e.g. cat, Chau *et al.*, 1998; Kiehn *et al.*, 1992; rat, Sqalli-Houssaini & Cazalets, 2000; Kiehn *et al.*, 1999). In the *Xenopus* tadpole NA has been shown to slow swimming by

enhancing release of glycine from the terminals of inhibitory interneurons McDearmid *et al.*, 1997; Figure 1.5). The aim of this chapter was to investigate whether the modulation of the spinal networks controlling locomotion by NA might utilise glycinergic and/or GABAergic inhibitory pathways via activation of specific adrenoreceptors. Hatchling *Xenopus* possess a well-characterised spinal locomotor system (Roberts & Clarke, 1982; Roberts, 2000) which is capable of generating self-sustaining fictive swimming without reliance on persistent drug application (Kahn & Roberts, 1982a). As shown in chapter three (see also Fischer *et al.*, 2001) the noradrenergic neuromodulation of swimming in tadpoles is predominantly mediated via both α_1- and α_2-adrenoreceptors, although β-receptors are also involved in the modulation of longitudinal delays (Fischer *et al.*, 2001). In this chapter, I have however focused only on the α-receptor subtypes, which between them influence all the principal parameters of swimming. A schematic diagram summarising my findings is shown in Figure 4.7.

In *Xenopus*, NA slows swimming and modulates the longitudinal co-ordination of the rhythm-generating network by decreasing the RC-delay in motor neuron activation. Although the mechanisms responsible for generating a RC-delay in firing are not fully understood, it is thought that the descending head-to-tail gradient in the strength of both excitation and inhibition may be involved (Tunstall & Sillar, 1993; Tunstall & Roberts, 1994). The existence of such a gradient in glycinergic inhibition is significant as, for example, it may affect the ability of neurons to fire as a result of post-inhibitory rebound (Roberts & Tunstall, 1990; Wall & Dale, 1994; Wolf & Roberts, 1995; Tunstall & Roberts, 1994; see also chapter 5). NA has previously been shown to exert at least part of its effects on cycle period, via an enhancement of glycine release from commissural interneurons (McDearmid *et*

al., 1997; Figure 1.5). By strengthening mid-cycle inhibition it is thought that neurons take longer to recover from this inhibition before starting the next cycle, and hence cycle periods increase. This effect on glycinergic inhibition is further supported here by the finding that using strychnine to pharmacologically block glycine receptors reduces the noradrenergic reduction of swimming frequency and RC-delays (Figure 4.1B,C). However, the glycinergic mechanisms underlying the NA-mediated modulation of the RC-delay are so far not fully described (but, see chapter 5).

The finding that strychnine did not completely reverse in magnitude the effects of NA on swimming frequency, suggests that the noradrenergic modulation of *Xenopus* swimming does not exclusively utilise glycinergic inhibitory pathways. For example, the $GABA_A$ receptor antagonist bicuculline applied alone significantly reduced the effects of NA on cycle periods (Figure 4.1D), as well as after the prior application of strychnine (Figure 4.5H). This suggests that both glycinergic and GABAergic pathways may be utilised by NA, and that both inhibitory pathways may be affected simultaneously. This matches previous findings in non-locomotor systems of vertebrates, where NA was reported to affect both glycinergic and/or GABAergic pathways (e.g. Baba *et al.*, 2000; Kawaguchi & Shindou, 1998).

As predicted from computer modelling experiments (e.g. Ekeberg & Grillner, 1999; Dale, 1995) and based on experimental data from work in this (McDearmid *et al.*, 1997) and other vertebrate systems (e.g. lamprey; Buchanan, 1982), swimming frequency is exquisitely sensitive to adjustments in the amplitude of the glycinergic mid-cycle inhibitory component. As discussed in chapter one, commissural interneurons in the spinal circuitry use glycine as a reciprocal inhibitory transmitter, which causes opening of chloride ion channels, membrane hyperpolarisation and

inhibition. NA is thought therefore, to reduce swimming frequency in *Xenopus* by enhancing release of glycine from the terminals of commissural interneurons and hence mid-cycle glycinergic inhibition. However, there is no obvious GABAergic component that is phase-locked to the swimming cycle, even though there are occasional sporadic GABAergic potentials throughout episodes of swimming. Since these IPSPs may be sufficiently long (~200ms; Reith & Sillar, 1997) to affect more than one cycle (~50-100ms), they may, over the course of an episode, summate to provide a background level of tonic inhibition (Reith & Sillar, 1999).

In chapter three I have shown that the noradrenergic modulation of the swimming activity is largely mediated via activation of α_1- and α_2-receptor (for other vertebrates see: e.g. Sqalli-Houssaini & Cazalets, 2000; Barbeau & Rossignol, 1991). NA affects both swimming frequency and the RC-delay in motor neuron activation through α_1-receptors, whereas α_2-receptors are involved only in the modulation of swimming frequency. In this chapter I have presented evidence that the selective pharmacological activation of either α_1- or α_2-adrenoreceptors specifically affects glycinergic and/or GABAergic pathways during rhythm generation. Firstly, blocking $GABA_A$ receptors with bicuculline did not counteract the effects of α_1-receptor activation on cycle periods or the RC-delay (Figure 4.3B-C), but did reverse in magnitude the α_2-receptor-mediated effects on swimming frequency (Figure 4.4C). Secondly, blocking glycine receptors decreased in magnitude the α_1- and α_2-receptor mediated effects on cycle periods and α_1-mediated effects on RC-delay (Figures 4.2A-C; 4.4A,B). Furthermore, blocking $GABA_A$ receptors did not occlude the effects of α_1-receptor activation (Figure 4.6A,B), whereas prior blocking of glycine receptors did prevent these effects (Fig. 4.5E-G). Since blocking glycine receptors reversed in magnitude (and not simply

opposed) the increase in cycle periods mediated by α_1- and α_2-receptors, and blocking GABA receptors reversed the α_2-mediated effects on cycle periods, this suggests that GABAergic and glycinergic pathways are indeed specifically utilised by NA following α_1- and α_2-adrenoreceptor activation. In addition, neither NA nor α_1- or α_2-adrenoreceptor agonists were able to modulate rhythmic activity in the presence of both GABA and glycine receptor antagonists simultaneously (Fischer *et al.*, 2001). This further indicates that the noradrenergic modulation of tadpole swimming appears to essentially utilise the GABA and glycinergic inhibitory pathways, however this does not exclude subtle complimentary effects on other synaptic or cellular properties.

These findings suggest that NA might exert its complex modulatory effects on these parameters of locomotor rhythm generation by simultaneously activating different adrenoreceptor subclasses that in turn are associated with particular inhibitory pathways. By targeting α_1-receptors, NA might control swimming frequency as well as RC-delays, by predominantly influencing glycinergic pathways, whereas α_2-receptors may be activated to preferentially influence swimming frequency through GABAergic pathways. It is not possible to completely exclude a role for GABAergic transmission in mediating the effects of NA on RC-delays, since blocking $GABA_A$ receptors reduces these delays (Reith & Sillar, 1999; Figure 4.6E).

In the previous chapter (see also Fischer *et al.*, 2001), the only developmental difference in the effects of noradrenergic modulation was in the duration of swimming episodes after activation of α_1-receptors. Blocking neither glycine nor $GABA_A$ receptors was able to reduce the noradrenergic decrease in swim episode duration. However, pre-application of bicuculline occluded the effects of NA, α_1- and α_2-receptor activation on episode duration (Figure 4.1E). At both developmental

stages, the termination of swimming episodes can be accomplished by the activation mhr GABAergic neurons (Boothby & Roberts, 1992a,b). In embryos, these neurons are normally activated via primary afferents innervating the rostral cement gland (Boothby & Roberts, 1992a,b; see also chapters one and three). In larvae the termination of swimming often occurs spontaneously, coincident with a barrage of GABAergic IPSPs, presumably resulting from an intrinsic activation of the mhr neurons (Reith & Sillar, 1997,1999). It is reasonable to propose that at both stages the control of swimming duration by both α_1- and α_2-adrenoreceptors utilises GABAergic inhibition, yet interestingly α_1-receptors (based on this study) appear not to use GABAergic inhibition to control any other parameter of swimming.

The functional implications of a possible interaction between GABAergic and glycinergic inhibitory transmission during the generation and, in particular, the modulation of vertebrate spinal locomotor patterns is not fully understood. In other motor systems, glycine and GABA can be co-localised in the terminals of pre-motor inhibitory interneurons (e.g. rat, Lahjouji *et al.*, 1996; cat, Taal & Holstege, 1994; Ornung *et al.*, 1994, 1996) where there is strong evidence that they can be co-released (Jonas *et al.*, 1998; Chery & De Koninck, 1999). However, there is some evidence against co-release of these two inhibitory transmitters in *Xenopus*. For example, TTX-resistant GABAergic IPSPs, but not glycinergic ones, are selectively modulated by classes of neurosteroids and are completely blocked by bicuculline, while the glycinergic ones were unaffected. Thus, there appear to be two non-overlapping populations of IPSPs (Reith & Sillar, 1997).

Whilst the role of adrenoreceptor activation in generating and modulating locomotion in vertebrate motor systems is widely established (Forssberg & Grillner, 1973; Kiehn *et al.*, 1992, 1999; Barbeau & Rossignol, 1991; Sqalli-Houssaini &

Cazalets, 2000), it is not possible to say for certain whether activation of these receptors might be functionally related to the modulation of inhibitory pathways. The present study suggests that a further degree of intrinsic flexibility within a locomotor network might be achieved by a selective interaction of adrenoreceptor subclasses with particular inhibitory pathways.

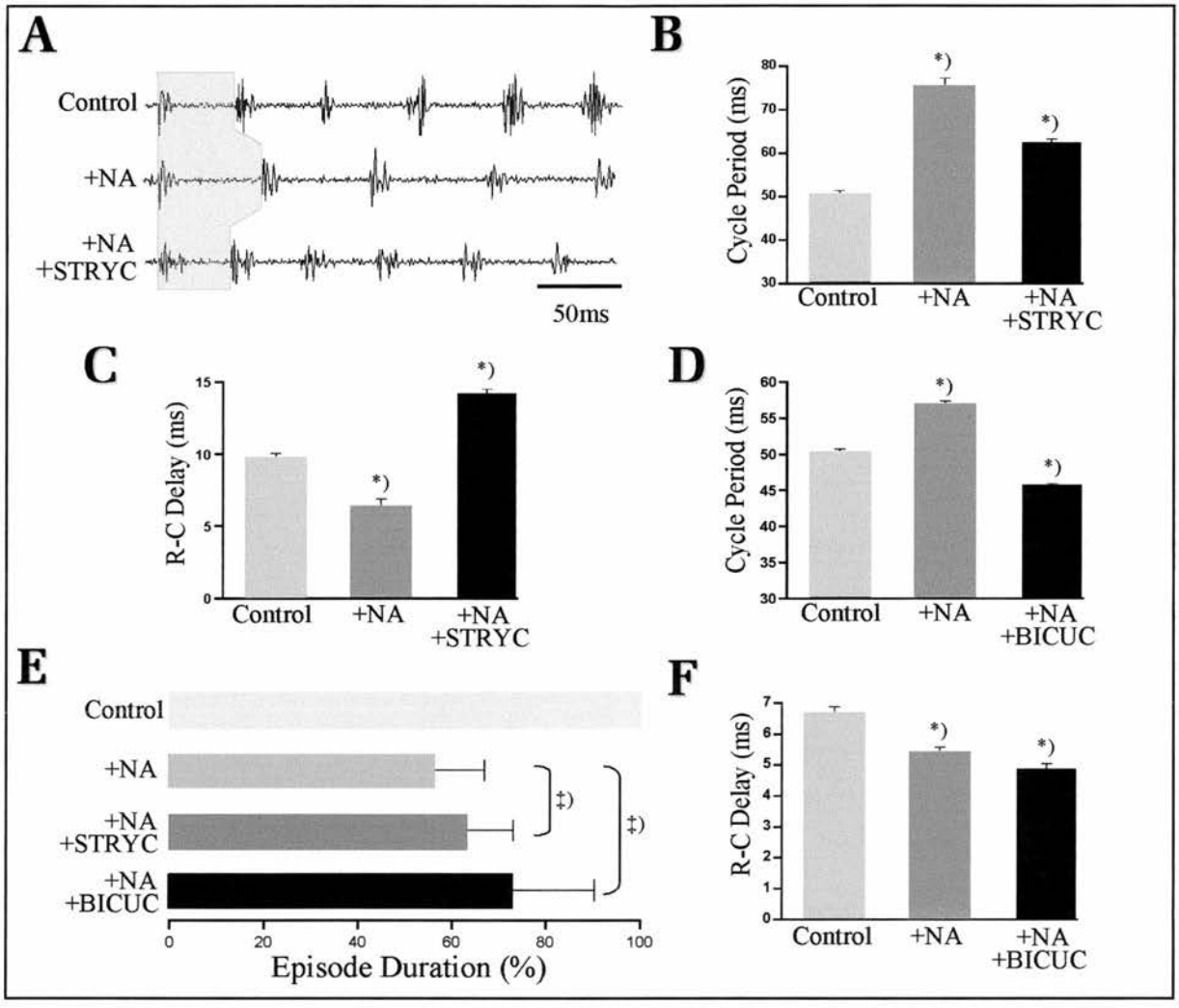

Figure 4.1: *Effects of blocking glycine receptors with strychnine or $GABA_A$ receptors with bicuculline on the noradrenergic modulation of swimming.* Data shown for two representative animals in (**A**)-(**C**) and (**D**,**F**). For each set of conditions, data were pooled from 3 swim episodes. Application of strychnine (STRYC) significantly counteracted the effects of NA on cycle period (**A,** grey shaded area, **B**) and the RC-delay (**C**). Bicuculline (BICUC) also significantly counteracted the NA effects on cycle period (**D**), but further decreased RC-delay (**F**). (**E**) NA significantly reduced episode durations. The effects of NA were not significantly reduced by strychnine (N=8) or bicuculline (N=7). *), indicates values significantly different from the control (P<0.01, n=3). ‡) Not significantly different (P>0.05).

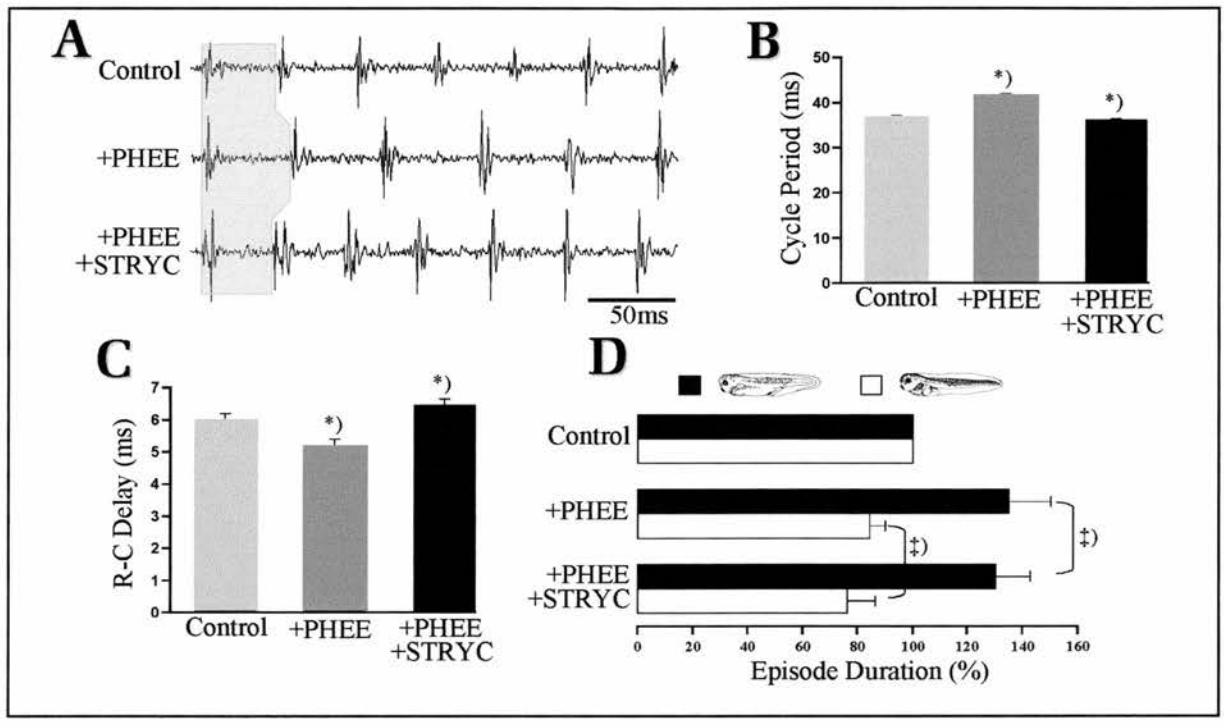

Figure 4.2: *Effects of blocking glycine receptors after the activation of α_1-adrenoreceptors by phenylephrine.* Data is shown for one representative animal in (**A-C**). Blocking glycine receptors with strychnine significantly counteracted the effects of phenylephrine on cycle period (**A**, **B**) and the RC-delay (**C**). (**D**) Phenylephrine significantly increased embryonic episode durations and shortened larval episodes. Strychnine did not counteract the effects of phenylephrine in embryos or larvae. P>0.05). *) P<0.01 (n=3); ‡) Not significantly different (P>0.05, n=3).

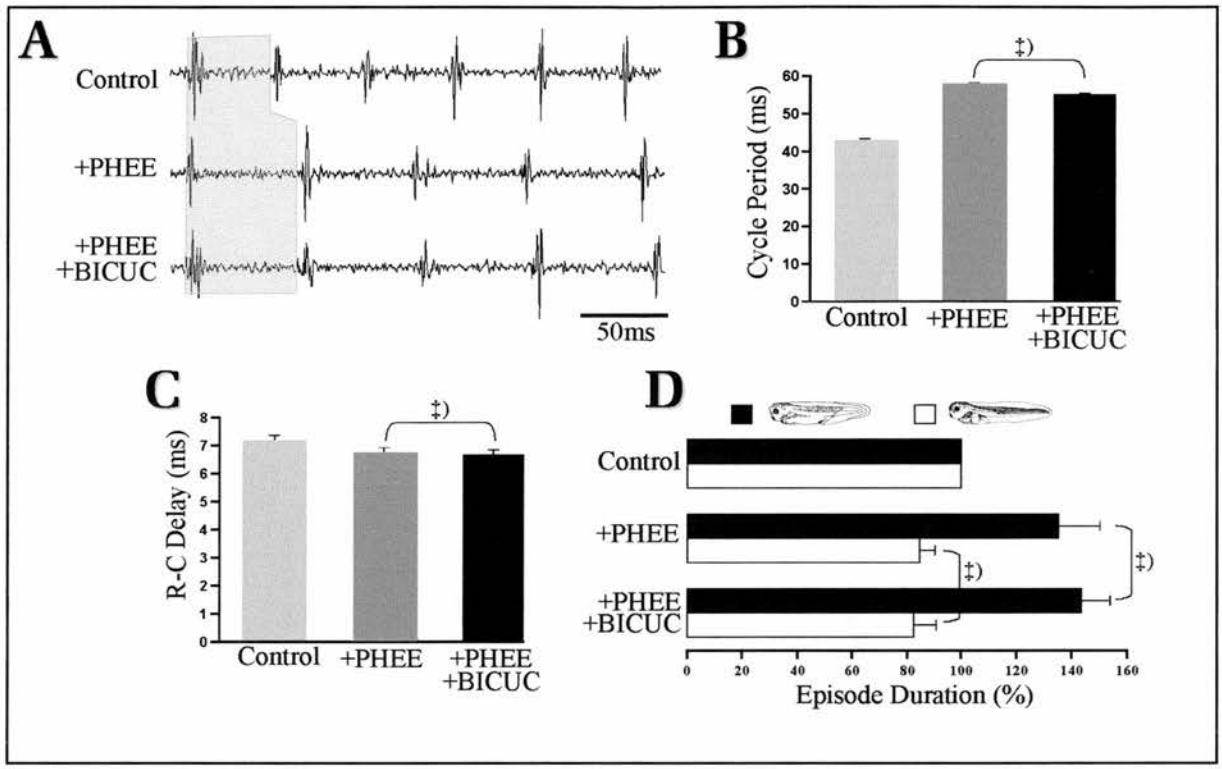

Figure 4.3: *Effects of blocking $GABA_A$ receptors after the activation of α_1-adrenoreceptors by phenylephrine.* Data are shown for one representative animal in (**A-C**). Blocking $GABA_A$ receptors with bicuculline was not able to counteract the increase in cycle periods (**A,B**), the decrease in RC-delays (**C**) or effects on episode duration following α_1-adrenoreceptor activation by phenylephrine. (**D**) ‡) Not significantly different ($P>0.05$, $n=3$).

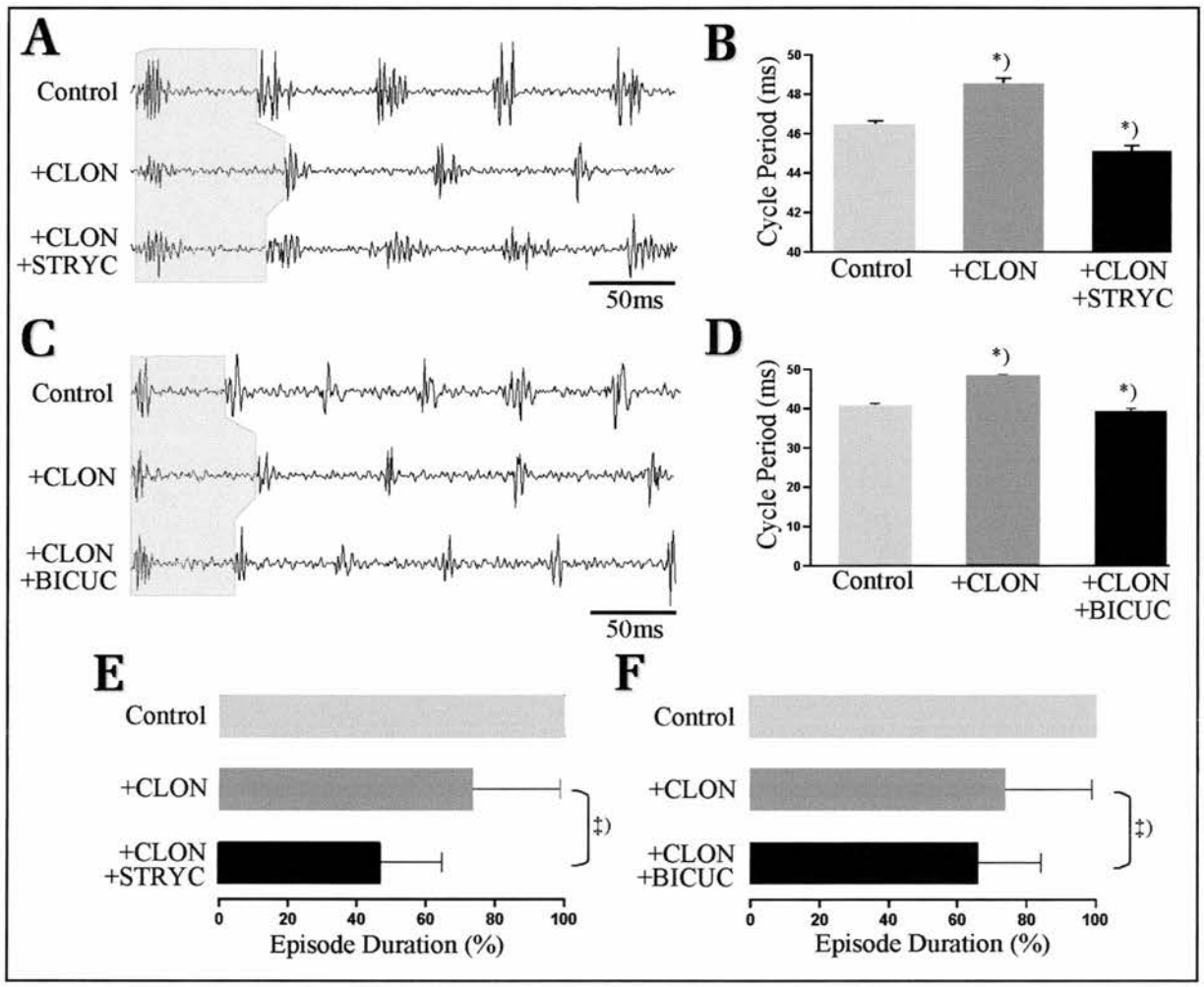

Figure 4.4: *Effects of blocking either glycine or $GABA_A$ receptors after the activation of α_2-receptors by clonidine.* Data are shown for two representative animals in (**A, B**) and (**C**, **D**). Blocking glycine receptors with strychnine (**A**, **B**) or $GABA_A$ receptors with bicuculline (**C**, **D**) significantly counteracted the effects of clonidine on cycle period ($P<0.05$). Furthermore, neither strychnine (**E**) nor bicuculline (**F**) counteracted the α_2-mediated decrease in episode duration. *) $P<0.01$ ($n=3$); ‡) Not different ($P>0.05$).

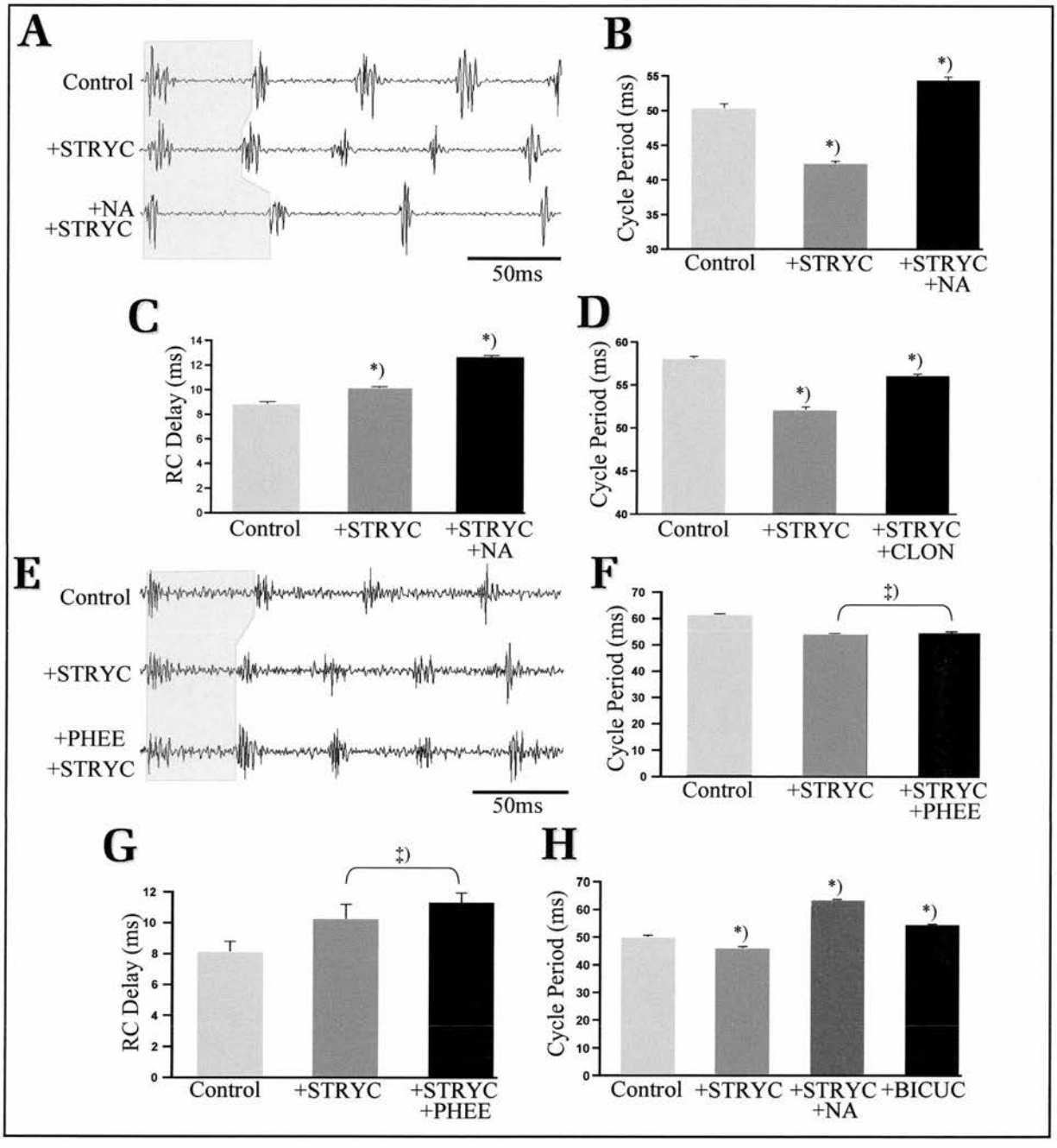

Figure 4.5: *Effects of NA, α_1-, and α_2-adrenoreceptor activation after blocking glycine receptors*. Data are from 4 representative animals (**A-C**, **D**, **E-G** and **H**, respectively). Blocking glycine receptors with strychnine did not occlude the effects of NA (**A**, **B**) or the effects of α_2-receptor activation by clonidine (**D**) on cycle period. However, NA could not reduce an increase in the RC-delay under strychnine (**C**). The application of the α_1-receptor agonist phenylephrine, had no significant effect on cycle periods (**E, F**) and RC-delay when glycine receptors were blocked by strychnine (**F**). (**H**) After the prior application of strychnine and NA, bicuculline counteracted the noradrenergic increase in cycle periods *) $P<0.01$ ($n=3$); ‡) Not different ($P>0.05$, $n=3$).

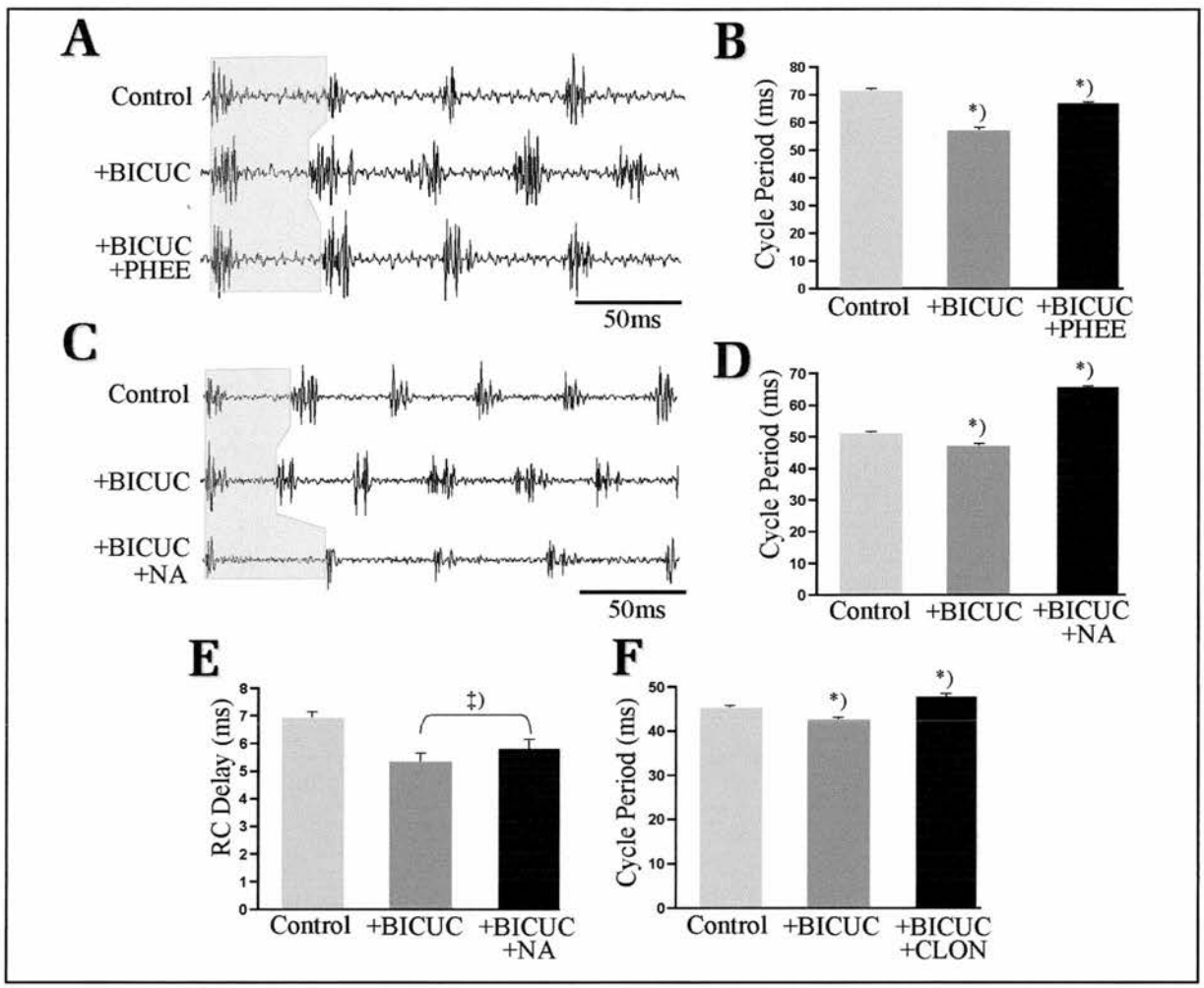

Figure 4.6: *Effects of NA, α_1- and α_2-adrenoreceptor activation after blocking $GABA_A$ receptors.* Data are from 3 representative animals (**A-B, C-E**, and **F**, respectively). The reduction in cycle periods by bicuculline was significantly reduced by the α_1-receptor agonist phenylephrine (**A**, **B**), NA, (**C**,**D**) and the α_2-agonist clonidine (**F**). NA did not significantly affect RC-delays in the presence of bicuculline (**E**). Data per animal were pooled from 3 episodes in each experimental condition. *) $P<0.01$ ($n=3$); ‡) Not different ($P>0.05$, $n=3$).

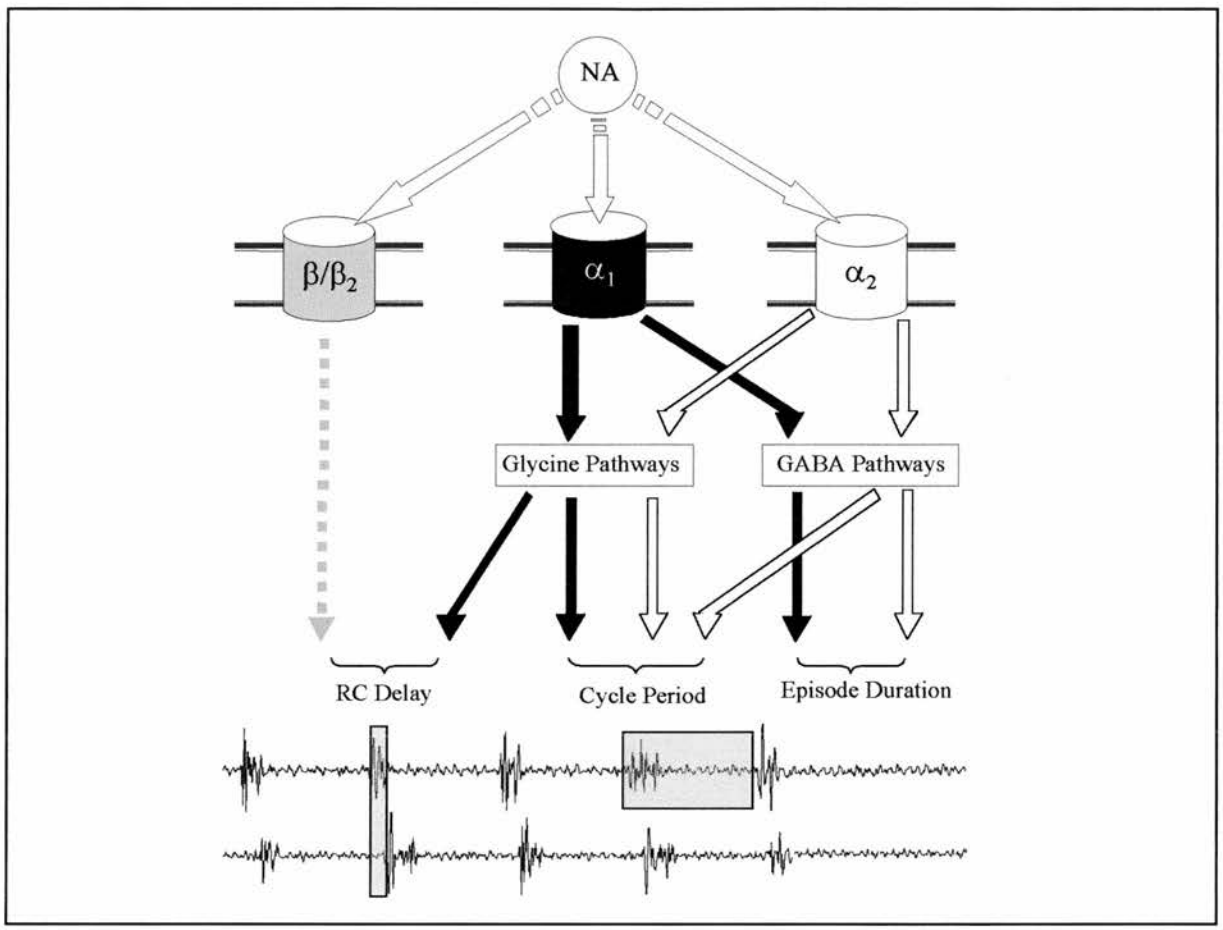

Figure 4.7: *Summary of the effects of adrenoreceptor activation and their putative interactions with inhibitory pathways.* Cycle periods are increased by selective pharmacological activation of α_1- and/or α_2-adrenoreceptors, whilst α_1-receptor activation additionally reduces the longitudinal delay. The effects of α_1-receptors on cycle period and RC-delay utilise glycinergic pathways, whilst α_2-receptors utilise both glycinergic and $GABA_A$ pathways to modulate swimming frequency. The control of episode duration by both α_1- and α_2-receptors also apparently involves $GABA_A$ergic inhibition. A recent study (Fischer *et al.*, 2001) shows that β-receptors modulate RC-delays, but the mechanism underlying this has yet to be addressed (as indicated by the dashed line).

Chapter Five

Synaptic and cellular mechanisms underlying the noradrenergic modulation of tadpole swimming

5.1 Summary.

The effects of NA on tadpole swimming are now well defined- slower swimming and a reduction in the RC-delay and episode duration. The receptor types via which these effects are mediated have also been identified. However, the cellular and synaptic mechanisms underlying these effects remain unclear. I have addressed this deficit in our understanding by combining a pharmacological approach with sharp microelectrode recordings from spinal motor neurons in the spinal cord of *Xenopus*.

When swimming in embryos was prematurely terminated by stimulation of the rostral cement gland, NA or activation of α_1- or α_2-adrenoreceptors could enhance the resulting barrage of PSP's, which were subsequently blocked by bicuculline, suggesting that they were GABAergic in origin. During the quiescent periods between episodes of swimming, spontaneous glycinergic and GABAergic IPSPs were present. NA increased the frequency, but not the amplitude or duration of spontaneous GABAergic and glycinergic potentials. The frequency of the bicuculline-sensitive GABAergic IPSPs was also increased in the presence of TTX, suggesting that NA can pre-synaptically enhance the release of GABA. The noradrenergic uptake inhibitor protriptyline increased the frequency of both glycinergic and GABAergic potentials, thus replicating the effects of exogenously-applied NA. Activation of α_1-adrenoreceptors pre-synaptically increased the frequency of spontaneous glycinergic, but not GABAergic IPSPs, whilst no consistent effects of α_2-activation on spontaneous release were detected. Activation of both α_1- and α_2-receptors reduced the frequency of swimming, although the cellular and synaptic effects accompanying this were paradoxical. α_1-receptors appeared to increased the amplitude of the mid-cycle IPSP, whilst α_2-receptors reduced the amplitude and increased the duration of these glycinergic IPSPs.

Finally, I investigated whether NA and α_1-receptor activation might affect post-inhibitory rebound firing, which has been implicated in the control of RC-delays. The synaptic inputs during swimming were simulated by injecting a depolarising current (tonic excitation) superimposed upon which were brief hyperpolarising currents (mid-cycle IPSPs). When these conditions were held constant throughout the experiment, NA and α_1-receptor activation enhanced the probability of rebound firing, indicating a direct effect on motor neuron membrane properties, in addition to any effects of enhanced synaptic inhibition.

These results suggest that the noradrenergic modulation of swimming is controlled predominantly via a pre-synaptic facilitation of glycine and GABA release. However, direct post-synaptic effects, such as the enhancement of rebound firing, may act in tandem with these pre-synaptic changes in synaptic strength to modulate swimming.

5.2 Introduction.

The inherent capability of neural circuits to alter their output in response to perpetually changing environmental conditions is crucial for an organisms survival. The generation of locomotory behaviours, such as walking, running or swimming relies upon the co-ordinated cyclical contraction and relaxation of antagonistic muscle pairs, whose rhythmic activation is co-ordinated by neuronal central pattern generators located within the CNS. The output of these pattern generators can be precisely and rapidly adjusted by changes in both the cellular properties of individual neurons and the synaptic interconnections between them. For example, in aquatic animals, swimming must be inherently flexible, both in its speed and intensity and thus the propagation of muscle contractions along the body must be co-ordinated in such a way that the body shape optimum for propulsion is be maintained. Such flexibility and control, which discounts the prevailing view that neural networks are 'hard-wired', can be achieved by the actions of neuromodulators.

Neuromodulation of motor behaviours (for reviews see: Kiehn & Katz, 1999; Pearson, 2001,1993), has now been investigated in a wide range of both invertebrate and vertebrate organisms, from feeding in molluscs (e.g. Morgan *et al.*, 2000), and digestion in crustaceans (e.g. Selverston *et al.*, 1998), to the control of respiration in bullfrogs (Hedrick *et al.*, 1998) and locomotion in, for example, crayfish (e.g. Pearlstein *et al.*, 1998) and lamprey (Buchanan, 2001).

In the spinal locomotor networks of vertebrates, the aminergic neuromodulators NA and 5-HT are able to initiate, sustain and modulate rhythmic motor activity. For example, in the neonatal rat, 5-HT can initiate alternating activity (Cazalets *et al.*, 1992) appropriate to induce hindlimb movements (Kiehn & Kjaerulff, 1992). However, in the cat, where 5-HT is unable to initiate motor

activity, it can modulate ongoing locomotion (Barbeau & Rossignol, 1991). In the lamprey, 5-HT reduces the frequency of motor bursts and increases the inter-segmental phase-lag during NMDA-induced activity (Harris-Warrick & Cohen, 1985; for a review see Buchanan, 2001), whilst in the *Xenopus* tadpole, over a 24-hour period during development, it confers the ability to generate more intense swimming, coincident with the developmental in-growth of serotonergic projections into the spinal cord (Sillar *et al.*, 1992b,c; for a review see Sillar *et al.*, 1998).

NA and the associated activation of adrenoreceptors is also capable of initiating motor activity. For example, activation of α_2-receptors using clonidine can initiate locomotion in acutely spinalised cats (Barbeau & Rossignol, 1991; Chau *et al.*, 1998) or, can modulate ongoing activity in the later stages after spinalisation (Barbeau *et al.*, 1991; for a review see Rossignol *et al.*, 1998). In the rat, whilst NA can initiate a slow, irregular rhythm, it more reliably modulates ongoing NMDA and/or 5-HT-induced activity by slowing the rhythm and increasing the duration of motor bursts (Kiehn *et al.*, 1999). These modulatory effects of NA in the rat spinal cord appear to be mediated via both α- and β- adrenoreceptors (Sqalli-Houssaini & Cazalets, 2000). In the *Xenopus* tadpole (as discussed in chapters three and four) NA can simultaneously reduce the frequency of swimming and the longitudinal delay in motor neuron firing (see also McDearmid *et al.*, 1997).

Despite the identification of a range of modulators in various locomotor systems, we are still surprisingly ignorant of the cellular and synaptic changes that underlie their effects. At the cellular level, 5-HT can confer a range of voltage-dependent, non-linear properties upon motor neurons. For example, in both the cat and the turtle, membrane bistability, which supports the presence of long-lasting Ca^{2+} dependent plateau potentials, relies upon 5-HT (Hounsgaard & Kiehn, 1989,

Hounsgaard *et al.*, 1988). It has also been reported than L-Dopa (a pre-cursor of dopamine and NA) can induce similar oscillations in the cat (Conway *et al.*, 1988; Kiehn, 1991). In *Rana temporaria* tadpoles, co-activation of 5-HT and NMDA receptors is required to induce membrane potential oscillations (even in the presence of TTX, thus discounting any synaptic influences; Sillar & Simmers, 1994b). Similarly, in the neonatal rat and the *Xenopus* tadpole, the induction of non-linear, voltage-dependent membrane potential oscillations relies upon both 5-HT and NMDA (Scrymgeour-Wedderburn *et al.*, 1997; Kiehn *et al.*, 1996; MacLean *et al.*, 1997, 1998; MacLean & Schmidt, 2001). Whilst an intrinsic role for such oscillatory phenomena still requires investigation, in most cases it seems likely that they contribute to increased excitability within the CPG, by accelerating neuron firing and producing faster, more intense activity. In the lamprey, the principal cellular effect of 5-HT appears to be the block of K_{Ca} channels responsible for both the slow after-hyperpolarisation in motor and lateral interneurons, and for the plateau phase of NMDA oscillations (Van Dongen *et al.*, 1986, Wallen *et al.*, 1989).

At the synaptic level, the fast inhibitory amino acid transmitters GABA and glycine are important targets for neuromodulators. The generation of motor activity in vertebrate locomotor networks is usually organised as antagonistic half-centres, coupled by reciprocal inhibition. This inhibitory mid-cycle component is principally glycinergic (but see Jonas *et al.*, 1994) and parameters of the motor pattern such as locomotor frequency, longitudinal co-ordination and burst duration may be controlled via an enhancement or suppression of glycinergic inhibition (Soffe, 1987; Dale, 1985; Cohen & Harris-Warrick, 1984; Buchanan, 1982; McDearmid *et al.*, 1997). Computer simulations support this experimental evidence. For example, in a simulation of the neural networks controlling locomotion in the lamprey, reciprocal

inhibition (from crossed contralateral inhibitory neurons) determines the alternating pattern of activity and also contributes to the control of burst frequency, such that increasing reciprocal inhibition slowed motor output (Hellgren *et al.*, 1992). Similarly, in a model of the *Xenopus* CPG, reciprocal glycinergic inhibition was shown to be important in determining swimming frequency, no matter what the level of excitation. This was tested experimentally in *Xenopus* embryos using strychnine, which also reduced swimming frequency. Furthermore, removing all inhibition from the network prevented self-sustaining activity (Dale, 1995).

The role of GABA in motor pattern generation has not been so widely investigated. Unlike glycine, it appears not to be involved in the timing of motor output, but is involved in the modulation of sensory afferent transmission during locomotion (Dubuc *et al.*, 1988; Stuart & Redman, 1991). However, GABA has been implicated in a number of motor systems. In the neonatal rat, GABA can dramatically slow NMDA-induced rhythmic activity, and with increasing doses can prevent rhythmic activity altogether (Cazalets *et al.*, 1994). In the lamprey, GABA has been shown to affect the frequency of motor bursts and the inter-segmental phase lag (Tegner *et al.*, 1993, Tegner & Grillner, 2000) as well as intra-limb co-ordination (rat; Cowley & Schmidt, 1995; Kremer & Lev-Tov, 1997). During swimming in *Xenopus* tadpoles, activation of $GABA_A$ receptors can modulate both swimming frequency and episode duration. Even though GABA seems not to contribute directly to mid-cycle reciprocal inhibition, it seems likely that it can reduce swimming frequency over several cycles through an increase in the tonic background levels of inhibition (Reith & Sillar, 1999). When the rostral cement gland is activated in embryos (as would occur when the tadpole encounters an obstacle) mhr GABAergic neurons fire and terminate swimming by activating $GABA_A$ receptors on spinal

neurons (Boothby & Roberts, 1992a,b). By larval stage 42, episodes of swimming often terminate with a barrage of apparently spontaneous GABAergic IPSPs, which closely resemble those evoked by cement gland stimulation at stage 37/38. This suggests that during development the mhr neurons may have been retained and recruited into an intrinsic stopping pathway (Reith & Sillar, 1999).

Despite both GABA and glycinergic pathways being integral transmitter systems within the vertebrate CNS, most of the reported aminergic modulation of these inhibitory pathways has been in non-locomotor systems (e.g. adrenergic modulation of respiration rhythm: Arata *et al.*, 1998; noradrenergic increase in GABAergic IPSC's in cortical cells of the rat: Kawaguchi & Shindou, 1998). However, there are some reported cases of aminergic modulation of synaptic properties in locomotor systems. In motor neurons of the lamprey spinal cord, where no noradrenergic innervation has been reported, dopamine reduced glycinergic IPSPs from CC interneurons (Kemnitz, 1997), although the overall effect of dopamine on locomotor output is, as yet, not certain (but see: Harris-Warrick & Cohen, 1985; McPherson & Kemnitz, 1994; Schotland *et al.*, 1995). The latter study also importantly showed that dopamine co-localises with 5-HT in interneurons in the ventro-medial aspect of the spinal cord. In the rat, although modulatory effects of NA have been reported (Kiehn *et al.*, 1999; Sqalli-Houssaini & Cazalets, 2000) the underlying mechanisms have not yet been determined.

In the *Xenopus* tadpole, the noradrenergic reduction in swimming frequency can be partly accounted for by an increase in the probability of glycine release from the terminals of pre-synaptic neurons. This enhances membrane hyperpolarisation and synaptic inhibition, and is thought to reduce swimming frequency by delaying recovery from mid-cycle inhibition and hence the onset of the next cycle

(McDearmid *et al.*, 1997). Furthermore, in the previous chapters, I have demonstrated that the noradrenergic modulation of *Xenopus* swimming may be mediated via α-adrenoreceptors which appear to utilise both glycinergic and GABAergic fast inhibitory pathways to modulate the frequency of motor bursts, their longitudinal propagation along the body and the duration of motor activity. In this chapter, using sharp microelectrode recordings from presumed motor neurons in the ventral spinal cord, I have now investigated further both the synaptic and cellular changes produced by NA and the associated activation of α-adrenoreceptors.

5.3 NA potentiates GABAergic inhibition following activation of the cement gland stopping pathway.

As discussed above, in embryos, when the rostral cement gland is activated, mhr GABAergic neurons fire and terminate swimming by activating $GABA_A$ receptors on spinal neurons (Boothby & Roberts, 1992a,b; see section for 1.7 for more detail). By larval stage 42, episodes of swimming often terminate with a barrage of apparently spontaneous GABAergic IPSPs which closely resemble those evoked by cement gland stimulation at stage 37/38. This suggests that during development the mhr neurons may have been retained and recruited into an intrinsic stopping pathway (Reith & Sillar, 1999). As I have shown in chapters three and four, NA consistently shortens swimming episodes and so my first approach was to examine whether this might be achieved by facilitating these known GABAergic pathways.

In 7 embryos, the effects of NA (4-8μM) were investigated following electrical stimulation of the cement gland (see Figure 2.1 for method). In 4/7 animals (no change in 3 animals), after cement gland activation, the duration of the inhibitory

barrage coincident with the termination of swimming was significantly increased, as measured from the stimulus artifact until the membrane returned to its resting level (data analysed per animal and for 3 episodes under each set of conditions; $P<0.05$; Figure 5.1A-B, 5.2A-B; note also in 5.2B that the IPSPs (asterisked) increase in amplitude under NA). In addition, there was a clear increase in the number of individual PSPs that continue after the membrane potential has returned to rest (e.g. asterisked in 5.1B; see also 5.2B). This effect was counteracted in 2 animals by returning to control conditions (Figure 5.1C). In 1 experiment, the $GABA_A$ receptor antagonist bicuculline was subsequently applied in the presence of NA and it counteracted the effects of NA. This supports the conclusion that the IPSPs enhanced by NA are GABAergic in origin (Figure 5.2C). Pooled data from 3 episodes under each condition reveal that the duration of the GABAergic barrage increased from an average of 934 ± 203ms in control conditions to 1733 ± 319ms after addition of NA. In a further 4 animals, NA was applied in the presence of strychnine to exclude the possibility that NA was potentiating glycinergic inhibition. In 3 of these animals, the GABAergic barrage following cement gland stimulation increased significantly after addition of NA, further suggesting that NA enhances GABAergic inhibition in response to afferent input via the cement gland (data analysed per animal and for 3 episodes under each set of condition; $N=3$, $P<0.05$; Figure 5.3A-C). There was no significant effect in the remaining animal. In the presence of NA, no consistent effects were observed however, following the natural termination of episodes in either embryos or larvae (data not shown). It should be noted that effects of each drug were considered on the basis of the time taken for the membrane potential to return to rest, rather than by counting individual PSPs as they often summated (e.g. Figure 5.3), making an accurate assessment of their frequency difficult. Even so,

these results suggest that NA can facilitate release of GABA from a known brainstem pathway.

5.4: Both α_1-and α_2-adrenoreceptor activation potentiate GABAergic inhibition following stimulation of the cement gland.

The finding that NA enhances the GABAergic barrage associated with the cement gland stopping response raises the possibility that activation of either α_1- and/or α_2-adrenoreceptors, which, as shown in chapters three and four mediate the effects of NA on episode duration, might be also produce this effect. The same experimental protocol was therefore used to examine the effects of phenylephrine and clonidine following cement gland stimulation (although, this has been investigated only once for each receptor type). As shown in Figure 5.4A-B, following activation of α_1-adrenoreceptors with 150μM phenylephrine, the GABAergic barrage following activation of the cement gland was enhanced and the time taken for the membrane potential to return to rest increased by 170 ± 35.5% (N=1, $P<0.05$). Similarly, when α_2-adrenoreceptors were activated using clonidine (120μM) the duration of the GABAergic barrage increased by an average of 54.2 ± 10.3 % (N=1, $P<0.001$; Figure 5.4C-D; data averaged from 3 episodes under each condition). In 7 additional embryos to which phenylephrine (N=2) and clonidine were applied (N=5), there were no consistent effects when episodes were allowed to terminate of their own accord.

Phenylephrine was then applied to 4 larvae, in 2 of which the duration of the GABAergic barrage, which is often associated with the termination of swimming at this stage of development (Reith & Sillar, 1999), increased by an average of 434 ±

72.5% (data analysed per animal and for 3 episodes under each set of conditions; N=2, P<0.05; Figure 5.5A-B). Subsequent addition of phentolamine to 1 of these animals counteracted the effects of phenylephrine and reduced the barrage to 200 ± 35.8% of the control (N=1, P<0.05; Figure 5.5C). Finally, in one larva in which α_2-receptors were activated with clonidine (150μM), no effect was observed as episodes terminated (data not shown).

Although the effects of α-adrenoreceptor activation have so far been investigated in only a few animals, the fact that both phenylephrine and clonidine could mimic the effects of NA by enhancing the GABAergic barrage following cement gland stimulation suggests that they are the likely receptor types through which NA mediates this effect. Similarly, the effect of α_1-adrenoreceptor activation on the termination of larval swimming episodes indicates that at the later stage of development, the function of this receptor class persists. Clearly through, further experiments are required to confirm these initial findings.

5.5 NA pre-synaptically increases the probability of GABA release during the quiescent periods between swimming episodes.

Having shown that NA can enhance the efficacy of a known GABAergic synaptic pathway, my next approach was to investigate whether NA might additionally facilitate GABA release under other conditions. When recordings from motor neurons are made using KCl-filled microelectrodes, frequent, depolarising IPSPs are observed during quiescent periods between swimming episodes. These represent the quantal release of both glycine and GABA from the terminals of inhibitory interneurons (Reith & Sillar, 1998). Glycinergic and GABAergic IPSPs can be distinguished either pharmacologically (using strychnine and bicuculline

respectively) or can be identified by their distinct time course- typically 20-80ms for glycine and 90-200 for GABA; Reith & Sillar, 1998; Figure 5.6E). NA has previously been shown to pre-synaptically increase the probability of glycine release, presumably from the terminals of commissural glycinergic interneurons (McDearmid *et al.*, 1997). In chapter four I showed that NA appears to additionally utilise GABAergic pathways during the modulation of swimming, therefore the release of spontaneous GABAergic potentials might be similarly affected.

The effects of NA on spontaneous GABA release were tested in 13 animals. In 5 animals there was no significant effect on GABAergic spontaneous IPSPs (sIPSPs) following addition of NA (N=5, P>0.05; data not shown), however in the remaining 8 animals, the frequency of presumed GABAergic sIPSPs was significantly increased after addition of NA (6-10μM). In the animal shown in Figure 5.6 (an embryo), the average number of GABA IPSPs per 30 second interval, fell by 13.7 ± 17.9% following the addition of 1μM strychnine to block glycinergic sIPSPs (Figure 5.6B), but increased significantly by 92.8 ± 25.0% after addition of 9μM NA (P<0.05; Figure 5.6C; data pooled from three, 30-second samples under each set of conditions; average number of IPSPs per 30-second interval under strychnine 16 ± 3.1). In 4 of these animals to which bicuculline (40-60μM) was subsequently applied, these GABAergic IPSPs were completely abolished (Figure 5.6D). Although this finding suggests that NA does increase the rate of GABAergic sIPSPs, these results were next confirmed in the presence of tetrodotoxin (TTX) to synaptically isolate the cell and confirm that the effects were not due to spike-induced transmitter release. In 9 animals, strychnine (1-2μM) and TTX (0.5-1μM) were applied. As shown in the example in Figure 5.7, this led to a dramatic reduction in both the frequency and amplitude of spontaneous release and only GABAergic

IPSPs (identified on the basis of their time course; Reith & Sillar, 1998) remained. After application of NA (6-10μM), in 6/9 animals tested, there was a significant increase in the frequency of spontaneous GABAergic IPSPs (Figure 5.7C). In 4 of these animals to which bicuculline was subsequently applied these IPSPs were completely abolished (Figure 5.7D). Whilst the frequency of GABAergic IPSPs increased under NA, there was no apparent effect on either their time course (Figure 5.7E) or their amplitude (Figure 5.7G). These experiments suggest that in addition to increasing the frequency of glycinergic sIPSPs, NA is also able to pre-synaptically enhance the release of GABAergic sIPSPs from pre-synaptic terminals.

As a preliminary attempt to examine the effects of endogenous NA on both glycinergic and GABAergic inhibitory pathways, the noradrenergic uptake inhibitor protriptyline was applied in 3 animals. In 2 of these animals, protryptiline (60-90μM) significantly increased the frequency of both glycinergic and GABAergic potentials (Figure 5.8A,B), without affecting the time course of these two populations of IPSPs (Figure 5.8Dii). In the animal illustrated in Figure 5.8, the average number of glycinergic potentials per 90-second interval increased from 53 ± 5.4 in control to 82 ± 6.5 after application of 90μM protriptyline, whilst the mean number of GABAergic potentials increased from 20 ± 3.7 to 47 ± 9.5 ($P<0.001$; data analysed for three 90-second samples under each set of conditions; Figure 5.8D). Furthermore, in this 1 animal, when phentolamine (50μM) was applied, the frequency of release of both glycinergic and GABAergic inhibition was subsequently reduced ($P<0.001$; Figure 5.8C, D). It should also be noted that the rate of glycinergic sIPSPs under phentolamine is less than in control, indicating that there might be a persistent noradrenergic tone in synaptic transmission (Figure 5.8Di). The ability of protriptyline and the associated potentiation of endogenous NA levels to

replicate the effects of exogenously-applied drugs provides good evidence that the utilisation of GABAergic and glycinergic inhibitory pathways is intrinsically involved in the noradrenergic modulation of locomotion.

5.6 α_1-adrenoreceptor activation increases the probability of glycine release during the quiescent periods between swimming episodes.

My next approach was to examine the effects of α_1-adrenoreceptor activation on the spontaneous release of GABA and glycine. As demonstrated in chapters three and four, the effects of NA appear to be mediated largely via α_1- and α_2-adrenoreceptor activation. The effects of α_1-receptor activation could be occluded completely by strychnine (Figure 4.5), but not by bicuculline, suggesting that their activation largely utilises glycinergic, but not GABAergic pathways. It is therefore possible that α_1-receptors might mimic at least some of the effects of NA on glycinergic pathways.

In 3 animals, the effects of α_1-receptor activation on glycinergic inhibition were investigated by the pre-application of bicuculline (40-60μM) to block GABAergic sIPSPs, whilst phenylephrine (100-200μM) was subsequently applied to activate α_1-receptors and investigate any effect on spontaneous glycinergic transmission. As shown in Figure 5.9, in all 3 animals, the frequency of spontaneous glycinergic potentials significantly increased following α_1-adrenoreceptor activation ($P<0.05$; Figure 5.9A-D), although there was no significant effect on their duration ($P>0.05$; Figure 5.9E). In a further 2 animals the effects of α_1-adrenoreceptor activation were investigated in the presence of TTX to determine whether this increase in glycinergic inhibition might be as a result of a pre-synaptic enhancement

of the probability of glycine release. As shown in Figure 5.10, addition of 1μM TTX reduced the frequency of spontaneous vesicular release, however glycinergic and GABAergic IPSPs could be easily determined on the basis of their time course. Subsequent addition of phenylephrine (100μM) increased the frequency of glycinergic ($P<0.05$), but not GABAergic IPSPs ($P>0.05$; Figure 5.10C,E). In 1 of these animals, phentolamine was subsequently applied, which counteracted the effects of phenylephrine on spontaneous glycinergic IPSPs ($P<0.05$; Figure 5.10D,Ei). The duration of glycine sIPSPs was unaffected by both phenylephrine and phentolamine (Figure 5.10Eii). These findings suggest that the utilisation of glycinergic pathways by NA is mediated, at least in part, by the activation of α_1-adrenoreceptors, which are presumably pre-synaptically located on the terminals of glycine neurons.

5.7 α_2-adrenoreceptor activation has no consistent effects on the spontaneous release of inhibitory transmitters.

In 5 animals, the effects of α_2-adrenoreceptor activation on the spontaneous release of glycinergic and GABAergic IPSPs was investigated. However, in none of these animals was a consistent effect observed on the frequency, duration or amplitude of either glycinergic or GABAergic IPSPs ($N=5$, $P>0.05$; data not shown). In a further two animals, the effects of clonidine on spontaneous release were investigated in the presence of TTX. Again, no consistent effects were detected ($N=2$, $P>0.05$; data not shown). Taken together, my findings suggest that NA can increase the frequency of both glycinergic and GABAergic sIPSPs, consistent with the results obtained in chapter four. In addition, α_1-adrenoreceptor activation can increase the frequency of glycinergic, but not GABAergic sIPSPs, which suggests

that NA achieves the increase in glycinergic inhibition by interaction with this receptor class. Its is perhaps surprising that α_2-receptors had no consistent effects on spontaneous release as, in light of the findings of chapter four, they might be expected to interact with both glycinergic and GABAergic pathways. Further experiments may help to resolve this apparent discrepancy.

5.8 Effects of α-adrenoreceptor activation during swimming.

Having looked at the effects of NA and α_1-adrenoreceptor activation on the spontaneous release of glycine and GABA, I next carried out a preliminary investigation of the effects of α-adrenoreceptor activation on evoked swimming. In chapters three and four I showed that both α_1- and α_2-receptor activation could slow swimming frequency. By using intracellular recordings I have begun to examine the mechanisms which underlie this effect.

The effects of α_1-receptor activation during swimming were investigated in 3 animals in which an increase in the amplitude of the mid-cycle IPSP was observed in 1 animal following application of phenylephrine (150μM; Figure 5.11). This was coincident with an increase in the cycle period and is similar to the reported effects of NA on mid-cycle inhibition and cycle periods (McDearmid *et al.*, 1997). The mid-cycle amplitude was measured by subtracting the peak IPSP amplitude from the resting membrane potential. If a spike accompanied the IPSP then the amplitude was measured directly after it. It was not, however, possible to examine the duration of the IPSP accurately as the fall of the inhibition in this animal was often masked by the onset of the excitation. In the remaining 2 animals no significant differences were found after the addition of phenylephrine. Further experimentation is now required to investigate the effects of α_1-receptors during swimming more fully.

In 4 animals (all embryos), the effects of α_2-adrenoreceptor activation during swimming were investigated. As shown in Figure 5.12A, after addition of 80-120μM clonidine, the duration of the mid-cycle component of the synaptic drive was elongated on virtually every cycle (as measured by the half-fall time). Pooled data of 30 cycles (3 different episodes) under both conditions, reveal that as the duration of each cycle increased from an average of 59.7 ±1.3ms in control to 71.9 ± 0.6ms under clonidine ($P<0.001$; Figure 5.12Cii), the half-fall time of the mid-cycle IPSP increased from 22.7 ± 0.7ms to 25.1 ± 0.5ms ($N=4$, $P<0.01$; Figure 5.12Ci). Whilst this effect parallels that reported for NA (McDearmid, 1998), in contrast to the reported effects on IPSP amplitude (McDearmid *et al.*, 1997), the amplitude of the mid-cycle inhibition (measured by subtracting the resting membrane potential from the maximum amplitude of the IPSP) was *reduced* in all 4 animals ($P<0.01$; Figure 5.11Ciii). These results partly support the finding of McDearmid (1998) who showed that NA *increased* the duration of the mid-cycle IPSP during swimming. However, in parallel, NA induces an increase in IPSP amplitude, thus delaying the onset of excitation at the end of each cycle and slowing swimming.

The use of α_1- and α_2-receptor selective ligands suggests that the effects on mid-cycle IPSPs are insufficient to fully account for the noradrenergic effects on fictive swimming. I therefore explored the possibility that NA additionally modulates the firing properties of spinal neurons and, in particular, post-inhibitory rebound.

5.9 NA enhances the probability of post-inhibitory rebound firing.

The phenomenon of post-inhibitory rebound has been implicated in the generation of RC-delays during swimming in *Xenopus* tadpoles (Tunstall & Roberts,

1994; 1991). It is suggested that increases in mid-cycle hyperpolarisation enable motor neurons to recover faster from on-cycle excitation through sodium channel de-inactivation and potassium channel inactivation. This raises membrane excitability when hyperpolarising input is decaying and promotes spike production (Tunstall & Roberts, 1994). To investigate the possible effects of NA on rebound firing, which might contribute to the noradrenergic effects on swimming, including the reduction of RC-delays, the synaptic inputs during swimming were simulated by injecting a positive current (tonic excitation) superimposed upon which were brief negative currents (mid-cycle IPSPs; Figure 5.13A). Spike threshold was first established by injecting brief (150ms) depolarising current pulses (average threshold 2.25 ± 0.4nA). 150ms hyperpolarising current pulses were then superimposed upon a sustained depolarising (just sub-threshold) current. The amplitude of the hyperpolarising pulses was maintained at just below the threshold required to initiate a post-inhibitory rebound spike (Figure 5.13Bi; average rebound spike threshold –1.7 ± 0.3nA). Even when these conditions were held constant throughout the experiment, application of 6-10μM NA enhanced the probability of rebound firing (N=6; Figure 5.13Bii) indicating a direct noradrenergic effect on motor neuron membrane properties, in addition to any indirect effect of enhanced synaptic inhibition on firing properties. The effects of NA on rebound firing could be counteracted by addition of 50μM phentolamine (N=2; Figure 5.13Biii). As shown in Figure 5.13Bii, in 4/6 animals, the spike threshold was reduced after addition of NA. Unfortunately, the experimental protocol prevented spike threshold from being repeatedly re-measured under NA, but as the level of depolarising current was the same under each set of conditions, a reduction in spike threshold can be inferred. There was however no detectable effect on either the resting membrane potential or membrane conductance.

For example, in the experiment shown in Figure 5.13, resting membrane potential was –67mV in control, -69mV after addition of NA and remained at –69mV in the presence of phentolamine. These results suggest that not-withstanding the effects of increased inhibition under NA, which might promote rebound firing, NA has a clear post-synaptic effect, reducing spike threshold and enhancing post-inhibitory rebound firing.

5.10 α_1- adrenoreceptor activation enhances the probability of rebound firing.

As the noradrenergic enhancement of rebound firing could be counteracted using phentolamine, this suggests that α-adrenoreceptor activation might underlie these effects. To examine this in more detail, the same protocol was next applied both before and after activation of α_1-adrenoreceptors. The effects of phenylephrine on rebound firing were examined in 6 animals. Under control conditions, average spike threshold was determined as 1.7 ± 0.8nA and the average threshold required to initiate rebound firing as 2.2 ± 0. 6nA. As before, the synaptic drive for swimming was then simulated by injecting sub-threshold depolarising current, superimposed on which were 150ms hyperpolarising current pulses (Figure 5.14Ai). In 4/6 animals, after application of 100-150μM phenylephrine, and when injecting the same levels of current as in control conditions, rebound firing occurred (Figure 5.14Aii). As was the case with NA, there was no detectable effect on membrane conductance or resting membrane potential (e.g. -62mV throughout the experiment in Figure 5.14), although in 3 of the 4 animals where rebound firing was enhanced, there was a clear reduction in spike threshold. In 1 animal to which phentolamine was subsequently added, the effect on both spike threshold and rebound firing was counteracted (c.f.

Figure 5.13Biii). Finally, the effects of α_2-adrenoreceptor activation on rebound firing have not yet been examined. The finding that α_1-receptor activation, in addition to NA, can enhance the probability of rebound firing, suggests that this receptor class may, in addition to a pre-synaptic location on glycinergic terminals, be located post-synaptically, where they mediate this direct effect on NA on motor neurons.

5.11 Discussion.

In chapters three and four I showed pharmacologically that α_1- and α_2-adrenoreceptors mediate the noradrenergic effects on swimming, and suggested that the likely mechanisms behind this response involved the utilisation of glycinergic and GABAergic inhibitory pathways. The aim of this chapter was to investigate these findings in more detail, using intracellular recording techniques in tandem with a pharmacological approach. I aimed to identify more precisely both the targets and possible locations of α-adrenoreceptors and to explore how such interactions might bring about the increase in swimming frequency, and reduction in RC-delays and episode duration which characterise the noradrenergic modulation of swimming. As discussed in the introduction, glycinergic and GABAergic pathways play pivotal roles in shaping motor output not only in *Xenopus*, but in a variety of vertebrates, and I will now discuss the utilisation of these pathways during the noradrenergic modulation of swimming and its functional implications.

At both stages of development studied (embryonic stage 37/38 and larval stage 42), NA shortens the duration of swimming episodes. In embryos, episodes of swimming can be terminated prematurely following activation of the rostral cement gland. This well-described (Boothby and Roberts, 1992a,b; Reith & Sillar, 1999)

afferent pathway is activated when the animal contacts an obstacle, such as the meniscus of the water surface, and instantly terminates swimming by releasing GABA from mhr neurons onto the motor components of the spinal network. This pathway begins to degrade as larval development progresses and by stage 42 its reliability is already dramatically reduced. The mhr neurons themselves are still present and it is possible that they become recruited into a new pathway controlling episode duration, such that, at stage 42, episodes of swimming often terminate spontaneously with a barrage of GABAergic potentials (Reith & Sillar, 1999). The results presented above indicate that NA and activation of α_1- and α_2-adrenoreceptors can enhance release of GABA following cement gland stimulation. In addition α_1-receptors enhance the GABAergic barrage at the end of larval episodes, coincident with a shortening of swimming episodes and consistent with my earlier findings that α_1-activation shortens the duration of larval episodes (Figure 3.3). The results obtained in this chapter suggest that through the activation of α_1- and α_2-adrenoreceptors, NA is directly affecting the release of GABA from the terminals of GABAergic neurons (the most likely candidates being the mhr neurons) in response to an afferent cue. From a behavioural point of view, the cement gland is often activated when, for example, the animal touches an object in its path or the surface of the water. This terminates swimming and the embryo hangs beneath the water surface, suspended by a mucus-like substance secreted from the cement gland. Presumably an increase in the response to cement gland activation, such as when levels are NA are raised, will increase the reliability of episode termination, but how the animal is advantaged in this way remains unclear. It may though be that any strong stimulus which activates swimming will cause a generalised increase in

arousal, including facilitation of this stopping pathway which causes the organism to hang motionless, invisible to predators.

In chapter three I demonstrated that NA and α_2-adrenoreceptor activation reduces episode duration in embryos in the absence of an extrinsic signal to terminate swimming (cement gland activation; Figures 3.1-2, 3.4). I have found no evidence in this chapter to suggest a mechanism by which NA or α_2-adrenoreceptors might reduce the duration of normal embryonic swimming episodes. Whilst the mechanisms responsible for the natural termination of swimming in embryos are at present unclear, two proposals have been out forward. Firstly, over the course of an episode there may be an increase in levels of calcium (e.g. as a result of calcium influx during action potentials), which might increase a known Ca^{2+}-dependent K^+ current, increase spike threshold and eventually prevent spike production altogether, thus terminating swimming (Wall & Dale, 1995). The second proposed mechanism suggests that over the course of an episode, levels of adenosine (a breakdown product of ATP) gradually increase, which blocks calcium channels, reduces excitability, and eventually terminates swimming (Dale & Gilday, 1996). In a more recent study, however, nitric oxide reduced embryonic swimming episodes, which terminated with a barrage of GABAergic IPSPs similar to those seen in larvae (McLean & Sillar, 2002). This raised the possibility that the mhr GABAergic neurons might be recruited by NO to terminate swimming. However, no such effect was observed in this study following NA or α_2-adrenoreceptor activation and any effect of NA on levels of ATP or Ca^{2+} will now need to be examined.

The second significant effect of NA on the *Xenopus* motor network is a reduction in swimming frequency (Chapters three and four; McDearmid *et al.*, 1997). In most vertebrates, the neural networks generating motor activity are

organised as two half-centres, coupled by reciprocal inhibition. As simulated in computer modelling experiments (e.g. Hellgren *et al.*, 1992; Dale, 1995), swimming frequency is most readily adjusted by affecting this reciprocal inhibitory component of each motor cycle. In *Xenopus*, it has previously been shown that NA pre-synaptically enhances mid-cycle glycinergic IPSPs during swimming, enhancing chloride influx, membrane hyperpolarisation and inhibition, thus reducing swimming frequency (McDearmid *et al.*, 1997). In chapter four, I showed that NA was still able to modulate swimming frequency in the presence of strychnine, although the subsequent addition of bicuculline counteracted these effects (Figure 4.5H), suggesting that GABAergic pathways might also be utilised to control swimming frequency. There is, however, no GABAergic component phase-locked to the each swim-cycle, so how would an increase in GABAergic inhibition bring about a reduction in swim frequency? During larval swim episodes there are occasional sporadic GABAergic potentials which, although not locked to any phase of the swim cycle, are sufficiently long in duration (90-200ms) to affect more than one cycle (~50-70ms), and indeed over the course of an episode may summate to produce a background level of tonic inhibition such that swimming frequency is reduced (Reith & Sillar, 1999). Whilst in this study I have not directly examined the appearance of such potentials during evoked swimming (largely because the majority of experiments were performed on embryos where such potentials are seldom present), I have shown that NA can enhance vesicular release of GABA during the periods between swimming episodes (Figures 5.6,5.7). Coupled with the effects of enhancement of the GABAergic cement gland response, NA is clearly able to up-regulate GABAergic transmission and thus could conceivably utilise GABAergic pathways to increase levels of inhibition during swimming, which, as just outlined,

may contribute to the slowing of swimming over the whole episode rather than on a cycle-by cycle basis. This hypothesis will now need to be confirmed using recordings from larval neurons, together with the pharmacological block of mid-cycle glycinergic inhibition using strychnine.

The noradrenergic enhancement of GABAergic inhibition persisted in the presence of TTX (thus excluding transmitter release evoked by sodium spikes), which is indicative of a pre-synaptic site of action, whilst the ability of bicuculline to counteract this effect suggests $GABA_A$ receptors as probable post-synaptic targets. This parallels the finding of McDearmid *et al.*, (1997) that NA pre-synaptically facilitates glycine release and taken together with the increase of both glycinergic and GABAergic spontaneous IPSPs by the noradrenergic uptake inhibitor protriptyline, it confirms my conclusions from chapter four that NA utilises both of these fast inhibitory pathways (Figure 4.1). From these experiments it is not possible to confirm the source of GABA, but of the eight known GABAergic neuron classes in the CNS, only the ascending interneurons and the Kölmer-Agduhr cells are located within the spinal cord (Roberts *et al.*, 1987; Dale *et al.*, 1987), although the mhr neurons also have descending projections into the cord. The mhr neurons would seem improbable candidates as these participate in the termination of swimming by releasing GABA onto the CPG (Boothby & Roberts, 1992a). Presumably any enhancement of GABA release from these neurons would merely serve to terminate swimming. The function of both the ascending and Kölmer-Agduhr neurons are unknown, but either or both of these cell types, with their somas located within the spinal cord would seem more probable sources of GABA. This could readily be tested using animals spinalised beneath the level of the mhr neurons, which would

demonstrate whether the GABAergic sIPSPs, whose frequency is increased by NA, were spinal in origin.

Activation of α_1-adrenoreceptors by phenylephrine pre-synaptically enhanced spontaneous release of glycine, *but not* GABA. This corroborates previous findings in three ways. First, I showed in chapter four that strychnine could counteract the reduction of swimming frequency and RC-delays by phenylephrine (Figure 4.2), whilst pre-application could occlude the effects of phenylephrine on all the parameters of swimming (Figure 4.5). Second, in parallel with the reported effects of NA during evoked episodes of swimming (McDearmid *et al.*, 1997), phenylephrine increased the amplitude of the mid-cycle IPSP, thus presumably contributing to the slowing of swimming. Third, and most important, the ability of α_1-adrenoreceptor activation to replicate the effects of NA on glycine release is direct and additional evidence that this receptor class is located on the terminals of glycinergic interneurons. The obvious conclusion is that activation of these receptors by NA would facilitate, via interpolated second messenger pathways, or by direct coupling to the release machinery, the vesicular release of glycine into the synaptic cleft. The effects of α_2-receptor activation were, however, less clear.

In contrast to both NA and α_1-receptor activation, I have so far found no consistent effects of α_2-receptors on the frequency, amplitude or duration of spontaneous IPSPs. During swimming, however clonidine had a consistent effect on the mid-cycle IPSP, *reducing* its amplitude, but simultaneously increasing its duration. This finding contrasts with the effects of both NA (McDearmid *et al.*, 1997) and α_1- receptors (this study) where the amplitude of the IPSP increased. However, the increase in the duration of the mid-cycle IPSP (as measured by its half-fall time) does parallel the findings of McDearmid (1998) for NA. The greater

proportion of each cycle occupied by inhibition can account for the reduction in swimming frequency following α_2-receptor activation, since prolonging the IPSP will delay the onset of the excitation and slow swimming. The elongation of mid-cycle IPSPs (in the face of declining IPSP amplitudes) is indicative of a post-synaptic site of action for α_2-receptors, either by modification of ion channel properties or by prolonging the action of glycine at its receptors. This hypothesis would be consistent with reports from some higher vertebrates where α_2-receptors are presumed to be located post-synaptically (e.g. cat, Chau *et al.*, 1998). The reduction in amplitude could be due to a pre-synaptic effect, directly reducing the probability of glycine release, but at present I have no data to support either of these hypotheses. In chapter three, I showed that clonidine reduced the amplitude of ventral root bursts during swimming (Figure 3.4F). This could indicate that α_2-adrenoreceptors are located on the axons of neurons (either motor neurons themselves, or on pre-motor interneurons). If α_2-receptor activation reduces the amplitude of action potentials, then the duration of calcium channel opening will be necessarily truncated and the frequency of inhibitory vesicular release from inhibitory terminals might be reduced, hence accounting for the reduction in mid-cycle IPSPs.

It is worth noting that α_2-receptors are known autoreceptors for noradrenergic transmission (Langer, 1974), responsible for negative feedback onto noradrenergic neurons and reducing release of NA. It remains possible that such a mechanism might account for the reduction of mid-cycle amplitude seen during this study, although clearly there must be a parallel, post-synaptic effect that increases the duration of the IPSP. The presence of auto-receptors now needs to be investigated using spinalised animals, which will, by definition, sever any feedback

to the isthmic region that might otherwise affect the release of NA. A final point of note is that in chapter four I have shown that activation of α_2-receptors appear to utilise both glycinergic and GABAergic inhibition to modulate the frequency of swimming. Whilst I have found no consistent effects on the spontaneous release of these transmitters which would support this finding, it is possible that, as discussed above, there might be an increase in GABAergic inhibition across the course of an episode, which could contribute to the elongation of cycle periods, even though the amplitude of the mid-cycle glycinergic IPSP appears to decrease. In support of this hypothesis, activation of $GABA_B$ receptors in *Xenopus*, using the agonist baclofen, has been shown to reduce both the duration of swimming episodes and the amplitude of ventral root spikes. It also concomitantly reduces the amplitude of the mid-cycle glycinergic IPSP (Wall & Dale, 1993). I have provided evidence to show that all three of these phenomena occur following the activation of α_2-adrenoreceptors, thus raising the possibility that through utilisation of GABAergic pathways, which then target $GABA_B$ receptors, the amplitude of the mid-cycle IPSP might be reduced. This hypothesis will now require further investigation using antagonists at $GABA_B$ receptors to assess, if any, their contribution to the observed effects of α_2-adrenoreceptor activation.

The ability of both NA and α_1-adrenoreceptor activation to enhance post-inhibitory rebound firing adds a further component to our understanding of how the effects of NA on motor activity are produced. The phenomenon of post-inhibitory rebound is reliant on the fact that inhibition allows motor neurons to recover more quickly from excitation through sodium channel de-inactivation and potassium channel inactivation. This raises membrane excitability while hyperpolarising input is decaying and promotes cell firing (Tunstall & Roberts, 1994). Clearly then, when

levels of inhibition are raised the probability of rebound firing should also be increased. In the spinal cord of *Xenopus* there is a rostro-caudal gradient of inhibition, with strong mid-cycle inhibition onto rostral neurons, which gradually declines along the body, such that in caudal cells mid-cycle inhibition is often absent. Post-inhibitory rebound has been suggested to play a role in the generation of RC-delays in *Xenopus*, although the exact mechanisms behind it are not known (Tunstall & Roberts, 1994). It has been shown that NA can enhance glycinergic inhibition along the entire length of the spinal cord, however, the effect is relatively greater in caudal neurons where inhibition is initially weaker, thereby reducing the gradient in inhibition (McDearmid, 1998). It follows therefore that with enhanced inhibition under NA, the probability of rebound firing will be increased along the whole spinal cord, but the effect will be more pronounced in caudal neurons, thus potentially advancing their firing relative to more rostral ones and reducing the RC-delay along the body.

Not-withstanding this possible enhancement of rebound firing due to increased inhibition, the results presented in this chapter indicate that NA (and α_1-adrenoreceptor activation) can enhance rebound firing through a direct effect on cell properties. The parallel decrease in spike threshold suggests that both these effects might be achieved through, for example, facilitation of the recovery of sodium channel in-activation, thus reducing spike threshold and promoting firing. That the effects of NA on rebound firing seem to be mediated, at least in part, by α_1-adrenoreceptors is an intriguing finding. It suggests that in addition to a pre-synaptic site of action (as described above), α_1-receptors might also be present on the post-synaptic membrane, where their activation modulates cell properties and enhances rebound firing. The effects of α_2-receptors on post-inhibitory rebound have yet to be

investigated, however, as shown in chapter three, they appear not to have consistent effects on RC-delays and as such it might be expected that they would not enhance rebound firing. This now requires investigation.

What other functional roles does an enhancement of rebound firing have? A key advantage of an enhancement of rebound firing is the ability to sustain longer cycle periods, such as produced by NA. Ordinarily, across the course of an episode, cycle periods lengthen, coincident with a 'drop-out' of pre-motor interneurons from the synaptic drive. Eventually, if enough interneurons drop out, then episodes may terminate, as the synaptic drive is insufficient to maintain ongoing activity. If post-inhibitory rebound were enhanced, as a result of raised inhibition and/or a direct post-synaptic effect under NA, then the firing of another action potential is promoted from the falling phase of the mid-cycle IPSP and the probability of swimming continuing will be increased, despite the slower swimming frequency. NA has previously been shown to recruit otherwise silent inhibitory neurons during swimming (McDearmid, 1998), which can be explained by an increase in rebound firing, promoting excitability and recruiting pre-motor interneurons to the swim-generating network. This might allow for the maintenance of swimming even at the longer cycle periods produced by NA. As discussed in chapters six and seven, such a parallel effect supports the hypothesis that NA might be involved in motor pattern selection, switching to produce struggling activity, which is characterised by longer cycle periods than swimming and where neuron firing propagates caudo-rostrally.

Taken together, the results obtained in this chapter have provided important new details, not only of which specific fast inhibitory pathways are utilised by NA, but also of the possible location of the α-adrenoreceptors through which these effects are mediated. By adopting both a pharmacological and physiological approach I

have been able to examine the individual effects of these receptors, and have established their functional roles in modulating specific parameters of swimming. However, the combined effects and interactions of specific adrenoreceptors must now be considered.

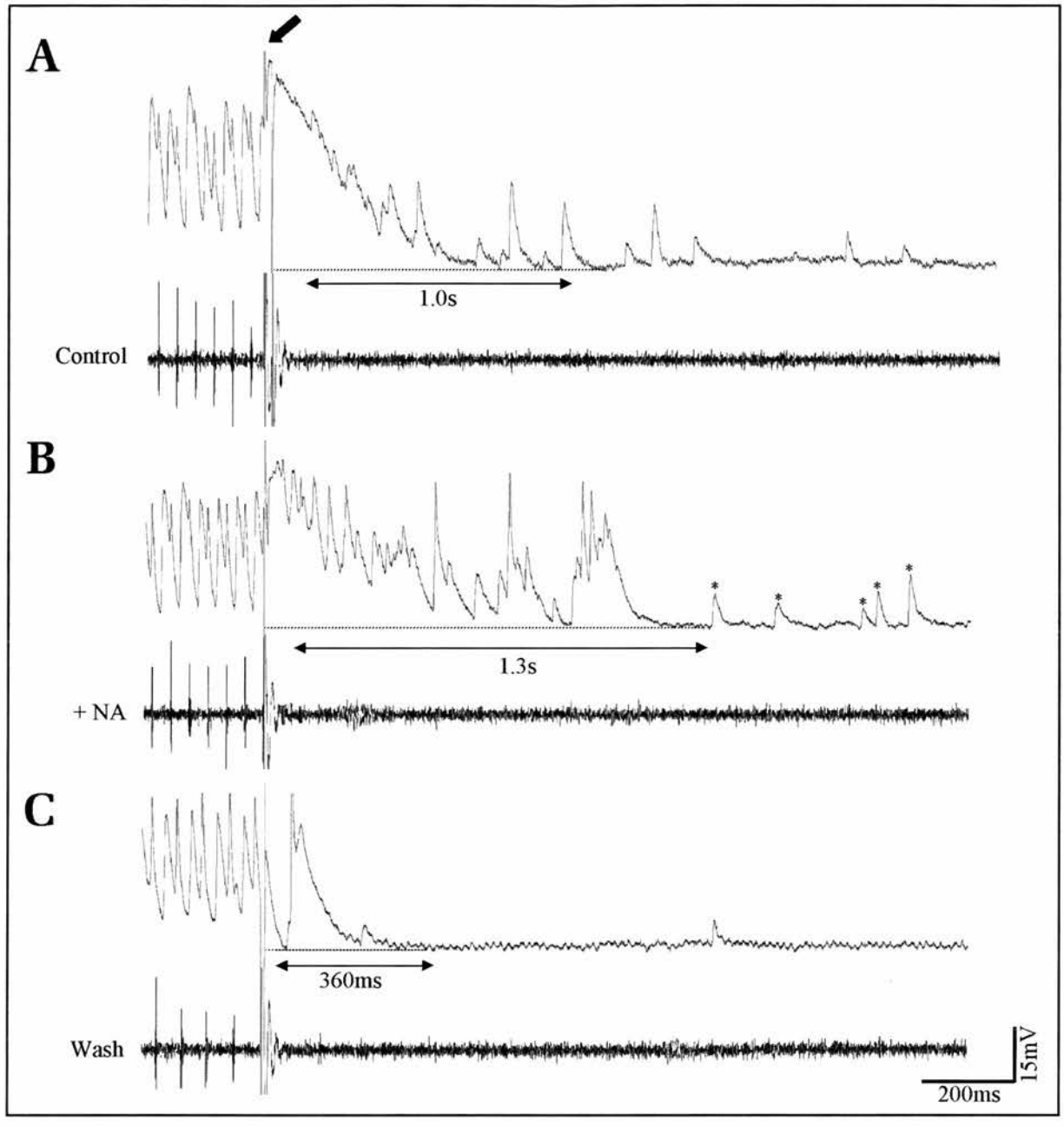

Figure 5.1: *NA enhances the GABAergic barrage following cement gland stimulation in embryos.* (**A**) Swimming in embryos can be terminated prematurely by stimulation of the rostral cement gland (artefact arrowed in A). This activates mid-hindbrain reticulospinal GABAergic neurons, releasing GABA onto the CPG for swimming, causing the termination of swimming (see text in section 1.7 for further details). (**B**) In the presence of NA, stimulation of the cement gland results in a potentiation of presumed GABAergic IPSPs. Note also that these potentials can continue even after the membrane has returned to its resting potential (asterisked) (**C**) The effect of NA can be counteracted by returning to control conditions.

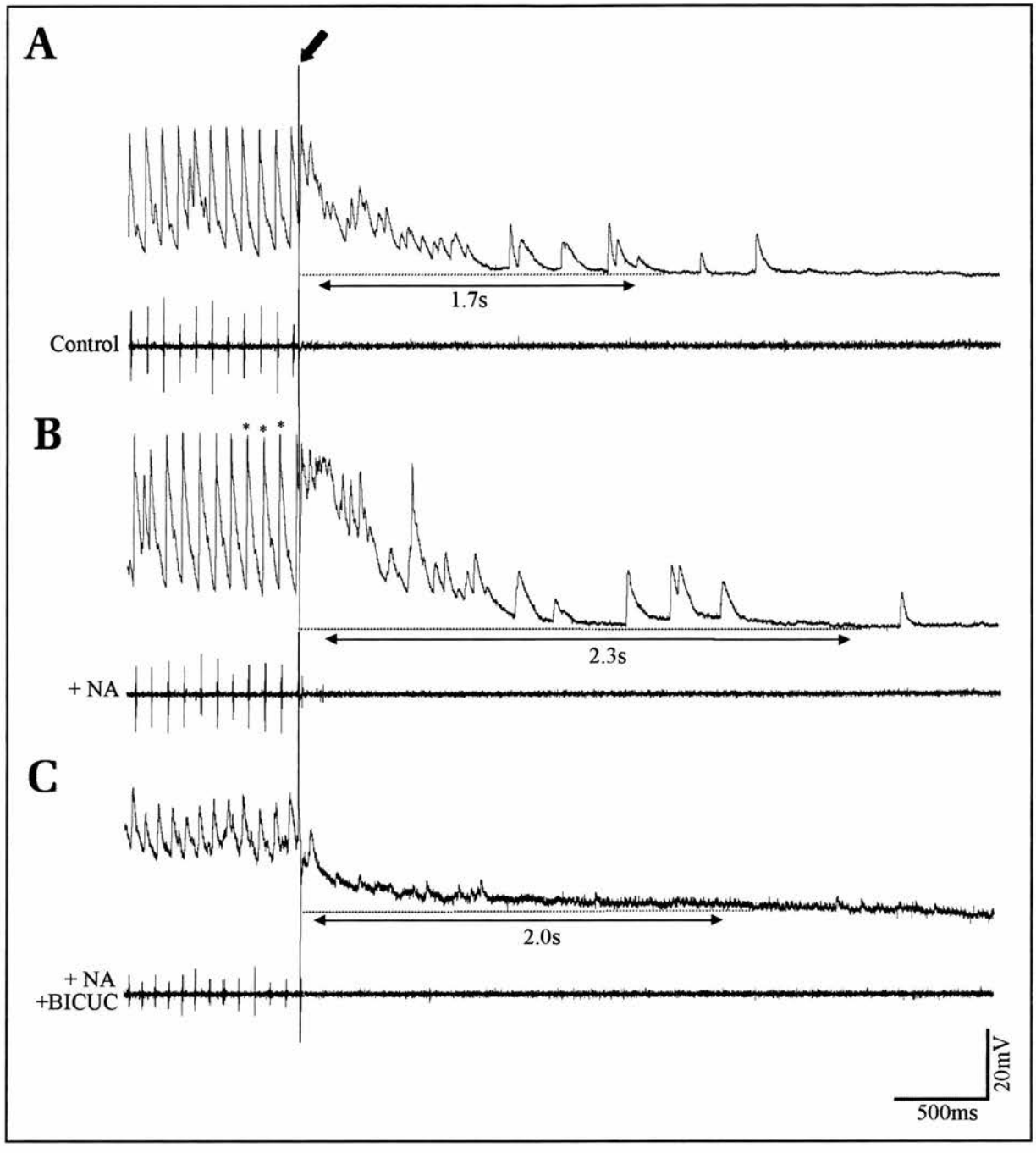

Figure 5.2: *Bicuculline counteracts the noradrenergic enhancement of the cement gland stopping response.* **(A)** As shown in Figure 5.1, cement gland stimulation in embryos terminates swimming with a barrage of IPSPs. **(B)** In the presence of NA, there is a large potentiation of this inhibitory barrage and there is a greater delay before the membrane potential returns to rest. Note also the increase in amplitude of the mid-cycle inhibition under NA (asterisked in B) **(C)** The subsequent addition of the $GABA_A$ receptor antagonist bicuculline counteracts the effects of NA (despite the quality of the recording diminishing), indicating that the IPSPs potentiated by NA are GABAergic in origin.

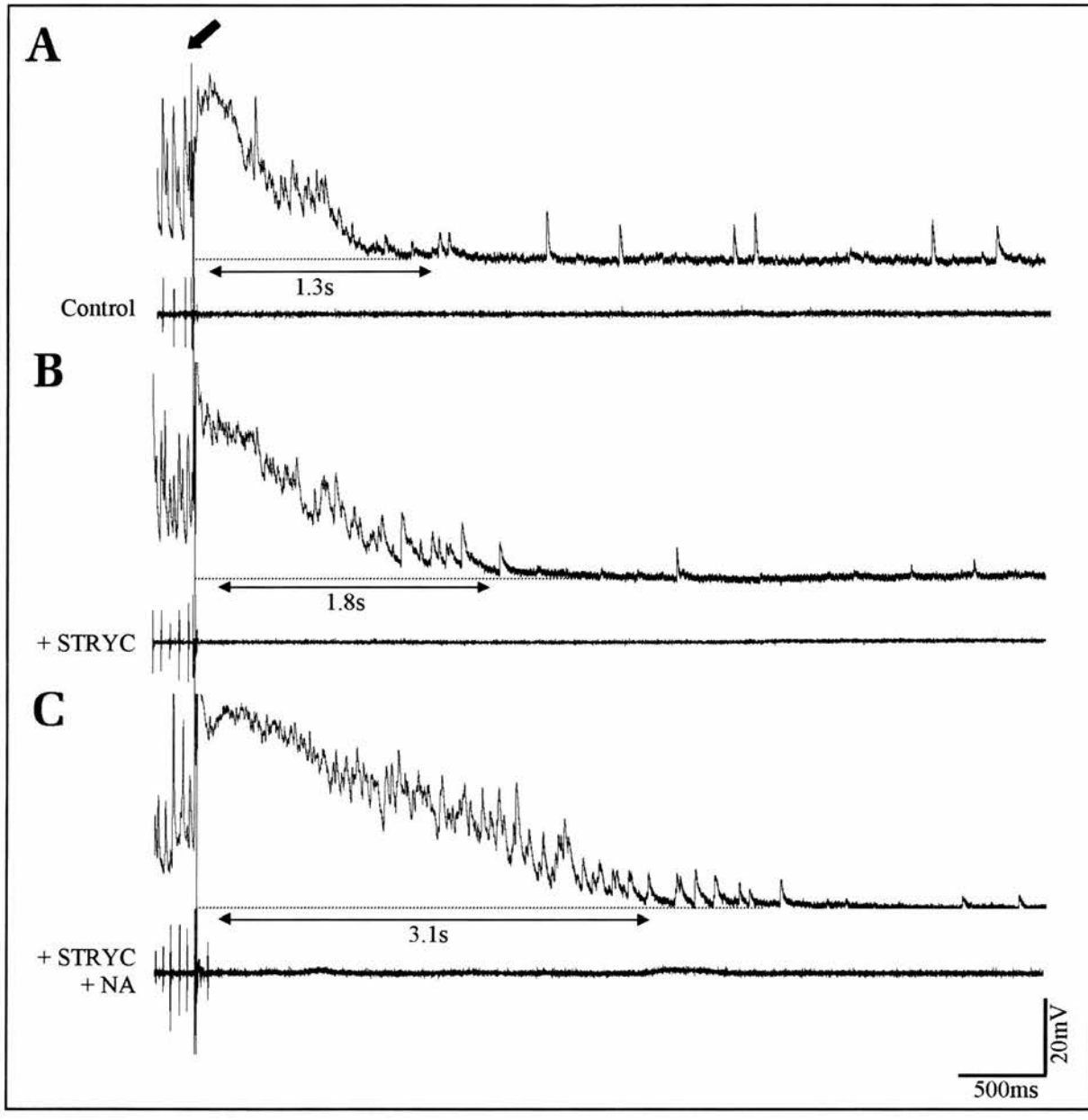

Figure 5.3: *Strychnine does not occlude the noradrenergic enhancement of the GABAergic barrage following cement gland stimulation.* (**A**) Stimulation of the cement gland terminates swimming with a flurry of GABAergic IPSPs. (**B**) Addition of strychnine does not prevent the termination of swimming following cement gland activation and the GABAergic IPSPs persist. (**C**) In the presence of NA, the GABAergic barrage in response to cement gland stimulation is more pronounced and the time taken to return to the resting potential is increased.

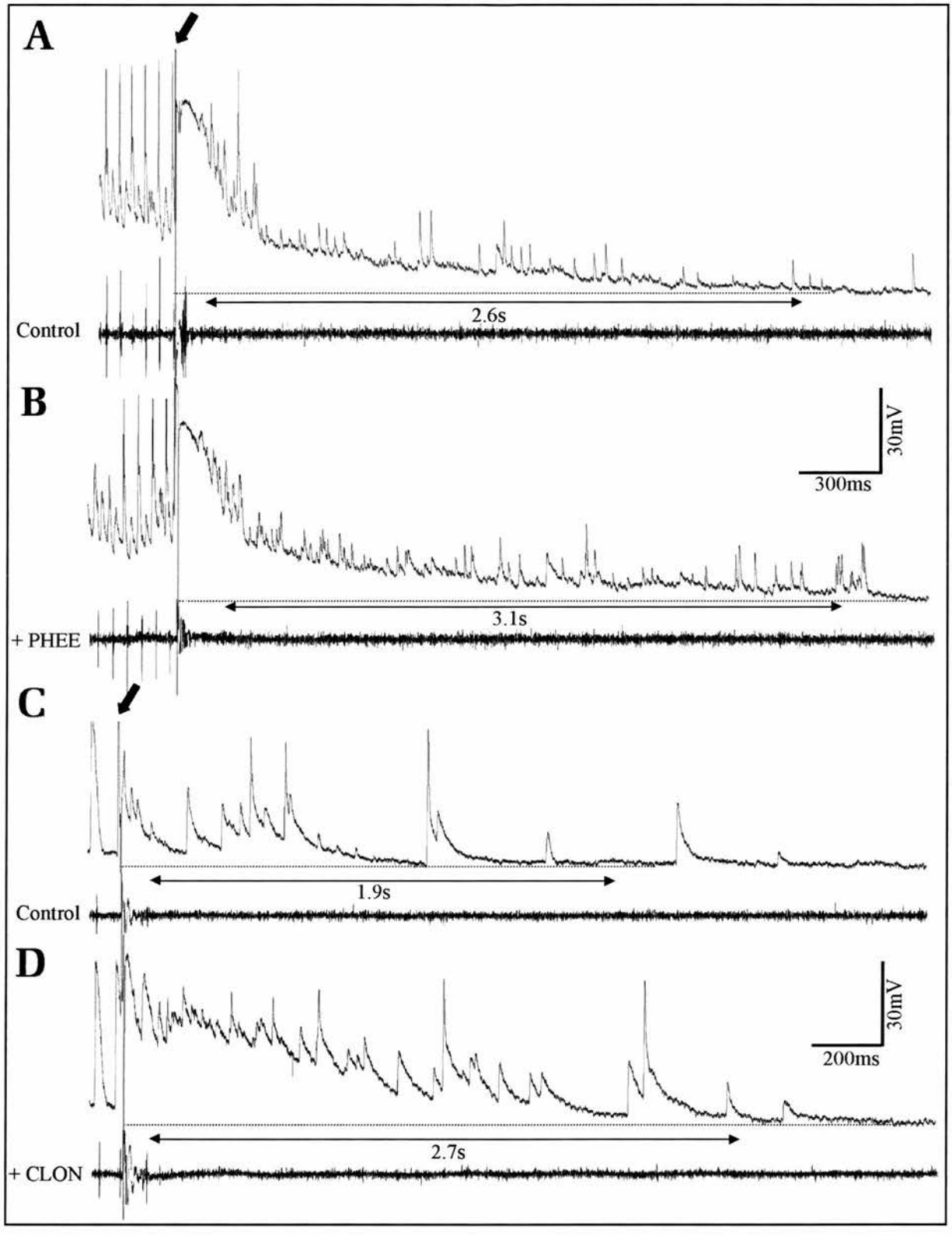

Figure 5.4: *α_1- and α_2-adrenoreceptor activation enhances the GABAergic barrage following cement gland activation.* **(A, C)** As shown in Figures 5.1-5.3, swimming in embryos can be terminated by stimulation of the rostral cement gland (artefact arrowed in A and C). Following activation of α_1-receptors by phenylephrine **(B)** and α_2-receptors using clonidine **(D)**, the GABAergic barrage elicited by cement gland stimulation is enhanced and the time taken for the membrane potential to return to rest is increased .

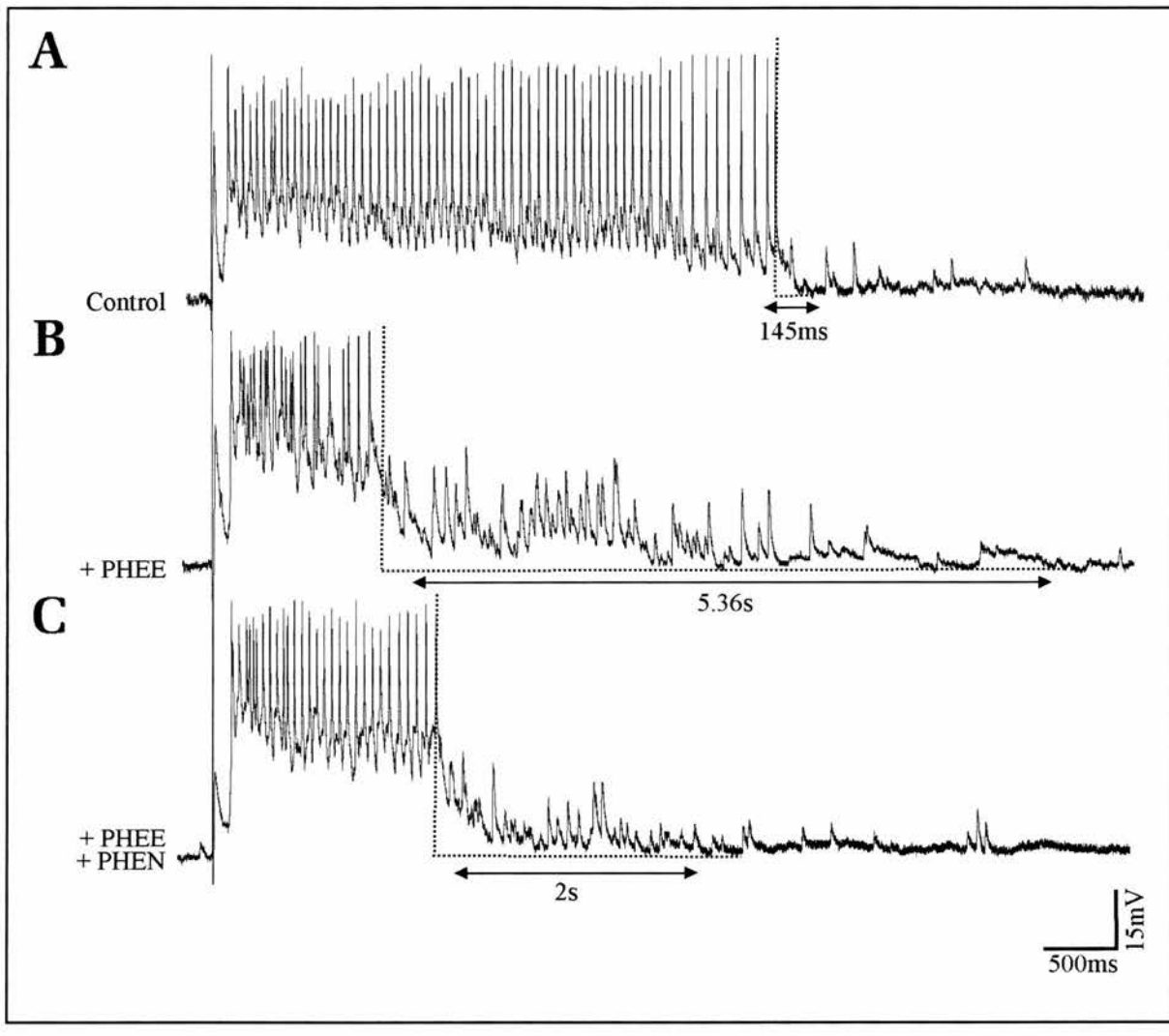

Figure 5.5: *Phenylephrine enhances the GABAergic barrage coincident with the termination of swimming in larvae.* (**A**) In larvae, episodes of swimming often terminate with a barrage of GABAergic IPSPs (see section 1.7 for more details). (**B**) After application of 150μM phenylephrine, the duration of this barrage was significantly increased. Note also the reduction in episode duration under phenylephrine; see chapter three and Figure 3.3A. (**C**) Subsequent application of the general α-adrenoreceptor antagonist phentolamine (40μM) counteracted the effects of phenylephrine.

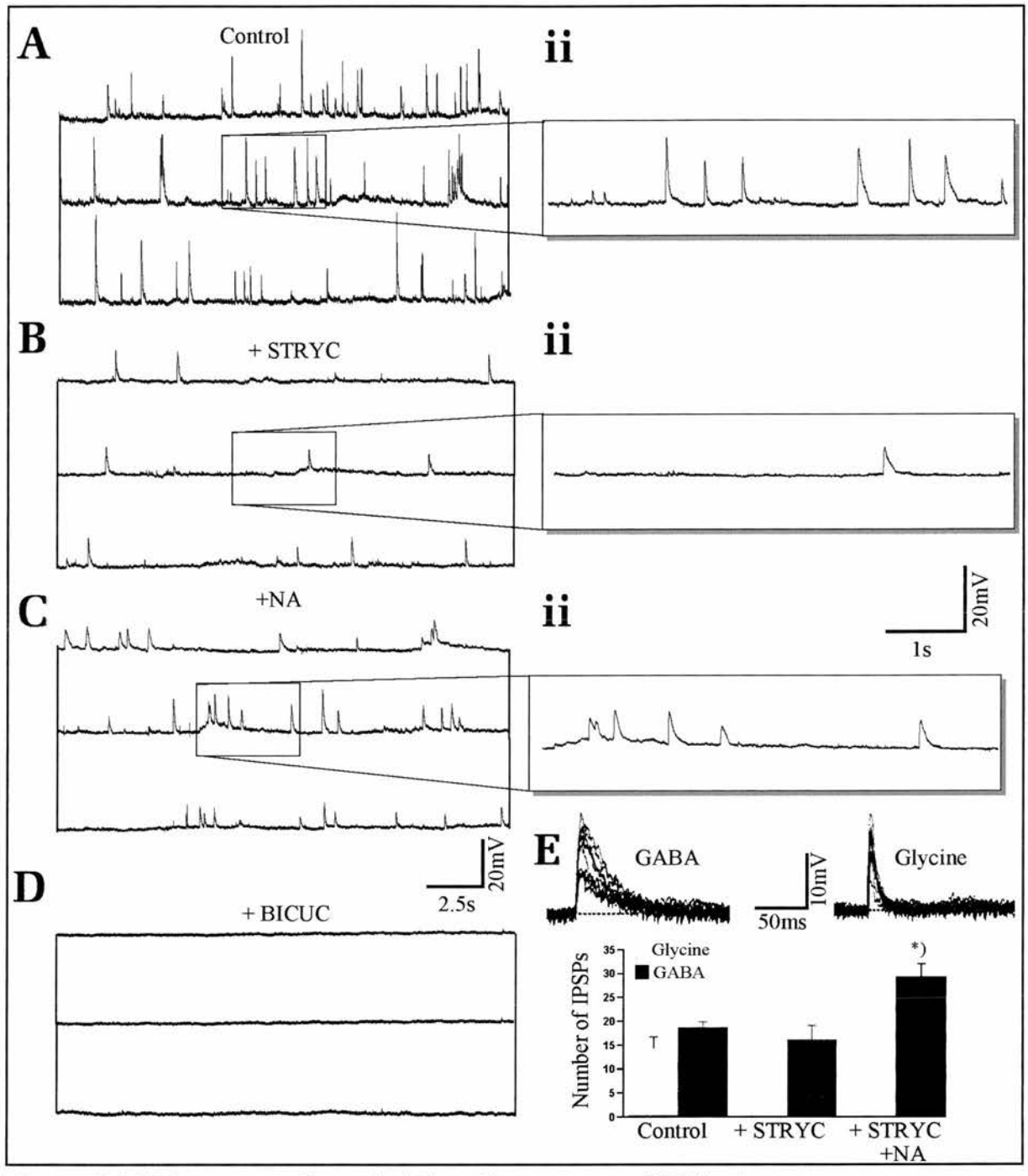

Figure 5.6: *NA increases the probability of spontaneous GABA release onto motor neurons.* (**A**) Recording from an embryo motor neuron, using KCl-filled electrodes during the quiescent periods between swimming episodes reveals glycinergic and GABAergic IPSPs (these can be distinguished on the basis of their time course and pharmacology (see **E;** also Reith & Sillar, 1998). (**B**) Application of strychnine blocks inhibitory glycine receptors and only GABAergic IPSPs remain. (**C**) After addition of 9μM NA, the frequency of spontaneous GABA potentials increased significantly. (**D**) The GABAergic origin of these potentials was confirmed by abolishing them by the subsequent application of bicuculline. (**E**) Overlays of GABAergic and glycinergic potentials, revealing their distinct time courses- GABAergic IPSPs are typically 90-200ms in duration, whilst glycinergic ones are between 20-80ms (adapted from Reith & Sillar, 1998). Bottom panel shows plot of average number of glycinergic and GABAergic potentials for three, 30-second intervals under each set of conditions. *) $P<0.01$

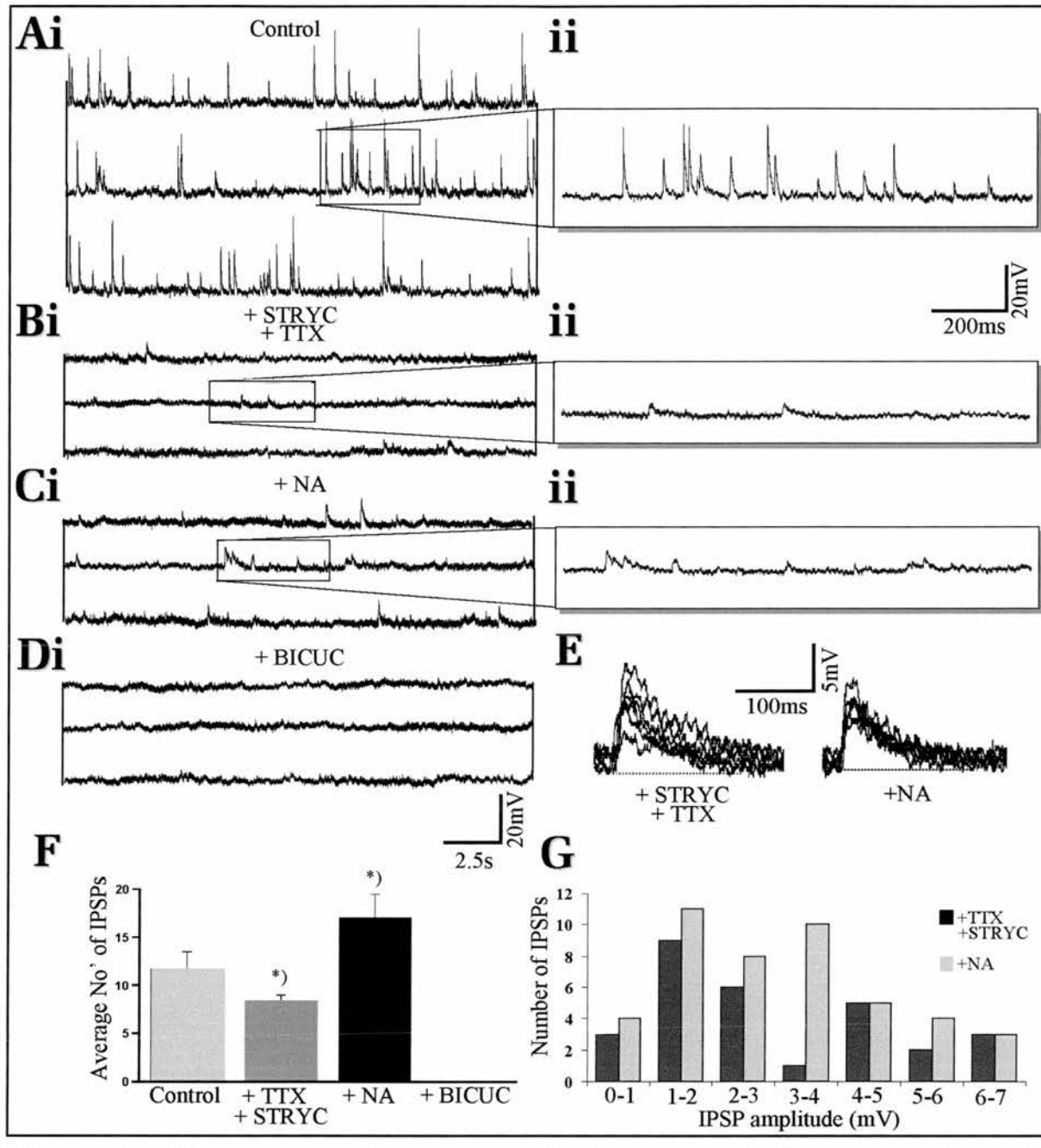

Figure 5.7: *NA pre-synaptically increases the frequency of GABA release.* (**A-B**) To investigate whether the noradrenergic enhancement of GABAergic inhibition (shown in Figure 5.6) is due to a pre- or post-synaptic effect, the same experiment was performed in the presence of strychnine and TTX, to synaptically isolate the neuron being recorded. (**C**) Under TTX and strychnine, addition of NA significantly increased the frequency of spontaneous GABA release. (**D**) These potentials were blocked following addition of bicuculline, proving that they were GABAergic in origin. (**E**) Overlaps of 6 GABAergic potentials under TTX/strychnine and NA, show that NA does not affect the duration of these IPSPs (c.f. Figure 5.6E). (**F**) Plot of number of GABA IPSPs under each set of conditions and their amplitude distributions (**G**). *) $P<0.05$

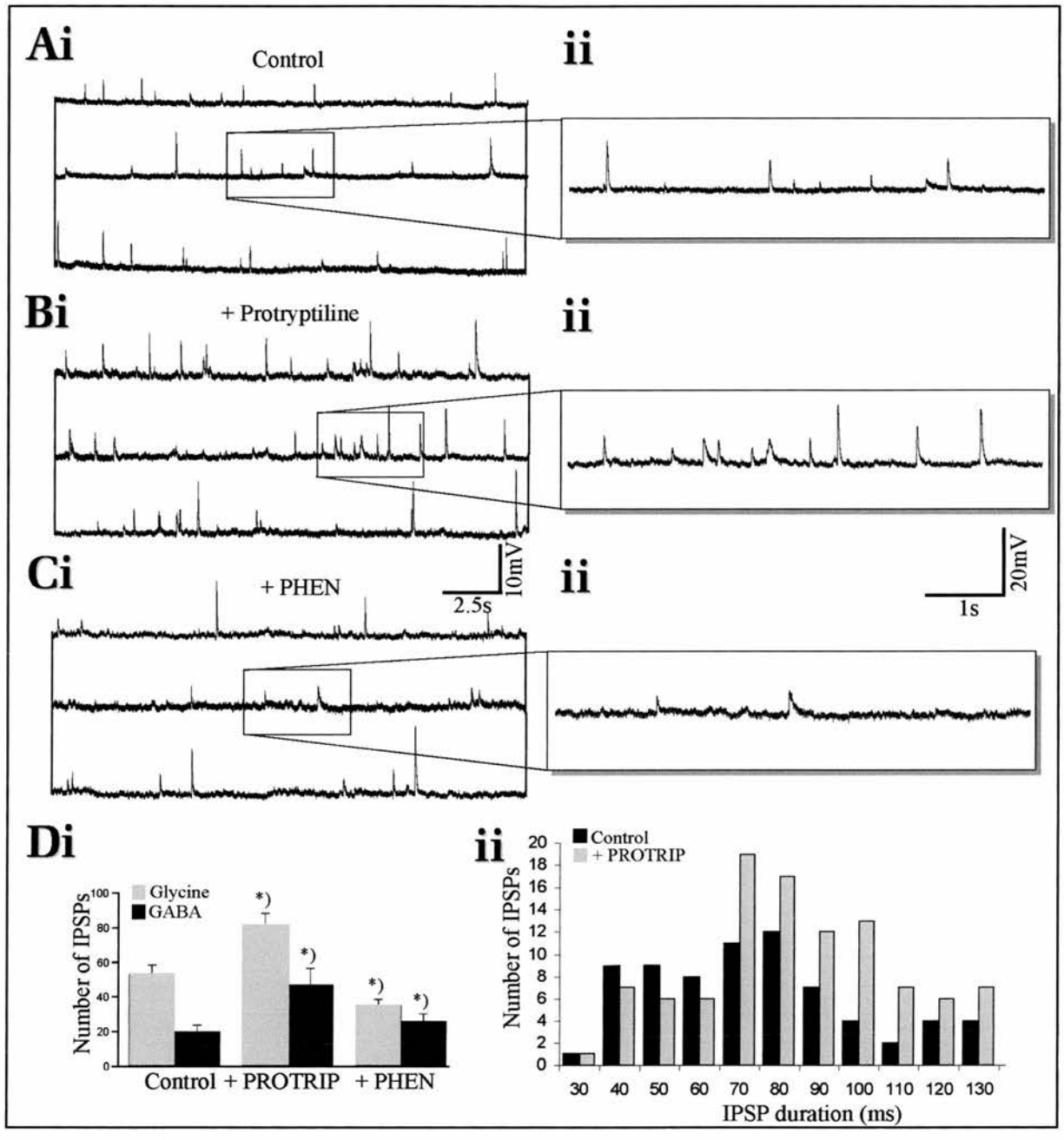

Figure 5.8: *The noradrenergic uptake inhibitor protriptyline increases the frequency of GABAergic and glycinergic spontaneous IPSPs.* (**A**) Recording from a larval motor neuron, using KCl-filled electrodes during the quiescent periods between swimming episodes reveals depolarising glycinergic and GABAergic IPSPs. (**B**) In the presence of the noradrenergic uptake inhibitor protriptyline (90μM), the frequency of both glycinergic and GABAergic potentials is significantly increased ($P<0.001$). (**C**) Subsequent application of the general α-adrenoreceptor antagonist phentolamine significantly counteracts the effects of protriptyline. (**Di**) Average numbers of glycinergic and GABAergic potentials in control and under protriptyline during 90-second sample periods. (**Dii**) Plot of number of IPSPs grouped by their duration (10ms bins), showing that protriptyline increases the frequency, but not the duration of both glycinergic and GABAergic potentials. *) $P<0.05$

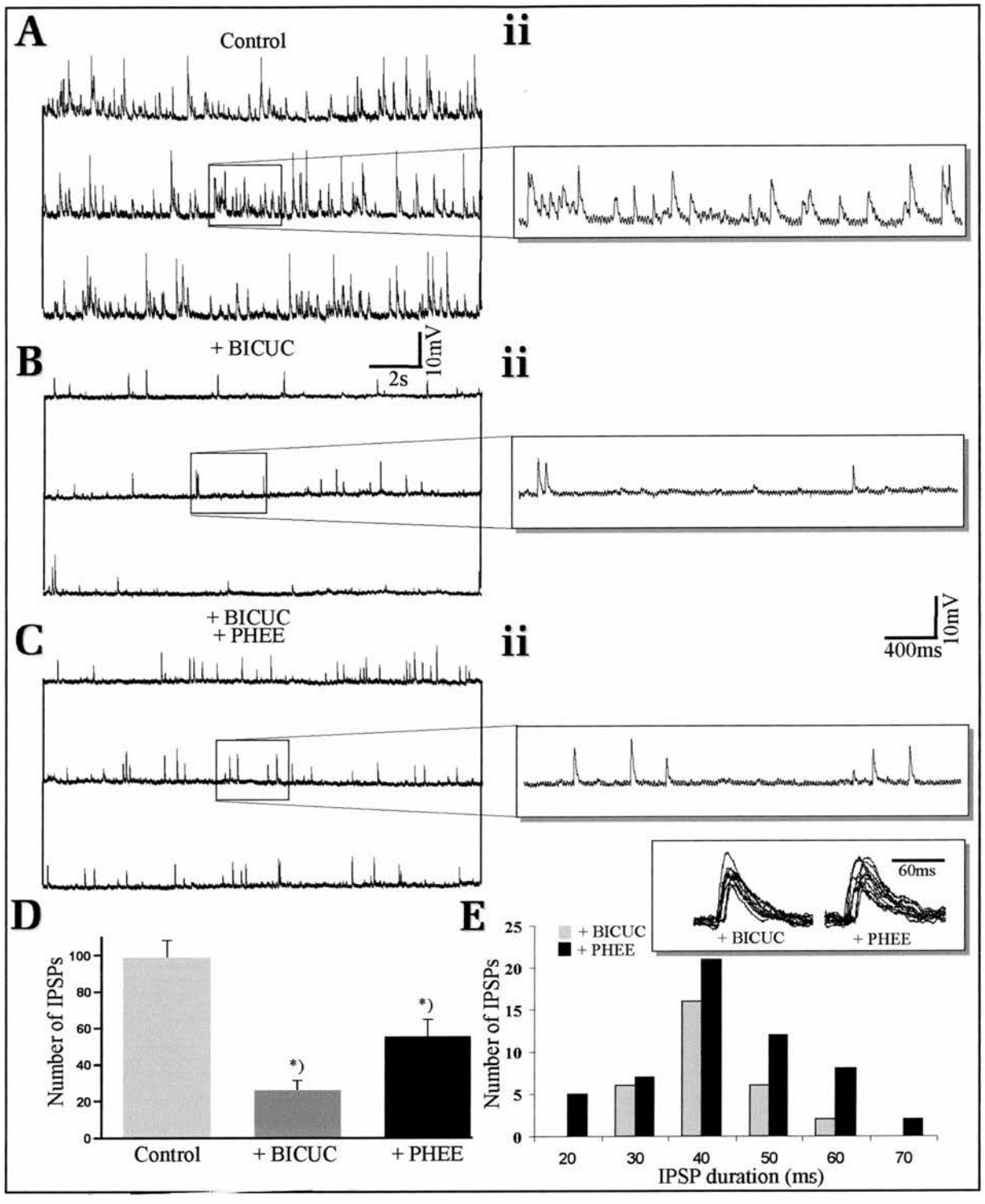

Figure 5.9: *Phenylephrine increases the probability of spontaneous glycine release.* (**Ai-ii**) Recording from a larval motor neuron using a KCl-filled electrode revealing spontaneous glycinergic and GABAergic IPSPs. (**Bi-ii**) Addition of bicuculline eliminates GABAergic potentials, leaving only glycinergic ones, and the rate of spontaneous release is consequently reduced. (**Ci-ii**) Subsequent application of 150μM phenylephrine significantly increases the rate of spontaneous glycine release ($P<0.05$). (**D**) Average number of glycinergic IPSPs per 30-second interval. (**E**) Plot of number of IPSPs against duration, revealing that although the frequency of spontaneous glycinergic IPSPs is increased, their time course is unaffected (see also inset showing 10 overlays of glycinergic IPSPs under bicuculline and phenylephrine). *) $P<0.05$

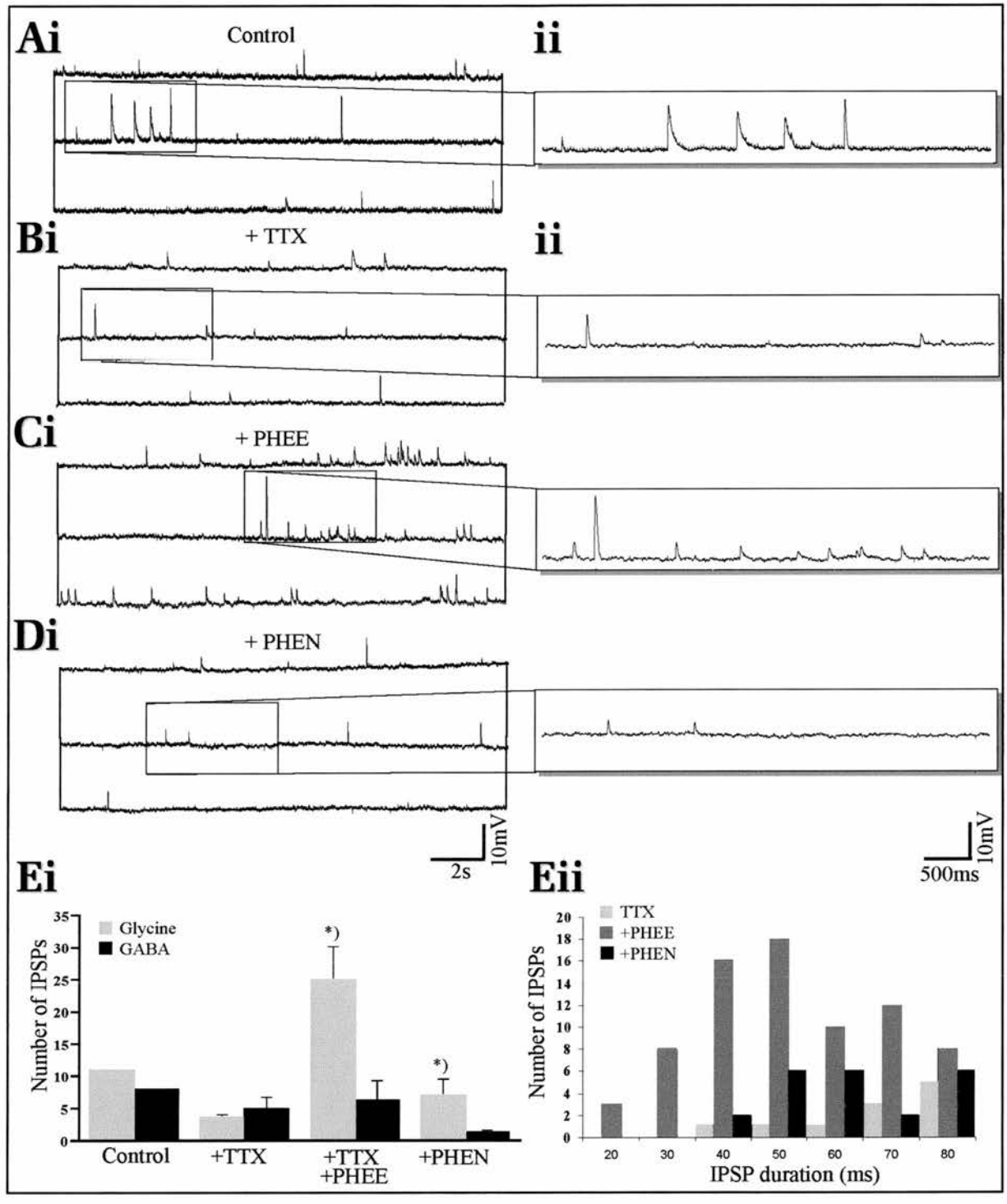

Figure 5.10: *Phenylephrine pre-synaptically enhances the spontaneous release of glycine.* (**Ai-ii**) Recording from an embryonic motor neuron during the periods between swimming episodes reveals glycinergic and GABAergic IPSPs. Following addition of TTX (**Bi-ii**) and application of phenylephrine (**Ci-ii**) increased the frequency of glycinergic, but not GABAergic potentials. This effect was counteracted by addition of phentolamine (PHEN) (**Di-ii**). (**Ei**) Plot of average number of IPSPs per 30-second sample time under each set of conditions. (**Eii**) Plot of the duration of glycinergic potentials under each set of conditions shows that although their duration is more variable under phenylephrine there was no elongation of their time course. *) $P<0.01$

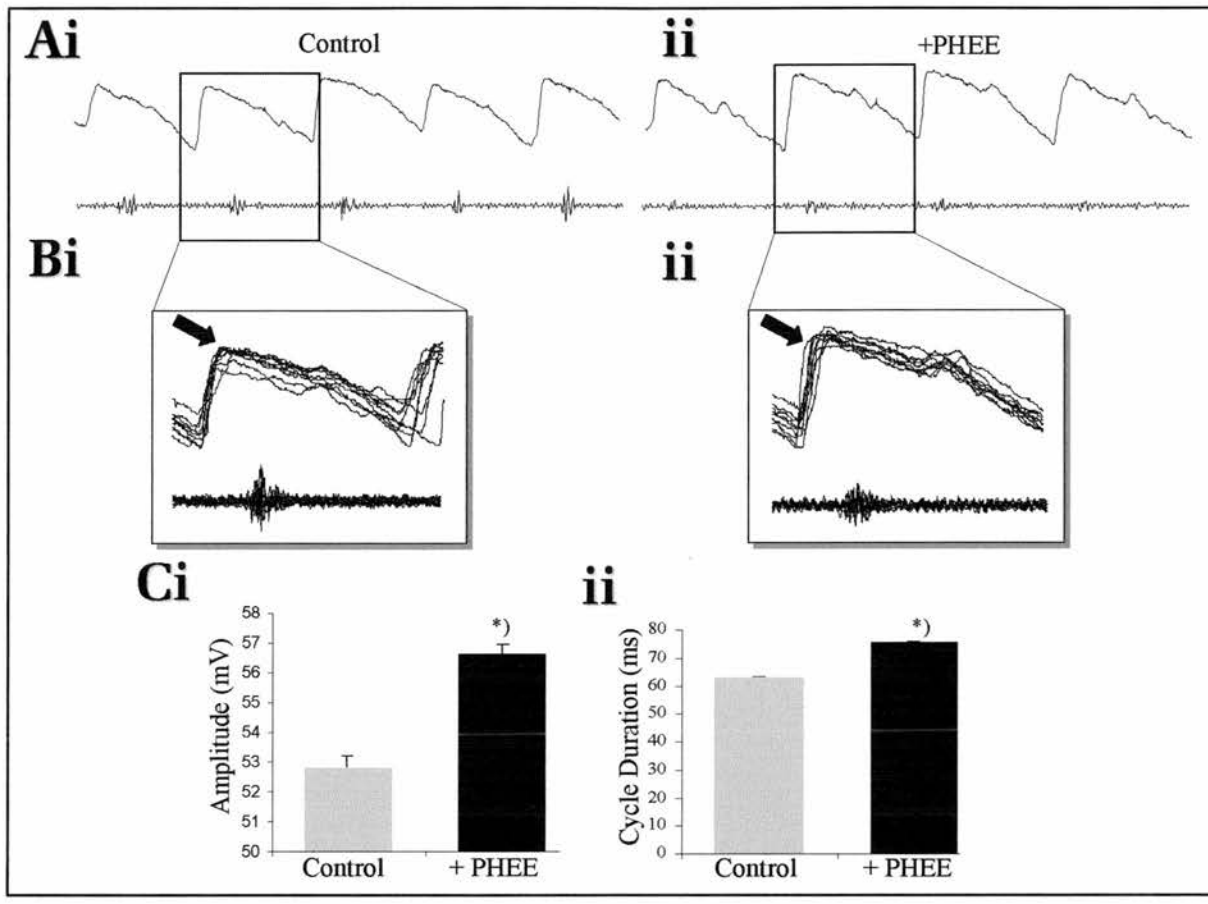

Figure 5.11: *Phenylephrine increases the amplitude of mid-cycle IPSPs during swimming.* (**A**) Phenylephrine reduces swimming frequency in *Xenopus* (see also chapters three and four). (**B**) Overlaps of 10 representative cycles under control conditions (**Bi**) and after addition of 150μM phenylephrine (**Bii**) reveal that swimming becomes slower, associated with an increase in the amplitude of the mid-cycle inhibition (arrowed). Pooled data from 3 episodes during control and under phenylephrine reveal that the amplitude of the mid-cycle IPSP increases under phenylephrine (**Ci**) co-incident with an increase in the cycle duration (**Cii**). Note that although the amplitude of the ventral root is reduced under phenylephrine, this is most likely as a result of the recording quality, as a more detailed study in chapter three showed that phenylephrine has no significant effects on burst amplitudes. *) $P<0.01$

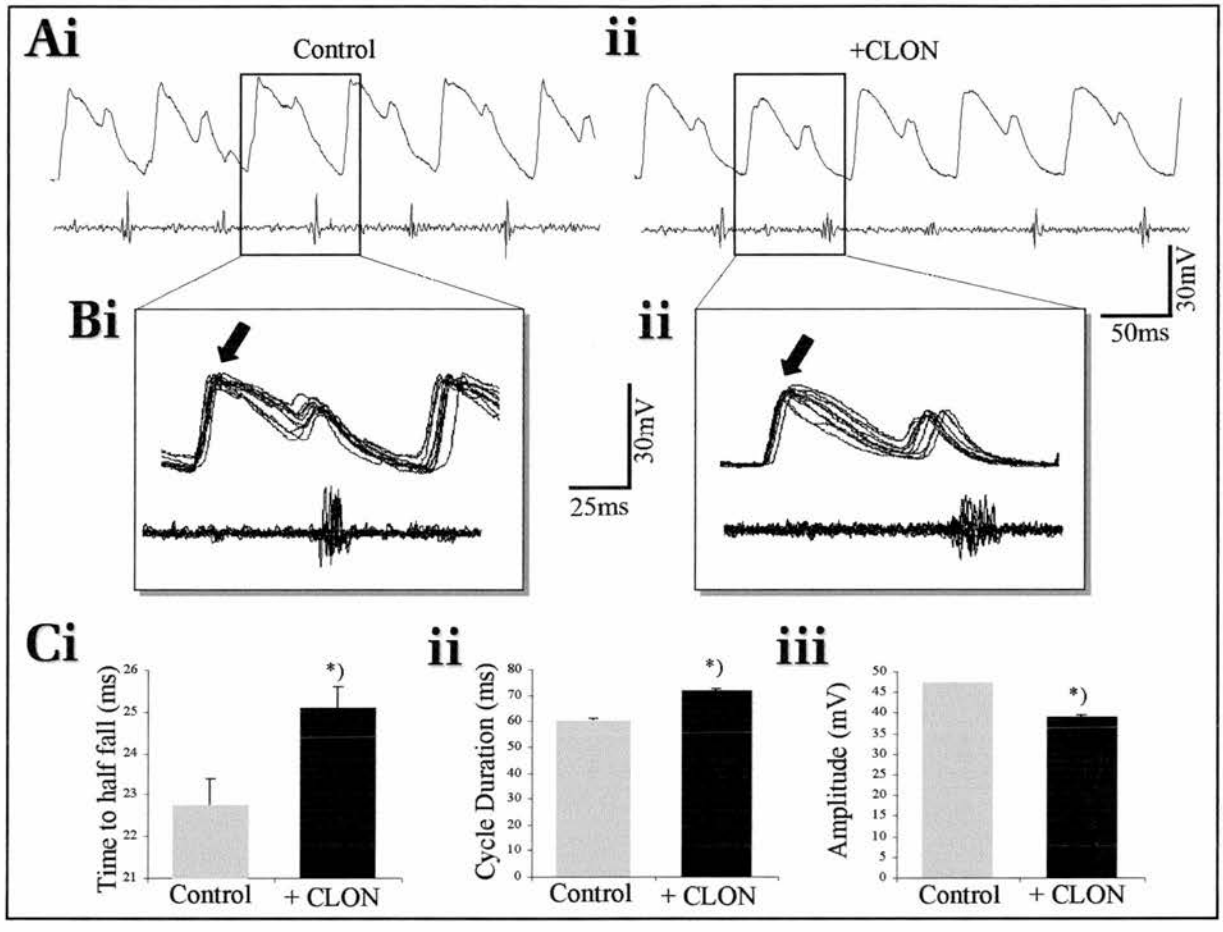

Figure 5.12: *Clonidine slows swimming by prolonging mid-cycle inhibition.* (**A**) Clonidine reduces swimming frequency in *Xenopus* (see also chapters 3 and 4). Note also the reduction in burst amplitude in the presence of clonidine (see Figure 3.4F) (**B**) Overlaps of 10 representative cycles under control conditions (**Bi**) and after addition of 120μM clonidine (**Bii**) reveal that clonidine slows swimming and prolongs the duration of the mid-cycle IPSP (arrowed), which delays the onset of the excitation. (**Ci**) Pooled data from 3 episodes of swimming during control and under clonidine (10 cycles per episode) reveal that that time to half fall of the mid-cycle IPSP increases under clonidine (N=4, P<0.0.01), as does the total cycle duration (N=4, P<0.001; **Cii**). The amplitude of the mid-cycle IPSP is also reduced after addition of clonidine (**Ciii**). *) P<0.05

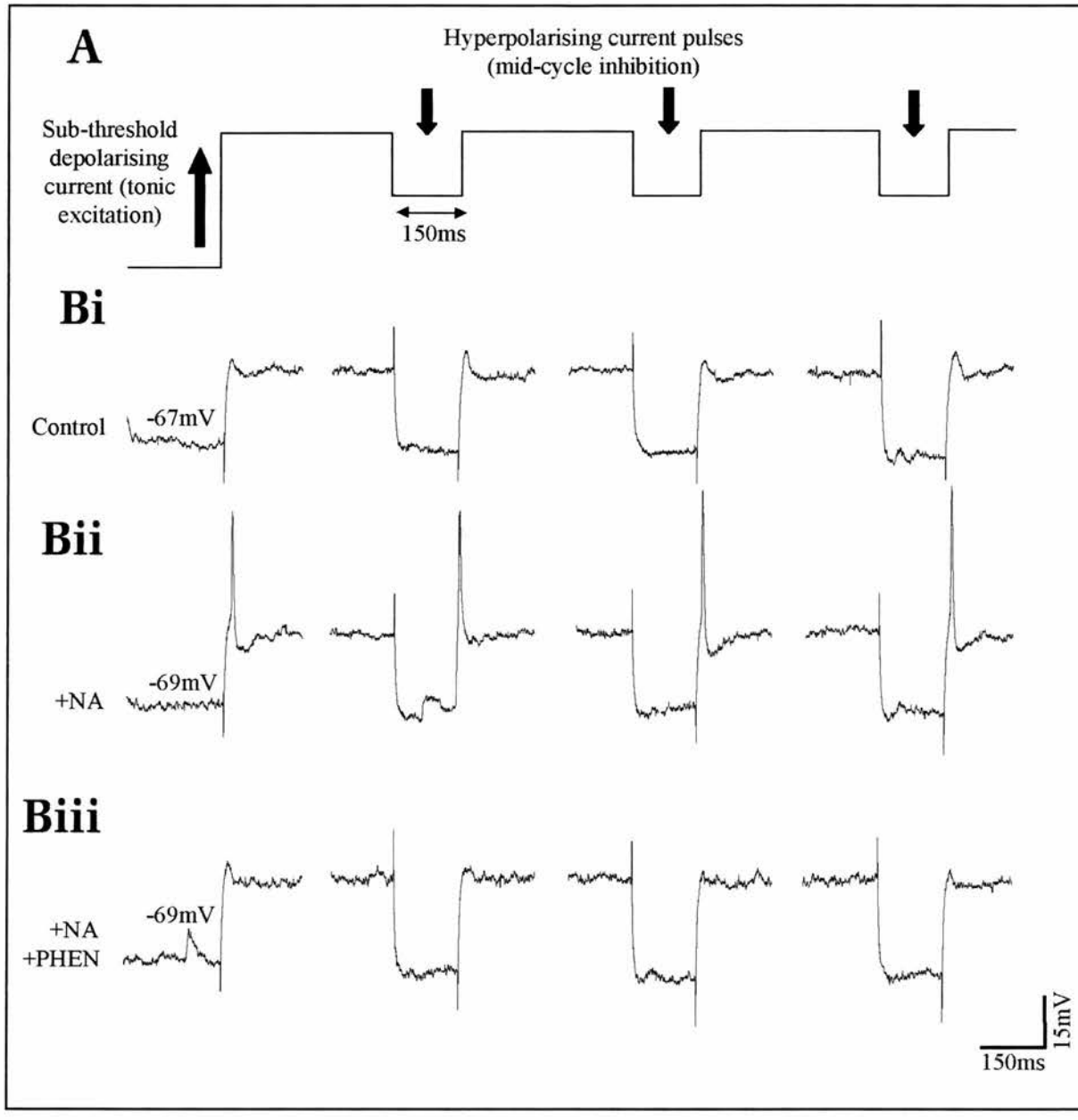

Figure 5.13: *NA enhances the probability of post-inhibitory rebound firing.* (**A**) The synaptic inputs during swimming were simulated by injecting a positive current (tonic excitation) just below spike threshold, superimposed upon which were brief (150ms) negative currents (mid-cycle IPSPs), at just below the threshold required to produce a rebound spike. (**Bi**) The protocol described above during control conditions does not elicit either a spike at the onset of the depolarising current or at the end of the hyperpolarising pulses. (**Bii**) After application of 10μM NA, even when the above conditions were held constant, the probability of rebound firing is enhanced and a post-inhibitory rebound spike is produced. Note also the reduction in spike threshold under NA, as shown by the spike at the onset of the depolarising current. (**Biii**) These effects could be counteracted by application of phentolamine.

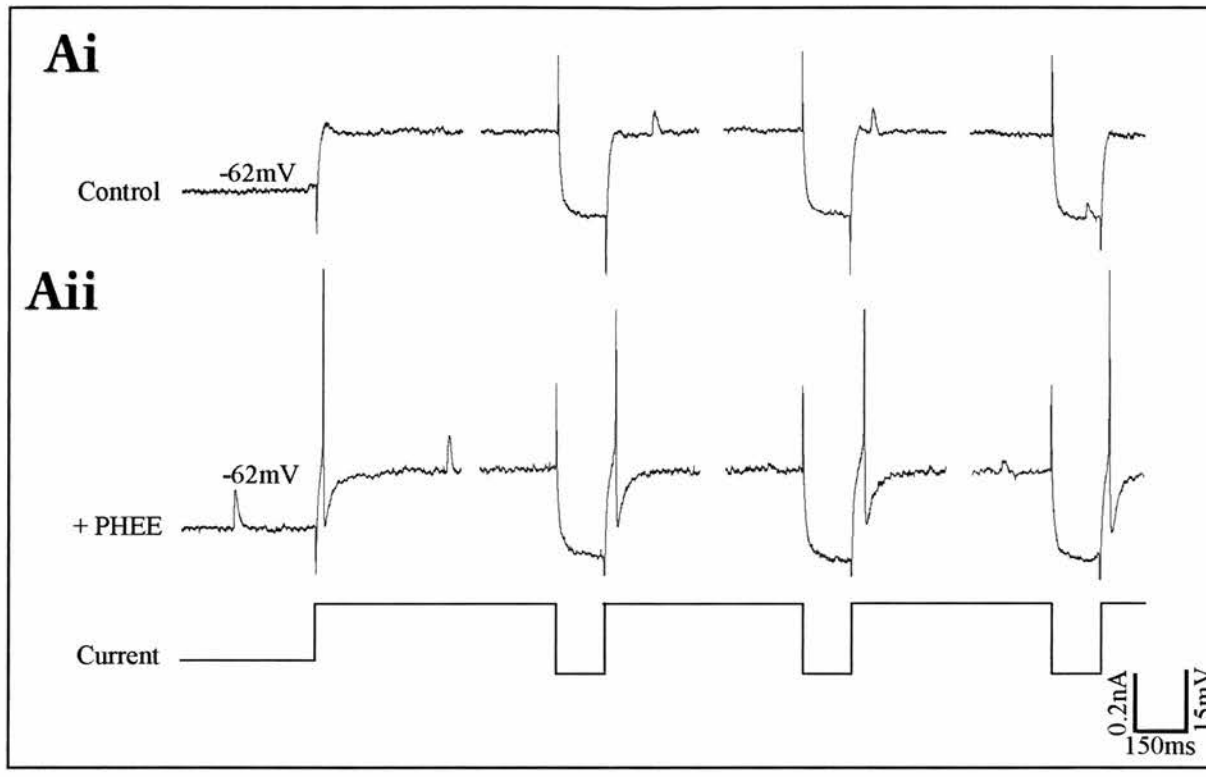

Figure 5.14: *α_1-adrenoreceptor activation enhances post-inhibitory rebound firing.* (**Ai**) As described in detail in the text and illustrated in Figure 5.13, the synaptic drive for swimming was simulated using depolarising and hyperpolarising current pulses. (**Aii**) After activation of α_1-adrenoreceptors by application of phenylephrine, spike threshold was reduced and rebound firing was enhanced. There was no effect on the resting membrane potential (-62mV throughout), or on membrane conductance as measured by amplitudes of the voltage deflections incurred following hyperpolarising current pulses.

Chapter Six

Noradrenergic modulation of sensory processing and the gating of behavioural selection in *Xenopus*

6.1 Summary.

In this chapter I have investigated the noradrenergic modulation of sensory processing in the spinal cord of *Xenopus* and examined its possible role in gating afferent inputs to elicit different behavioural responses. Whilst brief stimulation of the skin can elicit swimming (characterised by alternating spikes of activity progressing along the body with a rostro-caudal delay), more prolonged stimulation can induce struggling- longer bursts of motor activity which propagate caudal to rostral along the body.

Bath application of NA, or α-adrenoreceptor agonists, could elicit bouts of struggling activity, both at the start, and within episodes of fictive swimming elicited by brief stimulation of the tail skin. When struggling was induced following more prolonged activation of skin sensory cells, blocking α-adrenoreceptors with phentolamine reduced both the frequency of motor bursts and the caudo-rostral delay in their propagation. Similarly, antagonism of β-adrenoreceptors with propranolol also reduced both burst frequency and caudo-rostral delays. Furthermore, both antagonists reversibly reduced the ability to induce struggling behaviour following even repetitive and prolonged stimulation of the skin. When phentolamine and propranolol were co-applied, both the frequency of motor bursts and the caudo-rostral delay in their propagation were again reduced. Often, however, the frequency of motor bursts was comparable with that during swimming and the firing pattern of neurons had switched to become rostral-to-caudal, a characteristic of swimming rather than of struggling. These findings suggest that adrenoreceptor activation might play a pivotal and intrinsic role in gating behavioural selection between swimming and struggling within the spinal cord of *Xenopus*.

In the presence of NA, or following α-adrenoreceptor activation, the threshold stimulus intensity required to initiate swimming increased. This effect could be counteracted using phentolamine or by returning to fresh control saline. Both GABAergic and glycinergic pathways also appear to be utilised during this noradrenergic modulation of sensory processing. Furthermore, intracellular recordings from motor neurons reveal bicuculline-sensitive IPSPs, in the presence of NA, when the ipsilateral tail skin is stimulated just below the threshold required to initiate swimming. This suggests that NA unmasks a previously silent inhibitory pathway that controls the sensitivity of the R-B afferent pathway. In combination with my earlier findings, this evidence suggests that NA is a ubiquitous neuromodulator within the spinal cord of *Xenopus*, acting at multiple levels within the pattern generating network to modulate and gate sensory inputs whilst additionally modulating the motor outputs which these stimuli elicit.

6.2 Introduction.

It is well recognised that central pattern generators for locomotion can generate activity even when isolated from sensory feedback (e.g. Delcomyn, 1980; Marsden *et al.*, 1984). However, in intact animals a close correspondence must be maintained between the central pattern generators responsible for generating locomotion and the sensory neurons from which they take their cues. Such a relationship rapidly allows an appropriate behavioural response to be extracted from different sensory inputs, providing a fast response to sudden environmental changes, such as an impending threat from predators. Neuromodulators are thought to be important, in part, for altering the integration of sensory cues by modulating both individual membrane properties and the synaptic interactions between them.

Afferent information has three main roles to play in the production of motor patterns: timing motor activity, controlling the transition from one phase of movement to another (e.g. cat, Grillner & Rossignol, 1978; lamprey, Grillner *et al.*, 1981; locust, Pearson, 1995; Reye & Pearson, 1988; crayfish, Sillar *et al.*, 1986) and reinforcing ongoing motor activity (for example, load-sensitive afferents reinforce motor neuron activity during the stance phase of walking- e.g. Stephens & Yang, 1999; Fouad & Pearson, 1997; for more general reviews see MacKay-Lyons, 2002; Rossignol *et al.*, 1988; Pearson, 1993). As well as adapting ongoing motor activity, different afferent inputs may also allow switching between two distinct motor outputs, thus generating different behaviours. There is some evidence that single neurons can trigger behavioural switching as a function of their firing rate. For instance, in the stomatogastric motor networks of the lobster, the anterior gastric receptor, a primary mechanosensory neuron, can permit either a synchronous activation of all power-stroke gastric teeth motor neurons, or, when firing more

strongly, can elicit a different pattern in which medial and lateral teeth motor neurons fire in antiphase (Combes *et al.*, 1999). Similarly, in *Aplysia*, the central pattern generator for feeding can produce two distinct behaviours- ingestion and egestion, the production of which are controlled by two neurons, CBI-2 and CBI-3. Whilst CBI-2 neurons can produce egestion or ingestion, after recruitment of CBI-3 neurons, the motor pattern switches to produce only ingestion (Jing & Weiss, 2001). This effect could be mimicked by addition of the neuropeptide APGWamide which has been detected within CBI-3 neurons- thus suggesting that release of this peptide may underlie the switch between these two behaviours (Morgan *et al.*, 2002). Another well-defined example of motor pattern switching in response to changing afferent information is in *Xenopus*, where brief activation of mechanosensory R-B neurons can induce swimming, whilst more prolonged stimulation initiates struggling (Kahn & Roberts, 1982c; Soffe, 1991).

Struggling is characterised by alternating bursts of motor activity which propagate caudal to rostral along the body, in contrast to the alternating single motor neuron spikes of swimming along the spinal cord in a rostro-caudal drive (Figure 1.2; Figure 6.1B). Relatively little is known of the mechanisms that underlie the switch between swimming and struggling. Soffe (1993) showed that the pattern of synaptic drive is similar in both behaviours, i.e. a background excitation upon which alternating cycles of excitation and inhibition are superimposed. Since levels of excitation and inhibition appear to be critical factors in determining the outcome of motor activity, it is possible that subtle changes in excitation or inhibition might be effected by a neuromodulator.

As described in detail in chapter one, hatchling *Xenopus* possess a comparatively simple nervous system whose sensory pathways include receptors

primarily responsible for mechanoception and photoception (Figure 6.5A; Figure6.9; Roberts, *et al.*, 1983). As yet, no receptors specific for nociception have been identified. The behavioural response of *Xenopus* to these stimuli is easily studied by observing the nature of their motor output in immobilised animals. The primary mechanosensory receptors are the R-B cells that innervate the skin of the tadpole, which interestingly also show immuno-reactivity for Substance P in both their somas and their axons (Clarke *et al.*, 1984; Gallagher & Moody, 1987). The dorsolateral commissural interneurons and the dorsolateral ascending interneurons are the two classes of sensory interneuron that receive direct synaptic excitation from the axons of R-B cells (Figure 6.5A; Clarke & Roberts, 1984). Neither R-B nor dorsolateral interneurons are then involved in any further processing of this primary sensory response; the interneurons being actively inhibited during swimming. Swimming can be initiated either in response to a brief sensory stimulus which activates this R-B pathway, or when the illumination is reduced. Reduced illumination, such as when a shadow is cast over the water, excites pineal photoreceptors, which activate pineal ganglion cells. These in turn excite diencephalic/mesencephalic-descending interneurons that excite each half centre and initiate swimming (Figure 6.9A; Roberts *et al.*, 1998)

In the previous three chapters I have focused upon the neuromodulation of ongoing swimming activity in *Xenopus* by NA and the adrenoreceptor subtypes through which these effects are most likely mediated. However, in this chapter I consider an additional role for NA within the spinal cord. Firstly, NA may play an intrinsic gating role in priming the switch between swimming and struggling in response to the prevailing environmental needs. In addition, NA appears to modulate sensory processing within the spinal cord, desensitising the pattern-generating

network to further sensory inputs, possibly as part of an escape mechanism. The possible levels at which this modulation occurs and the mechanisms that might underlie it are discussed.

6.3 NA and α-adrenoreceptor activation can induce bouts of fictive struggling.

In the preceding chapters, I have shown that NA and α-receptor activation (using the specific agonists phenylephrine and clonidine) modulates ongoing swimming activity. The concentration ranges at which these effects occur were determined as 4-6μM for NA, 50-100μM for phenylephrine and 35-110μM for clonidine. However, at concentrations just above these levels, application of NA or activation of α-adrenoreceptors using these agonists can illicit bouts of fictive struggling either at the start of, or during episodes of evoked swimming (e.g. Figure 6.2B). Furthermore, the noradrenergic uptake inhibitors protriptyline (N=4) and maprotiline (N=3) at concentrations of 80-100μM could also induce bouts of struggling during swimming. Indeed, with prolonged application of these drugs, struggling became the only response to either brief or more sustained sensory stimulation (Figure 6.2C).

These preliminary observations raised the intriguing possibility that NA and the associated activation of adrenoreceptors might play a role in affording the behavioural selection between swimming and struggling. This hypothesis has therefore been examined in more detail, looking specifically at the effects of both NA and activation of adrenoreceptor classes during mechanically-induced fictive struggling.

6.4 Mechanically induced struggling is less reliably evoked in the presence of α- or β-adrenoreceptor antagonists.

In the following series of experiments, pressure applied to the skin overlying the head of immobilised animals (see Figure 6.1A) induced bouts of fictive struggling. Whilst the amount of pressure applied was not quantified, comparable pressure was applied under each set of experimental conditions. The experiments below were performed on both embryos and larvae as no developmental difference in the ability to induce struggling was detected.

Pressure applied to the head skin of 14 animals produced 2-10 cycles of struggling under control conditions. However, in the presence of the α-adrenoreceptor antagonist phentolamine (>50μM, *N*=4), the β-receptor antagonist propranolol (>40μM,*N*=5) or co-application of phentolamine and propranolol (*N*=5), the force required to initiate fictive motor activity was much greater than during control conditions. This effect, although not possible to quantify without measurement of the force applied, was partially reversed upon a return to control saline. Often, when applying pressure comparable to that used under control conditions, only swimming, rather than struggling could be induced in the presence of adrenoreceptor antagonists.

6.5 Effects of α-adrenoreceptor antagonists on the parameters of struggling.

Not only was the probability of struggling reduced by phentolamine and propranolol, but, when it was induced, both antagonists could modulate the parameters of struggling activity. In 4 animals, the broad-spectrum α-adrenoreceptor antagonist phentolamine (70-120μM) was applied. Following pressure to the head

skin, comparable to that required in control conditions to induce struggling, cycle periods were reduced in the presence of phentolamine by 30.5 ± 13.7% (N=4, P<0.05; Figure 6.3; average cycle period in control 141.3 ± 13.8ms). In 3 of these animals, the frequency of motor bursts under phentolamine (~13Hz) was comparable to the range normally produced during swimming (10-20Hz), whereas the frequency of motor bursts during struggling is typically less than 10Hz. In 2 animals, which were returned to control conditions, the effects of phentolamine on cycle periods were counteracted (N=2, P<0.01; Figure 6.3B,C). The caudo-rostral delay in motor neuron activation was also reduced by 40.3 ± 14.4% after application of phentolamine (N=4, P<0.05; Figure 6.3B,D; average delay in control 21.3 ± 2.4ms). However, in none of the animals tested did the propagation of neural activity switch to progress rostro-caudally along the body. The effect on delays was counteracted in 2 animals by washing with control saline (N=2, P<0.05; Figure 6.3D). Phentolamine did not have any significant effects on the duration of motor bursts during struggling (N=4, P>0.05; data not shown; average burst duration in control 66.5 ± 3.3ms).

The finding that phentolamine reduces both the frequency of struggling motor activity and the delay with which this activity progresses along the body parallels the effects on these parameters during swimming, where activation of both α_1- and α_2-adrenoreceptors modulates swimming frequency, with α_1-receptors additionally affecting the delay in motor neuron activation. The next approach, therefore, was to examine whether β-receptor activation (which modulates RC-delays during swimming; Fischer *et al.*, 2001) might also be involved in the noradrenergic modulation of struggling.

6.6 Blocking β-adrenoreceptors reduces cycle periods and caudo-rostral delays during mechanically-induced struggling.

In 5 animals, the broad-spectrum β-adrenoreceptor antagonist propranolol (70-120μM) was bath applied. After inducing bouts of struggling using mechanical stimulation of the head skin, cycle periods under propranolol fell by 27.9 ± 4.1% (N=5, P<0.05; average cycle period in control 108.9 ± 12.7ms; data not shown). In 2 animals, a return to control conditions significantly counteracted this reduction in motor burst frequency (N=2, P<0.05). Under propranolol there was a greater reduction in caudo-rostral delays than with phentolamine. Delays were reduced by 52.7 ± 19.7% (N=5, P<0.001; average delay in control 17.7 ± 5.2ms; data not shown). In one animal, after addition of 65μM propranolol, caudo-rostral delays were significantly (P<0.001) reduced from an average of 16.1 ± 10.91ms in control to –11.27 ± 9.9ms (i.e. they became rostro-caudal). A return to control saline in 2 animals partly counteracted these effects on delays (N=2, P<0.05). As was the case with phentolamine, propranolol had no significant effects on the duration of motor bursts during struggling (N=5, P>0.05, data not shown). These results suggest that not only α-, but also β-adrenoreceptors appear to modulate both swimming and struggling. β-receptor mediated effects on longitudinal co-ordination have also been found during swimming (Fischer *et al.*, 2001; see also chapter 3), however, unlike during struggling, this receptor class was not found to affect motor burst frequency.

6.7 Simultaneously blocking α- and β-adrenoreceptors can elicit swimming in response to a sensory stimulus appropriate to induce struggling.

Having found evidence above that both α- and β-adrenoreceptor classes might play a role in modulating struggling, the next set of experiments used co-application of phentolamine and propranolol to investigate the combined role of α- and β-receptors, and to examine whether they might merely modulate ongoing activity or play a role in effecting the transition from swimming into struggling.

Co-application of phentolamine (50-100μM) and propranolol (30-50 μM) to 5 animals reduced cycle periods by 40.8 ± 5.2% (N=5, P<0.001; average in control 177.3 ± 8.5ms; Figure 6.4B,C). In 3 of the 5 animals, cycle periods were reduced such that motor bursts were produced at a rate comparable to that during swimming (~20Hz). The effects of phentolamine and propranolol were counteracted in 3 animals by returning to control conditions (N=3, P<0.05; Figure 6.4C). Caudo-rostral delays were reduced after the co-application of phentolamine and propranolol by 104.2 ± 17.2 (N=5, P<0.01; average delay in control 33.7 ± 9.2ms; Figure 6.4Bi,E). As is evident from this pooled data, the reduction in caudo-rostral delays greater than 100%, (in 1 animal as much as 143%) indicates that the firing pattern of neurons had switched to become rostro-caudal, a characteristic of swimming not of struggling. In 2 animals in which a wash was performed, these effects on longitudinal delays were counteracted and they became caudo-rostral again. Burst durations were also reduced in the presence of both propranolol and phentolamine by 37.9 ± 4.7% (N=5, P<0.001; average in control 67.8 ± 3.5ms; Figure 6.4B). Again, a return to control conditions in 2 animals counteracted this effect on burst duration.

The simultaneous blocking of both α- and β-adrenoreceptor classes reveals that not only do these receptors appear to be involved in modulating ongoing swimming and struggling, but their co-activation may facilitate (or even trigger) a switch between the two behaviours. The relevance of these findings is explored in more detail in the discussion below.

6.8: NA and α-adrenoreceptor activation decrease spinal sensory transmission.

In the experiments above I have identified a potential role for NA in gating sensory inputs to effect different behavioural outcomes, but NA also appears to have a wider modulatory role upon sensory transmission in the *Xenopus* spinal cord. Preliminary data by McDearmid (1998) showed that NA reduces sensory transmission in both embryonic and larval preparations. Thus the electrical stimulus to the tail skin required to initiate activity was increased, although the mechanisms which underlie this modulation were not fully investigated. The experiments that follow examine this additional role for NA in more detail.

The effect on threshold was examined by initiating swimming using an electrical stimulus via a suction electrode placed on the tail skin (see Figure 2.1). 1ms pulses were applied, beginning at 2V in amplitude and increasing in 2V increments until swimming was evoked. This was regarded as the threshold voltage. Within 5 minutes after addition, to both embryonic (N=15) and larval (N=15) animals, NA increased the threshold voltage required to evoke fictive swimming activity (c.f. McDearmid, 1998). The effect was more profound in larvae than in embryos, with 2-4μM of NA typically increasing the threshold by between eight and ten fold in the later stage animal. By comparison, addition of 2-4μM NA to embryonic preparations only increased the voltage threshold by around four fold.

These effects were fully reversible upon return to control saline at both stages of development. Furthermore, the effects of NA on sensory transmission could be counteracted using the general α-receptor antagonist phentolamine (*N*=5). For example, in Figure 6.5B, NA increased the voltage required to initiate swimming by approximately 2.5 fold, from an average of 10.5 ± 0.3V in control to 27.5 ± 0.3V after addition of 4μM NA. Subsequent addition of phentolamine reversed this effect and the swim voltage threshold fell to 11.6 ± 0.7V. The ability of a general α-adrenoreceptor antagonist to counteract the effects of NA suggests that activation of α-receptors may mediate this noradrenergic decrease in sensory transmission.

The specific α_1-agonist phenylephrine (*N*=4, Figure 6.5C) and the α_2-agonist clonidine (*N*=4; Figure 6.5D) were next used to examine the role of α-receptors in more detail. Both were able to mimic the effects of NA in increasing stimulus threshold and returning to control conditions could counteract their effects. For example, in Figure 6.5C, phenylephrine increased the swim initiation threshold from 16.5 ± 0.5V to 33.5 ± 3.3V whilst returning to control conditions reduced it again to 25.8 ± 4.1V.

These findings indicate that both α_1- and α_2-adrenoreceptors may participate in the noradrenergic modulation of sensory transmission. In the previous chapters I have investigated the interaction between these adrenoreceptor classes and inhibitory glycinergic and GABAergic pathways during the modulation of ongoing motor activity. The next series of experiments investigates whether utilisation of these pathways may additionally be involved in the noradrenergic modulation of sensory transmission.

6.9 Inhibitory pathways underlie the noradrenergic modulation of sensory transmission.

My first approach was to investigate the possible utilisation of glycinergic pathways by applying strychnine (*N*=3; 1μM) subsequent to an increase in threshold by NA. As shown in the example in Figure 6.6A, blocking glycine receptors could partially counteract the effects of NA. However, when strychnine was pre-applied (*N*=3; Figure 6.6B), it could not occlude the noradrenergic effect. Furthermore, addition of 20-40 μM of bicuculline (*N*=4) was also able to partially counteract the decrease in sensory transmission observed after application of NA (Figure 6.6C). These findings suggest that both GABAergic and glycinergic inhibitory pathways might be utilised by NA to modulate sensory transmission. This finding corroborates evidence obtained for the role of α-receptors in the modulation of swimming in which both α_1- and α_2-receptors results in the enhancement of glycinergic pathways, whilst α_2-receptor activation additionally results in the utilisation of GABAergic pathways. It should be noted, however, that as dlc interneurons are inhibited during swimming (by glycinergic inhibition; Figure 6.5A), it is perhaps not surprising that application of strychnine sensitises this sensory pathway.

Further evidence for the involvement of GABAergic pathways was provided by the ability of muscimol (*N*=5, 0.5μM; $GABA_A$ receptor agonist) to raise the swim-initiating threshold, an effect which was counteracted using bicuculline (40-50μM). For example, in Figure 6.6D, the threshold required to initiate swimming increased from 15.5 ± 0.4V in control to 25.0 ± 1.0V after addition of 0.5μM muscimol. Bicuculline reduced the threshold to 12.0 ± 0.0V. At higher (1-4μM) concentrations, muscimol reduced sensory transmission further, requiring repetitive

pulses at the maximum (100V) limit of the stimulator to initiate fictive swimming (data not shown). Finally, the GABA uptake inhibitor nipecotic acid (N=3; 200-400μM) could increase the threshold voltage required to initiate swimming. In the example shown in Figure 6.6E, the threshold increased from 7.6 ± 0.4V in control to 15.6 ± 1.5V after application of 300μM nipecotic acid. This effect was reversed after subsequent addition of bicuculline.

This series of experiments suggests that GABAergic inhibition, and presumably the activation of $GABA_A$ receptors, might be utilised during the noradrenergic modulation of sensory processing. However, whilst these tentative observations based on extracellular data strongly suggest that the sensory triggering of swimming is inhibited by NA, intracellular recordings of both sensory and motor/pre-motor neurons are required to tease out the levels of sensory/motor integration at which this modulation occurs.

6.10 NA modulates GABA release in the skin sensory pathway.

As a first approach to try to identify the level(s) at which NA may modulate sensory processing, sharp microelectrode recordings were made from presumed motor neurons in the ventral spinal. In response to a single electrical stimulus to the ipsilateral tail skin, at a threshold just below that required to initiate swimming, a single depolarising (when recording using KCl-filled microelectrodes; see Figure 1.4) post-synaptic potential could be recorded (N=13/17 (10 embryos, 3 larvae); Figure 6.7A). After addition of 4-8μM NA, the threshold required to initiate swimming increased (see section 6.8) and sub-threshold ipsilateral skin stimulation now evoked a flurry of depolarising PSP's (Figure 6.7B). This effect could be blocked either by the addition of phentolamine, suggesting that α-adrenoreceptor

activation underlies this effect of NA (Figure 6.7C), or the $GABA_A$ receptor antagonist bicuculline (Figure 6.8Aiii), suggesting that these potentials were IPSPs and GABAergic in origin. In addition, the time course of these PSP's (~100-200ms) also suggested that they were GABAergic (see overlays in Figure 6.8B, c.f. Reith & Sillar, 1998). Furthermore, these potentials were still present in the presence of strychnine (Figure 6.8A-C). This suggests that NA unmasks a previously silent inhibitory pathway that controls the sensitivity of the R-B to motor neuron afferent pathway.

6.11 NA restores the dimming response in larval tadpoles.

In *Xenopus* embryos, fictive swimming can be elicited either by brief stimulation to the head or trunk skin (discussed in detail above) or by dimming of the illumination (see section 6.2 above). This excites pineal photoreceptors, which excite pineal ganglion cells. These are thought to excite diencephalic/mesencaphalic-descending interneurons which activate each half-centre (Figure 6.9A). By larval stage 42, the reliability of this response was considerably reduced and swimming could not usually be elicited in this way. After application of NA (4-6μM) to embryos (N=10), there was no effect on the reliability of the dimming response (Figure 6.9B). In larvae (N=5/7), dimming of the illumination elicits either no response or a brief flurry of IPSPs (Figure 6.9C), however swimming cannot be evoked. In the presence of NA, dimming of the illumination can reliably and reversibly evoke episodes of swimming (Figure 6.9C).

6.12 Discussion

One of the most important attributes of neuromodulation is its ability to integrate sensory cues to ensure a suitable behavioural outcome, by modulating both the membrane properties of individual neurons and the synaptic interconnections between them. In the preceding chapters I have examined the mechanisms through which NA and adrenoreceptor activation finely tunes motor output in the *Xenopus* spinal cord. In this chapter I have examined the noradrenergic modulation of afferent inputs to the same spinal pattern-generating network and have considered a possible role for NA in gating the processing between these inputs and the generation of different motor outputs. The implication of these findings is now discussed in more detail.

Several lines of evidence provided above suggest that NA could play a role in gating the switch between the generation of swimming versus struggling behaviour. Firstly, both NA and activation of α-adrenoreceptors, (which in the preceding chapters I have shown to mediate the effects of NA), were able to induce bouts of struggling during episodes of swimming. Secondly, and conversely, application of α- and β-adrenergic antagonists significantly reduced caudo-rostral delays and cycle periods during mechanically-induced struggling. Thirdly, the reliability with which struggling was elicited using this method was greatly reduced. Indeed, when α- and β-adrenoreceptors were simultaneously blocked, swimming was often evoked rather than struggling. Whilst this evidence is clearly indicative of a pivotal role for NA in effecting the switch between the two behaviours, how sure can one be that these effects are not merely artifacts of, for example, increasing levels of inhibition, that a single switch might be activated by multiple routes, or that the intrinsic mechanism by which this switch occurs remains to be discovered?

In the preceding chapters and in an earlier study (McDearmid *et al.*, 1997), NA and α-adrenoreceptor activation have been shown to enhance both glycinergic and GABAergic inhibition during the modulation of swimming. As a result, swimming becomes slower and there is a shortening of the RC-delay. This slowing in the frequency of motor output and a concomitant change in the longitudinal firing properties of neurons are precisely the defining features of struggling versus swimming. Presumably, therefore, when NA or adrenoreceptor agonists induce bouts of struggling during swimming, they may be doing so by increasing levels of inhibition and conversely, use of antagonists at these receptors reduces inhibition and disrupts the generation of struggling. Indeed, reducing glycinergic inhibition either with strychnine or by surgical transection of the spinal cord, thus removing input from glycinergic commissural interneurons, can both disrupt struggling, whereas although reduced glycinergic transmission dramatically speeds up swimming, it does not prevent its generation (Soffe 1993, Green & Soffe, 1998).

Excitation is also important for the induction and maintenance of struggling. Excitation is stronger during struggling than swimming, and whereas only around 75% of motor neurons and their associated interneurons fire during swimming, they nearly all fire during struggling (Soffe, 1993). Furthermore, low doses of excitatory amino acids such as kainate and glutamate can evoke swimming, whilst at higher doses struggling is elicited. Struggling cannot however be evoked by NMDA, suggesting that this class of receptors are at least not required for its induction (Soffe, 1996). There is as yet no evidence that either NA or adrenoreceptor activation directly affects levels of excitation, either during swimming or struggling, but simultaneous effects on both excitation and inhibition cannot be ruled out.

In the previous chapter, I have shown that NA can directly affect the properties of motor neurons, enhancing their ability to fire from post-inhibitory rebound. In addition, greater inhibition under NA is also likely to enhance rebound firing (see section 5.11 for more details), thus raising the intriguing possibility that NA could promote recruitment of both excitatory and inhibitory interneurons during the transition from swimming to struggling. It has been suggested that post-inhibitory rebound contributes to the generation of rostro-caudal delays in *Xenopus* (Tunstall & Roberts, 1994). As I have argued in chapter five, it is possible that this phenomenon contributes to the reduction in delays by NA. Furthermore, when the frequency of motor output is dramatically reduced, such as under NA, and it becomes harder to sustain ongoing activity, rebound spike facilitation allows the maintenance of these longer cycle periods. Hence under elevated levels of NA, an enhancement of rebound firing alone (not withstanding other synaptic effects) can partially account for the longer cycle periods and changes in longitudinal firing during struggling.

It seems reasonable to conclude that controlling the fine balance between excitation and inhibition within the spinal cord defines the nature of the motor output produced, irrespective of the route via which this balance is altered. It is possible that rather than wholly gating the switch between these two motor patterns, NA could be simply priming and reconfiguring the network in such a way as to facilitate the switch between them. Certainly the reduction in swimming frequency and the delay in rostro-caudal firing are at one end of the normal range of activity characteristic of swimming and closer to that produced during struggling. Under NA, swimming frequency may be reduced to such an extent that swimming can no longer be viably sustained and the network is forcibly reconfigured to generate struggling.

Indeed, at higher concentrations of bath-applied NA, bouts of struggling were often observed during episodes of swimming.

The fact that sensory transmission is inhibited by noradrenergic modulation adds another intriguing layer of complexity to the hypothesis that NA underlies the switch between swimming and struggling in *Xenopus*. Despite the fact that sensory neurons do not fire during either fictive swimming or fictive struggling (Soffe, 1991), different temporal patterns of sensory stimuli can determine which behaviour is produced. In the presence of NA, the sensory pathway becomes less responsive to the light touch or brief stimuli required to initiate fictive swimming. When trying to initiate struggling through more sustained stimulation, the use of noradrenergic agonists desenstitises the sensory network to such stimuli. In many cases swimming as opposed to struggling is observed. It is therefore possible that NA is acting as a neuromodulator in response to mechano- and/or nociceptive inputs. In response to light stimuli, it suppresses the sensory components of the network (e.g. by increasing inhibition onto sensory and pre-motor interneurons). This could be an important component of an escape mechanism, whereby information from sensory inputs is reduced whilst the tadpole swims away from the source of the stimulation. Such a response has been illustrated in the crayfish escape system, where a single action potential in the lateral giant interneuron synaptically inhibits behavioural responses other than rapid flexion, which carry the animal away from the source of the stimulation (Wine, 1977a,b). When greater force is applied and more sensory (skin and R-B) cells are stimulated, this greater stimulus poses more of a threat to the animal and thus more intense and aggressive behaviour, struggling, is produced. Behaviourally, struggling is presumably an attempt to wriggle free from a grasp and it is possible that during it, the skin sensory pathway is desensitised to avoid the

animal self-activating skin receptors as it lashes about, thus allowing the attempt to escape to continue unhindered by further sensory inputs. The effects of the adrenergic antagonists on sensory transmission during struggling support this hypothesis. In short, the number of skin cells or R-B cells that are activated by a stimulus could control the release of NA. Low levels of NA, in response to a light stimulus, inhibit further sensory input and condition the animal for swimming. In contrast, a greater release of NA, resulting from prolonged stimulation, biases the motor pattern-generating network to produce struggling. This hypothesis would assume that not only R-B neurons, but also the locus coeruleus (or, as yet unidentified spinal sources of NA) receive direct afferent inputs, and as such the duration and intensity of the stimulus might control the amount of NA released. There is evidence that NA and substance P (thought to be present within R-B neurons; Gallagher & Moody, 1987) can be co-released (e.g. Kessler & Black, 1982). There is also evidence that NA is co-localised in afferent nerve cells with other neuropeptides such as somatostatin and neuropeptide Y (Franke-Radowiecka *et al.*, 2002). If this were the case in *Xenopus* (although there is, as yet, no evidence to suggest that it is), then it would be easy to see how activation of R-B neurons could increase the release of NA, which might then prime the network as described above, such as to make struggling more viable.

The results of this study provide more evidence on the mechanisms underlying the down regulation of sensory transmission in the R-B pathway. Firstly, the partial reversal of the effect of NA with the glycine receptor antagonist strychnine implies a possible role for glycinergic inhibition in the mediation of the R-B pathway, although as discussed above, this effect is not unsurprisingly since dlc neurons receive inhibition during swimming, which is presumably removed under

strychnine, thus allowing them to fire more readily. More interesting, however, is the effect of GABA receptor agonists and antagonists. After prior application of NA, the $GABA_A$ receptor antagonist bicuculline was able to prevent much of the decrease in sensory transmission. This is supported by the evidence that the $GABA_A$ receptor agonist muscimol could increase the threshold voltage required to initiate fictive swimming activity. These results suggest a role for GABA acting via $GABA_A$ receptors. Furthermore, the GABA uptake inhibitor nipecotic acid was, like NA, also able to decrease sensory transmission. Hence the effects of these GABAergic agents are consistent with NA decreasing sensory transmission by facilitation of GABAergic synapses. This hypothesis is supported by the appearance of bicuculline-sensitive IPSPs onto motor neurons after application of NA, co-incident with a decrease in sensory transmission. This suggests that NA is recruiting a previously silent inhibitory pathway, which inhibits the induction of motor activity, by directly inhibiting one or more types of neurons within the CPG. In addition, the broad-spectrum α-adrenoreceptor antagonist phentolamine was able to reverse the effects of NA on sensory transmission. This would imply α_1-and/or α_2-adrenergic receptors are the targets of NA. Indeed, this suggestion is reinforced by the finding that the α_1-adrenoreceptor agonist phenylephrine and the α_2-adrenoreceptor agonist clonidine could both increase the voltage threshold required to initiate swimming, although to a lesser extent than could NA. The fact that antagonising both of these receptors with phentolamine completely reverses the effects on sensory transmission, suggests that they may be co-activated by NA.

The mechanism by which NA is able to restore the response to dimming of the illumination in larval animals also remains to be elucidated. Although the basic pathway that underlies the initiation of swimming in response to reduced

illumination is known (Figure 6.9A), a detailed analysis of the synaptic interconnections within it has yet to be performed. Much of this processing is performed in the forebrain, with projections into the hindbrain from where the CPG's that produce swimming are activated. Clearly, NA could be acting at any of these levels and in restoring the ability to initiate swimming in larvae through this pathway is presumably unmasking what was previously a silent or inhibited pathway. At this later stage of development where swimming cannot be so reliably elicited by dimming the illumination, the pineal pathway might be inhibited, possibly as effects mediated by the eyes have superseded it, or because NA selectively facilitates a response from the eyes rather than the pineal gland. In support of this conclusion, it is also interesting to remember that by contrast, NA simultaneously reduces sensory transmission from skin cell afferents. As discussed above, the acquisition of a more flexible larval motor pattern parallels the development of serotonergic projections into the spinal cord. At the same time, the ability of this pathway to evoke swimming is also diminished. It feasible therefore that this might be a consequence of increased serotonergic innervation, upon which NA may have a reciprocally inhibitory effect.

In this chapter I have explored a range of modulatory roles for NA on the processing of sensory information within the spinal cord of *Xenopus*. Whilst a detailed investigation of the synaptic levels at which NA exerts its effects on these afferent pathways remains to be completed, it is clear from these results that NA has profound inhibitory effects on sensory processing, affording the animal the ability to ignore extraneous environmental cues, so that its behaviour can continue unhindered. More importantly though, there is good evidence that NA may be an intrinsic neuromodulator of both the sensory and motor components of the spinal pattern

generator, capable of effecting and sustaining a transitional switch between two distinct behaviours, and that these differential effects might be controlled by changing levels of NA, possibly as a direct result of sensory inputs.

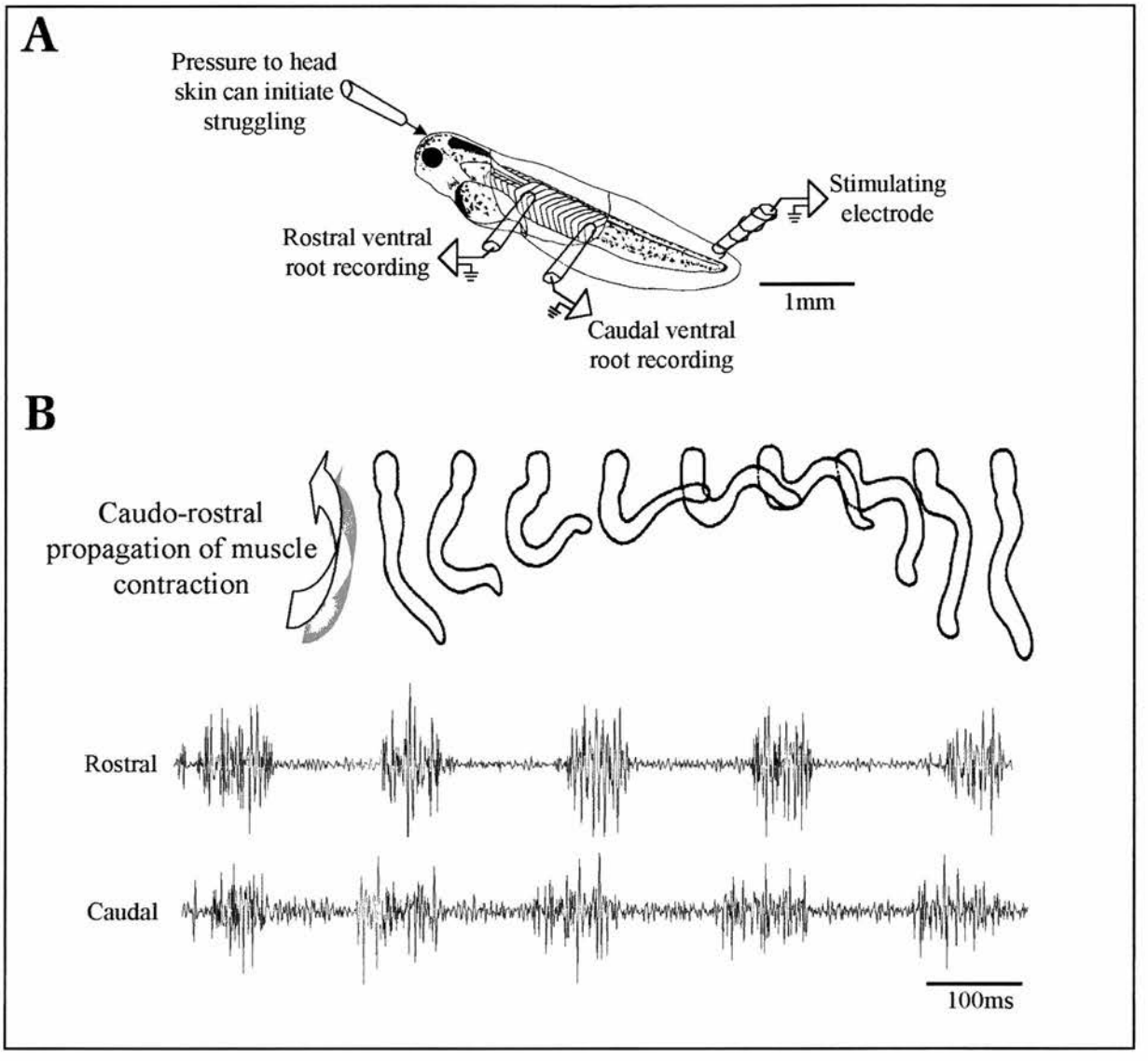

Figure 6.1: *Experimental induction of fictive struggling.* (**A**) Fictive struggling can be induced experimentally by sustained activation of multiple mechanoreceptors, following pressure applied to the head skin using a blunt glass probe. (**B**) Ventral root recordings of fictive struggling illustrate that it is characterised by prolonged (50-100ms) bursts of activity, with a frequency of typically <10Hz, which progress along the body with a brief caudo-rostral delay . Silhouettes of struggling in (B) modified from Kahn & Roberts, 1982c

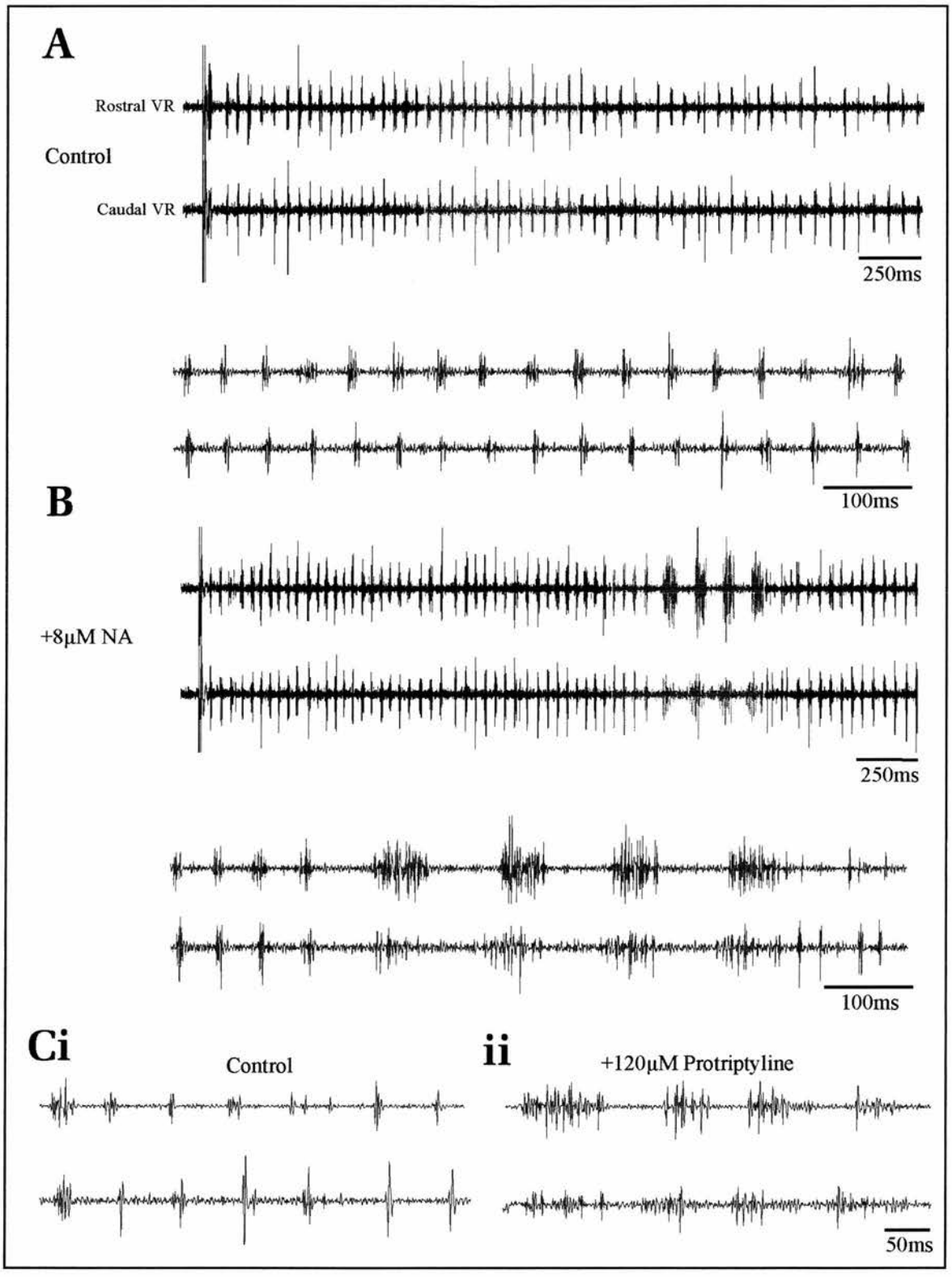

Figure 6.2: *NA can induce bouts of struggling within episodes of swimming* (**A**) Example of swimming activity, characterised by brief bursts of activity progressing along the body with a rostro-caudal delay. (**B**) Following application of NA (>6μM), episodes of swimming are often interspersed with bouts of struggling, characterised by prolonged bursts of activity, which progress along the body with a caudo-rostral delay. (**C**) Example of the ability of the noradrenergic uptake blocker protriptyline to induce struggling activity.

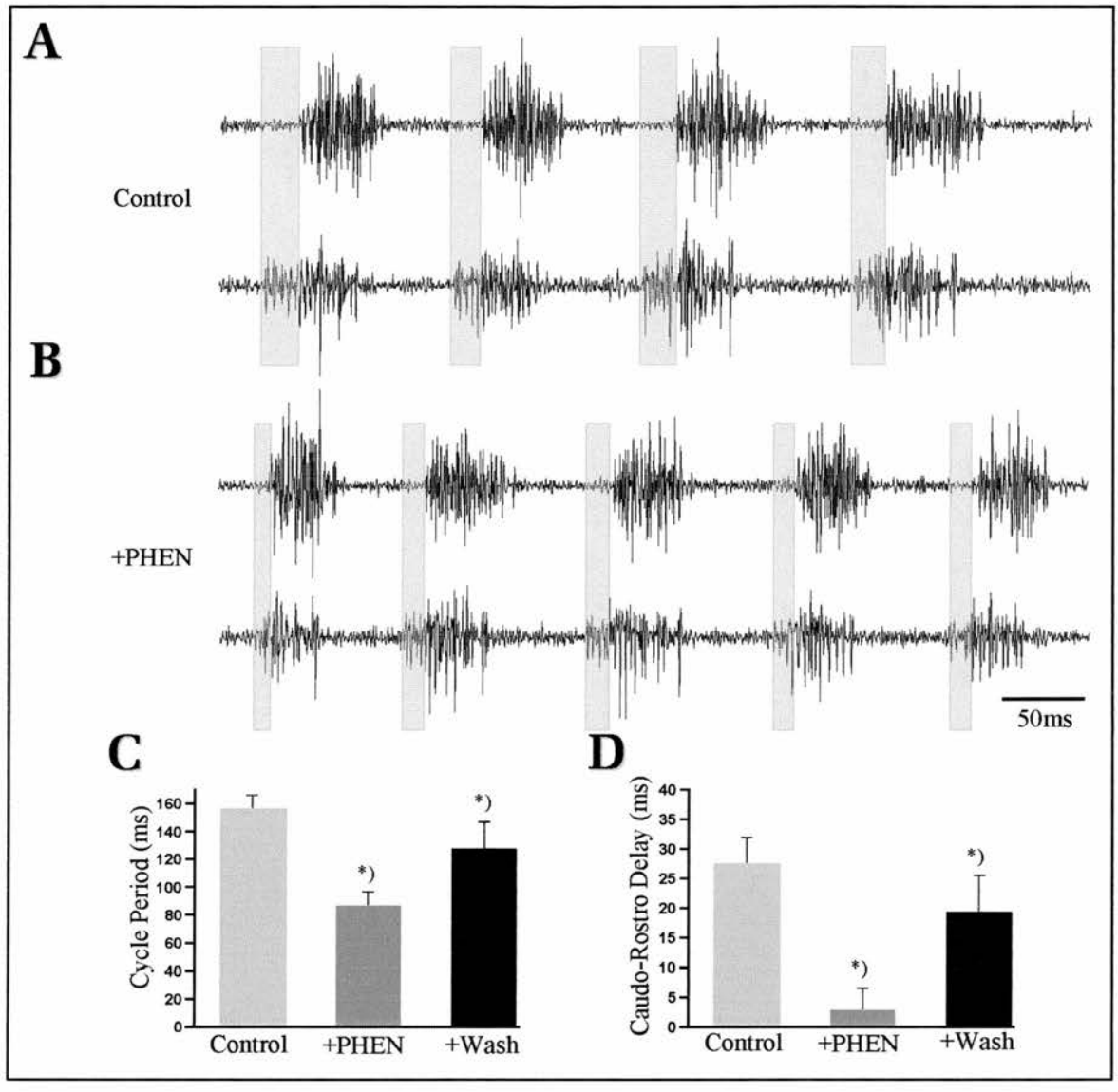

Figure 6.3: *The α-adrenoreceptor antagonist phentolamine reduces cycle periods and caudo-rostral delays during struggling.* (**A-B**) Phentolamine reduces the frequency of motor bursts and the caudo-rostral delay in motor neuron firing following prolonged stimulation of the head skin, appropriate to initiate struggling behaviour. The effects of phentolamine on cycle period (**C**) and caudo-rostral delay (**D**) could be substantially reversed by returning to control saline. *) $P<0.01$

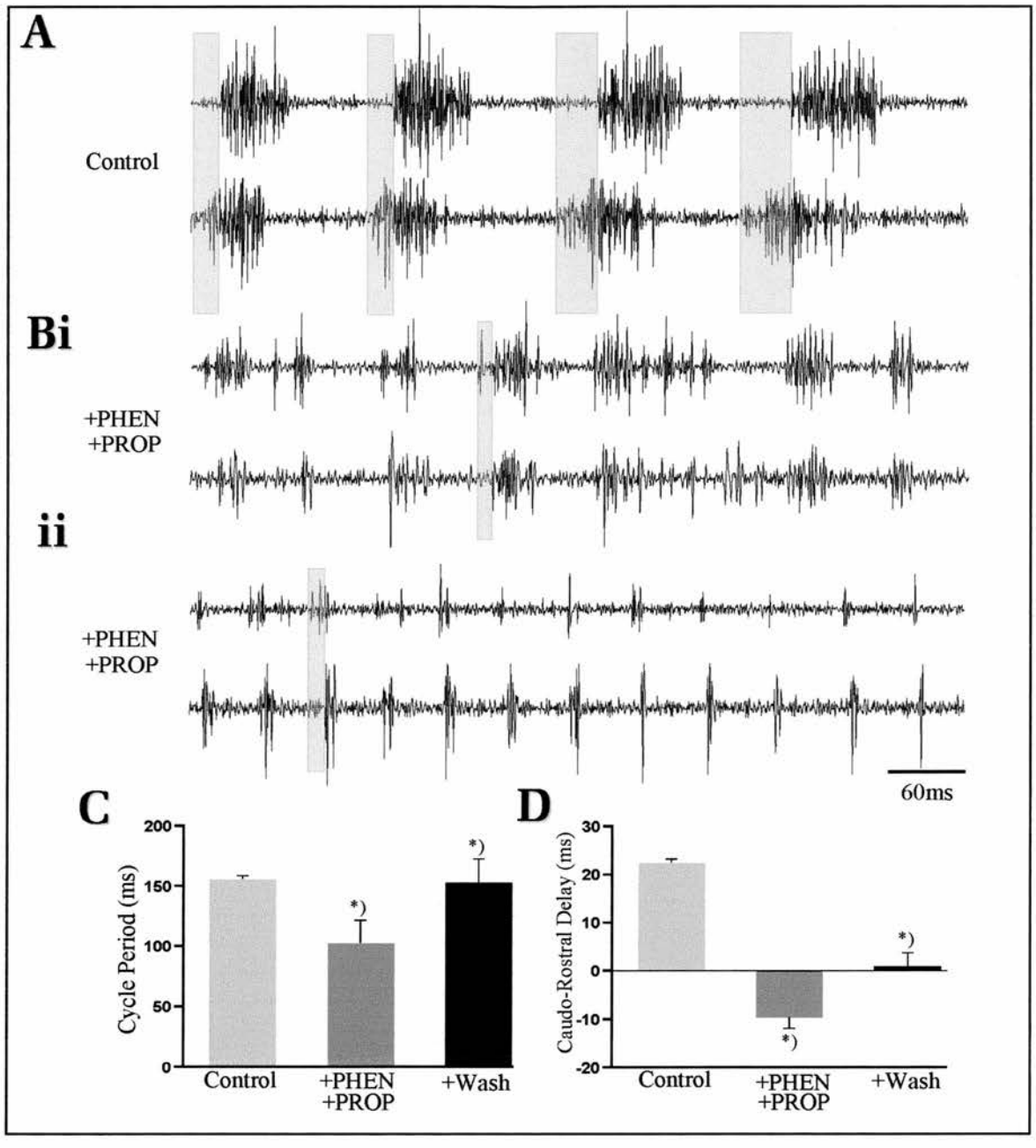

Figure 6.4: *Effects of phentolamine and propranolol on fictive struggling.* (**A-C**) After blocking α- and β-adrenoreceptors with phentolamine and propranolol respectively, the frequency of motor bursts and the caudo-rostral delay in muscle activation following prolonged stimulation of the head skin was reduced or reversed (**Bi**) such that eventually only swimming (**Bii**) is initiated. The effects of phentolamine and propranolol on cycle period (**C**) and caudo-rostral delay (**D**) could be partially counteracted by returning to control conditions. *) $P<0.01$

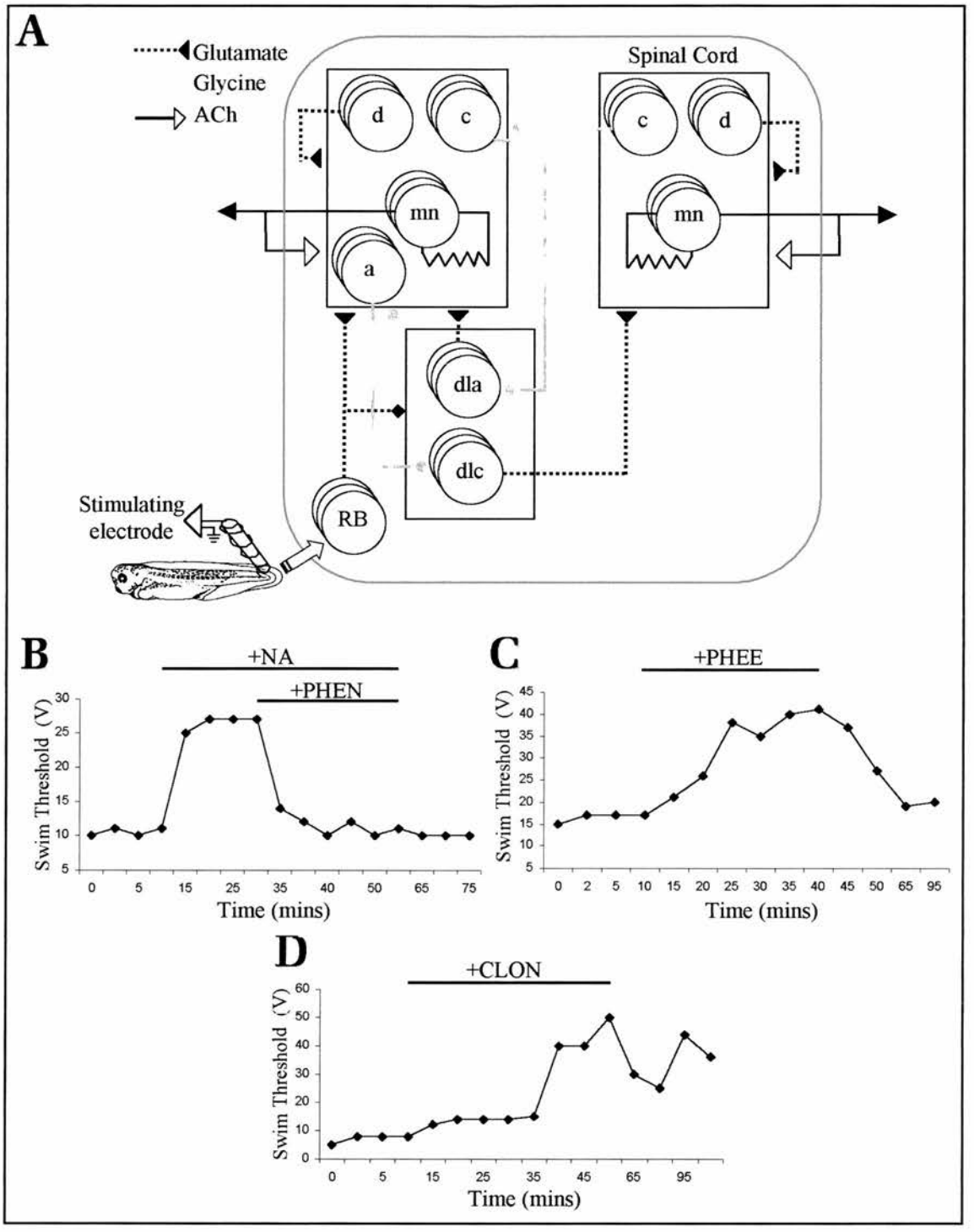

Figure 6.5: *NA decreases sensory transmission in Xenopus tadpoles.* (**A**) Swimming can be initiated following brief electrical stimulation of the skin and activation of Rohon-Beard (R-B) neurons. They in turn excite dorsolateral commissural (dlc) and ascending interneurons (dla), which excite the contralateral and ipsilateral motor half centres respectively. (**B**) NA decreases sensory transmission (as shown by the increase in the threshold voltage required to initiate swimming). This effect is counteracted by phentolamine. The decrease in sensory transmission can be mimicked by activation of α_1- and α_2-adrenoreceptors using phenylephrine (**C**) and clonidine (**D**) respectively. Diagram in (A) adapted from Roberts et al., 1998.

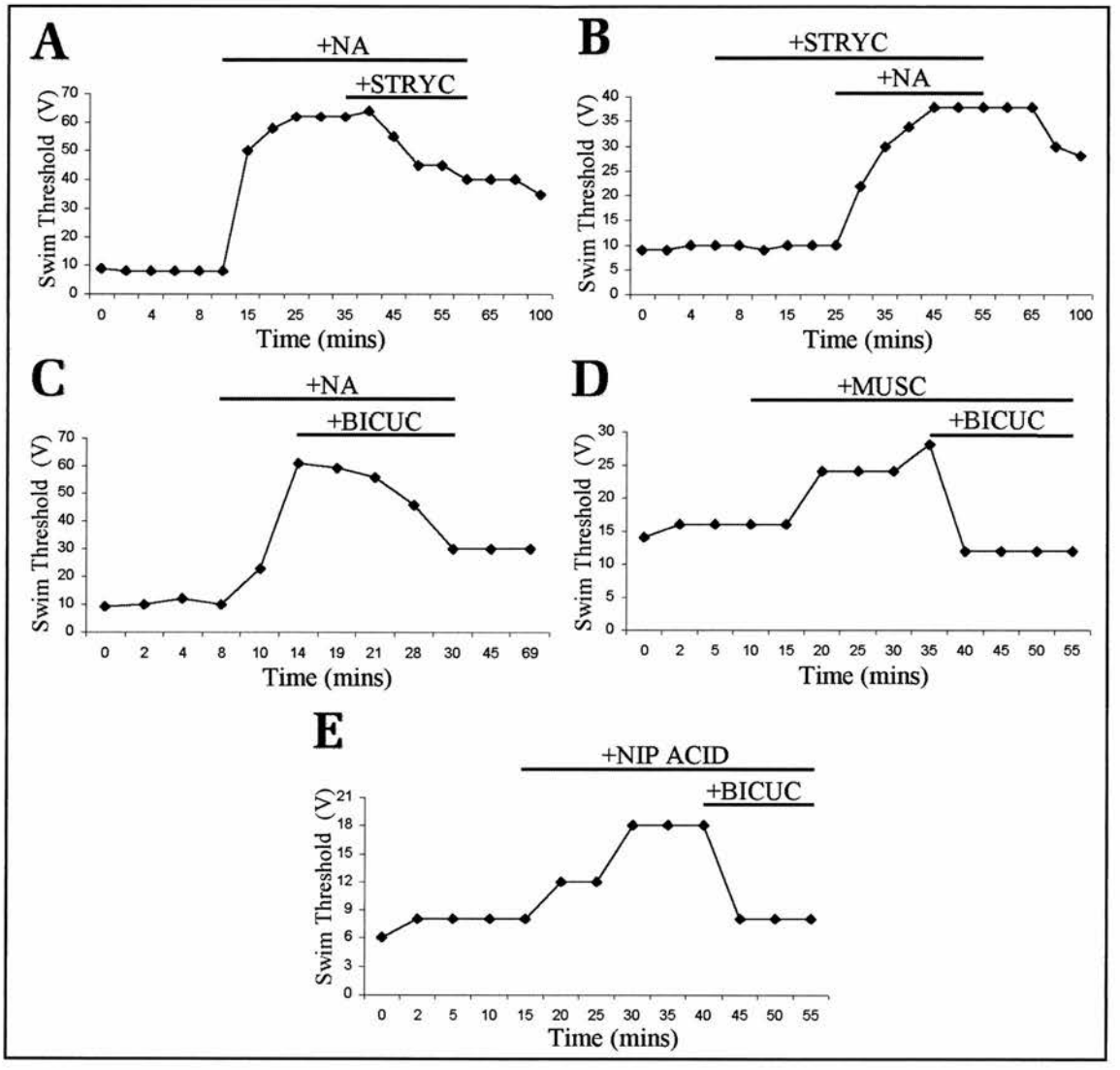

Figure 6.6: *Inhibitory pathways influence the noradrenergic modulation of sensory transmission*. Strychnine can partially counteract the noradrenergic decrease in sensory transmission (**A**), but pre-application of strychnine cannot occlude the effects of NA (**B**). (**C**) Bicuculline can partially counteract the effects of NA on the swim initiation threshold. The $GABA_A$ receptor agonist muscimol (MUSC) (**D**) and the GABAergic uptake inhibitor nipecotic acid (NIP ACID; **E**) decrease sensory transmission. Addition of the $GABA_A$ receptor antagonist bicuculline (BICUC) can counteract both of these effects.

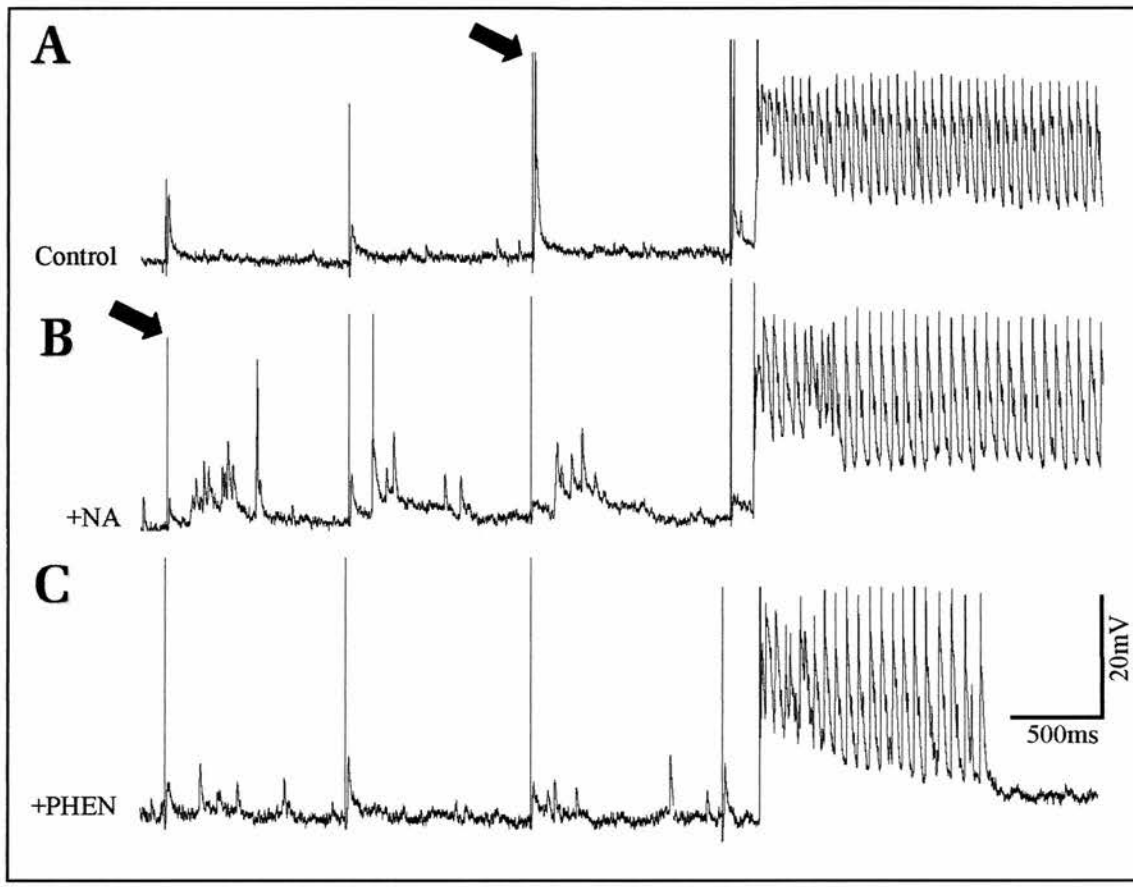

Figure 6.7: *NA elicits IPSPs in response to sub-threshold ipsilateral skin stimulation.* **(A)** Intracellular recordings from a motor neuron reveal a single depolarising post-synaptic potential in response to a single electrical stimulus to the ipsilateral tail skin, at a threshold just below that required to initiate swimming. **(B)** In the presence of NA, a sub-threshold skin stimulus now produced a flurry of depolarising PSPs. This suggests that NA unmasks a previously silent inhibitory pathway that controls the sensitivity of the R-B afferent pathway. Note that even at with a 10ms skin stimulus (arrowed in A), the response is negligible compared with that at a 1ms duration and smaller amplitude under NA (arrowed in B). **(C)**These potentials could be almost abolished in the presence of the general adrenoreceptor antagonist phentolamine.

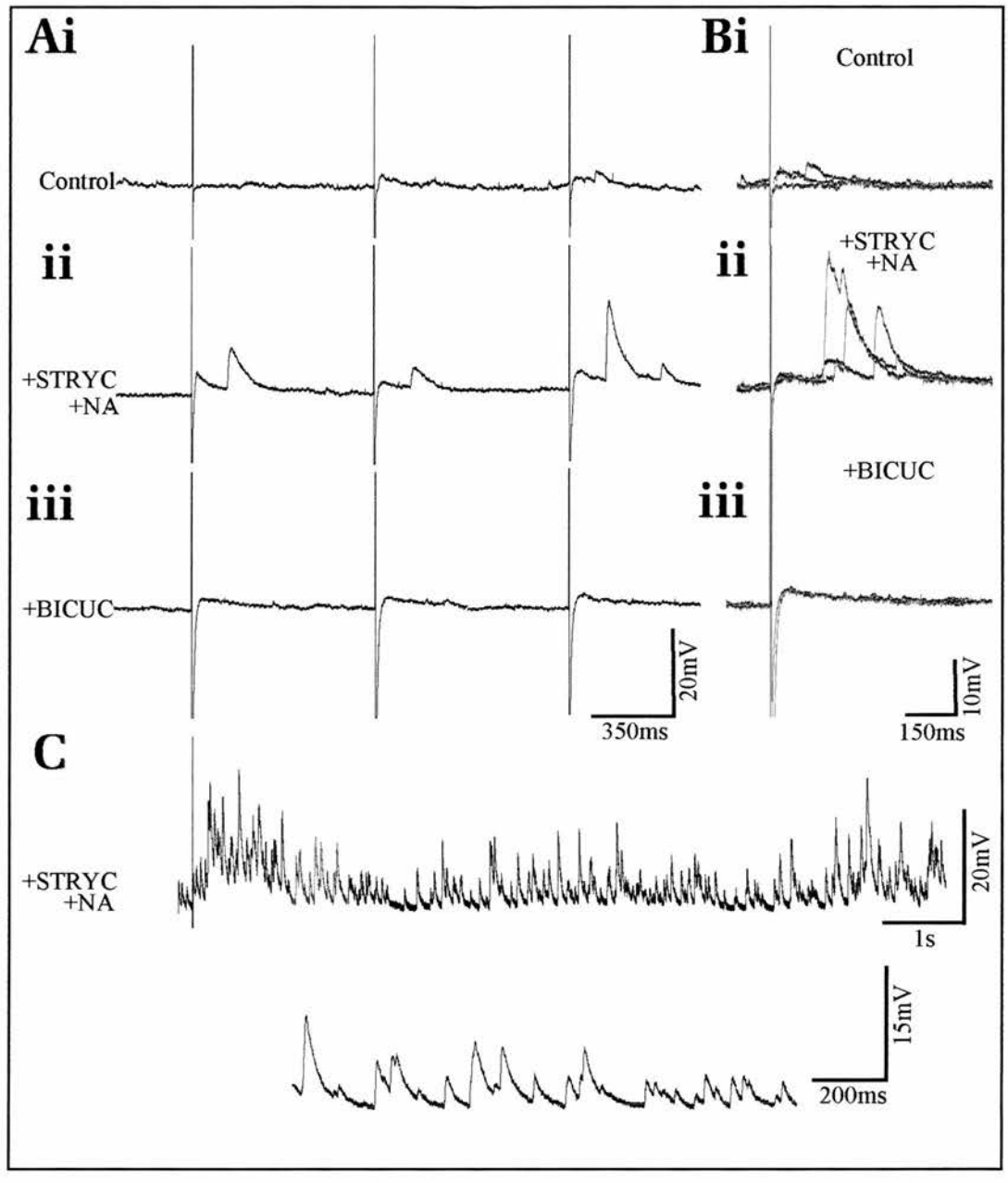

Figure 6.8: *NA elicits GABAergic IPSPs in response to sub-threshold ipsilateral skin stimulation.* **(Ai-ii)** Intracellular recordings from a motor neuron in the presence of NA and strychnine reveal depolarising IPSPs when the ipsilateral tail skin is stimulated just below the threshold required to initiate swimming. **(Aiii)** These potentials could be almost abolished in the presence of the $GABA_A$ receptor antagonist bicuculline, suggesting that these IPSPs are GABAergic in origin. **(B)** Expanded overlays of 3 sub-threshold stimuli in each condition. The time course of the PSPs (~150ms) is further evidence that they are GABAergic (c.f. Reith & Sillar, 1998). **(C)** A more extreme example of the effects of NA, in the presence of strychnine, with an intense barrage of IPSPs following a single stimulus, further suggesting that the IPSPs are GABAergic.

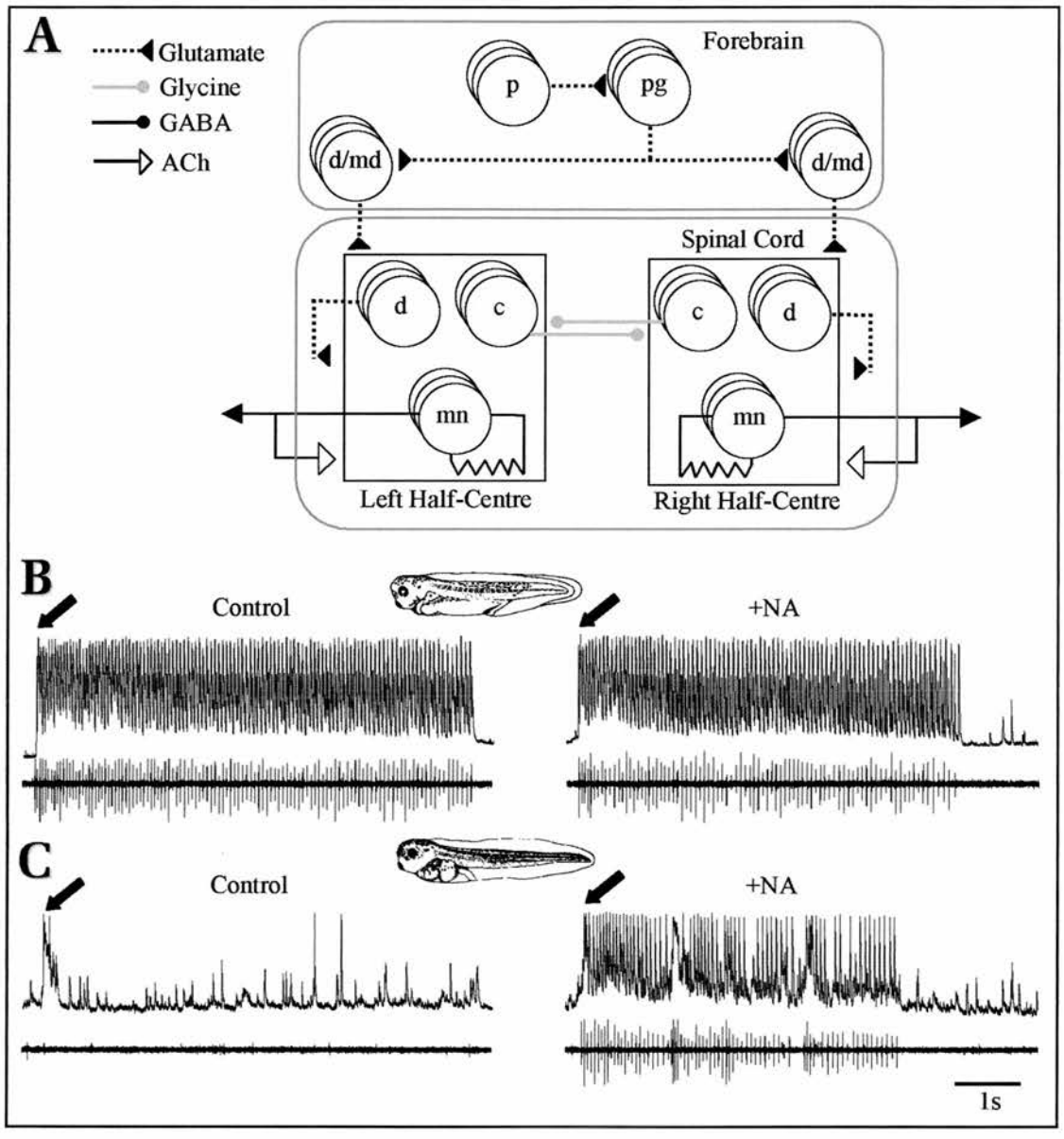

Figure 6.9: *NA restores a dimming response in larvae*. (**A**) Swimming in embryos can be initiated following dimming of the illumination. This excites pineal photoreceptors (p), which excite pineal ganglion cells (pg). These in turn are thought to excite diencephalic/mesencaphalic descending interneurons (d/md) which activate each half-centre. By larval stage 42, this pathway no longer reliably initiates swimming. (**B**) Dimming of the illumination (at arrows in B and C) in embryos can initiate swimming, both before and after the application of NA. (**C**) In the majority of larvae, swimming cannot be elicited by dimming the lights- instead a brief flurry of IPSPs is seen when recording from motor neurons in the spinal cord. However, after application of NA, dimming the lights can elicit swimming.

Chapter Seven

General Discussion

As discussed in the preceding chapters, efficient neuromodulation of neuronal membrane properties and their synaptic interconnections is essential if sustained alterations in the output of CPG's are to be achieved. Often such changes take account of the nature of sensory inputs, and the gating of this information is also a common target of neuromodulation. During this study, my aim was to investigate the noradrenergic neuromodulation of sensory processing and motor pattern generation by NA in a vertebrate spinal cord, using the intact nervous system of the *Xenopus laevis* tadpole as a model system.

The *Xenopus* preparation is ideally suited to such an investigation because of the relative simplicity of its nervous system, which allows different classes of neuron to be identified and recorded from with relative ease, and the fact that sustained fictive motor activity can be initiated and maintained with the nervous system both intact and *in situ*. This contrasts with other vertebrate preparations, where persistent application of excitatory amino acids are usually required to initiate a motor pattern, as the spinal cord is isolated from both the body and the brainstem. The advantage of using an intact nervous system has been highlighted in this study, where I have been able to investigate the noradrenergic modulation of motor activity and of the sensory cues which shape and/or select it.

An important outcome of this work is the elucidation of the receptor pharmacology that underlies the effects of NA in *Xenopus* (see summary in Figure 4.7 and Figure 7.1*)*. Activation of α_1- and α_2-adrenoreceptors can together account

for all the observed effects of NA on swimming. I have shown that the reduction in swimming frequency and episode duration are mediated via both α_1- and α_2-adrenoreceptors, whilst α_1-receptors are additionally involved in the noradrenergic modulation of longitudinal co-ordination. Furthermore, a recent study suggests that β-adrenoreceptors may additionally be involved in the modulation of these RC-delays (Fischer *et al.*, 2001). The involvement of adrenoreceptor activation in initiating and modulating locomotor activity have similarly been documented in a number of other vertebrates (e.g. cat, Forssberg & Grillner, 1973; Barbeau & Rossignol, 1991; rat, Sqalli-Houssaini & Cazalets, 2000). However, the pathways which are targeted by these receptors have not yet been explored. The findings of this study, therefore, provide an important insight into the likely targets of adrenoreceptor activation within the vertebrate spinal cord; namely the fast inhibitory glycinergic and GABAergic pathways. Not only this, but I have been able to show that different receptor subtypes utilise specific pathways to achieve different outcomes. For example, α_1-receptor activation appears to utilise glycinergic, but not GABAergic pathways to reduce the frequency of swimming and the RC-delay in motor neuron firing (Figures 4.2, 4.3, 4.5), which is supported by its ability to facilitate release of glycine, but not GABA. Both α_1- and α_2-receptor classes utilise GABAergic inhibition to reduce the duration of swimming episodes (chapter four; although a different or parallel mechanism must be employed by α_1-receptors in embryos where episode durations are increased). The ability of both receptor types to facilitate GABAergic inhibition following stimulation of the cement gland and of α_1-receptors to prolong the GABAergic barrage in larvae, coincident with a shortening of episodes, strengthens this finding and indicates that both receptor types are likely to be associated with the terminals of GABAergic mhr neurons.

The frequency of swimming naturally declines over the course of an episode, largely due to the 'drop-out' of pre-motor interneuron firing, which eventually leads to the termination of swimming when there are insufficient active interneurons to maintain ongoing activity (Sillar & Roberts, 1993; see for example Figure 1.3Bii of this study where the amplitude of the mid-cycle IPSP reduces across the course of the episode). Increases in inhibition, such as under NA, lengthen cycle periods and, in the absence of any other mechanism, might ordinarily be expected to terminate swimming for the reasons just described. However, under NA, a combination of increased inhibition, which as described in chapter five can enhance post-inhibitory rebound firing, and the direct post-synaptic effects of NA, which also enhance rebound, are likely to help maintain swimming despite the longer cycle periods. Any increase in rebound firing will hypothetically allow for the recruitment of interneurons (or prevent their drop-out in the first place) and thus allow longer cycle periods to be sustained. In addition, post-inhibitory rebound has been suggested as a mechanism by which longitudinal delays might be reduced. There is a longitudinal gradient of inhibition along the cord and it has been proposed that in rostral neurons, where inhibition and excitation are greater, spikes can easily be produced from the falling phase of the mid-cycle IPSP. In caudal neurons, where both excitation and inhibition are lower relative to rostral neurons, the probability of rebound firing will be reduced as neurons will be further from their firing threshold, thus introducing a longitudinal delay in neuron firing (Tunstall & Roberts, 1994). It follows, therefore, that any enhancement of inhibition, such as under NA, will presumably be relatively greater in caudal neurons. Such increases in inhibition are likely to promote rebound firing and in caudal neurons, where this increase in inhibition is greater, then the enhancement of rebound is likely to be greater. This will advance their firing relative

to rostral neurons and reduce, or potentially reverse, the RC-delay in firing, as occurs during struggling.

One inconsistency in the theory that NA and α-adrenoreceptor activation promote the maintenance of longer cycle periods is that episode durations are concomitantly reduced, which is consistent with the natural drop-out of neurons across an episode, rather than their recruitment. As shown in chapter five, NA appears to increase the efficacy of the intrinsic GABAergic stopping pathways and this might account for the shortening of episodes, rather than via the drop-out of interneurons. This will need to be investigated by recording from pre-motor interneurons to assess their relative contributions under NA, but it has been shown that in caudal motor neurons, where mid-cycle inhibition is often absent, NA can unmask silent synapses and induce mid-cycle inhibition on most cycles (McDearmid, 1998). In addition, I have shown here that NA can apparently unmask a silent GABAergic pathway which controls the sensitivity of the R-B pathway (Figures 6.7, 6.8). Taken together, this provides an interesting example of how and why both glycinergic and GABAergic pathways might be utilised by NA, in parallel with direct post-synaptic effects. Facilitation of glycinergic inhibition and the enhancement of rebound firing can account for the increase in cycle periods and the decrease in delays, whilst a concomitant increase in GABAergic inhibition seems to control the duration of episode durations.

The ability of different sensory cues to trigger different rhythmic motor patterns has been documented in several systems (e.g. stomatogastric system, Combes *et al.*, 1999; *Aplysia*, Jing & Weiss, 2001; *Xenopus*, Kahn & Roberts, 1982c). However, the mechanisms that underlie the ability of these cues to switch between different patterns of behaviour are less well understood. My results raise the

intriguing possibility that neuromodulation by NA might facilitate the switch between swimming and struggling, two distinct behaviours in *Xenopus*. Whilst it is not possible to conclude with certainty whether NA is the intrinsic activator of this switch, or whether it merely conditions the network such that struggling can be more easily produced, my findings do indicate that the targets of NA are critical for the switch to occur.

Firstly, the effects of NA on the parameters of swimming (a dramatic increase in cycle period and reduction in RC-delays) support the hypothesis that it can bias the spinal motor network away from swimming and towards struggling. Struggling is also characterised by long cycle periods and a caudo-rostral delay in motor neuron firing, which paradoxically might be initiated by default as a consequence of RC-delays becoming shorter, to the point where caudal neurons are able to fire before rostral ones. Indeed, increasing concentrations of NA, or α-receptor agonists and using noradrenergic uptake inhibitors at concentrations above those that modulate swimming, can induce bouts of struggling. It follows that at these concentrations, the effects on cycle periods and RC-delays are exaggerated to the extent that struggling might be induced.

In parallel, the ability of NA to enhance post-inhibitory rebound firing is likely to contribute to these effects and provides a plausible explanation for how the long cycle periods characteristic of struggling can be maintained. For the reasons described above, it is also possible to envisage how the longer cycle periods during struggling and the longitudinally distributed patterns of neuron firing could be induced and maintained by NA (although it should be noted that bouts of struggling are often unable to be sustained for long periods without additional sensory inputs). It has been shown that during swimming only about 75% of motor and pre-motor

neurons fire, whilst during struggling they nearly all do (Soffe, 1993). This would further support the idea that greater inhibition under NA causes recruitment of interneurons through enhanced rebound firing and would explain why strychnine, which will reduce inhibition, can disrupt struggling (Soffe, 1996).

These hypotheses are also supported by the ability of α- and β-adrenergic antagonists to disrupt struggling activity, as they will reduce the efficacy of NA through these receptors and counteract any increase in inhibition. Increased levels of excitation can also induce struggling (Soffe, 1996), although this can presumably be explained by the tonic increase in excitation promoting neuron firing, and possible recruitment of interneurons. One characteristic of struggling which cannot easily be accounted for by the effects of NA is the dramatic increase in burst durations compared to during swimming, as neither NA, nor activation of α-adrenoreceptors, significantly affected the duration of motor bursts. The multiple firing which underlies the increased burst durations during struggling is thought to derive from a greater tonic depolarisation than during swimming, which depolarises each neuron to such an extent that multiple firing can occur. NA does not appear to cause a depolarisation of the membrane potential, such as would be appropriate to allow multiple firing, although it did reduce the threshold required to initiate spiking, However, no evidence has so far been obtained to suggest that it can confer the ability to produce multiple spiking and indeed the lack of effect on ventral root impulses would suggest that it does not. In contrast, 5-HT does increase the duration of motor bursts, particularly in embryos, although it also remains to be investigated whether induction of a multiple spiking capability is responsible (Sillar *et al.*, 1992c; Wedderburn & Sillar, 1994)

A final point of note about the determining factor between swimming and

struggling is that the nature of the sensory stimulus is able to direct which pattern is produced- brief sensory stimulation initiates swimming, whilst more prolonged activation of sensory receptors produces struggling. I have shown in chapter six that NA can de-sensitise the R-B skin cell pathway in *Xenopus*, by unmasking GABAergic inhibitory synapses. It is conceivable that stimulation of the skin, or the associated activation of R-B neurons might directly trigger release of NA, which inhibits further inputs through this pathways, possibly as part of an escape response. If this were the case, in response to more prolonged stimulation of the skin, such as would be appropriate to induce struggling, increased levels of NA might result in struggling rather than swimming being produced. It should also be noted that repetitive stimulation of the skin, such as would trigger struggling, may release substance P from R-B neurons and NA might interact with this peptidergic spinal system to facilitate the switch between the two behaviours.

Despite having clearly shown in the preceding chapters how NA can shape motor activity and modulate sensory processing, it is important to remember that the influence of the noradrenergic system is not exclusive within the spinal cord of *Xenopus* and it undoubtedly interacts at many levels with other transmitters; indeed its interactions with GABAergic and glycinergic pathways have been well-highlighted here. An example of other potential interactions is with that of the serotonergic system. Whilst NA enhances glycinergic inhibition and slows swimming, 5-HT reduces this inhibition and produces more intense activity (McDearmid *et al.*, 1997). Behaviourally, these two amines probably afford the animal the ability to produce a range of swimming speeds and intensities, appropriate to different sensory cues, and the actions of one might impact upon the release and actions of the other. Indeed, preliminary experiments where 5-HT and

NA were bath-applied suggest that the order of drug application modulates swimming in different ways (Reith & Sillar, unpublished observations). *In vivo*, the conditions that will ultimately determine the relative contribution of the two amines and the nature of the motor output produced will presumably depend largely upon sensory inputs.

The nitrergic system has similar effects to NA on tadpole swimming, causing a slowing of swimming frequency and a shortening of swimming episodes (McLean & Sillar, 2000). The levels of interaction of this neuromodulator with other transmitters are more clear as three distinct brainstem clusters of nitrergic neurons have been identified, which appear to share their morphology with neurons of the serotonergic raphe nucleus, the noradrenergic isthmic region (homologous to the locus coeruleus) and the GABAergic mhr neurons (for a review see McLean *et al.*, 2000). Hypothetically, if NO were co-localised with any of these neuron populations then it would be easy to see how they might control the release of each others transmitters and thus direct the motor output to produce different patterns of swimming.

The interactions of the noradrenergic system with these other modulatory systems will now need to be addressed if a more complete picture of neuromodulation within the spinal pattern generator of *Xenopus* is to be formed. This will most easily be achieved by considering the role of each system in the presence of antagonists to known receptors against one or more of the others, and the effects of co-application of each transmitter, such as has been attempted for 5-HT and NA. A clear understanding of the location of receptors for NA will also make this task easier. Now that the receptor types through which NA is acting, have been identified (chapter three), antibodies against specific adrenergic receptors, combined with the

physiological evidence provided here, will elucidate the location of each receptor type and suggest interactions with other neurons and their associated processes, such as NO, whose morphology is better described (McLean & Sillar, 2000; 2001). In addition, mechanisms of auto-feedback are also likely to exist and must be investigated further. The role of α_2-receptors as noradrenergic autoreceptors has already been mentioned, and experiments on spinalised animals, which severs this negative feedback mechanism by removing ascending information to, and descending release of NA from, the brainstem will help to assess the contribution of auto-inhibition. Similarly, the endogenous role of NA must also be considered. In this study I have shown that the uptake inhibitor protriptyline can replicate the effects of NA by increasing the spontaneous release of both glycine and GABA (Figure 5.8) and also highlighted its ability to induce bouts of struggling during swimming, in a manner similar to NA (Figure 6.2). This preliminary evidence must now be augmented, using pharmacological antagonists at α-adrenoreceptors (such as phentolamine), which would be expected to counteract the effects of the synaptic potentiation of NA by uptake inhibitors. Finally, the combined activation of the different receptors for NA requires investigation. Clearly when NA is released endogenously or its levels are raised by exogenous bath application, more than one receptor class is activated and the relative contribution of each needs to be assessed. For example, in chapter three I have shown that whilst both α_1-and α_2-receptor activation can reduce swimming frequency, only α_1-receptors appear to mediate the reduction of RC-delays by NA. It seems logical to suggest that NA might simultaneously activate both receptor types and thus affect both swimming frequency and delays. However, whilst both pathways utilise glycinergic inhibition to reduce swimming frequency, α_2-receptors additionally target GABAergic

pathways, raising the possibility that selective activation of either, or both, receptor types might be possible, although this has yet to be examined. Despite these gaps in our knowledge, this investigation has yielded important information about the mechanisms that underlie the effects of a common neuromodulator in the nervous system of vertebrates. A summary of my major findings is shown in Figure 7.1 and in combination with the simplicity afforded by the tadpole preparation they now provide a useful platform to advance understanding of these principles on a more general level.

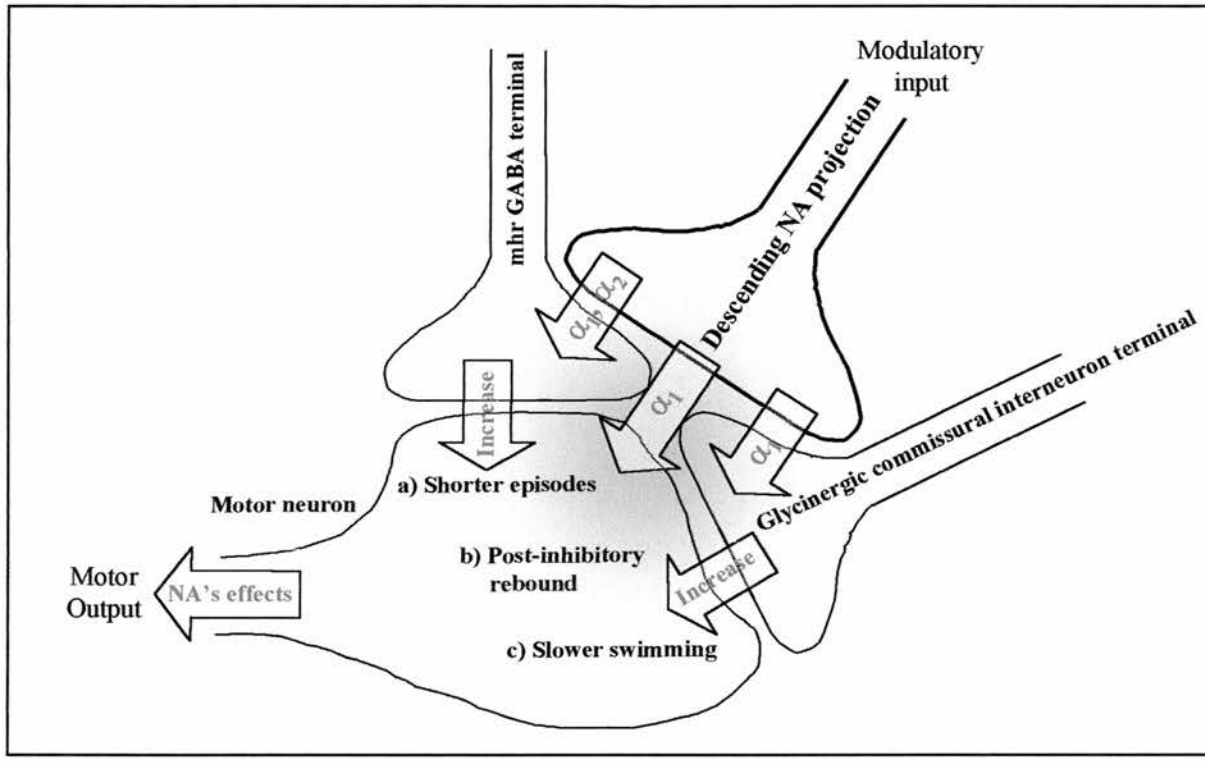

Figure 7.1: *Current hypothesis of the mechanisms underlying the noradrenergic modulation of swimming in Xenopus.* Descending noradrenergic projections release NA, which activates both α_1- and α_2-adrenoreceptors located on the the pre-synaptic terminals of mid-hindbrain reticulospinal GABA neurons. Activation of these receptors facilitates release of GABA onto motor neurons (such as in response to activation of the rostral cement gland in embryos) and terminates swimming. α_1-adrenoreceptors are also apparently located on the terminals of commissural glycinergic interneurons. From here, they pre-synaptically enhance the probability of glycine release, which strengthens the mid-cycle IPSP during swimming and reduces swimming frequency. In addition, α_1-receptors enhance the probability of rebound firing via a presumed direct post-synaptic effect on motor neurons. This may contribute to the reduction in RC-delays by NA and the ability to maintain longer cycle periods. Diagram courtesy of D.L.McLean.

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Appendix

Publications arising from this work

- Adrenoreceptor-mediated modulation of the spinal locomotor pattern during swimming in *Xenopus laevis* tadpoles.

  Fischer, H., **Merrywest, S.D.** & Sillar, K.T.

  European Journal of Neuroscience **13(5)**, 977-986. (2001)

- Alpha-adrenoreceptor activation modulates swimming via glycinergic and $GABA_A$ergic inhibitory pathways in *Xenopus laevis* tadpoles

  Merrywest, S.D, Fischer, H. & Sillar, K.T.

  European Journal of Neuroscience **15(2)**, 375-383. (2002)

Additional publications:

- McLean, D.L., **Merrywest, S.D.** & Sillar, K.T. (2000) The development of neuromodulatory systems and the maturation of motor patterns in amphibian tadpoles. *Brain Research Bulletin* **53(5)**, 595-603.
- Sillar, K.T., McLean, D.L., Fischer, H. & **Merrywest, S.D.** (in press) Fast inhibitory synapses: Targets for neuromodulation & development of vertebrate motor behaviour. *Brain Research Reviews*
- **Merrywest, S.D.** & Sillar, K.T. (2002) Enantioselective modulation of $GABA_A$ receptor-mediated synaptic inhibition in *Xenopus laevis* tadpoles by the general anaesthetic etomidate. *Journal of Physiology* **539P.**