GABAergic modulation of locomotor rhythmicity during post-embryonic development in *Xenopus laevis*.

A thesis submitted to the University of St. Andrews for the degree of Doctor of Philosophy.

by
Carolyn A. Reith

Gatty Marine Laboratory
Department of Biological and Medical Sciences
University of St. Andrews
St. Andrews
Fife

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To mum, dad, Kevin
and grandma.

Declaration

I, Carolyn Anne Reith, hereby certify that this thesis, which is approximately 35 000 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

date. 29th March '96 signature of candidate..

I was admitted as a research student under Ordinance No. 12 in October 1992 and as a candidate for the degree of Ph.D. in September 1993; the higher study for which this is a record was carried out in the University of St. Andrews between 1992 and 1996.

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

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Contents

Abbreviations

5β3α	5β-pregnan-3α-ol-20-one
5HT	5-hydroxytryptamine
ACh	acetylcholine
APV	5-amino phosphonvaleric acid
α-BTX	α-bungarotoxin
CACA	cis-4-aminocrotonic acid
CdCl2	cadmium chloride
CNS	central nervous system
CPG	central pattern generator
DAB	diaminobenzidine
DHKA	dihydrokainic acid
dla	dorsolateral ascending
dlc	dorsolateral commissural
DMSO	dimethyl sulphoxide
Ecl	chloride equilibrium potential
epsp	excitatory postsynaptic potential
GABA	gamma aminobutyric acid
H_2O_2	hydrogen peroxide
HRP	horse radish peroxidase
ipsp	inhibitory postsynaptic potential
K/A	kainate/AMPA
KAc	potassium acetate
KCl	potassium chloride
mhr	midhindbrain reticulospinal
MS222	3-aminobenzoic acid ethyl ester
NMDA	N-methyl-d-aspartate

PB	phosphate buffer
PDA	cis-2,3-piperidine dicarboxylic acid
P.O.M.	post otic myotome
R-B	Rohon-Beard
rh	rostral hindbrain
TBPS	t-butylbicyclophosphorothionate
TTX	tetrodotoxin
vc	ventral commissural

List of figures.

Abstract.

1. The role of the inhibitory amino acid, GABA acting at GABAa receptors during embryonic and larval fictive swimming in *Xenopus laevis* has been investigated.

2. Intracellular recordings, under tetrodotoxin, reveal two types of spontaneous inhibitory postsynaptic potential: short duration glycinergic ipsps and longer duration GABAergic ipsps.

3. The neurosteroid, 5β-pregnan-3α-ol-20-one (5β3α), enhances the presynaptic release of GABA and the postsynaptic effects of GABAa receptor activation, without affecting glycinergic ipsps.

4. At embryonic stage 37/8, GABAa receptor activation has an overall inhibitory action on the swimming pattern. 5β3α causes a decrease in the frequency and duration of fictive swim episodes.

5. By larval stage 42, GABAa receptor activation influences various parameters of fictive swimming (burst duration, cycle period and rostrocaudal delay) and thus finely tunes the motor output. Bicuculline-blockade of GABAa receptors causes swimming frequency and burst durations to increase and rostro-caudal delays to decrease.

6. GABA release from the descending projections of midhindbrain reticulospinal neurons also appears to play a role in the intrinsic termination of larval swimming, as opposed to their previous involvement in a reflex stopping response in the embryo.

7. By stage 42, the dimming response, which elicits swimming following a sudden dimming of the illumination, is declining. GABAergic neurotransmission plays a role in down regulating this sensory reflex.

8. As opposed to embryonic swimming, sustained larval swimming requires a certain level of inhibition since it is abolished when both glycine and GABAa receptors are blocked.

9. When GABAa and glycine receptors are blocked during NMDA-induced swimming activity, a slow network oscillation is revealed. This appears to result from the enhancement of a pre-existing low amplitude oscillation already present during NMDA-induced rhythmic activity.

10. Pharmacological evidence suggests that the slow network modulation of NMDA-induced swimming results from the expression of intrinsic 5HT-dependent membrane potential oscillations, since both are abolished by a specific 5HT1a antagonist, pindobind-5HT1a.

Chapter 1
General Introduction.

In 1957, a previously detected brain agent, known as factor 1 was identified as the amino acid, γ-amino butyric acid (GABA, Bazemore et al., 1957). GABA is now known to be one of the most important inhibitory transmitters in the central nervous system (CNS) with the possibility that 20 to 50% of all central synapses use GABA as their transmitter (Sieghart, 1995) and nearly every neuron as well as some glial cells, respond to the amino acid (Burt and Kamachi, 1991).

GABA is synthesised from its metabolic precursor glutamic acid and stored in vesicles until it is released by a calcium-dependent mechanism into the synaptic cleft. Reuptake is mainly into nerve terminals but also into some glial cells. Once released into the synaptic cleft the actions of GABA are mediated through two distinct receptors, the GABAa and GABAb receptor subtypes. The pharmacological division of these receptors is based on either their blockade by bicuculline (GABAa) or their activation by baclofen (GABAb, Bormann, 1988). The ionotropic GABAa receptor directly gates a chloride ion channel (Bormann, 1988), whereas the metabotropic GABAb receptor mediates its effects through a second messenger system and has been shown to decrease K^+ and Ca^{2+} currents in different brain regions (Dunlap and Fishbach, 1981, Newberry and Nicoll, 1986). A third GABA receptor has recently been characterised, the GABAc receptor (for review see, Bormann and Feigenspan, 1995). It is thought to act in a similar way to the GABAa receptor in gating a chloride ion channel and may, in fact, be structurally related to the GABAa receptor (Sieghart, 1995). The GABAc receptor is, however, insensitive to drugs that modulate either the GABAa or GABAb receptor and is selectively activated by cis-4-aminocrotonic acid (CACA).

This study focuses on the actions of GABA at the bicuculline-sensitive GABAa receptor. There is now a large amount of evidence to

suggest that the GABAa receptor is the site of action of several different classes of therapeutic agent which have sedative, anxiolytic, antiepileptic and anaesthetic properties. They include the benzodiazepines, barbiturates and certain steroids (see chapter 3). The GABAa receptor is also the binding site of plant-derived convulsants such as picrotoxin and t-butylbicyclophosphorothionate (TBPS).

The GABAa receptor.

The GABAa receptor is one of a superfamily of ligand-gated ion channel receptors which includes the nicotinic acetylcholine (ACh) receptor and the glycine receptor (Schofield et al., 1987). It has been proposed to be a macromolecular protein of around 200 to 300 kDa (Sigel et al., 1983), consisting of several different polypeptide units of approximately 55kDa. The initial view of the GABAa receptor was that it consisted of two alpha and two beta subunits, which were the binding sites for benzodiazepines and GABA respectively (Mamalaki et al., 1987). It is now known that there are at least 15 different subunits: α1-6, β1-4, γ1-3 and ρ1-2 (MacDonald and Olsen, 1994). There is 70-80% sequence homology between subunits within each subfamily and 30-40% homology between subunits of different subfamilies. The sequence homology between subunits of other members of the superfamily is approximately 20-30% (Burt and Kamachi, 1991). Each subunit is made up of around 450 amino acids with certain common elements: an NH_2 extracellular domain with two conserved cysteine residues thought to be important for ligand binding; three putative membrane spanning regions (M1-M3) and in analogy to the nicotinic ACh receptor, M2 is thought to line the ion channel (Olsen and Tobin, 1990); a

poorly conserved cytoplasmic loop (Schofield et al, 1987) and a fourth membrane spanning region near the COOH terminus.

Despite the range of subunits which have now been characterised, little is known about the combinations of subunits which make up naturally occurring GABAa receptor-chloride ionophore complexes. With 15 subunits and no rules on how they combine, there are 151,887 possible different combinations (Burt and Kamachi, 1991). It has been shown that cells making single subunit receptors can form GABA-gated chloride ionophores (Blair et al., 1988, Pritchett et al., 1988). The single subunit receptors show barbiturate potentiation and picrotoxin blockade but do not respond to benzodiazepines in the same way as native GABAa receptors (Burt and Kamatchi 1991). Therefore some functions of the receptor are the result of conserved sequences in all subunits, but it is unlikely that these single subunit receptors are produced *in vivo* since they form inefficiently (Sieghart, 1995). Similarly, αβ-complexes show either no potentiation (Levitan et al., 1988) or atypical potentiation (Moss et al., 1991) of GABA mediated chloride currents by benzodiazepines. Other studies have shown that they are also unlikely to form naturally as the αβ-complex is sensitive to zinc but in the presence of γ-subunits zinc sensitivity is lost (Draguhn et al., 1990). It would appear that for normal benzodiazepine binding, the presence of a γ-subunit is essential (Pritchett et al., 1989). Recent studies have begun to suggest some of the subunit combinations that exist using specific subunit selective antisera (see McKernan and Whiting, 1996, for review). Rat brain studies have identified the α1β2γ2 and α2β3γ2 as two of the main GABAa receptor subtypes which account for 75-85% of the diazepam-sensitive GABAa receptors (Benke et al., 1994).

The heterogeneity of the GABAa receptor is thought to be a reflection of different subunit compositions formed in different brain areas.

The distribution of some of the subunits is limited suggesting that they might impart important functions. For example, the α6 subunit is limited to the cerebellum (Kato, 1990) and the ρ-subunit is only found in the retina (Cutting et al., 1991). During development, the expression and distribution of different subunits can change, causing alterations in the characteristics of the receptor (Lanius et al., 1994). Post-translational modifications such as phosphorylation may add yet another level of modification on the function of the receptor (Moss et al., 1995), the effects of which may again change during development (Shaw and Lanius, 1992)

Gating of GABAa receptor channels.

The development of the single channel, 'patch clamp' recording technique (Hamill et al., 1983, Bormann et al., 1987, MacDonald et al., 1989) led to a description of the kinetics of the ligand-gated chloride channel. The GABAa receptor channel opens to multiconductance levels of 11-12pS, 17-19pS and the main conductance level of 27-30pS (MacDonald and Olsen, 1994) through which more than 95% of the current flows (Twyman and MacDonald, 1992). Currents through the GABAa receptor chloride channel have a burst-like appearance with openings to the same conductance level which are interrupted by short closings. Each burst is separated by a relatively long closed period. The main conductance level has three open conductance states (0.5ms, 2.6ms, 7.6ms), which change in proportion with increasing GABA concentrations, and multiple closed states (MacDonald and Olsen, 1994). The proposed model for the gating of a single channel incorporates the binding of two molecules of GABA with three open states, ten closed states and one desensitisation state (MacDonald and Twyman, 1992).

Drug actions at the GABAa receptor.

When GABA binds to the GABAa receptor it causes an increase in chloride ion conductance and since, in most cases, the concentration of chloride ions is lower inside cells, the chloride gradient will force chloride ions into the cell. This normally causes the membrane potential to hyperpolarise since the resting membrane potential is usually lower than the chloride equilibrium potential (E_{Cl}). The nett effect of GABAa receptor activation is to clamp neurons close to E_{Cl} and make them less responsive to excitation. There are some cases, for example during development, where the action of GABA has been shown to be excitatory, a phenomenon thought to result from a reversed chloride gradient at that time (Cherubini et al., 1991, Hosokawa et al., 1994)

In order to activate the chloride channel, GABA binds to high, low and very low affinity sites (Olsen et al., 1986). The physiological effects of GABA are exerted through the very low affinity sites thus ensuring that the chloride channel is only opened when there is a significant increase in transmitter in the synaptic cleft (Maconochie et al., 1994). The antagonist bicuculline competitively blocks the action of GABA by binding to the same site(s) (MacDonald et al., 1989) and the low affinity site is thought to be the preferred antagonist binding site. (Lan et al., 1991).

Most of the binding sites of the different agents acting on the GABAa receptor are physically separated. Benzodiazepines, such as flunitrazepam and diazepam, bind to high affinity sites on the GABAa receptor complex and enhance the action of GABA by increasing the frequency of chloride channel opening (Burt and Kamachi, 1991). The benzodiazepines increase GABA binding to low affinity sites through which GABA mediates its effect on the channel (Skerrit et al., 1982, Concas et al.,

1985). Most classical benzodiazepines have a similar affinity for GABA receptors in different brain areas, but some benzodiazepines have been identified which distinguish between different benzodiazepine sites and are thought to reflect differences in the structure of the α-subunit (Pritchett et al., 1989).

Like the benzodiazepines, barbiturates such as pentobarbital, also enhance GABA binding to low affinity sites (Yang and Olsen, 1987). However, they cause an increase in the average duration rather than frequency of channel openings by increasing the proportion of longer opening states (MacDonald et al., 1989). At the higher concentrations reached during anaesthesia, barbiturates can activate the chloride ionophore directly (Franks and Lieb, 1994), possibly suggesting two sites of action on the GABAa receptor complex. As well as enhancing the actions of GABA, barbiturates increase benzodiazepine binding and inhibit TBPS binding (Doble and Martin, 1992). These results therefore suggest that the barbiturates have a separate binding site to the benzodiazepines.

The actions of neurosteroids are discussed in detail in chapter 3 but they have been shown to act in a similar way to the barbiturates and increase the average open time of the channel. However, this effect is achieved by binding to a separate site distinct from the barbiturate site of action.

The convulsant plant extract, picrotoxin and other bicyclic cage compounds such as TBPS, antagonise chloride conductances but not by preventing GABA binding. They prevent the conformational change which causes the channel to open and decrease both the average open duration of the channel and the average burst duration (Twyman et al., 1989). They are thought to do so by binding to a site either in or near the channel (Gee et al,

1988), with more recent evidence tending to favour the latter (MacDonald and Olsen, 1994).

GABA and locomotion.

The distribution of GABA within the spinal cord of many different vertebrates is widespread suggesting that it plays important functional roles. However, the role of GABA transmission within the cord has remained unclear until relatively recently. Glycine is thought of as the major inhibitory transmitter in the spinal cord and has been shown to mediate reciprocal inhibition of alternating rhythmic activity in some vertebrate species (Buchanan, 1982, Cohen and Harris-Warrick, 1984, Dale, 1985, Alford and Williams, 1987, Soffe, 1987, Cazalets et al., 1994), whereas GABA appears not to be involved in the production of the basic motor output.

It was demonstrated some time ago and there is now a general agreement that spinal sensory afferents are presynaptically inhibited in the spinal cord by GABAergic neurotransmission (Eccles et al., 1963, Curtis and Malik, 1985, Christenson and Grillner, 1989). Additionally, presynaptic modulation of sensory inflow during fictive locomotion in the cat has been shown to be phasically related to the step cycle suggesting that the motor network may produce rhythmic GABAergic inhibition of primary afferents (Dubuc et al., 1988). This modulation acts to gate incoming information ensuring that reflexes do not interrupt rhythmic activity at an inappropriate time.

The GABAergic influence on locomotor activity was initially suggested to result from its action on descending systems such as the mesencephalic networks that trigger locomotion (Garcia-Rill and Skinner,

1986) but other studies have shown that GABA can act directly on the spinal network and that the GABA system is active to some extent during normal locomotor activity (Alford et al., 1990a,b, Tégner et al., 1993, Cazalets et al., 1994).

There is evidence to suggest that presynaptic inhibition is not limited to sensory transmission. The first report of presynaptic GABAergic inhibition on the motor system came from studies on the lamprey spinal cord preparation (Alford et al., 1990a). Recording from the axons of both excitatory and inhibitory premotor interneurons, showed a depolarisation of the membrane potential in response to bath applied GABA. This response remained unchanged in the presence of TTX indicating a direct action which resulted from the activation of both GABAa and GABAb receptors with GABA, muscimol and baclofen all causing an increase in membrane conductance. The action of GABA at the GABAa receptor was shown to result in an increased chloride conductance whereas the mechanism at the GABAb receptor was unclear. During rhythmic activity the axonal membrane potential oscillated, with the depolarised state coinciding with ventral root activity on the ipsilateral side. These oscillations were shown to result from the phasic endogenous activation of GABAa and GABAb receptors and could only be abolished by the simultaneous bath application of antagonists to both receptor types. At the network level, blocking either receptor type alone had little effect on rhythmic activity, but blocking both GABAa and GABAb receptors caused a disruption in the activity recorded from segments of spinal cord ten segments or more in length, but had no effect on the activity recorded from shorter segments. The GABAergic input responsible for these effects was assumed to be from spinal multipolar neurons since there are no known populations of GABA neurons in the lamprey brain with axons that descend into the spinal cord (Brodin et al.,

1990). In the lamprey spinal cord, inhibitory glycinergic interneurons have axons which extend over several segments and the suggestion was made that local GABAergic inputs may act to prevent glycine inhibition from influencing the rhythm at an inappropriate part of the movement cycle (Grillner et al., 1991).

Although initial studies found little effect of either GABAa or GABAb agonists or antagonists alone on locomotor activity in the lamprey, other studies have indicated different effects following GABAa or GABAb receptor blockade. By blocking glycinergic inhibition with the antagonist strychnine, a role for GABAa receptors was revealed. In the presence of strychnine, GABAa receptor blockade with bicuculline led to an increase in the duration of episodes of fictive swimming accompanied by an initial increase in the burst frequency (Alford et al., 1990b). At the cellular level, GABAa receptors appear to be important in the repolarisation of neurons especially in the absence of glycinergic inhibition, since blockade of GABAa receptors with bicuculline leads to rhythmic activity in which neurons are significantly depolarised above the resting potential seen before stimulation (Alford et al., 1990b). This effect was proposed to be presynaptic since voltage-clamp studies revealed no outward current in recordings from motorneurons, as would be expected if the action of GABA were directly on GABAa receptors on the postsynaptic membrane (Alford et al., 1990b)

In order to separate the action of GABA at the two different receptor types, the action of GABA on N-methyl-d-aspartate (NMDA)-induced activity was investigated (Tégner et al., 1993). The effects of the endogenous GABA system were enhanced with the uptake blocker nipecotic acid and the effects of specific antagonists at the GABAa and GABAb receptor were investigated to determine the contributions of each

receptor type. The blockade of the GABAb receptor with phaclofen or saclofen resulted in an increase in the burst frequency, but a regular rhythmic pattern of activity was maintained, whereas GABAa receptor blockade with bicuculline caused a very irregular pattern of activity as well as influencing the burst rate. From experiments on split bath preparations in which the rostral and caudal segments of the cord were isolated and bathed in different solutions, it was shown that GABA transmission could regulate intersegmental coordination with GABAb receptor activation modifying the phase lag and GABAa receptor activation reducing the variability in the timing of activity between segments (Tégner et al., 1993). Therefore the actions of the two different receptors influence different aspects of the lamprey locomotor rhythm and GABAa receptors appear to be important in maintaining a regular pattern of rhythmic activity.

In the neonatal rat, rhythmic locomotor activity generated in lumbar segments of the spinal cord does not require an inhibitory influence but like the lamprey, an increase in GABA inhibition caused either a slowing or a complete cessation of rhythmic activity (Cazalets et al., 1994). The effects of GABA were shown to be mimicked by GABAa and GABAb receptor agonists indicating once more that both receptors are involved in mediating the overall effect of GABA. Evidence for an endogenous role for GABA in the neonatal rat came from experiments which showed that perfusing the brainstem alone with NMDA could not elicit locomotor activity in lumbar segments of the spinal cord until either GABAa or GABAb antagonists were applied. This suggested that sustained rhythmic activity depends on a balance of excitatory and inhibitory influences and that the lumbar rhythm generators are tonically inhibited by GABAergic transmission (Cazalets et al., 1994). Differences in the roles of the two receptors types were confirmed from experiments which compared the effects of the antagonists.

Blockade of GABAa receptors with bicuculline led to a more stable long lasting pattern of activity, with decreased cycle periods and an increase in the amplitude of the locomotor rhythm (Cazalets et al., 1994). The effects of blocking GABAb receptors were much weaker giving more variable patterns of activity. Baclofen had a similar effect to bicuculline on the cycle period but had no apparent effect on the amplitude of the activity. This provided some evidence that GABAa receptors were located both pre- and postsynaptically whereas the majority of the GABAb receptors were located on the presynaptic membrane alone (Cazalets et al., 1994). There is some evidence to indicate that the GABAergic neurons involved in the response are spinal in origin as the GABAergic effect remained after any influence from supraspinal neurons was removed by bathing the thoracic and brainstem areas with an isotonic solution of sucrose (Cazalets et al., 1994).

Recent experiments have also been carried out on the neonatal rat preparation to determine whether GABA plays any role in reciprocal inhibitory interactions. In the presence of NMDA, either strychnine or bicuculline abolished both flexor and extensor activity as well as left-right alternation whereas the GABAb antagonists, phaclofen and 2-hydroxysaclofen, had no effect on the alternating rhythm. These results suggest that the activation of GABAa receptors in addition to glycine receptors is important for alternating activity in antagonistic motor pools (Cowley and Schmidt, 1995).

Taken together, these studies suggest that GABA has an overall inhibitory influence on locomotor activity and in addition can finely tune the timing of the central pattern generator (CPG, Delcomyn, 1980). This 'fine tuning' of the locomotor activity is thought to arise from the action of

GABA opposing the 'activatory' influences of transmitters such as 5HT (Cazalets et al., 1994, Wallén, 1995)

The *Xenopus* preparation.

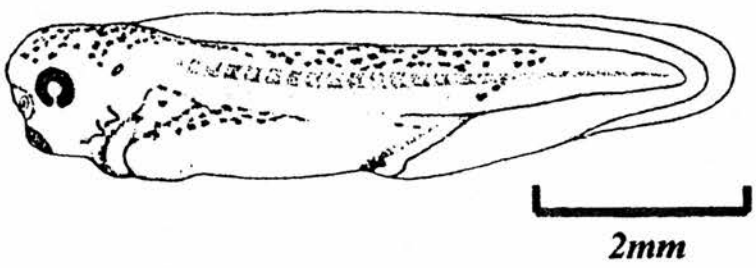
The CPG responsible for generating swimming behaviour in the stage 37/8 *Xenopus* embryo is one of the best understood vertebrate locomotor circuits, along with that of the adult lamprey. This simple system has allowed a detailed investigation of some of their membrane properties of the neurons and synaptic interconnections which are involved in the production of swimming near the time of hatching (for review, see Roberts, 1990). More recently, the preparation has also been exploited as an amenable model for studying developmental changes which occur in the swimming CPG subsequent to hatching (Sillar et al., 1995a).

However, the role of GABAergic neurotransmission in *Xenopus* swimming is still largely unknown, despite the presence of several populations of neuron which show GABA-like immunoreactivity (Dale et al., 1987, Roberts et al., 1987). So far only one of these populations, that which is involved in the neural pathway mediating the 'stopping' response, has been investigated in any detail (Boothby and Roberts, 1992a,b and see below).

Behaviour

Near the time of hatching, around two days into development (figure 1.1a), the 5mm long embryo of the African clawed toad, *Xenopus laevis,* will swim when touched on the trunk or tail or following sudden dimming of the illumination. Following a brief sensory stimulus, rhythmic

a.



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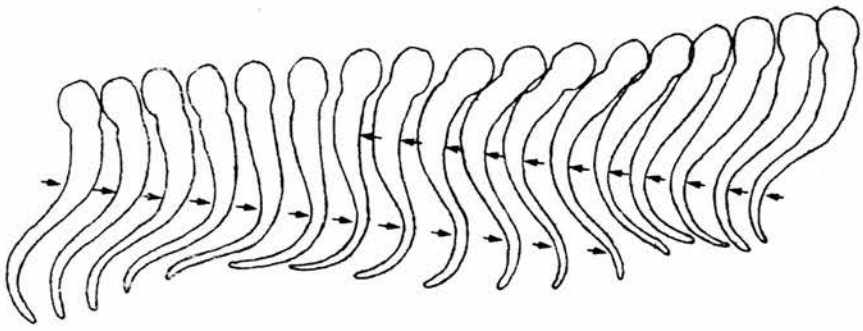

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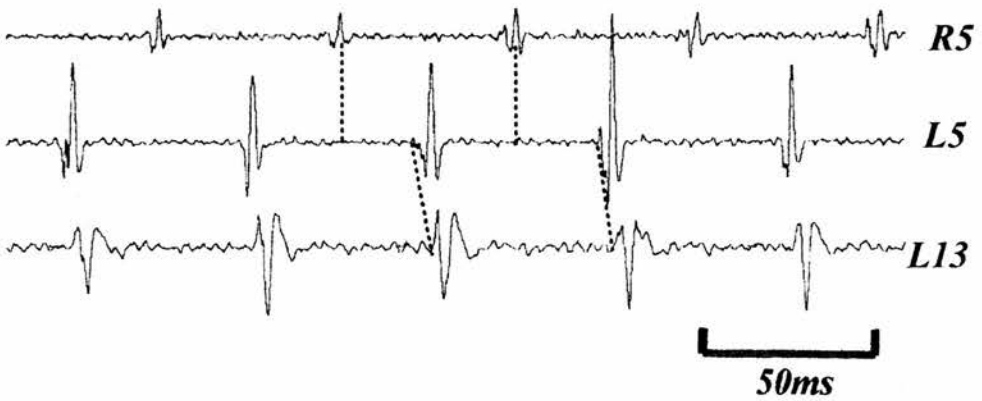


Figure 1.1. Swimming in the *Xenopus* embryo.

At the time of hatching, the *Xenopus* embryo **(a)** is capable swimming by lateral undulation via coordinated contractions of segmental myotomes. **(b)** Tracings of swim cycle from high speed video recordings. Swimming occurs at frequencies of 10-20Hz and a wave of bending passes down the length of the animal, first down one side and then the other, arrows mark the point of maximum body curvature (Drawing by J.F.S. Wedderburn). **(c)** Recordings from the ventral roots of an animal immobilised in the neuromuscular blocker, α-bungarotoxin, reveal a pattern of activity appropriate to drive swimming activity. One recording is made from a cleft on the right hand side of the animal, five intermyotomal clefts caudal to the otic capsule (R5), and the other two from the rostral (L5) and caudal (L13) end of the animal on the left hand side. The pattern of activity strictly alternates on the left and right sides and shows a rostrocaudal delay along the length of the animal on the same side (indicated by the dotted lines). (Data provided by K.T. Sillar)

swimming activity can be sustained for up to several minutes. This activity is achieved by a coordinated pattern of contractions in the segmented myotomal muscles, which alternates between the two sides and passes down the body from head to tail, at around $15cms^{-1}$ resulting in a propulsive wave of bending which moves the animal forward at about $5cms^{-1}$ (Roberts, 1990, figure 1.1b). Swimming terminates either spontaneously or after the animal encounters an obstruction in its path.

In embryos which have been paralysed with the neuromuscular blocking agent, α-bungarotoxin (α-BTX), extracellular recordings made from the ventral motor roots reveal a rhythmic pattern of activity which is suitable to drive swimming behaviour, called 'fictive swimming'. Fictive swimming activity can be initiated following a brief (0.5-1ms) electrical current pulse applied to the tail skin or by dimming the illumination. Since no proprioceptive input is required to maintain fictive swimming within the normal range of frequencies, it must therefore be centrally generated (Kahn and Roberts, 1982). The sustained rhythmic activity which occurs at frequencies of 10-30Hz strictly alternates between the left and right sides and progresses with a brief rostrocaudal delay down the length of the animal on the same side (fig 1.1c). Spinalisation studies, which remove any possible influences from the brain, have also shown that the spinal cord itself is sufficient to produce rhythmic activity (Roberts et al., 1986). Therefore, the CPG for swimming is located within the spinal cord. Fictive swimming activity can also terminate either spontaneously or after touching the head skin or rostral cement gland, as in the real behaviour.

Neuroanatomy.

In 1982, Roberts and Clarke used horseradish peroxidase (HRP) staining to anatomically define the classes of differentiated neurons contained within the embryo spinal cord and described just eight anatomically distinct types. Figure 1.2c, shows a schematic drawing of the position of the classes of neuron within the cord.

Rohon-Beard (R-B) cells are primary mechanosensory neurons whose large cell bodies occupy the medial dorsal cord. Single unmyelinated neurites arising from R-B cell bodies innervate the skin with free nerve endings (Hughes, 1957, Roberts and Hayes, 1977) and their dorsal ipsilateral axons bifurcate to ascend as far as the hindbrain and also descend within the spinal cord. Two classes of second order sensory interneurons are also located in the dorsal cord just beneath the R-B cells; dorsolateral ascending (dla) and dorsolateral commissural (dlc) interneurons. The cell bodies of both classes of sensory interneuron lie at the dorsal limit of the lateral tract and have dendritic branching which extends into the dorsal tract, where they are thought to synapse with R-B cells and into the dorsal part of the lateral tract. Axons of the dorsolateral ascending interneurons project rostrally in the lateral tract on the ipsilateral side, whereas the axons of the dorsolateral commissural cells cross the cord and ascend and descend in the lateral tract on the contralateral side.

Located dorsoventrally, in the middle portion of the cord, are three other classes of interneurons; commissural interneurons (c), descending interneurons (d) and ascending interneurons (a). The commissural interneurons have unipolar somata and a main neurite which runs ventrally along the inside edge of the lateral tract from which short radial dendrites extend into the lateral tract. The axon runs in the ventral commissure to the

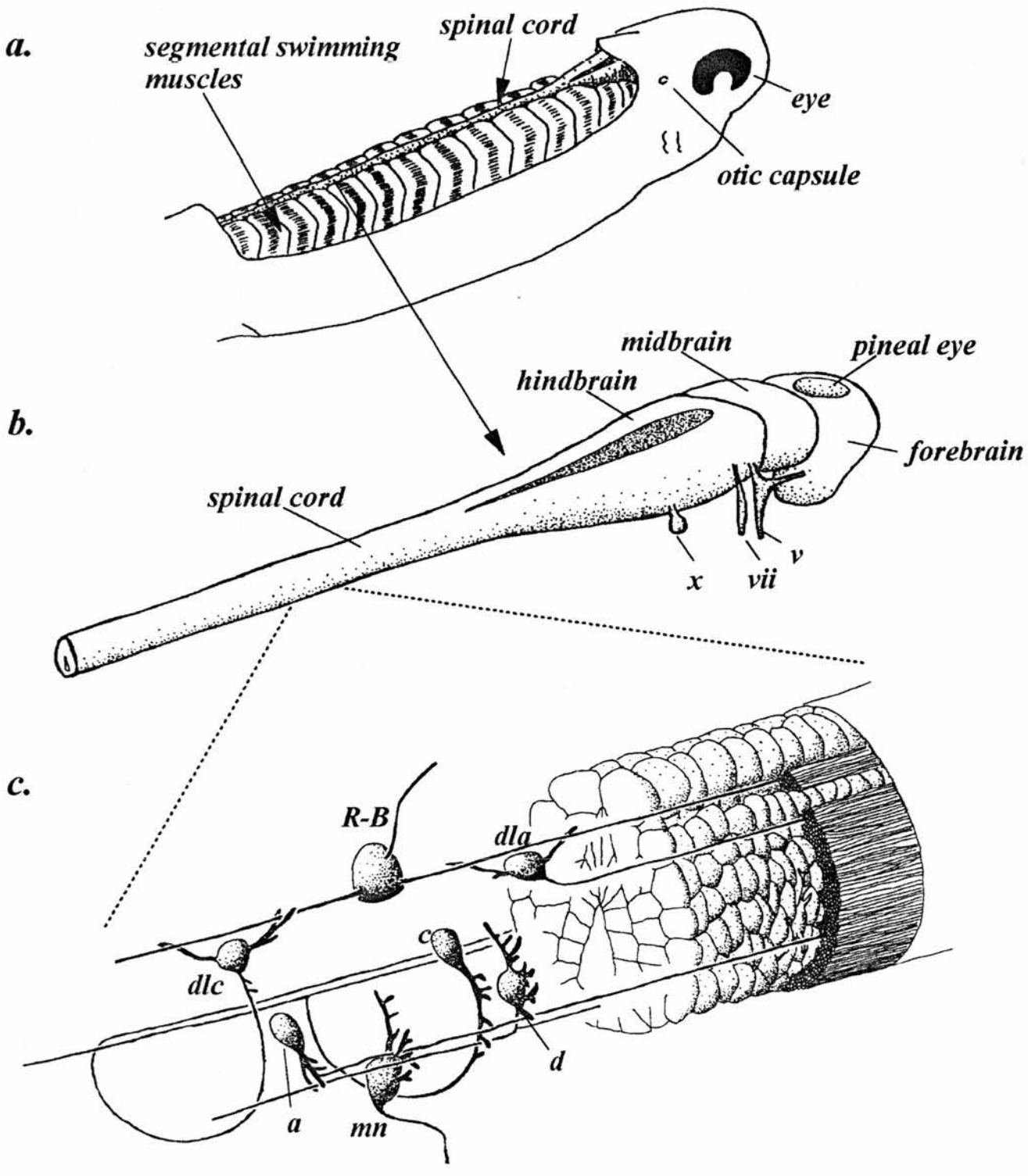

a.
segmental swimming muscles
spinal cord
eye
otic capsule
b.
midbrain
pineal eye
hindbrain
forebrain
spinal cord
x
vii
v
c.
R-B
dla
dlc
c
d
a
mn

Figure 1.2. The neuroanatomy of the *Xenopus* embryo spinal cord.

a. The spinal cord is a gently tapering tube which lies between the segmental myotomes.
b. The isolated embryo CNS consists of a forebrain, midbrain and hindbrain which tapers into the spinal cord. **v**, **vii**, **x** = fifth, seventh and tenth cranial roots.
c. An expanded section of the spinal cord showing examples of the anatomically defined differentiated classes of spinal neurons: **R-B** - primary sensory Rohon Beard cells; **dlc** - dorsolateral commissural sensory interneurons; **dla** - dorsolateral ascending sensory interneurons; **c** - commissural interneurons; **d** - descending interneurons; **a** - ascending interneurons; **mn** - motorneurons.
Drawings kindly provided by S.R. Soffe.

ventral part of the contralateral tract where it branches rostrally towards the hindbrain and caudally within the spinal cord. Descending interneurons have multipolar somata which lie in the inside border of the lateral tract. The dendrites reach all levels of the lateral and dorsal tracts and the axons run caudally in the lateral tract just dorsal to the motorneurons. The final population of neurons in this region of the cord are the ascending interneurons whose unipolar somata are located deep in the cord relative to the lateral tract. Dendrites which innervate the ventral half of the lateral tract arise from a single process which reaches the inner margin of the lateral tract and branches. The axons lie in the inner part of the lateral tract where they ascend to the hindbrain and also descend for a short distance.

The cell bodies of the motoneurons occupy the ventral most regions of the cord, lying along the inner edge of the lateral tract forming a column often more than one cell thick. The dendrites innervate the lateral tract but do not reach the dorsal tract. The axons tend to run caudally before turning obliquely to leave the cord. Some motorneurons possess two peripheral axons and can innervate more than one motor nerve. The motor nerves reach caudally, usually to the next intermyotomal cleft, where they innervate the myotomes.

The final class of differentiated cell types found in the embryo cord are the Kölmer Agdhur cells (not shown in figure 1.2c). These are ciliated ependymal cells which have also been described in many vertebrate species and which contact the neurocoel. The axons run in the lateral tract to reach the hindbrain. These cells show GABA-like immunoreactivity (Dale et al., 1987), but nothing is known about their function.

Basic rhythmic output underlying swimming activity

The detailed knowledge of the neuroanatomy of the embryo spinal cord combined with the relative ease of making intracellular recordings from individual neurons within the cord has facilitated progress towards defining the roles of different classes of neurons in the limited behavioural repertoire of the embryo. Of the eight classes of neuron described above, only three of them appear to be involved in the generation of swimming: motorneurons; descending ipsilateral interneurons and commissural interneurons.

Intracellular recordings made from motorneurons located in the ventral portion of the cord (Roberts and Kahn, 1982) have shown that the synaptic drive during swimming comprises three main components: i.) phasic excitation, causing the neurons to fire an action potential once per cycle of activity; ii.) tonic excitation, providing a background depolarisation upon which activity is superimposed and iii.) midcycle inhibition, during which the motorneurons are hyperpolarised when neurons on the opposite side of the cord are active (figure 1.3).

Both the phasic excitation and the background tonic depolarisation result from the release of an excitatory amino acid, probably glutamate, from just one population of rhythmically active interneurons, the descending interneurons (Dale and Roberts, 1985). There are two different subtypes of ionotrophic postsynaptic receptor which are activated by excitatory amino acid agonists: kainate/AMPA (K/A) receptor and the NMDA receptor (Watkins and Evans, 1981, see Lodge and Collingridge, 1990 for latest classification). These receptors subtypes are present on *Xenopus* embryo motorneurons (Dale and Roberts, 1984). The antagonist, cis-2,3-piperidine dicarboxylic acid (PDA) blocks the activation of both

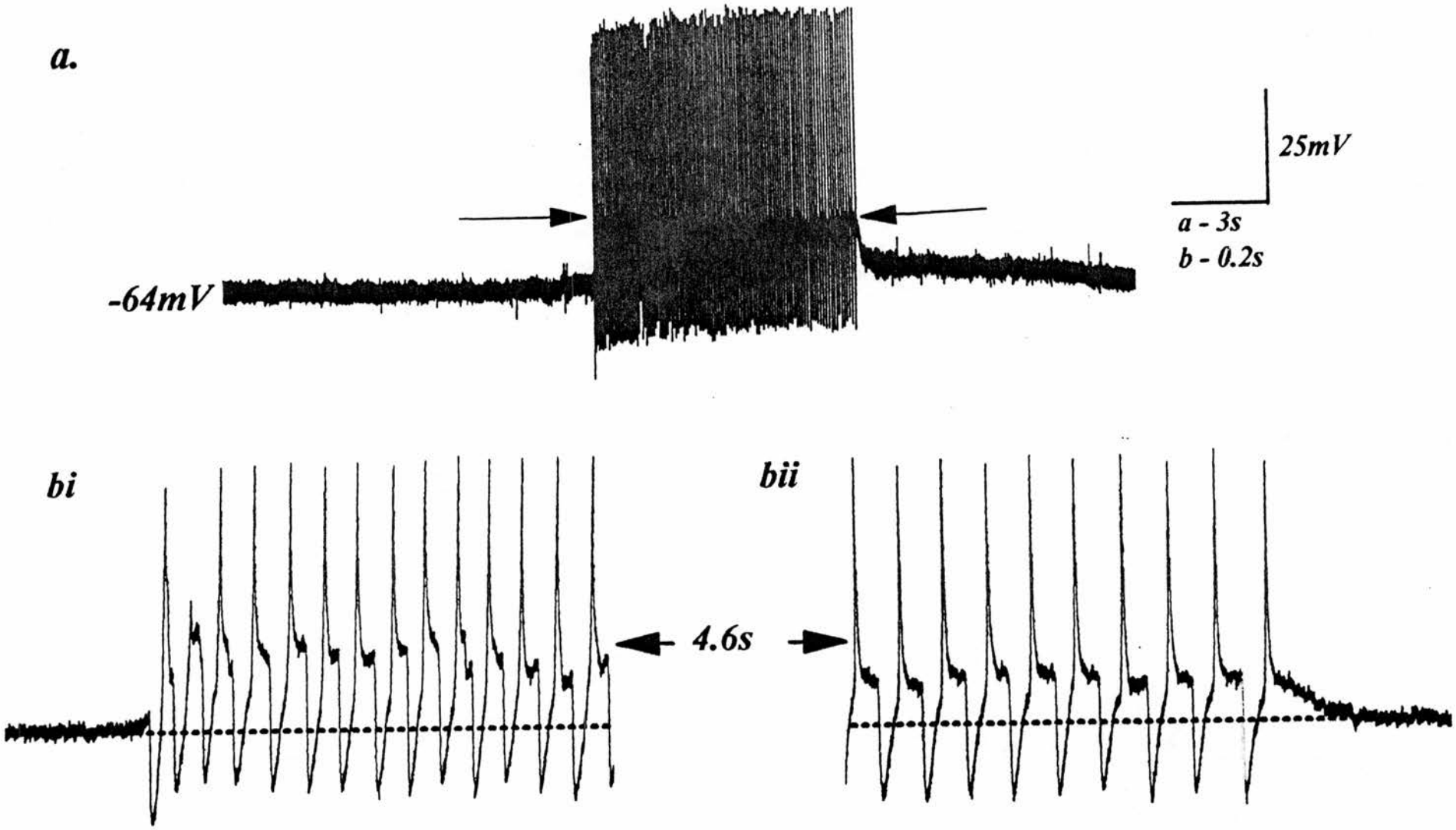

a.
25mV
a - 3s
b - 0.2s
-64mV
bi
bii
4.6s

Figure 1.3. The synaptic rhythmic drive underlying swimming activity.

a. Intracellular recording from ventrally positioned spinal neuron, presumed to be a myotomal motorneuron, during an episode of swimming activity in response to dimming the lights. Spiking is superimposed upon a level of tonic depolarisation (indicated by the arrows).
bi, ii. The three components of the rhythmic activity are clearly shown on the expanded traces of the beginning **(i)** and end **(ii)** of the episode. The dotted line indicates the resting membrane potential. Note spiking occurs from a depolarised level (at arrow). The neuron is inhibited midcycle to below the level of the resting membrane potential, when neurons on the contralateral side are activated. The tonic depolarisation is greatest at the start of the episode **(i)** and declines somewhat towards the end of the episode **(ii)**. (Data of K.T. Sillar).

receptor subtypes, while (±)-2-amino-5-phosphonovaleric acid (APV) selectively blocks the activation of the NMDA receptor. Using these antagonists, it has been shown that unitary epsps, evoked in motorneurons by extracellular stimulation of descending interneuronal axons are 'dual-component', having a fast rise but a slow fall time and are due to the combined activation of both K/A and NMDA receptors. Bath application of APV blocks the slow fall of the dual-component epsp with little effect on the peak amplitude suggesting that this slow component is due to the activation of the NMDA receptor. The remaining fast component is sensitive to PDA and is therefore mediated by the K/A receptor subtype (Dale and Roberts, 1985). The slow epsps resulting from activation of the NMDA receptor have a duration of around 200ms (Dale and Roberts, 1985) whereas the normal swim cycle period ranges between only 50 and 100ms. Thus, these longer duration NMDA-potentials will summate when evoked at normal swimming frequencies to produce a sustained tonic depolarisation like that seen in motorneurons during swimming. Meanwhile, the faster potentials resulting from the activation of K/A receptors are still available to provide the phasic excitation underlying impulse generation on each cycle.

Paired intracellular recordings from premotor interneurons and postsynaptic motorneurons have shown that action potentials generated by depolarising current injection into certain interneurons evoke epsps in the motorneurons with the same pharmacology as those which were evoked extracellularly. Neurons of this type which were stained intracellularly with HRP (Dale and Roberts, 1985) were found to have similar morphology to the descending interneurons described by Roberts and Clarke (1982; see earlier). The excitatory interneurons receive the same synaptic drive as the other rhythmically active neurons during swimming, making it seem likely that they will mutually re-excite themselves and computer modelling studies

have shown that this feature is essential to sustain rhythmic output (Roberts and Tunstall, 1990).

The initial view that all of the on-cycle excitation received by motorneurons was the result of glutamate release from descending interneurons and that motorneurons were just passive output elements has recently been challenged. The motorneurons themselves are known to make both chemical and electrical synapses in the spinal cord which contribute to on-cycle excitation during swimming (Perrins and Roberts, 1995a,b,c). Motorneurons make strong excitatory, nicotinic cholingeric and electrical synapses onto each other (Perrins and Roberts, 1995a) as well as weak cholinergic synapses onto premotor interneurons of the CPG (Perrins and Roberts, 1995c). Paired recordings have shown that action potentials in a rostral motorneuron elicits one-for-one short latency epsps in more caudal neurons, consistent with the presence of monosynaptic connections between neighbouring motorneurons (Perrins and Roberts, 1995a). From studies using local applications of nicotinic antagonists, it was determined that during rhythmic activity, 20% of the on-cycle excitation onto motorneurons was the result of nicotinic ACh receptor activation by endogenously released ACh (Perrins and Roberts, 1995b). A further 50% of the epsp was resistant to Cd^{2+} and therefore largely due to the electrical coupling (Perrins and Roberts, 1995b). Unlike chemical transmission, electrical connections are non-rectifying and are restricted to pairs of neurons located less than 70μm apart (Perrins and Roberts, 1995a). Therefore during swimming activity only around 30% of on-cycle excitation onto motorneurons is the result of the release of glutamate from descending excitatory interneurons. During swimming, the release of ACh will enhance fast on-cycle excitation of more caudal motorneurons, providing a possible explanation for the reliability of firing of embryonic motorneurons. In addition the electrical

connections will promote the synchronous firing of local populations of motorneurons (Perrins and Roberts, 1995a). Activation of nicotinic ACh receptors on premotor interneurons also increases the reliability of firing of premotor elements. As a result this will enhance tonic depolarisation during swimming which is dependent on the number of excitatory interneurons which fire (Perrins and Roberts, 1995c).

The normally hyperpolarising midcycle ipsps recorded in motorneurons are reversed in sign to become depolarising when recordings are made with KCl-filled microelectrodes indicating that they are chloride-dependent. The ipsps are also reduced or abolished by the glycine receptor antagonist, strychnine indicating glycine as the transmitter involved (Dale 1985; Soffe, 1987). Several lines of evidence indicate that the population of neurons mediating midcycle inhibition are located on the opposite side of the cord, in addition to the fact that no neurons have been found which fire midcycle on the ipsilateral side. Initial compelling evidence was gained from hemisection studies where the cord was firstly cut along the midline over a length of one myotome and the cord on one side then severed to prevent any descending input (Soffe and Roberts, 1982). Recordings made from motorneurons on the operated side, below the level of the severed cord showed only rhythmic inhibition and rhythmic excitation during swimming was abolished. Of the eight populations of differentiated neurons described by Roberts and Clarke (1982), only two of these populations have commissural axons: dorsolateral commissural neurons which have been shown to be inhibited during rhythmic swimming activity (Sillar and Roberts, 1992a) and commissural interneurons which show a similar pattern of activity to the motorneurons on the same side. Paired recordings in combination with intracellular HRP staining confirmed that those neurons which caused strychnine-sensitive inhibition of contralateral motorneurons,

had the same morphology as commissural interneurons (Dale, 1985). Subsequent immunocytochemical studies confirmed that the commissural interneurons show glycine-like immunoreactivity (Dale et al., 1986) and furthermore they appear to be the only population of glycinergic interneuron in the entire CNS.

Commissural interneurons project to various levels in the contralateral lateral tract and appear to inhibit all types of rhythmically active neurons on the opposite side of the cord, including contralateral commissural interneurons. They may therefore ensure that the rhythmic pattern of ventral root activity during swimming is strictly alternating and indeed bath application of strychnine weakens and eventually abolishes the reciprocal activation of motorneurons during swimming activity (Roberts et al. 1985). Computer modelling studies have also indicated a role for cross cord inhibition in 'post inhibitory rebound' (Roberts and Tunstall, 1990) whereby neurons will fire a rebound action potential after a brief hyperpolarisation. Furthermore, it has been shown experimentally that neurons will fire following a brief hyperpolarisation so long as they are already tonically depolarised (Roberts et al., 1986, Soffe, 1990). However, it has also been shown that after longitudinally dividing the CNS, thereby removing any cross cord influences, one side of the hindbrain and spinal cord can still generate sustained rhythmic motor discharge (Kahn and Roberts, 1982). Some glycinergic neurons also have ipsilateral axons (Dale, 1985) which produce on-cycle inhibition and in the half brainstem, spinal cord preparation, blockade of glycine receptors with strychnine does affect the ability of the rhythm generator to sustain rhythmic activity (Soffe, 1989). However, rhythmic activity can still be produced in the presence of strychnine, suggesting that glycinergic inhibition is not strictly necessary for rhythm generation in each half centre (Dale, 1985). Rhythm generation in

the half cord appears to rely on the voltage-dependence of NMDA receptors since rhythmic activity is only seen in the presence of extracellular magnesium (Soffe, 1989). These findings do not necessarily rule out the likely possibility that midcycle glycinergic inhibition still influences sustained rhythmic activity in the intact preparation.

The alternating pattern of rhythmic locomotor activity recorded from the *Xenopus* embryo resembles that seen in many other vertebrate species. For example, the neural activity underlying locomotion in the lamprey has also been used as a simple vertebrate model. Like the *Xenopus* embryo, the lamprey swims by lateral undulations of the body produced by contractions of the segmental myotomes and this pattern of activity appears to be generated in much the same way as swimming in the *Xenopus* embryo. The excitatory drive is produced by the coactivation of NMDA and non-NMDA glutamate receptor subtypes (Dale and Grillner, 1986) and reciprocal inhibition, which is essential for the alternation of activity between the two sides of the cord, is achieved by the release of glycine from commissural interneurons (Buchanan, 1982, Alford and Williams, 1987). Additionally, this formula for the generation of locomotor activity is not limited to lower vertebrates with axial based systems, as similar transmitters and receptors have been shown to be involved in the production of locomotor activity in neonatal and adult mammals (Kudo and Yamada, 1987, Fenaux et al., 1991, Douglas et al., 1993). This would suggest, therefore, that the *Xenopus* system is a valuable simple model in which to further explore the basic mechanisms which underlie rhythm generation.

Initiation of swimming activity.

Fictive swim episodes can be initiated in similar ways to the real swimming in the intact embryo, either by a mechanical or electrical stimulus applied to the trunk skin (Clarke et al., 1984) or by transiently dimming the illumination (Roberts, 1978). The skin sensory pathways involve the activation of the only known population of sensory afferents, the Rohon-Beard cells (Clarke et al., 1984) but in addition the skin cells themselves are electrically excitable, connected to each other via gap junctions and have access to the CNS via the trigeminal nerve providing an additional route for the initiation of swimming (Roberts and Smythe, 1974). It is, however, not yet known how this additional skin sensory pathway initiates swimming. The cell bodies of R-B neurons are positioned in the dorsal part of the cord and their unmyelinated peripheral neurites innervate the trunk and tail skin with free nerve endings. Each peripheral neurite extends fine branches between the cells of a small area of skin thereby creating an overlapping network of receptive fields over the trunk and tail. The free nerve endings respond transiently to pressure stimuli (Roberts and Hayes, 1977).

The central axons of R-B neurons ascend to the hindbrain and descend in the spinal cord within the dorsolateral tract (Roberts and Clarke, 1982). Since spinal embryos still swim in response to a skin stimulus, the R-B cells must make direct synaptic contact onto neurons within the cord. Two classes of sensory interneuron have been shown to be excited by R-B cells (Clarke et al., 1984, Roberts and Sillar, 1990, Sillar and Roberts, 1992a). The first population has been confirmed anatomically as the dorsolateral commissural (dlc) interneurons described by Roberts and Clarke in 1982 (Sillar and Roberts, 1988a, Roberts and Sillar, 1990). The

second population is assumed to be the dorsolateral ascending neurons, but their identity has yet to be confirmed (Sillar and Roberts, 1992a).

Intracellular recordings from dorsolateral interneurons showed that following electrical stimulation of unmyelinated R-B cell neurites, compound epsps are recorded with a latency which is consistent with a monosynaptic input from R-B cells (Clarke et al., 1984, Sillar and Roberts, 1988b). The epsps recorded from the postsynaptic neuron are similar to those already reported to occur in motorneurons (Dale and Roberts, 1985) in that they result from the release of glutamate which activates both NMDA and non-NMDA receptors resulting in a dual component epsp (Sillar and Roberts, 1988b).

The graded nature of the amplitudes of the epsps recorded in sensory interneurons in response to graded stimulus intensities, suggests that one dorsolateral interneuron receives an input from many R-B cells and also implies that one Rohon-Beard cell can make contact with many dorsolateral interneurons, thereby amplifying the brief impulse from an R-B cell. This means that sensory interneurons could be activated following a stimulus to any part of the trunk skin (Sillar and Roberts, 1988b). Furthermore, dorsolateral ascending neurons project to axons on the same side while dorsolateral commissural interneurons excite the opposite side, resulting in further amplification of the signal by allowing the brief signal from the R-B cells to spread to both sides of the cord. This also explains how axons of the R-B neurons which run up and down the spinal cord on the ipsilateral side can initiate a reflex muscular contraction on the opposite side to bend the animal away from the site of the stimulus (Sillar and Roberts, 1988a).

During rhythmic activity dlc neurons are rhythmically inhibited in phase with motor activity on the same side of the cord (Roberts and Sillar, 1990, Sillar and Roberts, 1992a). This inhibition is strychnine-sensitive and

its timing suggests that it results from inhibition from the ipsilateral processes possessed by a sub-population of interneurons which are part of the CPG (Sillar and Roberts, 1992b). The result of this inhibition is that a stimulus can only excite motorneurons on the opposite side when they are already active. Behaviourally this will mean that the crossed avoidance reflex can only modulate swimming when it would result in the animal turning away from the site of stimulation. Thus the sensory inflow to the spinal cord can both initiate fictive swimming and modulate the activity at a certain phase in the locomotor cycle. The CPG itself then modulates incoming sensory information.

The 'dimming' response appears to be a simple escape behaviour in which the embryo will swim following a sudden dimming of the illumination, as would occur when a potential predator casts a shadow. However, since the behavioural response to changes in light levels in free swimming embryos is relatively weak compared to that observed in immobilised animals, it may play a more subtle role in increasing the likelihood of swimming in response to other stimuli. The dimming response is most effective between stages 33 and 39 and declines until stage 44 when the animal no longer swims in response to sudden changes in light levels (Roberts, 1978; Foster and Roberts 1982). Experimental evidence implicates the pineal eye in this response (Foster and Roberts,1982). It is known to contain photoreceptors (Bagnara, 1965) similar to those found in the vertebrate retina which are most sensitive to wavelengths of light around 520nm. Recordings made from the pineal have shown that there is a continuous, irregular discharge of activity which is transiently increased following a sudden decrease in light levels (Foster and Roberts, 1982). However, the precise pathway involved in this response has not yet been fully described. Axons of the pineal run ventrally and superficially forming

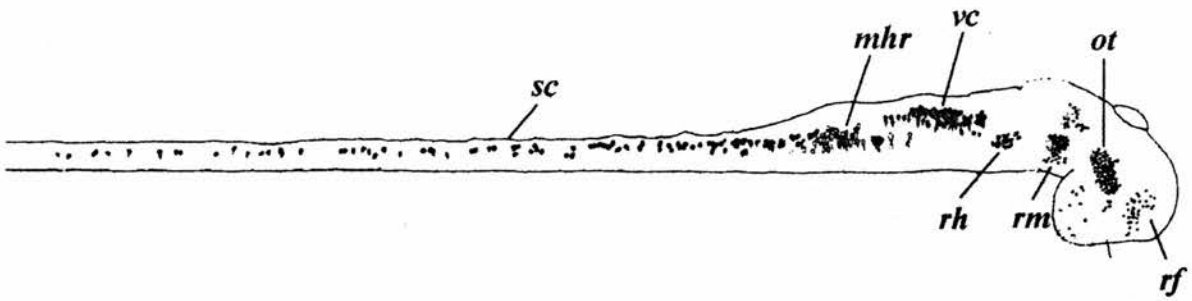
a groove between the mid- and forebrain to form a ventral commissure but they do not project directly to the spinal cord.

Stopping response - a role for GABA.

So far, studies on the *Xenopus* embryo have found no role for GABA in the generation or modulation of the locomotor rhythm (Soffe, 1987). Despite this, the embryo CNS has been shown to possess eight populations of neurons which show GABA-like immunoreactivity (figure 1.4a, Dale et al., 1987, Roberts et al., 1987). Two of the populations are spinal in origin: the ascending neurons and the K-A cells, whose anatomy have been described previously (Roberts and Clarke, 1982) and for which there is, as yet, no known role. The remaining six populations of neurons originate in the brain. Three of them are located in the hindbrain: the midhindbrain reticulospinal neurons (mhr); the vestibular complex neurons (vc) and the rostral hindbrain (rh) commissural neurons. The final populations located in the mid- and forebrain are the rostral midhindbrain neurons (rm), the optic tract neurons (oc) and the rostral forebrain neurons (rf). Of the six brain populations a role for only one of them, the mhr neurons, has so far been described.

At the time of hatching the embryo will usually cease swimming after contacting an obstruction in its path and then become attached to the object by a strand of mucus secreted from the rostral cement gland (figure 1.4bi). This 'stopping' response is seen in animals as young as stage 28, although its reliability is low. By stages 33/34 through to 40 it is at its most reliable after which the response gradually declines through to stage 46 when the animal now maintains its position in the water by 'hovering' via rhythmic undulations of the tail. During this time the cement gland

a.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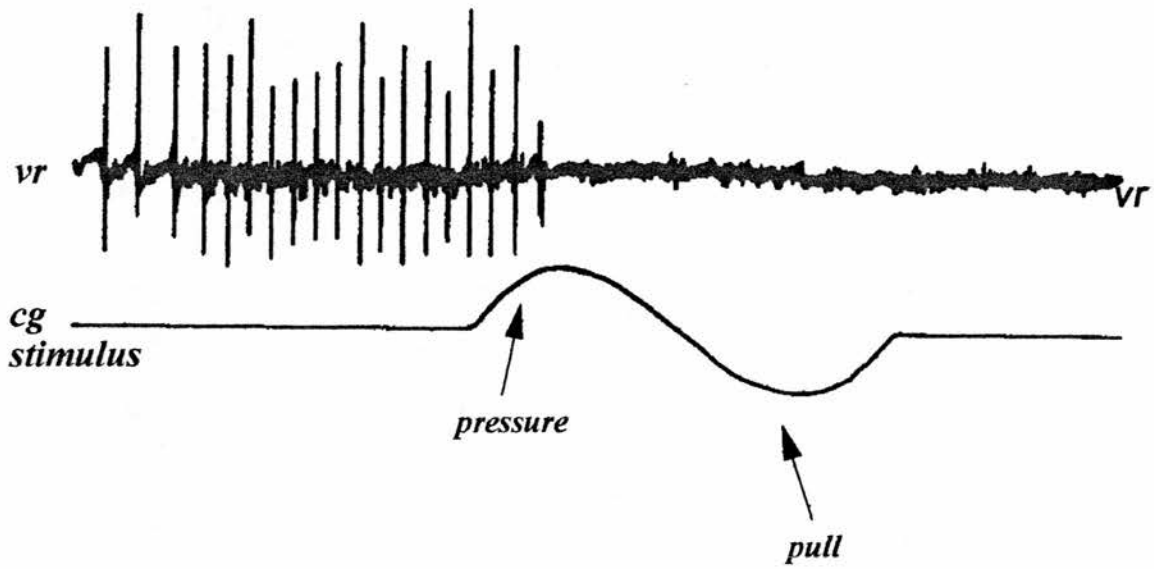
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ii

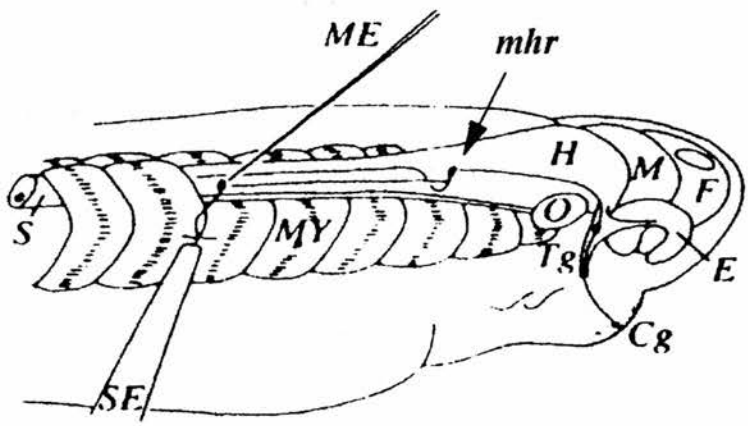
ME
mhr
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Figure 1.4. MHR neurons are involved in the stopping response.

a. Eight populations of neurons in the embryo spinal cord show GABA-like immunoreactivity. Two populations are located in the spinal cord (**sc**), ascending interneurons and ciliated Kölmer-Agdhur cells (not shown). The hindbrain contains the midhindbrain reticulospinal neurons (**mhr**), the vestibular complex neurons (**vc**) and the rostralhindbrain commissural neurons (**rh**). Located in the mid- and forebrain are the rostral midhindbrain neurons (rm), the optic tract neurons (**ot**) and the rostral forebrain neurons (**rf**). From Roberts et al. (1987).

bi. Fictive swimming activity can be stopped by applying pressure to either the head skin or cement gland of the animal. The top trace shows ventral root activity which is terminated following gentle pressure on the cement gland, the timing of which is indicated in the bottom trace. From Boothby and Roberts, (1992a).

bii. The primary afferents in the cement gland pathway are the trigeminal ganglion neurons whose sensory projections innervate the head skin and cement gland. They are thought to excite the GABAergic mhr neurons which have ipsilateral and contralateral descending axons that project to the spinal cord and inhibit CPG interneurons and motorneurons on both sides of the cord. **E** - eye, **F** - forebrain, **M** - midbrain, **H** - hindbrain, **O** - otic capsule, **Tg** - trigeminal ganglion, **Cg** - cement gland, **MY** - myotome, **ME** - microelectrode, **SE** - suction electrode, **S** - spinal cord. From Boothby and Roberts, (1992a).

structure itself is also deteriorating until, at stage 43, the structure is almost lost (Boothby and Roberts, 1992a).

In 1975, Roberts and Blight described how swimming in the embryo could usually be halted by applying pressure to any part of the head but especially the cement gland. A fictive correlate of the stopping response is also seen in immobilised embryos following cement gland or head skin stimulation and like the dimming response, it is also found to be more reliable than in free swimming animals (figure 1.4b). If both the head skin and cement gland are removed (Boothby and Roberts, 1992a) the stopping response is abolished but as long as one of these structures is intact the pathway is still functional.

Stimuli to the cement gland or the head skin activate of a class of movement detector neurons in the trigeminal ganglia (Roberts and Blight, 1975; Roberts, 1980) whose peripheral neurites were shown by HRP studies to innervate both the cement gland and head skin with pressure sensitive, free nerve endings (Hayes and Roberts, 1983). Simultaneous recordings of trigeminal neurons and ventral root activity (Boothby and Roberts, 1992b), have shown that trigeminal ganglion activity precedes the cessation of ventral root activity which occurs within one cycle period (50-100ms) of the first trigeminal ganglion spike. Therefore there is good circumstantial evidence that these neurons are the primary sensory neurons of the cement gland pathway. HRP studies performed by Hayes and Roberts (1983) further showed that the axons of trigeminal ganglion neurons run caudally through the hindbrain, but very few of the axons project all the way to the spinal cord. Given that immunocytochemical studies failed to reveal any neurons within the trigeminal ganglia which show either glycine or GABA-like immunoreactivity, it seemed probable that at least a disynaptic pathway was involved in the stopping response. Lesion experiments have indicated

that the pathway involved is located in the hindbrain (Boothby and Roberts, 1992a). The pharmacology of the response provided evidence for a role for GABAa receptor activation in the spinal inhibitory component of this pathway (Boothby and Roberts, 1992b). As described previously, the hindbrain of the embryo contains three populations of neurons with GABA-like immunoreactivity: mhr, vc and rh neurons (Roberts et al., 1987). Of these neurons, only the mhr population has been shown to have ipsilateral and contralateral descending axons (Roberts et al., 1987). In addition, the mhr neurons are the first population of GABA-immunoreactive neurons to develop, at around stage 25/26 (Roberts et al., 1987), with the stopping response becoming functional from stage 28. Therefore it seems likely that the mhr neurons are involved in the stopping response and are either activated directly by the trigeminal neurons or via an interposed excitatory interneuron (figure 1.4bii).

Aims of this study.

Recent studies have explored the postembryonic development of the swimming rhythm and have shown that only twenty four hours later in development (at stage 42) the pattern of activity is far more complex and flexible. These findings are reviewed in the introduction to chapter 4, but in summary, at stage 42 in larval development, the swimming rhythm involves longer duration bursts of motor activity rather than the single-spike per cycle pattern of activity displayed at stage 37/8. Larval swimming, therefore, more closely resembles the locomotor output generated by adult and higher vertebrates Since the activation of GABA receptors plays a potentially important role in finely tuning locomotor activity in these other preparations (eg. lamprey- Tégner et al., 1993, neonatal rat- Cazalets et al.,

1994) it is conceivable that during postembryonic development of the *Xenopus* swimming system, GABAergic neurons likewise become more involved in modulating swimming activity. The major aim of this study therefore, was to investigate whether, at the larval stage in development, GABA modulates the swimming rhythm via the activation of GABAa receptors.

GABAa receptor activation is apparently not involved in the production of the basic embryonic swimming output, yet a population of GABAergic neurons, the mhr neurons, is involved in the stopping response (Boothby and Roberts, 1992b). Their action at GABAa receptors on spinal neurons causes swimming to cease when the animal contacts an obstruction. In order to study the possible role of GABAa receptor activation in *Xenopus* fictive swimming, selective agonists and antagonists were required to analyse the effects of activating or blocking these receptors on swimming activity. Previous studies have shown that certain neurosteroids are potent agonists at the GABAa receptor and also have the advantage that they enhance the activation of the receptor only following binding of endogenously released GABA (see introduction to chapter 3). The first chapter of results investigates the action of a neurosteroid on the stopping response pathway to confirm the specificity and synaptic mechanism of action of the neurosteroid agonist in this preparation. The neurosteroid and the GABAa antagonist, bicuculline, were then applied during embryonic fictive swimming activity to re-examine the possible role of GABA transmission in locomotion. These agents are then utilised in the second results chapter to address whether there is a developmental increase in the contribution of GABAa receptor activation to larval swimming activity.

The final results chapter then examines the pattern of larval 'swimming' activity which results from excitatory synaptic input in the

absence of inhibitory transmission. One descending input which develops over the twenty four hour period between the embryonic and larval stages is the innervation of the cord with serotonergic projections from the raphe nucleus. 5HT has been shown to modulate the locomotor activity of many different vertebrate preparations via several cellular and synaptic actions, including the induction of intrinsic membrane bistability (see introduction to chapter 5). Intrinsic membrane bistability has recently been shown in *Xenopus* larval preparations to be induced in the presence of exogenous 5HT and NMDA but the role of membrane potential oscillations during locomotor activity has remained unclear due to their frequency being low in comparison with the range of fictive swimming frequencies. Results presented in the last chapter indicate a possible role for these intrinsic membrane potential oscillations during larval swimming revealed following the blockade of inhibitory transmission.

Chapter 2
Methods.

Animals

Experiments were carried out on embryos (stage 37/8) and larvae (stage 42) of the African clawed toad, *Xenopus laevis,* staged according to Nieuwkoop and Faber (1956). The animals were obtained by induced breeding following injection of human chorionic gonadotrophin (1000 units per ml, Sigma) into pairs of adult animals from a laboratory colony, with the female receiving 0.35ml and the male receiving 0.15ml. The eggs were reared in de-chlorinated tap water at 17 - 23°C until they reached the appropriate stages throughout the week.

Experimental preparation and electrophysiological techniques.

The tadpoles were initially anaesthetised in 3-aminobenzoic acid ethyl ester (MS222, Sigma) and their tail fins gashed using fine etched tungsten needles to enable rapid access of the paralysing agent to the nervous system. After recovery from the anaesthetic, the animals were then placed in a chamber containing 2mls of the nicotinic ACh receptor antagonist, α-bungarotoxin (1.25μM, Sigma), an irreversible neuromuscular blocking agent.

After around 30 minutes, when the animals were fully paralysed, they were transferred to a preparation bath (ca. 5mls) which was continuously recirculated, at a rate of approximately 2ml/min, with frog ringer of the following composition (in mM): NaCl, 115: KCl, 2.5; $NaHCO_3$, 2.5; HEPES, 10; $MgCl_2$, 1; $CaCl_2$, 2 (extracellular experiments), 4 (intracellular experiments); buffered to pH 7.4 with 1N NaOH. The saline was gravity fed from a stock bottle containing 100ml saline. The animals were secured on their right sides through the notocord, using fine

pins etched from tungsten wire (Clarke Electromedical Instruments), to the sylgard surface of a rotating perspex table located in the preparation bath.

The preparation is shown in figure 2.1. To make extracellular recordings of swimming activity, the trunk skin was removed using mounted etched tungsten wires and fine forceps, to reveal the underlying myotomes. Ventral root recordings were then made by placing glass suction electrodes in the intermyotomal clefts where motor axons leave the spinal cord to innervate the muscle. The position of each electrode was noted as its distance in clefts from the otic capsule. The electrodes were hand pulled using glass fibreless capillary tubes (outer diameter 1mm, Clarke Electromedical Instruments) and then cut using a diamond knife to give a tip diameter of approximately 50μm. The recordings were amplified (x10K) using an AM-Systems differential amplifier (model 1700). Fictive swimming activity was initiated by either dimming the illumination (Roberts, 1978) or by applying a brief (0.5-1ms) current pulse (using a Digitimer DS2 isolated stimulator) to the tail skin via a stimulating suction electrode (Clarke et al., 1984). Stimulating electrodes were manufactured in the same way as recording electrodes.

To prepare the animal for intracellular recordings, a rostral section of myotomes was removed, using tungsten needles, to expose the underlying spinal cord. Recordings were then made using glass microelectrodes pulled on a Campden Instruments moving coil microelectrode puller (model 753) from filamented glass capillary tubes (outer diameter 1mm). The electrodes were filled with 2M KCl and had resistances of around 100-150MΩ. Penetrations were made in the ventral portion of the spinal cord using capacity overcompensation. It was assumed that the majority of recordings were from motorneurons as the ventral portion of the cord is known to

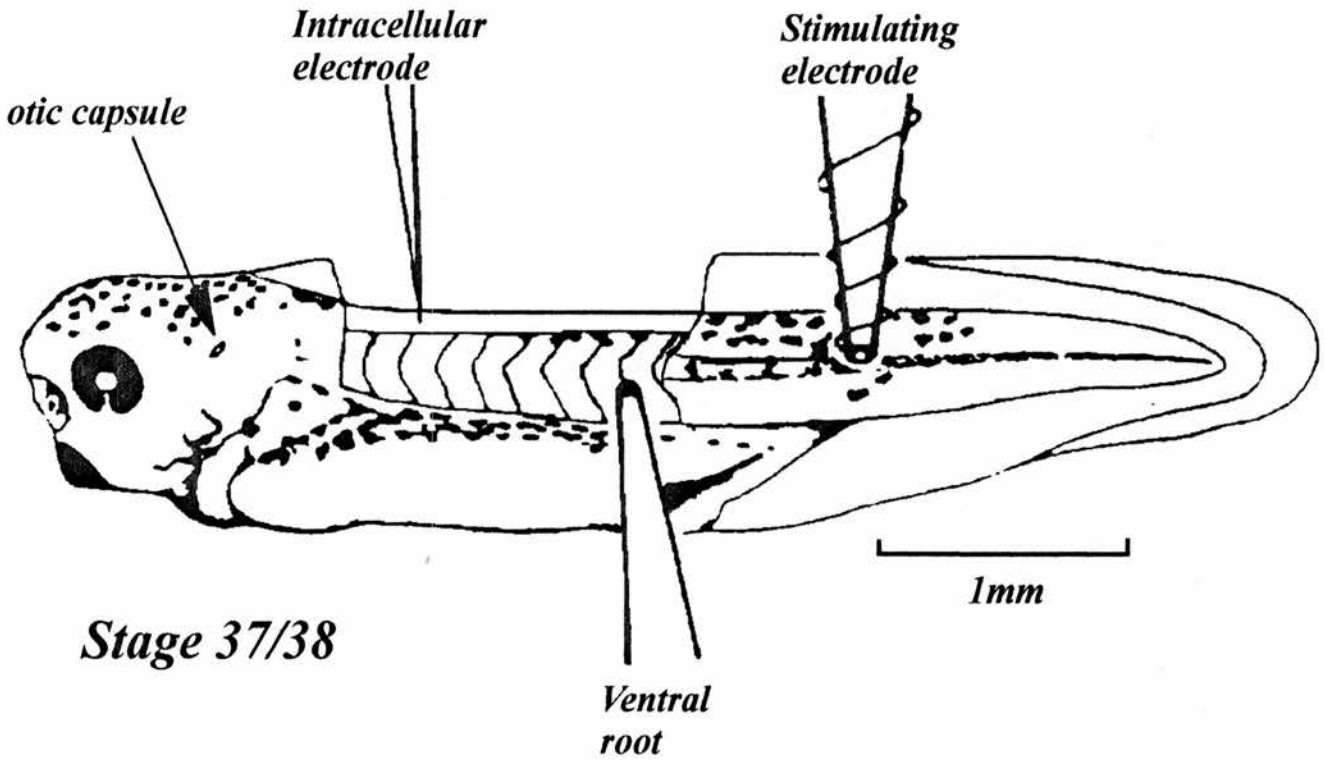

Intracellular electrode
Stimulating electrode
otic capsule
1mm
Stage 37/38
Ventral root

Figure 2.1. The preparation.

To make extracellular recordings, the trunk skin of the animal is removed and a suction electrode placed in an intermyotomal cleft to record from the axons of the motorneurons in a ventral root. After clearing away a rostral section of the myotomes which overlie the spinal cord, intracellular recordings are made, with microelectrodes, from the ventral portion of the cord which consists almost entirely of motorneurons. Episodes of swimming are initiated with a brief electrical current pulse delivered via a second suction electrode placed on the tail skin. The position of the electrodes is noted with reference to the otic capsule.

consist almost entirely of motorneurons (Roberts and Clarke, 1982). Recordings were amplified (x 10) using a laboratory made amplifier.

The experiments were recorded through a CRC VR-100B digital-to-analogue converter (Instrutech Corp.) onto video cassette and displayed on a Gould digital oscilloscope (model 1602). Permanent records were obtained from either a Graphtec thermal arraycorder or Gould colourwriter.

Since this project investigated the actions of GABA at the GABAa receptor, which is directly linked to a chloride-ion channel, intracellular experiments were carried out using 2M KCl-filled microelectrodes as this reverses and enhances chloride dependent ipsps. This effect is shown in figure 2.2, which compares recordings made from embryonic motorneurons with 3M KAc and 2M KCl electrodes. With 2M KCl as the electrolyte, the midcycle inhibition which is known to be the result of glycine-mediated chloride currents (Soffe, 1987) is reversed in sign to become depolarising and it can be seen that the cell can spike on top of the reversed inhibition.

Pharmacological agents were bath applied to the perfusate by adding known quantities to the stock bottle to achieve the desired final bath concentration. The drugs used in the course of this study are detailed below.

Drug	Concentration (μM)	Source
5β3α	0.7-10	Sigma
5HT	2-5	Sigma
bicuculline	20-50	Sigma
CdCl2	200	Sigma
DHKA	50	Sigma
Nipecotic acid	100-200	Sigma

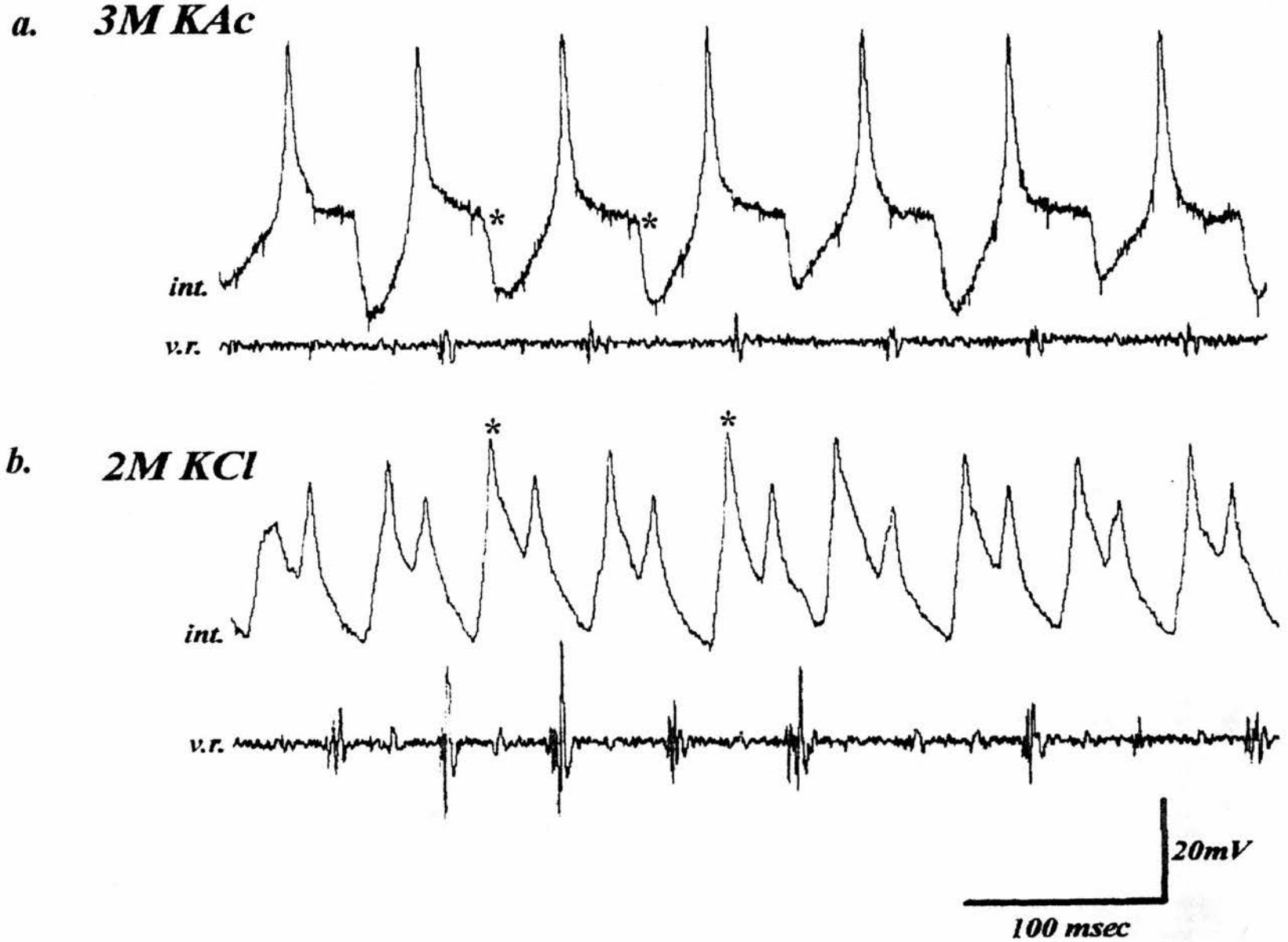

a.
3M KAc
int.
v.r.
b.
2M KCl
int.
v.r.
20mV
100 msec

Figure 2.2. KCl-filled microelectrodes reverse and enhance chloride-dependent IPSP's.

a. Rhythmic activity recorded from an embryoic motorneuron with a 3M KAc microelectrode showing that embryonic motorneurons fire once per cycle of activity and are inhibited midcycle by hyperpolarising ipsps (*).
b. 2M KCl filled microelectrodes causes a reversal of the chloride gradient thereby reversing and enhancing glycinergic midcycle inhibition, to the point that the cell sometimes spikes midcycle (*)

NMDA	50-100	Sigma
Pindobind-5HT1a	10-20	RBI
Strychnine	2-5	Sigma
TTX	0.5	Sigma

Data analysis.

To determine drug effects on ventral root activity, three basic parameters of the activity were measured:

i. burst duration - measured on both the rostral and caudal root as the length (in ms) of each individual discrete burst.

ii. cycle period - measured as the time interval between the onset of a burst of activity in one cycle and the start of the next ventral root burst on the same side.

iii. rostrocaudal delay - measured as the delay between the onset of activity recorded rostrally and the onset of activity at some more caudal position on the same side.

Averages were calculated from seventy five measurements of each parameter taken from three different episodes in control conditions, following bath application of a pharmacological agent and then after washing off the agent with control saline. The first 500ms of activity in each episode were ignored to avoid any influence from sensory effects.

Immunocytochemistry:

In order to reveal populations of GABA-immunoreactive neurons in embryo and larval CNS's, both the immunofluorescence and HRP methods

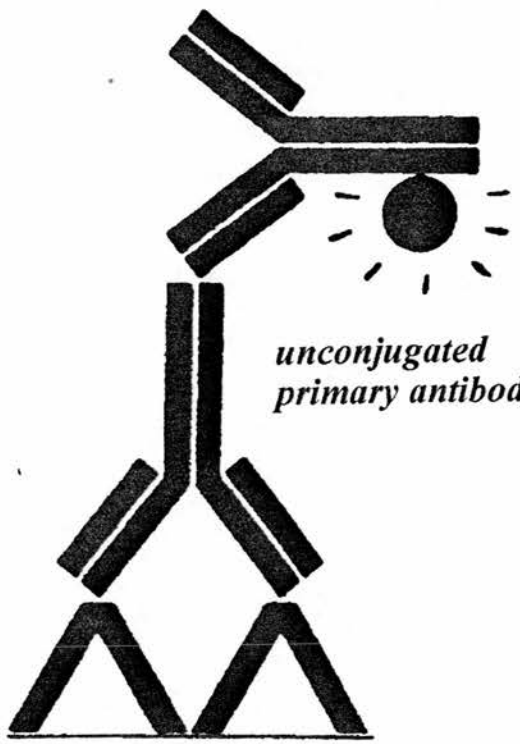
were adapted from Coons (1958) where an unconjugated primary antiserum is bound to the antigen in the tissue and then a labelled antiserum is directed against the primary antiserum (figure 2.3). Firstly animals were anaesthetised in tap water containing MS222 (Sigma) and transferred to 5% glutaraldehyde fixative diluted in 0.1M phosphate buffer (PB) for an hour. The animals were then pinned out on a rotating table in a 2ml preparation bath with 0.1M PB. The CNS was dissected clear of all the other tissue with finely etched tungsten pins under an Olympus microscope. After dissection the CNS's were postfixed, again in 5% glutaraldehyde. Following fixation, any remaining melanophores were bleached overnight in a solution of dents fixative (20% DMSO and 80% methanol) and 10% H_2O_2 at room temperature.

The tissue was then permeabilised to allow the antisera to penetrate the tissue. This was achieved by dehydrating the tissue through an ascending series of alcohols (50%, 70% and 100%), finishing with xylene. The tissue was then rehydrated and washed thoroughly with 0.1M phosphate buffered saline with 0.5% Triton-X (PBS-x).

The next stage was to place the specimens in a 3% solution of donkey serum for 2 hours in order to block any non-specific sites which could bind the specific antisera. Having blocked these sites the next stage was the primary incubation. The primary antibody was rabbit anti-GABA (a polyclonal antibody supplied by Seralab). Firstly, a 1:50 stock solution was prepared which contained 5% donkey serum and was diluted with PBS-x. The correct antiserum dilution was determined by carrying out a series of titration experiments. The best dilution was found to be around 1:150.

The specimens were incubated in eppendorf tubes with the diluted rabbit anti-GABA antiserum for a period of seven days at 4^{o}C. At the end

fluorescein isothiocyanite (F.I.T.C.) labelled secondary antibody



unconjugated primary antibody

antigen in tissue

Figure 2.3. The indirect immunofluorescence technique.

An unconjugated antibody is bound to the antigen in the tissue and fluoroscein isothiocyanate (FITC) secondary antiserum is directed against the primary antiserum.

of this time the preparations were washed thoroughly in PBS-x. At this stage the cords were then either incubated with fluorescein isothyocyanate labelled donkey anti-rabbit antiserum or with donkey anti-rabbit HRP conjugated antiserum. These were used at concentrations of 1:20 (at least ten times as concentrated as the primary antibody).

For immunofluorescence, the preparations were incubated in the secondary antibody for two hours at room temperature after which they were thoroughly washed in phosphate buffered saline (PBS) and mounted on cavity slides and coverslipped with permafluor (Biogenesis), an aqueous mounting gel. The HRP secondary antibody was left for a further 24 hours at room temperature before being washed in PB. Next the specimens were visualised with a diaminobenzidine (DAB) solution, containing 0.25 mg/ml DAB diluted with 0.1M PB and 0.003% hydrogen peroxide. Having observed a colour change in the CNSs, the process was stopped by washing in PB. The cords were then dehydrated through a series of alcohols to xylene and mounted in Canada Balsam (Sigma).

Negative controls were processed under the same conditions except that the primary incubation was substituted by normal (non-immune) serum from the same animal (i.e. rabbit serum). This kept all the constituents of the primary incubation the same, apart from the specific antibody, and therefore ensured that no other component was causing non-specific staining.

The HRP results were viewed under a Zeiss microscope and permanent records obtained from photomicrographs taken with an Olympus camera attachment, using colourslide film, or by drawings made using a camera lucida drawing tube.

The immunofluorescence results were also drawn using a Zeiss ultraviolet microscope with the camera lucida drawing tube and

photomicrographs were obtained from investigations using a laser scanning confocal microscope.

RESULTS

CHAPTER 3
The role of GABAa receptors during embryonic swimming activity.

Introduction

Endogenous and synthetic neuroactive steroids modulate the GABAa receptor.

In contrast to the classical, long term action of steroid hormones mediated through intracellular receptor-mediated changes in protein synthesis, some steroids have been shown to act within seconds to alter CNS excitability. This rapidity suggests that they act at recognition sites on neuronal membranes. A substantial amount of evidence has now been reported which shows that certain synthetic and naturally occurring steroids with anaesthetic properties are positive allosteric modulators of the GABAa receptor. In comparison to other classes of therapeutic agents known to act on the GABAa receptor complex, 'neurosteroids' are the most potent.

The first electrophysiological evidence for an action of neurosteroids on the GABAa receptor chloride-ionophore complex, came from extracellular recordings made from rat brain slice (Harrison and Simmonds, 1984). This study showed that the response to GABA was enhanced in the presence of the synthetic steroid alphaxalone. These initial data were confirmed by voltage-clamp studies on isolated bovine chromaffin cells (Cottrell et al., 1986) and mouse spinal neurons in culture (Cottrell et al., 1987) which demonstrated that whole cell currents evoked by GABA were potentiated by alphaxalone. These findings were later extended to structurally related endogenous steroids such as 5β-pregnan-3α-ol-20-one and 5β-pregnan-3,20-dione (Barker et al., 1986; Callachan et al., 1987; Peters et al., 1988) which have very similar effects on GABAa receptors.

The postsynaptic currents recorded under voltage-clamp conditions were found not to be significantly altered in either rise time or amplitude by

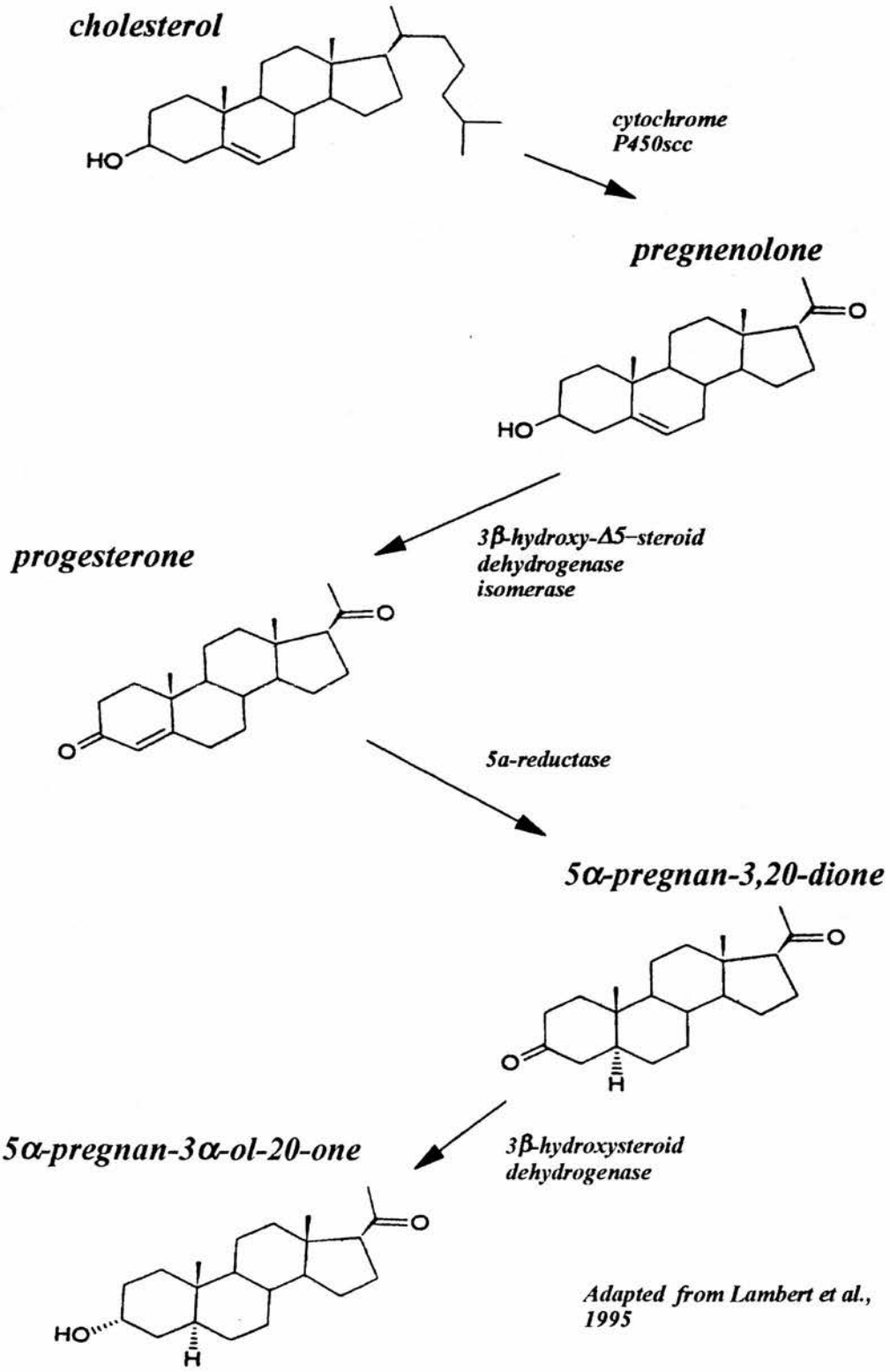
steroids but instead their duration was significantly increased (Harrison et al., 1987a). The underlying mechanism through which neurosteroids augment GABA-mediated chloride currents was elucidated from patch-clamp studies and was found to be similar to that of the barbiturates (Owen et al., 1986), where the mean open time of the channel is increased (Majewska et al., 1986, Callachan et al., 1987, Simmonds, 1991). In contrast, the other class of sedative, hypnotic agents which act at the GABAa receptor, the benzodiazepines, enhance the activity of GABA by increasing the frequency of channel opening (Skolnick and Paul, 1988). However, a more recent study by Twyman and McDonald, (1992) which explored the single channel kinetic properties of GABAa receptors on mouse spinal neurons in culture, demonstrated that the mean increase in the open time of the chloride channel was achieved by an increase in the probability of naturally occurring longer open states and that the steroid did actually cause an increase in the channel opening frequency similar to the benzodiazepines. So far there is no evidence for any other mechanism of action for the neurosteroids in that they do not affect the reversal potential or have any effect on the GABA uptake system (Callachan et al., 1987, Lambert et al., 1990). However, the augmentation of GABA activated chloride currents by steroids are seen at low concentrations, whereas at high concentrations, like the barbiturates, steroids can directly activate the chloride channel (Callachan et al., 1987, Cottrell et al., 1987, Lambert et al., 1990).

Synthesis

The primary sites of neurosteroid production are the adrenal glands (MacDonald et al., 1991), but it has been known for a long time that the

brain contains a substantial amount of steroid precursor and that some of the enzymes for metabolising these precursors are also present in brain tissue (Iwamori et al., 1976, Le Goasgone et al., 1987, Mellon and Deschepper, 1993, Mensah-Nygan et al., 1994). These enzymes have been shown to be primarily glial in origin (Jung-Testas et al., 1989). More recent evidence has revealed that steroids can actually be produced *de novo* in the CNS although the metabolic pathway would appear to be different to that in the periphery as some of the key enzymes have not been isolated from brain tissue (Baulieu and Robel, 1990, Mellon and Deschepper, 1993). Since steroids are found at much higher concentrations in the CNS than in plasma (Baulieu et al., 1987, Jung-Testas et al., 1989) it is likely that they play a functional role in the CNS.

The demonstration that steroids can be produced in the CNS as well as the periphery led to the introduction of the terms "neurosteroid" and "neuroactive steroid" to distinguish those steroids of central origin from those that are produced solely in the periphery but act on the CNS (Baulieu et al., 1987). Therefore, neurosteroids, although they may be produced in the periphery, can be produced *de novo* in the CNS. This distinction says nothing about the mechanism of action of the compounds which are all active in the CNS. Some of the most widely studied endogenous steroids which are active at the GABAa receptor are the metabolites of progesterone and deoxycorticosterone. Their metabolites are examples of both neurosteroids and neuroactive steroids, respectively, since progesterone is metabolised in the CNS as well as the periphery whereas there is no evidence for the metabolism of deoxycorticosterone in the CNS. The synthesis of the neurosteroid allopregnanolone (5α-pregnan-3α-ol-20-one) from cholesterol is shown in figure 3.1.



Adapted from Lambert et al., 1995

Figure 3.1. The synthesis of 5α-pregnan-3α-ol-20-one from cholesterol.

5α-pregnan-3α-ol-20-one is synthesised from cholesterol through three intermediate stages including the neurosteroid antagonist pregnenolone.

Steroidal modulation of the GABAa receptor is stereoselective.

Structure activity studies have established a marked stereoselectivity of the 3α-hydroxysteroids. All potency at the GABAa receptor is essentially lost if the hydroxyl group at the C3 position is in the β-configuration (Cottrell et al., 1987, Gee et al., 1988, Peters et al., 1988). Comparing the effects of the 3β-ol series with the 3α-ol series showed that at equimolar concentrations the 3β-ol series had no effect on GABA-evoked currents and that although there was a weak antagonism of GABA-evoked currents at higher concentrations, this effect was non-selective (Lambert and Peters, 1989). Similar results were observed for both the cis or trans A-ring compounds, determined by the orientation of the hydrogen at c5 of the reduced pregnane series, suggesting that either configuration can impart activity (Harrison et al., 1987b, Gee et al., 1988, Peter et al., 1988). Additionally a ketone group at C20 is not essential for activity but is nevertheless a feature of the most potent neuroactive steroids (Harrison et al., 1987b).

Site of action.

Although there are known sites on the GABAa receptor upon which the benzodiazepines and barbiturates act, experimental evidence has ruled out the possibility that neuroactive steroids act at either of these sites. Firstly, there is strong evidence that neurosteroids do not bind to the benzodiazepine site because the benzodiazepine antagonist, RO-15-1788, does not abolish the steroid action (Harrison and Simmonds, 1984, Callachan et al., 1987, Lambert and Peters, 1989). Additionally, steroids

are found to enhance, rather than prevent, benzodiazepine binding (Prince and Simmonds, 1992). Evidence against neurosteroids binding at the barbiturate site was less obvious due to the similarity in their mechanism of action as mentioned above. Like the steroids, barbiturates also enhance benzodiazepine binding and both classes of compounds prevent the binding of the channel blocker, t-butylbicyclophosphorothionate (TBPS) (Callachan et al., 1987, Gee et al., 1988, Peters et al., 1988). Despite these similarities, other studies showed that at maximal levels of barbiturates, steroids still enhance GABA currents and *vice versa* (Gee et al., 1988 Lambert et al., 1987). This additive effect would be inconsistent with a shared binding site although it might be possible that they act through a common effector mechanism (Twyman and MacDonald, 1992).

The exact binding site of neuroactive steroids is still unclear. Evidence from patch clamp studies has suggested an extracellular binding site because intracellularly applied neurosteroids have no effect on GABA-mediated chloride conductances (Lambert et al., 1991). Although the marked stereoselectivity suggests a protein site of binding and related studies have provided results in support of a protein binding site (Olsen et al., 1986, Majewska et al., 1990), other investigations have shown that treatment with phospholipase A2 also disrupts neurosteroid action (Demirgoren et al., 1991). A hypothetical model of the interaction of neuroactive steroids with the GABAa receptor was proposed by Majewska, (1992), in which the steroid binds to the fatty acid chain of the lipid membrane and then the electronegative atom at C20 or the hydroxyl group at C3 could be essential for the steroid binding to the GABAa receptor via hydrogen bonds (Im et al., 1990).

Attempts have also been made to determine the site of action of neurosteroids with regard to different subunits of the GABAa receptor.

Various combinations of subunits form ligand-gated channels which then differ in their sensitivity, especially to benzodiazepine binding (Schofield et al., 1987, Pritchett et al., 1989). Initial evidence suggested that steroid binding was not dependent on any specific GABA receptor subunit (Puia et al., 1990). More recent studies, however, have indicated certain subunits as being important although the results have been contradictory. There is little consensus on the role of the α-subunit. One study indicated the α1 subunit as being important in steroid augmentation of GABA-evoked currents (Shingai et al., 1991) while another showed that the presence of the α3 subunit enhanced the action of neuroactive steroids on benzodiazepine binding (Lan et al., 1991). The γ subunit appears not to be a crucial determinant of steroid function although the presence of the γ1 subunit in combination with the α1 and β1 subunit was shown to result in greater enhancement of GABA evoked chloride currents than other types of γ subunit (Puia et al., 1993).

Selectivity

Results from studies which have examined the selectivity of neurosteroids have shown that some of the neuroactive steroid agonists, as well as being highly potent, are also very selective. Despite the similarity of the GABAa receptor to the glycine receptor, neuroactive steroid agonists have no obvious effect on glycine mediated potentials (Harrison and Simmonds, 1984, Lambert et al., 1990, Woodward et al., 1991). Furthermore, under voltage-camp conditions, alphaxalone and 5β-preganan-3α-ol-20-one have also been shown to have no effect on NMDA or kainate induced currents (Lambert et al., 1990).

Antagonists.

In contrast to the allosteric agonists of the GABAa receptor, some neurosteroids act as noncompetitive antagonists of GABA-mediated chloride currents (Majewska and Schwartz, 1987, Mienville and Vincini, 1989). Included in this category of neuroactive steroids are pregnanolone sulphate and dehydroepiandrosterone sulphate which are metabolic precursors of neurosteroid agonists (figure 3.1). The antagonists tend to have mixed activity. Although, they inhibit GABA/muscimol induced chloride fluxes (Mienville and Vincini, 1989), this effect is seen at micromolar concentrations whereas at nanomolar concentrations they have been shown to enhance GABA activity like the agonists (Majewska and Schwartz., 1987). The barbiturate enhanced benzodiazepine binding is decreased in the presence of the antagonists but in the absence of barbiturates, antagonist neurosteroids again act much like the agonists and enhance benzodiazepine binding (Majewska and Schwartz, 1987).

There are thought to be several sites of action for neurosteroids antagonists which differ from those of the agonists. Pregnenolone sulphate has been shown to competitively displace TBPS binding and therefore may act at the picrotoxin/TBPS binding site (Majewska and Schwartz, 1987). However, dehydroepiandrosterone sulphate also displaces TBPS but via a different mechanism (Majewska et al., 1990). In addition to the differences in binding at the GABAa receptor, neurosteroid antagonists are also less specific than the agonists. They have been shown to exert their overall excitatory effects on the CNS not only by blocking GABAa receptor activity but also by inhibiting glycine release (Wu et al., 1990) and potentiating NMDA receptor activation. (Fahey et al., 1995).

Behaviour.

The anaesthetic actions of cholesterol have been known for a long time (Cashin and Moravek, 1927) as well as reports of the anaesthetic actions of progesterone and deoxycorticosterone and some of their metabolites (Seyle, 1942). This led to the development of synthetic intravenous anaesthetics which closely resembled endogenous neurosteroid agonists (Gymerk and Sokya, 1975). However, although the steroid anaesthetics had the advantage of a rapid onset in anaesthesia but with a short duration, these advantages were overshadowed by the side effects caused by the vehicle in which the steroids were dissolved and lead to their withdrawal from clinical use. Recently, there has been a renewed interest in steroid anaesthetics with the development of a nonantigenic solvent. *In vitro* studies are now examining the steroid, 5β-pregnan-3α-ol-20-one as a possible anaesthetic (Lambert et al., 1995).

In addition to the well described anaesthetic actions of neurosteroids, they have been implicated in other behavioural effects. Neurosteroid agonists have also been shown to protect against seizures induced by the convulsants picrotoxin and bicuculline (Belleli et al., 1989) but have little effect in preventing strychnine-induced seizures, which is consistent with the lack of effect of neurosteroid agonists on glycine transmission (Woodward et al., 1991). One type of epilepsy which is thought to be closely linked with circulating levels of endogenous neurosteroids is catamenial epilepsy. Some women have an increased susceptibility to seizures during the luteal phase of the menstrual cycle which is associated with low levels of progesterone and appears to be correlated with the variation in circulating levels of 5α-pregnan-3α,20α-diol (Finn and Gee,

1994). Studies on rats have also shown that there is cyclical variation in sensitivity to the anticonvulsant effects of 5α-pregnan-3α-ol-20-one (Finn and Gee, 1994). One of the problems of the endogenous neurosteroids which have been tested as anticonvulsants is that despite their potency they have a very short half life. One approach which is now being used to overcome this problem is to protect the 3α-hydroxy group of 5α-pregnan-3α-ol-20-one with a 3β-methyl group. The resulting compound has only a very slight reduction in affinity and at therapeutic doses appears not to interrupt cognitive functions (Gee et al., 1995).

Other studies have looked at the anxiolytic action of steroids and results from swim stress experiments in rats have provided evidence for increased levels of alloprenanolone in response to stress (Purdy et al., 1991). This response is thought to occur at later stages in the response to stress with antagonistic neuroactive steroids being involved in the initial response to stress by causing an increase in arousal (Baulieu et al., 1987). There is also some evidence that the steroid antagonist, prenenolone sulphate, can improve cognitive functions (Flood et al., 1992).

Aim of this study.

The *Xenopus* embryo preparation provides an excellent model for studying the action of the chosen neurosteroid since there is an identified GABAergic pathway with a clear function in behaviour (Boothby and Roberts, 1992b). Additionally the preparation was amenable for studying in some detail the synaptic mechanism of action of the steroid. It is clear from behavioural studies that, as well as the renewed interest in the properties of neuroactive steroids as anaesthetics, they are also potentially important therapeutic agents for a number of disorders including epilepsy and possibly

Alzheimers disease. Therefore, a clear understanding of the mechanism of action of neuroactive steroids is essential for the development of therapeutic agents with specific properties. The work carried out so far has provided a substantial amount of evidence regarding the cellular and molecular mechanism of action of different synthetic and endogenous neuroactive steroids. However, very few studies have addressed the action of the neuroactive steroids on synaptic transmission.

Having shown the specificity of the steroid action in this preparation, the remainder of this results section re-examines the effects of GABA transmission on embryonic swimming activity by using the neurosteroid to augment the endogenous activation of GABAa receptors and the specific GABAa receptor antagonist, bicuculline to block the activation of GABAa receptors.

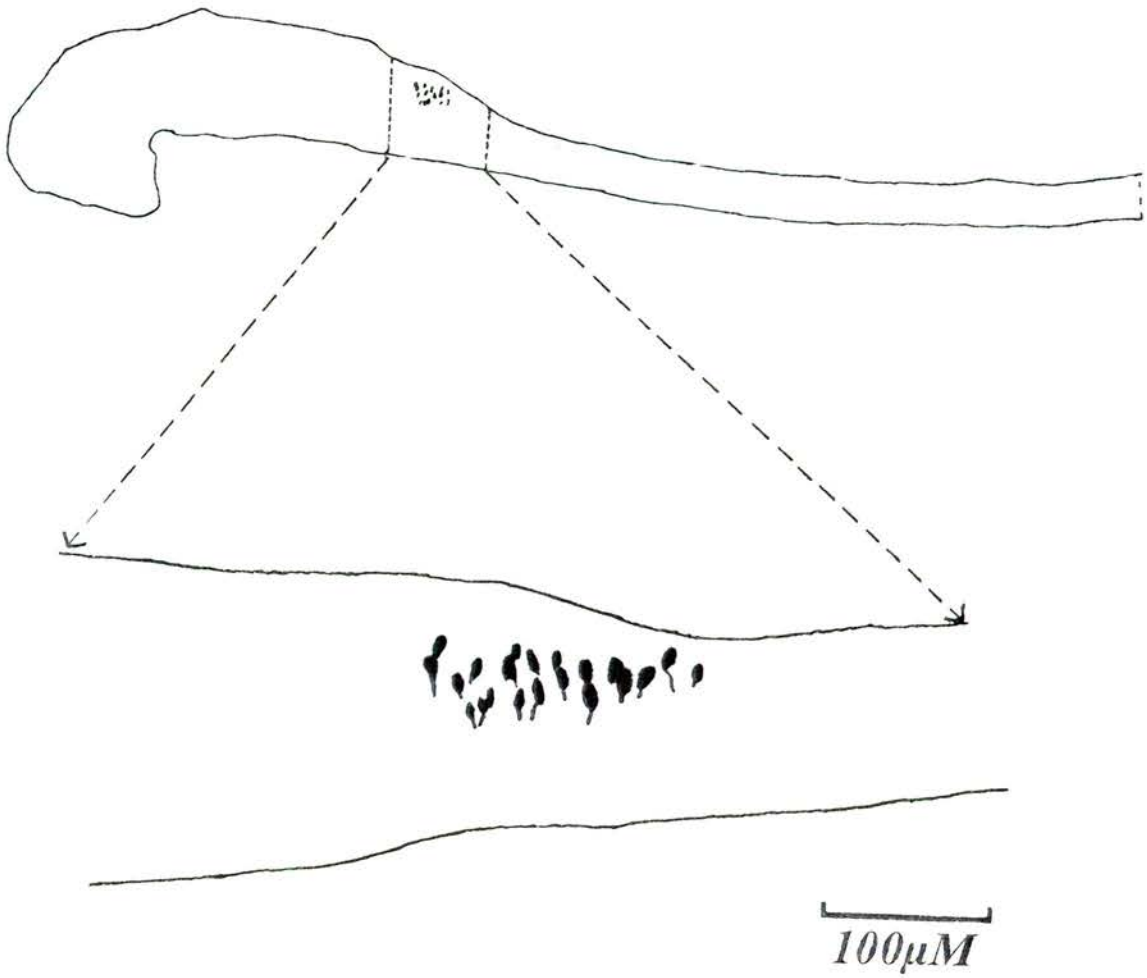
Results

1. The effects of 5β3α on the cement gland pathway.

This study used the endogenous neurosteroid 5β-pregnan-3α-ol-20-one (5β3α) which is the stereoisomer of 5α-pregnan-3α-ol-20-one shown in figure 3.1. 5β3α is known to be a potent agonist at the GABAa receptor and its properties are now being re-examined as a possible anaesthetic (Lambert et al., 1995). Initially the effects of 5β3α on the stopping response were investigated as it is known to involve a population of descending GABAergic neurons (Boothby and Roberts, 1992 a,b), the mhr neurons described in chapter 1 (Roberts et al., 1987). In the embryo these neurons form a strongly stained GABA immunoreactive group around 200μm from the caudal edge of the otic capsule which have both descending ipsilateral and contralateral axons (Roberts et al., 1987). Camera lucida drawings (figure 3.2 A) and a confocal image (B) of GABA-like immunoreactivity confirmed the position of the mhr neurons in the embryo CNS.

During episodes of fictive swimming activity, cycle periods generally increase towards the end of an episode when rhythmic activity stops apparently spontaneously. This is represented graphically in figure 3.3A where, at the beginning of the episode, the average cycle period was around 60ms, but by the end of the episode had increased to around 100ms. The fictive stopping response was evoked in immobilised embryos by applying a brief (1ms) current pulse via a glass suction electrode placed over the cement gland. Figure 3.3B, shows that swimming activity is prematurely terminated following electrical stimulation of the cement gland. Intracellular recordings from motorneurons during fictive swimming

A



B

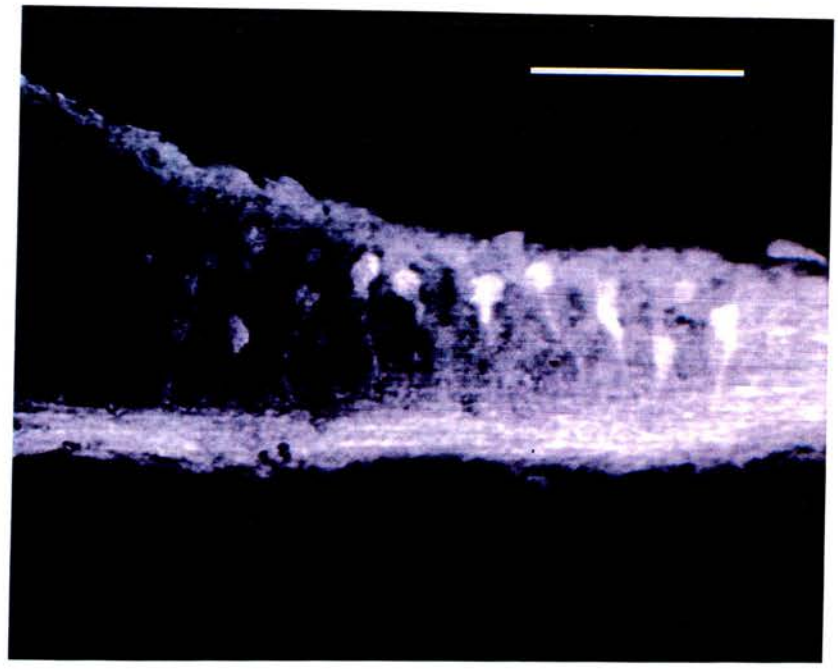


Figure 3.2. Hatchling *Xenopus* (stage 37/8): the mhr neurons.

A. Camera lucida drawing of the whole embryo CNS and an expanded area showing details of a population of GABA immunoreactive neurons lying just caudal to the otic capsule which are presumed to be the mhr neurons described by Roberts et al. (1987).
B. Confocal image of the brainstem and rostral spinal cord which clearly shows the same population of neurons (scale bar = 100μm).

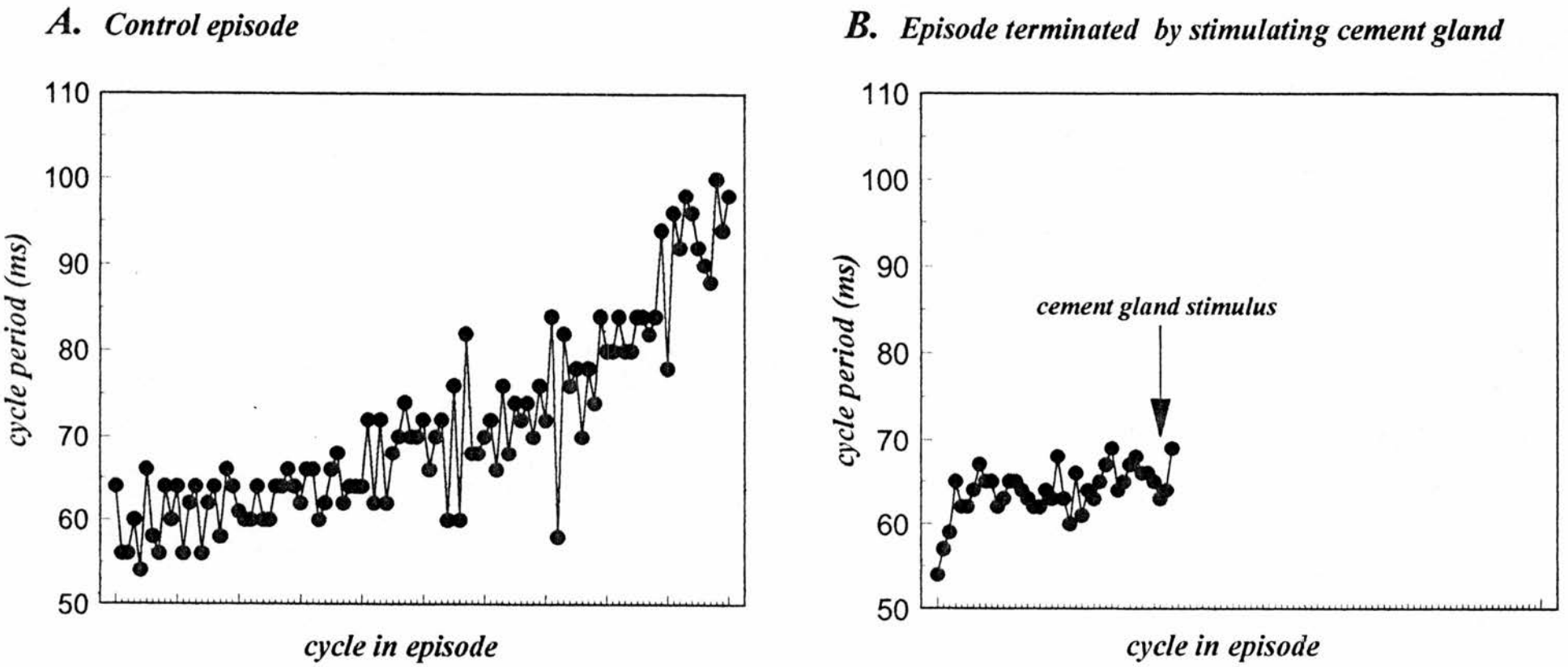

A. Control episode
110
100
90
80
70
60
50
cycle period (ms)
cycle in episode
B. Episode terminated by stimulating cement gland
110
100
90
80
70
60
50
cycle period (ms)
cement gland stimulus
cycle in episode

Figure 3.3. Cement gland stimulation abruptly terminates swimming.

A. Graph showing the gradual increase in cycle period throughout an episode of embryonic fictive swimming which terminates apparently spontaneously.

B. Graph of cycle periods during an episode which was terminated prematurely following electrical stimulation of the cement gland. Note that the episode was terminated abruptly following the cement gland stimulus.

revealed that, following the cessation of swimming in response to cement gland stimulation, a barrage of ipsps was observed. The ipsps were depolarising when recordings were made with KCl-filled microelectrodes (figure 3.4a). From the work by Boothby and Roberts (1992b), these were most likely due to the activation of GABAa receptors. In the presence of 5μM 5β3α, the duration of these inhibitory potentials increased to the extent that they summated (figure 3.4b) suggesting that 5β3α enhances the effects of endogenous GABAa receptor activation (n=3).

The pharmacology of this response was investigated further by mechanically stimulating the cement gland in the absence of fictive swimming activity. This was achieved by using an extracellular electrode which was placed just in front of the cement gland and moved forward manually to contact the gland (n=2). Depolarising ipsps were observed both in response to contacting and pulling away from the cement gland (figure 3.5a, indicated by the arrows). Following the bath application of 5μM 5β3α the responses were markedly increased in duration presumably reflecting the action of the steroid at the GABAa receptor (b). This was confirmed when the majority of the response was blocked following the subsequent bath application of the GABAa antagonist, bicuculline methiodide (50μM, c). Interestingly, however, bicuculline did not entirely abolish the response to stimulating the cement gland and bicuculline-resistant shorter duration depolarising potentials remained (c) These could be blocked by the glycine antagonist strychnine sulphate (5μM, d), suggesting that the cement gland stopping response also involves the activation of glycine receptors.

The main conclusion of these initial data is that the neuroactive steroid, 5β3α, enhances the action of GABA in a known GABAergic pathway in this preparation. The data also suggests that the cement gland

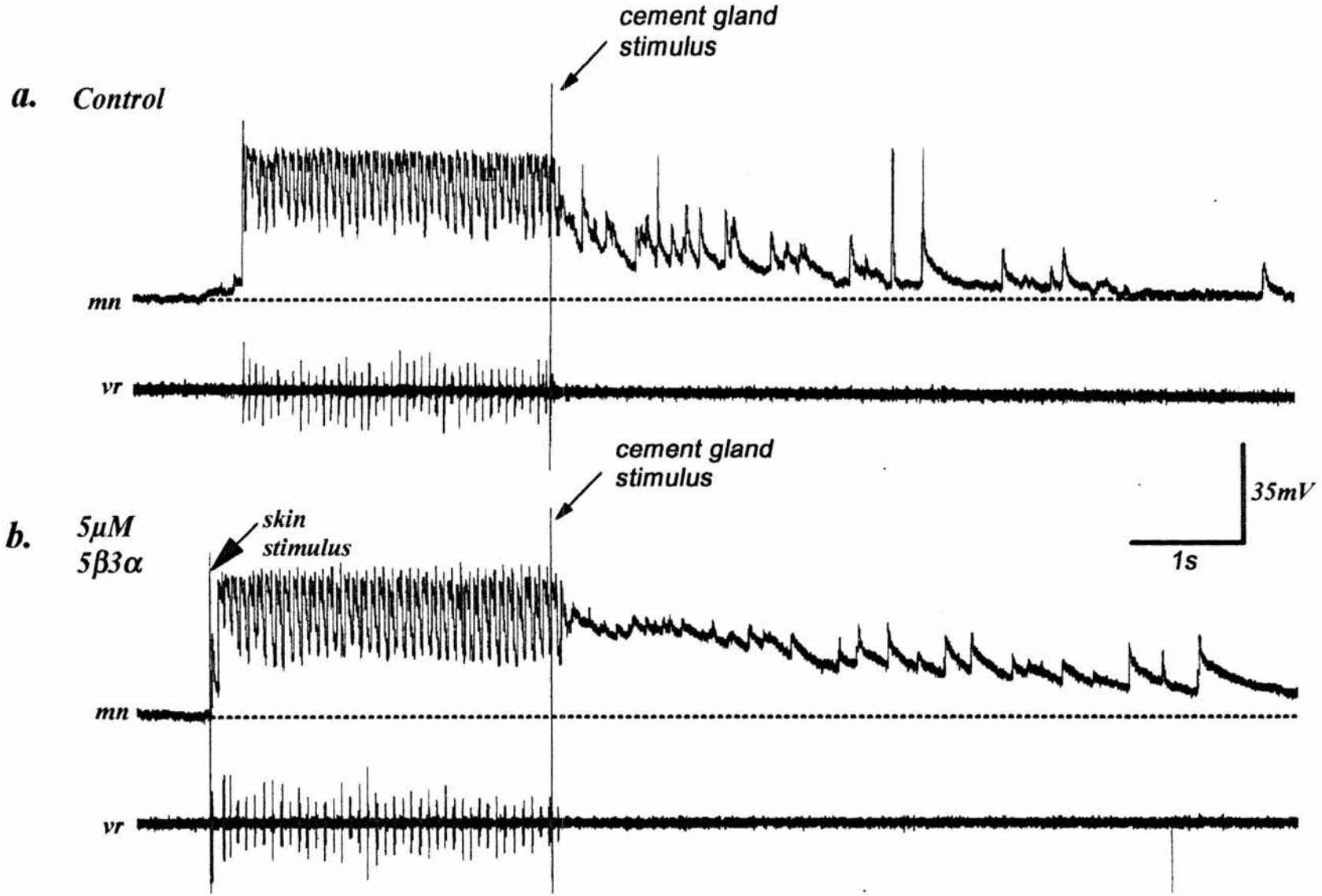

a.
Control
cement gland
stimulus
mn
vr
b.
5μM
5β3α
skin
stimulus
cement gland
stimulus
35mV
1s
mn
vr

Figure 3.4 5β3α potentiates the stopping response following cement gland stimulation.

a. Intracellular recording from an embryonic motorneuron, showing that a barrage of ipsps was observed following the termination of swimming by electrical stimulation of the cement gland

b. In the presence of 5μM 5β3α the summated ipsps increase the length of time for the membrane potential to return to the resting level (indicated by the dotted line).

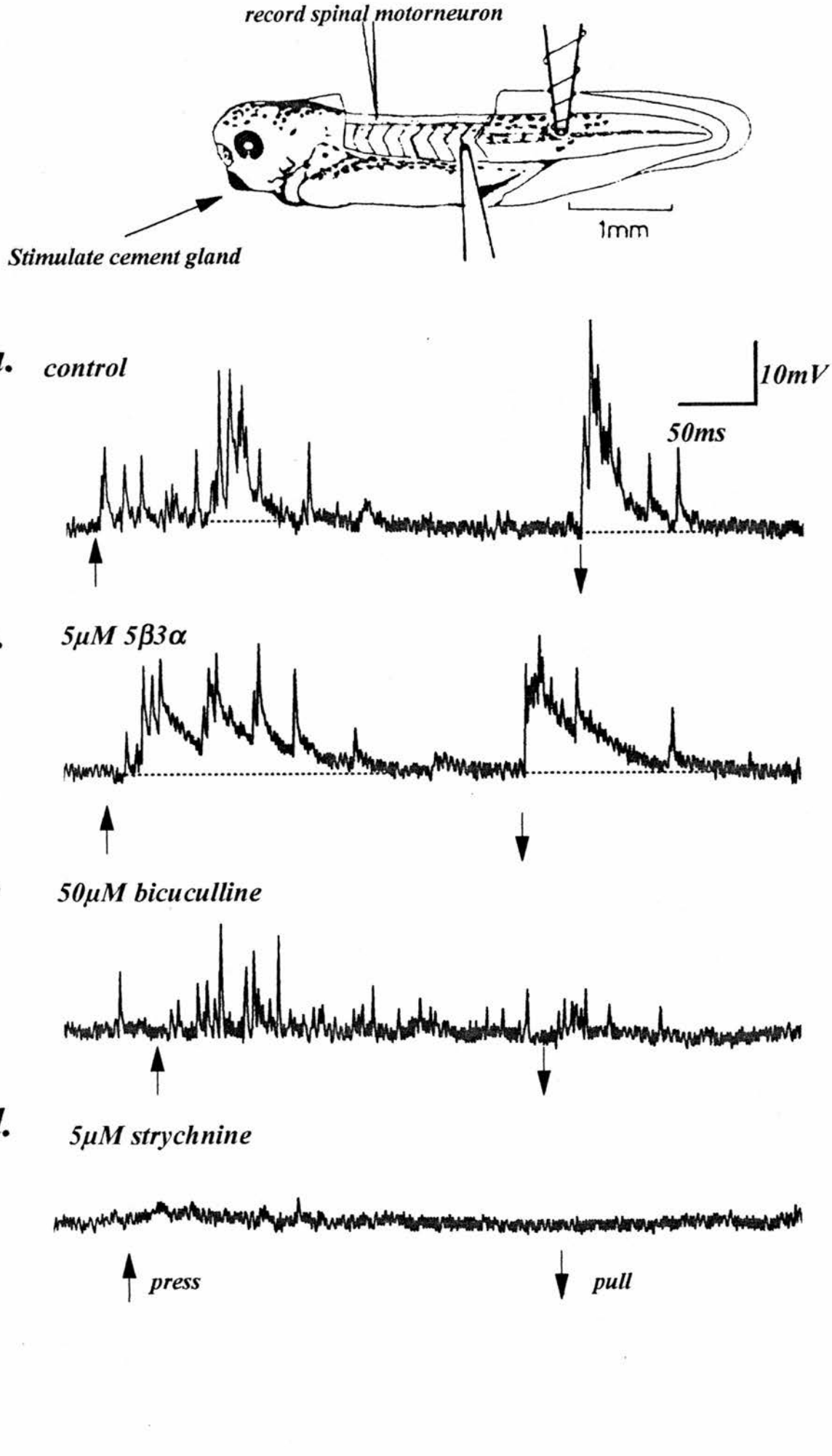

record spinal motorneuron
1mm
Stimulate cement gland
a.
control
10mV
50ms
b.
5μM 5β3α
c.
50μM bicuculline
d.
5μM strychnine
press
pull

Figure 3.5. Pharmacology of the cement gland response.

a. Intracellular recording from an embryonic motorneuron, in the absence of rhythmic activity, reveals inhibitory depolarising psps in response to mechanically pressing and pulling away from the cement gland.
b. Following the bath application of 5μM 5β3α the potentials increased in duration.
c. The majority of the potentials were blocked by the GABAa receptor antagonist, bicuculline, leaving shorter duration potentials.
d. The remaining potentials were blocked by the glycine antagonist strychnine.

stopping response involves dual inhibition with the activation of both GABAa and glycine receptors. This finding has not been reported previously. Furthermore, the data provides some evidence that 5β3α is a selective agonist at the GABAa receptor since, despite the fact that 5β3α increased the overall duration of the potentials elicited by mechanically stimulating the cement gland (figure 3.5b), following blockade of GABAa receptors (c) only very short duration glycine potentials remained suggesting that they were unaffected by 5μM 5β3α.

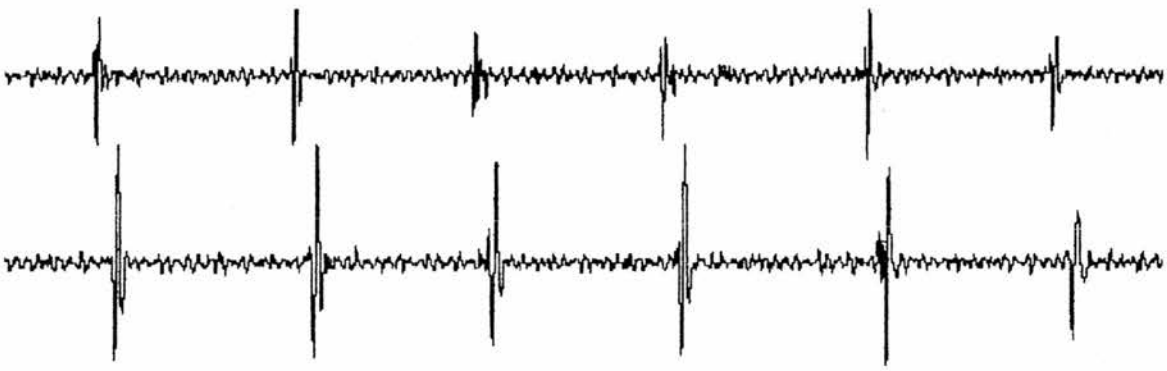
2. The effects of 5β3α on NMDA induced activity.

Continuous fictive swimming activity can be elicited following the bath application of the glutamate receptor agonist, N-methyl-D-aspartate (NMDA). Figure 3.6 compares control swimming activity, recorded following stimulation of the tail skin (a), with activity recorded in the same preparation in the presence of 100μM NMDA (b). Following application of NMDA, the basic pattern of rhythmic swimming activity is maintained; neither burst durations nor cycle periods are significantly affected and a rostrocaudal phase delay is present. Figure 3.7, shows the effect of 5β3α on the continuous pattern recorded from an intact embryo. Four minutes after the addition of 100μM NMDA, continuous fictive swimming was observed (a). Shortly after the addition of 2μM 5β3α the pattern of activity became regularly disrupted with periods when there was no discernible ventral root activity (b), an effect which was observed at concentrations of steroid as low as 500nM (not shown). Continuous activity was restored by blocking GABAa receptors with 20μM bicuculline (c).

To determine the location of the population of neurons involved in mediating the steroid response, preparations were spinalised at different

a. *Control*

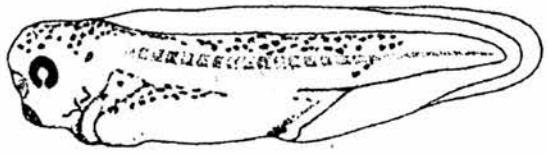
b. *100μM NMDA*



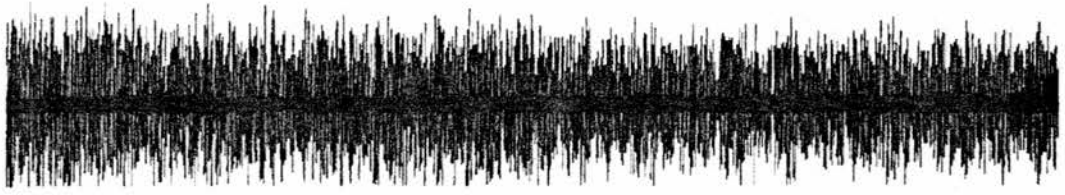
40 msec

Figure 3.6. Ventral root activity in the presence of NMDA.

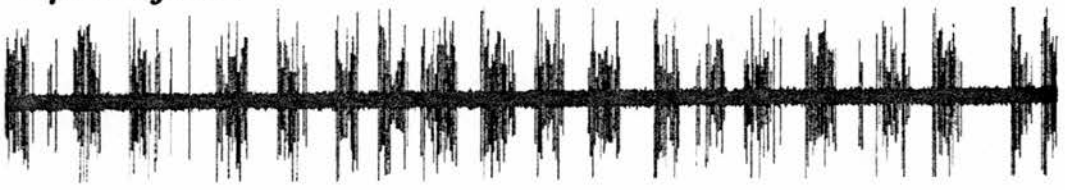
a. Following a brief current pulse to the tail skin of the animal, fictive swimming was recorded with a cycle period of around 60 - 70ms and a rostrocaudal delay between the two ventral roots positioned 5 myotomal clefts apart.
b. 100μM NMDA elicited continuous fictive swimming approximately ten minutes after being added to the control saline. The basic swimming pattern was similar to control with cycle periods still around 60 -70ms and the rostrocaudal delay maintained.



a. ***100μM NMDA***



b. ***2μM 5β3α***



c ***20μM bicuculline***

2s

Figure 3.7. Effects of 5β3α on an intact embryo.

a. Continuous fictive swimming activity induced by 100μM NMDA recorded from P.O.M. 5.
b. After ten minutes in the presence of 2μM 5β3α, rhythmic activity was regularly interrupted.
c. This effect of the steroid was reversed five minutes after bath applying the GABAa antagonist, bicuculline (20μM).

levels. Immunocytochemical studies on *Xenopus* embryos have shown that there are two populations of neurons present in the spinal cord and six populations occur in the brain which show GABA-like immunoreactivity (Dale et al., 1987, Roberts et al., 1987). Therefore, experiments were carried out on preparations which were spinalised either at the otic capsule or at the fourth postotic myotome (figure 3.8). Spinalisation at the level of the otic capsule (b) removes any input from fore- and midbrain populations as well as the vc and rh neurons in the hindbrain. This procedure leaves only the mhr neurons and the two populations of spinal neurons intact. Spinalisation at the level of the fourth post-otic myotome ensures that only the spinal cord populations are left intact (c). I will now refer to these two preparations as 'high' and 'low' spinal preparations, respectively.

In high spinal preparations, essentially the same discontinuous pattern of activity was recorded under 100μM NMDA and 3μM 5β3α as that recorded in intact animals (n=2, figure 3.9 cf figure 3.7). This suggests that the population of GABAergic neurons involved in modulating NMDA-induced activity in the presence of steroid is caudal to the otic capsule and excludes any involvement of populations of GABAergic neurons in the forebrain or midbrain, including the rostral midhindbrain neurons, the optic tract neurons and the rostral forebrain neurons as well as the vc and rh neurons in the hindbrain.

In low spinal preparations, the results obtained were very different. Continuous fictive swimming activity could still be elicited under NMDA with this level of spinalisation (figure 3.10a) even although some of the normal descending excitation must have been removed (Roberts and Alford, 1986). However, 5β3α now had no effect on the continuous activity induced by NMDA, even at concentrations of up to 10μM (b, n=3). These data from low spinal preparations suggest that despite immunocytochemical

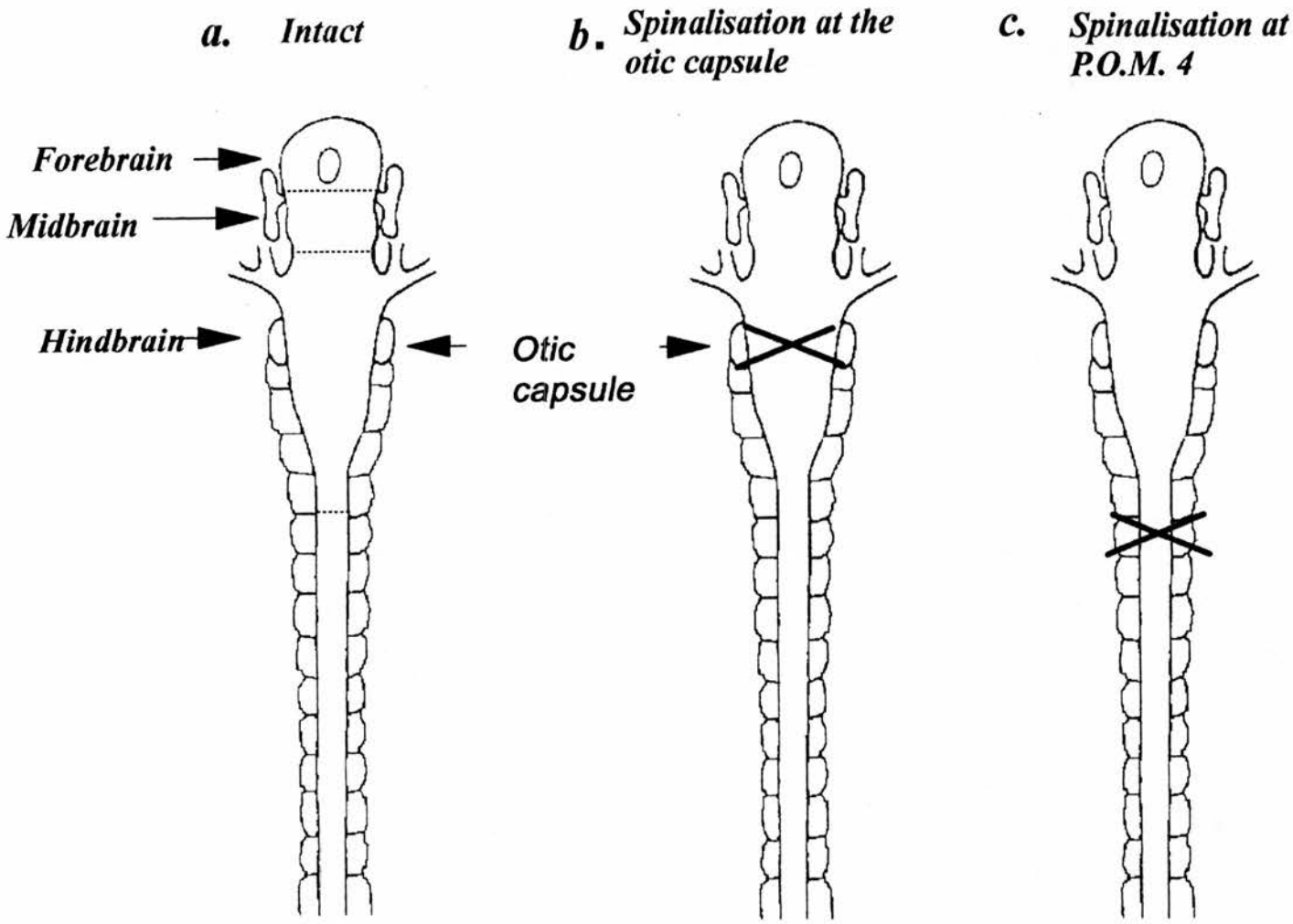

a. Intact
b. Spinalisation at the otic capsule
c. Spinalisation at P.O.M. 4
Forebrain
Midbrain
Hindbrain
Otic capsule

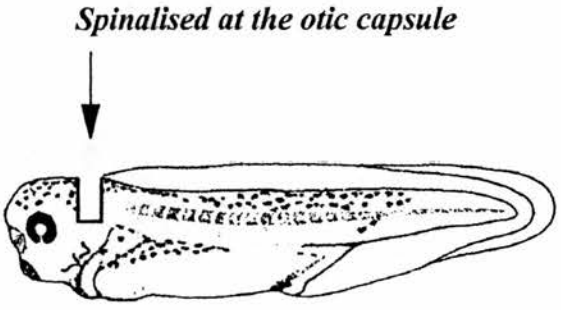
Figure 3.8. Levels of spinalisation.

Three different preparations were used to determine the location of the population of GABAergic neurons involved in mediating the steroidal modulation of NMDA-induced swimming.

a. Embryo CNS showing the extent of the fore-, mid- and hindbrain.

b. Embryos were spinalised at the level of the otic capsule, which removes all fore- and midbrain populations of GABAergic neurons, in addition to the vestibular complex and rostral hindbrain commissural neurons in the hindbrain.

c. Embryos spinalised at the level of the fourth postotic myotome, leaving only the two populations of spinal GABAergic neurons.



a. *100μM NMDA*

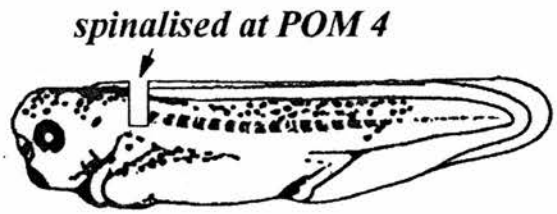
b. *3μM 5β3α*

c. *30μM bicuculline*

4s

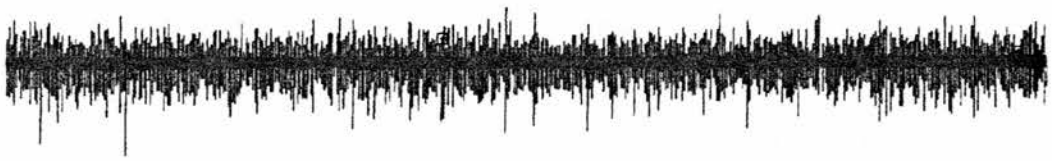
Figure 3.9. Effects of 5β3α on an embryo spinalised at the otic capsule.

Spinalisation at the level of the otic capsule did not abolish the steroid effect on NMDA-induced swimming. In the presence of 3μM 5β3α, the continuous ventral root activity induced by 100μM NMDA (**a**) was interrupted (**b**). Shortly after applying 30μM bicuculline, continuous fictive swimming activity was restored (**c**).



a.

100μM NMDA



b.

10μM 5β3α (10 mins)

4s

Figure 3.10. Effects of 5β3α on an embryo spinalised at P.O.M. 4.

(a) Continuous swimming activity induced by 100μM NMDA in a preparation spinalised at the level of the fourth post-otic myotome.
(b) 5β3α did not interrupt the continuous pattern of activity even at concentrations as high as 10μM.

evidence for two classes of GABA immunoreactive neurons in the embryo spinal cord, they are unlikely to be the source of the endogenously released GABA upon which steroidal modulation occurs. Additionally the negative result for the steroid on low spinal preparations also demonstrates that the concentration of steroid used in these experiments (up to 10μM) was probably not high enough to cause direct activation of the GABAa receptor, which has been shown to occur in other studies (Callachan et al., 1987, Cottrell et al., 1987, Lambert et al., 1990). Therefore the steroid effect was indeed a reflection of the enhancement of the endogenous activation of GABAa receptors.

Taking all the spinalisation results together, it would seem that 5β3α interrupts NMDA-induced fictive swimming activity by enhancing the effects of GABA released from the mhr neurons. During 'normal', stimulus-evoked episodes of swimming, the mhr neurons are inhibited from rest and receive midcycle glycinergic inhibition (Boothby and Roberts, 1992b). However, bath application of NMDA could activate NMDA receptors on these neurons causing a tonic depolarisation which would allow the mhr neurons to fire on rebound from inhibition. It has been shown that such a mechanism can occur in rhythmically active motorneurons (Soffe, 1990).

3. Mechanism of steroid action at GABAergic synapses.

Having established that the steroid, 5β3α enhances the actions of GABA within the GABAergic stopping pathway, the mechanisms by which 5β3α achieves its effect, at the level of the synapse, were then further investigated. When recording intracellularly from motorneurons with KCl-filled microelectrodes, in the absence of fictive swimming activity, apparently spontaneous depolarising potentials always occur (figure 3.11

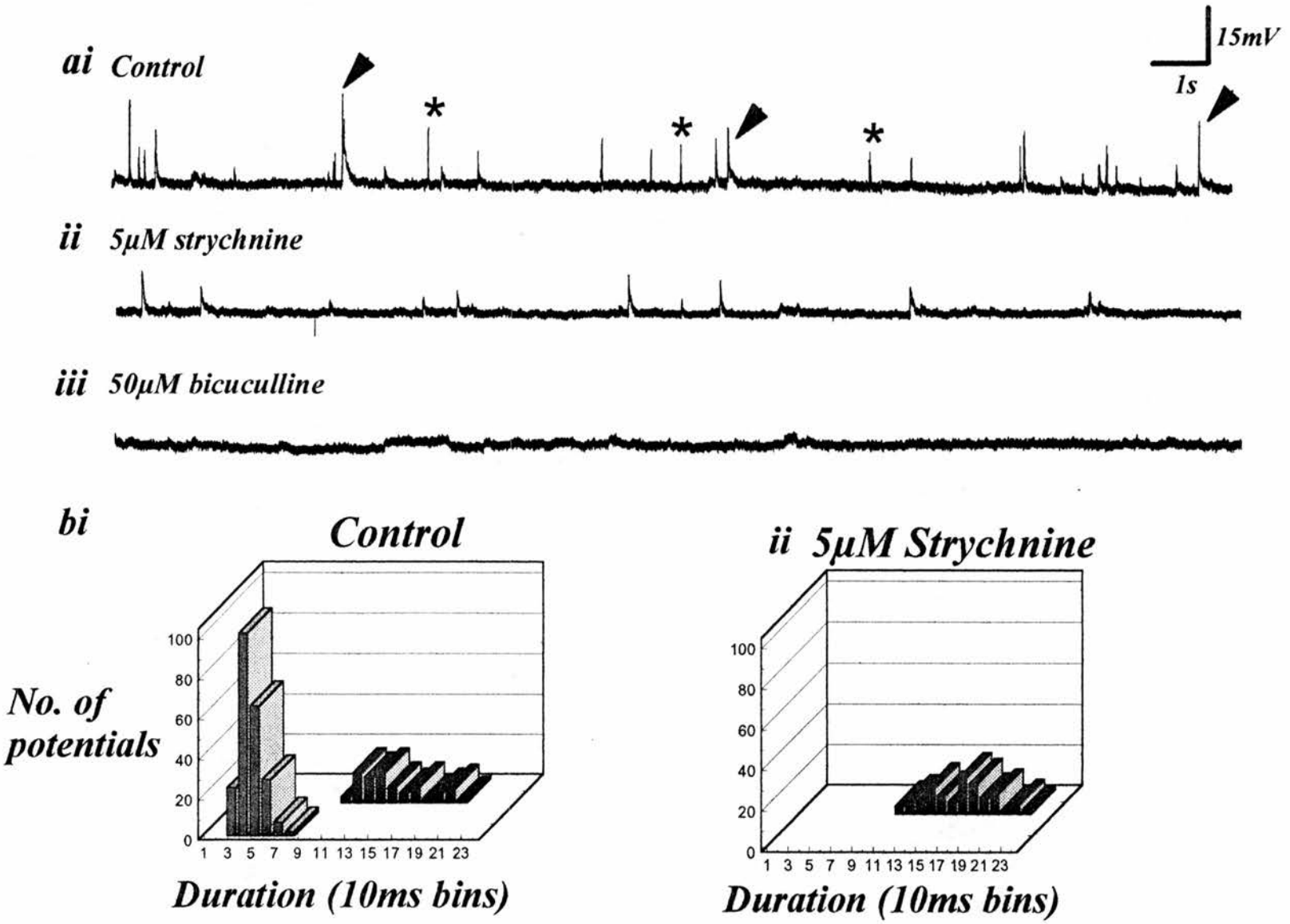

ai Control
15mV
1s
ii 5μM strychnine
iii 50μM bicuculline
bi
Control
ii 5μM Strychnine
No. of potentials
0
20
40
60
80
100
1 3 5 7 9 11 13 15 17 19 21 23
Duration (10ms bins)
Duration (10ms bins)

Figure 3.11. Inhibitory potentials in the absence of fictive swimming.

ai. In the absence of fictive swimming activity two types of depolarising inhibitory potential are recorded in motorneurons, separable by duration (indicated by the asterisks and the arrows).
ii. One class of inhibitory potentials are sensitive to the glycine antagonist strychnine (5μM).
iii. The remaining potentials are abolished by the GABAa receptor antagonist bicuculline (50μM).

bi. The shorter duration glycine potentials have durations which range from 20-80ms and the GABA potentials range from 120-230ms.
ii. Bath application of strychnine selectively blocks the population of inhibitory potentials with shorter durations.

ai). The durations of these potentials were found to fall into two categories, whereas previous reports had only ever described one population (Sillar and Soffe, 1987, Wall and Dale, 1993). In one population durations fall in the range of 20-80ms, while in the second population longer duration potentials occur in the range of 90-200ms (ai and bi). The faster potentials are abolished by 5μM strychnine sulphate (aii and bii) and are presumably therefore the result of glycine release from spinal commissural interneurons. The longer potentials, which are strychnine-insensitive, are blocked by bicuculline methiodide (50μM, aiii) and presumably therefore result from the release of GABA acting at the GABAa receptor subtype.

Bath application of tetrodotoxin (TTX, 0.5μM) blocks spike-evoked transmitter release from presynaptic terminals by preventing sodium-dependent impulses. TTX markedly decreased the number of inhibitory potentials (figure 3.12B cf. A), suggesting that some of them are the result of transmitter release evoked by sodium spikes. However, a steady release rate is maintained (n=6). This was assumed to reflect the spontaneous liberation of inhibitory transmitter from presynaptic terminals. Comparing the duration of the potentials which persisted in the presence of 0.5μM TTX (Cii and iv) with control (Ci and iii), suggested that the spontaneous ipsps resulted from the liberation of transmitter from both glycinergic and GABAergic terminals.

By investigating the effects of 5β3α on the duration, amplitude and frequency of these spontaneous GABAergic ipsps, the effect of the steroid on synaptic transmission could be further explored. Any effect on either the duration or amplitude of spontaneous potentials would be indicative of the steroid modulating the postsynaptic response of GABAa receptors to GABA. Alternatively, a change in the frequency of potentials, following

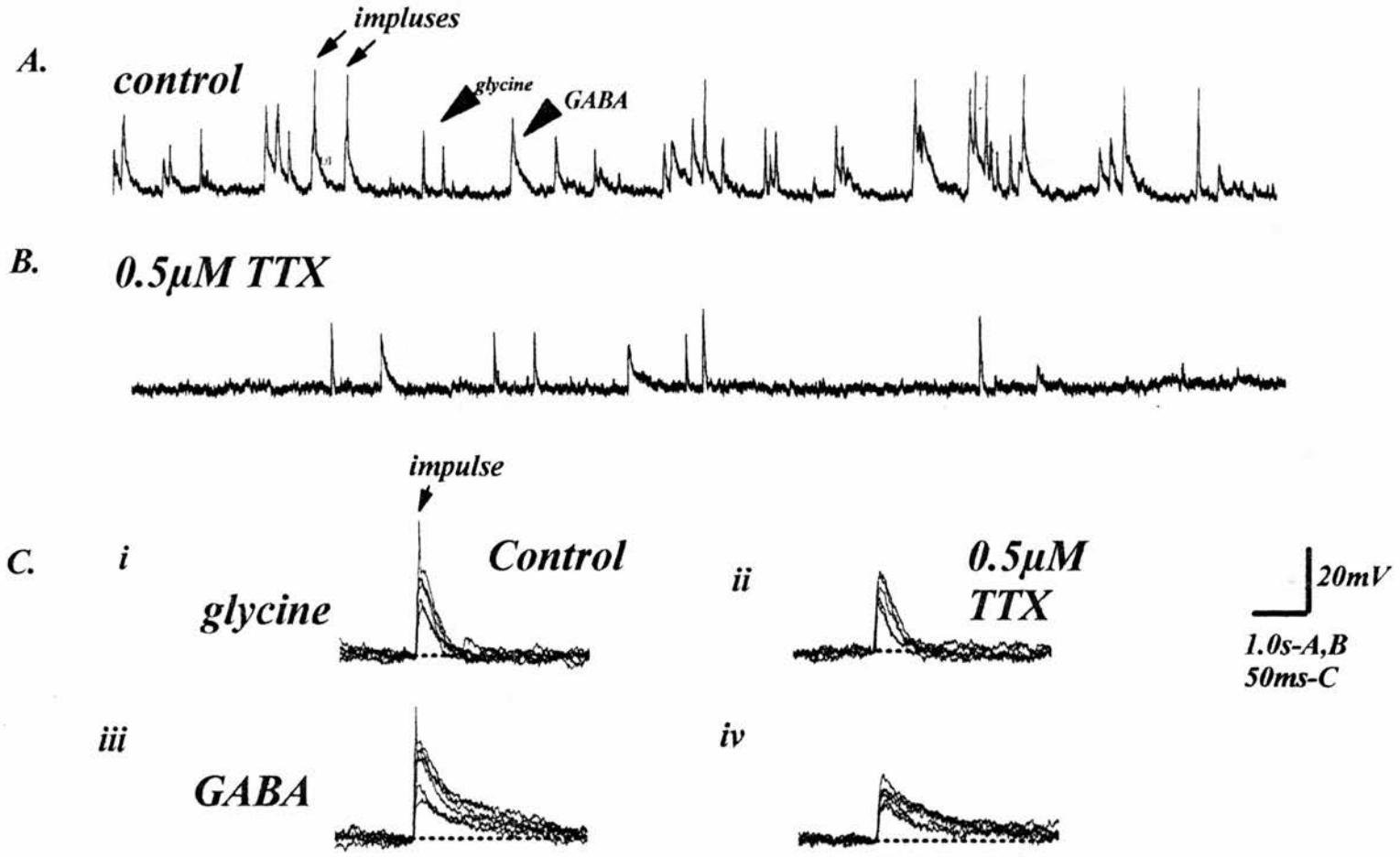

A.
impluses
control
glycine
GABA
B.
0.5μM TTX
impulse
C.
i
Control
glycine
ii
0.5μM
TTX
20mV
1.0s-A,B
50ms-C
iii
GABA
iv

Figure 3.12. TTX-resistant spontaneous ipsps.

a. Inhibitory potentials recorded in the absence of rhythmic activity resulting from both glycine and GABAa receptor activation.
b. Following the bath application of 0.5μM TTX, to block sodium-dependent spikes, the number of potentials significantly decreases, but two types of potential are still recorded, reflecting the spontaneous liberation of transmitter from GABAergic and glycinergic presynaptic terminals.
ci-iv. Six overlapped traces of both glycine and GABA potentials recorded before and after the addition of 0.5μM TTX, showing clearly the difference in duration of the potentials.

the addition of 5β3α, would suggest that the steroid could modulate the probability of transmitter release.

Initially, the effects of 5β3α on all spontaneous release (GABA and glycine) were investigated to determine the specificity of the steroid. Figure 3.13a, shows an example of spontaneous release following the bath application of 0.5μM TTX, where both types of potential were observed (n=3). Following the bath application of 5μM 5β3α (b), the duration of the longer bicuculline-sensitive potentials was increased further, but the duration of the glycinergic potentials was apparently unaffected. Analysis of the effects of 5β3α on the average duration of the population of glycine potentials confirmed the lack of effect of the steroid. In control saline, 74 glycine potentials were measured over a 3 minute interval which had an average duration of 42 ± 15ms. After the addition of 5μM 5β3α, 75 glycine potentials were measured over the same time interval and averaged 43 ± 13ms (t-test: $p > 0.05$). Thus, in keeping with previous reports (Woodward et al., 1991) and confirming the preceding results on the stopping response, 5β3α appears to be a selective agonist at the GABAa receptor in this system. Having shown a lack of effect of 5β3α on spontaneous glycine release, experiments were then carried out in the presence of 5μM strychnine sulphate to block glycine receptor activation and thereby isolate the spontaneous GABAergic ipsps. An example of the effect of 5β3α on the duration of spontaneous GABA potentials is shown in figure 3.14 (n=4). In the presence of 0.5μM TTX and 5μM strychnine, spontaneous (GABA) potentials were measured over a ten minute period before and 10 minutes after the addition of 5μM 5β3α to obtain an average duration under each condition. The duration of the spontaneous GABA potentials increased from 207 ± 100ms in control, to 589 ± 229ms after 10 minutes in 5μM 5β3α (t-test, $p < 0.0001$). The increase in the duration is immediately

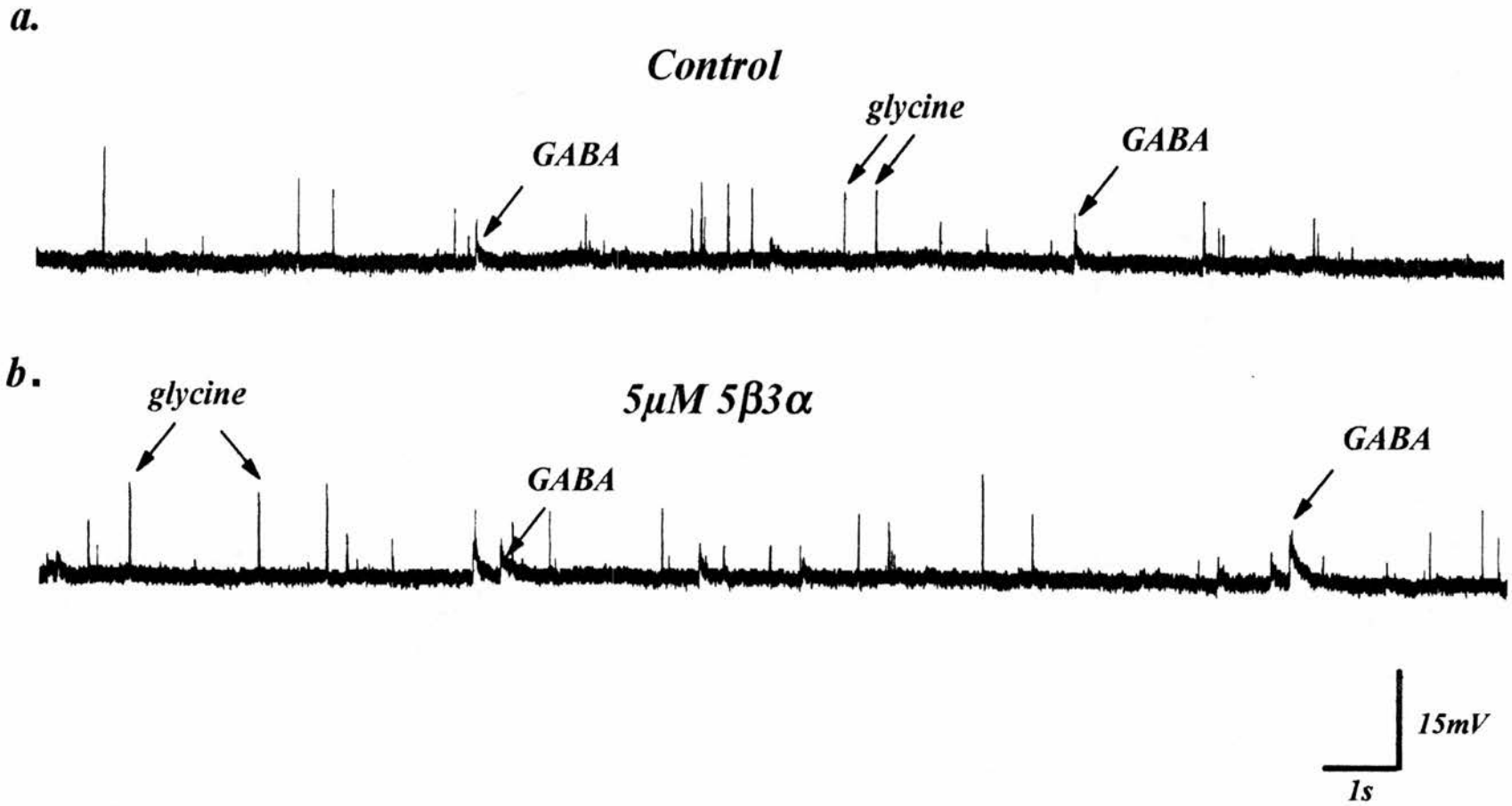

a.
Control
glycine
GABA
GABA
b.
glycine
5μM 5β3α
GABA
GABA
15mV
1s

Figure 3.13. 5β3α selectively enhances spontaneous GABA potentials.

In the presence of 5μM 5β3α, the longer duration GABA potentials are further increased in duration. The glycinergic potentials are apparently unaffected.

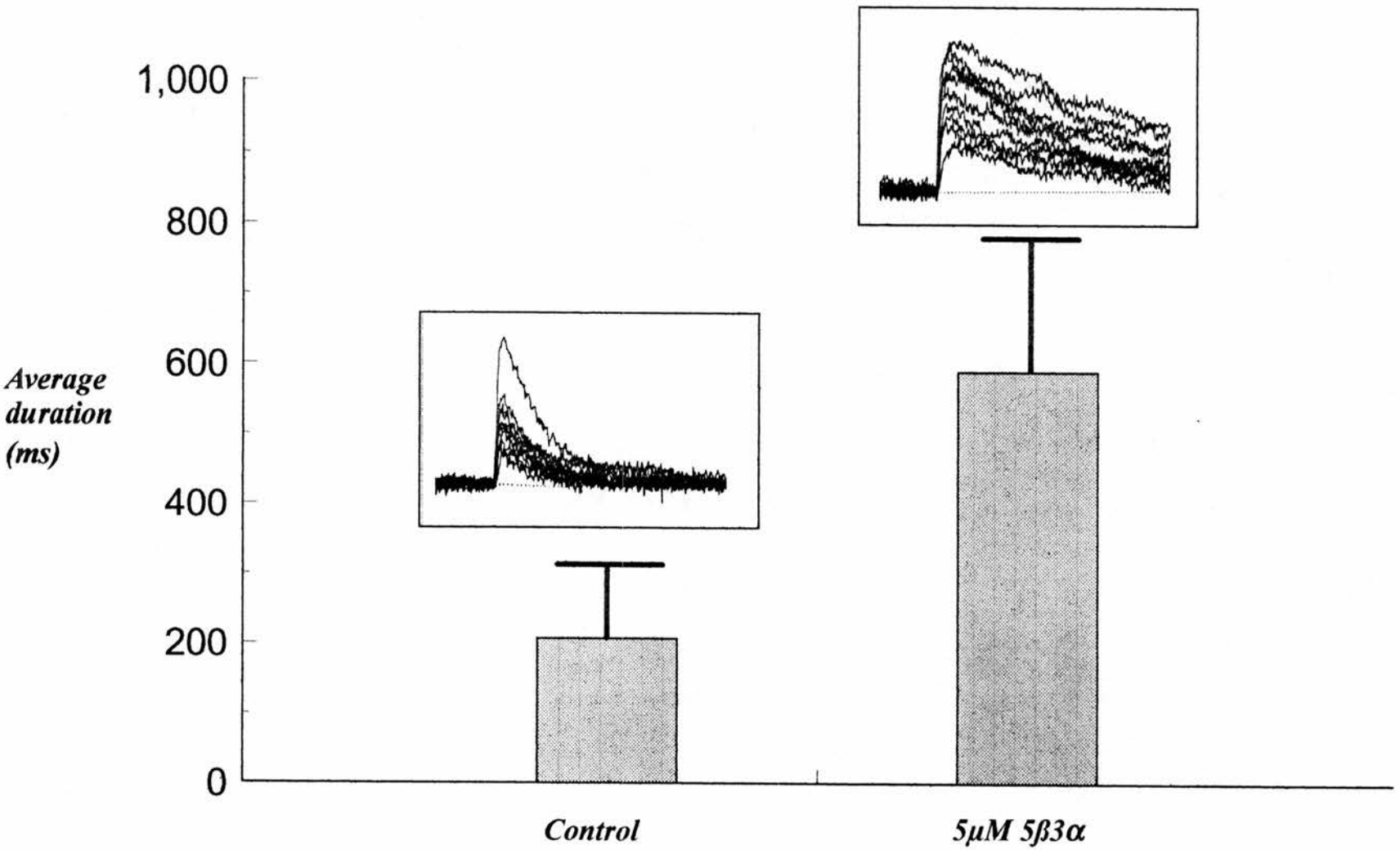

1,000
800
600
400
200
0
Average duration (ms)
Control
5μM 5β3α

Figure 3.14. The effect of 5β3α on the duration of GABA potentials.

Under control conditions, the average duration of GABA potentials recorded over a ten minute period was 207 ± 100ms. After bath applying 5μM 5β3α, the average duration calculated from potentials measured over the same time interval increased to 589 ± 229ms. The inserts above each histogram are 500ms of ten consecutive overlapped potentials recorded under each condition showing clearly the increase in duration. (Dotted line = resting membrane potential).

obvious from the inserts above each histogram which show ten consecutive superimposed potentials under each condition (the dotted line indicates the resting potential; 500ms sweep). After the addition of the steroid the rise time of the potentials appears to remain the same but the falling phase is greatly prolonged. This shows that in this system the steroid changes the response of the postsynaptic neuron following activation of GABAa receptors. This result is in keeping with previous reports on the effects of neurosteroids on GABA-activated chloride currents under voltage-clamp conditions (Twyman and McDonald, 1992).

In order to determine whether 5β3α had any additional postsynaptic effects, the range of GABA ipsp amplitudes was compared under control conditions and after the addition of 5β3α (n=4). The histograms in figure 3.15 indicate that 2μM 5β3α had no apparent effect on the distribution of amplitudes (again measured over ten consecutive minutes in control and under experimental conditions), suggesting that the steroid was having no other effect on GABA receptors on the postsynaptic membrane. The average amplitude of the potentials measured over ten minutes and was 3.5 ± 1.3mV in control and 3.7 ± 1.5mV after ten minutes in 2μM 5β3α. A students T-test confirmed that the addition of steroid caused no significant change in mean ipsp amplitude ($p > 0.05$). This lack of effect on the amplitudes of GABAa receptor mediated potentials suggests that the sensitivity of the postsynaptic membrane to GABA has not been affected. In addition, 5β3α did not detectably alter the resting membrane potential and thus its only postsynaptic action was to increase the duration of GABAa receptor mediated potentials by potentiating the actions of endogenously released GABA. Similarly, other studies also failed to find any other steroid-mediated postsynaptic changes in the GABA response (Callachan et al., 1987, Lambert et al., 1990)

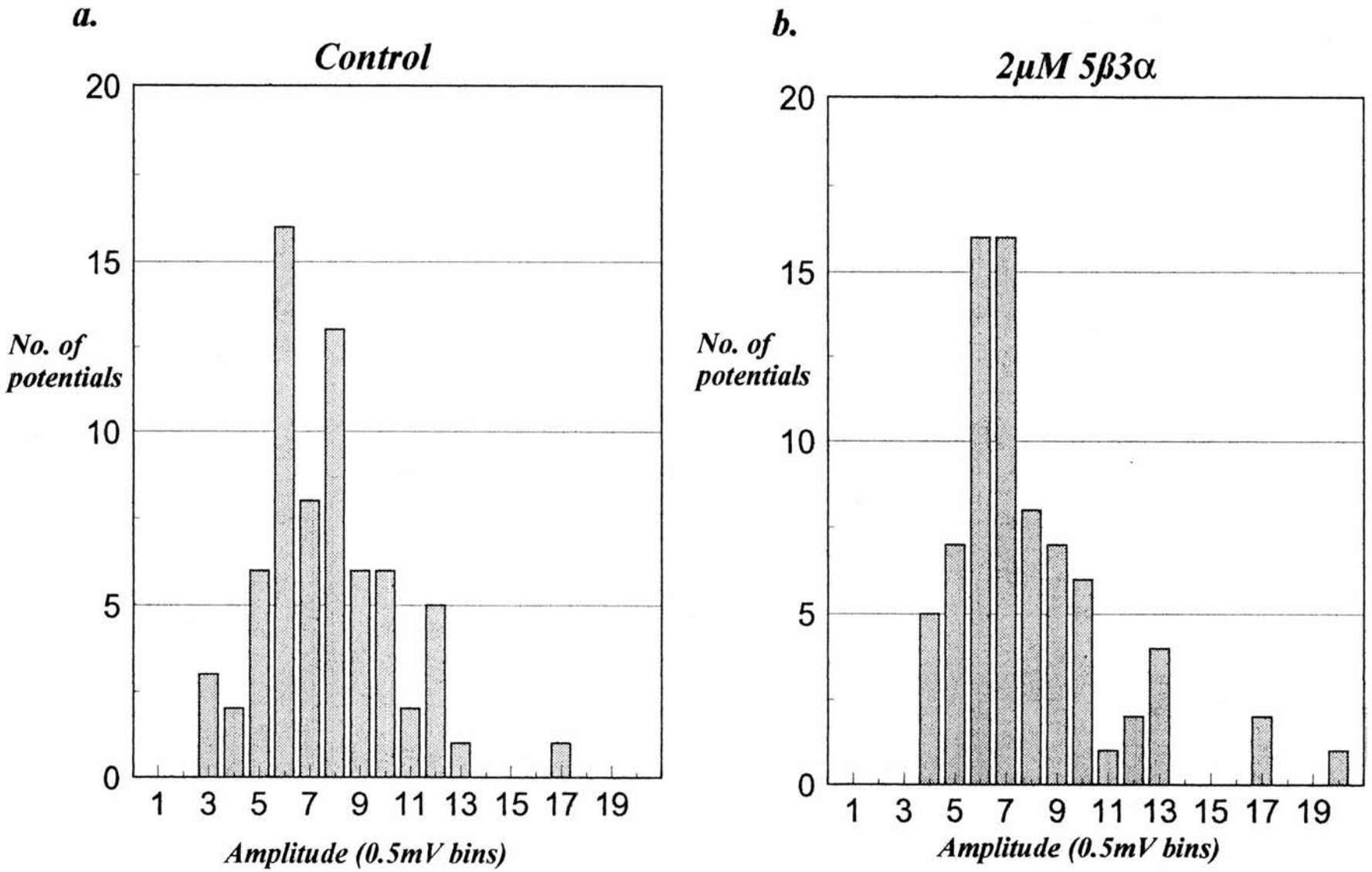

a.
Control
20
15
10
5
0
No. of potentials
1 3 5 7 9 11 13 15 17 19
Amplitude (0.5mV bins)
b.
2μM 5β3α
20
15
10
5
0
No. of potentials
1 3 5 7 9 11 13 15 17 19
Amplitude (0.5mV bins)

Figure 3.15. The effect of 5β3α on amplitude distribution.

a. Histogram showing the amplitudes of GABA potentials measured over 10 minutes under control conditions. The potentials were split into 0.5mV bins covering amplitudes ranging from 1.4 to 8.4mV.
b. Histogram showing the range of potentials measured over the same time interval after 2μM 5β3α had been circulating for 10 minutes. The amplitudes covered a similar range to control between 1.8 and 9.8mV.

However, an important additional effect of the steroid became apparent from recordings made under TTX in that 5β3α reliably increased the frequency of GABA potentials (n=10). A typical experiment illustrating this steroid effect is shown in figure 3.16. In the presence of 0.5μM TTX and 5μM strychnine sulphate, the rate of spontaneous GABA release is usually quite low (a), in this case less than 0.01Hz. Following the bath application of 4μM 5β3α, however, in addition to the increased duration of individual GABA potentials, the potentials also became far more frequent (b), approximately four fold in this example. All of the potentials were blocked by 40μM bicuculline (c) confirming that they result from spontaneous activation of GABAa receptors. The bicuculline effect was reversed after returning to control saline (0.5μM TTX and 5μM strychnine). However, the effects of 5β3α could not easily be reversed (d). In another experiment, the effect of 5β3α (4μM) on release rate is shown graphically (figure 3.17), where the interval between consecutive GABA potentials is measured and plotted against time (a). In control conditions, over a period of 60 seconds, the intervals between each of the 42 potentials occurring in this period ranged from 0.1s to 5.2s. The average interval, indicated by the dotted line, was 1.4s. Ten minutes after the addition of 4μM 5β3α, the intervals between 74 potentials were measured over a similar 60s time period. Now, the range of intervals decreased to between 0.1 and 2.9s with the average interval only 0.8s. The traces in b show 30s of the raw data that was measured to produce the graphs, clearly demonstrating the increase in both release rate and ipsp duration in the presence of 5β3α. Note also that the increase in frequency was sufficient to allow consecutive potentials to occasionally summate. This result shows that 5β3α, in addition to the previously described postsynaptic action, also acts presynaptically to facilitate the spontaneous release of GABA.

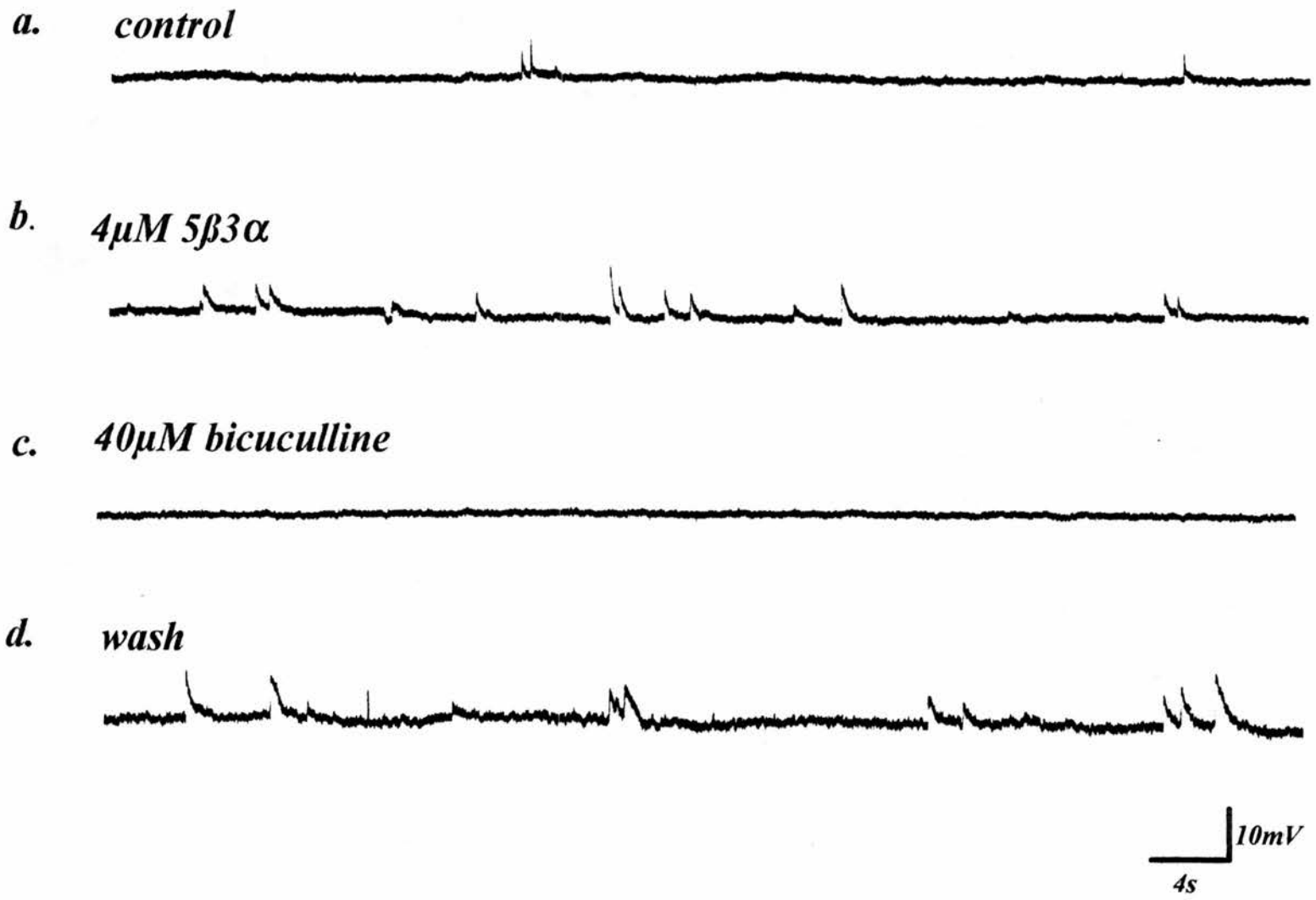

a.
control
b.
4μM 5β3α
c.
40μM bicuculline
d.
wash
10mV
4s

Figure 3.16. 5β3α increases the frequency of spontaneous GABA potentials.

Under control conditions in the presence of 0.5μM TTX the rate of spontaneous GABA release is low (**a**). Following the bath application of 4μM 5β3α, in addition to an increase in the duration of individual potentials, they are also far more frequent (**b**). All the potentials are blocked in the presence of 40μM bicuculline (**c**) but return after washing back into control saline (+0.5μM TTX) although the effects of 5β3α are not easily reversed (**d**).

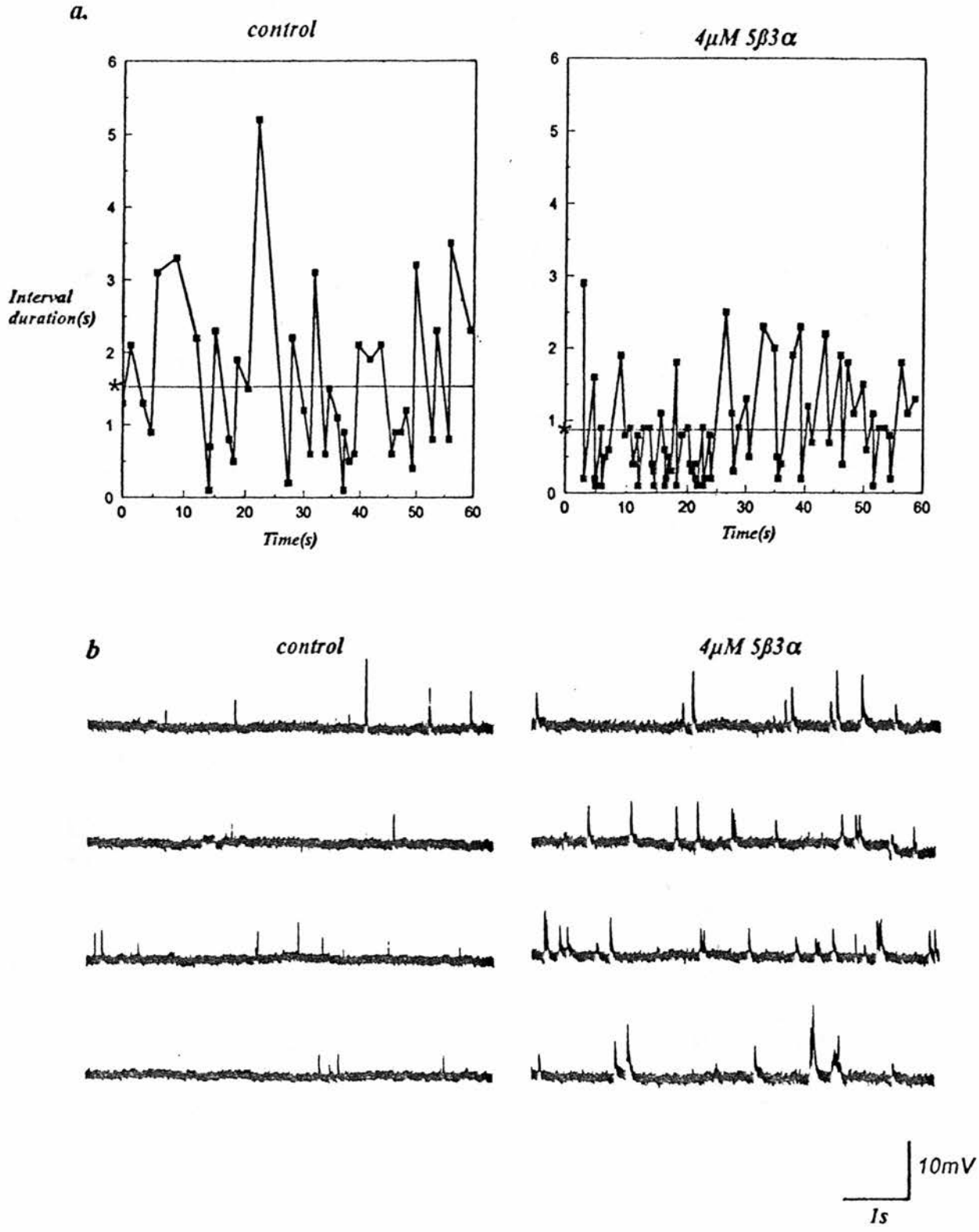

a.
control
4μM 5β3α
Interval duration(s)
Time(s)
Time(s)
b
control
4μM 5β3α
10mV
1s

Figure 3.17. Analysis of the effect of 5β3α on the frequency of GABA potentials.

a. The interval between each spontaneous GABA potential was measured over one minute and plotted against time before and after the addition of 4μM steroid. Under control conditions the interval between 42 potentials was measured, the average of which was 1.4s (indicated by the dotted line). 10 minutes after the addition of 4μM 5β3α, the interval between 74 potentials was measured with an average of 0.8s.
b. 30s traces of the raw data showing clearly the increase in frequency of spontaneous GABA potentials in the presence of 5β3α in comparison with control. The frequency increased to the extent that the potentials could sometimes summate.

4. The effects of 5β3α on fictive swimming.

From the preceding investigation into the synaptic actions of 5β3α, I conclude that this neuroactive steroid is a selective agonist at the GABAa receptor and acts by both increasing GABA release and by enhancing the postsynaptic response following activation of the GABAa receptors. Therefore, this agent has the advantage over other agonists in that it can be used to enhance the effects of endogenously active GABAergic systems. Thus, using 5β3α in combination with the specific GABAa antagonist bicuculline, the role of GABA during embryonic swimming activity was re-examined.

One clear effect which was reliably observed after bath application of the steroid was a marked decrease in the length of swim episodes in intact immobilised embryos (n=8). The histogram in figure 3.18 shows that episode length decreased in a dose-dependent fashion with increasing levels of 5β3α (1-4μM). In control conditions the episode length, averaged over five episodes, was 40.8 ± 21.1s whereas after the application of 4μM steroid the episodes only averaged 5.0 ± 2.2s. This effect on episode length could be reversed by the subsequent bath application of bicuculline (50μM, n=3), an example of which is shown in figure 3.19. The episode length in this experiment decreased from an average of 16.2 ± 7.2s in control saline, to 3.9 ± 0.8s after 30 minutes in 5μM 5β3α. Thirty minutes after the addition of 50μM bicuculline the episodes increased again to an average of 11.5 ± 2.6s.

There are known to be GABAa receptors on motorneurons (Soffe, 1987) and any enhancement of the effects of GABAa receptor activation

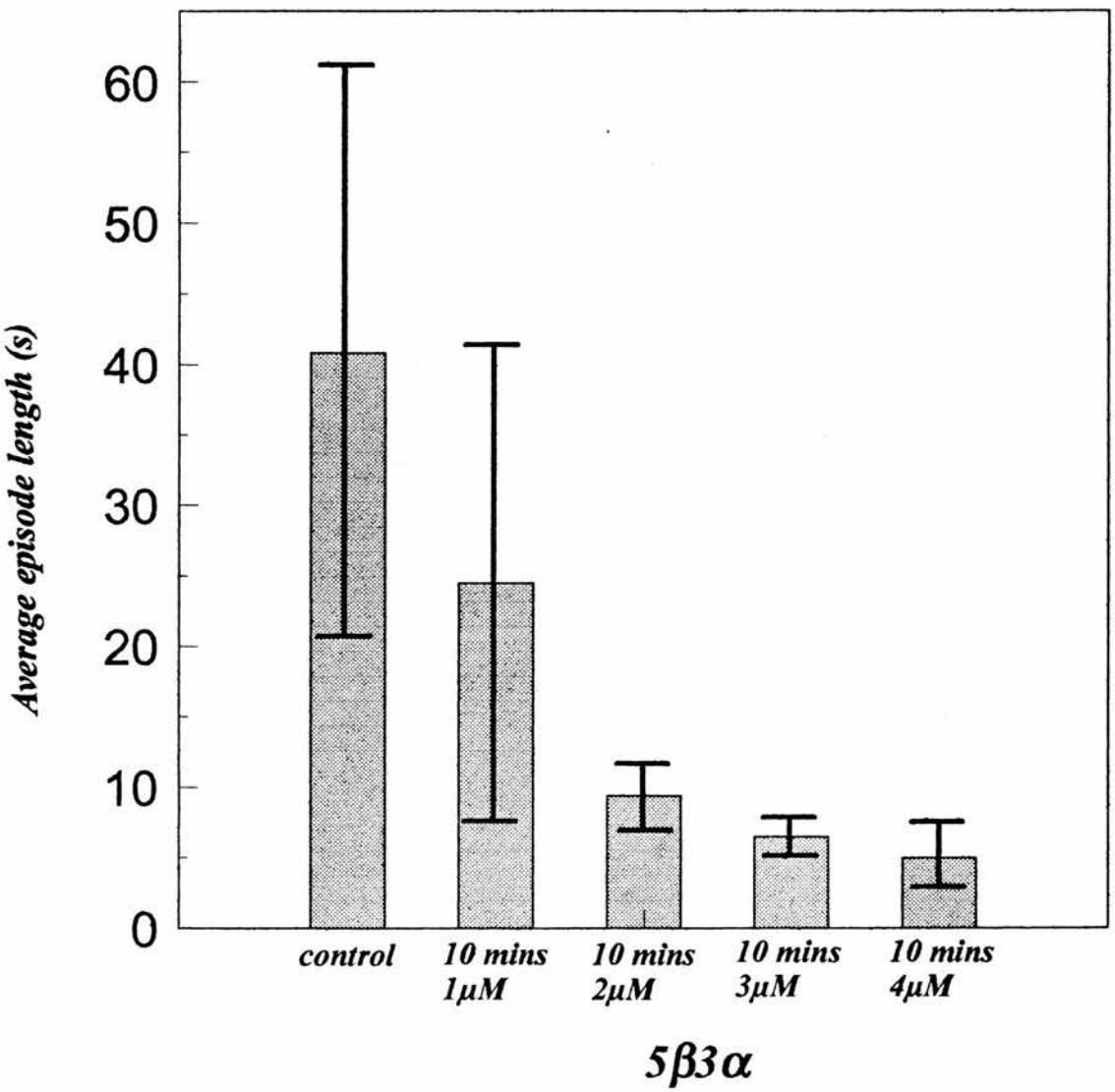

Average episode length (s)
60
50
40
30
20
10
0
control
10 mins 1μM
10 mins 2μM
10 mins 3μM
10 mins 4μM
5β3α

Figure 3.18. The effects of 5β3α on episode length.

Episode length was averaged over five episodes under each condition. In control saline episode length was 40.8 ± 21.1s, after 10 minutes in 1μM 5β3α the episode length was not significantly decreased but averaged 24.5 ± 16.8s (p=0.18). Ten minutes after the addition of 2μM 5β3α episode lengths were significantly decreased to 9.4 ± 1.8s (p=0.02). Ten minutes after adding 3μM 5β3α the episode length averaged 6.5 ± 1.1s (p=0.01) and in 4μM 5β3α it was 5.0 ± 2.2s (p=0.009).

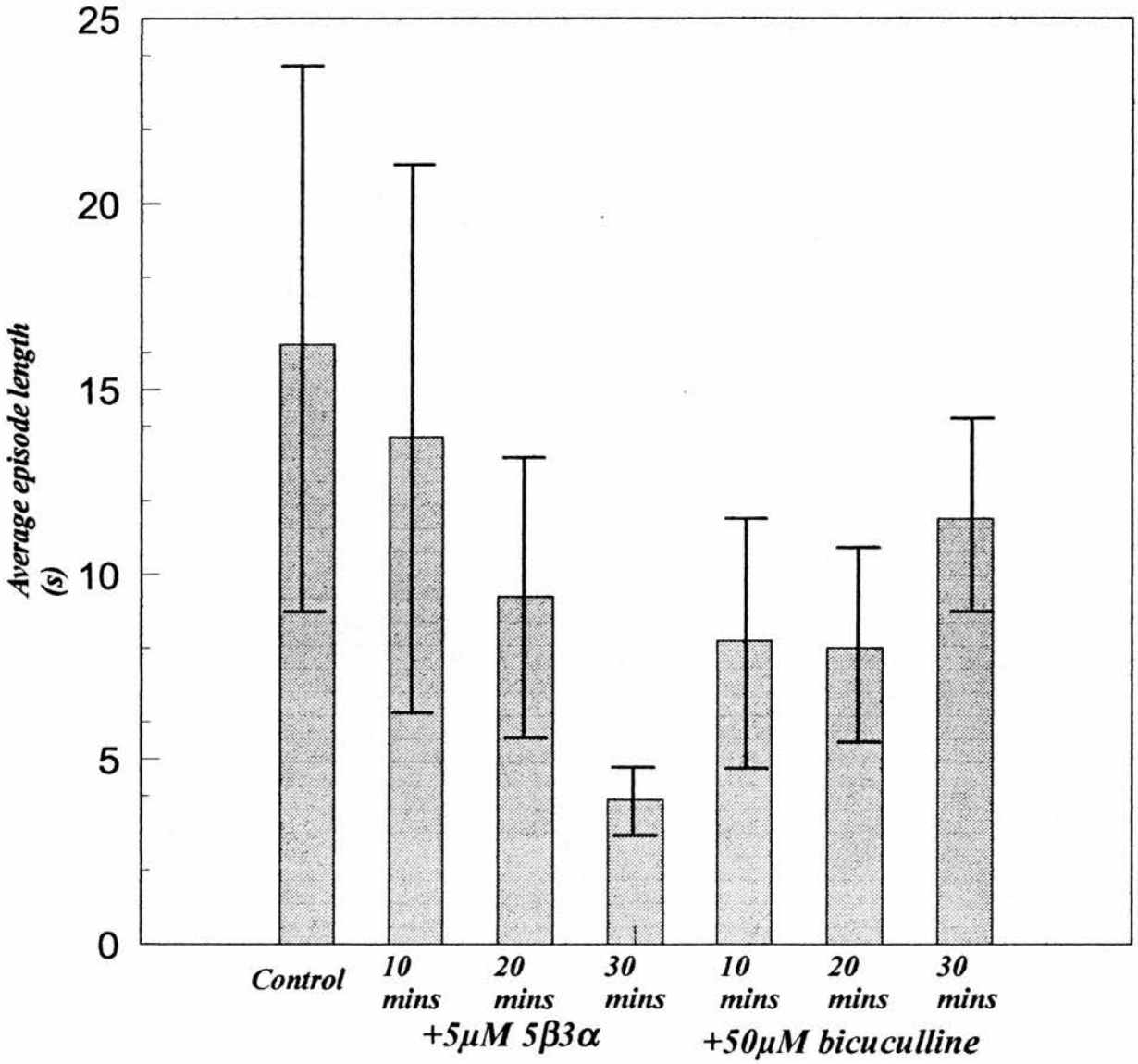
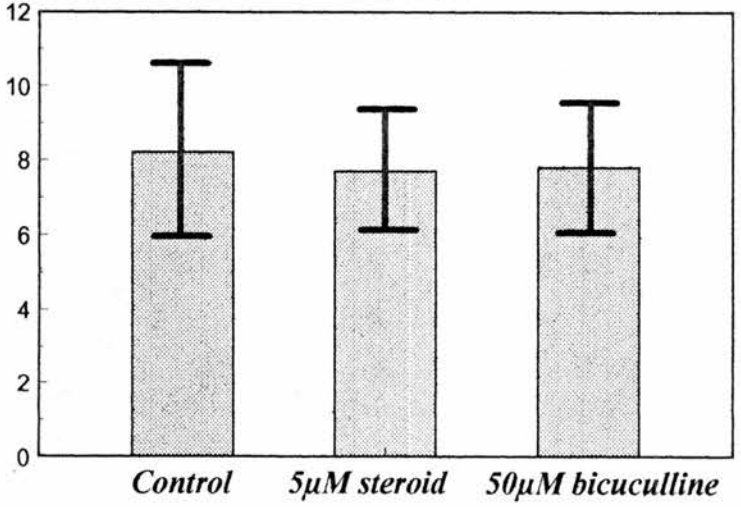

Average episode length (s)
25
20
15
10
5
0
Control
10 mins
20 mins
30 mins
10 mins
20 mins
30 mins
+5μM 5β3α
+50μM bicuculline

Figure 3.19. Bicuculline reverses the effects of 5β3α on episode length.

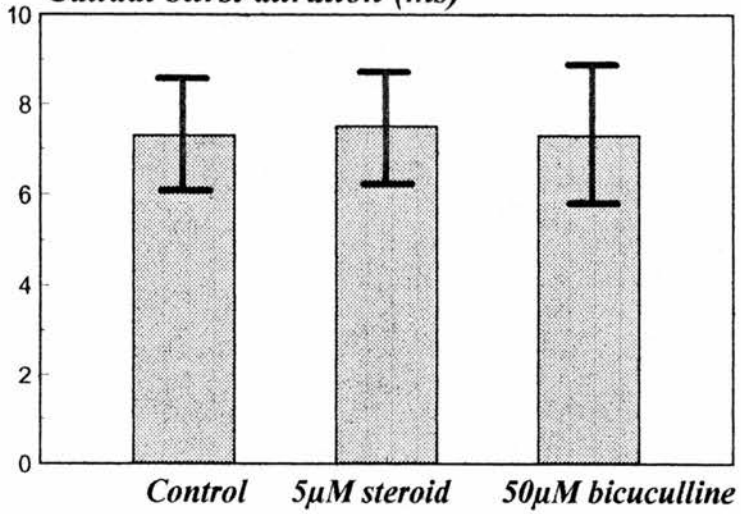
5μM 5β3α reverses the average episode length over time. Under control conditions, episode length was 16.2 ± 7.2s, 10 minutes after bath applying 5μM 5β3α, the episodes decreased to 13.8 ± 7.5s, at 20 minutes the average episode was 9.4 ± 3.7 and by 30 minutes, episodes were 3.8 ± 0.8s. After ten minutes in 50μM bicuculline the average episode was 8.2 ± 3.3s, at 20 minutes, the episodes were 8.1 ± 2.5s and after a further 10 minutes, the average episode was 11.5 ± 2.6s.

could cause a general decrease in their excitability resulting in the observed decrease in episode length. To determine whether the GABAergic system was having any direct effect on the central pattern generator, three basic parameters of fictive swimming (burst duration, cycle period and rostrocaudal delay) were compared under control conditions and following the bath application 5β3α (100nM - 5μM, n=7). The graphs in figure 3.20 show an example of a typical result following the bath application of 5μM 5β3α. The average burst duration recorded from the rostral electrode at P.O.M. 8 (a), was 8.2 ± 2.2ms in control, 7.7 ± 1.5ms 10 minutes after the bath application of 5μM 5β3α and 7.8 ± 1.7ms after blocking GABAa receptor activation with 50μM bicuculline. A students t-test confirmed that the bath application of steroid caused no significant change in rostral burst duration ($p > 0.05$). Similarly, the average duration of activity recorded in a caudal root (b), measured at P.O.M. 14, was also not statistically affected. Before bath application of the steroid the average burst duration was 7.3 ± 1.2ms, after the addition of 5μM 5β3α the average was 7.5 ± 1.2ms and after 10 minutes in 50μM bicuculline was 7.3 ± 1.4ms. Rostro-caudal delay, measured between the rostral electrode at P.O.M. 8 and the caudal electrode at P.O.M. 14 also remained unaltered with an average of 7.2 ± 1.8ms, 6.7 ± 1.3ms and 5.6 ± 1.6ms under control, 5μM 5β3α and 50μM bicuculline respectively (d). The only consistent, statistically significant change which was seen in the presence of 5β3α was that the cycle period increased (n=4). In the presence of 5μM 5β3α (c), the cycle period increased from 66.2 ± 5.3ms to 69.2 ± 4.0ms. Following the bath application of 50μM bicuculline, the cycle period significantly decreased to 58.3 ± 6.4ms ($p< 0.005$). Thus, it would appear that increasing the action of GABA at the GABAa receptor does cause a change in the frequency of fictive swimming activity and hence GABA may have a slight overall

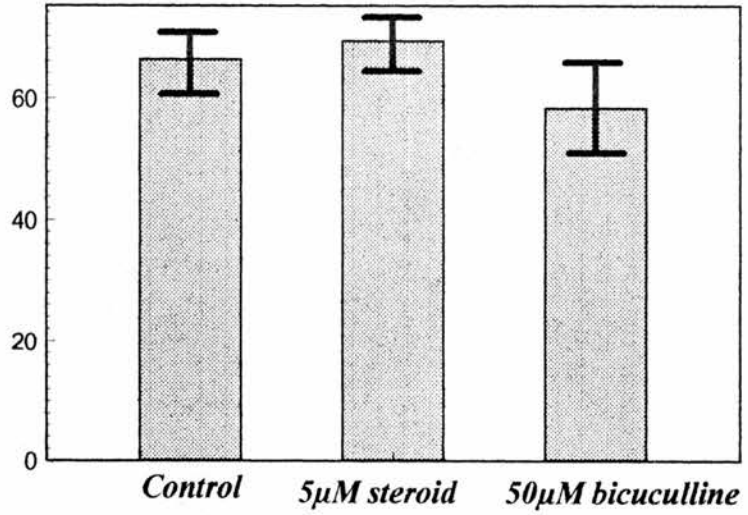
a. *Rostral burst duration (ms)*



b. *Caudal burst duration (ms)*



c. *Cycle period (ms)*



d. *Rostro-caudal delay (ms)*

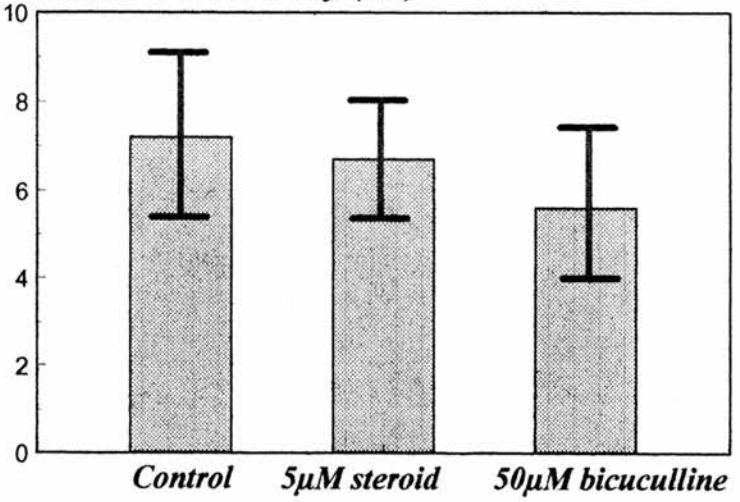
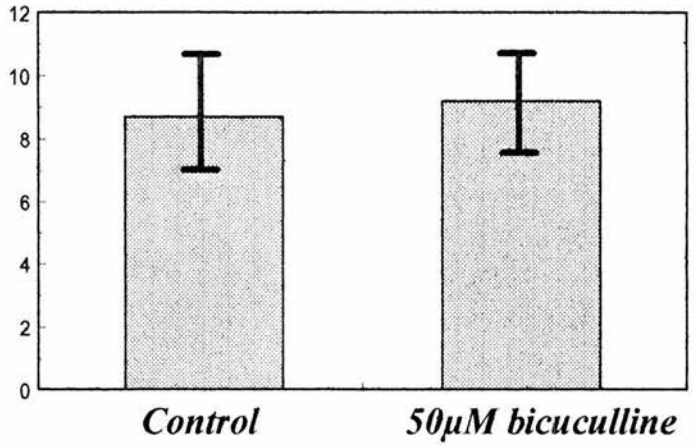


Figure 3.20. The effects of 5β3α on the basic parameters of ventral root activity.

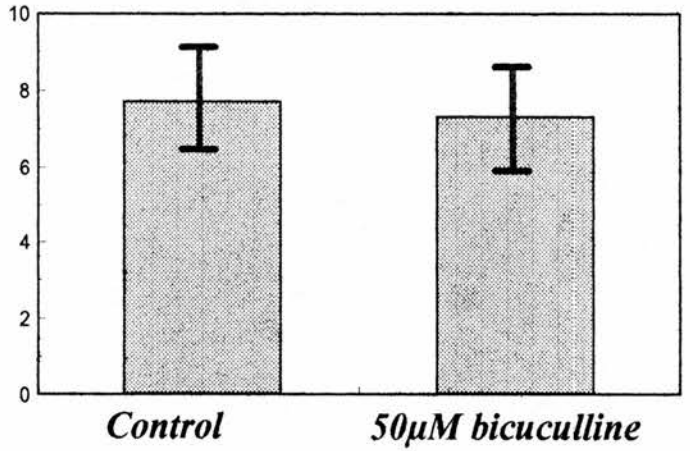
Rostral burst duration (**a**), caudal burst duration (**b**) and rostrocaudal delay (**d**) were not affected by 5μM 5β3α. Cycle period (**c**) was significantly increased in the presence of 5μM 5β3α from 66.2 ± 5.3ms to 69.2 ± 3.9ms ($p < 0.0001$). 50μM bicuculline reversed the effects of 5β3α and decreased the cycle period to below control levels to 58.3 ± 6.4ms ($p < 0.0001$).

inhibitory influence on the swimming CPG. In addition, bicuculline appeared to not only reverse the effect of the steroid, but it also caused a decrease in cycle period compared to those in control suggesting that a GABAergic system mediating the effect was active to some extent under control conditions. The effects of bicuculline, in the absence of steroid were therefore further investigated and an example of the antagonist's effects are shown in figure 3.21 (n=4). Again, the average rostral burst duration, caudal burst duration and rostro-caudal delay were not significantly affected even in the presence of a high concentration of bicuculline (50μM). Cycle period was the only parameter of fictive swimming to be significantly altered and 50μM bicuculline alone was again found to decrease cycle period, as was seen after blocking the steroid effect in the previous figure (3.15c). In the example shown in figure 3.16c, the average cycle period decreased from 53.9 ± 6.5ms in control to 48.5 ± 5.4ms, 10 minutes after the bath application of 50μM bicuculline ($p < 0.0001$). This result shows that the GABAergic system is active to some extent under control swimming and is enhanced in the presence of 5β3α.

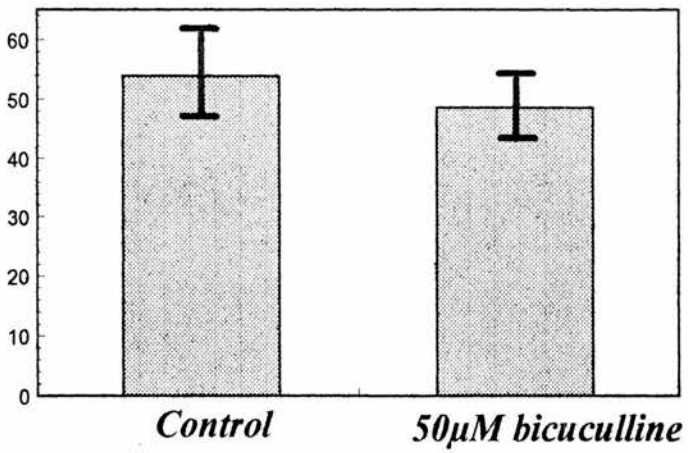
a. ***Rostral burst duration (ms)***



b. ***Caudal burst duration (ms)***



c. ***Cycle period (ms)***



d. ***Rostro-caudal delay (ms)***

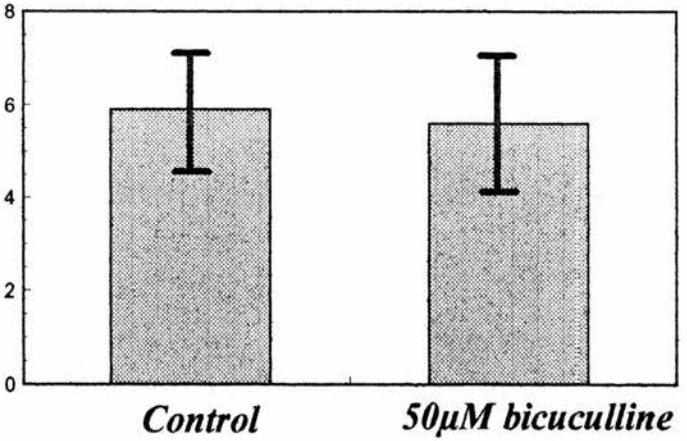


Figure 3.21. The effects of bicuculline on the basic parameters of ventral root activity.

Rostral burst duration (a), caudal burst duration (b) and rostro-caudal delay (d) were unaffected by the bath application of 50μM bicuculline. Cycle period decreased significantly from 53.9 ± 6.5ms in control to 48.5 ± 5.4ms in the presence of 50μM bicuculline ($p < 0.0001$).

Discussion.

In this chapter, I utilised the steroid, 5β3α as a means of enhancing the actions of GABA at the GABAa receptor to determine the effects of GABAa receptor activation on the swimming pattern of the *Xenopus* embryo. Initially however, the embryo preparation itself was utilised as a means of investigating the mechanisms of action of 5β3α at GABAergic synapses.

The steroid enhanced the effects of a known GABAergic pathway, namely the cement gland stopping response which causes the animal to stop swimming if it contacts an obstruction, from which it then remains suspended by a strand of mucus. Intracellular recordings from rhythmically active motorneurons showed that following cement gland stimulation, swimming terminated with a train of ipsps. Following the bath application of 5β3α, the duration of the ipsps was enhanced, allowing consecutive potentials to summate and increasing the time for the membrane potential to return to the resting level. Presumably, during this period the animal would be less likely to swim in response to a sensory stimulus. In the presence of NMDA, it would appear that the mhr neurons are activated periodically, possibly due to the neurons becoming depolarised by activation of NMDA receptors and then firing on rebound from micycle glycinergic inhibition as has been shown to occur in rhythmically active motorneurons (Soffe, 1990). The presence of 5β3α sufficiently enhanced the action of GABA to cause a regular disruption of fictive swimming activity.

Having shown that the steroid acts to enhance a known GABAergic pathway, the synaptic mechanism of action of 5β3α was further investigated. Analysis of depolarising inhibitory potentials recorded from

motorneurons with KCl-filled microelectrodes, in the absence of fictive swimming, revealed two populations of reversed inhibitory potentials separable by their duration. Previous studies had only reported one population which result from glycine receptor activation (Sillar and Soffe, 1987, Wall and Dale, 1993). In this study strychnine blocked potentials of short duration leaving longer duration potentials which were enhanced by steroid and blocked by bicuculline and therefore presumably the result of GABAa receptor activation. In the presence of TTX, the number of potentials recorded were far fewer but their durations still fell into two distinct populations. The potentials were therefore assumed to reflect the spontaneous release of inhibitory transmitter from GABA and glycine terminals. Both glycine receptors and GABAa receptors belong to a superfamily of ligand-gated ion channels and both directly gate a chloride ion channel. Perrins and Roberts (1995a) discuss several possible reasons for differences in the duration of potentials which result from direct ligand-gated ion channels. A difference in rise time could be indicative of differences in the opening kinetics of the channel. However, a comparison of spontaneous glycine and GABAa receptor-mediated potentials reveals no obvious difference in their rise time. The difference lies in the falling phase of the two types of potential. Additionally, the steroid augments the duration of GABA potentials by increasing the falling phase of the potential. This could indicate a difference in the binding of the agonist to the receptor with GABA binding for a more prolonged time than glycine or a difference in the breakdown of the transmitters in the synaptic cleft. Alternatively, the difference could arise from the distance of the synapses relative to the recording site. If GABA potentials were on the distal end of dendrites, cable properties would tend to slow the time course of the ipsp recorded in the cell body. However, Perrins and Roberts (1995a) suggest

that since spinal motorneurons have relatively short dendrites, cable properties would be unlikely to cause a discernible change in the time course of potentials at the recording site.

Analysis of the effects of 5β3α on spontaneous potentials revealed that the steroid caused no apparent increase in the duration of glycine potentials, suggesting that it is a selective agonist of the GABAa receptor. In comparison, 5β3α enhanced the duration of spontaneous GABA potentials without affecting their amplitudes and in addition significantly increased the number of GABA potentials occurring over a certain time interval. This suggests that in keeping with previous studies, 5β3α enhances the postsynaptic action of endogenously released GABA at the GABAa receptor (Twyman and MacDonald, 1992), but has little effect on the number of contacts involved or the quantal content of transmitter release. Additionally, the increase in frequency of GABA potentials is indicative of a presynaptic action of the steroid which increases the probability of transmitter release from the terminals of GABAergic interneurons. The mechanism by which 5β3α increases transmitter release is unknown. It is possible that the steroid acts on GABAa receptors on the presynaptic membrane. However, an action at GABAa receptors might be expected to **decrease** transmitter release and indeed some studies have indicated that neurosteroids can decrease GABA and glutamate release (Taubøll et al., 1993). Alternatively, there could be some feedback mechanism from the postsynaptic membrane whereby the enhanced postynaptic response generates some unknown retrograde signalling molecule acting on GABA terminals.

It also remains unclear whether the steroid directly or indirectly causes an increase in transmitter release by increasing the calcium flux into

the cell following the activation of voltage-dependent calcium channels or by acting directly on the transmitter release machinery. These two possibilities could be distinguished by examining the effects of 5β3α on transmitter release in the presence of cadmium chloride which blocks voltage-dependent calcium channels. If 5β3α still enhanced GABA release in the presence of cadmium it would suggest that it enhances release by acting directly on the release machinery. Clearly, further work needs to be carried out to elucidate the presynaptic actions of 5β3α. Since there is now a renewed interest in developing neurosteroids for clinical use, the mechanism behind this presynaptic action could be potentially important. Additionally, in light of this novel presynaptic site of action for 5β3α, it would be of interest to investigate whether other sedative agents which act at the GABAa receptor, such as the benzodiazepines and the barbiturates, have a similar effect on release rate or if this presynaptic site of action is unique to the neurosteroids.

Having shown that 5β3α is a specific agonist at the GABAa receptor which enhances the action of endogenously released GABA, the role of GABAa receptor activation during embryonic fictive swimming was re-examined by looking at the effects of both enhancing GABA transmission and blocking GABAa receptors with the antagonist bicuculline. In keeping with previous studies on *Xenopus* embryos (Soffe, 1987), I have shown that activation of GABAa receptors is not critically involved in generating the swimming rhythm. However, the steroid did decrease the length of swim episodes. Motoneurons are known to possess GABAa receptors and a decrease in the excitability of motorneurons could result in the observed decrease in episode length. However, 5β3α also caused a decrease in burst frequency and conversely the GABAa receptor antagonist, bicuculline

increased burst frequency. This suggests that GABA also has an inhibitory effect on the CPG and that a GABAergic system is active to some extent during fictive swimming. In both the lamprey and neonatal rat similar effects on cycle period have been reported during NMDA-induced locomotor activity following the bath application of GABA (Tégner et al., 1993, Cazalets et al., 1994). In both of these preparations GABA has a direct action on the locomotor CPG.

The results from spinalisation studies presented in this chapter suggested that the action of the steroid was to enhance the effects of GABA release from mhr neurons during NMDA-induced activity. However, the mhr neurons are not likely to release GABA during fictive swimming in the absence of NMDA since recordings made from these neurons during rhythmic activity have shown that they are phasically inhibited midcycle and do not fire impulses (Boothby and Roberts, 1992b). In the lamprey there are no known populations of GABA-immunoreactive neuron in the brain with axons that descend into the spinal cord, so the population of GABAergic neurons which act on the locomotor system are presumed to be located in the spinal cord (Brodin et al., 1990). The exact location of the population of neurons mediating the GABAergic effects on the neonatal rat spinal cord has not yet been determined. There is, however, some evidence for GABAergic neurons which are intrinsic to the spinal cord since GABA release still occurs in the absence of supraspinal spiking influences (Cazalets et al., 1994). In *Xenopus* embryos there are known to be two populations of GABA immunoreactive neurons located in the spinal cord (Dale et al., 1987, Roberts et al., 1987) and it could be that the overall inhibitory action of GABA is mediated by one or both of these populations. Since GABAa receptor activation does not have a marked influence on the

embryonic swimming pattern, these effects may be masked in the presence of 100μM NMDA.

Finally, the concentrations of 5β3α which were used in this study are somewhat higher than those used previously (eg. Barker et al.,1986, Callachan et al., 1987, Lambert et al., 1987). However, previous studies were carried out on isolated cells and thus GABAa receptors were fully exposed to the steroid. The *Xenopus* preparations is essentially an intact preparation and there could be access problems for the steroid. It is lipid soluble and therefore may be taken up by surrounding tissue. Although concentrations of around 2-5μM 5β3α were added to the stock bottle, there is no way of knowing the exact concentration which actually reaches the spinal cord and GABAa receptors. Despite the high concentrations, spinalisation studies presented in this chapter showed that bath applying 10μM 5β3α did not interrupt NMDA-induced activity therefore suggesting that the steroid was not directly activating the receptor-ion channel complex. In previous studies, direct effects were seen at around 1μM steroid (Callachan et al., 1987). Moreover, had there been any direct effect of the steroid causing activation of all GABAa receptors, a membrane depolarisation would have been observed in intracellular recordings from motorneurons. In addition, episode length experiments presented in this chapter suggested that although there was some decrease in episode length at concentrations of around 700nM (not shown), maximum effects were not seen until 2-3μM 5β3α was bath applied (figure 3.18).

CHAPTER 4
A developmental increase in the role of GABA transmission.

Introduction

The spinal CPG controlling swimming activity in *Xenopus* embryos has proven to be an excellent simple model for exploring neural mechanisms involved in vertebrate locomotion. Many of the mechanisms described first in the *Xenopus* embryo have subsequently been shown to contribute to the generation of locomotor activity in more complex vertebrate systems. There are, however, some atypical features of the embryonic rhythm. One of the most striking features is that motorneurons only fire once per cycle and they appear to do so on every cycle (Sillar and Roberts, 1993), making the system somewhat stereotyped and rather inflexible. One proposal is that the single impulse results from a slowly inactivating potassium current which prevents the neurons from recrossing the spike initiation threshold after they have fired and therefore from discharging multiply on each cycle of activity (Soffe, 1990). However, recent studies on isolated *Xenopus* embryo motorneurons have shown they can fire multiply when recorded using the whole cell patch clamp technique and it has been suggested the single firing recorded with intracellular microelectrodes, is due to the damaging effect of the electrodes which lowers the membrane resistance (Dale, 1995). Computer simulations of embryonic motorneurons could made more similar to intracellular recordings if this shunting effect was incorporated into the model (Dale, 1995). One problem with isolated neurons, though, is that they are no longer influenced by the same synaptic input and consequently their firing properties may be different to the intact preparation. Thus, neither theory has yet been conclusively proved or disproved.

Recent studies have investigated the postembryonic development of the swimming pattern from stage 37/38 through to stage 42, approximately

twenty four hours later in development (at 23°C, fig 4.1 Ai, Bi). By stage 42, fictive swimming activity has greatly increased in complexity (Sillar et al., 1991, 1992a): although the pattern of ventral root activity still strictly alternates across the body and progresses down each side with a brief delay between segments, discrete bursts of activity are now recorded replacing the biphasic impulses recorded in each cycle of embryo swimming (Sillar et al., 1991, see figure 4.1, Bii,iii cf. Aii,iii). Typically, larval motor bursts last up to or over 20ms, compared to embryonic ventral root bursts which average around 7ms in duration. Since, the cycle periods are essentially similar in embryonic and larval swimming, the bursts therefore occupy more and sometimes up to 50% of each larval swim cycle. Intracellular recordings from larval motorneurons have shown that the increase in burst durations reflects a change in the firing properties of rhythmically active neurons. Motorneurons are still active upon a background tonic depolarisation, but they will now often fire more than one impulse per cycle, especially at the beginning of an episode when activity is at its most intense (figure 4.1 Aiv, Biv, Sillar et. al., 1992a). Furthermore, while embryonic neurons tend to fire once on **every** cycle, larval neurons often cease firing on some cycles especially towards the end of an episode when their synaptic drive is weaker and the swimming frequency is slower (Sillar et al., 1992a).

In addition to the change in motorneuron firing during larval development, the associated synaptic drive underlying swimming activity has also been reported to be more complex with both on-cycle excitation and midcycle inhibition consisting of trains of PSP's rather than the single compound events recorded from embryonic motorneurons (Sillar et al., 1992a, figure 1.4 Biv). This indicates that rhythmically active, premotor interneurons also fire multiply during larval swimming. The increase in complexity of the larval rhythm is therefore largely achieved by a

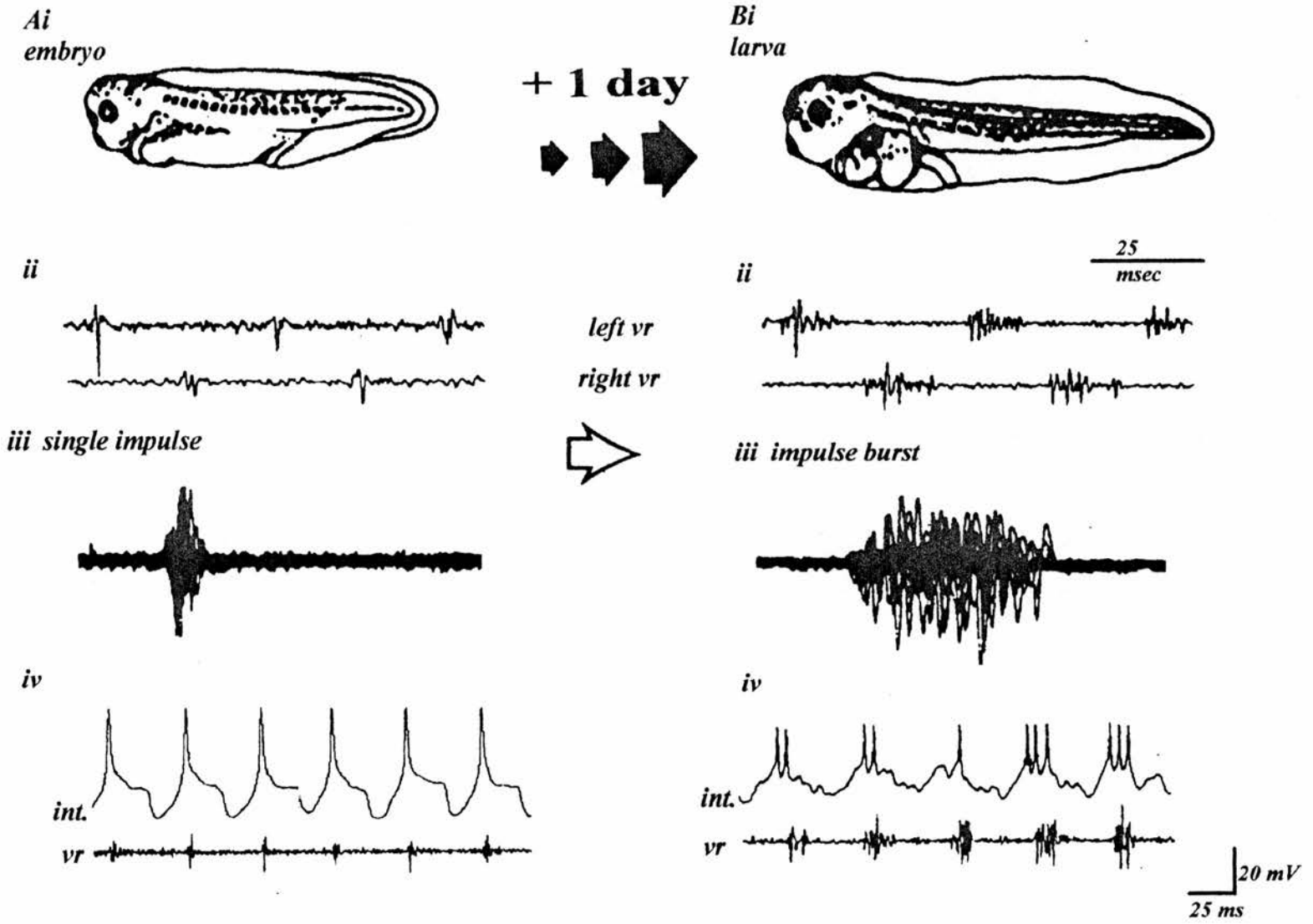

Ai
embryo
+ 1 day
Bi
larva
ii
left vr
right vr
25
msec
ii
iii single impulse
iii impulse burst
iv
int.
vr
iv
int.
vr
20 mV
25 ms

Figure 4.1. Postembryonic development of the motor pattern.

At larval stage 42 (**Bi**), 24 hours later in development, extracellular recordings show that brief impulse bursts seen in the embryo (**Aii, iii**) are replaced by much longer bursts of activity in each cycle (**Bii, iii**). Intracellular recordings at this stage show that motorneurons can now fire more than once per cycle and that the synaptic drive underlying swimming activity has become far more complex (**Biv** cf **Aiv**). Adapted from Sillar et al., 1995a.

developmental alteration in neuronal membrane properties rather than by any major reorganisation of the spinal network. The conclusion that the maturation process occurs without a change in the basic locomotor circuitry is not limited to this preparation. Other embryonic locomotor circuits have also been shown to be retained essentially intact during development even in less obvious circumstances where the actual behaviour generated by the circuit is no longer displayed. This has been shown to occur for the neural circuit underlying hatching in the chick. It might have been assumed that the circuitry underlying this form of locomotor behaviour would be lost shortly after hatching when the behaviour is no longer required, but evidence against this idea has arisen from the observation that a posthatching chick will display hatching activity when placed in a glass egg (Bekoff and Kauer, 1984). The hatching circuit may thus be re-used to produce walking behaviour even although its output is highly modified to enable the two legs to become alternating during walking when they were activated synchronously during hatching. These changes are thought to be produced by descending influences and sensory inputs which develop shortly after hatching. Hatching behaviour itself can be induced by proprioceptive signals arising from the neck being bent to either the right or left. Similarly, straightening the neck appears to be the signal to terminate hatching behaviour (Bekoff, 1992).

In *Xenopus*, the development of a more complex locomotor system has been shown to progress rostro-caudally along the body axis since ventral root recordings at an intermediate stage (40) revealed a transitional phase in which only the rostral roots display 'bursty' activity while the more caudal roots remain embryonic (Sillar et al., 1991). In the period after hatching, neurons whose processes descend into the spinal cord from higher centres, such as the brainstem, continue to develop. Immunocytochemical

studies in *Xenopus* revealed that one such population of neurons are the serotonergic raphe neurons located in the ventral medulla (van Mier et al., 1986). By the time of hatching, these neurons have already innervated the dorsal part of the spinal cord but are only just beginning to extend processes into the ventral portion of rostral segments. To explore whether a descending serotonergic influence might be involved in the developmental increase in complexity of the larval swimming rhythm, the effect of bath applied 5HT on ventral root activity at different stages was investigated. The results essentially showed that bath applied 5HT could modulate the pattern of activity of stage 37/38 and stage 40 animals so that it resembled that of an animal 12 hours older (ie, stage 40 and 42 respectively). Furthermore the sensitivity of the motor system to 5HT appeared to just precede the ingrowth of raphe spinal axons (Sillar et al., 1992b). By stage 42, the ventral root activity along the length of the cord is 'bursty' possibly reflecting its complete innervation by descending 5HT neurons and bath application of 5HT now modulated activity along the length of the body causing an increase in burst durations (Sillar et al., 1992b). A similar enhancing influence of 5HT on locomotion has also been reported in other adult vertebrate systems (Viala and Burser, 1969, Harris-Warrick and Cohen, 1985, Barbeau and Rossignol, 1990). Alternatively, the addition of a 5HT antagonist, NAN-190, could change the larval pattern of activity back to one which resembled an embryonic-like form (Wedderburn and Sillar., 1994a), once more showing that the basic network remained unchanged during development. The role of 5HT in modulating the larval rhythm is addressed in the next chapter.

Perhaps the most direct evidence that innervation of the spinal cord by the descending projections of raphe neurons is responsible for the developmental increase in the complexity of rhythmic swimming activity,

derived from experiments in which the serotonergic fibres were prevented from developing. Treating animals with a monoaminergic neurotoxin (5,7-dihydroxytryptamine) prior to the first appearance of raphe neurons (stage 25) prevented serotonergic axons from innervating the cord (confirmed by immunocytochemistry). When toxin treated animals are allowed to develop to the equivalent of stage 42, ventral root recordings showed that the pattern of activity remained embryonic (Sillar et al., 1995b). Thus deletion of descending 5HT projections prevents maturation of the swimming rhythm.

Although previous studies have shown that at least some of the basic circuits remain unchanged during vertebrate locomotor development, there have also been reports of a development change in the influence of different transmitters. In the chick for example, in contrast to *Xenopus*, GABA has been shown to be important in the production of the early embryonic output and although the general level of inhibition remains relatively constant during development, GABAergic inputs are superseded by glycinergic inputs (Antal et al., 1994). Similarly GABA immunoreactivity in the ventral horn of the rat spinal cord declines during the neonatal period (Ma et al., 1992). Thus, while studies have described a role for GABA during locomotor activity, including a role for GABAa receptor activation in maintaining alternating motor rhythms (Cowley and Schmidt, 1995), these results may be developmentally related. However, evidence from cat studies have suggested that the role of GABA **increases** during development and that locomotion in the adult spinal cat is inhibited to a greater degree than a newborn spinal animal (Robinson and Goldberger, 1986). The most marked recovery of locomotor activity, following intravenous administration of bicuculline was seen in adult spinal cats as opposed to those which had been spinalised at birth. This suggests that the

adult system is modulated to a greater extent by GABA than at earlier stages of development.

In *Xenopus* larvae, the pattern of activity is far more complex than in embryos and as a consequence can be modulated to a greater extent. 5HT has been shown to be important not only in the post-embryonic development of swimming but also in the intrinsic modulation of the larval swimming pattern, on which it has an overall excitatory effect. In other systems where 5HT has also been shown to also have an overall excitatory effect, it has been suggested that there is an opposing inhibitory system which results from GABA release (Cazalets, 1995, Wallén., 1995). The aim of the experiments presented in this chapter was to determine whether, in addition to the descending modulatory influence of 5HT neurons from the raphe nucleus, GABA transmission plays an increased role in modulating the pattern of larval swimming activity thereby providing an inhibitory influence which can finely tune larval rhythmic activity. After showing that GABAa receptor activation has a more marked influence on the larval spinal pattern generator, attempts were made to determine the location of the populations of GABAergic neurons mediating the response.

Results

1. A role for GABA during fictive swimming activity?

The first indication that GABA may modulate larval swimming to a greater extent than the embryo came from steroid experiments carried out in the presence of NMDA. It became apparent that there was a developmental difference in the pattern of activity observed following the bath application of bicuculline (20-50μM) to block the effects of 5β3α. At stage 38 bicuculline reversed the effect of 5β3α causing a resumption of continuous ventral root activity (chapter 3, figure 3.7) whereas, by stage 42 bicuculline itself interrupted ventral root activity much like the steroid (n=3). An example of this effect is shown in figure 4.2. After 4 minutes in 100μM NMDA continuous fictive swimming activity was observed (a). As in the embryo, rhythmic ventral root activity was periodically interrupted by 2μM 5β3α, with bursts of activity occurring around every 8s between which there was no discernible ventral root activity (b). The subsequent bath application of bicuculline (50μM) transiently reversed the effects of the steroid and continuous ventral root activity resumed (c). However 10 minutes later, bicuculline itself regularly disrupted rhythmic swimming activity with bursts of rhythmic activity occurring more frequently than in the presence of steroid, around every 6s (d). Experiments were therefore carried out to investigate the action of bicuculline on the continuous pattern of activity induced by either NMDA (100μM) alone or NMDA (70μM) in the presence of dihydrokainic acid (DHKA, 50μM). DHKA is a glutamate uptake blocker and has been reported to cause a more continuous pattern of fictive swimming when combined with NMDA (Smith et al, 1988). Figure 4.3, shows an example of the effect of bicuculline on NMDA/DHKA-

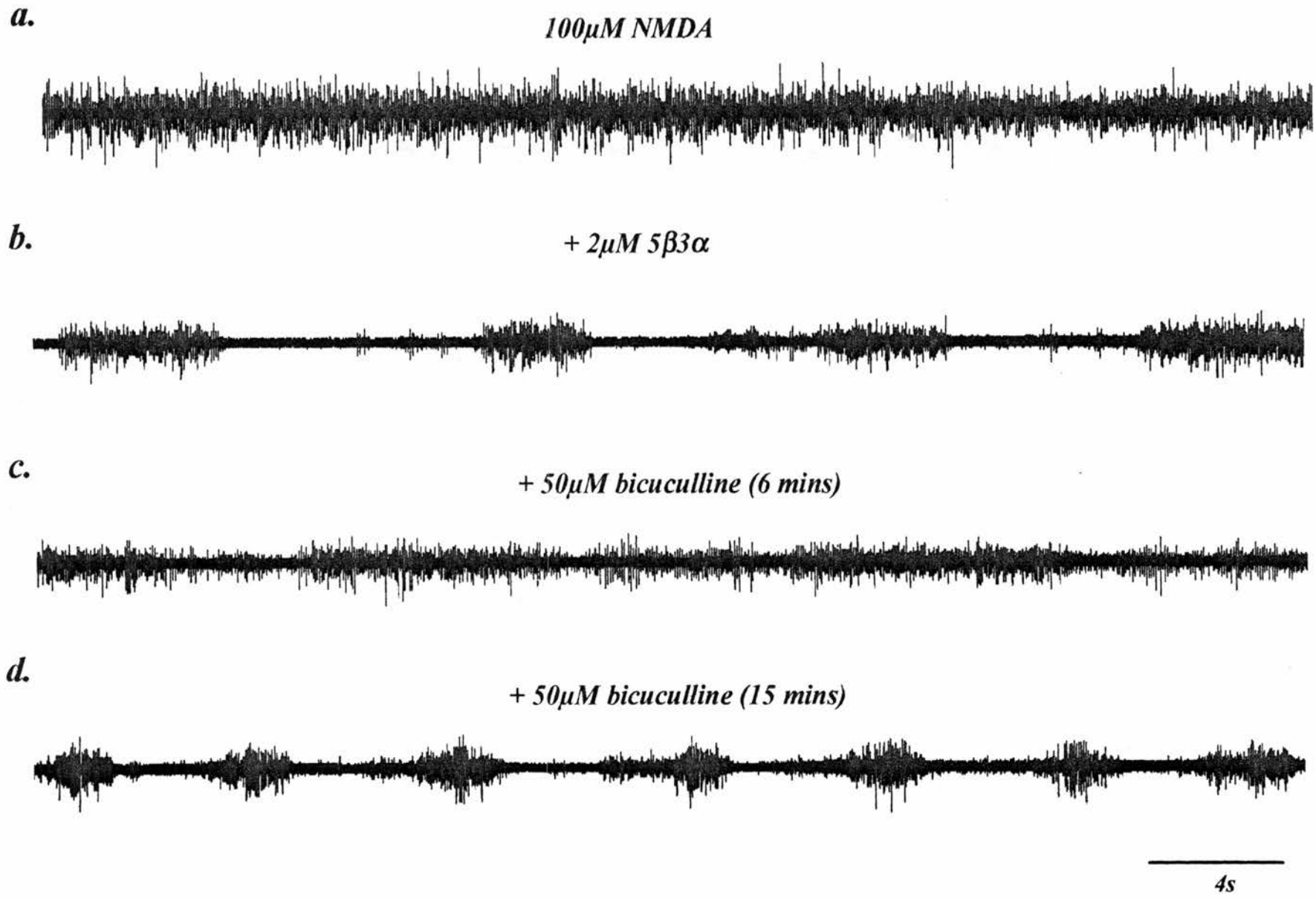

a.
100μM NMDA
b.
+ 2μM 5β3α
c.
+ 50μM bicuculline (6 mins)
d.
+ 50μM bicuculline (15 mins)
4s

Figure 4.2. The effects of 5β3α and bicuculline on NMDA/DHKA induced larval activity.

In the presence of 100μM NMDA (**a**), 2μM 5β3α interrupts continuous swimming (**b**). This effect is initially blocked (after 6 minutes) in 50μM bicuculline (**c**) and continuous fictive swimming resumes. After 15 minutes, 50μM bicuculline regularly interrupts larval swimming activity approximately every 2s (**d**).

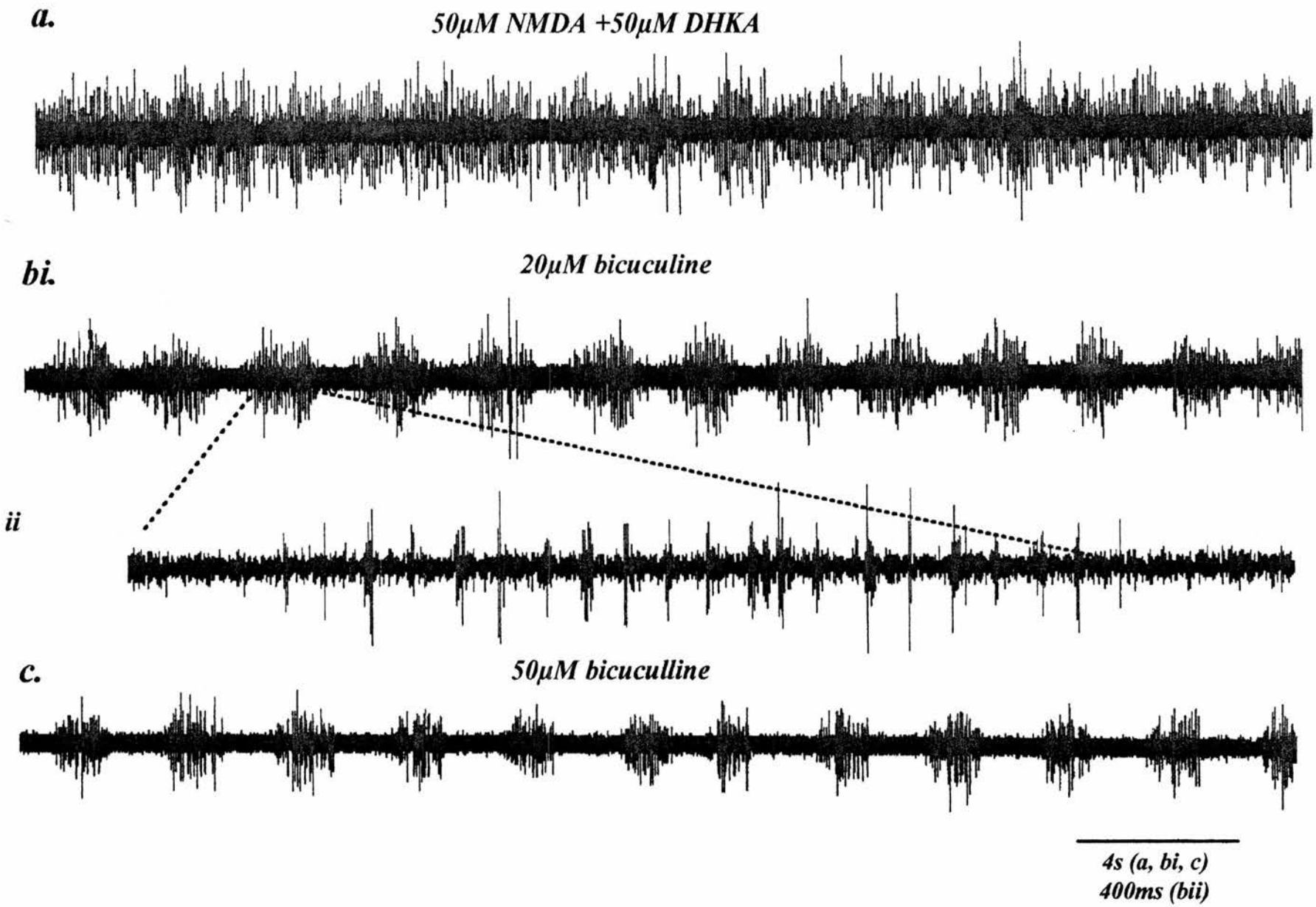

a.
50μM NMDA +50μM DHKA
bi.
20μM bicuculine
ii
c.
50μM bicuculline
4s (a, bi, c)
400ms (bii)

Figure 4.3. The effects of bicuculline on NMDA/DHKA induced larval activity.

From the continuous activity induced by 50μM NMDA and 50μM DHKA (**a**), 20μM bicuculline changes the activity to regular bursts of ventral root activity, with an interval of around 0.5s between each (**bi**). An expanded trace shows that the activity within each burst is still rhythmic (**bii**). Increasing to 50μM bicuculline, increases the interval between each burst of activity to around 1.3s.

induced activity at stage 42 (n=4). Following the bath application of 20μM bicuculline, the pattern of activity changes to regular 'bursts' which occur around every 2.5 seconds (bi). From the expanded trace illustrated in (bii), it can be seen that within each 'burst', rhythmic ventral root activity still persists. Thus bicuculline turns the continuous swimming pattern into shorter episodes of swimming with silent intervals between episodes. Increasing the concentration of bicuculline to 50μM causes the silent intervals to become slightly longer and the bursts of activity slightly shorter but they still occur around every 2.5s (c). These results differ from those obtained in embryos (figure 4.4), where NMDA-induced activity was only interrupted by higher concentrations of bicuculline (50μM, figure 4.4c, n=4) if at all (n=3).

2. Effects of GABAa receptor ligands on larval fictive swimming activity.

The preceding results suggest a developmental increase in ability of of endogenously released GABA to modulate swimming. However, the results were obtained in the presence of NMDA, an agonist which will excite many or all of the neurons in the locomotor network and could also excite sensory pathway neurons which are not normally active during swimming. It has already been shown in chapter 3, that the mhr neurons become active under NMDA. Therefore, to determine whether GABA plays a role during 'normal' episodes of evoked swimming activity, the effect of blocking GABAa receptor activation on the basic parameters of fictive swimming was analysed. Figure 4.5, compares the effects of varying concentrations of bicuculline (20 - 50μM) on the duration of embryonic and larval swim episodes averaged over 3 episodes in each condition. At both

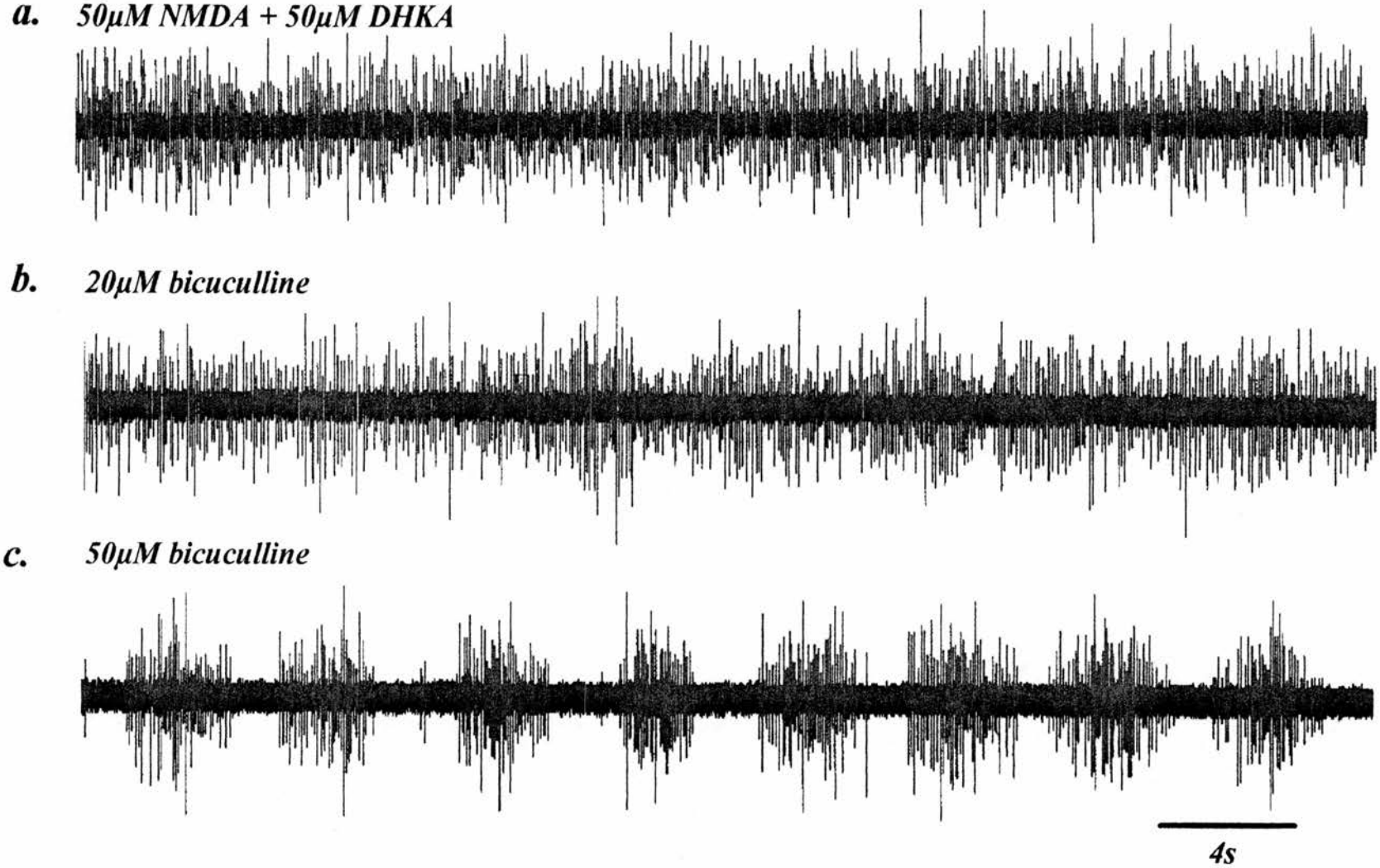
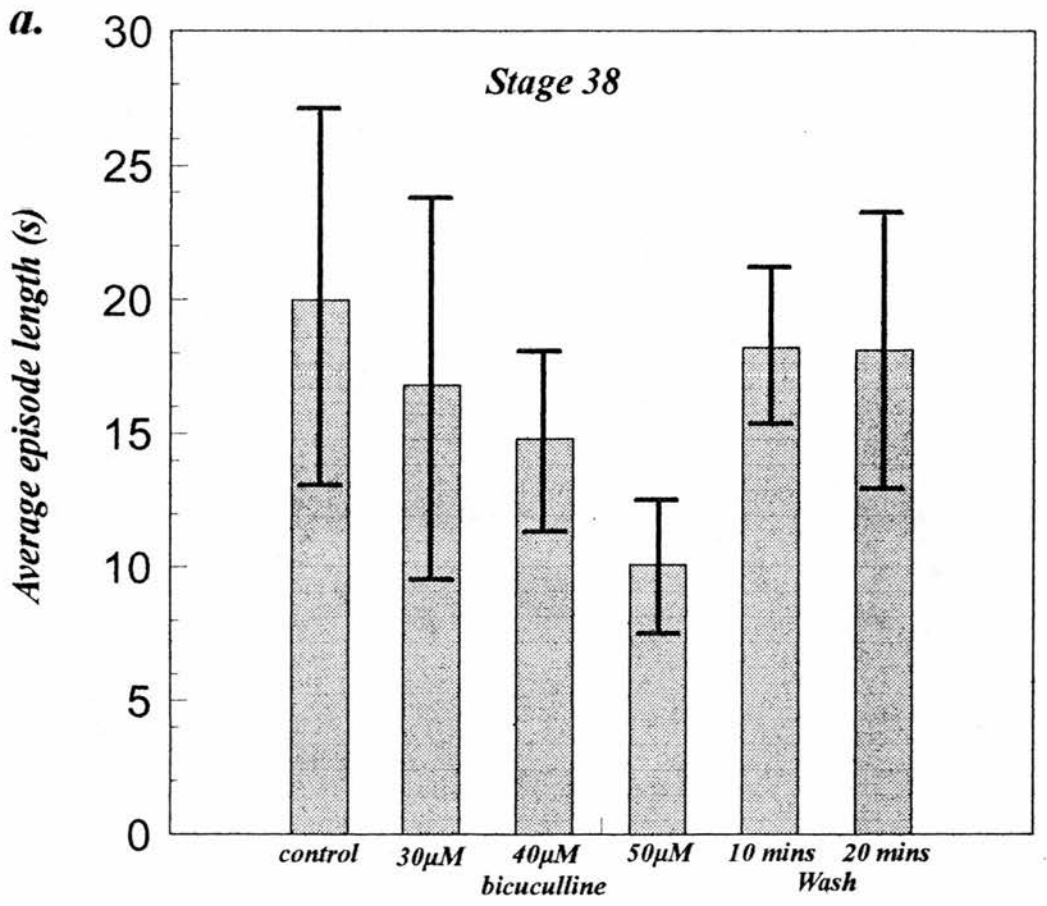

a. 50μM NMDA + 50μM DHKA
b. 20μM bicuculline
c. 50μM bicuculline
4s

Figure 4.4. The effects of bicuculline on NMDA/DHKA induced embryonic activity.

Continuous embryonic ventral root activity induced by co-application of 50μM NMDA and 50μM DHKA (**a**) was not affected by bath application of 20μM bicuculline (**b**). 50μM bicuculline caused bursts of activity around every 4s (**c**).


a.
Stage 38
Average episode length (s)
30
25
20
15
10
5
0
control
30μM
40μM
50μM
10 mins
20 mins
bicuculline
Wash

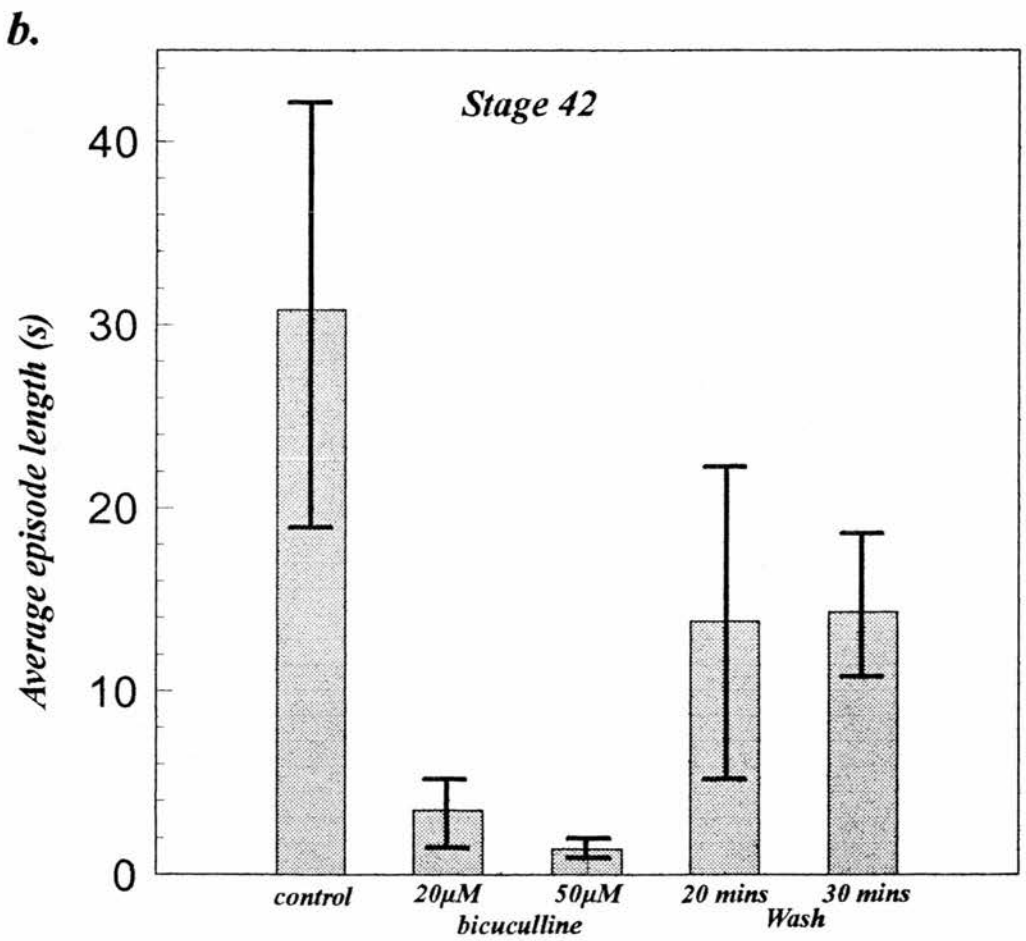

b.
Stage 42
Average episode length (s)
40
30
20
10
0
control
20μM
50μM
20 mins
30 mins
bicuculline
Wash

Figure 4.5. The effects of bicuculline on embryonic and larval swim episodes.

a. Only the highest concentration of bicuculline caused a significant decrease in the episode length of embryonic swim episodes. The average episode in control saline was 20.0 ± 6.8s, 16.8 ± 7.0s in the presence of 30μM bicuculline and 14.8 ± 7.0s ten minutes after increasing to 40μM bicuculline. At 50μM bicuculline, the episodes were significantly decreased, in compared to control, to 10.1 ± 2.3s ($p = 0.0028$). Episode length was increased following return to control saline to 18.2 ± 2.9s after 10 minutes and 18.1 ± 5.0s after 20 minutes.

b. Larval swim episodes averaged 30.8 ± 11.4s in control saline. 20μM bicuculline significantly decreased the length of swim episodes to 3.5 ± 1.6s ($p = 0.006$). Increasing to 50μM bicuculline further decreased the episodes to 1.4 ± 0.4s. After returning to control saline, episodes increased to 13.8 ± 8.3s after 20 minutes and 14.3 ± 4.0s after 30 minutes.

stages in development, episode durations was reversibly decreased. Graph a, shows an example of the effect of increasing concentrations of bicuculline over time on embryonic swim episodes. Before the bath application of bicuculline, the average episode was 20.0 ± 6.8s. After 10 minutes in 30μM bicuculline, the average episode decreased to 16.7 ± 6.9s and 10 minutes after increasing to 40μM bicuculline the average was 14.8 ± 3.2s. A students t-test however showed that at neither concentration was the average episode length significantly different to control ($p = 0.47$ and 0.18 respectively). Ten minutes after increasing to 50μM bicuculline, the episodes were significantly decreased, in comparison to control, to an average of 10.1 ± 2.3s ($p = 0.003$). Returning to control saline reversed the effects of bicuculline on episode length to 18.2 ± 2.9s and 18.1 ± 5.0s after 10 and 20 minutes in wash, respectively. In comparison, graph b shows the effects of bicuculline on larval swim episodes. Even at concentrations of bicuculline as low as 20μM, there was a significant decrease in the episode length measured 10 minutes after bath applying the antagonist. The average episode decreased from 30.8 ± 11.4s in control to 3.5 ± 1.6s ($p = 0.006$). Ten minutes after increasing to 50μM bicuculine there was a further decrease in the episodes which now averaged 1.4 ± 0.4s. Although returning to control saline did not fully reverse the effects of bicuculline back to control levels, there was a marked increase in swim episodes to an average of 13.8 ± 8.3s after 20 minutes and 14.3 ± 4.0s after a further ten minutes in control saline.

In order to determine whether these significant changes in episode length were paralleled by changes in the basic parameters of larval swimming, thereby indicating a role for endogenous GABA release in directly modulating the central pattern generator for larval swimming, burst durations, cycle periods and rostro-caudal delays were measured during

larval swimming activity in control conditions and after the addition of bicuculline. In contrast to the embryo (chapter 3, figure 3.21), bicuculline caused significant changes to all the parameters of swimming activity. Both rostral and caudal burst durations were typically increased while cycle periods and rostro-caudal delays were decreased. In the example shown in figure 4.6, rostral burst duration increased from 14.7 ± 3.5ms to 17.0 ± 4.0ms, caudal burst duration also increased from 12.8 ± 2.7ms to 15.6 ± 3.6ms, cycle period decreased from 52.8 ± 5.4ms to 45.6 ± 9.6ms and rostro-caudal delay decreased from 7.9 ± 1.6ms to 4.9 ± 2.4ms. A students t-test confirmed that all of these results are highly significant ($p < 0.0005$).

The above results suggest that endogenous GABA release does modulate larval swimming activity by acting directly on the central pattern generator to finely tune the pattern of activity. Consequently, the effects of GABA agonists would be expected to have the opposite effect on larval activity. Increasing GABA receptor activation, either by preventing the reuptake of GABA with nipecotic acid or by potentiating the actions of GABA at the GABAa receptor with 5β3α (see chapter 3) had consistent and significant effects on ventral root activity. Figures 4.7 and 4.8 show the effect of these agents on rostral and caudal burst duration. In contrast to the actions of bicuculline, both 200μM nipecotic acid (fig 4.7, n=4) and 3μM 5β3α (fig 4.8, n=2) significantly decreased average burst durations (students t-test, $p<0.0001$). The effects of 3μM 5β3α could also be reversed by applying 50μM bicuculline which significantly increased burst durations even above those recorded in control saline.

The main conclusion of these experiments is that by stage 42, an intrinsic central GABAergic system appears to significantly influence the larval pattern generator for swimming. Furthermore, the effects of bicuculline which blocks any endogenous activation of GABAa receptors

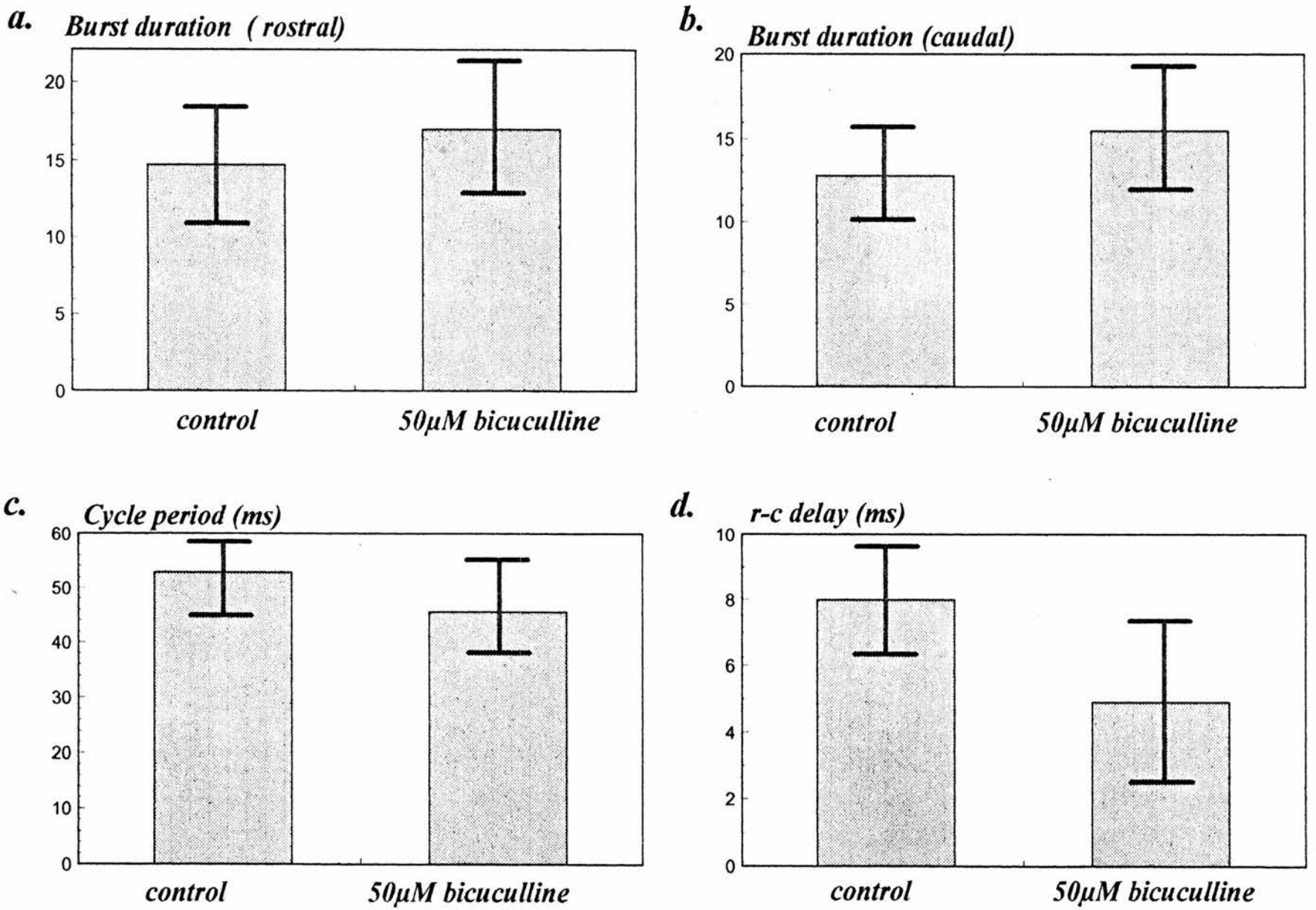

a.
Burst duration (rostral)
20
15
10
5
0
control
50μM bicuculline
b.
Burst duration (caudal)
20
15
10
5
0
control
50μM bicuculline
c.
Cycle period (ms)
60
50
40
30
20
10
0
control
50μM bicuculline
d.
r-c delay (ms)
10
8
6
4
2
0
control
50μM bicuculline

Figure 4.6. The effects of bicuculline on the basic parameters of larval swimming.

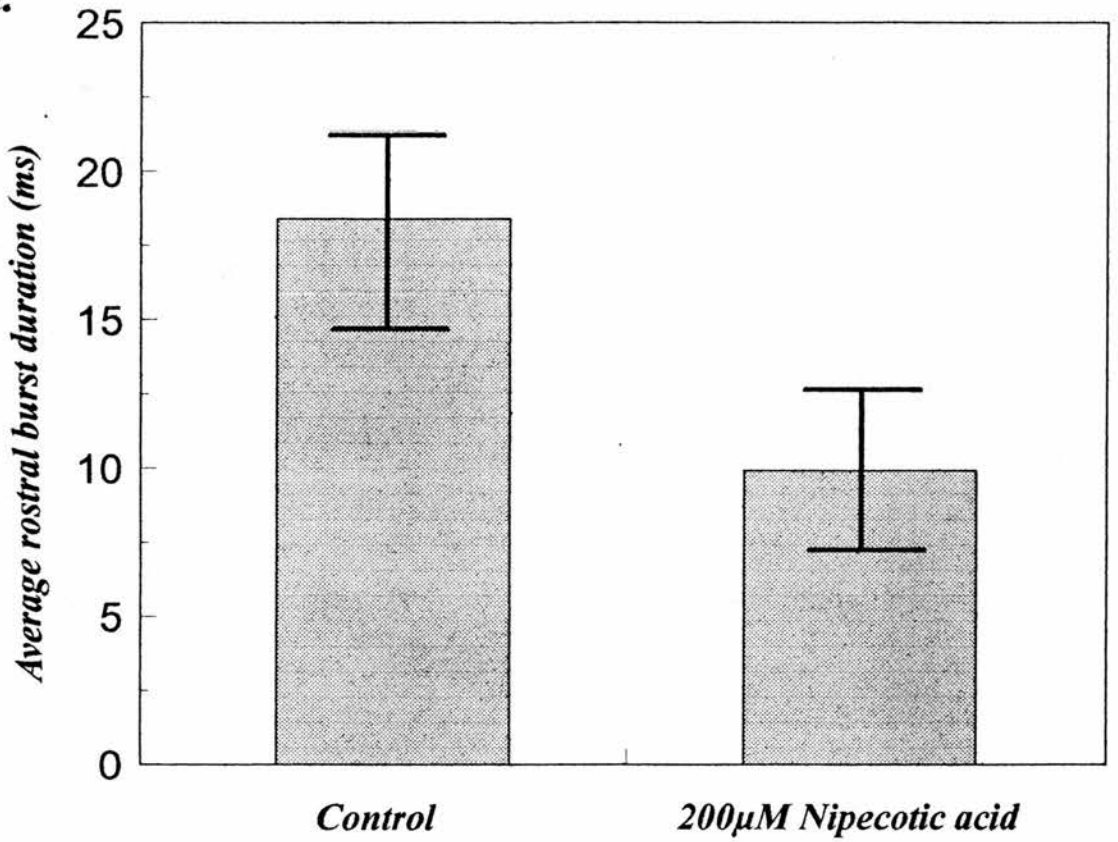
a. Rostral burst durations were significantly increased in the presence of bicuculline. The average burst duration in control saline was 14.7 ± 3.5ms which but increased to 17.0 ± 4.0ms in the presence of 50μM bicuculline (t-test, $p = 0.0003$).

b. Caudal burst duration was similarly increased from 12.8 ± 2.7ms before applying bicuculline, to 15.6 ± 3.6, ten minutes after bath applying 50μM bicuculline ($p < 0.0001$).

c. Under control conditions, the average cycle period was 52.8 ± 5.4ms. 50μM bicuculline significantly decreased the cycle period to an average of 45.6 ± 9.6 ($p < 0.0001$).

d. Rostrocaudal delay decreased from 8.0 ± 1.6ms in control to 4.9 ± 2.4ms in the presence of 50μM bicuculline ($p < 0.0001$).

a.


25
20
15
10
5
0
Average rostral burst duration (ms)
Control
200μM Nipecotic acid

b.

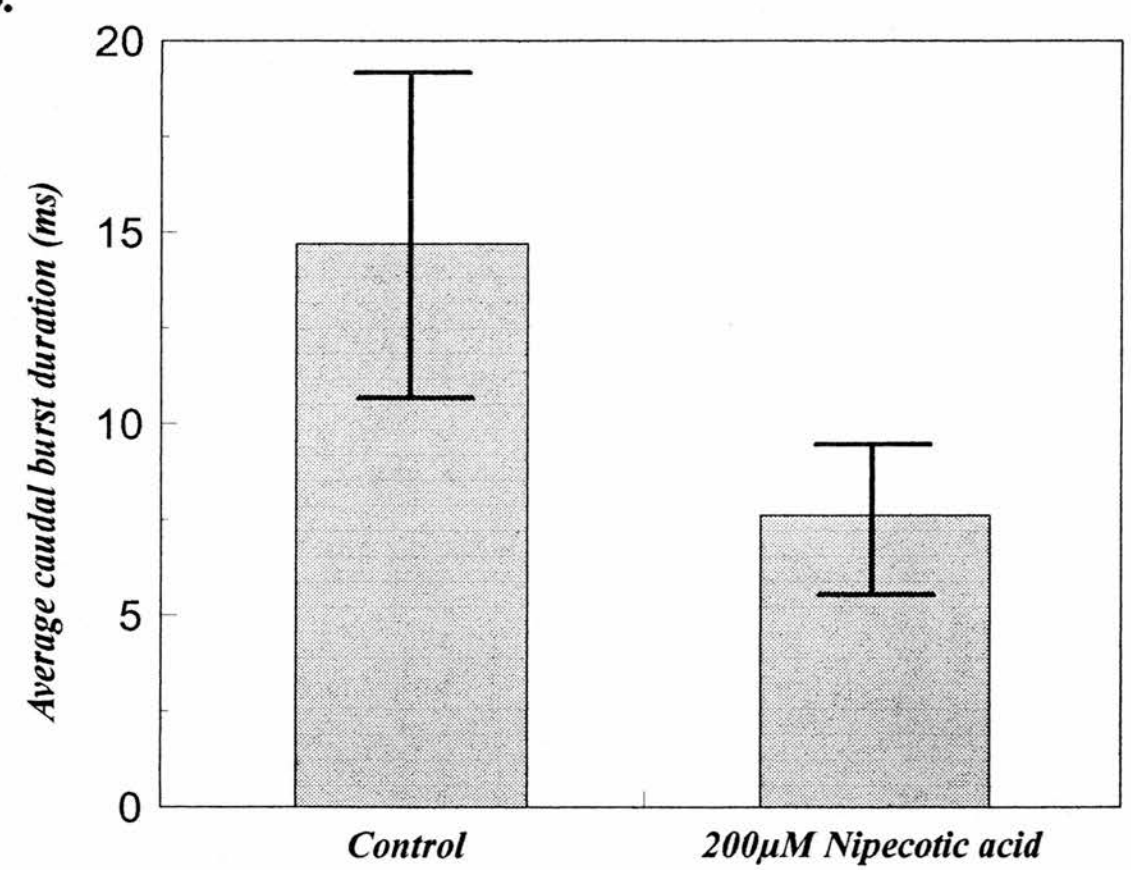
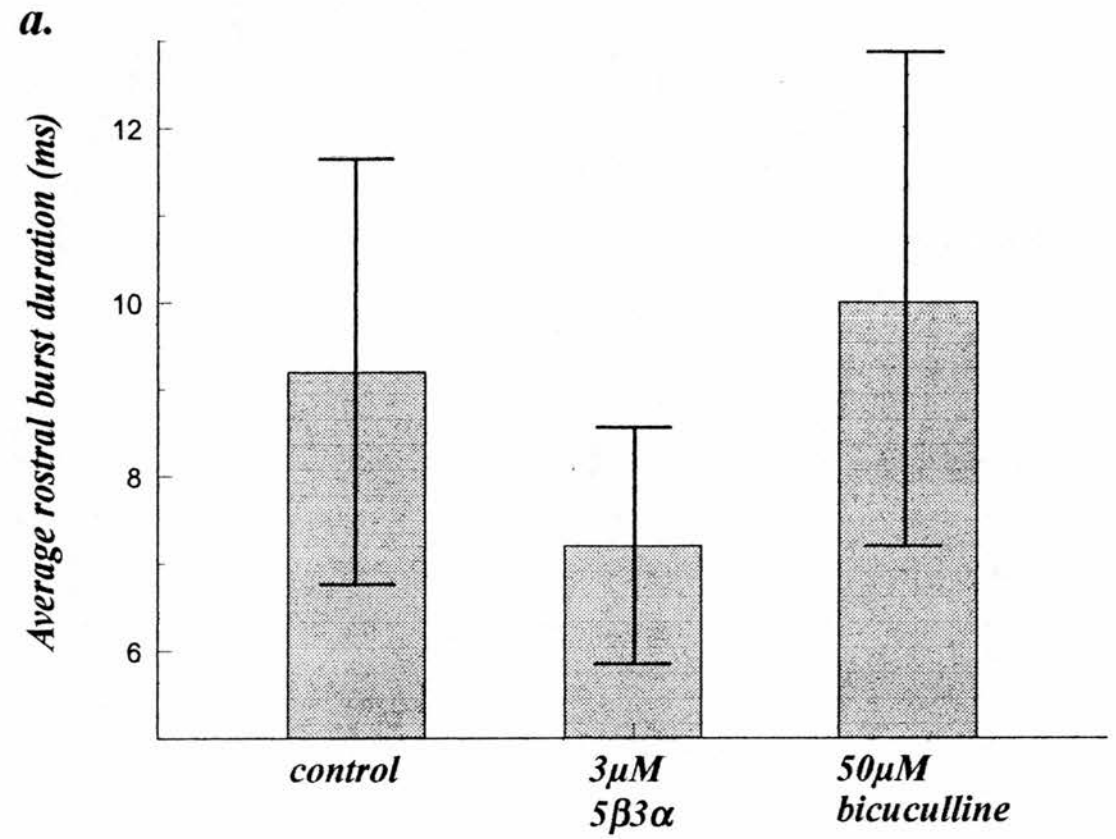

20
15
10
5
0
Average caudal burst duration (ms)
Control
200μM Nipecotic acid

Figure 4.7. The effects of nipecotic acid on larval burst durations.

The GABA uptake blocker, nipecotic acid, caused a significant decrease in burst durations.

a. rostral burst duration decreased from 18.4 ± 3.8ms to 9.9 ± 1.6ms, ten minutes after bath applying 200μM nipecotic acid ($p < 0.0001$).

b. caudal burst duration decreased from 14.7 ± 3.8ms in control saline to 7.6 ± 1.4ms in the presence of 200μM nipecotic acid ($p < 0.0001$).


a.
Average rostral burst duration (ms)
12
10
8
6
control
3μM
5β3α
50μM
bicuculline

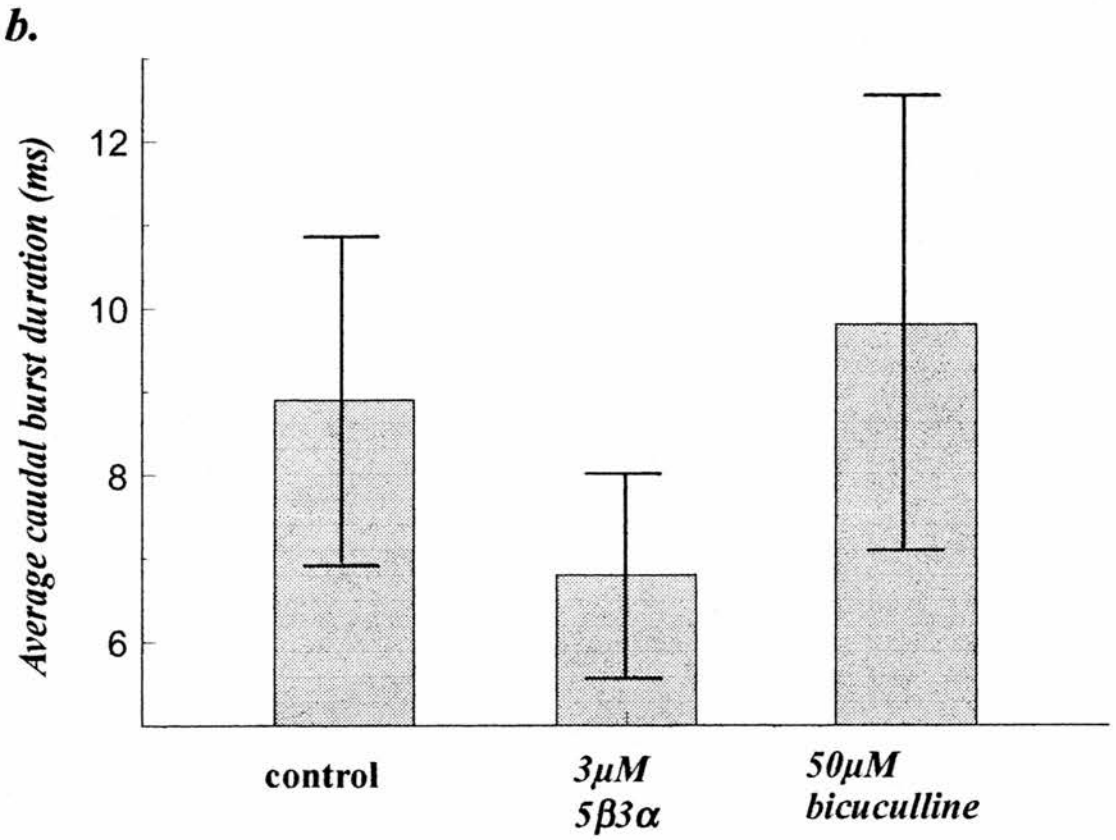

b.
Average caudal burst duration (ms)
12
10
8
6
control
3μM
5β3α
50μM
bicuculline

Figure 4.8. The effects of 5β3α on larval burst durations.

The GABAa agonist 5β3α reversibly decreased burst duration.
a. Rostral burst duration decreased significantly from 9.2 ± 2.4ms to 7.2 ± 1.3ms in the presence of 3μM 5β3α ($p < 0.0001$). 50μM bicuculline increased burst durations back to 10.0 ± 2.8ms.
b. Similarly caudal burst durations averaged 8.9 ± 1.9ms in control, 6.8 ± 1.2ms in the presence of 3μM 5β3α ($p < 0.0001$) and 9.8 ± 2.7ms after bath applying 50μM bicuculline.

strongly suggests that the system is active **during** episodes of fictive swimming activity.

3. A role for mhr neurons in modulating larval fictive swimming?

Imunocytochemical studies have revealed no new populations of GABAi neurons in the larval CNS (not illustrated), suggesting that the endogenous release of GABA during larval swimming must be from one of the existing populations described in the embryo CNS (Dale et al., 1987, Roberts et al., 1987). As was described in chapter 1, the cement gland stopping response is most reliable between stage 34 to 37 (Boothby and Roberts, 1992a) but then declines until it is completely lost by stage 45. The cement gland structure itself degenerates over the same time period and by stage 43 it has almost completely disintigrated. With the disappearance of the behavioural response, what is the destiny of the neurons, including the GABAergic mhr neurons which comprised the network mediating the response? If the neurons were no longer required they may degenerate. However, immunocytochemical studies on larval CNS's revealed a population of GABA-immunoreactive neurons in the same position within the midhindbrain as the mhr neurons described previously in the embryo. Figure 4.9 is a camera lucida drawing of the GABAergic neurons located just caudal to the otic capsule extending over approximately 200-300μm (cf fig. 3.2). The mhr neurons thus appear to still be present in the larval cns but due to insufficient data no direct comparison could be made between the numbers of neurons in the mhr population at embryonic and larval stages.

Alternatively, if the mhr neurons are not lost, they could become incorporated into a different pathway involved in modulating larval activity at later stages in development. To explore this possibility further

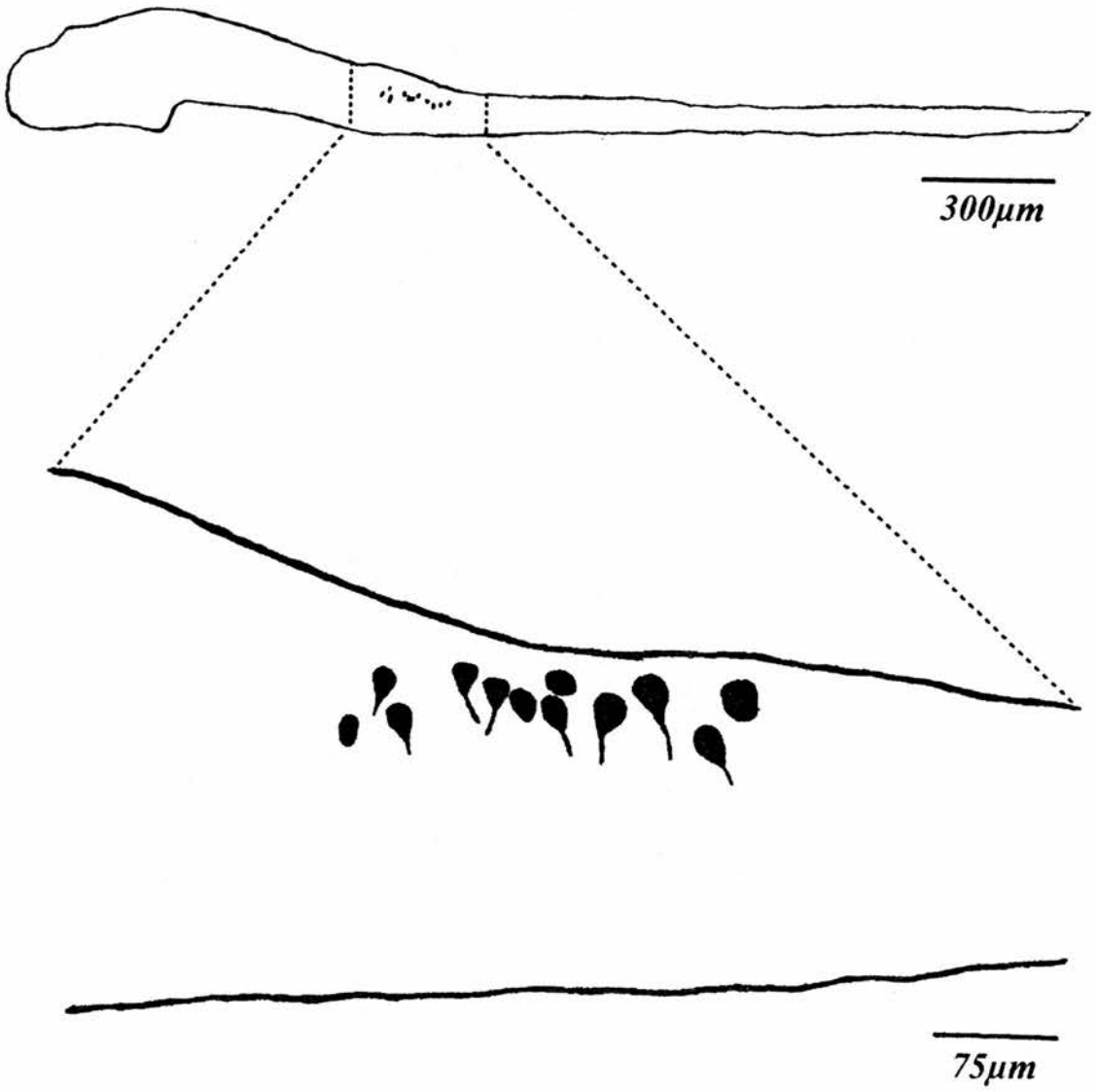

300µm
75µm

Figure 4.9. *Xenopus* larva (stage 42): the mhr neurons.

Camera lucida drawing of the stage 42 CNS and an expanded drawing of the area indicated showing the population of neurons in the midhindbrain which were revealed using HRP secondary antibodies visualised by DAB. These neurons were presumed to be the mhr neurons already described in the embryo (Roberts et al., 1987).

spinalisation experiments were carried out, at stage 42, similar to those described for the embryo in chapter 3 (figure 3.8) and the effects of blocking GABAa receptors investigated. The larvae were spinalised either at the otic capsule, to remove any influence from all but the mhr and spinal populations of GABAergic neurons, or at the level of the fourth postotic myotome to leave only the spinal cord circuitry intact. At the lowest level of spinalisation (figure 4.10, n=4), a continuous pattern of fictive swimming activity was recorded approximately five minutes after the co-application of 50μM DHKA and 70μM NMDA to the circulating saline (a). This pattern of activity was not noticeably affected by blockade of GABAa receptors with bicuculline even after prolonged exposure to high concentrations (50μM, b), left for up to twenty minutes (c). This result suggests that at stage 42 there is still no significant input to swimming from spinal populations of GABAergic neurons. However, in order to check whether any spinal GABAergic effects were being masked by spinal glycinergic inhibition, the effects of blocking both glycine and GABAa receptors with strychnine and bicuculline respectively, were investigated (n=3). The results of these experiments are shown in figure 4.11. Following the addition of 5μM strychnine, the continuous activity (a) was changed into bursts of rhythmic activity with a period of around 3s (b), similar to the effect observed in the presence of bicuculline in an intact larva (figure 4.3). The application of 50μM bicuculline in the presence of 5μM strychnine, further modified the pattern of ventral root activity (c). Firstly, the period between bursts of rhythmic activity was slightly increased (approximately 4s) and secondly, at regular intervals, the burst of activity became non-rhythmic (indicated by the arrow) following which there was an increased delay before the next burst. These changes in the pattern of activity were confirmed as the direct result of blockade of GABAa receptors, rather than

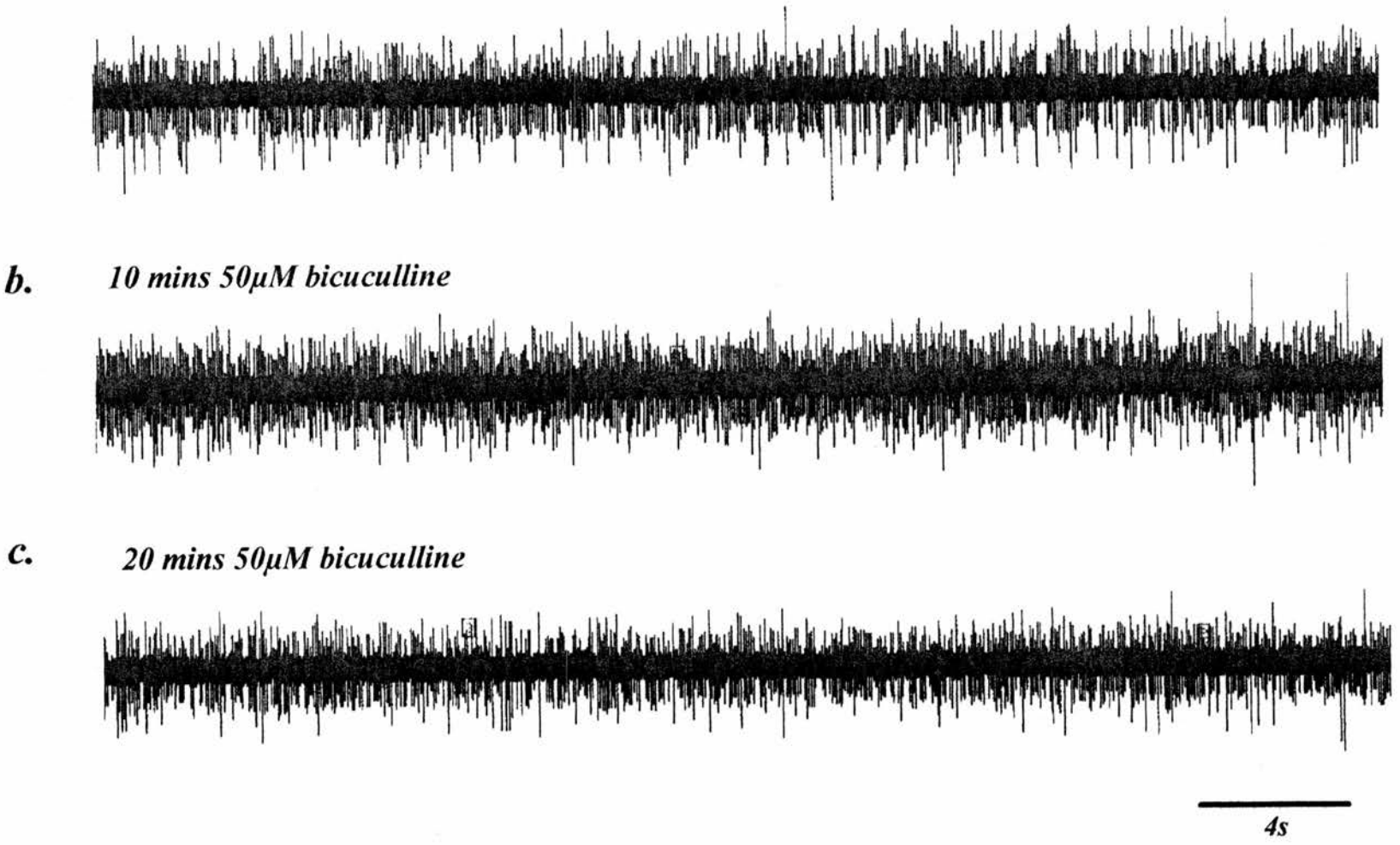

a.
70μM NMDA + 50μM DHKA
b.
10 mins 50μM bicuculline
c.
20 mins 50μM bicuculline
4s

Figure 4.10. The effects of bicuculline on a preparation spinalised at P.O.M. 4.

a. Continuous ventral root activity recorded from a larva spinalised at the 4th postotic myotome in the presence of 70μM NMDA and 50μM DHKA. **b**, **c**. 50μM bicuculline had no noticeable effect on the pattern of activity even after 20 minutes.

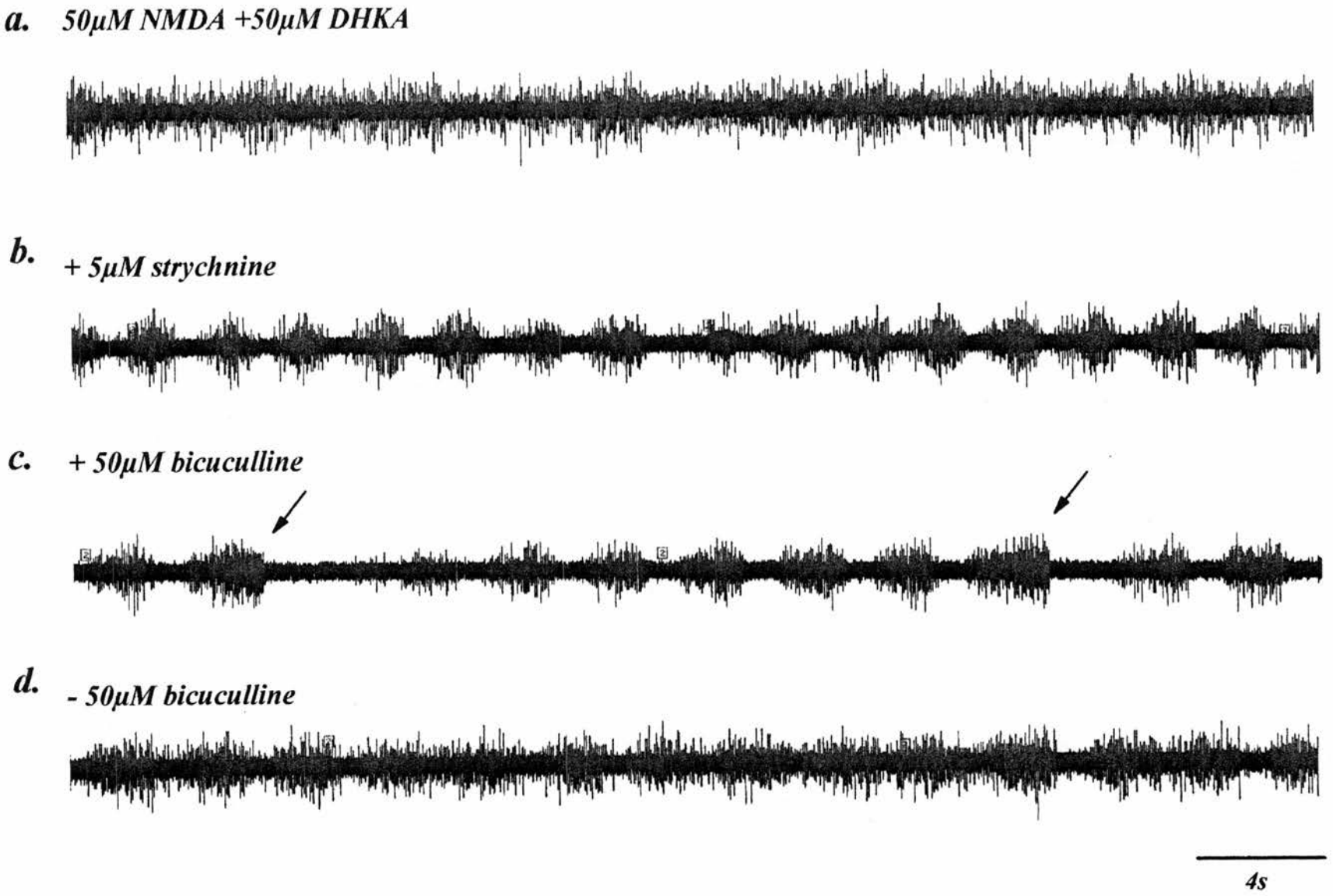

a. 50μM NMDA +50μM DHKA
b. + 5μM strychnine
c. + 50μM bicuculline
d. - 50μM bicuculline
4s

Figure 4.11. The effects of strychnine and bicuculline on a preparation spinalised at P.O.M. 4.

The continuous pattern of swimming activity induced by 50μM NMDA and 50μM DHKA (**a**) was regularly interrupted in the presence of 5μM strychnine which produced bursts of rhythmic ventral root activity approximately every second (**b**). Addition of 50μM bicuculline further modified the pattern of ventral root activity with rhythmic bursts still occurring around every second but around every seventh burst was intense and non-rhythmic after which there was a delay before the start of the next burst (**c**). The effects of bicuculline were reversed by washing (**d**).

any time-dependent change in the pattern of strychnine-induced activity, since they were reversed following the removal of bicuculline from the circulating saline (d). Thus co-application of the two inhibitory amino acid receptor antagonists suggest that there is an input from at least one of the two populations of GABAergic neurons in the spinal cord during NMDA-induced activity. However, whether these neurons are active during episodes of swimming in the absence of NMDA and DHKA is as yet unclear. Following spinalisation at the level of the otic capsule, essentially the same pattern of activity was observed to that in the intact animal (figure 4.12, n=5). Both 20 and 50μM bicuculline caused bursts of rhythmic activity in the presence of 50μM DHKA and 70μM NMDA. These results suggest that it may be the mhr neurons which play an important role during larval swimming.

In order to confirm that GABAergic transmission is involved in the modulation of fictive swimming in high spinal animals in the absence of exogenous NMDA, the effects of bicuculline on the parameters of stimulus evoked fictive swimming were analysed to determine whether blocking GABAa receptor activation still had significant effects on the ventral root activity similar to those of the intact preparation. Figure 4.13, shows the effect of bicuculline on the length of swim episodes in a high spinal preparation. It can be seen that bicuculline still causes a significant decrease in the length of episodes (n=4). In the illustrated example the episodes decreased from 14.3 ± 1.5s in control, to 4.4 ± 0.9s in 20μM bicuculline (p=0.002) and 3.7 ± 0.8s in the presence of 40μM bicuculline (p=0.008). Returning to control saline increased the episodes to 28.2 ± 5.8s reversing the effect of the antagonist. The following histograms (figure 4.14) demonstrate that increasing bicuculline concentrations reversibly and significantly increased both rostral (4.14a) and caudal (4.14b) burst

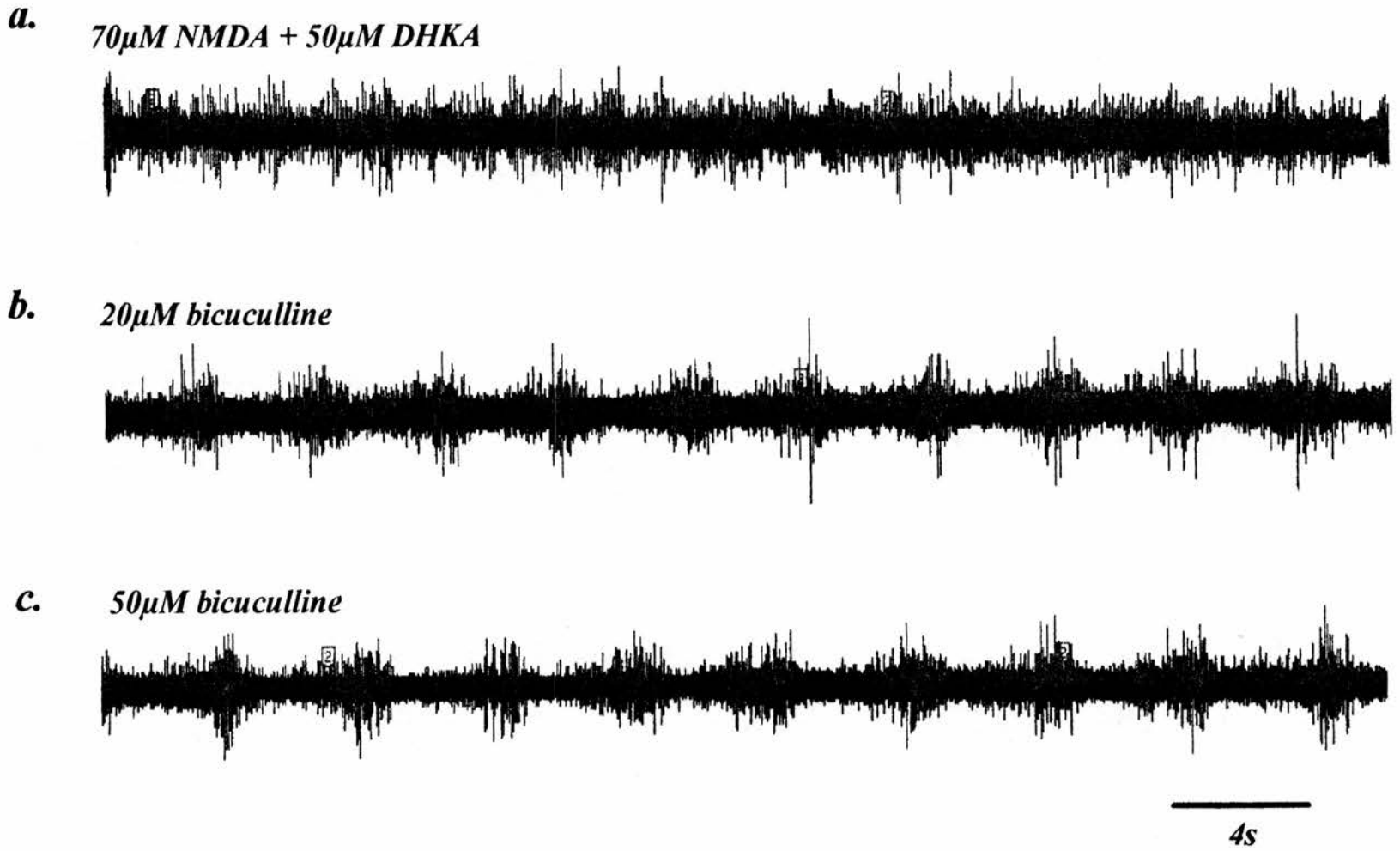

a.
70μM NMDA + 50μM DHKA
b.
20μM bicuculline
c.
50μM bicuculline
4s

Figure 4.12. The effects of bicuculline on a preparation spinalised at the otic capsule.

Bicuculline caused the continuous pattern of activity recorded in the presence of 70μM NMDA and 50μM DHKA (**a**) to become discontinuous. This effect occurred at 20μM bicuculline (**b**) and was not markedly changed by increasing to 50μM bicuculline (**c**).

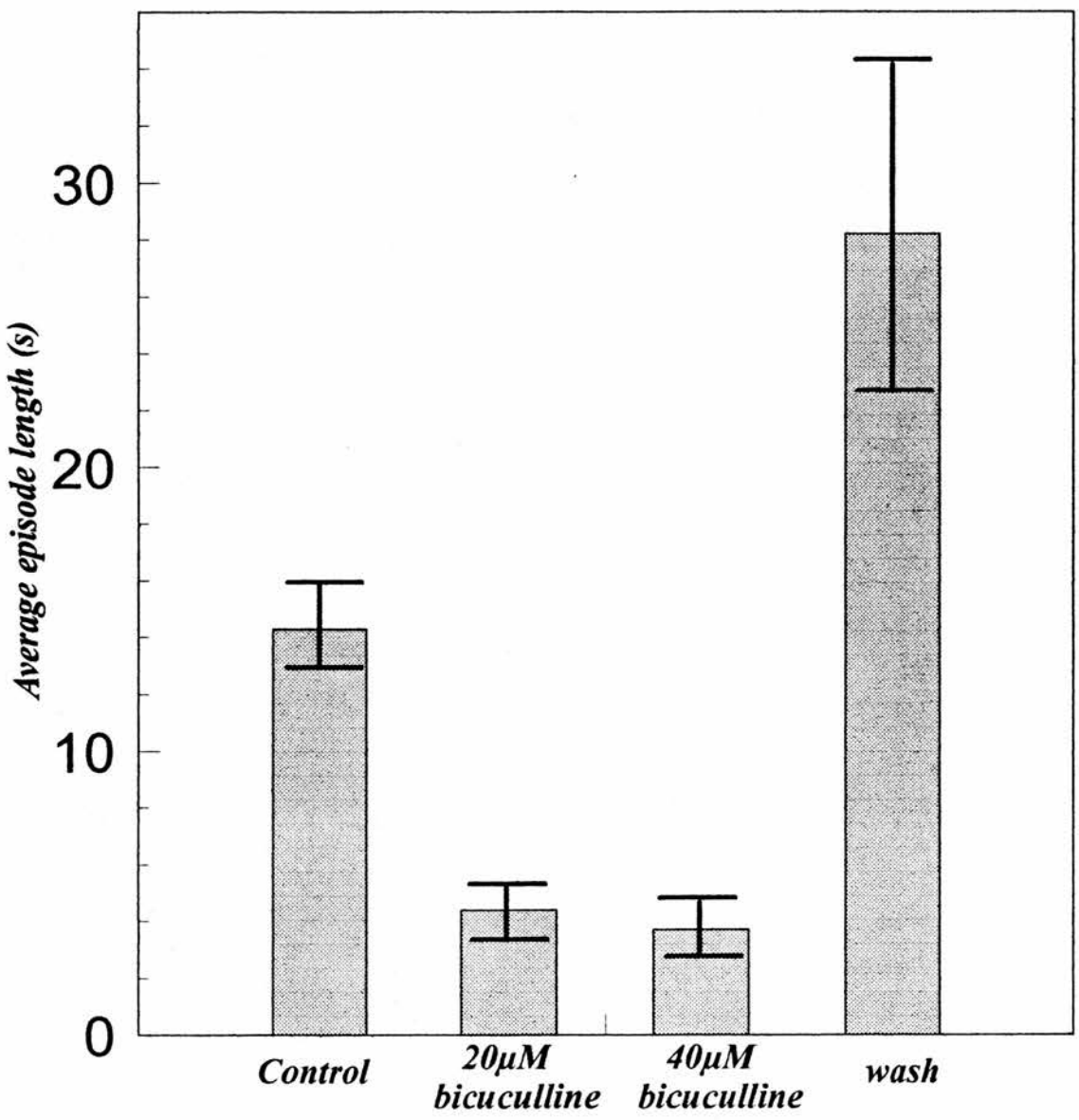
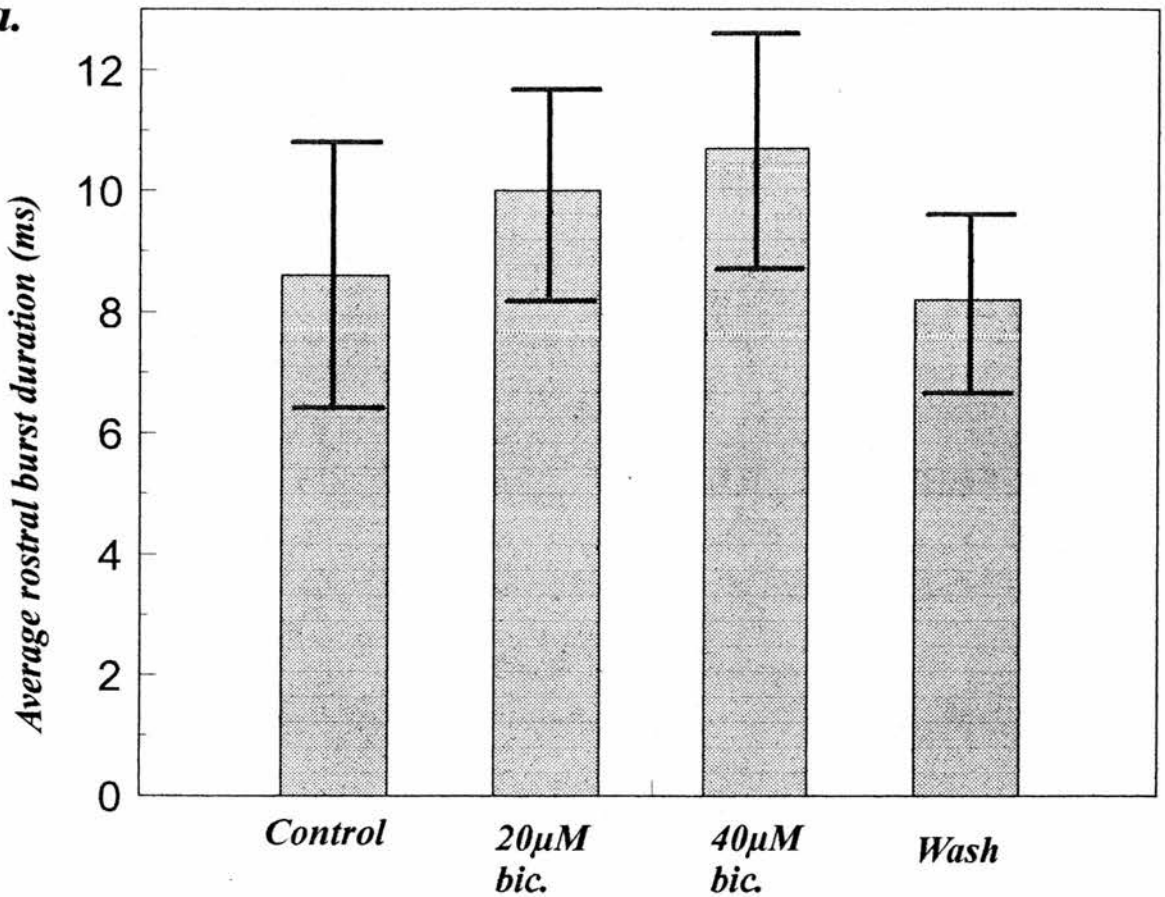

Average episode length (s)
0
10
20
30
Control
20μM bicuculline
40μM bicuculline
wash

Figure 4.13. Bicuculline reduces the length of swim episodes of a larval animal spinalised at the otic capsule.

Bicuculline significantly decreased the length of swim episodes of an animal spinalised at the otic capsule. The average episode in control saline was 14.3 ± 1.5s. Ten minutes after bath applying 20μM bicuculline, episodes decreased to 4.5 ± 0.8s ($p < 0.0001$) and after increasing to 40μM bicuculline episodes averaged 3.8 ± 0.7s. Episodes lengthened 20 minutes after washing off the bicuculline, to 28.3 ± 5.8s.

a.


Average rostral burst duration (ms)
0
2
4
6
8
10
12
Control
20μM bic.
40μM bic.
Wash

b.

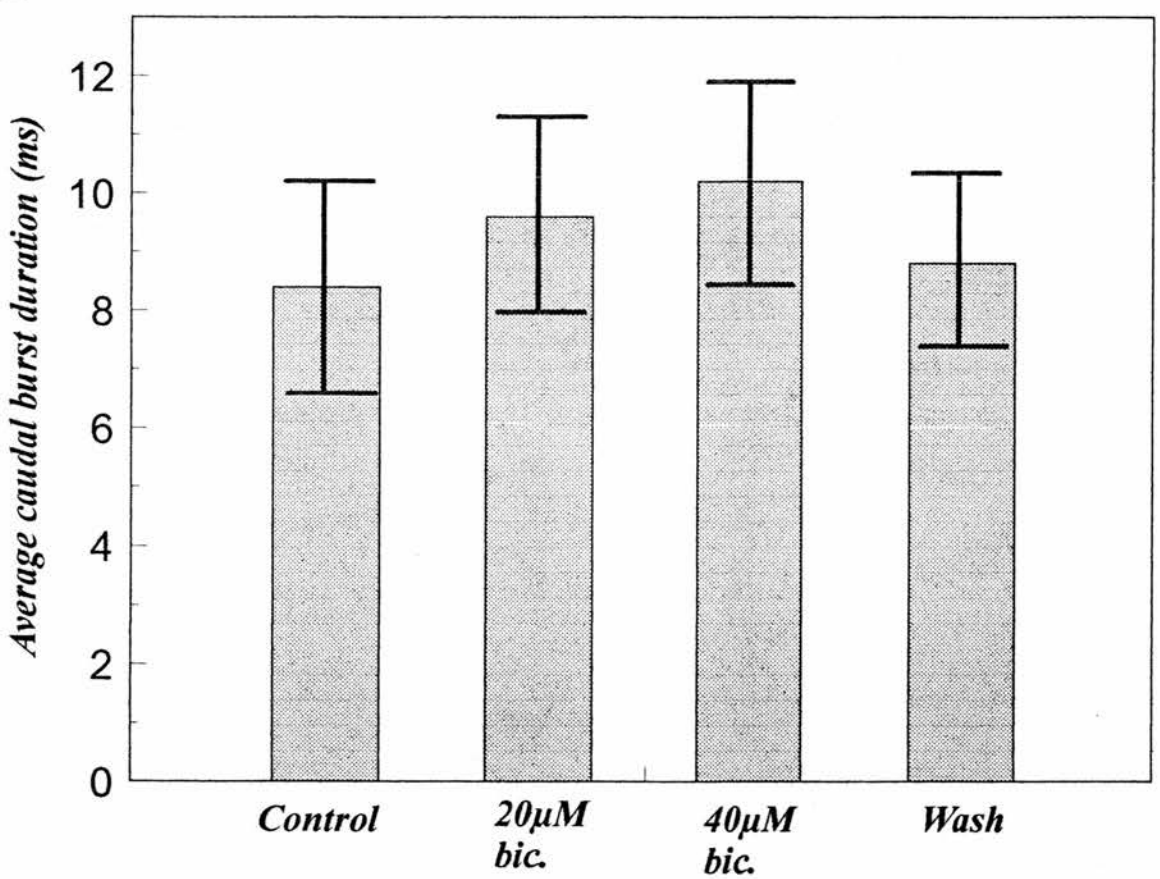

Average caudal burst duration (ms)
0
2
4
6
8
10
12
Control
20μM bic.
40μM bic.
Wash

Figure 4.14. Bicuculline increases burst durations during larval swimming in a high spinal animal.

a. The average rostral burst duration increased from 8.6 ± 2.2ms in control saline to 10.0 ± 1.7ms in the presence of 20μM bicuculline and 10.8 ± 1.8ms in the presence of 40μM bicuculline ($p < 0.0001$). Returning to control saline decreased the episodes back to 8.2 ± 1.4ms.

b. Caudal burst durations increased from an average of 8.5 ± 1.8ms to 9.6 ± 1.6ms in the presence of 20μM bicuculline and 10.2 ± 1.6ms after increasing to 40μM bicuculline. 10 minutes after washing off the bicuculline episodes decreased again to 8.8 ± 1.4ms.

durations (Students T-test; $p > 0.0005$). Average rostral burst durations increased from 8.6 ± 2.2ms, to 10.0 ± 1.7ms in 20μM bicuculline and 10.8 ± 1.8ms after increasing to 50μM bicuculline. After returning to control saline, burst durations were decreased back to 8.2 ± 1.4ms. Similar changes were observed in the caudal ventral root, where the average burst increased from 8.5 ± 1.8ms to 9.6 ± 1.6ms and 10.2 ± 1.6ms in increasing concentrations of bicuculline and were decreased back to 8.8 ± 1.4ms shortly after returning to control saline. Thus, bicuculline appears to have similar effects on the rhythmic activity produced by larvae spinalised at the otic capsule as it does on the activity recorded from intact preparations. These results suggest that the GABAergic neurons involved in modulating the locomotor output at stage 42 are the mhr neurons. Recordings from mhr neurons at stage 37/8 showed that they recieve glycine inhibition and do not fire during embryonic swim episodes (Boothby and Roberts, 1992b). The effects of bicuculline on larval swimming would therefore suggest that the input onto these neurons or their firing properties might change during development so that they are active and can modulate the swimming pattern intrinsically.

Further evidence to support the idea that the mhr neurons become involved in modulating larval fictive swimming came from the observation that episodes of fictive swimming activity often terminate with a barrage of depolarising psps in motorneurons (9 out of 20 preparations, figure 4.15b). This phenomenon was never observed in recordings from embryonic motorneurons, where swimming ends without an obvious synaptic input triggering its termination (figure 4.15a). However, the self-termination of swimming in some larval episodes (figure 4.15b) resembles the termination of embryonic swimming following activation of mhr neurons by cement gland stimulation (cf. chapter 3, figure 3.4a). Furthermore, experimental

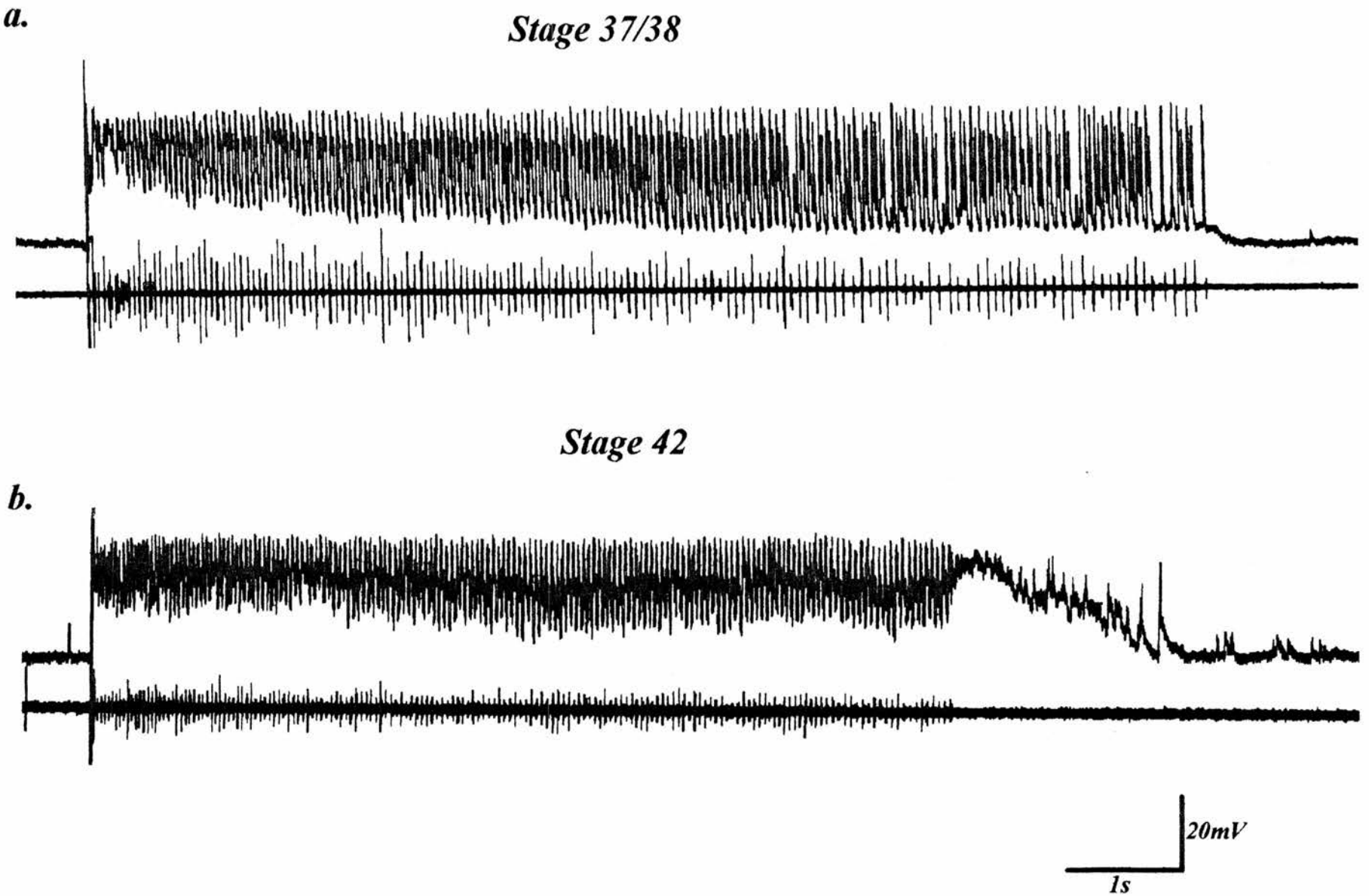

a.
Stage 37/38
Stage 42
b.
20mV
1s

Figure 4.15. Termination of swim episodes in embryonic and larval preparations.

a. Intracellular recording from an embryo motorneuron showing an episode of rhythmic activity which terminates spontaneously with no obvious synaptic input.
b. At stage 42, recordings from some motorneurons showed that swim episodes ended with a barrage of psps.

evidence suggests that the potentials at the end of larval swim episodes were also the result of GABAa receptor activation (figure 4.16). Firstly, they were long in duration and still observed at the end of episodes recorded in the presence of 5μM strychnine (a). Secondly, they were enhanced in the presence of 5μM 5β3α (b) and thirdly they were abolished by bath application of 50μM bicuculline (c). In addition, this figure also shows that GABAa receptor activation causes an increase in the apparent level of tonic depolarisation (since recordings were made with KCl-filled microelectrodes) during larval swim episodes since in the presence of 10μM 5β3α, the level is increased in comparison to control (b) and then markedly decreased in comparison to control after bath applying 10μM bicuculline (c). This observation provides support for the idea that these neurons are active during swim episodes and that GABA potentials could summate sufficiently to terminate some episodes of swimming. Of the nine preparations in which depolarising potentials were seen at the end of an episode, the majority (n=6) exhibited potentials at the end of every episode while the remainder (n=3) only had one or two episodes which ended with a barrage of psps. It was noted that in these recordings there was also a high rate of GABA release in the absence of fictive swimming, possibly suggesting that in these preparations GABAergic neurons made more extensive connections with the spinal motor system.

In combination, the preceding results together with those obtained from spinalisation experiments, indicated that a population of neurons situated in the midhindbrain are responsible for the release of endogenous GABA during fictive swimming. This strongly suggests that during postembryonic development, the mhr neurons become 'wired' into a pathway which is active during larval swim episodes.

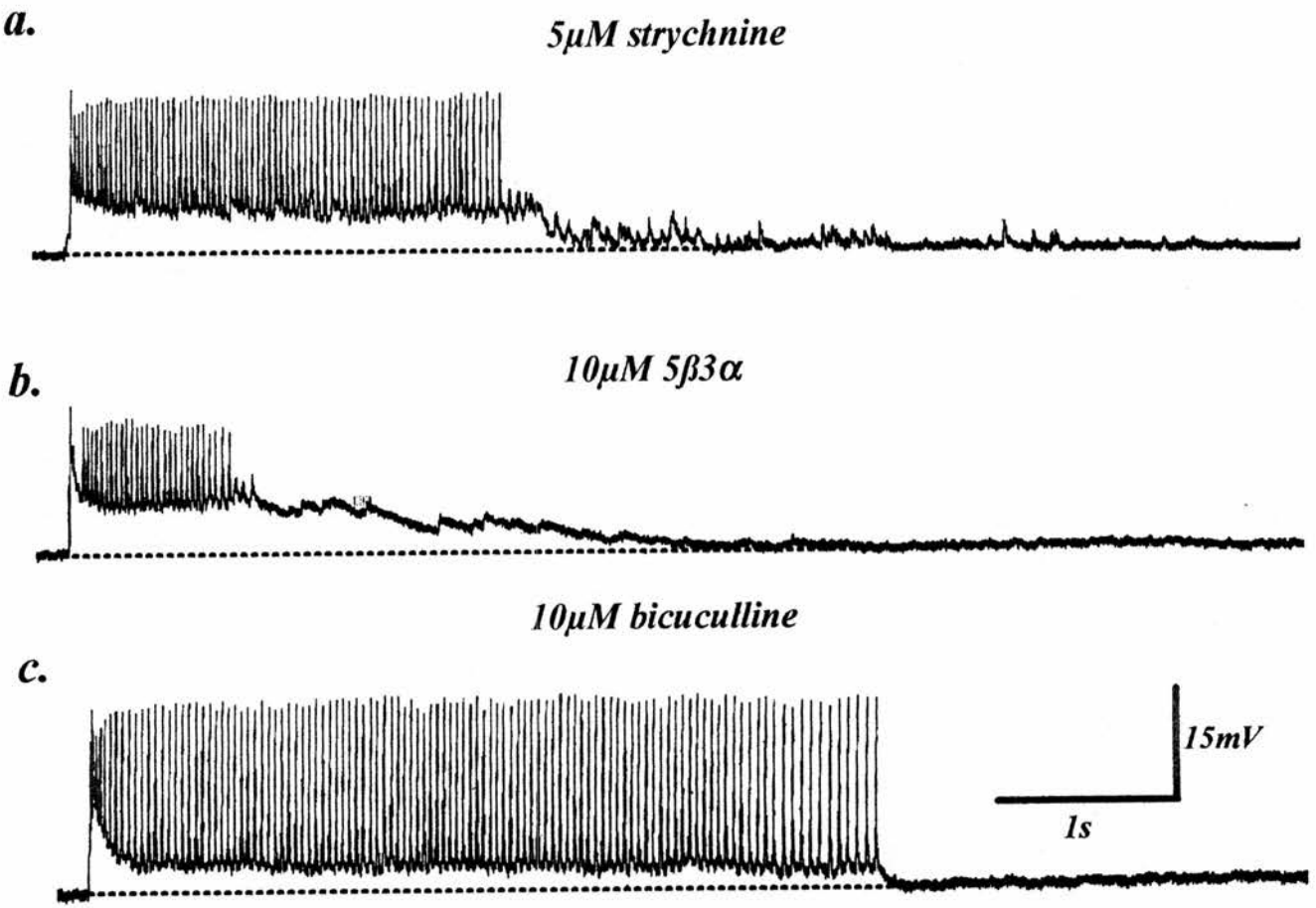

a.
5μM strychnine
b.
10μM 5ß3α
10μM bicuculline
c.
15mV
1s

Figure 4.16. IPSPs at the end of larval swim episodes are GABAergic.

a. Larval swim episode ending with ipsps.
b. 10μM 5β3α enhanced both the apparent tonic depolarisation during rhythmic activity and the ipsps occurring at the termination of swimming, causing them to summate.
c. 10μM bicuculline decreased the apparent tonic depolarisation during rhythmic activity in comparison with control and abolished all the ipsps at the end of the episode.

4. Effects of blocking all inhibition on rhythmic activity.

In order to examine the overall importance of inhibitory transmission on rhythm generation, the effects of strychnine and bicuculline on embryonic and larval rhythmic activity were compared. Figure 4.17 shows an example of the effects of blocking glycine and GABAa receptors on embryonic swimming activity recorded intracellularly from rhythmically active motorneurons and extracellularly on a neighbouring ventral root. Each trace shows rhythmic activity immediately following skin stimulation and then the pattern of activity occurring mid-episode. As expected from previous studies, bath application of strychnine (5μM) abolished all (reversed) midcycle ipsps (figure 4.17b cf.a) but rhythmic activity was sustained. Although the addition of 50μM bicuculline caused a non-rhythmic burst at the start of each episode, suggesting a possible role for GABAa receptor activation during sensory initiation, sustained rhythmic activity still persisted (n=8).

By stage 42, a very different picture emerges. Following the bath application of either 5μM strychnine (figure 4.18bi and ii) or 20-50μM bicucullline (not shown), rhythmic activity is still recorded. Like bicuculline, strychnine causes an increase in burst durations and a decrease in cycle period (bii). However, following the addition of 50μM bicuculline in the presence of strychnine (ci and cii) all rhythmic activity is abolished and only a burst of non-rhythmic activity is recorded in the ventral root. The expanded trace (cii) shows that although the activity is non-rhythmic, there is still a rostrocaudal delay indicating that the animal is not engaging in fictive struggling which would be accompanied by a reversal in the delay to caudal-rostral (Soffe, 1991). Rhythmic activity returned following wash

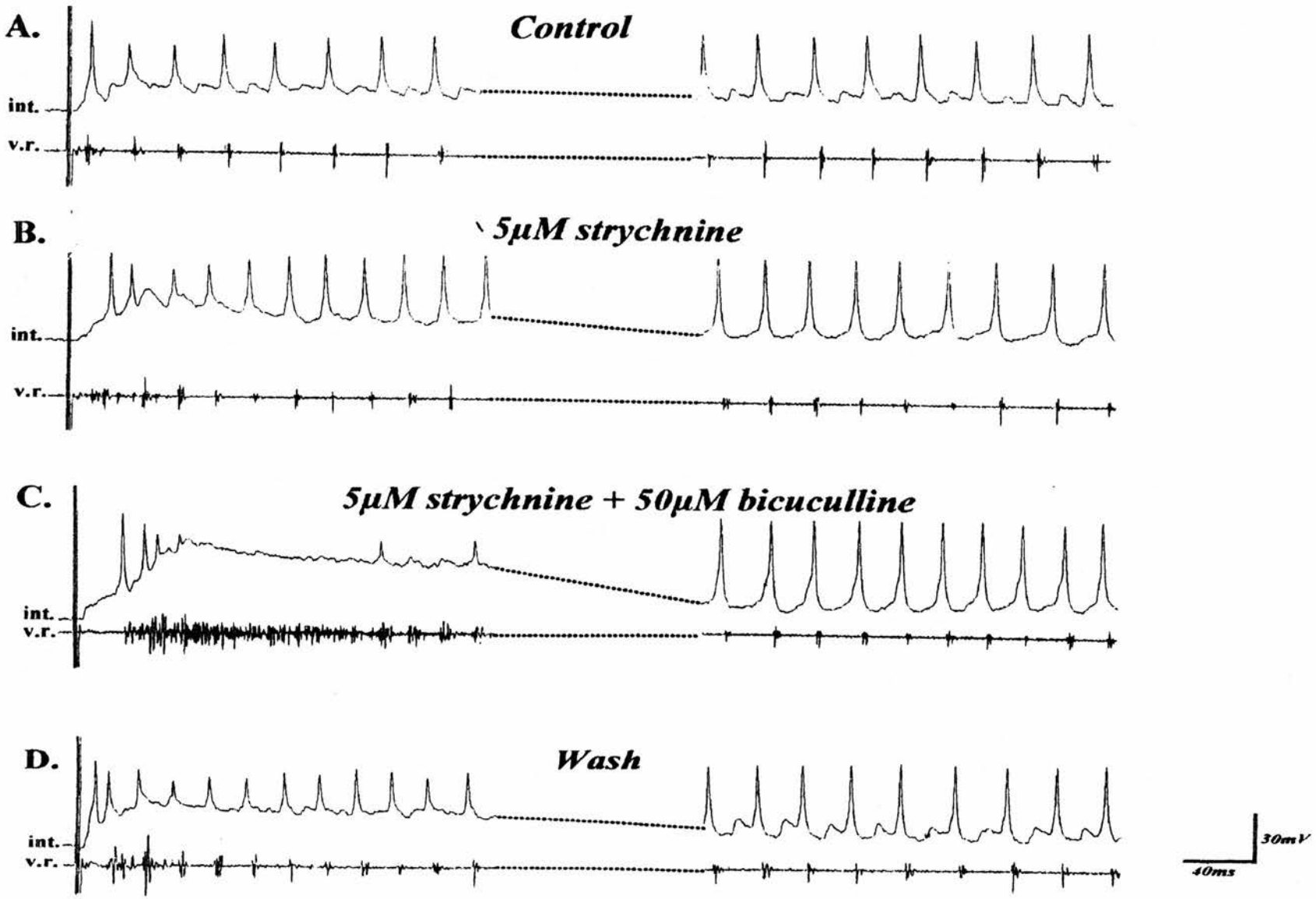

A.
Control
int.
v.r.
B.
5μM strychnine
int.
v.r.
C.
5μM strychnine + 50μM bicuculline
int.
v.r.
D.
Wash
int.
v.r.
30mV
40ms

Figure 4.17. The effects of strychnine and bicuculline on embryonic rhythmic activity.

a. Control embryonic rhythmic activity at the beginning of an episode (left) and sustained activity mid-episode (right).
b. 5μM strychnine abolishes depolarising midcycle ipsps but sustained rhythmic activity continues.
c. Subsequent addition of 50μM bicuculline elicits a non-rhythmic burst of activity following skin stimulation but rhythmic activity is sustained thereafter.
d. Returning to control saline abolishes the sensory effect of bicuculline and the midcycle inhibition, abolished by strychnine, resumes.

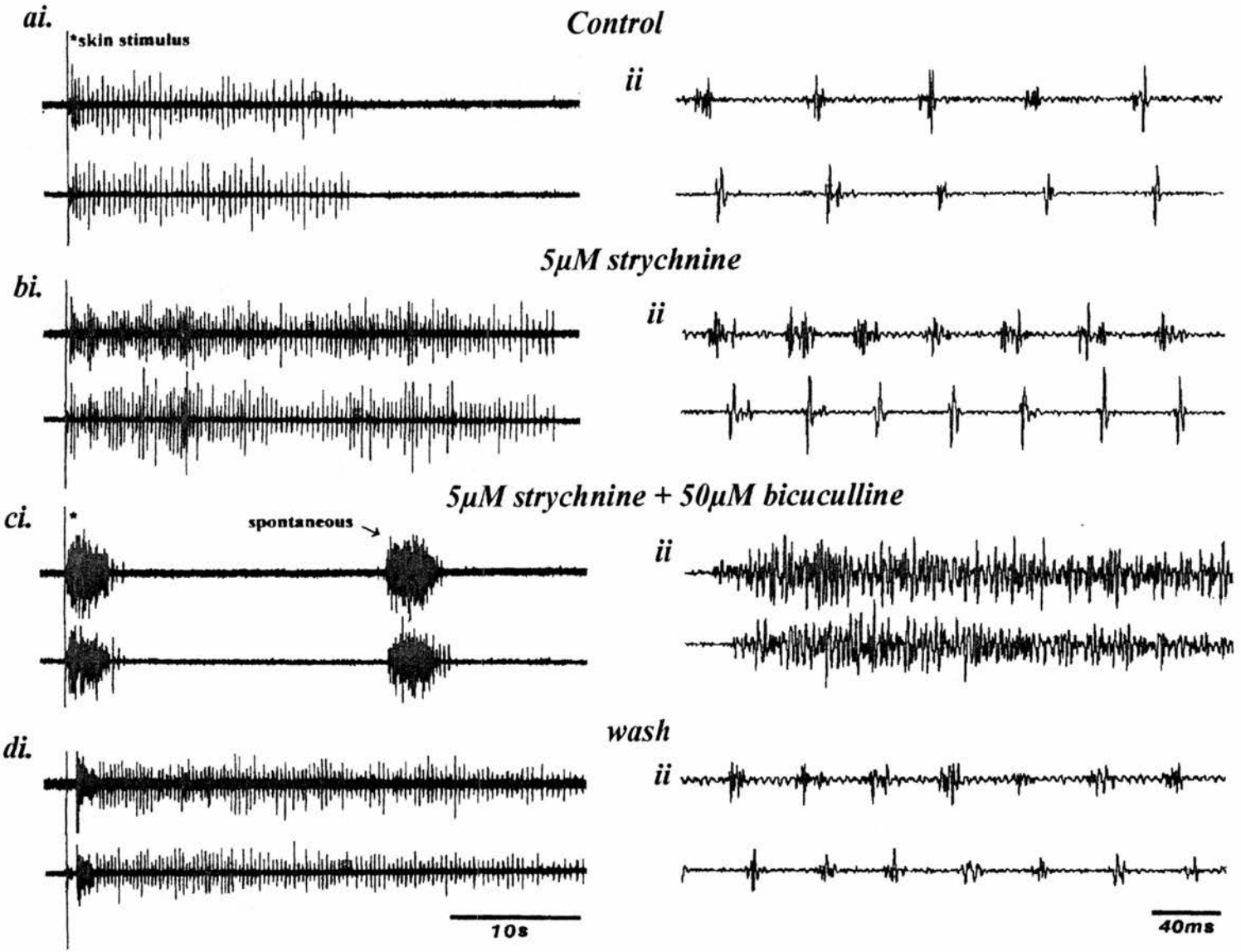

ai.
Control
*skin stimulus
ii
5μM strychnine
bi.
ii
5μM strychnine + 50μM bicuculline
ci.
*
spontaneous
ii
wash
di.
ii
10s
40ms

Figure 4.18. The effects of strychnine and bicuculline on larval ventral root activity.

ai. Control larval swim episode following a skin stimulus. **ii**. Expanded trace showing 350ms of data.
bi. Ventral root activity is sustained in the presence of 5μM strychnine. **ii**. Expanded trace shows that burst durations were increased especially in the rostral root and cycle period is decreased.
ci. Co-application of 50μM bicuculline abolishes rhythmic ventral root activity both in response to a skin stimulus and during spontaneous activity. **ii**. the non-rhythmic activity maintains a rostrocaudal phase delay.
di, ii. Shortly after returning to control saline, rhythmic ventral root activity resumes.

in control saline (di and dii). Equivalent intracellular recordings from larval motorneurons again show rhythmic activity persisting after bath applying strychnine although all midcycle inhibition is apparently abolished (figure 4.19b). This also suggests that there is no GABAergic component to midcycle inhibition. In the presence of both antagonists, the burst of non-rhythmic activity recorded from the ventral root is paralleled in the intracellular recording by the cell being locked in a depolarised state from which it eventually returns back to the resting level (c). Thus, as the animal develops, a certain level of inhibition appears to be essential for the generation of rhythmic activity. Blocking either glycine or GABAa receptors alone, as in the embryo, did not abolish rhythmic activity. However, in contrast to the embryo, blocking the activation of both types of receptor together prevented swimming activity.

5. Sensory role for GABA.

So far, this results chapter has described roles for GABA transmission in modulating and terminating fictive swimming activity. This final section describes an additional role for GABA during sensory transmission. The initiation of swimming activity can be achieved either by stimulating the trunk or tail skin to activate the Rohon-Beard pathway (Clarke et al., 1984), or by dimming the illumination which involves a pathway from the pineal eye (Roberts, 1978). During development the dimming response begins to decline. At stage 38 virtually all animals respond to a sudden dimming of the illumination but from stage 39 through to 44 the reliability of the response diminishes and fewer preparations respond to sudden changes in light levels (Foster and Roberts, 1982).

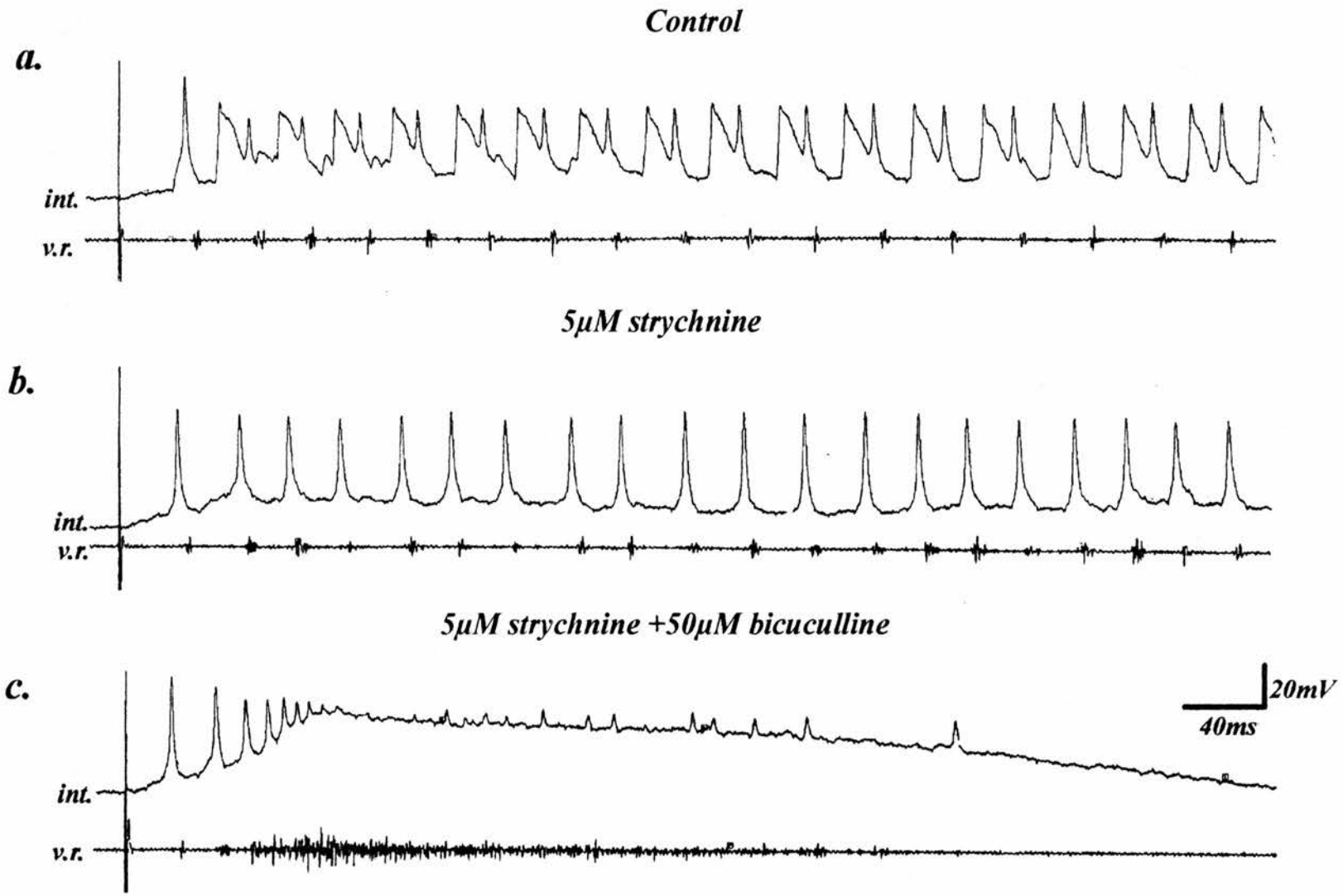

Control
a.
int.
v.r.
5μM strychnine
b.
int.
v.r.
5μM strychnine +50μM bicuculline
c.
20mV
40ms
int.
v.r.

Figure 4.19. Strychnine and bicuculline abolish larval rhythmic activity.

Intracellular recording showing the effects of blocking glycine and GABAa receptors.

a. Recording made from a larval motorneuron with 2M KCl-filled microelectrode which reverses the midcycle inhibition.

b. The midcycle inhibition is abolished in the presence of 5μM strychnine.

c. During non-rhythmic ventral root activity, the membrane potential became locked in a depolarised state from which it slowly returned to resting levels.

Several lines of evidence presented below suggest that GABAa receptor activation prevents this dimming response in some larval preparations. Firstly in larval preparations which do not display a dimming response under control conditions (ie. swimming is not initiated after dimming the lights), recording from motorneurons with KCl-filled microelectrodes showed that a sudden dimming of the illumination evoked a barrage of summating potentials. This is shown in figure 4.20A., in which the depolarising potentials were observed both in response to switching the lights off and then on again. Since the potentials were long in duration and resembled GABA ipsps (see chapter 3), the effects of bicuculline on these potentials were investigated. Figure 4.20B, again shows another larval preparation which did not swim in response to the lights suddenly being dimmed under control conditions and once more, depolarising potentials were observed in the motorneuron. However, after the bath application of 20μM bicuculline to block GABAa receptor activation, rhythmic activity was now recorded from the same motorneuron in response to a change in the light levels (n=4). Conversely, additional evidence implicating a role for GABA in preventing the dimming response, came from the effects of 5β3α on the reliable response to sudden changes in light levels seen in embryos. Ventral root activity recorded from stage 37/8 preparations is often more intense after dimming the lights than in response to a skin stimulus suggesting that it is a strong sensory cue for the initiation of embryonic swimming activity. Figure 4.21a, however, shows that the dimming response (a) could be abolished by bath applying 5β3α (3μM, b) but returned after blocking GABAa receptors with 20μM bicuculline (c, n=5). These results strongly suggest that by stage 42, a GABAergic input reduces the probability that swimming will occur in response to sudden changes in light levels.

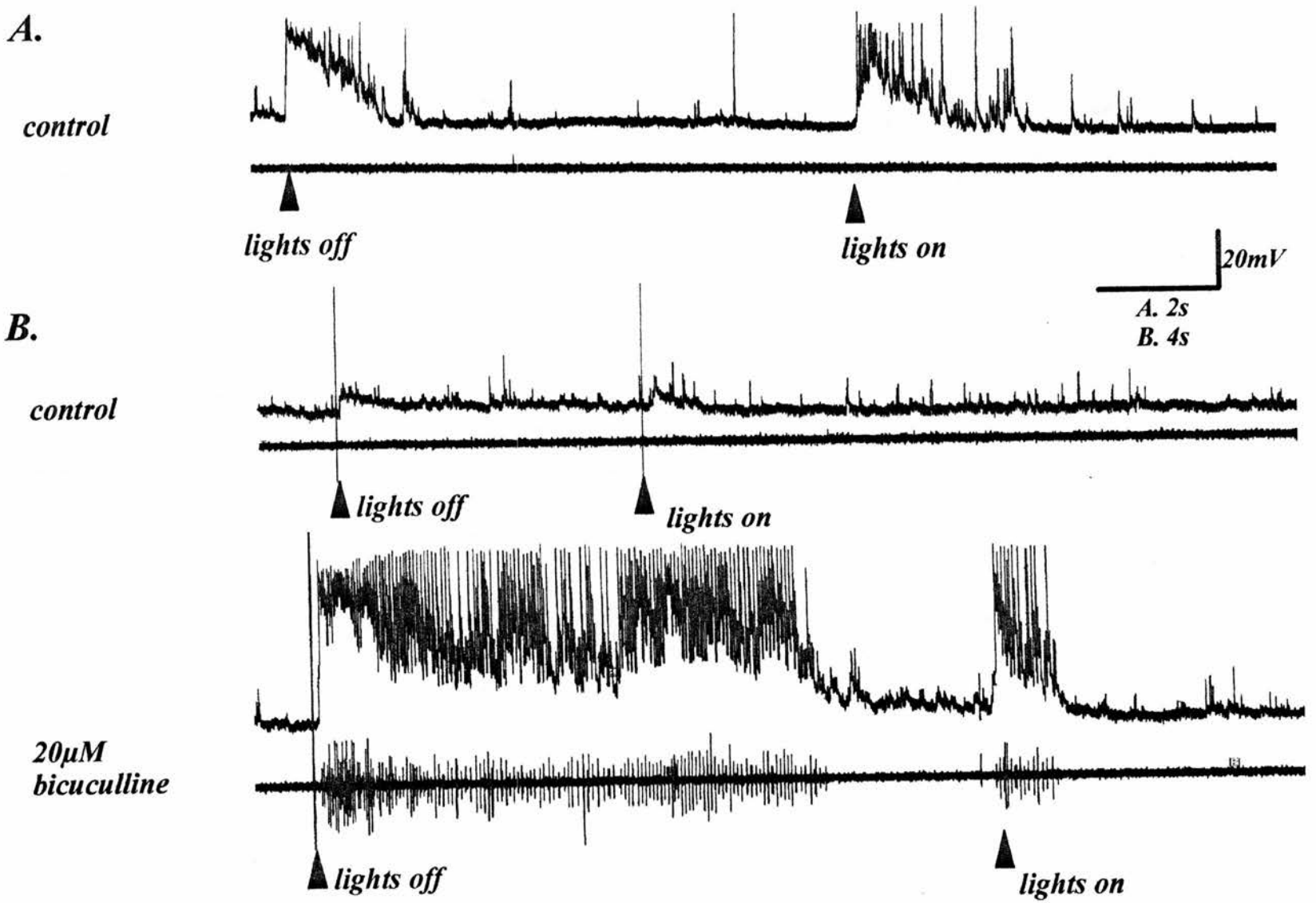

A.
control
lights off
lights on
20mV
A. 2s
B. 4s
B.
control
lights off
lights on
20μM
bicuculline
lights off
lights on

Figure 4.20. GABAa receptor activation prevents the dimming response at stage 42.

A. Intracellular recording from a larval motorneuron in a preparation which did not swim in response to dimming illumination. In response to either switching the lights off or on (indicated by the arrows), depolarising psps were recorded.

B. Larval preparation which did not respond to changes in illumination in control saline. Following the bath application of 20μM bicuculline, rhythmic swimming activity was recorded both in response to switching the lights off and on.

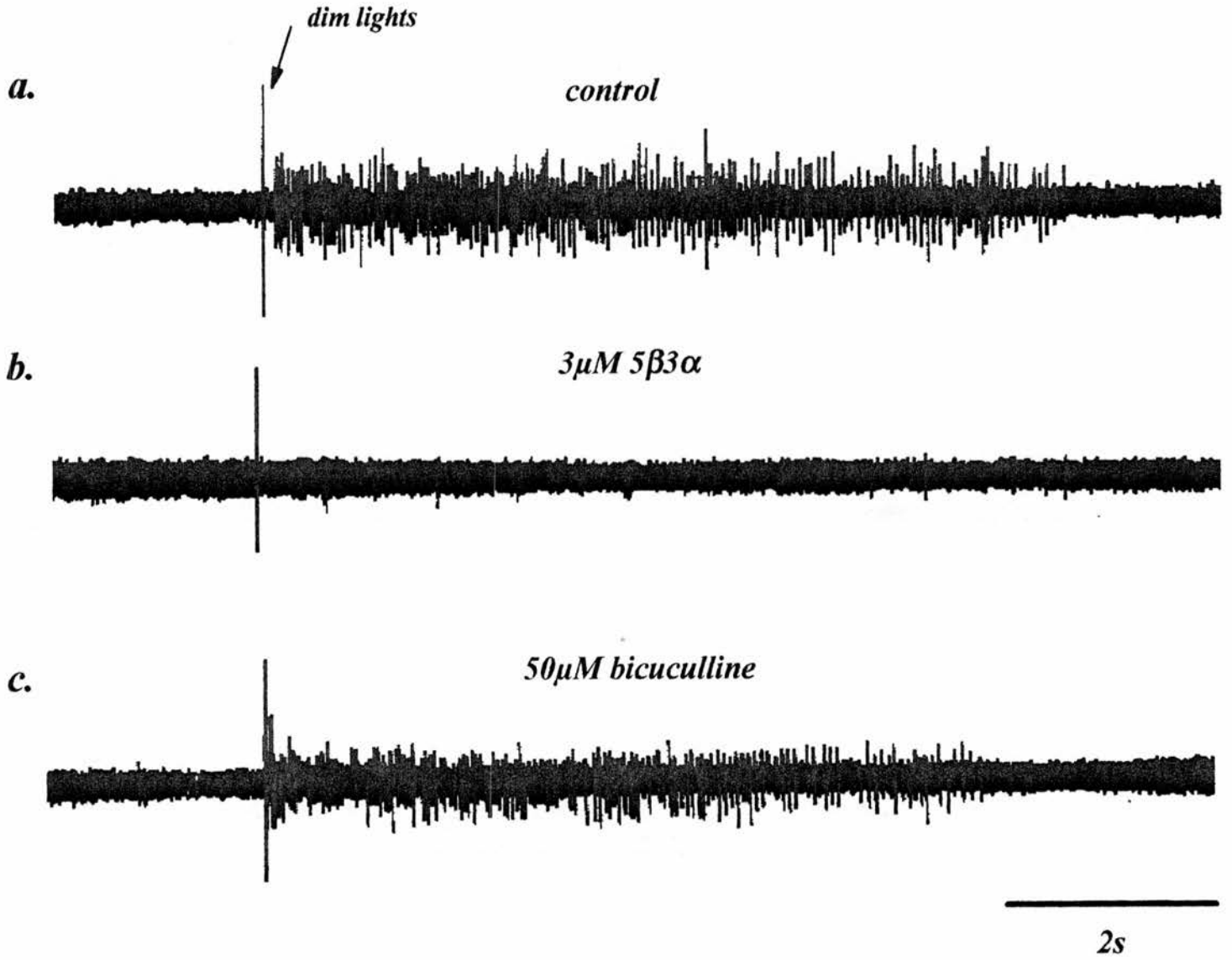

dim lights
a.
control
b.
3μM 5β3α
c.
50μM bicuculline
2s

Figure 4.21. 5β3α prevents the dimming response in embryonic preparations.

a. Embryonic swimming initiated by dimming the lights
b. In the presence of 3μM 5β3α, the dimming response is abolished.
c. Following blockade of GABAa receptors with 20μM bicuculline, the dimming response is restored.

Discussion.

The results presented in this chapter have revealed that over the 24 hour period between stage 37/8 and 42, GABAergic transmission becomes increasingly important in modulating rhythmic activity. By stage 42, blockade of GABAa receptors influences all of the parameters of ventral root activity investigated, causing an increase in burst durations at all levels in the cord and a decrease in both cycle periods and rostrocaudal delays. Additionally, GABAa receptor blockade **shortens** the length of swim episodes. If GABA had an overall inhibitory effect on the swimming system which generally decreased the excitability of motorneurons and interneurons of the CPG, then removing that inhibition might have been expected to increase the length of swim episodes as a result of the increased excitability of the network. Therefore it would seem that GABAergic transmission plays some role in finely tuning rhythmic activity to allow sustained episodes of fictive swimming.

Related studies on other vertebrates have suggested that the overall influence of GABAergic transmission is the result of both GABAa and GABAb receptor activation which modulate different aspects of the locomotor rhythm (Tégner et al., 1993, Cazalets et al., 1994). This study has concentrated on the effects of GABAa receptor activation. However, a few inital experiments explored the effects of GABAb receptor blockade with the antagonist 2-hydroxysaclofen and failed to detect any obvious effects on the larval swimming rhythm. Other recent studies have looked at the effect of GABAb receptor activation on the embryonic swimming rhythm (Wall and Dale, 1993). Bath application of a GABAb agonist caused a reduction in the length of swim episodes coupled with a decrease in the amplitude of ventral root spikes. Intracellular recordings further

revealed that there is a decrease in the reliability of action potential firing in motorneurons which could result in the observed decrease in ventral root amplitude (Wall and Dale, 1993). Additionally, baclofen also caused a decrease in the reliability of midcycle inhibition in part through a decrease in transmitter release from presynaptic terminals of commissural interneurons (Wall and Dale, 1993). However, although these effects result from the activation of GABAb receptors, little effect was noted after their blockade. It is thus unclear what role, if any, endogenous GABAb receptor activation has on embryonic swimming activity. Further work would be required to determine whether GABAb receptor activation influences larval swimming and if activation of the two receptor types influence different aspects of swimming as in the lamprey (Tégner et al., 1993) and in the neonatal rat (Cazalets et al., 1994).

In the lamprey, GABAa and GABAb receptor activation has been shown to modulate rostro-caudal phase delays with GABAa receptors specifically reducing their cycle by cycle variability (Tégner et al., 1993). This study has shown that GABAa receptor blockade decreases the overall rostrocaudal delay but further analysis would be required to determine whether GABA modulates rostrocaudal delay independently or whether the effect results from the accompanying decrease in cycle period. At stage 42, at high swimming frequencies the rostrocaudal delay is short and towards the end of an episode, as swimming frequency declines, rostrocaudal delay increases in proportion to the cycle period (Tunstall and Sillar, 1993). Therefore the decrease in rostrocaudal delays seen here may have been an indirect consequence of the increase in swimming frequency.

In both the lamprey (Alford et al., 1990b) and the neonatal rat (Cazalets, 1994), there is evidence that the GABAergic input during

locomotor activity is spinal in origin. In the lamprey, immunocytochemical studies have revealed that the GABAergic input is from a population of multipolar neurons with local arborisations, extending only to nearby segments of the CPG (Brodin et al. 1990). However although in the neonatal rat preparation there is some electrophysiological evidence for a spinal population of neurons mediating the GABA response, it has also been established that projections from the lower brainstem to the lumbar spinal cord also contain GABA which could modulate the locomotor CPG (Holstege et al., 1991). Spinalisation studies carried out in this chapter on *Xenopus* larval preparations have provided some evidence for a GABAergic input from both spinal and descending neurons. Experiments carried out on low spinal larva suggested that there is some input from spinal neurons at least during NMDA induced activity. It would have been difficult to determine whether these neurons play a role during stimulus evoked swimming activity since transecting the cord at such a low level results in episodes which only last a few cycles thus making it difficult to assess any effect of a GABAa antagonist. However returning to the embryo preparation, it was shown in chapter 3 that there is an overall inhibitory influence over the swimming cpg and this is unlikely to be due to GABA release from mhr neurons (see below). Therefore it is possible that one of the two populations of spinal neurons, the ascending interneurons (Roberts et al., 1987) or the Kölmer-Agdhur cells (Dale et al., 1987) provide an overall inhibitory influence over both larval and embryo swimming.

The descending GABAergic influence on larval swimming would appear to be from the mhr neurons and immunocytochemical evidence indicates that there is still a population of mhr neurons with GABA immunoreactivity in the stage 42 CNS despite the developmental decline in the stopping response. During embryonic fictive swimming, recordings

from mhr neurons have shown that they are inhibited from rest and phasically inhibited midcycle (Boothby and Roberts, 1992b). These, neurons are therefore being inhibited by the CPG and do not fire during embryonic swimming. This suggests that if these neurons become involved in modulating larval fictive swimming, their firing pattern must change during development either following a change in the synaptic input onto these neurons or following a change in their intrinsic membrane properties. It would be of considerable interest to record from the mhr region to determine the firing pattern of these neurons during larval swimming activity. To determine the fate of these neurons conclusively would require an immunocytochemical study on animals up to stage 45 and beyond, at which stage the stopping response is entirely abolished, to establish conclusively whether a population of mhr neurons is still present or not.

The results presented in this chapter also suggest that the influence of mhr neurons could increase sufficiently during development to play a role in the intrinsic termination of swimming. In the embryo it has been suggested that swimming may terminate due to an increase in the activation of a K_{Ca} current resulting from a build up of intracellular calcium during an episode. This in turn would decrease the input resistance and membrane time constant of neurons in the locomotor network thereby increasing their firing threshold during an episode to the point where they stop firing and swimming terminates (Wall and Dale, 1995). These authors suggest, however, that the spontaneous termination of swimming could feasibly result from several mechanisms one of which could be a build up in inhibitory transmission. Although they found little evidence for such a mechanism in terminating embryonic swim episodes, evidence presented here suggests that the termination of larval swimming could result, at least

in part, from an increase in GABA release towards the end of an episode. Following skin stimulation, swimming activity tends to have a high frequency and longer burst durations and as a consequence a short intersegmental phase delay. This is similar to the effects seen when GABAa receptors are blocked. It is conceivable that there is less of a GABAergic influence over rhythmic activity at the beginning of an episode. As each episode progresses, cycle periods increase and burst durations tend to decrease which could result partly from an increase in GABA release from mhr neurons. GABA potentials have been shown to have a much longer duration than glycine potentials, some of which had duration of 200ms or more. GABA potentials are thus longer than swimming cycle periods, which range from around 50ms, at the onset of an episode to around 100ms near the end of an episode. Therefore, like NMDA receptor-mediated potentials which summate during swimming activity to provide a level of tonic depolarisation (Dale and Roberts, 1985), GABA potentials could summate towards the end of an episode to provide a tonic inhibition which eventually terminates swim episodes. Thus, by stage 42 it would appear that as well as a role in finely tuning swimming activity, a GABAergic input may also terminate episodes of larval activity.

GABA potentials coinciding with the end of swimming episodes were only seen in about 50% percent of larval preparations possibly reflecting the variability in the strength of GABA synapses at different levels within the spinal cord. Comparing the level of intracellular recording, with reference to the otic capsule, of motoneurons in which GABA potentials were present at the end of an episode with those in which they weren not, provided no obvious differences relating to the position of the neuron in the spinal cord. Therefore within each motorneuron pool

innervating a given myotome, there may be neurons which are modulated to a different extent by GABAergic inputs. Alternatively, since the intrinsic role of GABA in terminating fictive swimming is presumably still developing as the stopping response declines, the observed differences could result from developmental differences in individual preparations.

By stage 42, a certain level of inhibition is required to sustain rhythmic swimming activity, in that blocking both GABAa and glycine receptors abolished all swimming. A similar pattern of bursting activity has been recorded from neonatal rat motorneurons in the absence of inhibitory transmission (Bracci et al., 1996). In contrast, however, blocking both GABAa and glycine receptors in the lamprey produced an increase in the burst duration at the beginning of an episode to the point where there was no clear interburst interval, but rhythmic activity was not abolished and an increase in episode length was observed (Alford et al., 1990b). As described in the introduction to this chapter, many changes take place during postembryonic development in *Xenopus*, including the innervation of the ventral portion of the cord by projections from serotonergic raphe neurons. Removing inhibition could lead to increased excitation to the point where swimming can no longer be sustained. Thus sustained rhythmic activity may depend on the balance between inhibition and excitation, as has already been suggested in other vertebrate preparations (Cazalets et al., 1994). The effects of increased excitation in the absence of inhibition are investigated in the next chapter.

GABA acting at the GABAa receptor is also involved in modulating the inflow of sensory information. The dimming response is most reliable at stage 37/8 and by stage 42 fewer animals respond to a sudden decrease in

light levels. Experimental evidence presented in this chapter suggests that dimming the lights fails to initiate swimming in some larval preparations because of an increase in GABA release activating GABAa receptors on motorneurons. At stage 42, the dimming response is restored following bath application of bicuculline and conversely at stage 38, the dimming response can be abolished by the bath application of the GABAa receptor agonist, 5β3α. Although the response is declining at stage 42 it is not lost completely until stage 44 and beyond. Therefore this role for GABA may only be transient, during the time when the response is declining after which the dimming response pathway may degenerate. The action of GABA in inhibiting the pathway may be part of that process.

CHAPTER 5.
A role for 5HT-dependent intrinsic oscillations.

Introduction

The results of the preceding chapter suggest that by the larval stage (42), reached approximately one day after hatching, GABAergic transmission plays a greater role in swimming and that in comparison with the embryo, inhibition, in general, is vital for the maintainment of a coordinated pattern of activity. In other preparations the idea of a dual mechanism which finely tunes the locomotor rhythm has been suggested (Wallén, 1995, Cazalets, 1995), where the final output is a balance of excitatory and inhibitory modulation. In the neonatal rat preparation the locomotor pattern generator is under the control of both GABAergic (inactivatory) and serotonergic (activatory) transmission (Cazalets, 1995).

Recordings of the locomotor output from several different vertebrate preparations have shown that the intensity and duration of motor bursts are increased during fictive locomotor activity when 5HT is exogenously applied (lamprey-Harris-Warrick and Cohen, 1985, *Xenopus*- Sillar et al., 1992a,b, rat-Cazalets et al., 1992, rabbit-Viala and Buser,1969, cat-Barbeau and Rossignol, 1990). In adult vertebrate preparations such as the lamprey, the effects of 5HT on the locomotor output have been explained in terms of its action on calcium-dependent potassium channels (K_{Ca}) which mediate the slow after-hyperpolarisation following an action potential, thereby resulting in a decrease in spike accommodation and an increase in firing frequency. This action goes some way towards explaining the enhancement of ventral root bursts recorded in the presence of 5HT (Van Dongen et al., 1986, Wallén et al, 1989). This effect of 5HT on spike activity has also been reported in cat (Hounsgaard et al., 1988) and turtle neurons (Hounsgaard and Kiehn, 1989). However, there is now some doubt over whether the global actions of 5HT on locomotor networks can be

explained entirely by this one cellular mechanism. More recent findings from the lamprey preparation have shown that apamin, which also abolishes the slow afterhyperpolarisation, has little or no effect on ventral root activity (Hill et al., 1992, Meer and Buchanan, 1992). In *Xenopus*, the enhanced burst durations observed in the presence of 5HT could be explained by a similar mechanism of action of the amine on spike generating capability and 5HT has been shown to increase the firing capability of motorneurons during swimming (Wedderburn J.F.S, PhD thesis). However, in addition to the increase in burst durations, 5HT also decreases interburst intervals, which suggested that amine may also modulate reciprocal inhibition. Recordings from motorneurons during fictive swimming have shown that in the presence of 5HT, midcycle inhibition can fail (Sillar and Wedderburn, 1994). An investigation into the effects of 5HT on spontaneous glycinergic ipsps has shown further that the amine presynaptically inhibits the release of transmitter from the terminals of commissural interneurons (Sillar and Wedderburn, 1994). 5HT has been shown to modulate transmitter release in other preparations. For example, it depresses the excitatory drive onto motorneurons in the lamprey (Buchanan and Grillner, 1991, Shupliakov et al., 1995) and in the adult frog it has been shown to enhance glutamate mediated responses (Cardona and Rudomin, 1983).

In addition to the action of 5HT on the duration of locomotor bursts during *Xenopus* larval swimming, the duration of swim episodes is also markedly reduced in the presence of 5HT. 5HT has been shown to elicit a TTX-resistant membrane hyperpolarisation, and this general decrease in the excitability of motorneurons could be responsible for the shortening of swim episodes.

Membrane potential bistability has also been reported, in several preparations, to be dependent on a source of 5HT (turtle- Hounsgaard and Kiehn, 1985, cat-Hounsgaard et al., 1984, 1986, 1988, Crone et al., 1988, Conway et al., 1988). In the acute spinal cat for example, these plateau potentials are only expressed after application of the 5HT metabolic precursor, 5-hydroxytryptophan indicating their dependence on descending 5HT projections (Hounsgaard et al., 1988, Kiehn, 1991). Schwindt and Crill (1980) were the first to describe plateau potentials in motorneurons during penicillin-induced seizures which resulted from a persistent inward calcium current. Plateau potentials allow the neuron to flip between two relatively stable states, one at rest and one at a more depolarised level. They are the result of a region of negative slope conductance mediated by a slowly inactivating current (Flatman et al., 1986) which results in a range of membrane potentials which are highly unstable (MacDonald et al., 1982).

In lamprey spinal neurons, in the presence of NMDA and TTX, membrane potential bistability is expressed as continuous oscillations between two stable states (figure 5.1; Wallén and Grillner, 1987). In this preparation, however, the possibility that their expression requires an exogenous source of 5HT has not been explored. The oscillations rely on the unique properties of the NMDA receptor ionophore complex which is both ligand- and voltage-gated. The voltage-dependency is due to the channel being blocked by magnesium ions, at membrane potentials close to rest (Nowak et al., 1984, Mayer and Westbrook, 1984). Oscillations occur, so long as NMDA is present, because the magnesium block of the channel is less than 100% at the membrane resting potential. The membrane will thus begin to depolarise (figure 5.1, 1) until the point where the magnesium block can be rapidly alleviated resulting in further rapid depolarisation of the membrane (2). A plateau is then reached when voltage-dependent

NMDA-induced TTX-resistant oscillations

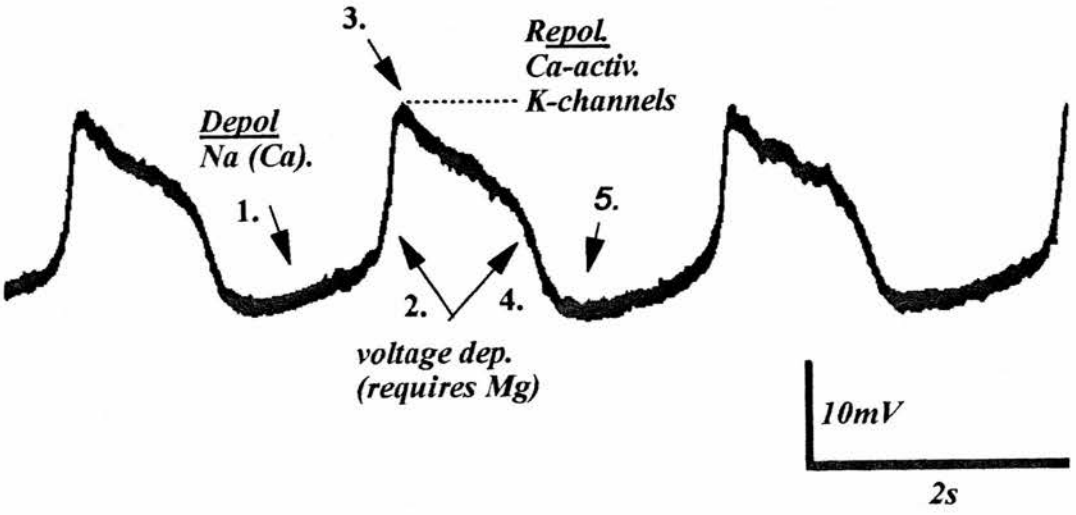


Figure 5.1. TTX-resistant NMDA receptor mediated voltage oscillations in lamprey rhythmic neurons.

Lamprey oscillations rely on the voltage dependence of the NMDA channel. **1.** The main current through the channel is carried by sodium ions but the channel is also permeable to calcium ions. **2.** As the cell begins to depolarise, a membrane potential is reached where there the magnesium block of the channel is alleviated causing a rapid depolarisation. **3.** The entry of calcium ions activates K_{Ca} channels which cause the membrane to begin to repolarise. **4.** The membrane potential is reached where the voltage dependent block resumes. **5.** K_{Ca} channels close as calcium levels decrease and a new cycle starts as the membrane again begins to depolarise. (Adapted from Wallén and Grillner, 1987).

potassium channels are activated by the depolarisation. Although the current flowing through the activated NMDA receptor ion channels is mainly carried by sodium ions, the channel is also permeant to calcium and potassium ions (Mayer and Westbrook, 1985, Ascher and Nowak, 1988). Consequently, calcium ions enter the neuron to activate K_{Ca} channels which results in slow membrane repolarisation (3). Eventually a level is reached where the magnesium block can be re-established, causing the membrane potential to repolarise towards the resting potential (4). The cycle will then repeat itself so long as NMDA is present (5). NMDA receptor mediated oscillations are not limited to the primitive vertebrate systems like the lamprey. For example, in the neonatal rat, both rhythmically active motorneurons, and interneurons surrounding the central canal demonstrate intrinsic oscillations in the presence of TTX and NMDA (Hochman et al., 1994a,b). Similarly, neurons in the adult rat nucleus tractus solitarii which control rhythmic activities such as breathing and swallowing (Tell and Jean, 1991, 1993) undergo intrinsic membrane potential oscillations.

In all of these preparations, the normal physiological role of NMDA-induced oscillations is open to speculation. However, indirect evidence suggests that they may contribute to the generation of rhythmic activity. In both the lamprey and neonatal rat preparations, the frequency of intrinsic NMDA-dependent oscillations overlaps with the frequency of locomotor rhythmic activity (Grillner and Wallén, 1985, Wallén and Grillner, 1987, Hochman et al., 1994a,b) and it has recently been confirmed that those neurons surrounding the central canal in the neonatal rat spinal cord which oscillate, do so in phase with locomotor activity (MacLean et al.,1995). In the lamprey, recordings from motorneurons during fictive locomotion have also provided evidence inconsistent with rhythmic activity being produced

entirely from synaptic input (Wallén and Grillner, 1987). TTX-resistant membrane potential oscillations are strongly influenced by injected current and during rhythmic activity in the absence of TTX, depolarising or hyperpolarising current resulted in either an earlier or later onset of the depolarising phase of each cycle of rhythmic activity, respectively. In similar experiments carried out in Mg^{2+}-free saline there was no longer any change in the timing of the depolarising phase following current injection (Wallén and Grillner, 1987). These results are consistent with the involvement of voltage-dependent membrane properties during swimming activity. It has thus been proposed that NMDA-dependent intrinsic membrane potential oscillations contribute to lamprey swimming on a cycle by cycle basis. However, the evidence derived from rhythmic activity induced by NMDA and therefore, it is still not clear whether intrinsic oscillations are involved in normal swimming activity in the absence of the agonist.

Despite the presence of voltage-dependent NMDA receptors on motorneurons of *Xenopus* embryos and embryos of the closely related amphibian species, *Rana temporaria*, intrinsic (NMDA-type) oscillations have not been recorded under similar experimental conditions to the lamprey (Sillar and Simmers, 1994a,b, Wedderburn and Sillar, 1994b). In the presence of TTX, to block spike mediated synaptic transmission, bath application of NMDA causes substantial (20-25mV) membrane depolarisation but does not lead to intrinsic oscillations. Further investigations demonstrated that in *Rana* membrane bistability could occur, but that continuous oscillations, akin to those observed in lampreys and elsewhere, were only induced following the addition of 5HT to the bathing medium (Sillar and Simmers, 1994a,b). In contrast to the *Xenopus* embryo, the ventral portion of the *Rana* spinal cord at the time of hatching, is

already innervated by 5HT projections from the raphe nucleus (Woolston et al, 1994). Subsequent experiments carried out on different developmental stages in *Xenopus* have also revealed a 5HT-dependency for the expression of intrinsic membrane oscillations and a developmental increase in the proportion of neurons which would oscillate under these conditions (Wedderburn and Sillar, 1994b). Presumably this reflects the expression of 5HT receptors on motorneurons which occurs just before the innervation of the spinal cord by descending 5HT fibres from the raphe nucleus (Sillar et al., 1992b). Therefore, in keeping with other vertebrate preparations described above, whose neurons show membrane bistability in the presence of 5HT, it would appear that two different amphibian preparations require the presence of the amine to express bistable membrane properties.

Although, these preparations required the additional presence of 5HT, the oscillations had a similar waveform to those already described in the lamprey and displayed many of the same properties. Oscillations were never seen in the presence of 5HT without NMDA, or in magnesium free salines, suggesting that the continuous oscillations still rely upon the voltage dependent properties of the NMDA channel. Furthermore, the oscillations could be abolished by the bath application of the NMDA receptor antagonist, D-2-amino-5-phosphonovaleric acid (APV). The oscillations reported in *Xenopus* and *Rana* motorneurons do differ from those recorded in lamprey neurons in that only their amplitude and not their frequency can be altered by current injection. The most likely explanation for this finding is that in both amphibian species, motorneurons have been shown to be electrically coupled (*Xenopus*, Perrins and Roberts, 1995a *Rana*- Sillar and Simmers, 1994c).

Although lamprey neurons do not appear to require the presence of 5HT to induce membrane bistability, 5HT nevertheless modulates the

frequency of the oscillations by slowing the repolarising phase and reducing the level of hyperpolarisation reached. This modulatory effect of 5HT is again thought to be due to a direct action of 5HT on K_{Ca} channels (Wallèn et al, 1989). As a result of the reduction of K_{Ca} currents by 5HT in the turtle, low voltage activated, L-like calcium channels remain open for a prolonged period and are thought to underlie the induction of membrane bistability by 5HT (Hounsgaard and Kiehn, 1985, 1989). Similarly, a T-like calcium current has been shown to be enhanced by 5HT in neonatal rat motorneurons *in vivo* which could facilitate membrane oscillations (Berger and Takahashi, 1990). In *Xenopus* larval motorneurons, when 5HT is bath applied to a preparation already in the presence of TTX and NMDA , the membrane hyperpolarises (Wedderburn and Sillar, 1994b) where in other preparations 5HT causes a depolarisation of the membrane (Hounsgaard and Kiehn, 1985). As a reduction in a potassium current would be expected to have the opposite effect on the resting membrane in *Xenopus*, it is unlikely that 5HT induces membrane potential oscillations by reducing K_{Ca} currents. Experimental evidence now suggests that 5HT receptor activation directly modulates the voltage-dependence of the NMDA-receptor ionophore complex by facilitating the magnesium block itself (Wedderburn and Sillar, 1994b). In the presence of TTX, NMDA causes membrane potential depolarisation and a large increase in conductance. However, injecting negative current pulses reveals an apparent conductance decrease presumably because the conductance pulse brings the membrane potential into the region where magnesium ions block the NMDA channel so reducing the current flow. Additional bath application of 5HT enhances the apparent conductance decrease suggesting that the amine facilitates the ability of magnesium ions to block the channel. A likely result of this

action is that the region of negative slope resistance will be steepened which is presumably what allows the neuron to oscillate.

There are now known to be at least seven distinct groups of 5HT receptors ($5HT_{1-7}$) within which further subtypes exist (for review see- Zifa and Fillion, 1992). In the rat the actions of 5HT have been shown to be abolished by either 5HT1 or 5HT2 receptor antagonists (Cazalets et al, 1992). In some preparations the action of 5HT on these two receptor subtypes has been shown to involve opposing effects, with the activation of 5HT1 receptors causing membrane hyperpolarisation while the activation of 5HT2 receptors leads to membrane depolarisation (Holohean et al, 1990, Wang and Dunn, 1990). However, although a range of effects have been reported to be activated by 5HT in *Xenopus*, which combine to mediate the overall effect of the amine on locomotor activity, all of the cellular and synaptic effects studied so far, appear to be mediated by the action of 5HT at a single receptor which most closely resembles the mammalian 5HT1a receptor in its pharmacological profile. This has been shown for the modulatory effects of the amine on ventral root bursts (Wedderburn and Sillar, 1993, 1994a) and on the presynaptic release of transmitter (K.T. Sillar and J.F.S. Wedderburn, unpublished observations). The subtype of receptor involved in *Xenopus* oscillations has not yet been determined but those recorded in *Rana* motorneurons are also sensitive to the 5HT1a receptor antagonist, NAN-190 (Sillar and Simmers, 1994b).

Despite the expression of intrinsic oscillations in many different preparations under certain experimental regimes, there is still a lack of direct evidence on what contribution, if any, they might make to locomotion. In the lamprey, it has been speculated that they may contribute to swimming activity on a cycle by cycle basis. Their frequency is slow in

comparison to the range of swimming frequencies but it is suggested that the intrinsic oscillations could be entrained by synaptic input (Grillner and Wallén, 1985, Sigvardt et al, 1985, Wallén and Grillner, 1987). However, this discrepancy in frequency is even greater in *Xenopus* where swimming occurs between a frequency of 10-30Hz, but the oscillations occur at a similar frequency to those described in the lamprey (approximately 0.5Hz) making it seem unlikely that they could influence swimming on a cycle by cycle basis. Chapter 4 concluded that sustained larval swimming activity relies on a certain level of inhibitory input in comparison to embryonic swimming, presumably to balance the input from excitatory pathways. Between the two stages in development, projections from the serotonergic raphe neurons innervate the ventral portion of the spinal cord and exert an overall excitatory influence on the motor system. By examining the pattern of activity recorded in the absence of inhibitory transmission, this chapter reveals a possible role for intrinsic 5HT-dependent oscillations during larval swimming activity.

Results

1. The effects of blocking inhibition on NMDA induced activity.

At the end of chapter 4, I showed that blockade of GABAa and glycine receptors, abolished larval swimming activity. Similar experiments carried out in the presence of NMDA (figure 5.2 bi and ii, n=5) show that bath application of strychnine (ci and ii) produces a similar pattern of activity to that recorded in the presence of NMDA and bicuculline (chapter 4, figure 4.3), where regular bursts of rhythmic activity occurred at a frequency of around 0.5Hz. The additional blockade of GABAa receptors with 20μM bicuculline, changed the activity to regular bursts of activity (figure 5.2 di) within which there were no longer discernible interburst intervals between individual ventral root bursts (dii). These 'non-rhythmic bursts' occurred at a slightly slower frequency of around 0.4Hz. After washing off the bicuculline (figure 5.2ei and ii), bursts of rhythmic activity resumed and the effects of strychnine were also partially reversed after returning to control saline (figure 5.2fi and ii). This non-rhythmic pattern of NMDA-induced activity, seen in the presence of strychnine and bicuculline, presumably results from a reduction in inhibitory transmission, thus causing an enhancement of excitatory synaptic drive.

It is noticeable that a slow modulation of the ventral root activity already exists in the presence of 100μM NMDA alone, involving a slow but regular waxing and waning of the amplitude of the ventral root activity recorded (figure 5.2bi). Intracellular recordings from larval motorneurons under equivalent conditions revealed that the ventral root activity recorded in the presence of 100μM NMDA reflects regular oscillations in the amplitude of the excitatory synaptic drive to motorneurons (figure 5.3A).

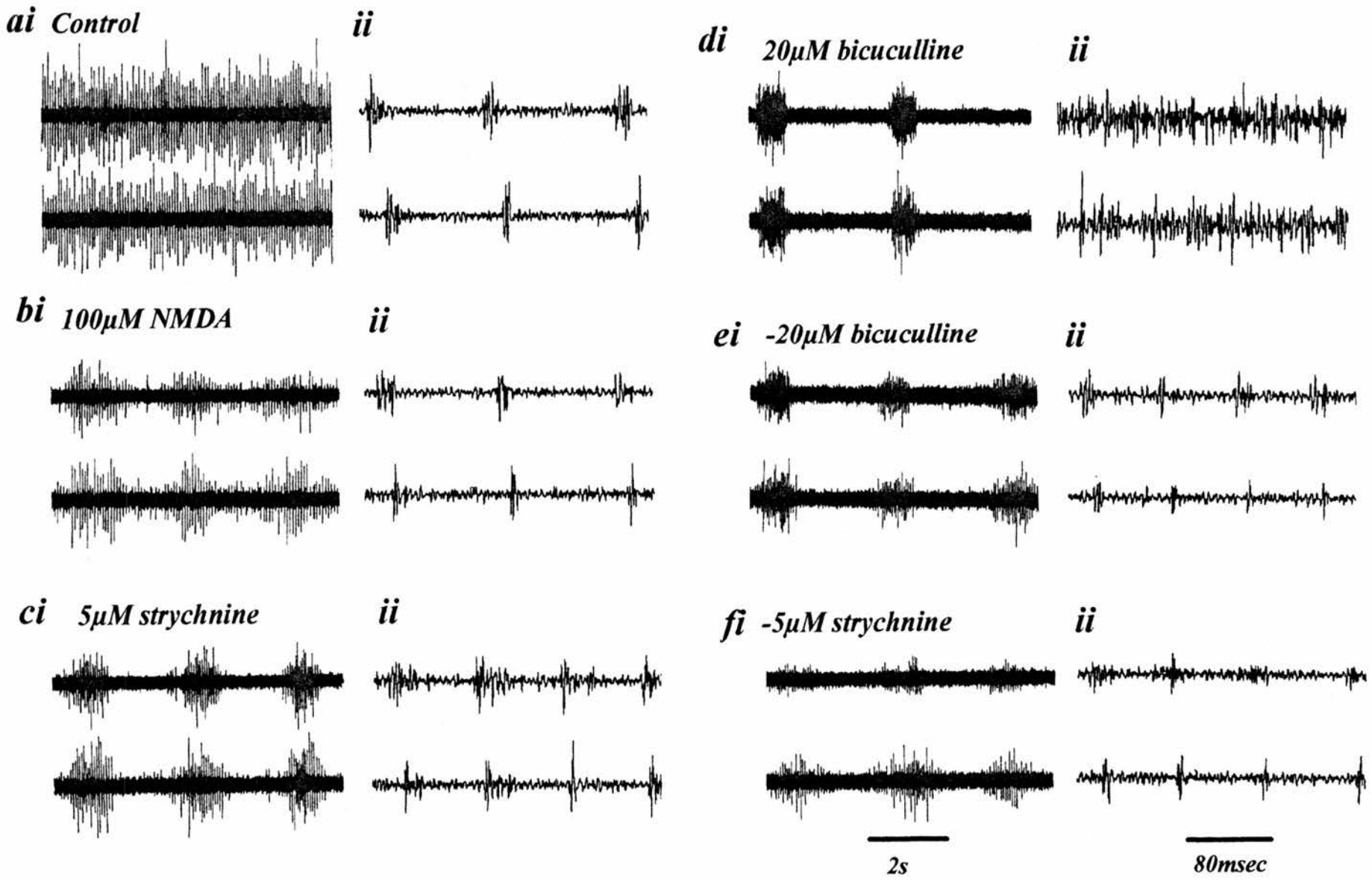

ai Control
ii
di 20μM bicuculline
ii
bi 100μM NMDA
ii
ei -20μM bicuculline
ii
ci 5μM strychnine
ii
fi -5μM strychnine
ii
2s
80msec

Figure 5.2 The effects of blocking glycine and GABAa receptor activation on NMDA-induced larval swimming activity.

ai. Control activity recorded following skin stimulus. **ii**. expanded trace to show clearly the cycle period, burst duration and rostrocaudal delay.
bi. Continuous activity recorded in the presence of 100μM NMDA with an underlying slow waxing and waning in the amplitude of the ventral root activity. **ii**. expanded trace showing that a regular pattern of activity is, however, still maintained.
ci. In the presence of 5μM strychnine, the pattern of activity becomes discontinuous and regular bursts of ventral root activity are seen. **ii**. strychnine causes a decrease in the cycle period and burst durations are increased.
di. Co-application of 20μM bicuculline changes the pattern of activity to regular bursts of non-rhythmic activity. **ii**. the expanded trace shows that there are no longer discernible bursts of activity.
ei. Washing off bicuculline leads to the resumption of bursts of rhythmic ventral root activity. **ii**. cycle periods and burst durations are similar to those seen originally in the presence of strychnine alone.
fi. The effects of strychnine could not be fully reversed and bursts of rhythmic activity were still recorded. **ii**. however the effects of strychnine on burst duration were reversed although cycle periods were still shorter than control.

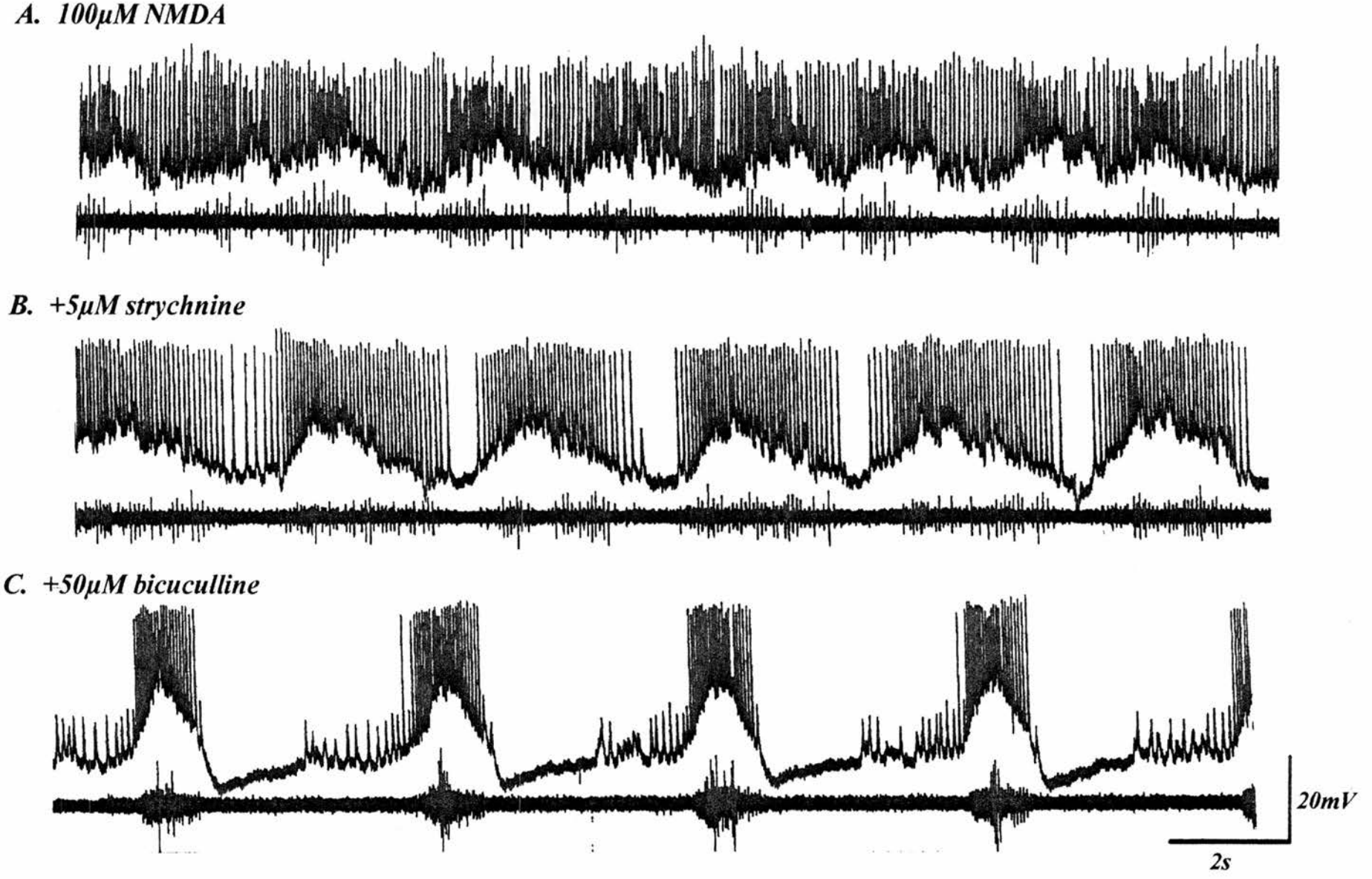

A. 100μM NMDA
B. +5μM strychnine
C. +50μM bicuculline
20mV
2s

Figure 5.3. Intracellular recording in the presence of strychnine and bicuculline.

A. Activity recorded from a rhythmically active larval motorneuron in which fast oscillatory swimming activity occurred on top of an underlying slow oscillation of the membrane potential with a frequency of around 0.3Hz.
B. The presence of 5μM strychnine enhanced and slowed the oscillatory activity to a cycling frequency of around 0.25Hz.
C. After the subsequent addition of 20μM bicuculline, the oscillatory activity was further enhanced with spike activity occurring on top of the depolarised phase. The frequency of the oscillations was around 0.2Hz in the absence of inhibitory transmission.

5μM strychnine further enhanced the amplitude of these oscillations and slowed their frequency from around 0.3Hz to around 0.25Hz (B). The additional blockade of GABAa receptors with bicuculline (50μM, C), decreased the frequency of the oscillations still further to 0.2Hz and bursts of spikes were now superimposed on top of the depolarising phase of each slow oscillation. Figure 5.4 shows another intracellular recording from a larval motorneuron firstly in the presence of 100μM NMDA (A) in which again there was a small but detectable oscillation underlying the continuous rhythmic activity. Following the bath application of 5μM strychnine and 20μM bicuculline slow oscillations were elicited (Bi). The expanded trace (Bii) shows more clearly the spike activity superimposed on the depolarising phase of the slow rhythm. Underlying each burst of spikes, trains of epsps could be detected indicating that presynaptic neurons in the spinal network were also firing rhythmically, in phase with the slow oscillations. I will refer to these slow rhythmic bursts, obtained after blockade of inhibitory receptors as 'network oscillations'.

To begin to test whether the network oscillations might result from intrinsic membrane potential oscillations or entirely from the synaptic drive, the effects of hyperpolarising current on network oscillations were examined. Figure 5.5 shows the effects of varying levels of hyperpolarising current on the network oscillations recorded from another larval motorneuron. It can be seen that initially increasing levels of hyperpolarisation up to about 0.2nA, caused an increase the amplitude of the underlying oscillations. However, at 0.2nA large oscillations alternated with lower amplitude oscillations. The average of ten oscillations measured with no current injection was 11.2 ± 2.3mV and at 0.2nA the average of the high amplitude oscillations was significantly increased to 16.2 ± 1.6mV ($p > 0.001$). Increasing the level of injected current to 0.3nA resulted in only

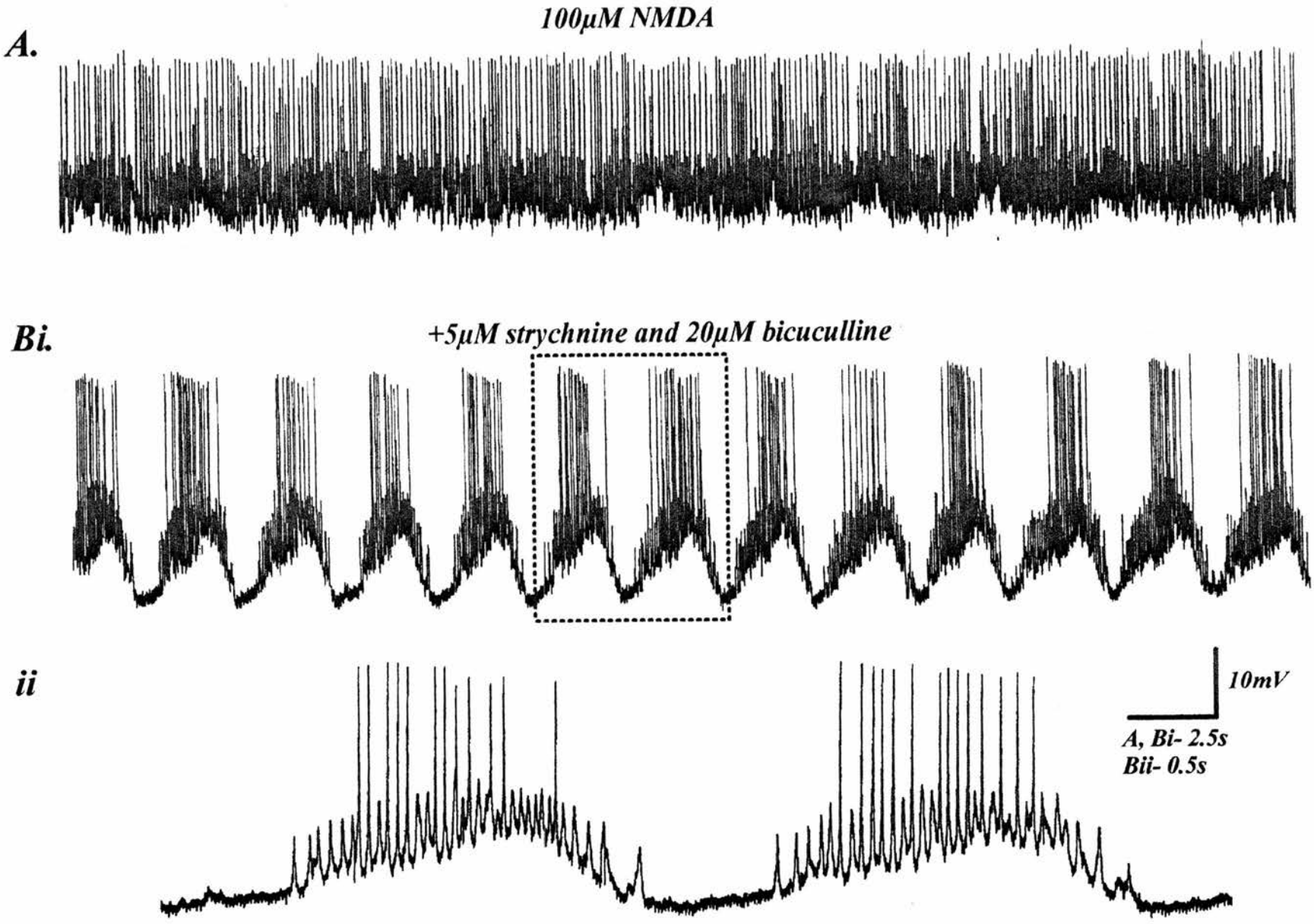

100μM NMDA
A.
Bi.
+5μM strychnine and 20μM bicuculline
ii
10mV
A, Bi- 2.5s
Bii- 0.5s

Figure 5.4. Network oscillations in the absence of inhibitory transmission.

A. Activity recorded from a larval motorneuron in the presence of 100μM NMDA. The underlying slow oscillation was less obvious but still apparent in this motorneuron.
Bi Following the bath application of 5μM strychnine and 20μM bicuculline however, a clear network oscillation was revealed. **ii**. The expanded trace shows the spike activity occurring on top of the depolarising phase with underlying trains of epsps in phase with the slow oscillations.

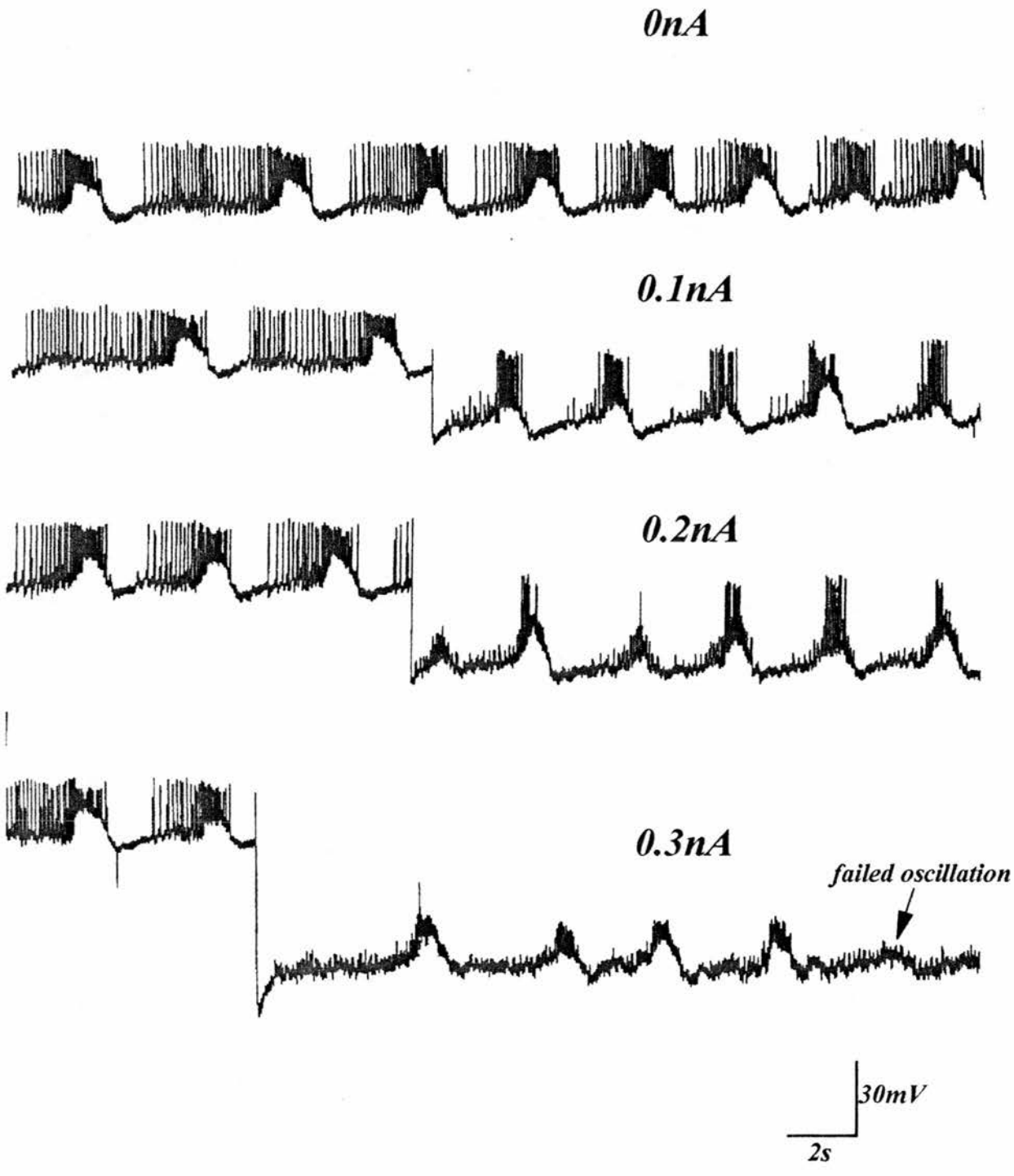

0nA
0.1nA
0.2nA
0.3nA
failed oscillation
30mV
2s

Figure 5.5. The effects of hyperpolarising current on network oscillations.

Network oscillations recorded at different levels of injected hyperpolarising current. Increasing the level of hyperpolarising current initially increases the amplitude of network oscillations although at 0.2nA the large oscillations are interspersed with lower amplitude oscillations. By 0.3nA, the oscillations were generally lower in amplitude and may occasionally fail altogether.

lower amplitude oscillations which were far less regular and appeared to sometimes fail. This result resembles the effect of hyperpolarising current on intrinsic membrane potential oscillations in which the injected current brings the membrane potential into the region where voltage-dependent regenerative blocking and unblocking of the NMDA receptor by Mg^{2+} can occur optimally. Increasing the levels of hyperpolarising current further takes the membrane potential out of that region and so reduces the amplitude of the oscillations (Wedderburn and Sillar, 1994b). This result is therefore consistent with the notion that voltage-dependent membrane properties are involved in the expression of network oscillations. Another piece of supporting evidence which is consistent with an involvement of voltage-dependent membrane properties is the effect of hyperpolarising current on the waveform of the network oscillations. This is shown clearly in the expanded traces in figure 5.6. The upper traces at each current level show the raw data. Underneath each trace is an outline of the profile of the underlying oscillations. In the absence of any hyperpolarising current (a) the oscillations have a sharp rising phase followed by a shallow plateau phase then a sharp falling phase back to the resting membrane potential. Following injection of 0.2nA of constant hyperpolarising current alternating low and high amplitude oscillations are seen (b). In comparison to control conditions the high amplitude oscillations now have a slow rise and fall with little or no plateau phase. These results could not be readily explained if the membrane potential oscillations were due entirely to synaptic inputs, suggesting that there is some involvement of intrinsic bistable membrane properties in the network oscillations.

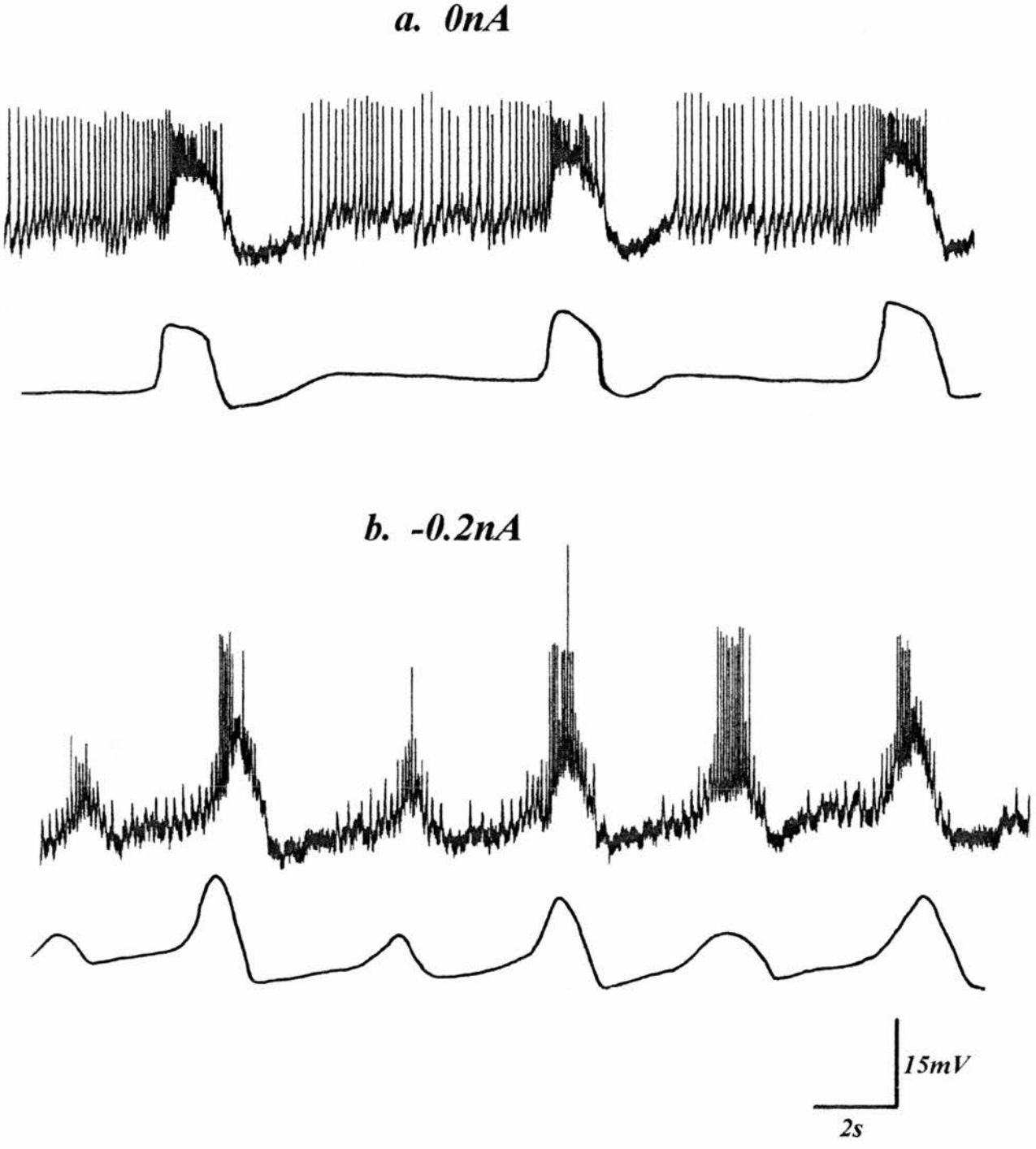

a. 0nA
b. -0.2nA
15mV
2s

Figure 5.6. Hyperpolarising current injection changes the profile of network oscillations.

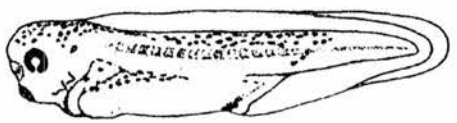
Before current is injected into the cell the oscillations had a fast rise, followed by a plateau and terminated rapidly. Injecting a constant level of hyperpolarising current firstly causes the network oscillations to alternate between high and low frequency and secondly the large amplitude oscillations now have a rather slower rise and fall phase with no apparent plateau phase.

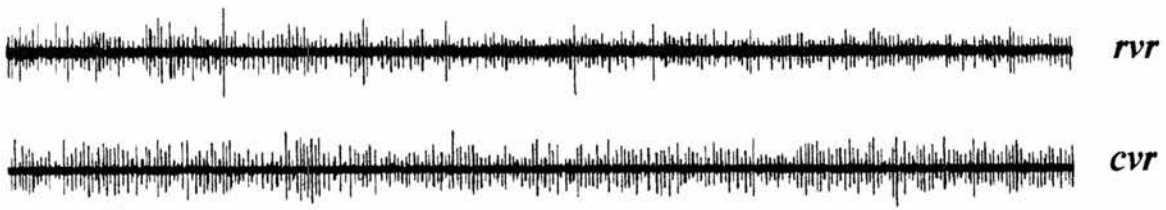
2. Developmental difference in the pattern of NMDA induced activity.

The preceding results show that blocking GABAergic and glycinergic inhibition in the presence of NMDA results in a slow oscillation in the central synaptic excitation to motorneurons which in some preparations appeared to enhance an already underlying slow modulation of swimming activity. The indirect evidence presented in figures 5.5 and 5.6 suggest that the network oscillations could result, at least in part, from voltage-dependent membrane properties. Closer examination of the activity recorded in larval preparations in the presence of NMDA, revealed that 66% of preparations (n=21) displayed an underlying slow modulation in the amplitude of ventral root activity during locomotor activity, an example of which is shown in figure 5.7B. By comparison, only 15% of recordings from embryonic (n=15) preparations showed any indication of a slow waxing and waning of ventral root amplitude during NMDA-induced swimming. In the majority of embryo preparations, in the presence of NMDA, the pattern of activity was relatively constant with little variation in the amplitude (figure 5.7A). These observations therefore suggest that the modulatory influence on the NMDA-induced swimming pattern results from an excitatory system which develops between stage 37/8 through to stage 42.

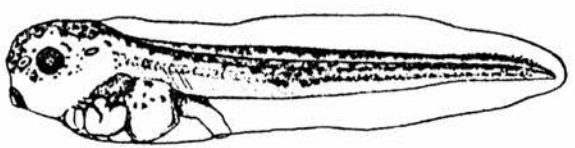
In order to characterise the slow modulation of activity in more detail, changes in amplitude, cycle periods and burst durations were measured of modulated NMDA-induced larval swimming. The graph in figure 5.8 is a plot of consecutive amplitudes measured over an 8s time interval, from the trace above the graph, where amplitude increased to a maximum approximately every 2s. An example of the accompanying

A. stage 37/8





B. stage 42



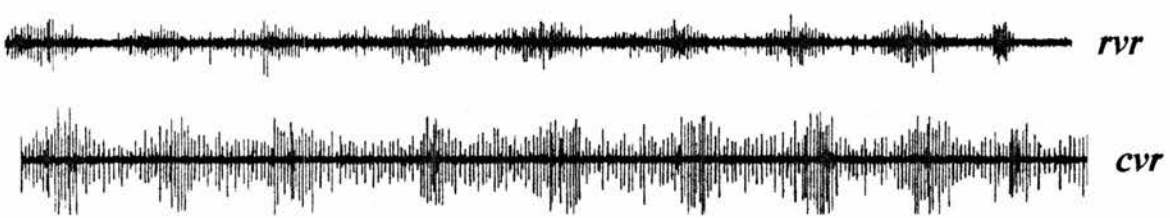


Figure 5.7. Developmental difference in the ventral root activity recorded in the presence of NMDA.

A. At stage 37/8, in most preparations (85%) bath application of NMDA elicits continuous fictive swimming with little variation in the amplitude.
B. Around 66% of larval ventral root activity induced by NMDA showed a slow waxing and waning of the ventral root amplitude, which increased to a maximum around every 2s.

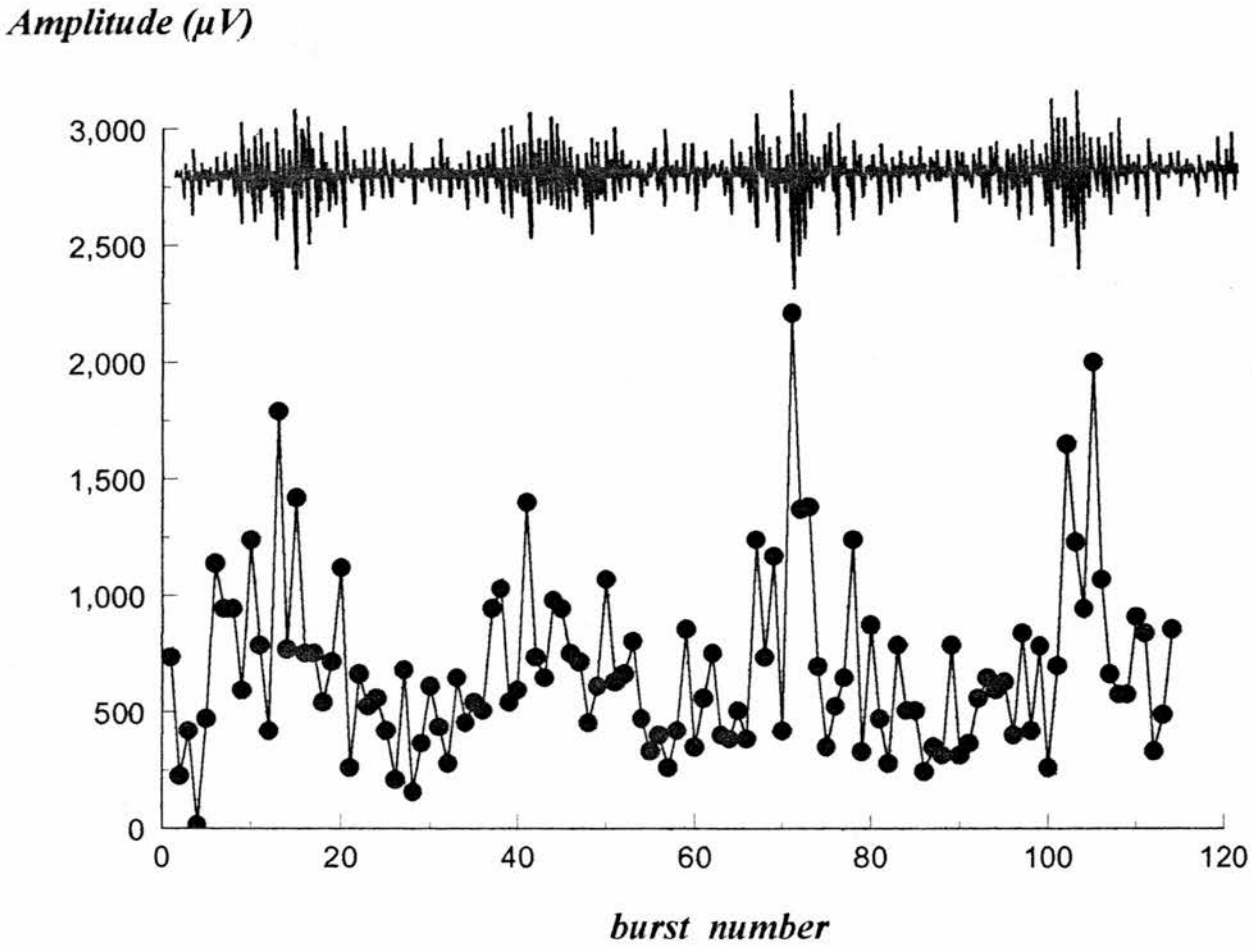

Amplitude (μV)
3,000
2,500
2,000
1,500
1,000
500
0
0
20
40
60
80
100
120
burst number

Figure 5.8. Variation in the amplitude of ventral root activity recorded in the presence of NMDA.

Graphical representation of the variation in amplitude of larval ventral root activity measured over an 8s period from the trace above the graph. Amplitudes increased to a maximum approximately every 2s.

variations in cycle period and burst duration is shown in figure 5.9. The graphs (bi and bii) display data measured from the boxed area of the ventral root activity displayed (a). Cycle periods decreased towards the point of maximum ventral root amplitude, from around 100 to around 60ms (bi). In bii, both rostral (o) and caudal (•) burst durations are plotted in sequence and were found to increase as the amplitudes increased and cycle periods decreased.

3. A role for intrinsic oscillations?

A proportion of larval motorneurons (~ 60%) which are normally rhythmically active during fictive swimming display intrinsic membrane potential oscillations in the presence of TTX which are dependent on NMDA and 5HT receptor co-activation (Wedderburn and Sillar, 1994b). However, although membrane potential bistability has also been described in other vertebrate species, the contribution of this membrane property to locomotion in general and especially to *Xenopus* larval fictive swimming activity has remained unclear. The reason for this is that there is a very large discrepancy between the frequency of swimming (10-30Hz, figure 5.10A) and that of the intrinsic oscillations (ca. 0.5Hz, B) recorded from *Xenopus* larval preparations. This makes it highly unlikely that the intrinsic oscillations could contribute to the cycle by cycle excitatory drive occurring during swimming. The results presented in the preceding sections describe a modulatory influence which enhances NMDA-induced fictive swimming over several consecutive cycles. Indirect evidence suggests that this slow modulation may result from the 5HT-dependent intrinsic membrane potential oscillations described by Wedderburn and Sillar (1994). Firstly, there is a remarkable similarity in the frequency of intrinsic- and network-

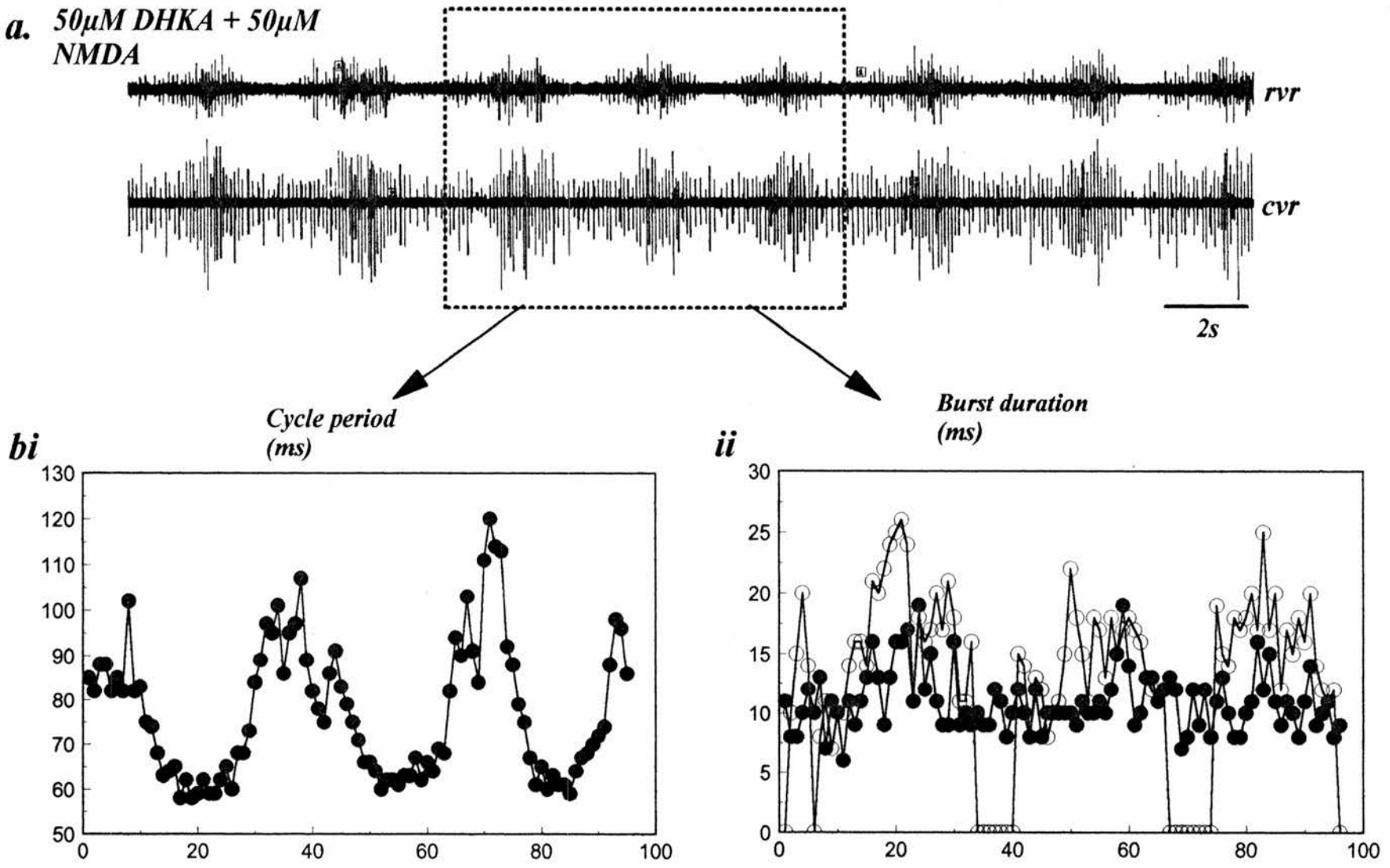

a.
50μM DHKA + 50μM NMDA
rvr
cvr
2s
bi
Cycle period (ms)
130
120
110
100
90
80
70
60
50
0
20
40
60
80
100
ii
Burst duration (ms)
30
25
20
15
10
5
0
0
20
40
60
80
100

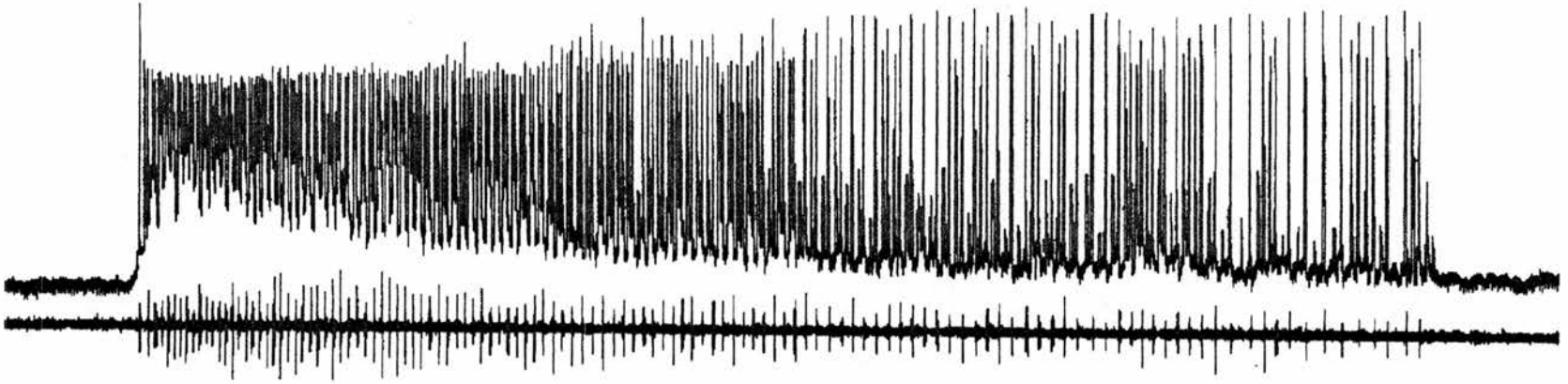
Figure 5.9. Changes in cycle period and burst duration of ventral root activity induced by NMDA.

a. Larval fictive swimming activity, induced with 50μM NMDA and 50μM DHKA, which showed a clear underlying slow modulation. Cycle periods and burst durations were measured from the boxed area.

bi. Graph of consecutive cycle periods measured over 9s. Cycle period decreased towards the point of maximum amplitude from 100ms and greater to 60ms or less.

ii. Graph of rostral and caudal burst durations measured over the same time interval. Both rostral (o) and caudal (•) burst duration increased towards the point of maximum ventral root amplitude and minimum cycle period.

A. *Network driven rhythmic activity recorded from a larval motorneuron*



B. *Intrinsic TTX resistant oscillations recorded from the same motorneuron*

Figure 5.10. Discrepancy between the frequency of rhythmic swimming activity and intrinsic oscillations.

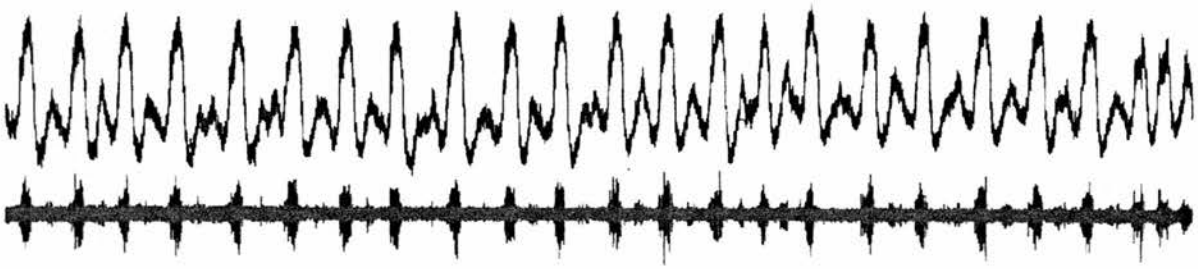
A. Episode of fictive swimming (125 cycles) in a stage 42 larva recorded from a motorneuron with an accompanying ventral root.
B. Intrinsic membrane potential oscillations (6 cycles) in same motorneuron in the presence of 0.5μM TTX, 100μM NMDA and 5μM 5HT.
Note the difference in frequencies of swimming an intrinsic oscillations.

oscillations, both of which have cycle period of approximately 2s. Secondly, there is a developmental increase in the number of preparations which display an underlying slow modulation of NMDA-induced rhythmic activity. This increase coincides the expression of 5HT receptors on postsynaptic receptors which just precedes the innervation of the cord with projections from serotoneric raphe neurons. Finally, it was speculated from experiments which explored the effects of hyperpolarising current on network oscillations, that voltage-dependent membrane properties might contribute to the network oscillations. I therefore set out to obtain more direct evidence that the slow modulation of swimming which is accentuated after blocking inhibition did indeed result from intrinsic 5HT-dependent membrane oscillations.

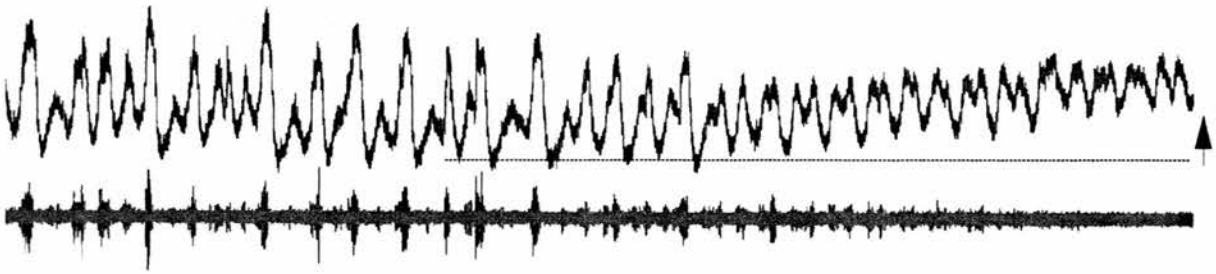
4. Network oscillations are blocked by TTX.

If the network oscillations are driven by intrinsic oscillations then it might be expected that they would persist in the presence of TTX. However, after the addition of 0.5μM TTX, the network oscillations (figure 5.11a) diminished, the membrane depolarised (figure 5.11b) and within 4 minutes of adding TTX to the stock bottle the oscillations had been completely abolished (iii). However, since TTX prevents spike activity, it could also prevent the release of endogenous 5HT from serotonergic interneurons upon which the intrinsic oscillations may depend. To test this idea, 5μM 5HT was added to the circulating saline after the network oscillations had been abolished by 0.5μM TTX (fig. 5.12a). As 5HT reached the preparation (b), the neuron hyperpolarised again and around three minutes after adding 5HT, intrinsic oscillations were initiated with a frequency of 0.5Hz (c).

a. 100μM NMDA, 5μM strychnine, 20μM bicuculline



b. 2 mins in 0.5μM TTX



c. 4 mins 0.5μM TTX

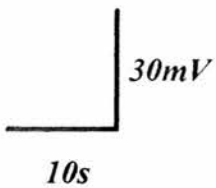
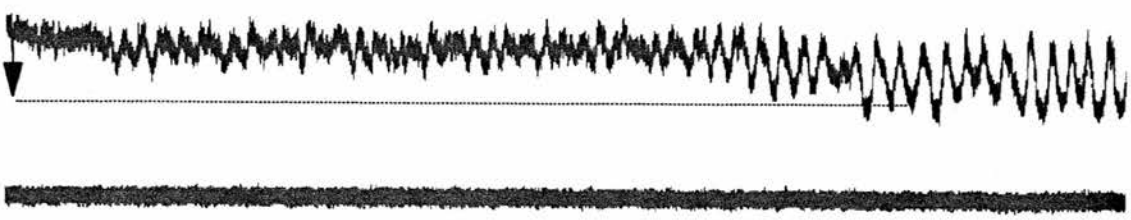


Figure 5.11. Network oscillations are abolished by TTX.

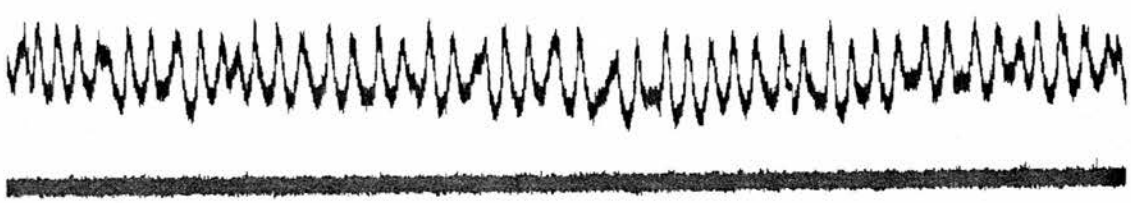
a. Network oscillations in the presence of 100μM NMDA. 5μM strychnine and 20μM bicuculline recorded from a larval motorneuron.
b.. Two minutes after adding 0.5μM TTX to the circulating saline, network oscillations began to diminish and the membrane potential depolarised by 10mV.
c. After 4 minutes in 0.5μM TTX, the network oscillations were completely abolished and the membrane potential became locked in a depolarised state.

a. 4 mins 0.5μM TTX

b. 2 mins 5μM 5HT



c. 3 mins 5μM 5HT



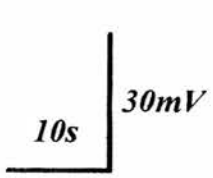


Figure 5.12. TTX resistant membrane potential oscillations are induced by 5HT.

No intrinsic oscillations were seen in the presence of 100μM NMDA and 0.5μM TTX (**a**). Shortly after the addition of 5μM 5HT to the stock bottle the membrane potential hyperpolarised by 10mV and intrinsic membrane potentials were initiated (**b**) and maintained as long as 5HT was present (**c**).

5. Pharmacology of the intrinsic oscillations.

One way of determining more directly whether the intrinsic oscillations underlie the slow modulation of NMDA-induced activity would be to test whether a specific 5HT-receptor antagonist, known to block intrinsic oscillations, also abolished the slow modulation of continuous NMDA-induced activity. However, the receptor subtype which meditates the intrinsic oscillations has not yet been investigated. Developmental studies have shown that 5HT has many roles firstly in promoting the development of the more complex larval rhythm in the twenty four hour period after hatching and subsequently in maintaining the rhythm. Despite the wide array of 5HT receptor subtypes which have now been classified so far, only the 5HT1a receptor has been implicated in all of these effects. Additionally, it has been shown that similar 5HT-dependent oscillations in the closely related amphibian species *Rana temporaria* also result from the activation of 5HT1a receptors. One specific antagonist of this subtype of receptor is pindobind-5HT1a. Therefore the effect of this antagonist on intrinsic membrane oscillations was investigated (n=1). Figure 5.13 shows clearly that intrinsic membrane potential oscillations induced by prior application of 5HT (A) were abolished shortly after the addition of 10μM pindobind-5HT1a to the circulating saline. As pindobind-5HT1a washed on to the preparation (B) the membrane potential gradually depolarised, reversing the hyperpolarising effect of 5HT seen during the induction of the oscillations (figure 5.12b). The oscillations then ceased completely after a further two minutes exposure to pindobind-5HT1a (figure 5.13C). The effect of pindobind-5HT1a was at least partly reversed by washing off the

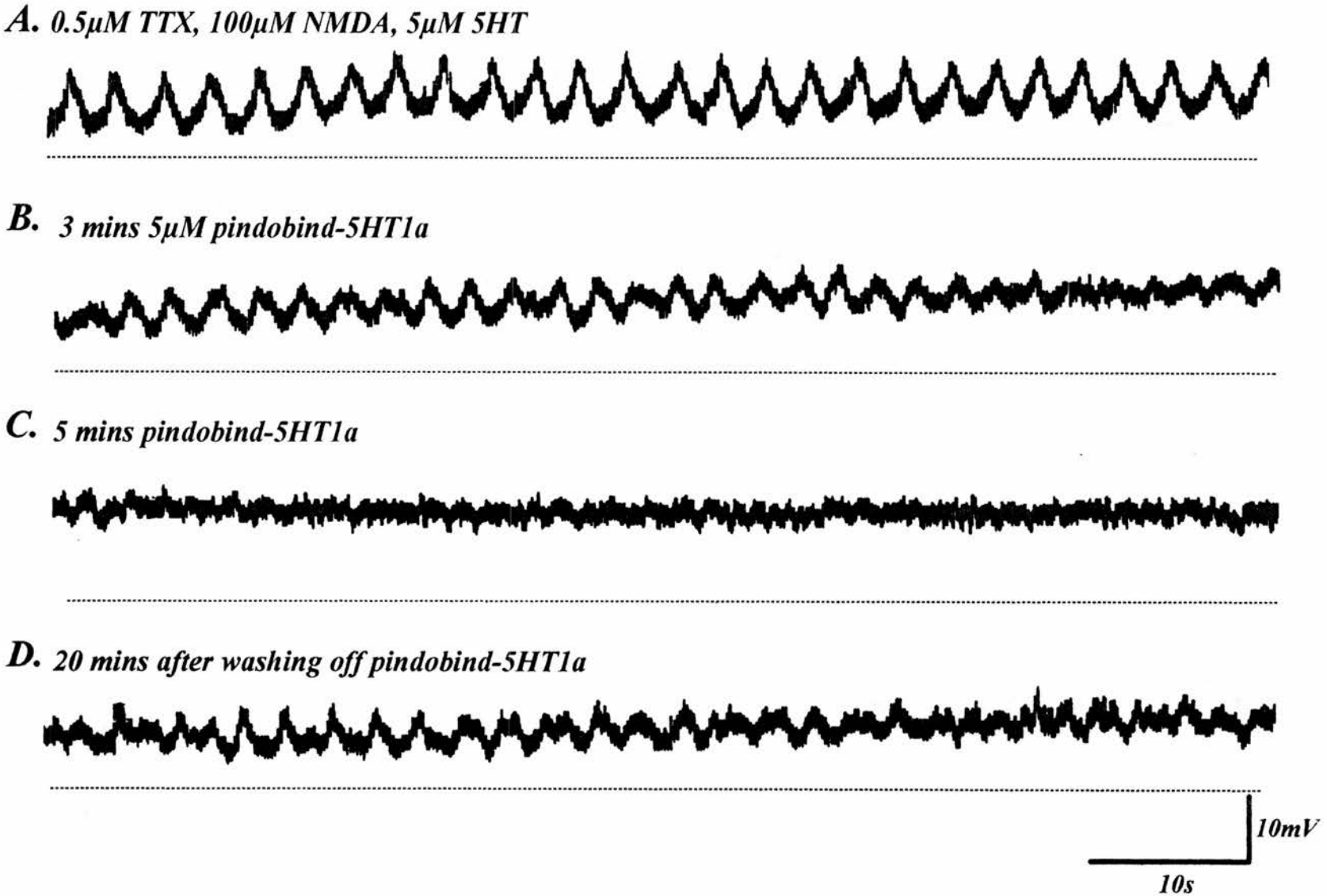

A. 0.5μM TTX, 100μM NMDA, 5μM 5HT
B. 3 mins 5μM pindobind-5HT1a
C. 5 mins pindobind-5HT1a
D. 20 mins after washing off pindobind-5HT1a
10mV
10s

Figure 5.13. Intrinsic membrane potential oscillations are blocked by a 5HT1a antagonist.

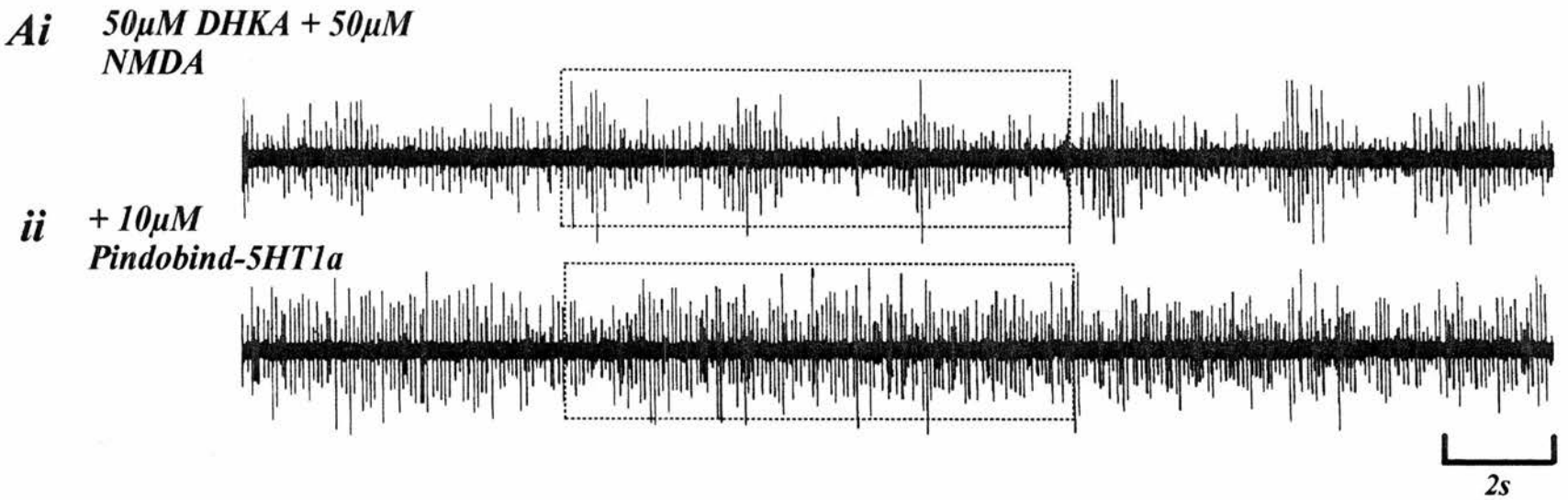
A. Intrinsic membrane potential oscillations recorded in the presence of 0.5μM TTX, 100μM NMDA and 5μM TTX.
B. 3 minutes after bath applying the 5HT1a receptor antagonist, pindobind-5HT1a, the membrane depolarised by 10mV.
C. After 5 minutes in the presence of pindobind-5HT1a intrinsic membrane potential oscillations were abolished.
D. Twenty minutes after removing pindobind-5HT1a from the circulating saline, there was a partial recovery of intrinsic membrane bistability.

antagonist (D). Thus the induction of 5HT-dependent oscillations appears to be mediated by a receptor with 5HT1a-like pharmacology.

6. The effects of pindobind-5HT1a on the slow modulation of larval activity.

I was now in a position to assess the possible contribution of intrinsic oscillatory membrane behaviour to NMDA-induced activity by using pindobind-5HT1a to block the expression of intrinsic oscillations. The effect of pindobind-5HT1a on NMDA-induced activity was investigated in preparations which clearly exhibited the underlying slow modulation (n=2). The results of these experiments are shown in figure 5.14. The slow modulation of swimming activity under control conditions is shown in the ventral root recordings (Ai) and also in the graph of cycle period (Bi) which shows that cycle period (measured from the data in the boxed area) varied between a maximum of 110-120ms and a minimum of around 60-80ms. After bath applying 10μM pindobind-5HT1a, the modulation of the rhythm was clearly abolished and the activity resembled that of the embryonic activity shown in figure 5.7A. This is reflected by the relatively constant cycle period shown in figure 5.14 Bii which was measured over the same time interval to control (boxed area in Aii). The average cycle period was now around 70ms. The increase in swimming frequency is consistent with the removal of oscillatory properties causing a nett depolarisation of rhythmic neurons (cf fig. 5.11b)

If the network oscillations were also due to an enhanced underlying slow modulation following blockade of glycine and GABAa receptors, they would also be expected to be abolished by pindobind-5HT1a. The effect of the antagonist on network oscillations is shown in figure 5.15. The


Ai
50μM DHKA + 50μM NMDA
ii
+ 10μM Pindobind-5HT1a
2s

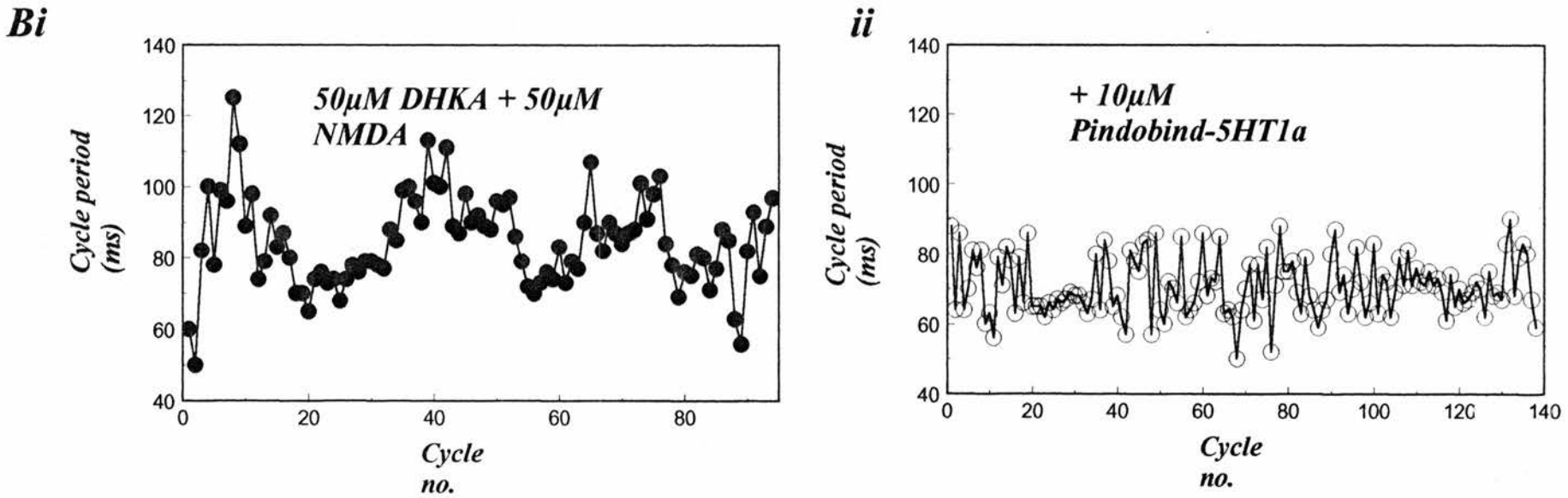

Bi
50μM DHKA + 50μM NMDA
Cycle period (ms)
Cycle no.
ii
+ 10μM Pindobind-5HT1a
Cycle period (ms)
Cycle no.

Figure 5.14. Pindobind-5HT1a abolishes the slow modulation of NMDA-induced ventral root activity.

Ai. Larval fictive swimming activity induced by 50μM DHKA and 50μM NMDA with an underlying slow modulation.
ii. After bath applying 10μM pindobind-5HT1a the underlying slow modulation was abolished as amplitudes became less variable.

Bi. Graph of cycle periods measured from the boxed are in Ai. Cycle periods varied from over 100ms to around 70ms and decreased towards the point over maximum amplitude.
ii. Graph of cycle periods measured from the boxed area in Aii. Cycle periods no longer showed the same cyclical variation and were within a much narrower range of around 60-80mV.

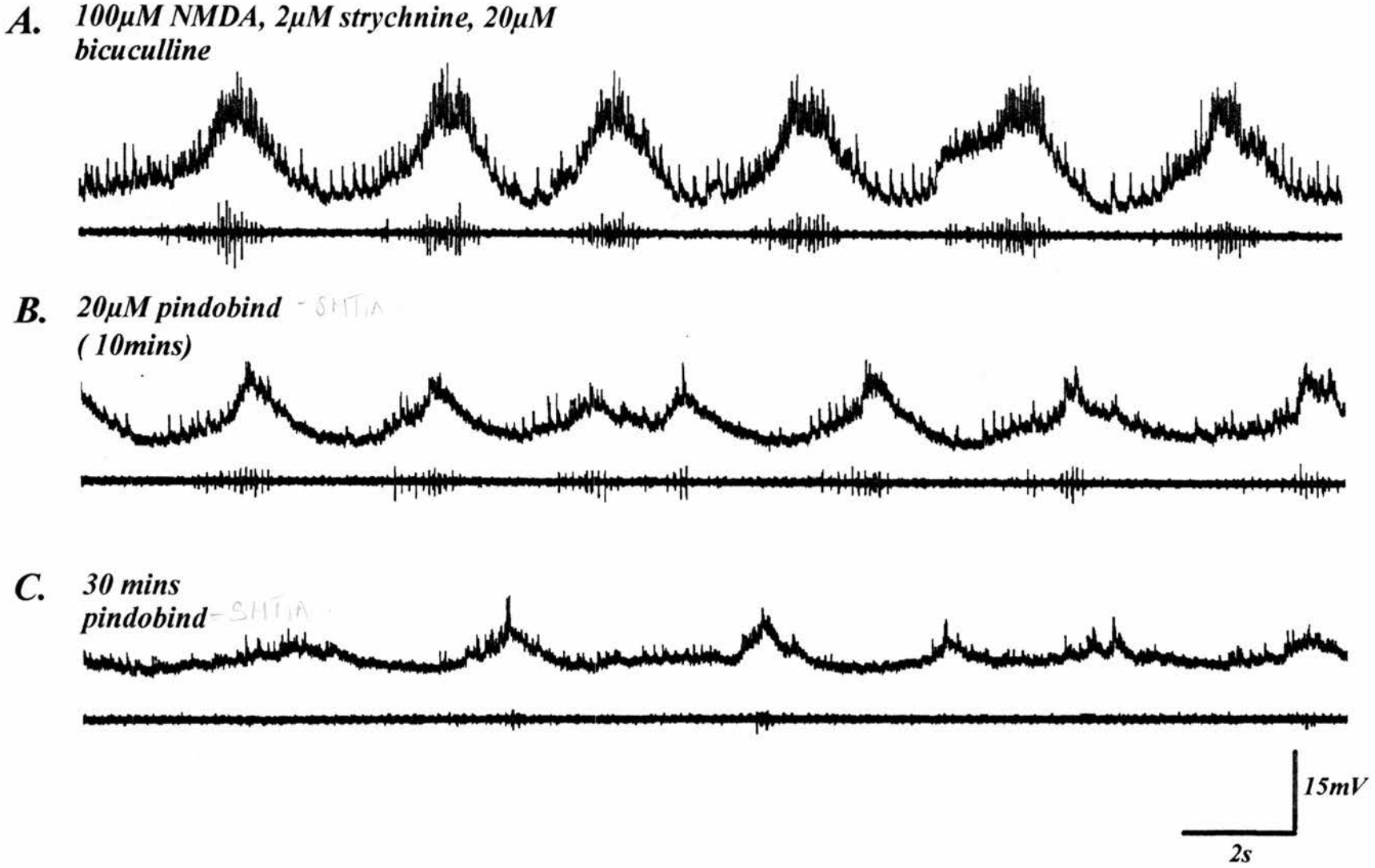

A.
100μM NMDA, 2μM strychnine, 20μM bicuculline
B.
20μM pindobind
(10mins)
C.
30 mins
pindobind
15mV
2s

Figure 5.15. Network oscillations are abolished in the presence of pindobind-5HT1a.

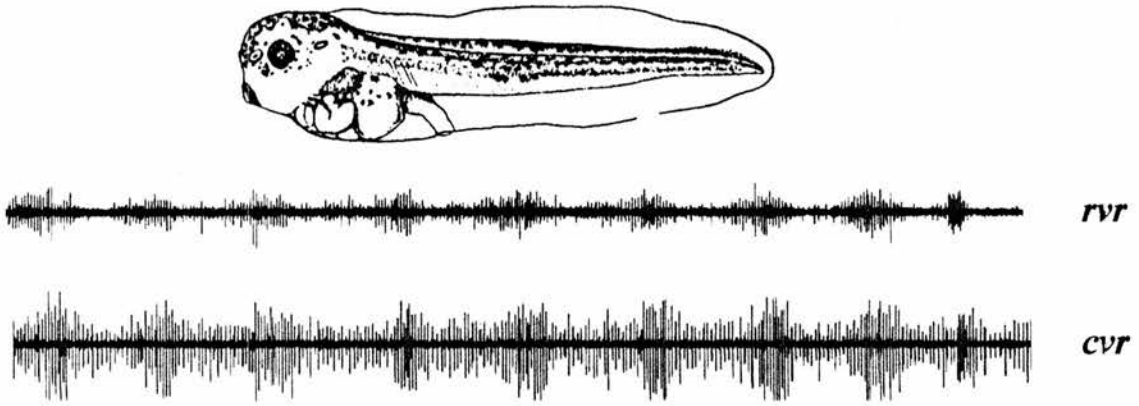
A. Network oscillations in the presence of 100μM NMDA, 2μM strychnine and 20μM bicuculline.
B. 10 minutes after bath applying 20μM pindobind-5HT1a, the oscillations were decrease in amplitude and less regular.
C. By 30 minutes in pindobind-5HT1a both the network oscillations and the ventral root activity were virtually abolished.

oscillations produced in the presence of 100μM NMDA, 2μM strychnine and 20μM bicuculline (A) began to diminish shortly after bath applying 20μM pindobind-5HT1a. 10 minutes after applying the antagonist the oscillations decreased in amplitude and became less regular (B). After 20 minutes, pindobind-5HT1a had virtually abolished both ventral root activity and the network oscillations (C).

The final piece of experimental data providing evidence for the requirement of an endogenous source of 5HT mediating membrane bistability during swimming activity in NMDA, came from spinalisation studies. Brainstem raphe neurons are thought to be the only source of 5HT in the *Xenopus* CNS (van Mier et al., 1986), so spinalisation at the level of the otic capsule should remove all sources of intrinsic 5HT to the spinal motor system. Figure 5.16 compares the activity recorded in an intact larva in the presence of 70μM NMDA and 50μM DHKA (A) with the activity of a spinalised larva under the same conditions (B). It can be seen that following the removal of all descending influences from the fore- and midbrain, including the raphe nucleus, the slow modulation of swimming activity is no longer observed (n=7).

Taken together, these results provide strong evidence that the slow modulation of swimming activity occurring in the presence of NMDA results from intrinsic membrane properties, which are expressed following endogenous release of 5HT from raphe neurons. This in turn suggests that the raphe neurons are activated by NMDA and release 5HT into the spinal cord.

A. Intact stage 42



B. Spinalized stage 42

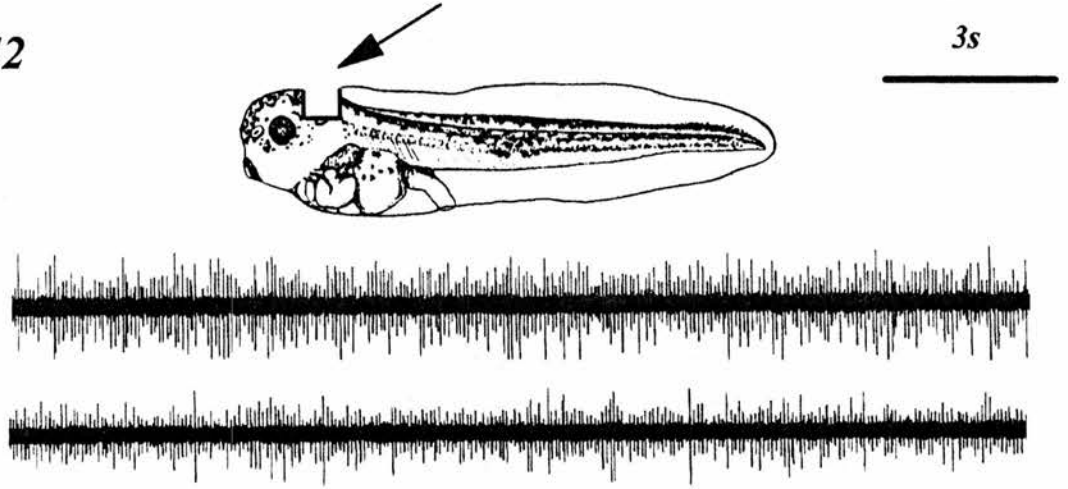


Figure 5.16. Spinalisation abolishes the slow modulation of NMDA-induced ventral root activity.

In comparison with the modulated ventral root activity recorded from intact larva in the presence of NMDA (**A**), after spinalising an animal at the level of the otic capsule, NMDA induced a continuous pattern of activity with little variation in the ventral root amplitude (**B**).

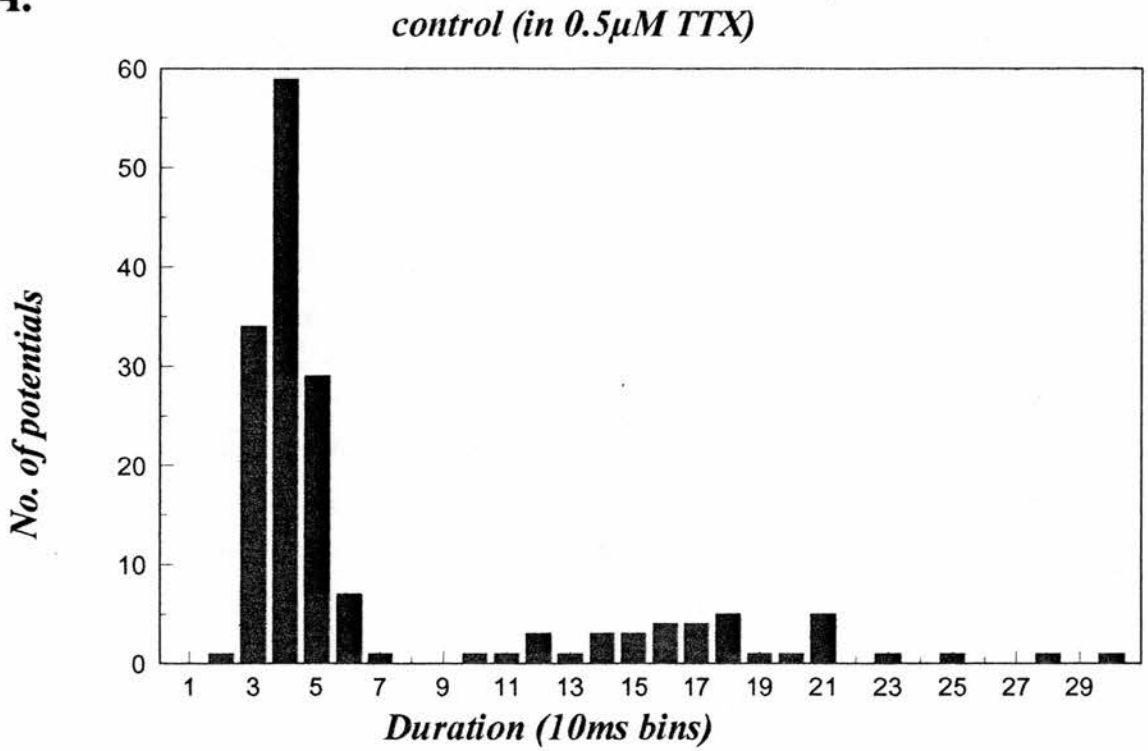
7. 5HT modulates GABA release.

The evidence presented above suggests that intrinsic oscillations can influence swimming activity over several consecutive cycles and that its effects on swimming activity are more pronounced if inhibitory transmission is reduced. It has already been reported that 5HT presynaptically reduces the amount of glycine released from the terminals of commissural interneurons in the spinal cord (Sillar and Wedderburn, 1994, see introduction) which may contribute to the observed increase in ventral root burst durations seen as their amplitudes increase. These results were obtained by examining the effect of 5HT on the spontaneous release of glycine in the presence of TTX and bicuculline, to block spontaneous GABA release. The analysis showed that after the addition of 5HT, the amplitudes of spontaneous ipsps were not significantly altered but that the occurrence of spontaneous glycinergic potentials was greatly decreased. This therefore suggested that 5HT was not altering the efficacy of postsynaptic glycine receptors but was causing a reduction in the liberation of transmitter from presynaptic terminals.

The previous results chapter showed that GABA plays an increasingly more important role in modulating larval swimming, generally opposing the effects of 5HT. I have therefore investigated whether the global action of 5HT includes a similar reduction in GABA release to that reported for glycine. Initial experiments were carried out in the presence of TTX alone, leaving both GABA and glycine spontaneous potentials present in intracellular recordings from motorneurons. This allowed the already established data of 5HT's effect on glycine release to be used as an indication of the potency of the amine. 5HT is a light sensitive chemical and therefore a negative result for GABA could have been due to a lack of

potency of 5HT. The two types of potential were however still separable by their different durations (as previously described in chapter 3). An example of the results obtained is shown in figure 5.17 (n=4). Graph A, shows the range of amplitudes of spontaneous ipsps measured over a 3 minute time interval. Potentials with durations ranging from 18-60ms were the result of glycine receptor activation. Longer duration potentials, of which there were far fewer, ranged from 92-296ms and were the result of GABAa receptor activation (cf. figure 3.11). Ten minutes after bath applying 5μM 5HT, measurements were again made of the duration of spontaneous potentials over the same time interval. As can be seen from graph B, the total number of spontaneous potentials was significantly decreased. The number of glycine potentials decreased from 131 in control to 62 in the presence of 5μM 5HT. Similarly, the number of potentials resulting from GABAa receptor activation were reduced from 36 to only 8 potentials over the same time interval under 5μM 5HT. These results provide evidence for a presynaptic site of action for 5HT resulting in a decrease in the rate of spontaneous GABA release. However, the number of GABA potentials was always too small in the presence of 5HT to determine whether the range of amplitudes was significantly affected and therefore I cannot rule out any parallel action of 5HT on postsynaptic GABAa receptors. In order to determine whether the presynaptic action of the transmitter resulted from an indirect decrease in the amount of calcium entering the terminal through voltage-dependent calcium channels or by a direct action on the release machinery, similar experiments were carried out in the presence of TTX and $CdCl_2$ to block calcium entry. The results are shown in figure 5.18 (n=2). Figure 5.18a, shows 80s of raw data in the presence of 0.5μM TTX, 200μM $CdCl_2$ and 5μM strychnine to block spontaneous glycine release. Clearly spontaneous GABA release does not rely on a calcium influx. After

A.


control (in 0.5μM TTX)
No. of potentials
0
10
20
30
40
50
60
1
3
5
7
9
11
13
15
17
19
21
23
25
27
29
Duration (10ms bins)

B.

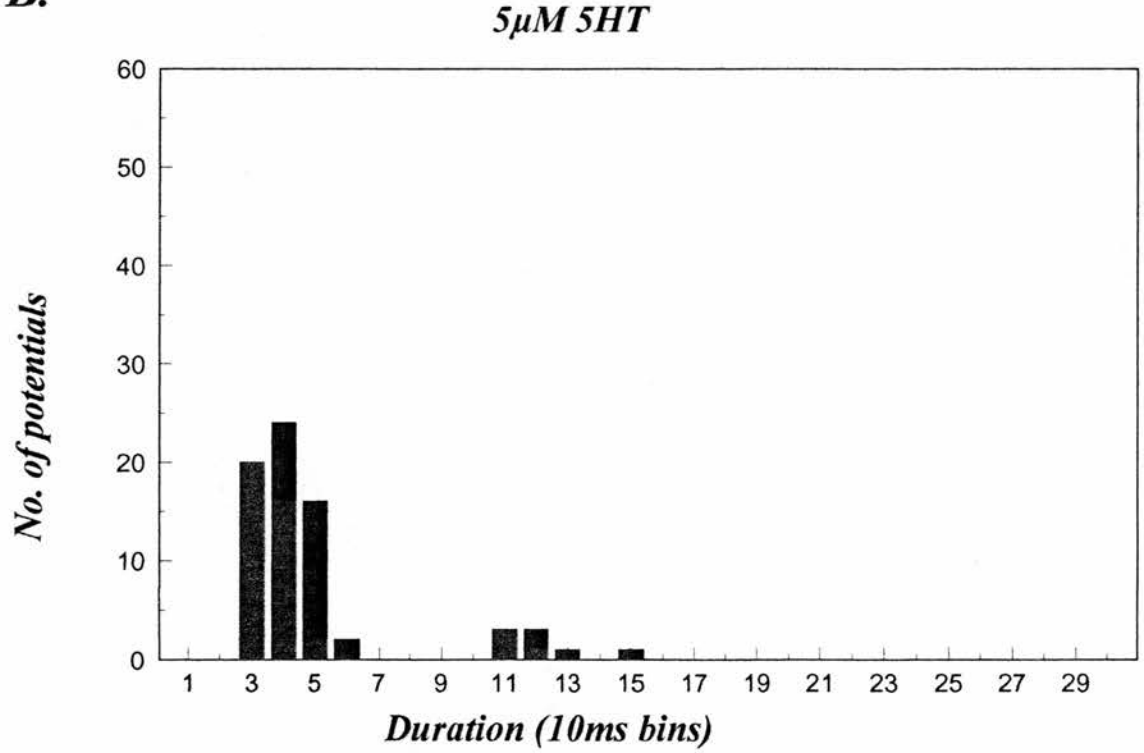

5μM 5HT
No. of potentials
0
10
20
30
40
50
60
1
3
5
7
9
11
13
15
17
19
21
23
25
27
29
Duration (10ms bins)

Figure 5.17. 5HT reduces spontaneous inhibitory transmission.

A. Graph of the duration of spontaneous inhibitory potentials recorded over a 3 minute time interval in control saline. 131 spontaneous glycine potentials were measured which ranged from 18-60ms and 36 GABA potentials were measured which ranged from 92-296ms.
B. After bath application of 5μM 5HT, 62 glycine potentials and 8 GABA potentials were measured over the same time interval.

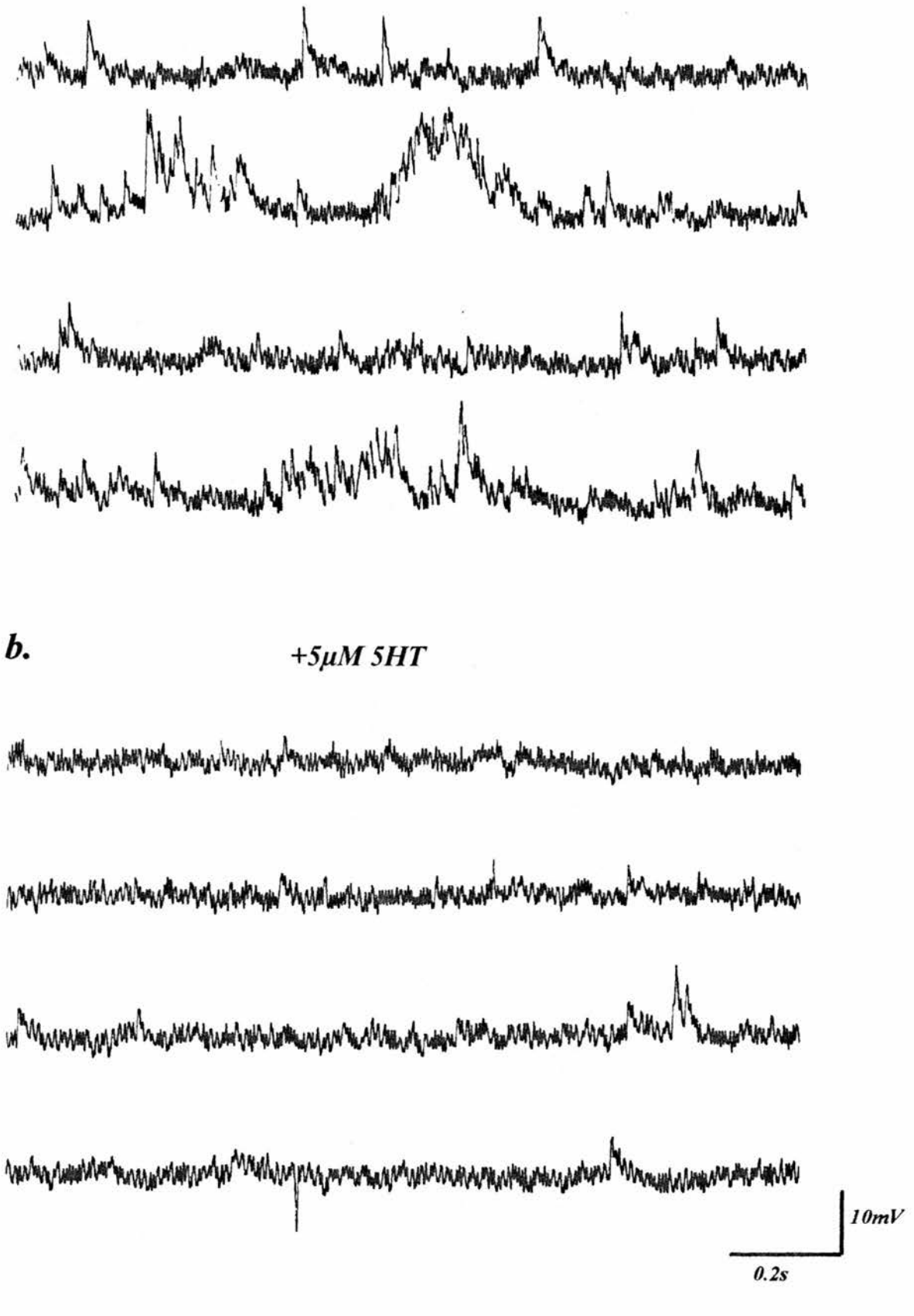

a.
0.5μM TTX, 200μM CdCl2, 5μM strychnine, 5μM 5β3α
b.
+5μM 5HT
10mV
0.2s

Figure 5.18. 5HT reduces spontaneous GABA release in the presence of TTX and $CdCl_2$.

a. Spontaneous GABA release was recorded in the presence of 0.5μM TTX, 200μM $CdCl_2$, 5μM strychnine and 5μM 5β3α. Their numbers were increased sufficiently to summate.
b. In the presence of 5μM 5HT the frequency of spontaneous release is markedly reduced.

bath applying 5μM 5HT the number of GABA potentials was still significantly decreased, suggesting that the mechanism of action of 5HT on the presynaptic GABA terminals involved a direct action on the release machinery. The presence of cadmium chloride also caused an increase in the frequency of spontaneous GABA ipsps which is thought to result from an increase in intracellular calcium due to its toxicity on the Na/Ca exchange pump at higher concentrations (Kimura et al., 1987). However, this still did not allow a detailed investigation into any possible actions of 5HT on the postsynaptic response of GABAa receptor activation because the number of potentials was increased to the extent that consecutive ipsps summated. This made it impossible to discriminate and measure the amplitudes of individual potentials to determine whether or not 5HT had any additional postsynaptic effects.

The results in this section show that 5HT regulates the amount of inhibitory transmitter available for release by presynaptically inhibiting GABA and as well as glycine release. As yet, the 5HT receptor mediating the effects on GABA release have not been investigated but evidence from the experiments investigating the pharmacology of 5HT's action on glycine release have indicated that like the intrinsic oscillations, the amine's effects are mediated through the 5HT1a receptor subtype (Sillar and Wedderburn, 1994).

Discussion.

The data presented in this chapter strongly suggest that 5HT-dependent oscillations in membrane potential, which can be recorded from motorneurons in the presence of TTX and NMDA, contribute to NMDA-induced fictive swimming, and that the slow pattern of rhythmic activity produced following blockade of GABAa and glycine receptors involves an enhancement of a pre-existing slow 5HT-dependent oscillatory mechanism. Indirect evidence, including the effects of current injection into motorneurons during network oscillations, pointed to a role for intrinsic membrane properties in the slow modulation of fictive swimming, seen when inhibitory transmission is blocked, as similar effects are seen following hyperpolarising current injection during intrinsic 5HT-dependent membrane potential oscillations, recorded from motorneurons in the presence of TTX and NMDA. In addition, there is a developmental increase in the number of preparations in which a clear underlying slow modulation of swimming activity was recorded in the presence of NMDA. This indicates that the slow rhythm might result from modulatory influences which develop during the first day after hatching, as does the innervation of the spinal cord by serotonergic raphespinal fibres (van Mier et al., 1986). It was also noted that, in some preparations, removing inhibition had a more profound effect on recordings from more rostral roots than more caudal roots (eg. figure 5.9A), which might also indicate that the modulatory influence descends from the brainstem during development. Even at stage 42, it has previously been noted that rostral burst durations tend to be longer than caudal burst durations, possibly reflecting a greater modulatory influence of 5HT on rhythmically active neurons at the rostral end of the cord (Sillar et al., 1991).

From the above data, it seemed feasible that, in the presence of NMDA, 5HT-dependent oscillations might rhythmically modulate swimming. More direct evidence for a role for 5HT-dependent intrinsic membrane potential oscillations was obtained from experiments that showed that the same antagonist which abolished intrinsic membrane potential oscillations also abolished the slow modulation of NMDA-induced ventral root activity and network oscillations (figures 5.14 and 5.15). The intrinsic membrane potential oscillations were blocked by a specific mammalian 5HT1a receptor antagonist, pindobind-5HT1a. Although I have only shown that intrinsic membrane potential oscillations are abolished by pindobind-5HT1a on one occasion, this result has subsequently been repeated (J.F.S. Wedderburn, unpublished observation). Thus, in keeping with the other cellular and synaptic actions of 5HT on the larval swimming system, intrinsic NMDA-receptor mediated membrane potential oscillations are mediated by a receptor which resembles the mammalian 5HT1a receptor. In the mammalian CNS, 5HT1a receptors usually mediate a down regulation of adenylate cyclase via a pertussis toxin sensitive Gi protein (Beer et al., 1993). It has recently been shown that the effects of 5HT on the lamprey spinal cord, including the reduction in K_{Ca} currents, are also mediated through a receptor with similar pharmacology to the mammalian 5HT1a receptor. However, the second messenger system appears to be different and it has been suggested that the binding of 5HT to the receptor may activate a pertussis toxin insensitive G-protein which is directly linked to an ion channel (Wikström et al., 1995).

The final piece of direct evidence for a role for 5HT-dependent oscillations, came from experiments in which animals were spinalised at a level of the otic capsule to remove any influence from raphe neurons and as

a result the slow modulation of NMDA-induced activity was also abolished. Although the subsequent bath application of 5HT would be expected to reinstate the slow modulation, this effect was not observed and in fact swimming activity was completely abolished by the amine (not shown). A explanation for this paradoxical effect emerged from a recent study which showed that although 5HT has an nett facilitatory effect on swimming activity in the intact animal, not all of its individual effects are excitatory. For example, bath application of 5HT to high-spinalised larval preparations actually inhibits swimming (Sillar and Woolston, 1995) and intracellular recordings also show that 5HT causes membrane hyperpolarisation (see figure 5.12). These results contrast with reports in other vertebrate preparations of the direct effect of 5HT on motorneurons. In the neonatal rat, 5HT has been shown to have a direct excitatory effect on motorneurons which is thought to be mediated by the 5HT1a receptor (Takahishi and Berger, 1990) and in turtle motorneurons, bath application of 5HT depolarises rather than hyperpolarises the membrane (Hounsgaard and Kiehn, 1989). In *Xenopus* larval preparations, 5HT has been proposed to play a dual role involving a direct inhibition of motorneurons in parallel with the facilitation of descending excitatory pathways. In the intact preparation, the facilitatory effect is presumably sufficient to overcome the spinal inhibitory action, resulting in an overall excitatory influence of 5HT. However, following spinalisation, the inhibitory action appears to become dominant. This proposal could explain why bath application of 5HT to a spinal preparation does not reinstate the slow modulation of NMDA induced activity.

Thus, the overall excitatory effect of 5HT on swimming probably results from several different cellular and synaptic effects occurring in parallel. It has already been shown that 5HT presynaptically inhibits

glycine release from commissural interneurons (Sillar and Wedderburn, 1994) by directly affecting the availability of vesicles for release. In this chapter, I have also shown that 5HT similarly decreases the probability of GABA release from presynaptic spinal terminals (although an additional action on the postsynaptic membrane cannnot yet be ruled out entirely). This overall decrease in inhibitory transmission is likely to further enhance the expression of intrinsic 5HT-dependent membrane properties. A decrease in midcycle inhibition following a reduction in glycine release could contribute to the increased burst durations which coincide with the increased amplitude of ventral root activity. Additionally, since GABAa receptor activation has been shown to have an increasing modulatory influence over larval swimming (see chapter 4) this could also prevent the expression of membrane bistability. Thus, the ability of 5HT to decrease the amount of GABA release into the synaptic cleft is also likely to enhance the expression of intrinsic membrane bistability, especially towards the end of an episode when GABA appears to have the greatest influence over swimming (see also chapter 4 discussion).

A similar slow modulation of swimming of activity has also been reported during lamprey swimming activity in the presence of NMDA and strychnine, in that blocking glycine receptors reveals a slow waxing and waning of ventral root burst amplitude (McPherson et al., 1994). As in *Xenopus* larvae, lamprey burst durations also increase, with the maximum burst amplitudes coinciding with the minimum cycle periods. Initially these authors assumed that the slow rhythm seen in the presence of strychnine was not present during control activity. However, on closer examination, cycle periods were also shown to undergo cyclical variation in control conditions, even when swimming activity appeared quite regular. In

Xenopus larvae the slow modulation of rhythmic ventral root activity in the presence of NMDA was only apparent in around 66% of preparations, however, closer analysis of the cycle period in the remaining preparations may have revealed an underlying slow modulation since all preparations did show a similar pattern of activity following the bath application of either strychnine or bicuculline regardless of whether there was already an obvious slow modulation in the presence of NMDA alone. Although the slow modulation of swimming activity in the lamprey has not been linked with 5HT (McPherson et al., 1994), these authors do not exclude the possibility that the slow modulation could result from endogenous properties of motorneurons and comment that it has been shown that 5HT can cause a slow rhythm in the firing of fin motorneurons during swimming (Macpherson et al., 1994). It may be of importance that although the intrinsic oscillations which have been reported in the lamprey do not require exogenous 5HT, serotonergic neurons are present in the spinal cord (Christenson et al., 1990). In other species, including *Xenopus,* they are confined to the raphe. Consequently, in the lamprey, in the presence of TTX and NMDA there may still be sufficient spontaneous release of 5HT in the spinal cord to enable the intrinsic oscillatory mechanism.

In *Xenopus* larvae, 5HT-dependent intrinsic oscillations do not appear to influence swimming on a cycle-by-cycle basis since swimming continues in the presence pindobind-5HT1a and NAN-190 which block intrinsic oscillations. This already seemed unlikely due to the discrepancy between the frequency of the intrinsic oscillations (ca. 0.5Hz) and the range of frequencies during fictive swimming (10-20Hz). The intrinsic oscillations do, however, slowly modulate the NMDA-induced swimming rhythm over several consecutive cycles, causing a periodic increase in the

intensity and amplitude of ventral root activity. This suggests that there is a cyclical increase in the excitatory drive to motorneurons and a recruitment of motorneurons from within the pool that innervates each myotome. However, these results were obtained from experiments carried out in the presence of NMDA and under these conditions the raphe neurons themselves may be constantly activated for which there is no evidence during stimulus evoked swimming activity in the absence of NMDA. In chapter 4, it was shown that skin stimulation elicits only a single burst of non-rhythmic activity in the presence of strychnine and bicuculline. This could reflect the induction of membrane bistability by endogenous release of 5HT from raphe neurons activated by the stimulus. However, although there was a general increase in spontaneous bursting activity, presumably due to the excitable state of the network, regular network oscillations like those seen under NMDA were not evoked despite the preparation being intact. This suggests that the underlying intrinsic membrane potential oscillations occurring under NMDA, could well be an artefact of the experimental conditions in which 5HT neurons are persistently activated by the agonist.

A possible role for 5HT-dependent membrane potential bistability is illustrated in figure 5.19. In general, cycle periods increase during the course of a swim episode. However, in most larval preparations (but not embryonic preparations), sudden accelerations in swimming frequency, accompanied by an increase in the intensity and duration of motor bursts lasting for several consecutive cycles are often observed (eg. at the arrow). Intracellular recordings show that the membrane potential over this period of increased activity follows a similar time course and profile to that of an intrinsic oscillation. I propose, therefore, that such a change in frequency could occur as the result of endogenous 5HT release triggering bistable

Modulation of rhythmic activity during an episode of fictive swimming

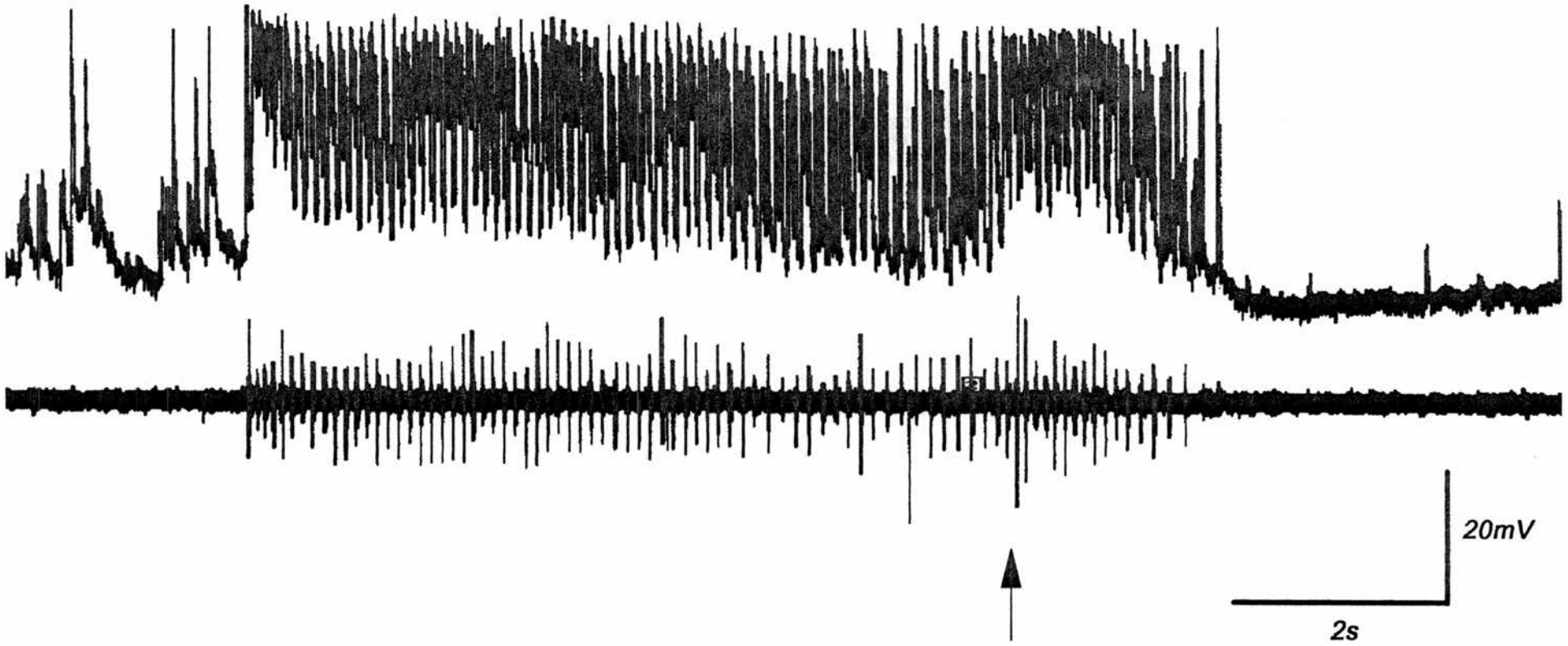


Figure 5.19. A role for membrane bistability during larval fictive swimming?

In general, cycle periods increase during the course of swim episodes. However, it has been noted that sudden increases in frequency can occur (eg. at arrow) which last for a few seconds. This transient increase in swimming frequency could occur as the result of endogenous 5HT release triggering bistable membrane properties within components of the locomotor network.

membrane properties within components of the locomotor network. These results are in contrast to those reported in the neonatal rat, in that although similar bursts of activity are recorded after blocking glycine and GABAa receptors (see chapter 4 discussion), the authors show that the activity results from large synchronous synaptic events rather than the result of intrinsic membrane properties (Bracci et al., 1996), which can be modulated but are not dependent on 5HT.

The possible role of intrinsic membrane properties, during *Xenopus* larval fictive swimming, could be assessed directly by examining the effects of pindobind-5HT1a during stimulus evoked fictive swimming activity. According to my proposal, this should prevent such sudden changes in swimming frequency. Additionally, a further piece of evidence which would be instrumental in determining the role of 5HT-dependent intrinsic membrane bistability during swimming would be to investigate the firing properties of serotonergic raphespinal projection neurons during swimming activity and then correlate these with spontaneous changes in the intensity and frequency of swimming. Experiments on cats have linked the firing activity of neurons in the raphe with locomotor activity and have shown that in a 'resting' state raphe neurons fire tonically at a low rate which can then increase and even become rhythmically active with increasing levels of motor activity (Jacobs, 1994). Experiments carried out on freely moving cats also confirmed that serotonergic neurons are responsive to specific motor challenges rather than an indirect increase in arousal (Veasey et al., 1995).

CHAPTER 6.
General Discussion.

The embryo of the African clawed toad, *Xenopus laevis*, has proven to be an excellent model for investigating some of the basic neural circuitry controlling locomotion; circuitry which has subsequently been shown to be remarkably similar to that which drives more complex locomotor activity in higher and adult vertebrates. Once released from its egg membrane, the embryo spends around 98% of the time stationary, suspended by a strand of mucus, secreted from the head cement gland, to the surface of the water or to obstructions which it has contacted. It has no need to seek food, being nourished entirely by its yolk sack. It is perhaps not surprising then, that the locomotor pattern generated at this early stage in development, though well coordinated and effective, is somewhat stereotyped and lacks the precision and flexibility required of more mature locomotor systems.

Around twenty four hours later, the animal is about to embark on an extended, free feeding and free swimming larval stage. The yolk sack is almost completely depleted and the mouth and gut have now developed. The larva has increased in length by around 40%, existing myotomes have been enlarged and new myotomes have been added caudally (Tunstall and Sillar, 1993). The reliability of both the stopping response and the response to dimming of the illumination are declining. Presumably these are no longer appropriate behaviours for a free-feeding larva. As a consequence, the animal requires a more adaptable and powerful swimming system and indeed, fictive swimming recorded at this stage shows a far greater complexity and flexibility (Sillar et al., 1992b). The increased complexity does not appear to result from any major changes in the wiring of the spinal CPG but instead results, at least partly, from the action of descending modulatory systems which develop over the first twenty four hours of larval life. One such descending influence is from serotonergic raphe spinal projections (Sillar et al.,1992b, Sillar et al., 1995b). 5HT appears to be

instrumental both in the development of the more complex pattern of locomotor activity and in exerting an overall excitatory modulatory influence on the larval swimming CPG. The present study has shown that, over the same time period in development, the inhibitory amino acid GABA also plays an increasingly more important role where it not only acts like a 'brake' to terminate fictive swimming activity, but can also finely tune the pattern of sustained rhythmic activity.

In keeping with results from other preparations (eg. neonatal rat - Cazalets, 1995, lamprey - Wállen, 1995), larval fictive swimming is apparently modulated by both excitatory and inhibitory influences and the final motor pattern then depends on the balance between the two. Consistent with this idea, I noted that it was far more difficult to elicit continuous swimming following the bath application of NMDA to intact larval preparations when compared to either low spinal larval preparations or intact embryonic preparations. In intact larval preparations a discontinuous pattern of rhythmic activity was often elicited after bath applying NMDA. In contrast, shortly after applying NMDA to low spinal preparations, continuous activity was always recorded. This suggests that descending modulatory influences, which are removed by spinalisation, could be preventing continuous activity. This could either be due to an NMDA-mediated increase in GABA transmission which prevents the swimming pattern from becoming continuous, or alternatively, through increased excitatory influences leading to a similar pattern of interrupted activity. The continuous activity elicited by NMDA in intact embryos would then be explicable by the fact that the modulatory influences have yet to become properly established. Similarly in the neonatal rat, the pattern of activity recorded in the presence of high levels of NMDA and low levels of bicuculline is very similar to those recorded with low levels of NMDA and

high levels of bicuculline (Cazalets et al., 1994). It was also found that bicuculline or strychnine could elicit a weak synchronous activity but usually required the bath application of NMDA, ACh or 5HT to promote sustained rhythmic activity (Cowley and Schmidt, 1995).

During development it would appear that layers of complexity are added to basic locomotor circuitry which is established very early in ontogeny (Sillar, 1994). The relatively simple *Xenopus* preparation has initially allowed the basic embryonic circuitry to be described in considerable detail (see Roberts, 1990 for review). This then provided a platform for subsequent developmental studies of the modulatory influences that enable the locomotor system to become more flexible during a comparatively brief developmental period (Sillar et al., 1995a). In many vertebrate systems the basic spinal circuits are formed very early in embryogenisis and are then increasingly influenced by higher brain centres during development so that they acquire the precision and flexibility which is important for adult life (Sillar, 1994). In the rat, coordinated movement patterns can be observed before birth (Bekoff and Lau, 1980) but until around postnatal day 15, the locomotor pattern lacks agility and fluidity (Westerega and Gramsbergen, 1990). The rapid development of a more adult-like pattern of activity is thought to occur at least partly via the maturation of supraspinal descending control systems (Westerega and Gramsbergen, 1993). Similarly, maturation of human locomotion is also regarded as a process whereby spinal circuits become increasingly more influenced by higher brain centres which continue to develop over the second half of the first year after birth. These influences can turn on or off the spinal rhythm generators which may initially have been controlled by spinal reflexes (Forssberg et al., 1991). Similarly, in the *Xenopus* embryo, the mhr neurons are involved in a reflex pathway which stops the animal

swimming when it contacts an obstruction (Boothby and Roberts, 1992b). In this study I have shown that by stage 42, as the dimming response is declining, mhr neurons are becoming involved in an intrinsic descending pathway which terminates episodes of larval fictive swimming.

Growing evidence suggests that myotomal motorneurons in *Xenopus* are not just passive output elements of the spinal CPG. Apart from intrinsic bistable membrane properties, recorded from larval motorneurons, which contribute to their own output (Wedderburn and Sillar, 1994), motorneurons have been shown to make both electrical and cholinergic synapses and contribute to the on-cycle excitation recorded during fictive swimming (Perrins and Roberts, 1995b,c, see general introduction). Additionally, by stage 42, it would appear that myotomal motorneurons no longer constitute a single homogeneous population. In contrast to the embryo swimming rhythm in which each motorneuron appears to fire only once on every phase of the swim cycle, only some larval motorneurons show this pattern of firing, while others only appear to be recruited during intense activity and can drop out toward the end of an episode (Sillar et al., 1992b). This facility for motorneuron recruitment may provide another layer of flexibility on the final output from the swimming generator, allowing the animal to increase the intensity of motor ouput and engage in more powerful swimming. Furthermore, if the intensity of activity on one side is increased relative to the other, this may allow the animal to turn in appropriate directions. Thus, there would appear to be a difference in the firing capability of motorneurons, possibly resulting from differences in the intrinsic membrane properties of individual neurons. Moreover, there appear to be differences in the level of synaptic input from modulatory influences such as 5HT and GABA onto individual motorneurons. For

example, only some larval motorneurons (around 45%) receive GABA potentials at the end of an episode of swimming and only a proportion of motorneurons (approximately 60%, Wedderburn and Sillar unpulished observations) oscillate in the presence of NMDA and 5HT. Similar percentages of neurons have been reported to express membrane bistability in the presence of 5HT in other vertebrate preparations. In the cat, the proportion of neurons which express 5HT-dependent membrane bistability was reported to be 64% (Hounsgaard et al., 1988) and in turtle the proportion of neurons is approximately 60% (Hounsgaard and Kiehn, 1989). Intrinsic membrane potential oscillations were also observed in around 60% of lamprey motorneurons (Wállen and Grillner) and in 66% of neonatal rat motorneurons (Hochman et al., 1994b) in the presence of TTX and NMDA. In turtle motorneurons there was found to be no difference in the number of motorneurons in lumbar compared with cervical regions which express bistability (Hounsgaard and Kiehn, 1989) suggesting all regions of the cord are similarly modulated by the amine. Likewise, over the range of positions of recordings made from different *Xenopus* larval motorneurons (P.O.M. 3 to P.O.M. 8), there also appeared to be no direct relationship between the level of the recording site and whether or not GABA potentials were recorded at the end of an episode. However, no recordings have yet been made from motorneurons at the very caudal end of the cord. Thus, within each motorneuron pool, there could be a range of motorneurons which differ not only in their firing properties, but also in their synaptic inputs from modulatory neurons.

A major advantage of the *Xenopus* swimming system over all other vertebrate preparations is that fictive locomotion can reliably be elicited by natural sensory stimulation without the requirement of electrical activation

of the brain or pharmacological intervention. The data presented in this thesis suggest that endogenous modulators may be released during NMDA-induced swimming activity where they either would not normally fire (eg, mhr neurons firing during NMDA-induced embryonic swimming) or are rhythmically activate where they might normally only fire irregularly (eg, raphe neurons continuously releasing 5HT during larval NMDA-induced activity). Thus, where NMDA is used to activate locomotor networks the resulting rhythm may be wholly unphysiological. It has been shown that the pattern of activity induced in the neonatal rat preparation by NMDA is rhythmic but which, on closer inspection, would not necessarily be suitable to drive locomotor activity. In the majority of preparations, either left-right alternation occurred but there was co-activation of intralimb flexors and extensors or rhythmic co-activation was recorded from all electroneurograms (Cowley and Schmidt, 1994). Also, in the lamprey, the discrepancy between the effects of apamin on NMDA induced activity reported by Hill et al. (1992) and Meer et al. (1992, see introduction to chapter 5) has been suggested to result from the differences in the concentration of NMDA used. The effects of apamin are more pronounced at low swimming frequencies, but are less obvious when the excitatory drive is high (El Manira et al., 1994). Therefore, the effects of neuromodulators can be masked depending on the levels of excitation within the network. In this study, I have suggested that the GABAergic influence on embryonic swimming activity could result from GABA released from spinal neurons. However, no effect was observed when bicuculline was added in the presence of 100μM NMDA. It is possible that an effect of GABA may have been more obvious if lower concentrations of NMDA had been used.

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