THE DEVELOPMENT OF LOCOMOTOR RHYTHMICITY IN POST-EMBRYONIC *XENOPUS LAEVIS*

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

by

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Dedication

In memory of my dear mother who died in December 1988.

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Contents

Abbreviations

1) AHP	Afterhyperpolarisation
2) α-BTX	α-Bungarotoxin
3) CGS maleate	7-Triflouromethyl-4(methyl-1-piperazinyl)-pyrrolo[1,2-a]quinoxaline maleate
4) CNQX	6-cyano-7-nitroquinoxaline-2,3-dione
5) cns	Central nervous system
6) cpg	Central pattern generator
7) 5CT	5-Carboxamidotryptamine
8) 5,7-DHT	5,7-Dihydroxytryptamine
9) dla	Dorsolateral ascending
10) dlc	Dorsolateral commissural
11) EAA	Excitatory amino acid
12) epsp	Excitatory post-synaptic potential
13) GABA	Gamma-amino butyric acid
14) HCG	Human chorionic gonadotrophin
15) 5HT	5-Hydroxytryptamine
16) 5HTP	5-Hydroxytryptophan
17) Hz	Hertz
18) ipsp	Inhibitory post-synaptic potential
19) NAN-190	1-(2-Methoxyphenol)-4-[4-(phthalimido)butyl]piparazine
20) NMDA	N-Methyl-D-aspartate
21) PKC	Protein kinase C
22) R-B	Rohon-Beard
23) 8-OH-DPAT	8-hydroxy-dipropylaminotetralin

24)	TFMPP	N-(Triflouromethylphenyl)piparazine
25)	TTX	Tetrodotoxin

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25) 5HT decreases episode length
26) 5HT suppresses the skin cell pathway
27) 5HT suppresses R-B cell pathway
28) Nan-190 reverses the effects of 5HT on swimming
29) Effects of Nan-190 on 5HT-enhanced swimming
30) Effects of Nan-190 on larval swimming
31) Effects of 5CT on swimming
32) Nan-190 reverses the effects of 5CT
33) Effects of R(+)-8OH-DPAT on swimming
34) 5HT increases the probability of multiple spiking in rhythmic neurons during fictive swimming
35) 5HT reduces the rate of spontaneous glycinergic inhibitory potentials
36) 5HT reduces the rate of spontaneous ipsp's and hyperpolarises the membrane
37) 5HT reduces evoked midcycle ipsp's during swimming activity
38) 5HT induces TTX-resistant membrane potential oscillations in the presence of NMDA and extracellular Mg^{2+}
39) Membrane potential oscillations are voltage-dependent
40) Membrane potential oscillations
41) Apparent membrane resistance is greater in NMDA+5HT than in NMDA alone

Summary of results

1) The post-embryonic development of rhythmic motor output underlying swimming in the amphibian, *Xenopus laevis* has been investigated.

2) By recording extracellularly from ventral roots in immobilised animals, dramatic changes in fictive swimming are shown to occur in a brief twenty-four hour period after hatching (between developmental stages 37/38 & 42; Nieuwkoop & Faber, 1956). These changes involve the acquisition of longer and more variable ventral root burst durations on each cycle of activity.

3) The development of ventral root bursts occurs in a rostro-caudal sequence. Near the time of hatching (stage 37/38), ventral root activity consists of brief (5-7ms) biphasic impulses on each cycle, although rostral activity is on average slightly longer in duration than caudal. This difference becomes much more apparent twelve hours after hatching (stage 40), when bursts of discharge lasting between 10 and 20ms occur in rostral ventral roots, while more caudally, the activity remains 'embryonic-like'. 24 hours after hatching (stage 42) ventral root bursts have developed also in caudal roots so that their activity resembles that of the rostral ventral roots.

4) These observations suggest a descending influence on the development of the spinal locomotor network. This suggestion is supported by the results of surgical removal of descending interneurons, since spinalisation of stage 42 larvae results in the

swimming rhythm resuming embryonic features.

5) The more complex and variable pattern of ventral root discharge in the stage 42 larva is associated with a considerably more flexible swimming pattern which permits manoeuvres such as turning and acceleration.

6) The properties of rhythmic spinal neurons (presumed to be myotomal motorneurons) of the locomotor network change during post-embryonic development. Most notably, they acquire a multiple firing capability during swimming and in response to depolarising injected current.

7) The synaptic drive onto motorneurons also acquires complexity consistent with the parallel development of a variable and multiple firing capability in rhythmically active premotor interneurons.

8) The locomotor network begins to acquire sensitivity to 5-hydroxytryptamine (5HT) around the time of hatching. The most notable effect of bath applied 5HT (2-5μM) is to enhance the duration of ventral root discharge in each cycle of fictive swimming activity.

9) Sensitivity to 5HT develops in a rostro-caudal sequence. When bath applied to different developmental stages, it enhances the ventral root discharge so that the pattern of swimming activity closely resembles that of an animal some twelve hours older in control conditions. Also, sensitivity to 5HT occurs before

functional innervation from descending serotonergic fibres.

10) The metabolic precursor to 5HT, 5-hydroxytryptophan (5HTP) (5-10μM) was utilised to enhance endogenous release of 5HT. 5HTP causes an increase in ventral root burst durations only at levels of the cord where 'burstiness' has already developed.

11) The neurotoxic ablation of serotonergic fibres with 5,7 dihydroxytryptamine resulted in the larval swimming pattern failing to develop. This suggests that they may have a central role in the development of locomotor function.

12) Bath-applied 5HT suppresses activation of swimming activity via both the Rohon-Beard cell and skin cell sensory pathways.

13) The pharmacological profile of receptors involved in the serotonergic modulation and development of swimming was studied by investigating the effects of a broad range of antagonists and agonists on the swimming pattern. Antagonists which act at $5HT_2$ and $5HT_3$ receptor families such as ketanserin and MDL72222 have no effect on the 5HT-enhanced rhythm. The specific $5HT_{1a}$ antagonist Nan-190 blocks the effects of 5HT on swimming, while the high affinity, non-specific $5HT_1$-receptor agonist 5CT, mimics the effects of 5HT on the swimming rhythm. These results indicate the involvement of a $5HT_{1a}$ receptor in the development and modulation of swimming.

14) Transient dimming of the illumination initiates fictive

swimming activity via activation of the pineal photoreceptor pathway in all stages examined. The bath application of either 5HT or 5CT blocks this light dimming response, while the $5HT_{1a}$ receptor antagonist buspirone restores it. This suggests a role for a $5HT_{1a}$ receptor in the serotonergic modulation of the pineal photoreceptor pathway.

15) The effects of 5HT on the membrane properties of rhythmic spinal neurons was investigated:

a) 5HT hyperpolarises the cell membrane by 5-10mV.

b) 5HT reduces the rate of spontaneous inhibitory potentials and the magnitude of evoked glycinergic midcycle ipsp's.

c) 5HT induces TTX-resistant membrane potential oscillations in the presence of NMDA and extracellular Mg^{2+}.

CHAPTER 1

General Introduction

FOREWORD

The immense complexity of adult vertebrate nervous systems, especially in mammals, has necessitated a number of simplifying approaches in attempts to gain an understanding at the cellular level. For example, the properties of individual neurons can be studied in isolation either by first growing them in culture or by acutely dissociating them; tissue slices offer the opportunity to look at limited circuits; spinalisation, reduced spinal cord preparations and lesion experiments permit the investigation of the properties of more intact circuits. Alternatively, developmentally and/or phylogenetically simple organisms offer the advantage that they permit a more complete understanding of how circuits and neuronal properties combine to generate actual behaviour.

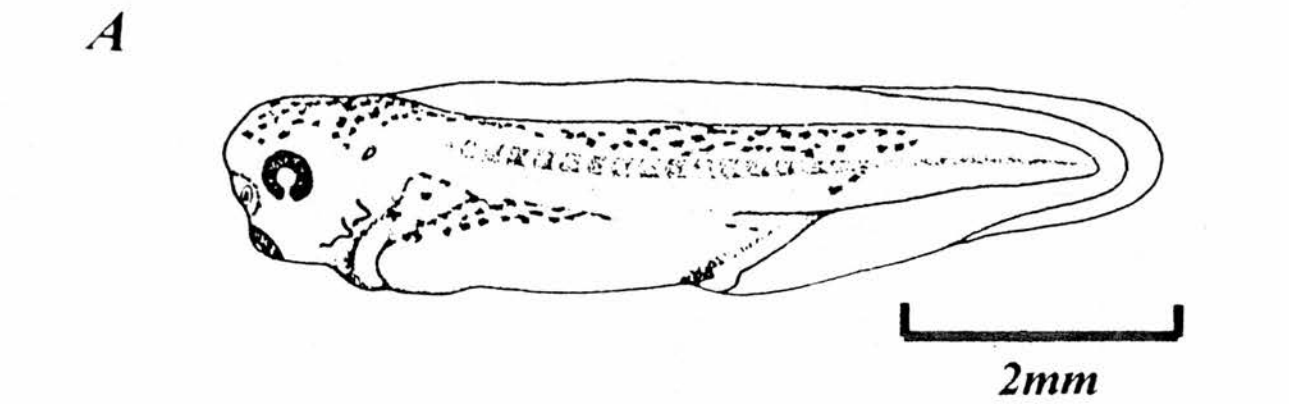
Although invertebrate preparations have played a central role in developing ideas about neural circuits and their modulation (for reviews, see Dickenson, 1989; Harris-Warrick & Marder, 1991), they necessarily have their limitations with respect to understanding vertebrate spinal cord networks. On the basis of a shared evolutionary ancestry, all vertebrates are likely to have common fundamental features underlying rhythmic locomotor behaviour. The late embryo of the amphibian *Xenopus laevis* (stage 37/38; Nieuwkoop & Faber, 1956) was developed as a model system on account of its remarkable neuroanatomical simplicity, its vigorous and well co-ordinated swimming activity and its accessibility to neurophysiological and anatomical techniques. I will now describe in some detail the *Xenopus* embryo preparation, its spinal neuroanatomy, and the circuitry and cellular

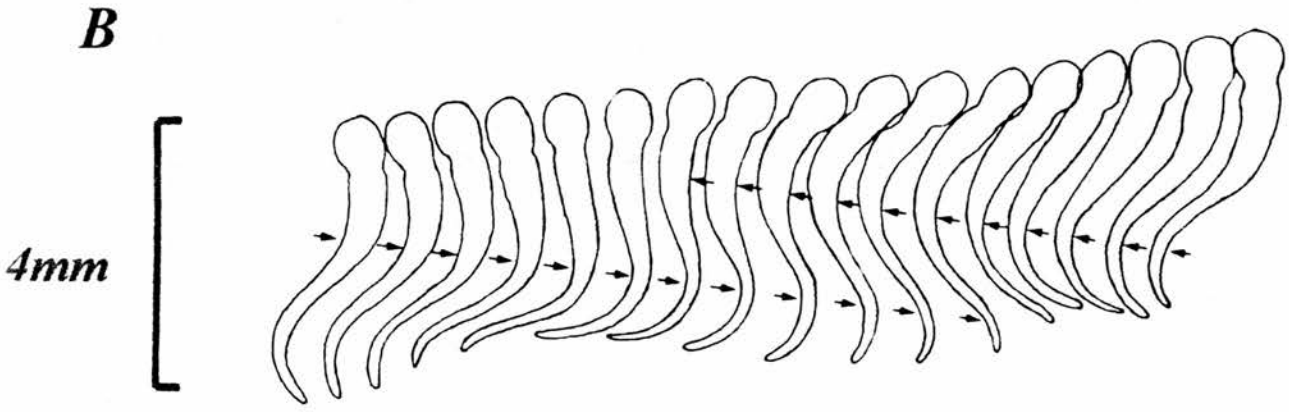
mechanisms underlying the generation of rhythmic swimming activity.

i) The Xenopus embryo preparation

Embryos of the African clawed toad *Xenopus laevis* hatch from their egg membranes after about two days of development (developmental stage 37/38; Nieuwkoop & Faber, 1956; fig 1 A). At this early stage, they are already capable of sustained episodes of rhythmic swimming behaviour. Swimming occurs at frequencies of 10 to 20 Hz and is accomplished by rhythmic, co-ordinated contractions of the segmented myotomal muscles. These contractions occur sequentially passing from head to tail first down one side of the body and then the other (Kahn *et al*, 1982; fig 1 B). This results in a travelling wave of body curvature which acts against the surrounding water to generate a reactive force which propels the animal forwards (Gray, 1933).

A rhythmic motor pattern suitable to drive swimming behaviour can be readily examined by first paralysing the embryos in a neuromuscular blocking agent, such as α-bungarotoxin (α-BTX) or d-tubocurarine (curare), and then recording extracellularly from motorneuron axons in the ventral roots as they pass between the myotomes. The pattern of discharge recorded from the ventral roots during episodes of this so-called 'fictive swimming' pattern appears to be entirely appropriate for the generation of real swimming behaviour; the activity is rhythmic, occurs at 10-20 Hz, passes down the body with a brief delay between segments and strictly alternates between the two sides (Roberts & Kahn, 1982;


A
2mm


B
4mm

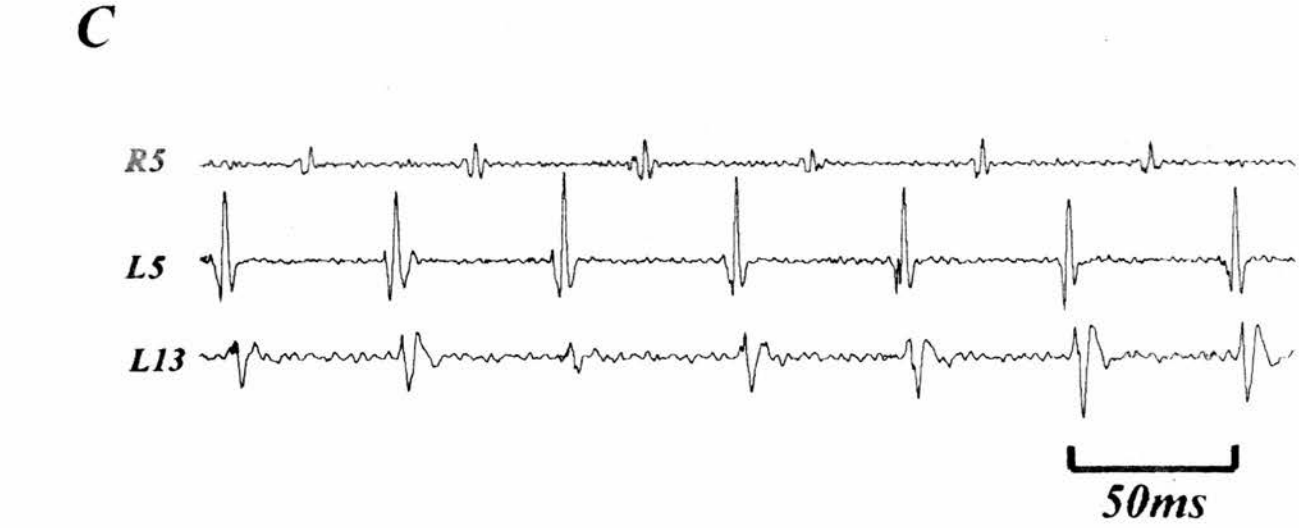

C
R5
L5
L13
50ms

fig 1. Swimming in the Xenopus embryo

The embryo, when it hatches from its egg membranes, is about 5mm long (A). It swims at frequencies of 10-20 Hz by generating propulsive waves of bending which pass first down one side of the body and then the other (B; drawing from Kahn & Roberts, 1982, arrowheads indicate points of maximum body curvature). Following immobilisation with a neuromuscular blocking agent, the underlying ventral root activity can be recorded using glass suction electrodes placed on the intermyotomal clefts. In C, three recordings have been made, one contralateral from the 5th post-otic intermyotomal cleft (R5), and two ipsilateral from L5 and L13 (see fig 5A for description of cleft positions). The activity appears entirely appropriate for driving real swimming since it passes first down on side of the body and then the other with a brief intersegmental delay, and occurs at a frequencies of between 10 and 20 Hz.

occur without sensory feedback (Kahn & Roberts, 1982a & b) and therefore must be generated within the central nervous system (cns). Fictive swimming also persists after spinalisation and so can be generated by a limited network of neurons intrinsic to the spinal cord alone (Roberts *et al*, 1986). However, a proportion of the interneurons contributing to rhythm generation are located in the brain stem, and rhythm generating capability is progressively impaired with more caudal levels of spinal transection (Roberts & Alford, 1986). Nevertheless, from these early studies it has been concluded that sufficient neural circuitry is present within the spinal cord alone to generate the basic swimming pattern. The 'central pattern generator' (cpg; Delcomyn, 1980) for swimming is therefore located within the spinal cord.

The spinal cord is neuroanatomically very simple, comprising just a few classes of anatomically distinct neuron (Roberts & Clarke, 1982). The accessibility of these spinal neurons to intracellular recording and staining techniques, has enabled the *Xenopus* embryo swimming circuit to be used very successfully as a model system for investigating the basic mechanisms underlying the generation of vertebrate locomotion. Consequently, since the first publication describing fictive swimming thirteen years ago (Roberts *et al,* 1981), a detailed knowledge of the circuitry and mechanisms which underlie swimming has accumulated, and it is now probably the best understood vertebrate cpg.

ii) The neuroanatomy of the Xenopus embryo spinal cord

The detailed anatomical study of the spinal cord of late embryonic *Xenopus laevis* by Roberts & Clarke (1982) provides one of the most complete descriptions of any vertebrate spinal cord. The cord is very simple in that it contains only eight classes of differentiated neuron (Roberts & Clarke, 1982). This excludes a class of **extramedullary** cells whose somata lie outside and dorsal to the spinal cord. These cells have axons projecting to the hindbrain and peripheral neurites which pass between the myotomes and extend under the skin (Hughes, 1957). They are probably sensory neurons whose somata have migrated out of the cord (see below), but beyond this nothing is known of their function. Neurons which are intrinsic to the spinal cord are more easily studied, and the function of the majority of the eight classes is now well known and will be described in later sections. A brief description of their anatomy now follows.

Rohon-Beard (R-B) cells are primary mechanosensory neurons (Clarke *et al,* 1984) with large cell bodies (*ca* 15-20μM) occupying the medial dorsal cord to form an almost continuous double row (Hughes, 1957; Roberts & Hayes, 1977; Clarke *et al,* 1984; fig 2B). Most have a single unmyelinated neurite which innervates the skin with free nerve endings and central axons which ascend and descend the dorsolateral tract of the spinal cord on the same side as the soma. R-B neurons and extramedullary neurons are therefore anatomically very similar except for the location of their cell bodies.

Also located in dorsal regions of the cord, are two classes of second order sensory interneurons; **dorsolateral ascending** (dla)

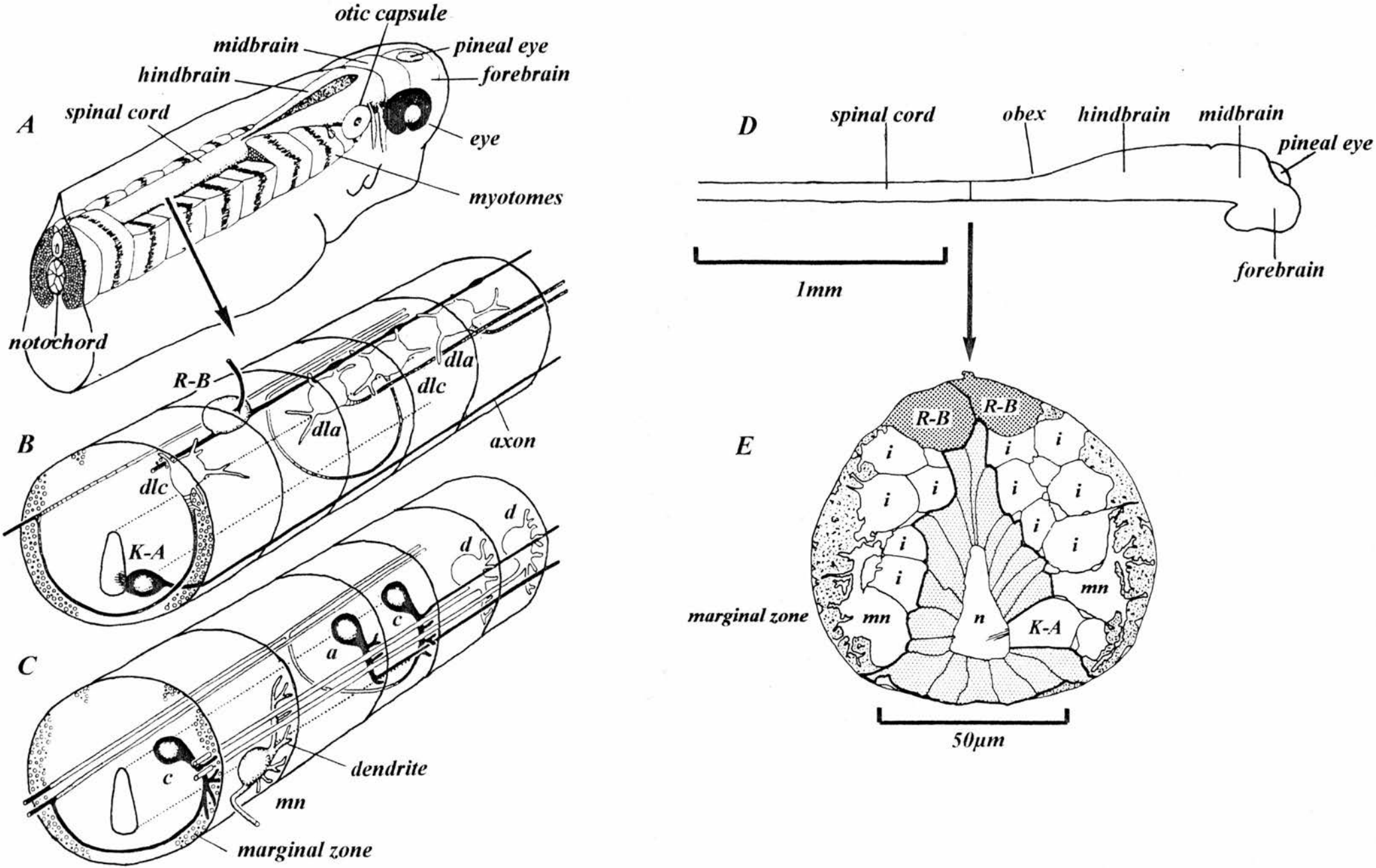

otic capsule
midbrain
pineal eye
hindbrain
forebrain
A
spinal cord
eye
myotomes
notochord
R-B
dla
dlc
dla
axon
B
dlc
K-A
d
d
c
a
c
C
c
dendrite
mn
marginal zone
D
spinal cord
obex
hindbrain
midbrain
pineal eye
forebrain
1mm
E
R-B
R-B
i
i
i
i
i
i
i
i
i
i
i
mn
marginal zone
mn
n
K-A
50μm

fig 2. The neuroantomy of the Xenopus embryo spinal cord

The spinal cord is a gently tapering tube lying immediately above the notochord and between the myotomes (A). Along the medial dorsal margin of the cord, Rohon-Beard (R-B) neurons form an almost continuous double row. They have long ascending and descending axons which make *en passent* synapses with two types of sensory interneuron, dorsolateral commissural (dlc) and dorsolateral ascending (dla) (B). Kölmer-Agdur (K-A) cells are ventrally positioned with their ependymal surface projecting into the neurocoel, and they have long ascending axons (B). In addition to the sensory interneurons, there are three other classes of interneuron, descending (d) and commissural (c) which are involved in rhythm generation, and ascending interneurons whose function is not known (C). Motorneurons occupy almost exclusively the ventrolateral part of the cord (C). D is a longitudinal drawing of the brain and rostral cord illustrating the gross anatomical features. E is a cross-sectional drawing through the cord at the level indicated in D. It illustrates the relative positions of Rohon-Beard neurons (R-B), interneurons (i), Kölmer-Agdur (K-A) cells, motorneurons (mn), the neurocoel (n), and the marginal zones comprising the dorsolateral and lateral tracts. Drawings reproduced by kind permission from Alan Roberts and Steve Soffe.

and **dorsolateral commissural** (dlc) interneurons (fig 2B). Both types of neuron are multipolar and have somata which lie close to the dorsolateral surface of the cord. The principle anatomical feature which distinguishes the two types is that dlc's have axons which cross the cord ventrally then either ascend to the hindbrain in the ventral half of the opposite lateral tract, or bifurcate so that they also have a descending projection. In contrast, dla's have axons which ascend in the dorsal part of the lateral tract on the same side of the cord (see Roberts & Clarke, 1982). The dendritic branching of both classes of sensory interneuron extends into the dorsal part of the lateral tract and dorsally into the dorsolateral tract, where they are thought to make *en passant* synapses with the central axons of sensory Rohon-Beard neurons (Roberts & Clarke, 1982).

Located more medially in the cord, are three other anatomically distinct classes of interneuron, two of which are known to be involved in rhythm generation, and whose roles will be described in later sections. Firstly, **descending** interneurons (d) have multipolar cell bodies positioned on the edge of the lateral tract and have descending axons (fig 2C). Dendrites emerge from the somata and extend into the lateral and dorsal tracts where they radiate extensively. Their axons project caudally in the lateral tract, just dorsal to motorneuron axons. Secondly, **commissural** interneurons (c) whose main distinguishing anatomical feature is that their axons pass ventrally along the inside edge of the lateral tract, emitting short radial dendrites, and then cross ventrally to reach the ventral part of the lateral tract on the opposite side of the cord (fig 2C). There they either ascend to the hindbrain or bifurcate to ascend and descend the cord. Their cell bodies are

unipolar lying along the edge of the lateral tract (Roberts & Clarke, 1982). Thirdly, **ascending** interneurons (a) which have long ascending axons and are immunopositive for GABA (Dale *et al*, 1987b), but their function is not yet known (fig 2C).

The cell bodies of myotomal **motorneurons** are relatively large (*ca.* 15 μm), occupy the ventral most regions of the cord and lie along the ventral and medial edges of the lateral tract to form an almost continuous longitudinal column which is sometimes more than one cell deep (Roberts & Clarke, 1982; fig 2C)). Their dendrites extend into the lateral tract where they radiate extensively, with prominent dorsally directed dendrites. The axons project caudally within the lateral tract, passing close to the ventrolateral dendrites of more caudal motorneurons, before branching outwards to leave the cord in groups where they are bundled together to form a motor nerve (ventral root). Some motorneurons, however, have an additional central axon which descends for a short distance and does not leave the cord. Motor nerves reach caudally, usually to the next intermyotomal cleft, where they innervate the myotomes. The motorneurons which innervate post-otic myotomes 1-3 are situated not in the cord, but in the caudal hindbrain. In addition to the main features shared with spinal cord motorneurons, these cells also have unusually long axons projecting up to 700μm down the lateral tract which do not leave the cord (Roberts & Clarke, 1982).

The final class of spinal neuron is the ciliated ependymal cell (Hughes, 1957), subsequently termed **Kölmer-Agduhr** (K-A) cells (Dale *et al*, 1987a; fig 2B). They have a ventrolateral position deep in the cord, and their ependymal surface projects into the neurocoel and is covered with short cilia. Their axons ascend to the

hindbrain in the most ventral part of the lateral tract. They are also immunopositive for GABA, but nothing is known about their function (Dale *et al*, 1987a).

iii) Locomotor rhythm generation in the Xenopus embryo

As described above, the spinal cord of the *Xenopus* embryo is remarkably simple in that it contains only eight differentiated neuron classes (Roberts & Clarke, 1982) of which only three appear to be involved in producing swimming activity. These are motorneurons, excitatory descending interneurons with axons descending on the same side of the cord, and inhibitory commissural interneurons whose axons cross the cord (fig 2C). Intracellular recordings of these neuron types reveal that they are rhythmically active and receive essentially indistinguishable synaptic drive during swimming. This synaptic drive is characterised by three main components; at the onset of each cycle, rhythmic neurons fire a single action potential driven off an excitatory post-synaptic potential (epsp) following which they receive a midcycle inhibitory post-synaptic potential (ipsp). There is also a sustained level of background excitation upon which the alternating excitation and inhibition is superimposed (Roberts & Kahn, 1982; Soffe & Roberts, 1982; Soffe *et al*, 1984).

The source and nature of these three main components of the synaptic drive have now been studied in some detail. Excitatory interneurons provide excitatory synaptic drive to rhythmic cells on the same side of the cord and use an excitatory amino acid (EAA), probably glutamate, or a closely related molecule, as a

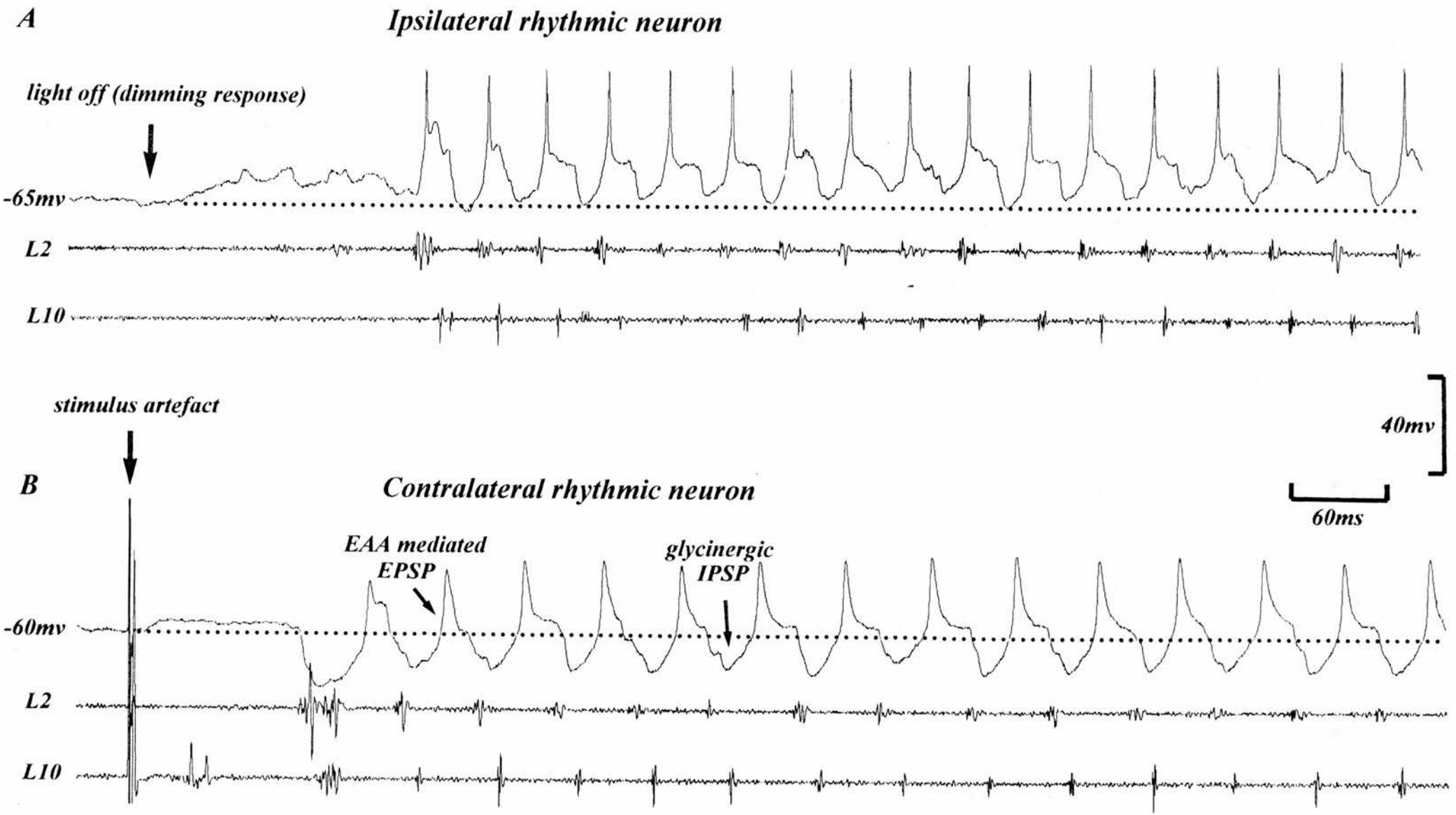

A
Ipsilateral rhythmic neuron
light off (dimming response)
-65mv
L2
L10
stimulus artefact
40mv
B
Contralateral rhythmic neuron
60ms
EAA mediated
EPSP
glycinergic
IPSP
-60mv
L2
L10

fig 3. Locomotor rhythm generation in the Xenopus embryo

Two examples of fictive swimming (A & B) both with ipsilateral (left) ventral root recordings at L2 and L10. In A, intracellular recording of ipsilateral motorneuron, and in B contralateral motorneuron, both at about the level of the 5th post-otic myotome. Swimming activity initiated by a transient dimming of the illumination (A) and by an electrical current pulse to the tail skin (B) (at arrows). The activity in the motorneuron consists of alternating phases of excitatory amino acid (EEA) receptor-mediated excitation and glycinergic inhibition superimposed on a tonic depolarisation (dotted lines indicate resting potential). A single action potential is driven off the excitatory post synaptic potential (epsp), and note also that the ipsilateral motorneuron fires an impulse in phase with the ventral root activity on the same side (A), while the contralateral motorneuron is inhibited (B).

neurotransmitter (Dale & Roberts, 1985). There are essentially two pharmacologically distinct types of EAA receptor (Watkins & Evans, 1981), N-methyl-D-aspartate (NMDA) and non-NMDA (kainate/quisqualate), both of which are present on *Xenopus* embryo motorneurons (Dale & Roberts, 1984). Their co-activation by excitatory interneurons results in a mixed epsp comprising a slow rise and fall (NMDA), and a fast rise and fall (non-NMDA) component. The phasic (non-NMDA) excitation triggers a single action potential on each cycle of swimming, while the slow (NMDA) epsp's (which last around 200ms), summate over consecutive cycles (cycle periods range from 50-100ms) to provide the tonic background excitation (Dale & Roberts, 1985). Reciprocal inhibitory synaptic drive is provided by commissural interneurons which stain with antibodies to glycine (Dale *et al*, 1986) and whose axons cross the cord to make strychnine-sensitive synaptic connections with rhythmic cells on the opposite side (Dale, 1985). Since commissural interneurons discharge rhythmically during fictive swimming, they are presumed to be responsible for the reciprocal midcycle inhibition which ensures strict alternation and tight coupling between the two halves of the cord.

Until recently, vertebrate motorneurons were thought to be essentially passive elements representing the output of the cpg and not therefore involved in rhythm generation *per se*. Although this notion was not tested experimentally in *Xenopus* embryos, in the lamprey no evidence for a rhythm generating role for motorneurons could be found (Wallén & Lanser, 1984). However, this view has recently been challenged in *Xenopus* embryos since motorneurons have now been shown to make cholinergic synapses with other

neurons in the spinal cord. While these connections may contribute to the synaptic drive underlying swimming activity, they are clearly not necessary for rhythm generation since an apparently normal swimming rhythm continues after these synapses have been blocked with cholinergic antagonists (Perrins, 1993).

What then are the mechanisms that permit this network of essentially two cell types to generate and sustain rhythmic locomotor activity? Since excitatory interneurons receive the same EAA-mediated excitatory drive as other rhythmically active cells (Dale & Roberts, 1985), it seems probable that they mutually re-excite themselves. Recent computer modelling suggests that this feature is essential for the maintenance of rhythmicity (Roberts & Tunstall, 1990). The model, however, also requires 'post-inhibitory rebound'. Experimental manipulation has shown that rhythmic cells will fire a rebound action potential following a brief hyperpolarising current pulse if the cell membrane is first depolarised by constant current injection (Roberts *et al*, 1986; Soffe, 1990). Thus, during swimming, tonically excited cells may be triggered to fire an action potential on rebound from a mid-cycle ipsp. This mechanism, however, is not **necessary** for rhythmicity in the *Xenopus* spinal cord since experiments in which the cns is surgically divided along the sagittal midline (thus removing cross cord coupling), reveal that the two halves of the cns are capable of sustaining rhythmic activity **without** reciprocal inhibition (Kahn & Roberts, 1982a). Moreover, activity continues in the presence of strychnine, which not only pharmacologically uncouples the two halves of the cord, but also blocks on-cycle glycinergic inhibition (see below). Since reciprocal inhibition appears to be the only cross cord influence during swimming, this also suggests it has an

important coupling role in the intact animal. Furthermore, the network of neurons within a single half of the spinal cord, is capable of sustaining rhythmic motor discharge, since removal of the hindbrain little affects the activity (Soffe, 1989).

Pharmacological manipulations have been of critical importance in revealing the underlying mechanisms of locomotor rhythm generation. Gamma amino butyric acid (GABA) is an inhibitory transmitter whose role, if any, in embryo rhythm generation is not well understood. All the early work on *Xenopus* embryos was carried out using curare as the immobilising agent which blocks GABA responses (Bixby & Spitzer, 1984). However, fictive swimming activity in curarised animals does not appear to be any different from that in animals immobilised in α-bungarotoxin, which does not block GABA receptors (see Soffe, 1987). Moreover, the GABA antagonist bicuculline also has no obvious effect on the locomotor rhythm (Soffe, 1987). However, if all known inhibition is blocked by the simultaneous application of the GABAergic and glycinergic antagonists, bicuculline and strychnine respectively, the half spinal cord of this pharmacologically reduced preparation continues to produce a pattern of rhythmic motor output (Soffe, 1989). This is important since there are inhibitory connections on the same side which could contribute to rhythmicity in a single half of the spinal cord (Dale, 1985). The basic rhythmicity exhibited by one side of the cord in the absence of inhibition appears to require activation of NMDA receptors, since the NMDA receptor antagonist D-2-amino-5-phosphonovaleric acid (APV) blocks all activity (Soffe, 1989). It also requires the presence of extracellular magnesium (Soffe, 1989). In the presence of physiological levels of extracellular

magnesium, the NMDA receptor ionophore is voltage-gated because Mg^{2+} ions block the opening of the channel at membrane potentials near rest (Nowak *et al*, 1984; Mayer & Westbrook, 1984). The importance of the voltage-dependant Mg^{2+} block of the NMDA receptor ion channels on naturally occurring fictive swimming in the intact animal is not clear, since a stable rhythm occurs with or without Mg^{2+}. However, in reduced half-cord preparations in which the hindbrain has been removed (Soffe 1989), and also in spinalised animals with an intact cord, extracellular Mg^{2+} is necessary for stable rhythm (Soffe & Roberts, 1989). Interestingly, activity will occur in the absence of extracellular Mg^{2+} in a surgically reduced half cord preparation which includes the hind brain. Crossed reciprocal inhibition, although undoubtedly a significant contributory mechanism, cannot be regarded, therefore, as a fundamental component of the rhythm generator, unlike NMDA receptor-mediated excitation, which is of central importance. It is probable, however, that commissural interneurons are responsible for the antiphasic coupling between the two halves of the cord by ensuring that when one side is active the other is not, since apart from some hindbrain GABA neurons whose axons cross and then descend into the cord (Roberts *et al*, 1986), inhibitory commissural interneurons provide the only known functionally active cross cord connections during fictive swimming.

iv) Longitudinal co-ordination

Although there can be little doubt that reciprocal inhibition is the major mechanism responsible for cross-cord co-ordination, the

mechanisms underlying longitudinal co-ordination remains perhaps the most enigmatic feature of the *Xenopus* embryo locomotor output. There are two essential features; the intersegmental delay between segments is constant down the length of the animal, and there is no significant correlation between the magnitude of the delay and cycle period (Tunstall & Roberts, 1991). Although a constant longitudinal delay for any given swimming frequency is also seen in other vertebrates, such as the lamprey (Wallén & Williams, 1984) and the dogfish (Grillner, 1974), the lack of correlation between delay and cycle period in *Xenopus* embryos is very unusual. In the lamprey the relationship between cycle period and segmental delay is thought to be important in conserving a constant wavelength of movement during swimming irrespective of the speed. As with other features of locomotor rhythm generation, longitudinal delay occurs in the absence of sensory feedback and so must be centrally programmed. Also, as in the lamprey, it probably cannot be explained in terms of axonal conduction and synaptic delays since calculations suggest that delays resulting from such mechanisms are much larger than those observed (Tunstall & Roberts, 1991; Tunstall & Sillar, 1993).

v) Initiation and modulation of swimming

A brief mechanical or electrical stimulus to the trunk skin, or a transient dimming of the illumination can initiate an episode of swimming in *Xenopus* embryos, which far outlasts the duration of the stimulus. There are two skin sensory systems comprising the R-B and skin cell pathways. R-B cells are the primary

mechanosensory afferents which form a double longitudinal column in the dorsal spinal cord. Each R-B cell innervates the skin via a single unmyelinated neurite with free nerve endings, thereby creating an overlapping network of receptive fields. Skins cell are electrically coupled so that following stimulation, a single impulse spreads rapidly over the entire epithelium (Roberts & Smyth, 1974), although it is not known how it excites the locomotor network. The dimming response is mediated via the pineal pathway, and it is likewise a poorly understood pathway, although it has been shown that the pineal eye acts as a luminance detector (Foster & Roberts, 1982).

Following a brief electrical or mechanical stimulation of their receptive fields in the skin, R-B neurons usually discharge a single impulse (Clarke *et al,* 1984), and this is usually followed by an episode of swimming activity. Moreover, evoking impulses by current injection in a single R-B cell is sometimes sufficient to elicit fictive swimming (Clarke *et al,* 1984). Although the axons of R-B cells project to the hindbrain (Roberts & Clarke, 1982), spinalised embryos also respond to skin stimulation with episodes of fictive swimming. This implies that R-B cells must directly contact neurons within the spinal cord itself. These are likely to be the two classes of dorsolateral interneurons which have dendrites in the dorsolateral tract. Following skin stimulation, intracellular recordings from dorsolateral sensory interneurons reveal a compound epsp which sometimes triggers an action potential and whose latency also suggests direct R-B to interneuron connections (Clarke & Roberts, 1984). The nature of the compound epsp's in dorsolateral cells suggests converging input from those R-B cells whose receptive fields overlap beneath the stimulating electrode.

This in turn implies that each R-B cell excites many dorsolateral cells, thereby greatly amplifying the primary afferent signal. Transmission between R-B cells and dorsolateral interneurons is EAA receptor-mediated, involving activation of both NMDA and non-NMDA receptors (Sillar & Roberts, 1988a).

Both classes of dorsolateral cell both have long axons which ascend to the hind brain, those of the dlc's first crossing the cord. They could, therefore, make *en passant* synaptic contact with excitatory interneurons and hence provide a mechanism for second stage amplification, and a means of exciting both sides of the locomotor network. Evidence for this has been obtained by intracellular recordings from excitatory interneurons and motor neurons which both receive a short latency epsp in response to a contralateral skin stimulus. Since R-B cells cannot directly contact rhythmic cells on the opposite side, it is probable that this cross-cord excitation is mediated by dlc's (Clarke & Roberts, 1984; Roberts & Sillar, 1990). Also, rhythmic interneurons on the same side of the cord as the stimulus, receive an ipsp. This connectivity may account for the 'crossed avoidance reflex' whereby a brief mechanical stimulus to one side will cause the muscles on the opposite side to contract, flexing the body and turning the animal away from the point of stimulation. Therefore, as swimming is initiated, this initial reflex orientates the animal so that it swims away from the stimulus (Sillar & Roberts, 1988b)

Cutaneous sensory input via the R-B cell pathway is also important in ensuring that ongoing swimming is itself adaptive to the surrounding environment. During fictive swimming, the two classes of dorsolateral cells are rhythmically inhibited (Sillar & Roberts, 1992a). Dlc's are inhibited in phase with ventral root

discharge on the **same** side so that sensory input to the contralateral locomotor network during that phase of the swim cycle is gated out (Sillar & Roberts, 1992a). Inputs however can occur on the opposite phase thereby increasing the excitatory drive of the contralateral locomotor network causing enhanced contraction of the muscles on the side furthest from the stimulus, so that, as in the crossed avoidance reflex, the animal turns and swims away from the point of stimulation (see, Sillar & Roberts, 1988a & b).

Besides this phase-dependent reflex, skin stimulation during swimming also results in an increase in swimming frequency which lasts for several cycles. Dlc's probably make direct contact with motorneurons and also with excitatory interneurons on the opposite side of the cord. Since excitatory interneurons relay excitation down the cord and also excite themselves in a positive feedback loop (Roberts *et al,* 1986), they provide an additional means of amplification and distribution of the excitatory synaptic input from dlc's to the contralateral locomotor network on the appropriate phase of the swim cycle, thereby increasing the gain of the reflex (Sillar & Roberts, 1992b). Not only therefore does a sensory gating mechanism exist which underlies a turning manoeuvre away from the point of stimulation, but also a mechanism which raises the excitability over a few cycles so that the animal also accelerates away.

As previously mentioned, swimming can also be initiated via the skin cell pathway (Roberts & Smyth, 1974). Skin cells are electrically coupled by gap junctions and when the skin is stimulated either mechanically or with a brief current pulse, they normally fire a single impulse (resembling a mammalian cardiac

action potential in waveform), which spreads rapidly from cell to cell over the entire surface of the embryo (Roberts & Stirling, 1971). When isolated surgically from the R-B cell pathway, swimming activity can be initiated via activation of the skin cell pathway alone. It is not known, however, how it excites the locomotor network since a skin impulse does not evoke short-latency epsp's in dorsolateral cells (Clarke & Roberts, 1984).

Swimming can also be initiated in response to a sudden dimming of the illumination, a response mediated by photoreceptors in the pineal eye (Roberts, 1978; Foster & Roberts, 1982). The pineal eye comprises a dorsal invagination of the diencephalic roof at the junction between the fore and mid-brain (fig 2A, D), and is connected to the brain via a stalk. Axons emerge from underneath the pineal vesicle and run ventrally between the mid- and forebrain to form a ventral commissure. Some axons also branch and form an ascending tract running around the optic stalk. There does not appear to be a descending tract, and it is unclear where and how pineal axons terminate (Foster & Roberts, 1982). Although the pathway by which the locomotor network is excited is not known, extracellular recordings from the caudal side of the exposed pineal reveal an inverse correlation between rate of discharge and light intensity. Moreover, a sudden dimming results in a burst of impulses in pineal axons, subsequently followed by a new sustained increase in firing frequency (Foster & Roberts, 1982). The pineal eye acts therefore as a luminance detector. The light dimming activation of swimming could be an escape response normally evoked as a predator casts its shadow over the embryo. It may also be that the pineal eye pathway plays a more subtle role in regulating the

excitability of the locomotor network, thereby affecting the animal's distribution and diurnal habits.

vi) Is the Xenopus embryo a good model for vertebrate locomotor rhythm generation?

The most completely described vertebrate spinal locomotor network in terms of anatomy, circuitry, pharmacology and cell properties is probably that controlling swimming in the *Xenopus* embryo. The question remains, however, has it been a good model system for exploring mechanisms of general significance in vertebrate locomotion?

The locomotor rhythm of, for example, the adult lamprey, contrasts with that of the *Xenopus* embryo in that it is more complex and variable, and occurs over a quite different frequency range. However, both animals share a number of fundamental features, and some striking similarities exist in the organisation of their motor circuits. Apart from myotomal motorneurons, identifiable neurons in the ventral horn of the lamprey spinal cord include lateral interneurons (Buchanan & Cohen, 1982), crossed caudal interneurons (Buchanan, 1982) and excitatory interneurons (Buchanan & Grillner, 1987). Rhythmically active motorneurons receive alternating EAA-dependent excitation and glycinergic inhibition during swimming. The EAA transmitter acts at both NMDA and non-NMDA receptors producing the same two types of epsp as have been described in the *Xenopus* embryo (Dale & Grillner, 1986). Also like *Xenopus,* both inhibitory and excitatory interneurons are rhythmically active during swimming (Dale,

1986). Moreover, in the reduced spinal cord preparation, pattern generation requires the application of EAA agonists which act at NMDA and/or kainate receptors (but not quisqualate) (Grillner *et al*, 1981; Brodin *et al*, 1985). Glycinergic inhibition plays an essential role in cross-cord co-ordination since the application of strychnine abolishes left-right alternation (Cohen & Harris-Warrick, 1984; Alford & Williams, 1987, 1989).

The basic features of locomotor rhythm generation are therefore shared by the lamprey and the *Xenopus* embryo. The lamprey is an Agnathan - a direct descendent of the first vertebrates to evolve and considered to be one of the most primitive today. In evolutionary terms the lamprey is separated from *Xenopus* by hundreds of millions of years. Both of these species are used as simple vertebrate models; the simplicity of one a consequence of its evolutionary inheritance, and of the other by virtue of its early stage in development. The remarkable similarities of their locomotor rhythms lend powerful credence to the assumption that basic features of vertebrate rhythmic locomotor activity are preserved as complexity is acquired both in evolutionary and developmental terms. This is even more apparent when comparisons are made with more complex vertebrate systems.

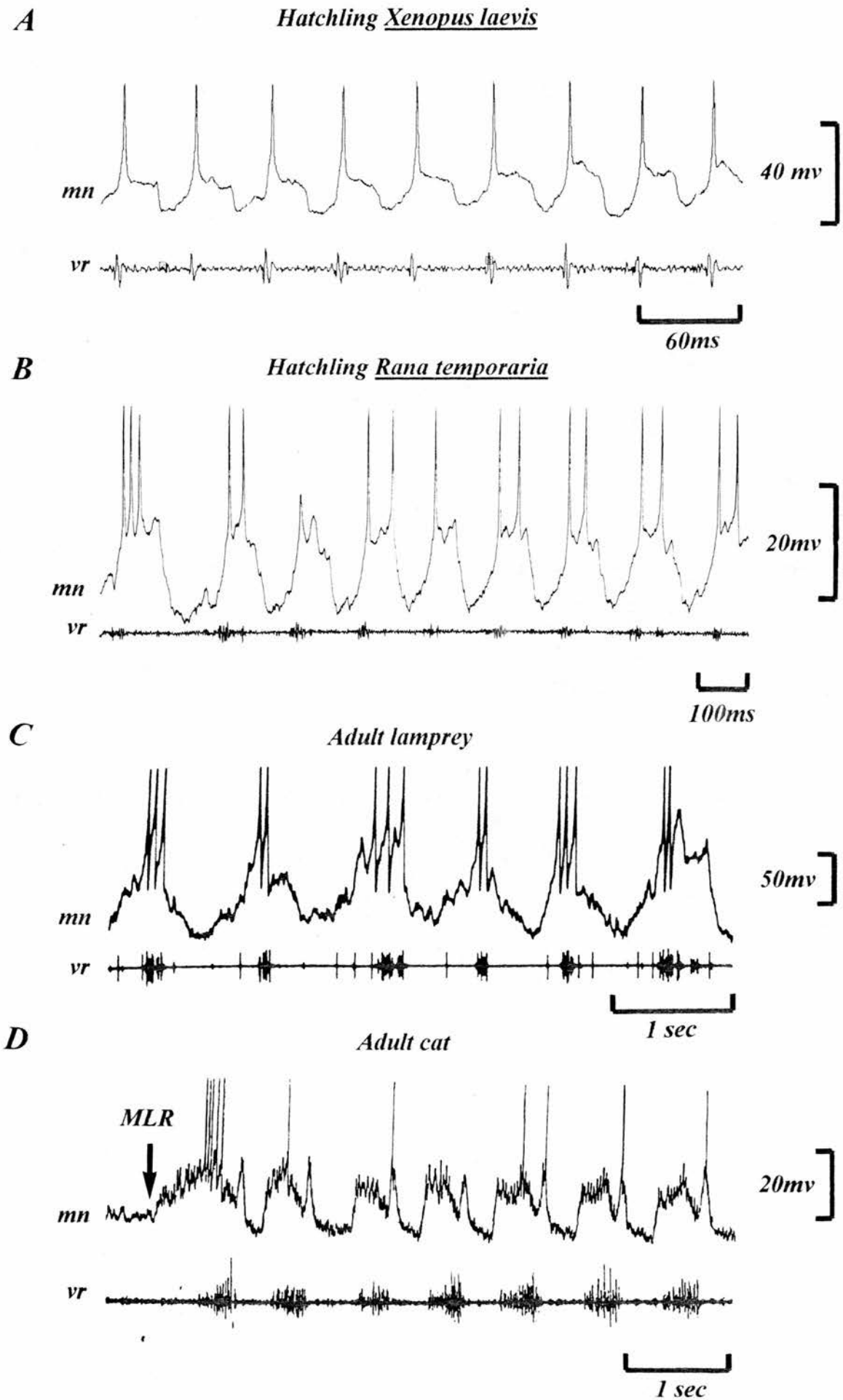
Although it is not yet feasible to explore adult mammal rhythm generators in similar detail as so-called 'simple' preparations, sufficient knowledge exists to suppose that they also share common fundamental features (see fig 4). Most of the evidence available for direct comparisons has derived from work on early developmental stages of more advanced vertebrates. For example, in the isolated chick spinal cord, rhythmic locomotor output spontaneously arises in the absence of descending

influences or sensory feedback (Landmesser & O'Donovan, 1984; O'Donovan & Landmesser, 1987; Beckoff *et al*, 1989). Motorneurons are rhythmically active and receive both inhibitory and excitatory synaptic drive from a network of premotor interneurons (O'Donovan, 1989; O'Donovan *et al*, 1992). Moreover, the application of NMDA or glutamate enhances spontaneous episodes of motor activity, which is reversibly blocked by EAA antagonists (Barry & O'Donovan, 1987). In both neonatal rat (Kudo & Yamada, 1987; Cazalets *et al*, 1990) and mouse (Hernandez *et al*, 1991), application of NMDA to the isolated spinal cord results in a rhythmic motor pattern appropriate for walking. Also in the mouse, glycine has been shown to have an important role in locomotor pattern generation (Tao & Droge, 1992; Droge & Tao, 1993). Thus, the involvement of EAA-mediated excitation and reciprocal glycinergic inhibition appear to be features of locomotor rhythm generation in a wide range of vertebrate species.

Evidence that the same basic features of locomotor pattern generation are preserved throughout vertebrates, is provided by the cat. Transection of the cat's lower thoracic spinal cord isolates the hind-limb circuits from descending input, and if supported the cat is capable of near normal walking on a moving treadmill (Grillner & Shik, 1973). This suggests that, as in the *Xenopus* embryo, the lamprey, the chick, and neonatal rat and mouse, more complex adult vertebrates also have circuits intrinsic to the cord, which generate rhythmic locomotor activity. Moreover, during fictive locomotion in the decerebrate cat, α-motorneurons receive alternating excitatory and inhibitory postsynaptic potentials (Shefchyk & Jordan, 1985; Pratt & Jordan, 1987) similar to those

fig 4. The Xenopus embryo as a model for vertebrate locomotor rhythm generation

A comparison of the activity underlying fictive locomotion in the *Xenopus* embryo (A) with those of hatchling *Rana temporaria* (B), the adult lamprey (C) and the adult cat (D). Each example comprises an intracellular recording from a motorneuron (mn) and an accompanying ventral root (vr). In common with the swimming activity of the *Xenopus* embryo, each cycle of locomotor activity in the other animals consists of alternating phases of excitation and inhibition. Unlike the *Xenopus* embryo, however, their locomotor activities are more complex. In particular motorneurons have a variable and multiple spike capability, while in stage 37/38 *Xenopus* they fire strictly a single action potential per cycle, and this also is reflected in the ventral root activity which consists mainly of brief biphasic impulses as opposed to bursts of discharge. Complexity appears to be added over the top of a fundamental pattern which is effectively stripped bare in the *Xenopus* embryo, thereby permitting detailed analysis of basic mechanisms thought to be common to all vertebrates. In C, locomotion was initiated by NMDA application, and in D via stimulation of the mesencephalic locomotor region (MLR) (arrow). The lamprey recording was reproduced from Wallén & Grillner, 1987, and the cat from Schmidt *et al,* 1988


A
Hatchling Xenopus laevis
mn
vr
40 mv
60ms
B
Hatchling Rana temporaria
mn
vr
20mv
100ms
C
Adult lamprey
mn
vr
50mv
1 sec
D
Adult cat
MLR
mn
vr
20mv
1 sec

seen in lamprey and *Xenopus* embryo motorneurons (Fig 4). The ipsp's appear to be glycinergic since last order Ia inhibitory interneurons make strychnine-sensitive synapses onto motorneurons, which when blocked abolish the ipsp's during locomotor activity (Pratt & Jordan, 1987).

Based on the above comparisons, the *Xenopus* embryo has clearly proven to be an excellent model for the study of vertebrate rhythm generation. However, certain features of its locomotor rhythm are atypical. Most notably, motorneurons and premotor interneurons which are rhythmically active during swimming normally fire a single action potential on each cycle of activity, and the pattern of synaptic drive also lacks the complexities which would arise from a multiple and variable discharge pattern in presynaptic neurons (fig 4A). In more adult systems, motorneurons and premotor interneurons fire bursts of impulses and the pattern of synaptic drive is consequently much more complex and variable (fig 4C). The simplicity of the *Xenopus* embryo locomotor rhythm has important implications with regard to the flexibility of the network. Other than modulation via phase dependent cutaneous reflexes (Sillar & Roberts, 1992a), this rigid simplicity provides little substrate upon which other more tonic 'gain-setting' inputs might act, such as those descending from the brain. One important mechanism which limits rhythmic neurons to a single action potential in each cycle is a slow-activating and sustained voltage-dependent potassium conductance which renders the membrane refractory after the single impulse in each excitatory phase of the cycle (Soffe, 1990). It is also possible that other cell properties of rhythmic spinal neurons in the *Xenopus* embryo differ from those of more adult vertebrates. In contrast to the lamprey,

for example, (Wallén & Grillner, 1987), there is no published evidence that rhythmically active spinal neurons exhibit intrinsic bistable membrane properties which might enable their membrane potential to oscillate between two relatively stable states. The expression of bistable membrane properties which have so far been described in vertebrate neurons, is conditional upon the presence of neurotransmitter molecules and is not therefore an endogenous membrane property (Kiehn, 1991). In the decerebrate cat, for example, bistability is dependent on the activity of descending fibres of brain stem serotonergic and noradrenergic interneurons (Kiehn, 1991). Similar bistable membrane properties have also been described in the turtle, the expression of which also appears to be dependent on the presence of the neuromodulator 5-hydroxytryptamine (5HT, serotonin) (Hounsgaard & Kiehn, 1985, 1989). In the presence of tetrodotoxin (TTX) to synaptically isolate cells by blocking Na^+-dependent impulse generation, and following the activation of NMDA receptors, lamprey motorneurons and interneurons can exhibit membrane potential oscillations. Although there is no strong evidence that they occur in the absence of TTX, it is thought that they might be an important mechanism in the generation of swimming activity, particularly at low rhythm frequencies. (Sigvardt *et al*, 1985; Grillner & Wallén, 1985; Wallén & Grillner, 1987). Unlike the turtle or cat therefore, the induction of bistable membrane properties in lamprey rhythmic spinal neurons does not appear to require 5HT, although addition of the amine to the bathing medium does modulate the frequency of the oscillations (Wallén *et al*, 1989), and the possibility that endogenous 5HT triggers and modulates their expression cannot be discounted. In the light of

these observations, the lack of published information on either the presence or absence of intrinsic oscillatory membrane properties in *Xenopus* embryos is curious. This may be simply due to a lack of detailed investigation into their presence, or perhaps a consequence of embryonic neurons being developmentally too immature. However, this latter possibility seems unlikely since motorneurons are endowed with NMDA receptors as in the lamprey. On the other hand, it may be that a neuromodulator such as 5HT is lacking.

TTX-resistant membrane potential oscillations in the lamprey occur largely as a consequence of voltage-dependent block of the NMDA receptor ionophore in the presence of physiological levels of Mg^{2+} (Nowak *et al*, 1984; Mayer & Westbrook, 1984). In the presence of NMDA, and since the Mg^{2+} block is always less than 100%, the membrane potential begins to gradually depolarise until it enters the region where the voltage-dependent block by Mg^{2+} is rapidly removed in a regenerative fashion causing further rapid depolarisation. A plateau is reached when voltage-dependent K^+ channels are activated. Although the current flowing through the activated NMDA receptor ion channels is carried mainly by Na^+ ions, Ca^{2+} also enters the cell and activates calcium-dependent potassium (K_{Ca}) channels so that the membrane potential begins to repolarise. The Mg^{2+} block of the NMDA receptor ionophore is then re-established, also in a regenerative fashion, so that the membrane rapidly repolarises back towards its normal resting potential. Since, however, the ligand is still present and K_{Ca} channels have closed due to a fall in intracellular calcium levels, the cycle repeats itself.

A number of questions arise from these observations and comparisons. For example, is the single spike per cycle of *Xenopus* embryo rhythmic neurons a characteristic imposed by their immaturity? And is the lack of evidence for TTX-resistant membrane potential oscillations, simply a consequence of the early stage of development of the nervous system? If so, at what stage do *Xenopus* larvae acquire a more adult-like locomotor pattern, and what are the mechanisms which might underlie such a change? Finally, do brainstem projections play a developmental role in the maturation of this very simple spinal circuit for locomotion?

vii) Field of study and Objectives

The simplicity of the *Xenopus* embryo preparation, both in terms of its neuroanatomy and locomotor activity, has been central to its success as a model system for the investigation of basic mechanisms underlying vertebrate locomotion. The connectivity of its spinal locomotor circuit and the underlying mechanisms are now very well understood in comparison with mammals. The broad aim of this study was to extend the use of this 'simple' model system to encompass a developmental perspective. Since the mechanisms of swimming in the stage 37/38 embryo are well described, there is a sound base from which to explore the post-embryonic development of the locomotor system. The cellular and synaptic mechanisms involved in the development of mammalian locomotion are so complex as to prohibit detailed analysis. However, the principle of using a relatively simple model system can with equal justification be applied to addressing developmental questions, since it is likely

that all vertebrates share not only basic features of locomotion (as outlined above), but also the underlying developmental mechanisms. Indeed, it is generally accepted that the basic spinal circuits responsible for the generation of locomotor activity are established at a very early stage of development, and that they are progressively influenced by higher brain centres during development. For example, in the human embryo, rhythmic limb movements have been observed in foetuses after only ten weeks of gestation, and descending influences on the locomotor circuits from higher centres continue for about six months following birth (Forssberg *et al*, 1991).

At about 24 hours prior to hatching, stage 27 *Xenopus* embryos are capable of rhythmic swimming-like movements (van Mier *et al*, 1986b). About a further 24 hours into development, the embryo (stage 37/38) hatches from its egg membranes and swimming is vigorous and well co-ordinated. Although at this stage, the embryonic swimming activity shares many of the principle features seen in adult vertebrates, it is nonetheless unusual because rhythmic neurons fire only a single action potential in each cycle of activity. The rhythm is therefore essentially an inflexible one with little scope for cycle by cycle modulation. This contrasts with more adult systems where the discharge pattern of motorneurons is highly variable, as is the ventral root activity (see fig 4). It is as though the *Xenopus* hatchling is equipped with an elementary 'starter-kit' which awaits developmental modifications that will ensure flexibility and survival.

This study addresses a number of questions arising from these observations. **Firstly**, what developmental changes occur to

the swimming activity after hatching in post-embryonic *Xenopus* larvae? Is there, for example, any change in the pattern of ventral root discharge which might indicate an acquisition of complexities normally associated with other vertebrate systems? **Secondly,** is there evidence that the locomotor circuit is being increasingly influenced by higher brain centres? In the *Xenopus* hatchling, the simple stereotyped swimming activity suggests very limited, if any, supraspinal modifications. This is also clear following spinalisation when the resulting swimming activity is almost indistinguishable from that of the intact animal. Since a number of descending brain stem interneurons are developing rapidly at this time, it may be that they are involved in post-embryonic modifications of the swimming circuit. **Thirdly**, are there developmental changes to the membrane properties of the component neurons of the spinal swimming circuit? The simplicity of the embryonic swimming pattern is imparted mainly by the strictly single spike capability of rhythmic neurons, and the tight synchrony of discharge among the motorneurons whose axons exit the same ventral root. Might an acquisition of additional complexity and variability in the swimming rhythm be associated with a multiple and variable spike capability as seen in other vertebrates, and might this be accompanied by a desynchronisation of spiking in motorneurons?

CHAPTER 2

Materials and Methods

Materials and Methods

Experiments were performed on embryos (developmental stages 32-37/38; Nieuwkoop & Faber, 1956) and early post-hatching larvae (stages 40 & 42) of the African clawed toad, *Xenopus laevis*. They were obtained by induced breeding following injection of human chorionic gonadotrophin (HCG) into breeding pairs from an adult laboratory colony.

Animals were initially anaesthetised in a dilute solution of MS222 and the dorsal fin gashed to permit subsequent access of the immobilising agent. Upon recovery from the anaesthetic and with swimming movements fully restored, they were immobilised with the neuromuscular blocking agent, α-bungarotoxin (α-BTX; 1.25μM). They were then pinned, with their left flank uppermost, with finely etched tungsten pins through the notochord to a rotatable Sylgard-coated perspex platform within a saline bath (volume *ca* 5ml). 100ml frog ringer's solution (composition in millimoles per litre; 115 NaCl, 2.5 KCl, 4 CaCl, 1 MgCl, 2.4 NaHCO3; pH 7.6) was gravity fed from a stock bottle and continuously recirculated through the bath with a flow rate of about 2ml per minute. The flank skin was then removed with finely etched tungsten dissecting pins to expose the underlying myotomes and the clefts between them in which motorneuron axons lie. Glass suction electrodes were then manipulated onto the clefts to record extracellularly the activity of motor axons in the ventral roots (see fig 5B). The electrodes were hand-pulled using a mini-Bunsen from 1mm outer diameter fibreless capillary tubes, and had tip diameters of around 50μM. Usually two or three recordings were made simultaneously, either one contralateral (right) and two

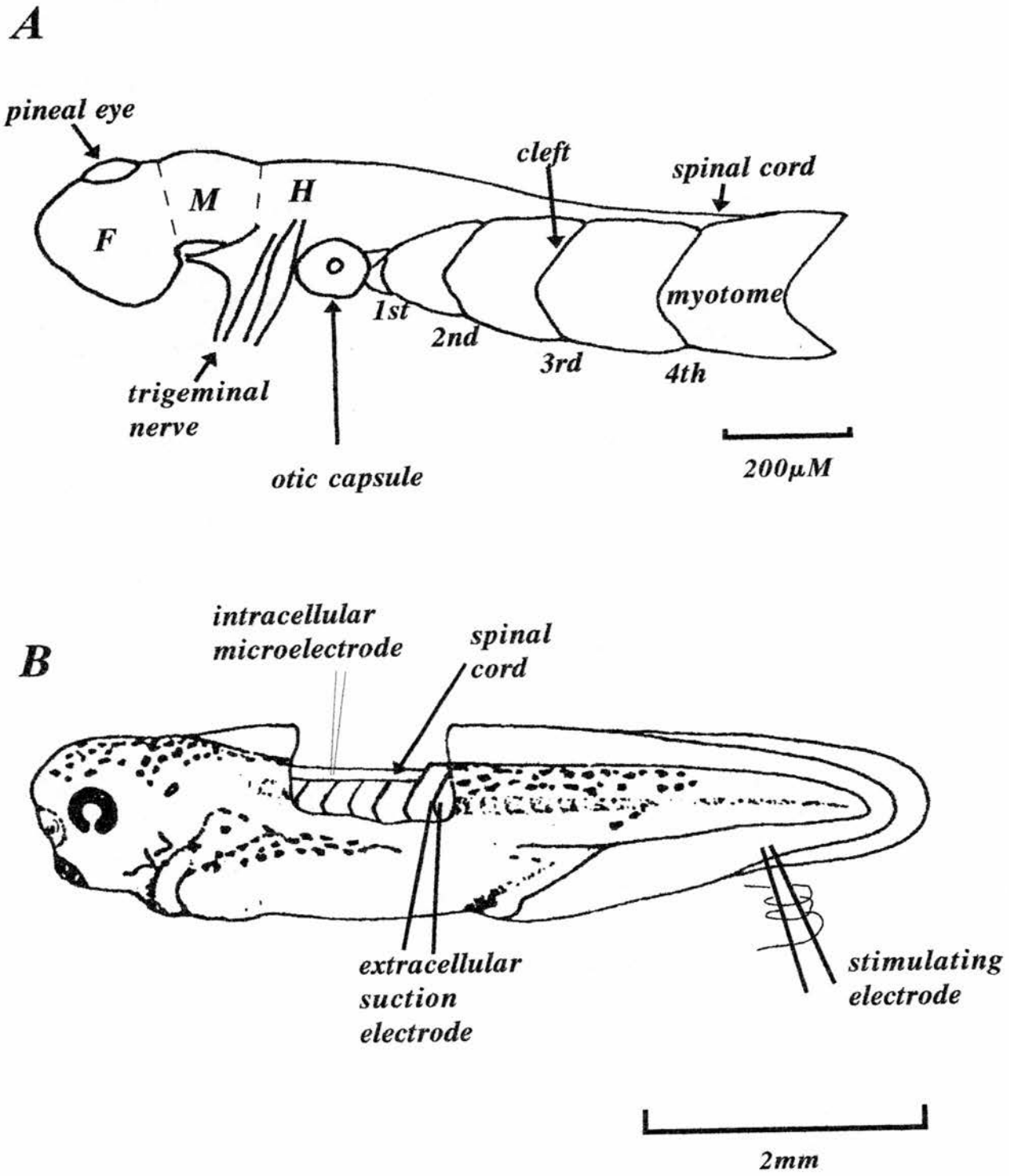

A
pineal eye
cleft
spinal cord
M
H
F
myotome
1st
2nd
3rd
4th
trigeminal nerve
otic capsule
200μM
B
intracellular microelectrode
spinal cord
extracellular suction electrode
stimulating electrode
2mm

fig 5. The preparation

(A) Drawing of the brain and rostral spinal cord of a *Xenopus* embryo showing also the otic capsule and the first few myotomes. F, forebrain; M, midbrain; H, hindbrain. The intermyotomal clefts are numbered, post-otically, as the 1st, 2nd *etc.* Glass suction recording electrodes are positioned onto the clefts to record extracellularly the activity in the ventral roots. (B) illustrates the dissected preparation. An area of trunk skin is removed to expose the underlying myotomes thereby permitting extracellular recording of ventral root activity from the intermyotomal clefts. To record intracellularly from rhythmic spinal neurons, myotomes overlying a small part of the spinal cord were removed to allow microelectrode access. Fictive swimming was elicited by applying a brief current pulse to the tail skin via a glass suction stimulating electrode or by dimming the illumination.

ipsilateral (left), or just two ipsilateral. Ventral root activity was amplified (x 10K) using an A-M systems high gain differential AC amplifier (model 1700). The clefts recorded from were identified as either left or right and were numbered rostro-caudally from the level of the otic capsule (fig 5A). For experiments in which rostro-caudal differences in locomotor activity were being investigated, clefts numbered 1-6 were deemed 'rostral' and 8-14 'caudal', with the proviso that there was a separation of at least five clefts.

Intracellular recordings of rhythmic spinal neurons were made by first removing the overlying myotomes from the rostral spinal cord with fine dissecting pins (fig 5B). Recordings were made with glass microelectrodes filled with either 2M potassium acetate or 3M potassium chloride and had resistances between 100 and 200 MΩ. They were pulled using a Campden Instruments micropipette puller (model 753). Only neurons in the ventral quarter of the cord were penetrated and this was achieved by capacitance overcompensation. Since this region of the cord is occupied almost exclusively by the cell bodies of motorneurons (Roberts & Clarke, 1982), I assumed that the vast majority of recordings from rhythmically active spinal neurons were from myotomal motorneurons. An A-M systems neuroprobe DC amplifier (model 1600) was used to amplify (x 10) the intracellular activity.

All experiments were recorded using a video cassette recorder coupled to a Medical Systems PCM-4 analogue to digital converter. Activity was displayed on a Gould digital 1604 oscilloscope, which was also linked to a Gould Colorwriter digital plotter and a Graphtec Thermal Arraycorder to enable permanent records to be made.

Drugs were bath applied by adding known quantities to the stock bottle to achieve the desired concentration in the bathing medium (listed below on page 32 are the drugs used in the course of this study, their concentrations and the name of the suppliers). To test the effects of 5HT on the fictive swimming pattern, 1-10μM of the amine was bath applied to the different developmental stages. The underlying ventral root activity was subsequently analysed to assess changes in average ventral root burst durations and cycle periods. Samples of fictive swimming used for analysis normally comprised twenty cycles taken from near the onset of swimming. The initial 500ms of activity were discarded to exclude any effects caused by the sensory stimuli. A range of 5HT receptor antagonists and agonists were bath applied in the same way to establish the pharmacology of the receptor subtypes involved, and their effects on the fictive swimming pattern similarly analysed. A number of experiments were also carried out on animals spinalised at the level of the otic capsule in order to remove supraspinal influences on locomotor rhythmicity. Spinalisations were done with fine dissecting pins, following which the animal was left to recover for thirty minutes.

A series of experiments were also conducted in collaboration with A-M Woolston involving neurotoxic ablation of serotonergic neurons using the selective neurotoxin 5,7-dihydroxytryptamine. Six animals were raised in 1 mM toxin from about stage 25, at which time brain stem serotonergic neurons are first detectable (van Mier *et al,* 1986). Since the neurotoxin is light sensitive, their subsequent development up to stage 42 was carried out in the dark alongside six control animals. At developmental stage 42, they were immobilised and the ventral root activity during fictive

swimming recorded and analysed.

The cellular and synaptic mechanisms of serotonergic modulation of both passive and active membrane properties of spinal neurons were investigated using both KCl and KAc-filled micro-electrodes. The principle advantage of using KCl electrodes, is that chloride leakage from the electrode into the neuron causes chloride-mediated inhibitory potentials to become massively depolarising, and this greatly facilitates analysis of inhibitory events. For example, frequency and amplitudes of spontaneous inhibitory potentials before and after 5HT application were analysed and plotted as amplitude versus frequency of occurrence histograms (*eg* fig 35). Estimates of relative changes in membrane resistance following drug applications were performed by injecting trains of eight 200ms hyperpolarising constant current pulses. Any change in the resulting voltage deflection, therefore, reflect changes in the membrane resistance. Before impalement and with the tip of the electrode in the bathing medium, bridge balancing was achieved by injecting current pulses and then negating any voltage deflection due to electrode resistance by use of the bridge balance control. However, since these fine-tipped, high resistance electrodes are susceptible to damage and/or blockage, accurate bridge balancing was difficult. Intrinsic membrane properties were also investigated by application of 1μM tetrodotoxin (TTX), which synaptically isolates cells from one another by blocking Na^+-dependent impulse generation. Stable penetrations of rhythmic spinal neurons in stage 42 larvae was considerably more difficult than in stage 37/38 embryos, possibly on account of a thicker sheath surrounding the cord.

Drugs used in the course of this study

Drug	Concentration (μM)	Source
5HT	1-10	Sigma
5HTP	5-20	Sigma
TTX	0.5-1	Sigma
5,7-DHT	100	Sigma
Bicuculline	30	Sigma
Strychnine	1-10	Sigma
d-tubocurarine	100	Sigma
NMDA	100	Sigma
5CT	0.05-1	RBI
R(+)-8-OH-DPAT	2-10	RBI
(+)-8-OH-DPAT	10-50	RBI
Buspirone	10-50	RBI
CGS maleate	10-50	RBI
TFMPP	20	RBI
Mianserin	50-100	RBI
Cyproheptadine	20-80	RBI
Ketanserin	10-100	RBI
Nan-190	10-100	RBI
Mesulergine	10-50	RBI
MDL 72222	10-100	RBI
Ondansetron	10-20	Glaxo
Methysergide	2-20	Sandoz

RESULTS

CHAPTER 3

The development of locomotor rhythmicity

INTRODUCTION

As has been described in the introductory section, the *Xenopus* embryo swimming system has proven to be an excellent model system for investigating the mechanisms of vertebrate locomotor rhythm generation. It is, however, a remarkably simple system generating a basic and essentially inflexible motor pattern. It seems likely that this simplicity is a consequence of its early developmental stage, rather than a species-specific phenomenon. In a wide range of other vertebrate species, the basic neural networks for locomotor rhythm generation appear to be established very early in embryogenesis, and then are progressively modified so that locomotion not only remains appropriate throughout development, but also conferring upon it the flexibility and responsiveness necessary for adult life. The development of locomotion has been studied in a range of vertebrates which include agnathans (Cohen *et al,* 1990), fish (Batty, 1984), amphibians (Kahn & Roberts, 1982a & b), birds (Beckoff, 1992) and mammals (Kudo & Yamada, 1987; Hernandez *et al,* 1991). These studies have revealed a number of common features regarding the development of locomotor rhythmicity which strongly suggest that immature circuits are the lineal precursors of the complex adult ones. Rhythmic 'locomotor-like' movements have been observed at very early stages in development often during embryogenesis when actual locomotion is impossible. The circuits which generate these movements are intrinsic to the spinal cord and require neither sensory feedback nor descending inputs, since following spinalisation, activity can occur either spontaneously or

in response to pharmacological stimulation. Thus in this regard, they are similar to adult locomotor systems. An other important observation is that basic co-ordination of motor output remains unchanged throughout development. Perhaps the best example of this is provided by the chick. Even although hatching and walking are distinct behaviours, the underlying motor activity is very similar, sharing the same muscle synergies, with only subtle changes in the phasing (Bekoff, 1992). This strongly suggests that the same basic circuits are involved, and are modified during development to produce the slightly different motor patterns required of different stages. In the light of these observations, therefore, I examined the swimming activity of developing *Xenopus* larvae over a brief 24 hour period following hatching. Since the circuitry and mechanisms underlying swimming in the stage 37/38 hatchling are relatively 'simple' and well described, it was hoped that post-embryonic modifications to the basic swimming rhythmicity could be observed and the underlying mechanisms revealed.

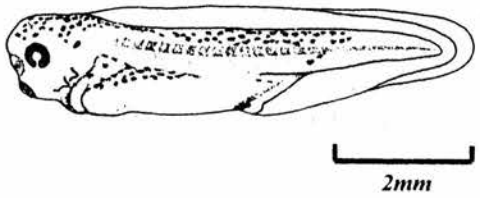
RESULTS

i) Development of ventral root activity underlying swimming

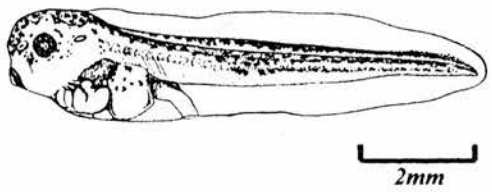
Episodes of fictive swimming, initiated by either a brief current pulse to the tail skin or by dimming the lights, were recorded extracellularly from the ventral roots of α-BTX-paralysed stage 37/38 *Xenopus* hatchlings. Ventral root activity usually consisted of brief (5-7ms) biphasic impulses alternating on opposite sides of the body and propagating rostro-caudally with a brief intersegmental delay of about 1-2ms (measured near the beginnings of episodes; see 'Materials & Methods') (fig 6 Ci). Cycle periods ranged from about 50-100ms, with swimming frequencies fastest (*ca* 20Hz) at the beginnings of episodes and slowest (*ca* 10Hz) towards the ends. The stereotyped nature of the ventral root discharge on each cycle is apparent when several consecutive cycles of activity are superimposed (see fig 7 Ci). The activity recorded in α-BTX-immobilised embryos (Boothby & Roberts, 1988) appears indistinguishable from that described in hatchlings paralysed in curare (Kahn & Roberts, 1982a & b).

However, after only about twenty-four hours of further development (at 23° C; Nieuwkoop & Faber, 1956), at stage 42, the ventral root activity underlying swimming is dramatically different, consisting of bursts of discharge lasting up to or occasionally more than 20ms in each cycle (fig 6 Cii). Thus, during fictive larval swimming, ventral root bursts are on average much longer in duration than in embryos. In the illustrated example (fig 7), the average burst durations of rostral ventral root

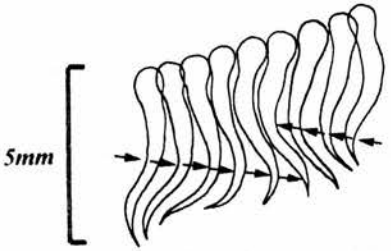
Ai ***Swimming in the stage 37/38 embryo***



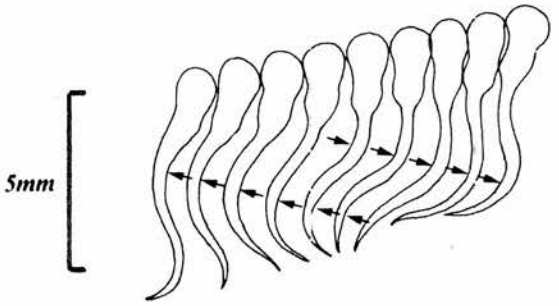
Aii ***Swimming in the stage 42 larva***



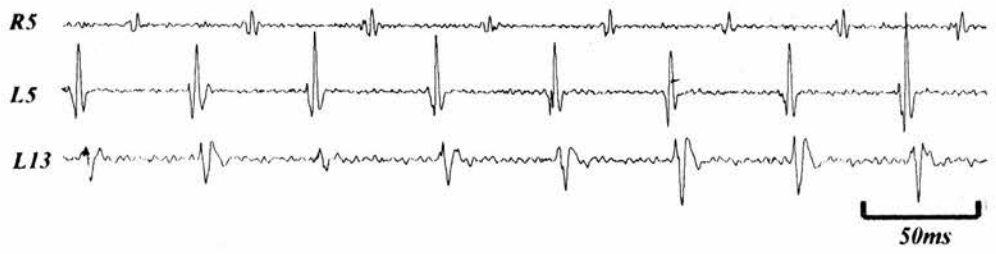
Bi



Bii



Ci



Cii

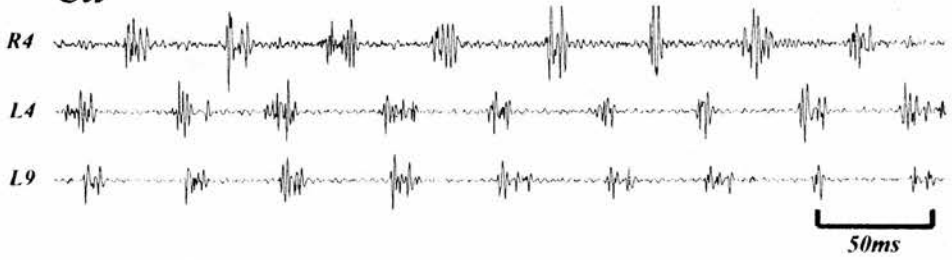


fig 6. Swimming in the stage 37/38 embryo & stage 42 larva

(A) Twenty-four hours after hatching (stage 37/38; Ai), the stage 42 *Xenopus* larva (Aii) is some 40% bigger, the yolk sac is almost entirely depleted and the gut has developed. (B). Outline drawings of swimming movements at stage 37/38 (Bi) and stage 42 (Bii) traced from high speed video images with 5ms between frames (arrowheads indicate points of maximum body curvature). In both stages, swimming comprises alternating propulsive waves of bending passing first down one side of the body and then the other, and occurs at similar frequencies (note the differences in the size and shape of the embryo and the stage 42 larva). (Ci & Cii) The co-ordination of the ventral root activity underlying swimming appears unchanged in that it alternates and passes down the body with a brief segmental delay. However, the ventral root activity itself is very different in the two stages. In the embryo (Ci), it consists of brief biphasic impulses of about 7ms duration, while in the larva (Cii), there are long busts of ventral root discharge of variable duration lasting up to and sometimes more than 20ms.

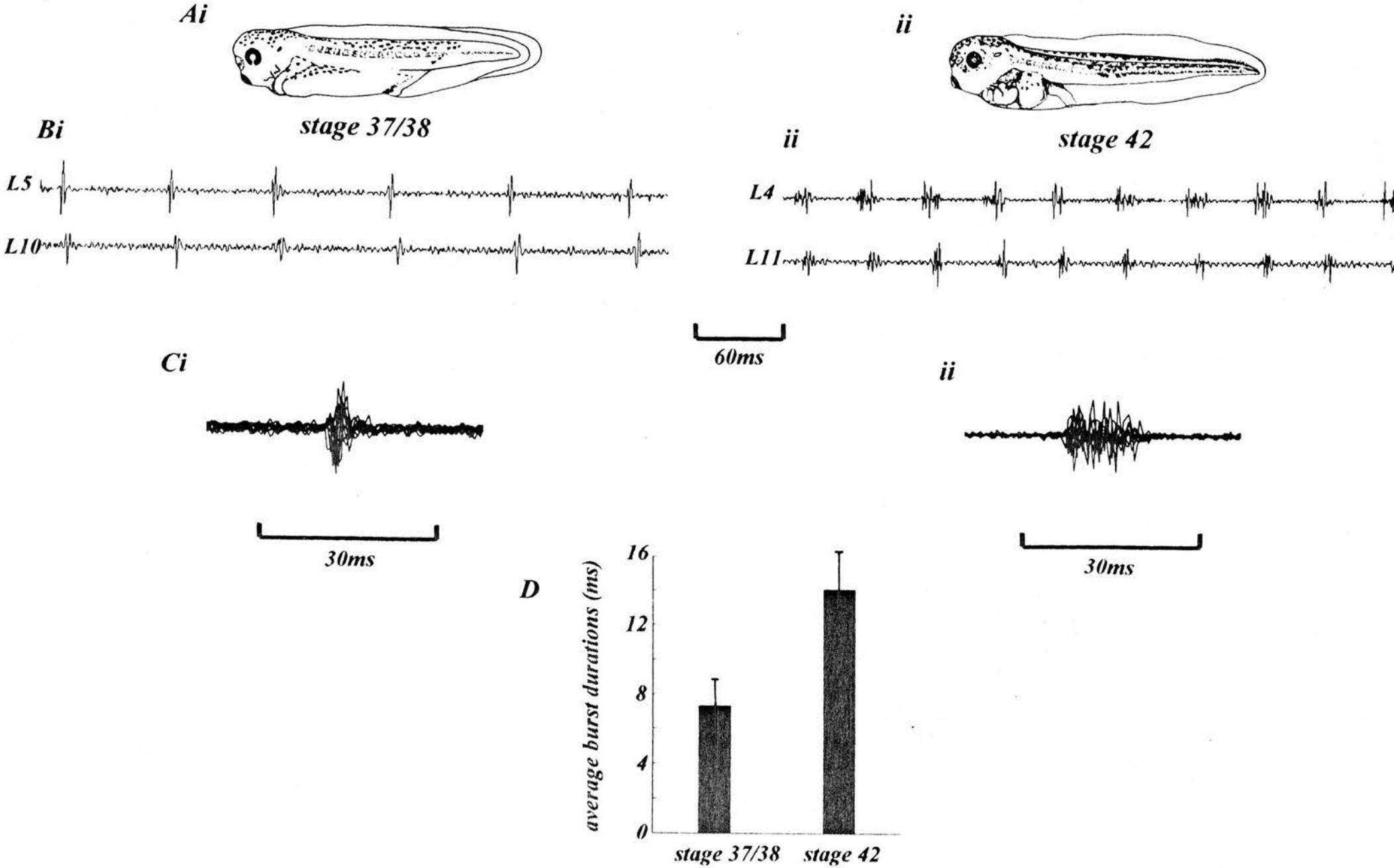

Ai
ii
Bi
stage 37/38
ii
stage 42
L5
L10
L4
L11
60ms
Ci
ii
30ms
30ms
D
average burst durations (ms)
16
12
8
4
0
stage 37/38
stage 42

fig 7. The development of locomotor rhythmicity

(Bi & Bii) The ventral root activity underlying swimming in the stage 37/38 embryo and the stage 42 larva. When the first ten cycles of an episode at each stage are superimposed, the differences in burst structure and durations are readily apparent (Ci & Cii). D is a plot of the average burst durations in the two stages (n=15). Note that, not only are average burst durations very much longer in the stage 42, but they are usually more variable. In this and subsequent figures error bars are standard deviation.

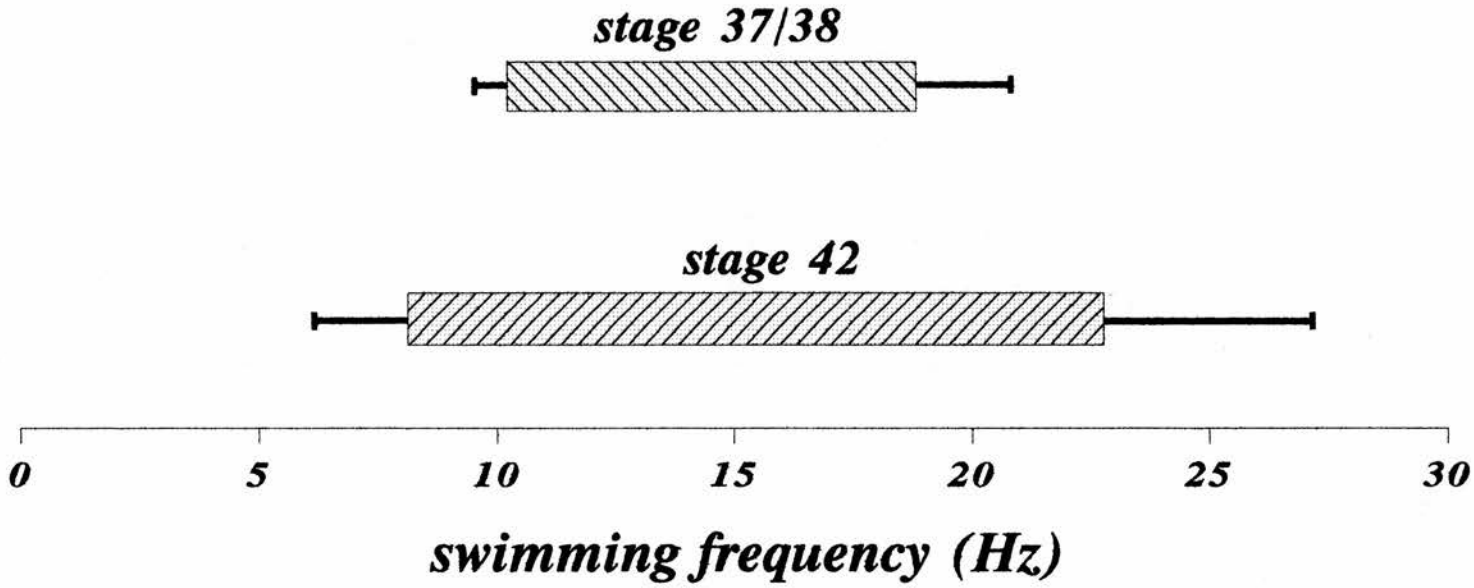

stage 37/38
stage 42
0
5
10
15
20
25
30
swimming frequency (Hz)

fig 8. Fictive swimming frequencies in stage 37/38 embryos & stage 42 larvae

The average range of fictive swimming frequencies in stage 37/38 embryos and stage 42 larvae. The maximum and minimum swimming frequencies were measured from the beginnings and ends of 23 episodes of embryonic swimming in ten animals, and from 30 episodes of larval swimming, also from 10 animals. The plot shows the average range of swimming frequencies (Hz) in the two stages with error bars (sd). In the embryo, the average minimum and maximum frequencies were 10.5 $^{+}/_{-}$ 0.96 and 18.33 $^{+}/_{-}$ 3.51Hz respectively, and in the stage 42 they were 8.8 $^{+}/_{-}$ 1.72 and 23.1 $^{+}/_{-}$ 4.9Hz.

activity measured over the first 15 cycles in the embryo was 7.3 ± 1.6 ms (sd), while it was nearly double that value in the stage 42 larva (14.1 ± 2.14 ms; fig 7D), a difference which is highly significant (students *t*-test, $P<0.005$). There is also increased variability in burst duration on a cycle by cycle basis. Superimposed traces of the first ten cycles illustrate these differences between the two stages more clearly (fig 7 Ci, ii). Moreover, the changes in the ventral root activity appear to occur without significant changes in locomotor frequency (see, however, below), so that by stage 42 each burst occupies a greater proportion of its cycle period (see fig 10). The older animal does, however, appear to exhibit a greater range of cycle periods than the embryo, and this is most apparent at the beginnings and ends of episodes. For example, the embryo typically swims at frequencies of 10-20Hz, while the stage 42 larva can swim at 7-30Hz (fig 8).

Although dramatic changes in the locomotor output clearly occur in a brief 24 hour period following hatching, the underlying co-ordination at stage 42 is apparently unaltered (fig 6). As in the stage 37/38 embryo, ventral root activity at stage 42 alternates between the two sides of the body and passes rostro-caudally with a brief intersegmental delay (*cf* fig 6 Cii & Ci). Just as the basic co-ordination of the fictive swimming activity is unaltered, so too is the co-ordination of real swimming. High speed video film shows that swimming in the two stages (fig 6 Bi, ii) similarly involves travelling waves of curvature passing first down one side of the body and then the other, which generates a reactive force against the surrounding water to propel the animal forward (Gray, 1933).

The major conclusion which can be drawn from these data is that a dramatic change in the structure of motor bursts underlying

swimming occurs in a very brief period (*ca* 24 hours) of post-embryonic development. The nature of the change and its possible consequences for swimming behaviour are now addressed in detail in the following sections.

ii) Rostro-caudal development of ventral root bursts

Insights into the temporal acquisition of ventral root bursts were obtained by recording fictive swimming in the stage 40 larva, about 12 hrs after hatching (n=65). At this stage of development, an intermediate pattern of ventral root activity is observed since there are clear differences in the duration of bursts in rostral ventral roots compared to caudal. Recordings from intermyotomal clefts near the front of the animal (intermyotomal clefts 1-6; see 'Materials and Methods') reveal burst durations of typically 10-20ms, similar to those of stage 42 bursts but different from the embryo. By contrast, caudal ventral roots (clefts 8-14) display a pattern of activity which is still essentially embryonic, having brief biphasic impulses of about 7ms in duration. In the illustrated example (fig 9), the rostral ventral root (L5) has an average burst duration of 11.82 ± 1.93 ms (n=20) compared with 5.57 ± 1.74 ms in the more caudal (L10) (n=20). The differences between the activities in the two roots is significantly different (Students *t*-test, $p < 0.005$) and this is even more evident when 15 cycles of activity near the start of an episode are superimposed (fig 9 C). Detailed analysis of burst durations in rostral and caudal ventral roots in the three developmental stages - hatchling, 12 hours post hatching and 24 hours post hatching - clearly reveal a rostro-caudal progression

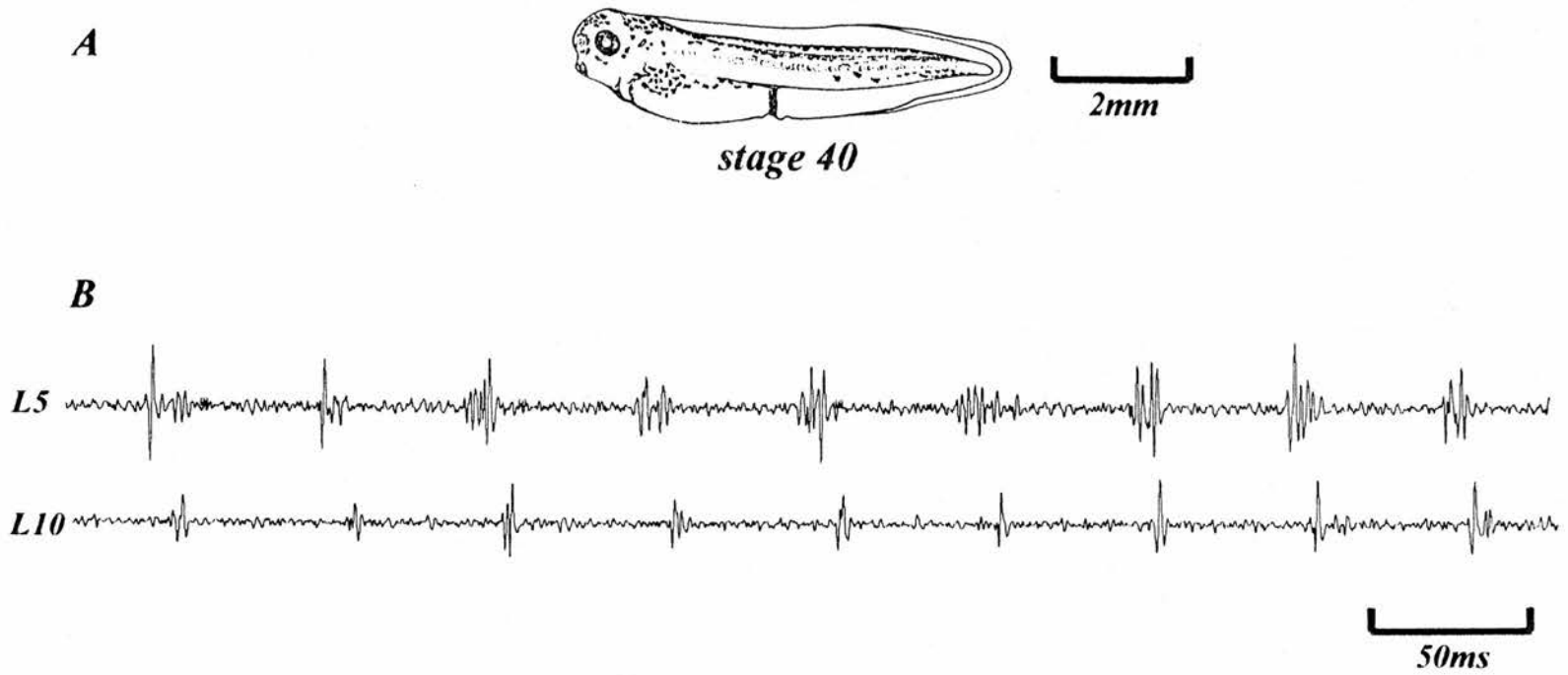

A
2mm
stage 40
B
L5
L10
50ms
C
rostral
caudal
20ms
D
average burst durations (ms)
12
8
4
0
rostral
caudal

fig 9. Swimming activity in the stage 40 larva

(A) Stage 40 larva. (B) Activity recorded from rostral (L5) and caudal (L10) ventral roots. Rostral ventral root discharge has burst durations of between 10-20ms. By contrast the caudal activity is 'embryonic-like', comprising mainly of brief biphasic impulses. (C) 15 superimposed cycles illustrate the differences between rostral and caudal ventral root activity more clearly (note that the apparent rostro-caudal delay in C is only an approximate representation). D is a plot of the average burst durations in 20 cycles of activity near the beginning of the episode.

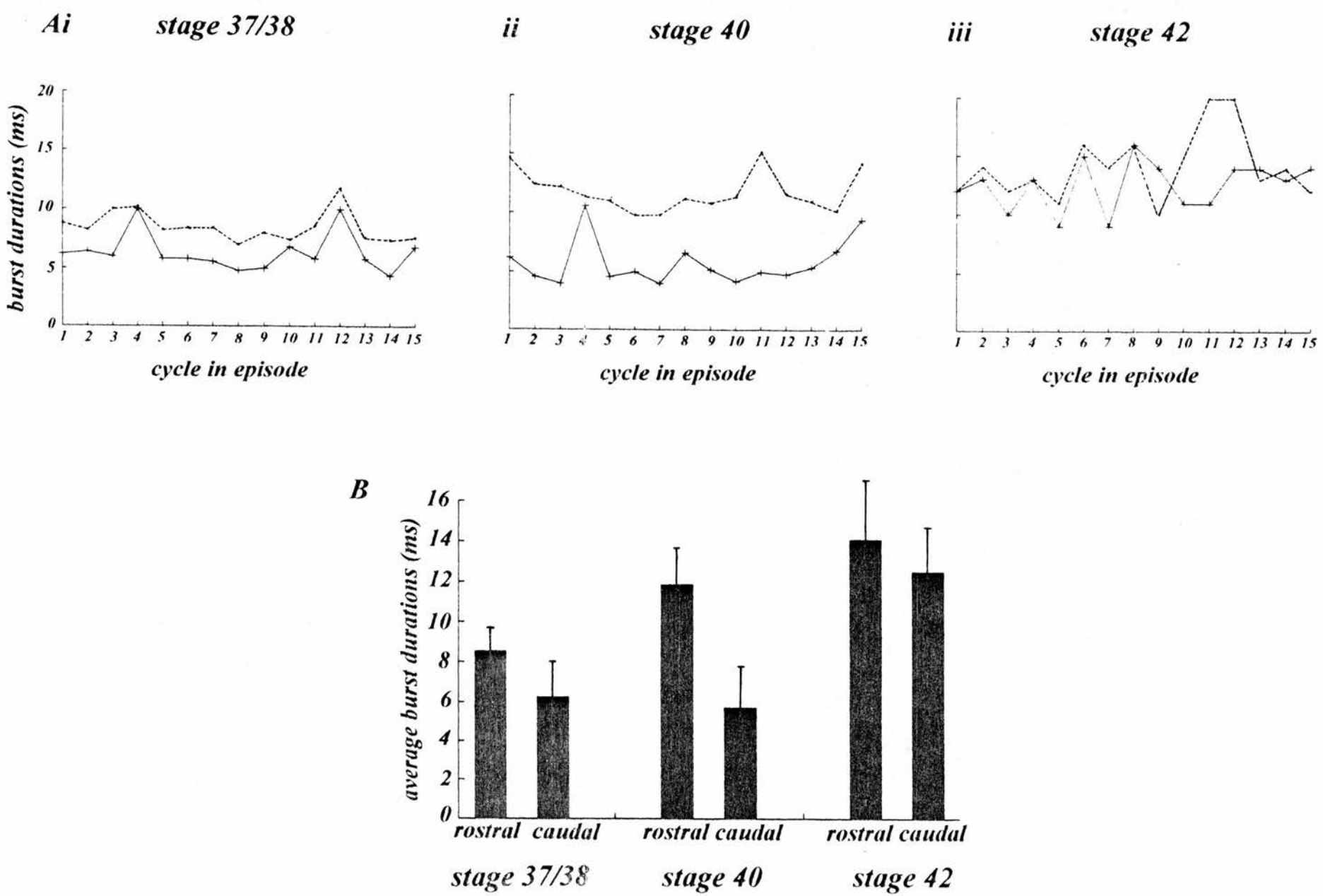

Ai
stage 37/38
ii
stage 40
iii
stage 42
burst durations (ms)
cycle in episode
cycle in episode
cycle in episode
B
average burst durations (ms)
rostral caudal
rostral caudal
rostral caudal
stage 37/38
stage 40
stage 42

fig 10. Development of locomotor rhythmicity occurs rostro-caudally

(A) Plots of the first 15 cycles of rostral and caudal ventral root burst durations from an episode of swimming in each of the three stages. Rostral ventral root activity is depicted by points and broken lines, and the caudal by crosses and solid lines. (Ai) In the stage 37/38 embryo, the activity in the rostral root is consistently, but only slightly longer than in the caudal. (Aii) At stage 40, this difference between rostral and caudal ventral root activity is more striking. The rostral ventral root activity is yet more 'bursty', while the caudal is 'embryonic like'. (Aiii) By stage 42, a marked rostro-caudal difference is less apparent, since caudal ventral root activity has acquired burst durations similar to the rostral. (B) Histogram of average rostral and caudal ventral root burst durations in the three stages, illustrating the progressive rostro-caudal increase in ventral root discharge durations during the first twenty-four hours of larval life.

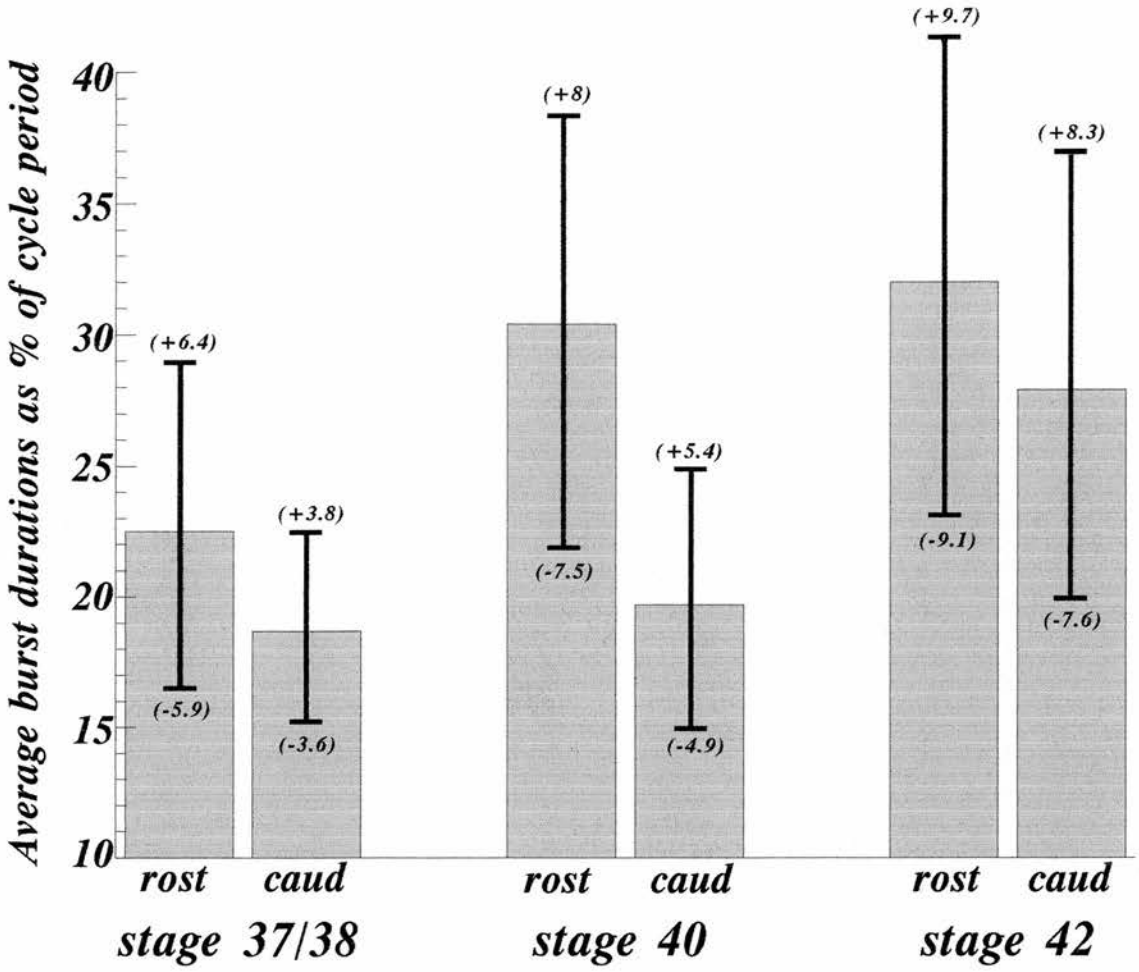


fig 11. Average burst durations as a percentage of cycle periods

Post-embryonic development of locomotor rhythmicity occurs rostro-caudally and involves an increase in ventral root burst durations without significant changes in cycle periods. Therefore, when burst durations are expressed relative to cycle periods (%), the proportion of the cycle period occupied by activity progressively increases in a rostro-caudal sequence as the animal develops. The plot illustrates that at stage 37/38, the relative burst durations are on average greater in the rostral ventral root than in the caudal. At stage 40, this difference is considerably more apparent since the relative burst durations in the rostral root have increased further, which is in contrast to the caudal where they appear to have remained embryonic-like. In the stage 42 larva, relative burst durations in the rostral and caudal roots more closely resemble each other. In the rostral root they have remained similar to those of the stage 40, while in the caudal they have increased. Note the increased variability which accompanies the increase in relative burst durations. These data derive from experiments not previously illustrated, and means and standard deviations were obtained using the arcsine transformation which transforms percentages and proportions into a variable which meets the criteria for analysis of variance. As a consequence, error bars are not symmetrical about the mean. Relative burst durations were calculated from thirty consecutive cycles near the beginnings of episodes.

of ventral root burst acquisition during rhythm development (fig 10). In the stage 37/38 embryo, rostral ventral root activity is on average slightly, but not significantly, longer and more variable than the caudal (Ai, B), however twelve hours later at stage 40, this difference is considerably more apparent (Aii, B). The stage 40 larva is clearly in a transitional phase of locomotor development; rostral ventral root activity closely resembles that of a stage 42 while the caudal is essentially embryonic-like. By stage 42, caudal ventral root bursts have also increased in duration and more closely resemble those of the rostral root, although a slight rostro-caudal gradient is still evident (Aiii, B). When ventral root burst durations are also expressed as a percentage of cycle period, the rostro-caudal acquisition of 'burstiness' is equally apparent (fig 11). This analysis also illustrates that swimming frequencies do not fundamentally change over this period, although the stage 42 larva does exhibit a greater range than the stage 37/38 embryo (see fig 8)

The development of locomotor rhythmicity thus occurs rostro-caudally, suggesting that a developmental influence progressively invades the spinal locomotor network in a rostro-caudal sequence to enhance the motor output. This is consistent with (but by no means proof of) the notion that descending brain stem systems influence and modify immature spinal locomotor networks in the course of development. The nature and mechanisms of this descending developmental influence is addressed in chapter 4. However, a second important implication can be drawn from these data. The embryonic rhythm is remarkably stereotyped in that it exhibits very little variability on a cycle by cycle basis, and thus lacks flexibility. This is in dramatic contrast to the stage 42 larva in which ventral root activity

comprises bursts of discharge of variable duration. It appears therefore to offer, at least in principle, greater scope for altering the drive to the muscles on a cycle by cycle basis and thereby permit a swimming behaviour with greatly enhanced flexibility. This notion is explored in the following section.

iii) Flexibility of the larval swimming pattern

Although embryonic fictive swimming frequency can vary within an episode, the neural output does not permit altered strengths of myotomal muscle contractions. This simplicity is imparted mainly by the strictly single spike capability of motorneurons during swimming, which fire reliably on every cycle of an episode (Sillar & Roberts, 1993). Those which exit the same ventral root also appear to fire synchronously so that the ventral root activity recorded during swimming represents the compound discharge of all the motorneurons in that 'segment'. This pattern of input to the myotomes must inevitably lead, therefore, to a fixed and stereotyped swimming rhythm with little scope for intrinsic modulation. By contrast, the ventral root activity at stage 42 comprises bursts of discharge of variable duration. Consequently there is now a neural substrate on which modulatory inputs might act to alter the pattern of motor output to the muscles, thereby enabling manoeuvres in response to environmental cues. Is there any evidence, however, that this does in fact occur?

During fictive swimming in the stage 42 larva, a pattern of ventral root activity which seems appropriate for a turning

manoeuvre is occasionally observed (n=5). Thus, when recording from both contralateral and ipsilateral ventral roots, activity can be suppressed on one side of the body and simultaneously enhanced on the other (fig 12). This would presumably result in a more powerful muscle flexion on one side of the body, thereby causing the animal to turn with minimal disruption to the basic underlying swimming rhythm. It is difficult to envisage such a phenomenon occurring in stage 37/38 embryos where the relative durations and intensities of brief ventral root impulses cannot be altered. Analysis of ventral root activity at this stage in development provides no evidence for such a capability, except when appropriately timed skin sensory input occurs (Sillar & Roberts, 1988b).

Also apparent at stage 42, but absent in the embryo, is the ability to switch rapidly to a faster swimming frequency (fig 13). This fictive acceleration can occur over a single cycle and involve an approximate doubling of swimming frequency (n=7). Figures 13A & B illustrate two examples of acceleration in the same animal, where the change in fictive swimming frequencies in A (at asterisk) occurs more rapidly than in B. Acceleration also involves an increase in the intensity and durations of the ventral root bursts, and where the acceleration is more rapid, the increase in burst durations and intensities is correspondingly greater (*cf* A & B). In the behaving animal, this pattern of motor activity could conceivably underlie a more rapid, higher amplitude propulsive wave of bending down the body. Although changes in the frequency of embryonic fictive swimming can occur following sensory stimulation (Sillar & Roberts, 1992b), it appears to lack the flexibility described above.

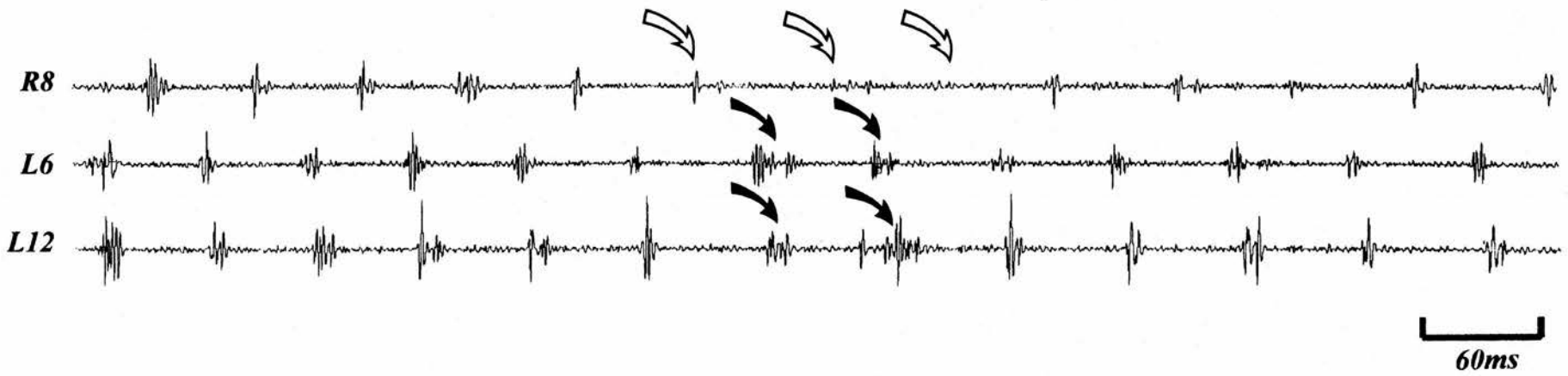
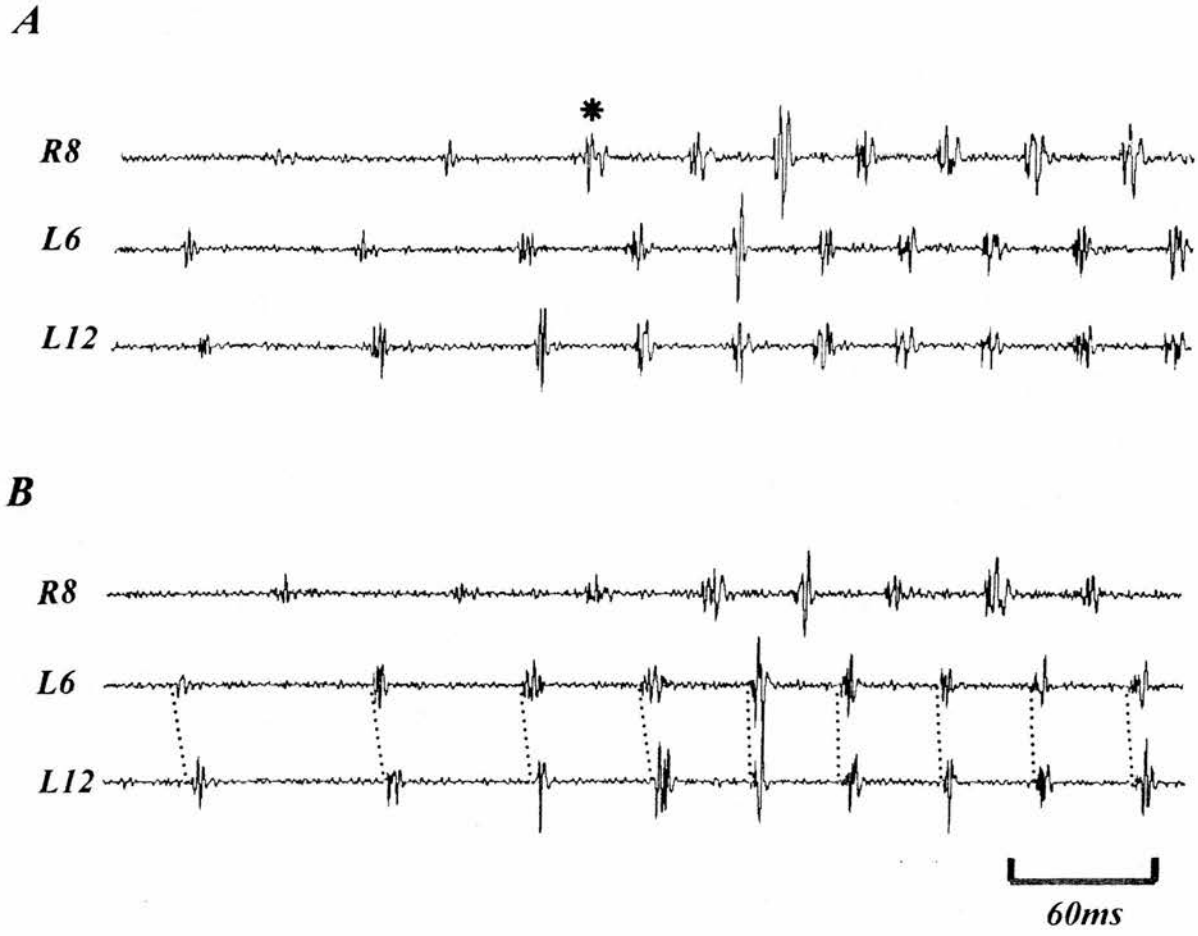

R8
L6
L12
60ms

fig 12. Fictive turning in the stage 42

When simultaneous recordings are made from both contralateral (R8) and ipsilateral (L6 & L12) ventral roots at stage 42, a spontaneous pattern of activity is occasionally observed which appears appropriate for a turning manoeuvre. Over a period of a few cycles, the activity is suppressed on one side (unfilled arrows), while at the same time it is enhanced on the other (filled arrows). This would presumably result in a relatively more powerful muscle flexion on one side of the body, causing the animal to turn. Note that the enhanced activity on the left side is distributed along the body, appearing at L6 and L12.


A
R8
L6
L12
B
R8
L6
L12
60ms

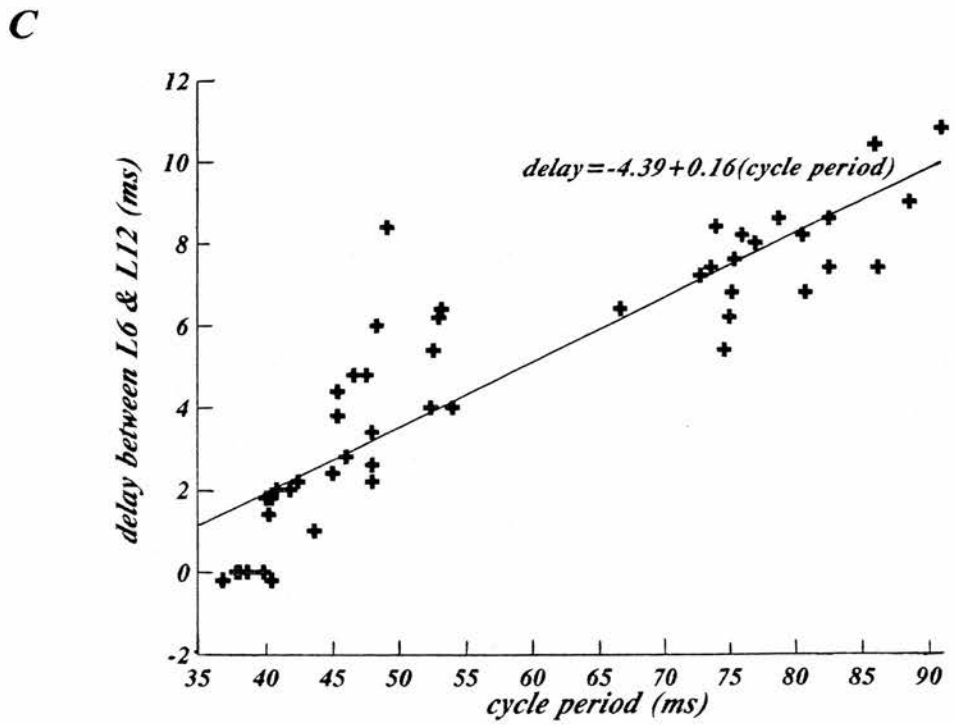

C
delay=-4.39+0.16(cycle period)
delay between L6 & L12 (ms)
cycle period (ms)
12
10
8
6
4
2
0
-2
35
40
45
50
55
60
65
70
75
80
85
90

fig 13. Acceleration & intersegmental delays in the stage 42

(A & B) Two examples of fictive swimming taken from the same stage 42 larva, in which the frequency and intensity of ventral root activity rapidly and dramatically increases in a way suggestive of acceleration. (A) Acceleration (onset at asterisk) appears more rapid than in (B), and involves nearly a doubling of swimming frequency over one cycle. In B, dotted lines join the onset of activity in L6 & L12, and their slope provides an indication of the delays between the two roots. They illustrate that at higher swimming frequencies following the onset of acceleration, the delay is reduced. (C) A plot of delays (ms) between the L6 & L12 against cycle period. Two clusters of points are evident, one cluster depicting the passage of swimming before acceleration occurred, where cycle period and delays were long, and the other after, when cycle periods and the corresponding intersegmental delays were brief. The two distinct clusters of points also emphasise the nature of the dramatic switch from slow to fast swimming. The fitted regression line has the equation: delay=-4.39+0.16(cycle period). The correlation coefficient (r) = 0.89.

iv) Development of intersegmental co-ordination.

The nature of the intersegmental co-ordination is another unusual feature of the stage 37/38 swimming rhythm compared to other swimming systems, in that the magnitude of rostro-caudal delay in motor output is not related to the cycle period (Tunstall & Roberts, 1991). In the lamprey (Wallén & Williams, 1984), the leech (Pearce & Friesen, 1984, 1985) and in a closely related amphibian species at an equivalent stage of development, *Rana temporaria* (Soffe, 1991), rostro-caudal intersegmental delays are correlated with cycle period. Is this unusual feature of the hatchling *Xenopus* motor rhythm also a consequence of the developmental simplicity of its swimming rhythm generator? This seems likely since by stage 42, the relationship between cycle period and intersegmental delay has adopted a more typical form in that the two have become correlated (Sillar & Wedderburn, 1993; reviewed in Tunstall & Sillar, 1993). The examples of acceleration described in the previous section involving a sudden switch in swimming frequencies, also serve well to illustrate this relationship between cycle period and segmental phase delay in larvae. Figure 13 C is a plot of cycle period against delay between L6 and L12. It comprises combined data from the two examples of acceleration where cycles of swimming immediately before and after the switch in frequencies were used for analysis (n=50). The data points clearly fall into two distinct clusters corresponding to low and high frequencies before and after the switch. At the onset of the acceleration, where cycle periods are brief (35-45ms), intersegmental delays are also correspondingly brief. Indeed, over

the initial few cycles of rapid acceleration (as in fig 13 A), they can be either close to zero or even reversed. Immediately before the switch, however, when cycle periods are about 80ms, delays are correspondingly longer (8-10ms). It is noticeable that at cycle periods of about 45ms delays are about 2ms, whereas at about 80ms, they are in the region of 8ms, which is in close agreement with previous findings (Sillar & Wedderburn, 1993; reviewed in Tunstall & Sillar, 1993).

v) Effects of spinalisation on larval swimming

Two important and related questions arise from these observations; firstly, are the changes mediated by a descending neuronal input, and secondly, to what extent is the developmental transformation in swimming due to the spinal circuit being permanently modified? These questions were addressed by examining the effects of spinalisation on the stage 42 rhythm (n=12).

Following transection of the spinal cord at the level of the otic capsule, the stage 42 larval rhythm appears to resume embryonic features in a number of regards. Most noticeably, ventral root burst durations resumed a brief and usually biphasic form with durations of typically about 7ms. In the illustrated example (fig 14), the rostral ventral root activity (L4) of a stage 42 typically comprised burst durations of between 10 and 20ms. However, following spinalisation, they resembled those of the embryo consisting usually of brief biphasic impulses of about 7ms duration (B). Superimposed traces of 15 cycles of rostral ventral

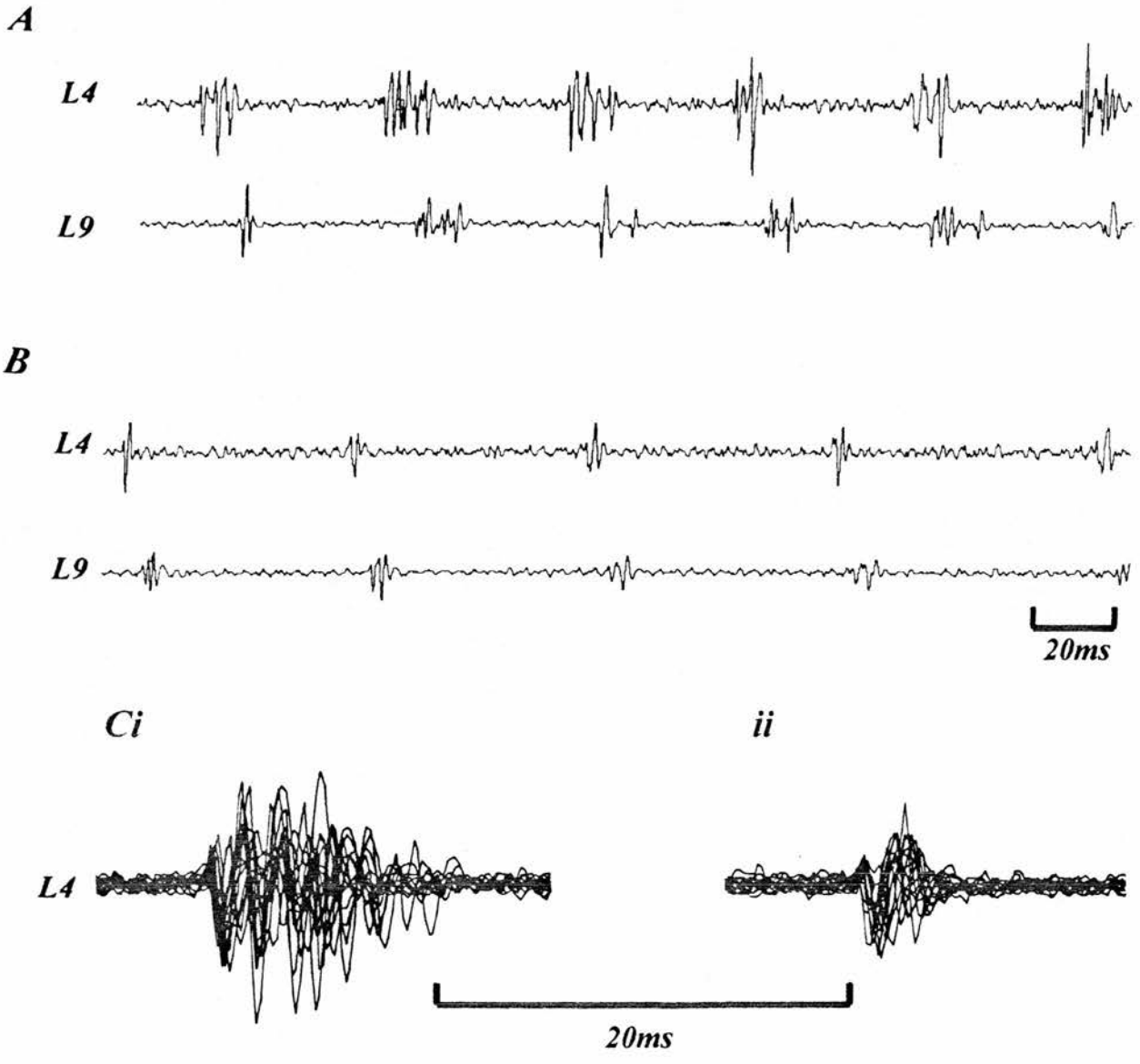

A
L4
L9
B
L4
L9
20ms
Ci
ii
L4
20ms
D
15
10
5
0
delay (ms)
30
50
70
90
110
cycle period (ms)
control
spinal

fig 14. Effects of spinalisation on swimming activity in the stage 42 larva

(A) An excerpt of fictive swimming in a stage 42 larva. (B) Swimming in the same animal fifteen minutes following spinalisation at the level of the otic capsule. Note that it appears 'embryonic-like', with brief ventral root activity. (C) 15 superimposed cycles of rostral ventral root (L4) activity before (i) and after (ii) spinalisation. (D) Plot of intersegmental delays (n=95) between L4 & L9 reveal a good correlation with cycle period before spinalisation (r=0.83), and the equation of the fitted regression line is; delay=1.36+0.083(cycle period). Following spinalisation (n=101) the relationship is lost (r=-0.12) and the regression line has the equation; delay=5.65-0.007(cycle period).

root activity before and after spinalisation clearly shows the contrast (fig 14Ci, ii). It has to be considered, however, that transection of the cord at the level of the otic capsule could remove a small proportion of descending excitatory drive for swimming (Roberts & Alford, 1986), and it may be that this loss might contribute to briefer ventral root activity post-spinalisation (see, however, 'Discussion'). Spinalisation also resulted in the loss of the relationship between cycle period and rostro-caudal intersegmental delays. In short, the intersegmental co-ordination of the spinalised stage 42 larva appeared indistinguishable from that of the intact stage 37/38 embryo. This is illustrated in fig 14 D, in which delays between L4 and L9 were plotted against cycle periods before and after spinalisation, and demonstrates clearly the change in the relationship between cycle period and intersegmental delay following the loss of descending inputs.

These data, taken together with those presented in the previous sections, offer good additional evidence that the progressive influence of a developing descending system first modifies the output of the spinal locomotor networks in post embryonic *Xenopus* larvae, and thereafter continues to maintain and modulate it. The most likely mechanism underlying the developmental change in ventral root activity is the acquisition of a multiple spike capability in rhythmic spinal neurons. The post-embryonic development of cell properties was therefore explored by making intracellular recordings from spinal neurons and this is described in the following section.

vi) The development of cell properties and synaptic drive

In *Xenopus* embryos (stage 37/38) immobilised in curare, intracellular recordings from rhythmic spinal neurons during fictive swimming, reveal a characteristic pattern of activity which is very similar for all the cell types of the motor circuit (Roberts & Kahn, 1982; Soffe & Roberts, 1982; Dale, 1985; Dale & Roberts, 1985). The activity consists of phasic excitatory amino acid mediated (EAA) excitation triggering a single action potential on each cycle, alternating with mid-cycle glycinergic inhibition, superimposed upon a level of sustained tonic excitation. This latter input results from the temporal summation of NMDA receptor-mediated synaptic potentials (Dale & Roberts, 1985). Moreover, embryonic swimming in α-BTX-immobilised animals appears indistinguishable from that of curarised animals, despite the possibility that a cholinergic component may contribute (see introduction).

In figure 15, motorneuronal activity was recorded with a 2M potassium acetate- (KAc) filled electrode and clearly illustrates the alternating phases of excitation and inhibition in each cycle of activity (see also figs 3, 4A). Also apparent is that motor neurons fire only once in each cycle (Kahn & Roberts, 1982a; Soffe & Roberts, 1982). In addition, the underlying synaptic drive lacks the complexity which would normally be associated with variable firing patterns of premotor interneurons (*cf* fig 4), suggesting that they too fire only once in each cycle of activity, which is consistent with previous findings (Dale, 1985; Dale & Roberts, 1985). In records of motorneuronal activity using potassium chloride- (KCl)

filled electrodes, chloride-mediated inhibitory potentials become reversed in sign. This is presumably the result of chloride leakage from the electrode tip into the neuron. In the illustrated example (fig 15), the reversed midcycle ipsp clearly comprises a single compound depolarising potential occurring midcycle. Similarly, the excitatory drive also consists of a single compound epsp off which the single action potential is driven.

The single impulse on the excitatory phase of each cycle of activity is unusual when compared to motorneurons of more adult systems such as the cat (Jordan, 1983), the late embryo of the common frog *Rana temporaria* (Soffe & Sillar, 1991) and the lamprey (Wallén *et al,* 1985) (see fig 4), all of whose motorneurons have a multiple spiking capability and fire bursts of discharge in each cycle of locomotion. In stage 37/38 *Xenopus*, rhythmic neurons normally respond to depolarising current injection with a single impulse at threshold, and increasing the level of depolarising current pulse does not usually increase the number of impulses evoked (fig 15C). This cell property presumably accounts for the brief and inflexible pattern of ventral root activity which underlies fictive swimming in the embryo. After only 24 hours of further development, however, ventral root activity consists of bursts of discharge of variable duration lasting around 20ms. Two mechanisms could account for this; either motorneurons acquire a multiple spiking capability, or they each continue to fire once per cycle but the population discharge becomes desynchronised. I therefore recorded intracellularly from stage 42 spinal neurons during fictive swimming, and examined their responses to injected depolarising current pulses.

Recordings from ventrally positioned neurons in stage 42 larvae during fictive swimming reveal a pattern of alternating phasic activity superimposed on a level of tonic depolarisation (fig 16 Aii), as in the embryo. In contrast to the embryo, however, they often fire more than one action potential in the excitatory phase of each cycle (Biii). Moreover, the number of impulses per cycle is variable, with multiple impulses being more apparent at the onset of episodes when synaptic drive is presumably at its highest, and often failing altogether particularly towards the ends of episodes as synaptic drive weakens. This contrasts with a stage 37/38 neuron which not only is limited to a single impulse per cycle, but continues to fire in every cycle throughout the episode (fig 16Ai, Bi, Bii). The synaptic drive, although appearing to comprise the same basic pattern of alternating excitation and inhibition, is also more complex and variable in the stage 42, consisting of trains of excitatory and inhibitory psp's rather than single compound potentials (fig 16 Biii, iv). This suggests that like the motorneurons, premotor interneurons have also acquired multiple firing capability and it argues against the possibility that damage inflicted upon the motorneuron during impalement might have caused it to fire multiply.

In the absence of fictive swimming, depolarising current injection also evokes a different response in stage 42 motorneurons. Although like the embryo, a single impulse occurs at threshold, subsequent small increases in the depolarising current pulse evokes multiple spiking (fig 16 Cii), and the number of spikes elicited is approximately proportional to the amplitude of the current pulse. This contrasts with the embryo where increasing the amplitude of

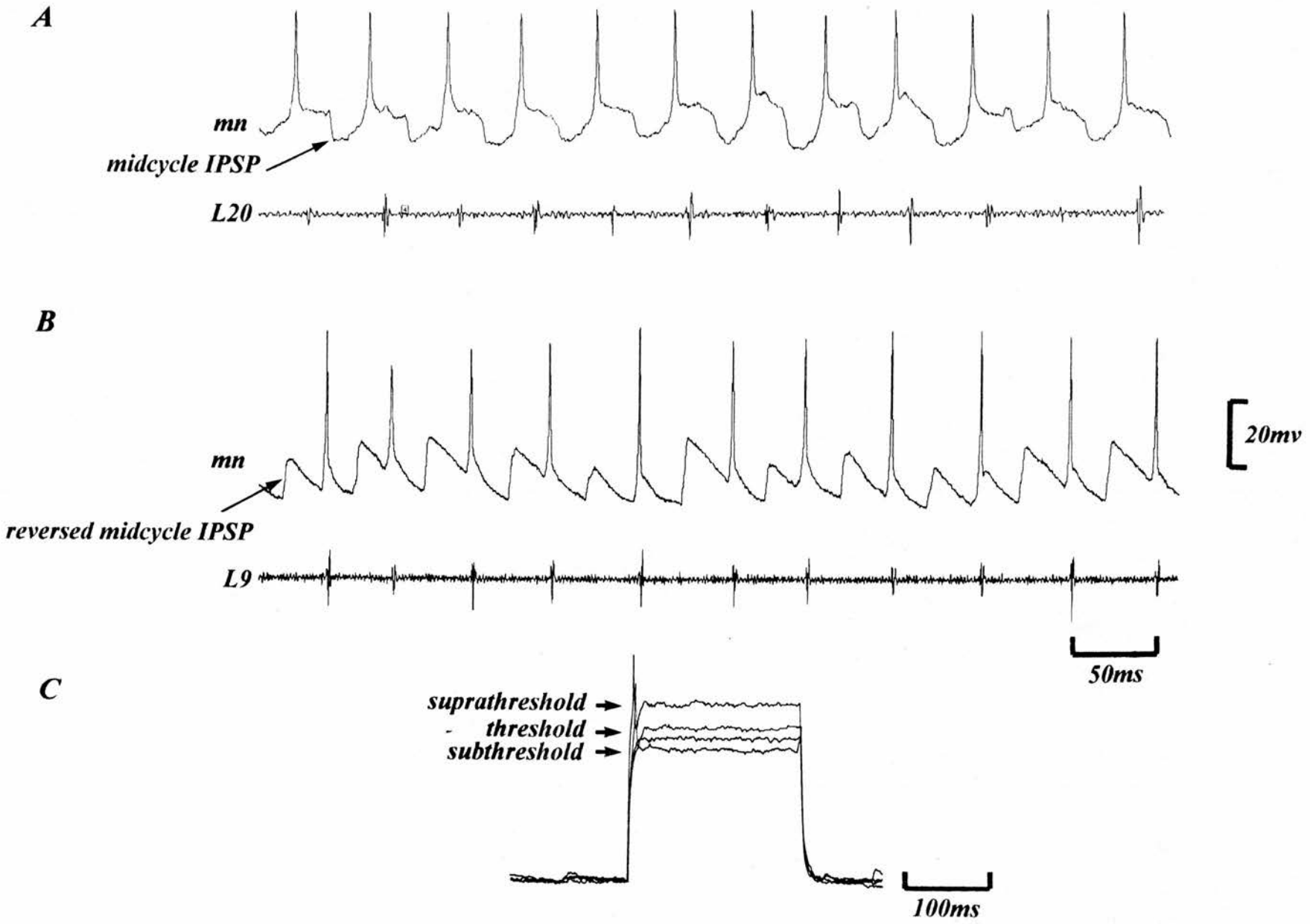

A
mn
midcycle IPSP
L20
B
mn
reversed midcycle IPSP
L9
20mv
50ms
C
suprathreshold
threshold
subthreshold
100ms

fig 15. Cell properties of stage 37/38 rhythmic neurons

(A) An excerpt of swimming showing the activity of one ventral root (L20) and a motorneuron recorded with a KAc electrode. The ventral root activity comprises of brief biphasic impulses, and the motorneuron fires a single action potential in each cycle. The excitatory and inhibitory phasic synaptic drive appears to comprise of single compound events suggesting synchronous and single firing in presynaptic interneurons. (B) Also an excerpt of swimming in an embryo showing the same basic features, except that the intracellular recording is with a KCl electrode which strongly reverses the midcycle inhibition. It illustrates more clearly the single compound nature of the midcycle ipsp. (C) Embryonic motorneurons only fire a single impulse in response to suprathreshold depolarising current pulses (*cf* Soffe, 1990).

Ai

mn

L2

30mv

1sec

ii

mn

L3

20mv

Bi

ii

50ms

iii

iv

Ci

suprathreshold (0.3na)

threshold (0.2na)

subthreshold (0.1na)

100ms

ii

suprathreshold (0.25na)

threshold (0.2na)

subthreshold (0.15na)

fig 16. The cell properties of the stage 37/38 embryo and the stage 42 larva

A direct comparison between the properties of rhythmic neurons in a stage 37/38 embryo and a stage 42 larva. (Ai) The beginning and end of a sustained episode of embryonic swimming activity on a slow time base, showing that the motorneuron fires throughout the episode. (Aii) An entire episode of larval swimming in which the motorneuron does not fire on every cycle and ceases completely towards the end of the episode. (B) Swimming on a faster time base showing the beginnings and ends of episodes in the two stages. (i & ii) The embryo motorneuron fires a single impulse throughout the episode. (iii) Close to the beginning of the episode, the larval motorneuron either does not fire an impulse in each cycle, or fires singly or multiply. (iv) Towards the end of the swim episode, it ceases altogether. (Ci & ii) In contrast to the embryo, suprathreshold depolarising current pulses elicit multiple spiking in stage 42 motorneurons. (current injection was carried out on a stage 42 neuron in a different experiment).

the current pulse beyond threshold usually fails to elicit more than a single impulse, although the delay to the impulse often decreases with increasing amplitudes of current (Ci, see also fig 15 C).

Although all stage 42 rhythmic neurons tested fired multiply to suprathreshold current pulses, they sometimes differed in their activity during swimming. Since the animal is developing very rapidly, this may have been due to slight differences in the relative 'ages' of the neurons. For example, in figure 17, both ventral root and intracellular activity during fictive swimming lacks the complexity normally associated with the older animal (*cf* fig 16). The neuron spikes only once on the excitatory phase of each cycle, and the ventral root activity is correspondingly brief, although the synaptic drive is more complex than that of an embryonic neuron. However, when injected with a small amount of depolarising current during fictive swimming, unlike the embryo, the neuron now fires multiply in the excitatory phase of each cycle (*cf* Sillar *et al*, 1992a).

The primary conclusion that can be drawn from these experiments is that the firing properties of spinal neurons change during post-embryonic development so that neurons can now fire multiply. When motorneuronal activity is compared directly with the activity in the ventral roots, there is some evidence in support of this notion. Figure 18 for example, illustrates a passage of swimming in which the ventral root activity is initially brief. Although the motorneuron does not spike (*cf* fig 16 Aii), the excitatory drive consists of trains of epsp's which summate with an amplitude which seems to correlate with the durations of the ventral root bursts. For example, the train of epsp's marked with

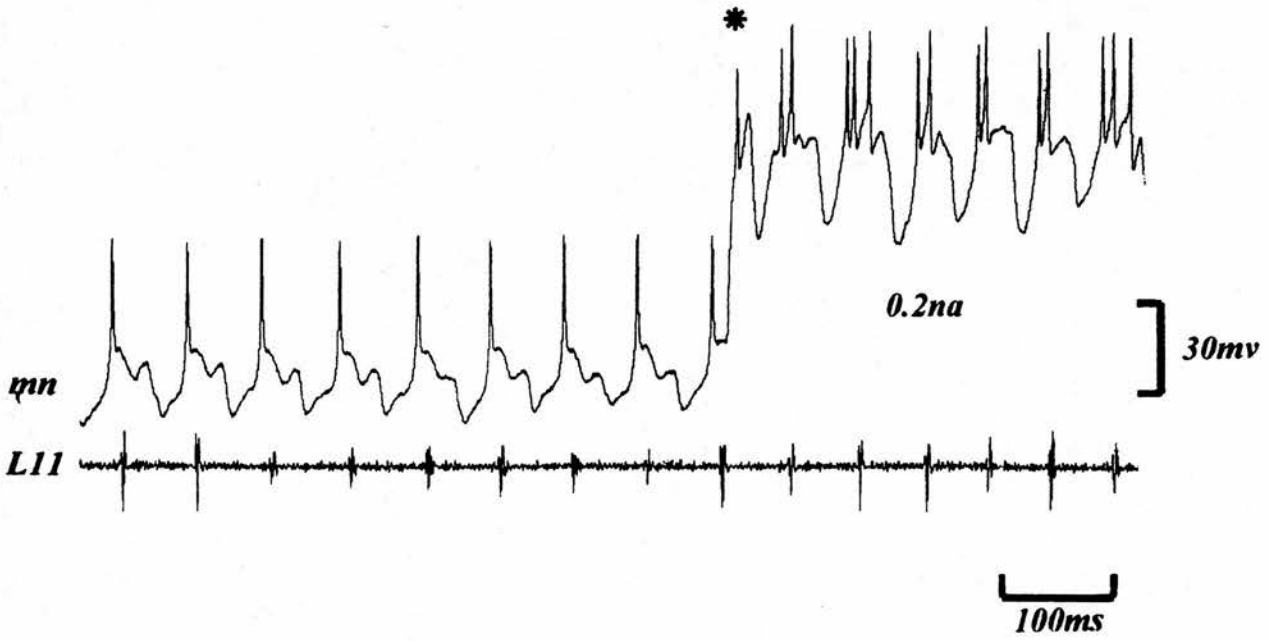

*
0.2na
30mv
mn
L11
100ms

fig 17. Effects of current injection in a stage 42 rhythmic neuron

Stage 42 motorneurons can differ in their activity during swimming. (A) The activity lacks the complexity normally associated with stage 42 rhythmic neurons in that it spikes only once in each cycle of activity. Also, although the synaptic drive is more complex than in an embryo, it nonetheless lacks the complexity normally associated with the stage 42 larva, and the ventral root activity is also correspondingly brief. Unlike an embryonic neuron, however, it has a capability for multiple spiking during swimming which is revealed by a small amount of tonic depolarising current injected into the neuron (onset at asterisk).

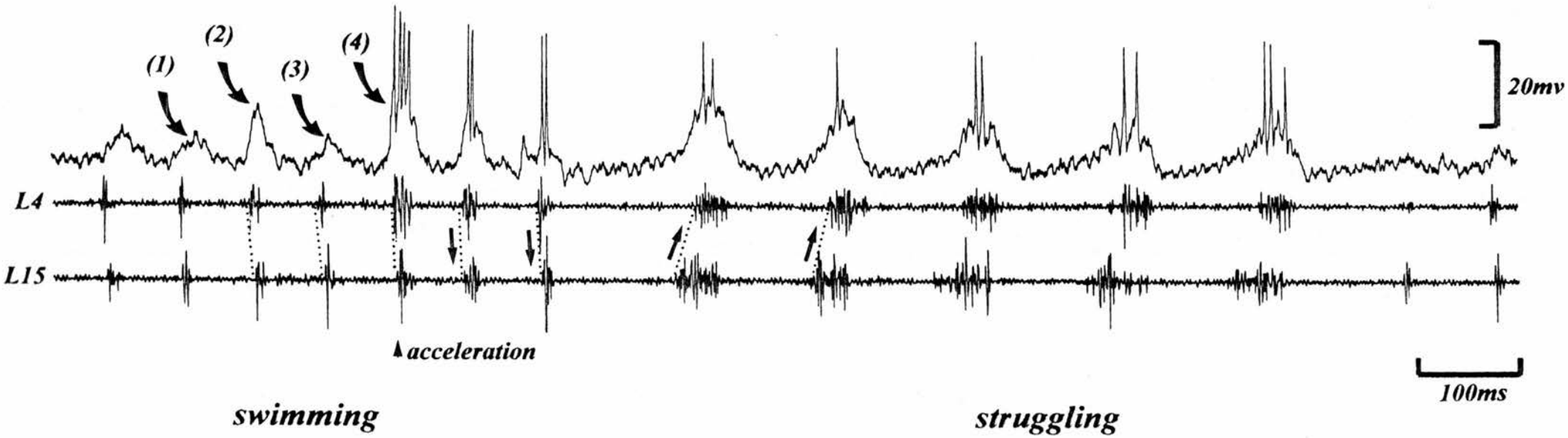

(1)
(2)
(3)
(4)
20mv
L4
L15
acceleration
100ms
swimming
struggling

fig 18. Activity of a stage 42 rhythmic neuron during fictive swimming and struggling

An excerpt of fictive swimming activity containing an apparently spontaneous switch to struggling, recorded intracellularly from a motorneuron at about the level of the 6th post-otic myotome, and extracellularly from ventral roots L4 and L15. The magnitude of the excitation in each cycle of motorneuronal activity appears to correlate with the intensity and duration of the corresponding ventral root discharge. The epsp's marked with arrow (2) appear to have summed to a higher amplitude than (1) and (3), and the corresponding activity in ventral roots L4 and L15 is longer on that cycle. There then occurs an apparently spontaneous change in the swimming pattern which suggests acceleration (arrow-head) involving a decrease in cycle period and an increase in the duration and intensity of the ventral root discharge. On that cycle, the motorneuron receives enhanced excitatory drive so that the membrane crosses threshold and fires a high frequency train of four impulses (arrow (4)). On the subsequent two cycles, the ventral root activity declines, as does the excitatory drive to the motorneuron, and although the membrane potential still crosses threshold, fewer impulses are elicited. This is then followed by a brief period of struggling (see also Kahn & Roberts, 1982c) which occurs at considerably lower frequency than swimming, with a caudo-rostral intersegmental delay (dotted lines & arrows) and much enhanced ventral root bursts.

arrow (2) summates with a higher amplitude than (1) and (3), and the activity in both the ipsilateral ventral roots (L4 & L15) is also correspondingly longer on that cycle. There then occurs an apparently spontaneous change in the pattern of ventral root activity with a dramatic increase in both amplitude and duration. This is also accompanied by a decrease in the cycle period. On that cycle, the motorneuron receives enhanced excitatory drive so that the membrane potential crosses spike threshold and fires a high frequency train of four impulses. The neuron is thus recruited during more intense, high frequency swimming activity. On the following two cycles, the ventral root activity declines in duration as does the excitatory drive to the motorneuron. Although the membrane potential still crosses spike threshold, the weaker excitatory drive results in fewer impulses being elicited. This is followed by a brief period of struggling which occurs at a considerably lower frequency than swimming (see Kahn & Roberts, 1982c), the ventral root activity having instead a caudo-rostral phase delay and enhanced burst durations.

DISCUSSION

A major consequence of the brief and essentially invariable rhythmic ventral root activity on each cycle of fictive swimming in *Xenopus* embryos, is that there is little scope for altering the intensity of motor output and therefore the relative power of muscle contractions on a cycle by cycle basis. With the exception of phase-dependent reflexes following cutaneous stimulation (Sillar & Roberts, 1988b; 1992a & b; see also introduction), and the ability to sustain locomotor rhythmicity over a wide frequency range (*ca.* 10-20 Hz), the embryonic motor pattern provides little scope for adaptive swimming movements. Clearly this is in marked contrast to the stage 42 larva which, though only 24 hours older, potentially at least, has a much more adaptive swimming capability.

Following hatching, the larva is not free swimming until after about 24 hours. During this time, it feeds entirely from internal stores in its yolk sac and hangs either from the surface layer or from the side of the tank, attached by mucus secreted from its cement gland. Usually it only swims in response to a light touch to the trunk or to sudden dimming of the illumination, and will normally continue to swim until it encounters an obstacle in its path from which it will then remain hanging. This stopping response can also be elicited under experimental conditions; fictive swimming ceases in response to a light pressure applied to the cement gland (Boothby & Roberts, 1988, 1992). After about twenty-four hours of post-embryonic development, the yolk sac is much depleted, the mouth and gut have developed and the animal is about to embark on a free swimming, suspension feeding

lifestyle (Nieuwkoop & Faber, 1956). It may be that the more sedentary lifestyle of the early post-hatching larva is in part be due to its limited swimming capability, especially in terms of avoiding predation; hanging immobile to an appropriate surface may therefore be a preferred strategy. Clearly an active suspension feeder has a requirement for a more adaptive and flexible swimming capability, and analysis of the ventral root activity underlying swimming suggests that the larva rapidly acquires this capability in a 24 hour period after hatching. It is also likely that there would be an additional requirement for muscle power as the tadpole increases its size and mass; during the first day of larval life new myotomes are added and existing one grow in size (van Mier *et al*, 1986b). As a consequence of increased body size, there will be an increase in the ratio of inertial forces to viscous ones (Reynolds number). The requirement for increased muscle power therefore would become increasingly important at the onset of swimming, and indeed a consistent feature of larval swimming is that the ventral root activity at the beginnings of episodes tends to have the longest durations and the greatest intensities, despite cycle periods being at their shortest.

What then might be the mechanisms underlying these dramatic developmental changes in the swimming motor pattern? It may be that the spinal locomotor network undergoes a major reconfiguration However, it is now generally regarded that spinal locomotor circuits are established very early in vertebrate embryogenesis, and rather than being dismantled and replaced by new circuits, they are retained and modified during the course of development. In the human, for example, rhythmic limb movements have been observed in foetuses after only about ten

weeks of development, and new-born infants will exhibit locomotor-like limb movements when placed on a moving treadmill with their weight supported. About a year passes before unsupported locomotion is possible and thereafter it takes many more years before the full range of adult locomotor behaviours become possible (Forssberg, 1985; Forssberg *et al*, 1991).

Descending control systems, which continue to develop after birth or hatching and are increasingly incorporated into the spinal networks, appear to play a central role in vertebrate locomotor development. An apparently universal feature of vertebrate locomotor circuits is that their development proceeds rostro-caudally suggesting a descending supraspinal influence (Ho & O'Donovan, 1993; Westerga & Gramsgergen, 1993; for recent review, see also Sillar *et al*, 1993). In neonatal rats, for example, the major propulsive force is provided by the fore-limbs. During the ensuing weeks, however, the hind-limbs play an increasing role until they become the dominant propellers (Westerga & Gramsgergen, 1993). As with other vertebrate systems therefore, it appears probable that the acquisition of a more flexible and potentially adaptive swimming capability in post-embryonic *Xenopus* larvae, is due to the progressive influence of developing brainstem interneurons which modify a pre-existing motor circuit in the spinal cord. Certainly the results described above support this idea in a number of ways. Firstly, although the developmental changes are rapid and profound, the basic underlying co-ordination remains unaltered in the stage 42 larva, in that it consists of alternating activity which progresses rostro-caudally with a brief intersegmental delay (see fig 6). Secondly, the acquisition of complexity and variability occurs rostro-caudally. A plausible

explanation for this is that the locomotor circuit, rather than being fundamentally altered, is being progressively influenced by the development of descending brain-stem interneurons. Thirdly, spinalisation of stage 42 larvae results in swimming activity which closely resembles that of an intact stage 37/38 embryo in two critical regards; it causes a resumption of the brief ventral root spikes on each cycle, and the loss of the correlation between segmental delay and cycle period. This could be explained by the removal of the descending input which has developed subsequent to hatching and which plays a central role in the ontogeny and modulation of swimming activity. In the spinalisation experiments on stage 42, the cord was transected at the level of the otic capsule. Although in embryos, descending excitatory interneurons do not appear to be located as rostrally as the otic capsules (Roberts & Alford 1986), it is not inconceivable that by stage 42 a few might be located at that level. Spinalisation may have removed or damaged, therefore, a small number of descending excitatory interneurons from the total population resulting in weaker synaptic drive and a reduction in ventral root burst duration. This explanation, however, is unlikely to account for the dramatic effects both on burst structure, and on the relationship between cycle period and intersegmental delays. Firstly, swimming frequency is probably dependent on the number of descending interneurons which are active. Following spinalisation, ventral root burst durations are nevertheless very much briefer compared to those of the intact animal at the same swimming frequencies, when presumably an equivalent number of excitatory interneurons would be active. Secondly, the correlation between cycle period and delay was also lost after spinalisation. It seems improbable that this

relationship would be in any way altered as a consequence of some interneurons being surgically removed from the circuit, since in any event excitatory interneurons tend to drop out of the circuit especially at lower swimming frequencies (Sillar & Roberts, 1993). An important observation, however, is that the fictive swimming activity of the spinalised stage 42 larva closely resembles that of the intact stage 37/38, strongly suggesting that the spinal locomotor circuit is basically unaltered in the older animal.

It appears therefore, that rather than involving a fundamental reconfiguration of the circuit, the development of locomotor rhythmicity primarily involves changes in properties of the component neurons, and that these changes are initiated and maintained by the influence of descending supraspinal input. Intracellular recordings support this view, since they demonstrate that changes in the membrane properties of motorneurons have occurred. For example, at stage 42, motorneurons are capable of firing multiply during fictive swimming activity, and in response to depolarising current injection. This finding does not exclude the possibility that the individual motorneurons whose axons exit any one ventral root, may also lose the tight synchronisation in their firing, which is evident in the stage 37/38 embryo. Indeed the transition from a single to a variable and multiple spike capability, would itself cause a desynchronisation of firing among motorneurons of each 'segment'. It has been demonstrated recently that electrical coupling exists between motorneurons in stage 37/38 embryos (Perrins, 1993). This coupling extends up to 200μm in both rostro-caudal and caudo-rostral directions. Thus, spiking in one motorneuron induces short latency excitation in its close

neighbours, and electrical coupling may therefore be an important mechanism ensuring the reliable and synchronous firing which is evident in embryonic motorneurons during swimming. If similar coupling exists in larval motorneurons, it may have a role in terminating bursts of high frequency spike discharge amongst neighbouring motorneurons. Conversely, post-embryonic development could conceivably involve a down regulation of electrical coupling to enable desynchronisation of activity to occur.

Despite these differences between embryonic and larval motorneurons, the underlying synaptic drive appears to share the same basic components, comprising an excitatory phase which drives impulses, a midcycle phase of presumed inhibition, and a tonic level of depolarisation. However, the complexity and variability of the synaptic drive is very much greater in the older animal. Instead of single compound events, impulse generation is driven off a train of summating epsp's, and the midcycle inhibition also appears to comprise a similar summating train of ipsp's (fig 16 Biii). This is likely to be a consequence of the premotor interneurons, like motorneurons, acquiring a multiple discharge capability. It must be added, however, that in the absence of direct evidence from intracellular recordings, it may be that desynchronisation has occurred amongst premotor interneurons which excite and inhibit a given motorneuron.

The post-embryonic modifications to the swimming circuit appear to impart much greater flexibility on larval swimming permitting manoeuvres such as turning and acceleration. An important contributory mechanism may be the recruitment of premotor and motorneurons into and out of the circuit. I have obtained some evidence for this since the novel discharge

capabilities of rhythmic neurons in the network provide the capability for this to occur. In the embryo, it has been shown that motorneurons fire reliably but that firing in premotor interneurons may progressively fail as frequency drops during the course of a swimming episode (Sillar & Roberts, 1993). In the larva, it may be that the multiple and variable spiking capabilities of premotor interneurons enhances both positive feedback in the network, and excitatory drive to the motorneurons. Certainly at the level of the motorneuron, the degree of the synaptic drive varies considerably and dictates not only the spike discharge frequency, but whether or not the neuron even reaches spike threshold. Thus variability in the durations and intensities of ventral root bursts could be a consequence of either changing discharge frequencies in motorneurons and/or some motorneurons dropping out of the network altogether. Intracellular recording suggest that both these mechanisms may be important. Figure 16 shows that larval motorneurons, unlike those of the embryo, do not spike throughout the episode, but on some cycles cease to fire and drop out completely towards the end of the swim episode as synaptic drive weakens. However, the motorneurons still receive rhythmic synaptic drive, so that potentially they could be readily recruited back into the circuit. Figure 18 illustrates an example where this has occurred, and it appears to be associated with an acceleration since cycle period was transiently reduced. Recruitment of motorneurons back into the circuit is paralleled by enhanced ventral root activity on the same cycles, whose intensity and duration appears broadly to correspond to the intensity and duration of motorneuronal discharge. On the basis of the data illustrated in figure 18, recruitment would also appear to be

important in the switch to an alternative motor program (struggling; Kahn & Roberts, 1982c). However, clearly a minimum number of presynaptic interneurons and motorneurons must be continually active for the maintenance of the swimming rhythm, and it may be that a proportion of rhythmic larval neurons have rather different cell properties to ensure this. It seems, therefore, that the flexibility of larval swimming is dictated by a balance of two mechanisms, one involving recruitment (or drop out) of motorneurons, and the other involving a regulation of discharge frequencies in active motorneurons. If larval motorneurons have relative differences both in their membrane properties and in their responses to modulatory inputs, this would facilitate, for example, the subtle interplay between the regulation of spike discharge frequency, and recruitment.

In view of the slow-activating and sustained voltage dependent potassium conductance which renders embryonic rhythmic cells refractory after a single impulse in each cycle (see Soffe, 1990), it is tempting to suggest that during development, this conductance is somehow reduced to enable multiple firing. Since the development of locomotor rhythmicity occurs rostro-caudally and is reversed by spinalisation, this suggests a tonic descending neuronal influence on the motor circuit. It is possible, therefore, that both the development and intrinsic modulation of cell properties in the spinal locomotor circuit, derives from descending inputs from developing brain stem interneurons. In the next chapter, I pursue this idea further and present evidence on the nature and function of these interneurons.

CHAPTER 4

The role of 5HT in the development and modulation of swimming activity

INTRODUCTION

i) Nature and distribution of 5HT

The results of the preceding chapter strongly suggest the involvement of a descending neuronal influence which imparts rostro-caudal modifications to the output of the swimming circuit in post-embryonic *Xenopus*. In the present chapter, I explore this notion and critically test the hypothesis that developing spinal projections emanating from serotonergic interneurons located in the brainstem raphe nucleus, play a causal role in the development of the swimming pattern. However, I will first review the properties and distribution of serotonin (5-hydroxytryptamine, 5HT) in vertebrates, and then present the evidence that this neuromodulator may be important in the development and modulation of vertebrate locomotion.

5HT is an indoleamine which is widely distributed in various tissues of both vertebrates and invertebrates. It was first discovered and isolated from beef serum in 1948 during the search for the identity of a vasoconstrictor released when blood clotted (Rapport, *et al*, 1948a & b). It was termed serotonin to denote its origin (*serum*) and action (*tonus*), a name still widely used today. At around the same time, the presence of a substance termed enteramine was identified in a wide range of species and tissues which included enterochromaffin cells of the mammalian gastrointestinal mucosa, salivary glands of two species of octopus, and amphibian skin (see Erspamer & Ghiretti, 1951). Enteramine

was considered to be a true hormone directly affecting, for example, the motility of the mammalian gut (hence its name) and kidney function. In invertebrates, it was shown to stimulate molluscan heart (Erspamer & Ghiretti, 1951). In 1952, it was established that serotonin and enteramine were one and the same, and identified as 5-hydroxytryptamine (Erspamer & Asero, 1952), which had been prepared synthetically the previous year (Hamlin & Fisher 1951) (for a highly authoritative contemporary review see also Page, 1958). It was, however, the pioneering work of John Welsh during the 1950's which established the importance of serotonin as a neurohumour (later termed neurotransmitter) in the cns of invertebrates and vertebrates. It was then considered to be the third neurohumour, the other two being acetylcholine (Ach) and noradrenaline. Since those pioneering days, 5HT has been implicated in a vast array of neural functions. It is now more often referred to as a neuromodulator rather than a neurotransmitter since, while not appearing to be essential for basic neural function and rarely involved in fast synaptic transmission, it profoundly shapes and modulates neural activity. In the vertebrate cns, its influence is extraordinarily widespread, exerting a tonic regulatory influence on almost all behavioural processes. For example, it is involved in neuroendocrine function (Collu *et al,* 1972), the sleep-wake cycle (Jouvet, 1972), pain perception (Jessel and Kelly, 1991), temperature regulation (Chase & Murphey, 1973) and locomotor function (Barbeau & Rossignol, 1991).

5HT is accumulated in the nerve terminals of serotonergic neurons via a high-affinity uptake mechanism, and also by synthesis from the precursor amino acid, tryptophan. The action of the enzyme tryptophan hydroxylase, which is confined to

serotonergic neurons, converts tryptophan to 5-hydroxytryptophan. This in turn is converted to 5HT by the action of a non-specific decarboxylase. 5HT is stored in granular storage sites in a 'bound' form. This provides protection for the amine against oxidative deamination by monoamine oxidase. Only the 'free' form is subject to uptake, inactivation and interaction with receptor sites. The activity of monoamine oxidase ensures a rapid turnover of the amine, and inhibitors of its activity are important tools in revealing the presence and physiological importance of 5HT (for reviews Folk & Long, 1988; Jacobs & Azmitia, 1992).

The first major advances in understanding the neuroanatomy of vertebrate serotonergic systems were made by employing the histochemical fluorescence technique. This enabled, for example, a detailed description of the localisation of serotonergic neurons in the rat cns (Dahlstrom & Fuxe, 1964; 1965). Subsequently autoradiography (Descarries, Beaudet & Watkins, 1975) and immunohistochemical (Steinbusch *et al*, 1978) techniques enabled more detailed description of the neuroanatomy of the serotonergic system. Notwithstanding some inter-species differences, the somata of serotonergic neurons appear to be ubiquitously located in the raphe nuclei of the brain stem, near the midline (*raphe* derives from the Greek meaning 'seam'). They are relatively few in number; in the adult mammalian cns, for example, they constitute only about 1/1,000,000 of all neurons. However they have a myriad of processes which ramify throughout the cns (Jacobs & Azmitea, 1992).

ii) Multiple receptors for 5HT

5HT exerts a diversity of effects on its target neurons by acting at multiple receptor subtypes on the post-synaptic cell membranes (Peroutka, 1991). It was known in 1957 that there were two pharmacologically distinct types of receptors in the guinea-pig ileum, one mediating contraction of the smooth muscle (the 'D' type), and the other mediating depolarisation of the cholinergic nerves (the 'M' type) (Gaddum & Picarelli, 1957). Early studies using extracellular recordings and iontophoresis also suggested the involvement of more than one receptor type mediating the effects of 5HT in the cns. These studies demonstrated that, although there was a predominantly inhibitory role for this transmitter, there were also excitatory responses which, unlike the 5HT-induced inhibitions, could be blocked by ergot alkaloids (see, Andrade & Chaput, 1991). Ever since then, an ever expanding number of 5HT receptor types have been described. To facilitate the understanding of receptors and the mechanisms by which 5HT exerts its regulatory effects on membrane excitability, a classification scheme was required. Such a scheme was initially proposed by Peroutka and Snyder in 1979, and this has been expanded and adapted into a system which still has broad acceptance today. It is, however, also recognised as an imperfect system given the enormous complexity of 5HT pharmacology, frequent interspecies differences and the lack of potent selective ligands required for detailed characterisation. The initial characterisation involved the use of radiolabelled ligands, and two distinct binding sites were described, one of which had high affinity for 5HT (in the nanomolar range) and the other for

spiperone. These were termed $5HT_1$and $5HT_2$ binding sites respectively. However, based on further ligand studies, it soon became apparent that there were several types of high affinity $5HT_1$ receptors, each displaying different affinities and selectivities for a range of ligands. These have now been further subdivided into $5HT_{1a}$, $5HT_{1b}$, $5HT_{1c}$ and $5HT_{1d}$ subtypes (although the pharmacological profile the $5HT_{1c}$ receptor more closely resembles that of the $5HT_2$ family of receptors; see below). They are clearly not, therefore, a homogeneous group and many lack definitive characterisation. One of the problems concerns a feature of the $5HT_1$ binding site which was described by Peroutka and Snyder in 1979; it had a low affinity for the then 'classical' 5HT receptor antagonists such as methysergide and cyproheptadine. Now it is recognised that these antagonists are predominantly $5HT_2$-receptor antagonists, and that there still remains a lack of specific antagonists with high potency and selectivity for members of the $5HT_1$-receptor family. An exception is Nan-190, which acts specifically at $5HT_{1a}$ binding sites. Since an important criterion for the definition of any receptor is that the agonist response should be blocked by a high potency and highly selective antagonist, it has been suggested that $5HT_1$-receptors should simply be referred to as '$5HT_1$-like' (see Bradley *et al*, 1986).

The $5HT_2$ receptor family, originally classified as those with binding sites with relatively low affinity for 5HT and high affinity for spiperone, have likewise been subdivided to distinguish between $5HT_{2a}$ and $5HT_{2b}$ subtypes. It has also been suggested that the $5HT_{1c}$ receptor subtype be included in this family on account of shared molecular, pharmacological and biochemical

characteristics (notwithstanding its high affinity for 5HT; see Peroutka, 1991).

The $5HT_3$ family of receptors was originally regarded as a homogenous group restricted to the periphery, but $5HT_3$ receptor binding sites have now been identified in the rat cortex (Kilpatrick *et al,* 1987). An important and unique feature of the $5HT_3$ receptor is that it constitutes a ligand-gated ion channel. This sets it apart from all other 5HT receptor types which are G-protein-linked. Its activation results in a fast excitatory potential not dissimilar to the fast excitation mediated by nicotinic acetylcholine receptors (Derkach *et al,* 1989; for recent review, see Fozard, 1992). It is also now thought that several subtypes of this group exist which appear to be primarily species-dependent, and in the past few years it has been reported that many agents act potently at $5HT_3$ receptors (Fozard, 1992)

In addition to these three major families of 5HT receptor subtypes, there is also evidence of other less completely characterised subtypes, which cannot, as yet, be placed within the characterisation scheme outlined above. These so called 'orphan' receptors include $5HT_{1e}$, $5HT_{1r}$, $5HT_{1p}$ and $5HT_4$ subtypes (see Peroutka, 1991; see also Clarke *et al,* 1989)

iii) Role of 5HT in locomotion and development

One behavioural process in which 5HT has been shown to exert a similar modulatory effect across a range of species, is that of locomotion. In 1969, Viala and Buser showed that administration of the metabolic precursor 5HTP, enhances

locomotor activity in the rabbit. Since then 5HT has been shown to exert a similar effect on the locomotor rhythms in a wide range of other vertebrates such as the cat (Barbeau & Rossignol, 1990; 1991) and the lamprey (Harris-Warrick & Cohen, 1985; Wallén *et al* 1989). Most notably, 5HT enhances the output of the locomotor network and increases the durations of the ventral root activity. There are some striking similarities between the developmental changes which occur to the *Xenopus* larval swimming pattern, and the effects that 5HT exerts on the fictive swimming activity of the adult lamprey. For example, in the lamprey, 5HT enhances ventral root activity and alters inter-segmental co-ordination (Christenson *et al*, 1989; Matsushima & Grillner, 1992). These two features are dramatically changed during a brief 24 hour period of post-embryonic development in *Xenopus* (see previous chapter). At a cellular level, 5HT enhances spike discharge frequency in lamprey motorneurons. Similarly, in the stage 42 *Xenopus* larva, motorneurons exhibit a variable multiple discharge capability, in contrast to the single spike capability of stage 37/38 motorneurons. As described in the preceding chapter, the rapid development of locomotor rhythmicity in post-embryonic *Xenopus*, involving an acquisition of ventral root bursts, occurs rostro-caudally, suggesting a descending neuronal influence on the locomotor circuit. This notion was further supported by the results of the spinalisation experiments, in which the bursty larval swimming pattern resumed an embryonic form shortly after surgical removal of descending inputs. A number of populations of supraspinal interneurons progressively invade the spinal cord around this time (Norlander, 1984; van Mier & ten Donkelaar, 1984). For example, by stage 36 there is a more extensive innervation of the spinal cord

by reticulospinal interneurons, and also the first vestibulospinal and raphespinal projections are evident (van Mier & ten Donkelaar, 1984), and one, or all, of these could conceivably play a role in the rostro-caudal development of swimming activity. The interneurons of the raphe nuclei are located near the midline in the rostral ventral medulla. As in all other vertebrates thus far studied, the vast majority of these are serotonergic and they comprise the only known population of serotonergic neurons in the developing cns of *Xenopus* (van Mier *et al*, 1986a). From about developmental stage 28, descending spinal projections develop rapidly and progressively invade the cord, extending to the most caudal part by stage 45 (van Mier *et al*, 1986a). There are striking similarities between the developmental changes in the post-embryonic *Xenopus* swimming pattern, and the 5HT-induced changes in, for example, adult lamprey swimming. This suggested the possibility that endogenous 5HT released at progressively more caudal levels in the cord by developing descending serotonergic fibres, could play a causal role in the development of swimming activity.

It also should be considered that post-embryonic *Xenopus* tadpoles are relatively immature and develop rapidly. In the developing cns many transmitters and neuromodulators, including 5HT, have different and/or additional roles from the ones they have in the adult system, in that they can also act as developmental signals or regulators (for review, see Whitaker-Azmitea, 1991). Is there any evidence, however, that the serotonergic system has such a role in the development of locomotor spinal circuits? Certainly, in a variety of vertebrates including *Xenopus* (van Mier *et al*, 1986a) and the chick (Okado *et al*, 1992), descending serotonergic fibres begin to differentiate very early in embryogenesis suggesting

that they might also have a developmental role. It is also notable that post-synaptic 5HT receptors on neonate rat motorneurons are expressed before functional innervation (Ziskind-Conhaim *et al*, 1993). It has also been suggested that 5HT has a role in regulating cell division (via activation of a $5HT_{1c}$ receptor) and cell differentiation (via a $5HT_{1a}$ receptor). Moreover, 5HT receptors have different distributions, serve different and/or additional functions and have different pharmacological profiles in the developing cns. All of this suggests an important if not fundamental developmental role for the amine (see Whitaker-Azmitea, 1991).

These observations, therefore, led to the hypothesis that a causal link exists between the ingrowth of descending serotonergic axons into the spinal cord, and the development of a more flexible and 'adult-like' swimming pattern in post-embryonic *Xenopus laevis*. To test this hypothesis, I examined first the effects of bath applied 5HT (2-10μM) on the developing neural network for swimming in pre-hatching (stages 32 - 36), hatching (stage 37/38) and post-hatching (stages 40 - 42) *Xenopus laevis*. The application of 5HT resulted in a pattern of ventral root activity normally associated with animals some twelve hours older, since it revealed a developing rostro-caudal sensitivity gradient to 5HT, which appeared to precede functional innervation by the descending serotonergic system. These initial results, therefore, established the feasibility of the hypothesis. In order to establish the importance of endogenous release of 5HT, I next bath applied the metabolic precursor 5-hydroxytryptophan (5HTP), which increases the amount of 5HT available for release from the

terminals of serotonergic fibres (see Introduction). Fictive swimming before and after drug application was analysed to assess affects on ventral root burst durations, cycle period and longitudinal co-ordination. These results derive from experiments on seven embryos at developmental stages 32-36 and twenty-eight at stage 37/38, and thirty-five stage 40 and forty-six stage 42 larvae. A series of experiments were also performed using the selective serotonergic neurotoxin 5,7 dihydroxytryptamine (5,7-DHT) to ablate raphe neurons (see 'Materials & Methods'). Finally, experiments were carried out to identify the receptors types which mediate 5HT-induced changes to the swimming pattern. A range of serotonergic agents selective for many different 5HT receptor types (see table, page 33) were bath applied at differing concentrations, in order to observe their effects on the swimming pattern. The results strongly implicate a major role for $5HT_{1a}$ receptors in the development and modulation of swimming.

RESULTS

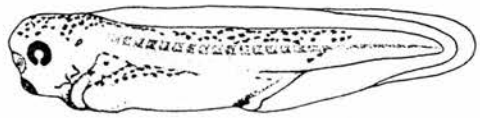
i) 5HT mimics development of swimming activity

The bath application of 1-10μM 5HT to stage 37/38 embryos caused a marked increase in the durations of bursts during swimming activity, but only in the most rostral ventral roots, leaving the caudal unaffected. In the illustrated example (fig 19), average rostral (L3) burst durations increased significantly ($p < 0.005$, *t*-test) from 7.63 +/- 1.04 (sd) ms to 11.98 +/- 2.24 ms. When expressed as a percentage of cycle period, burst durations increased on average from about 17% to 30%. In contrast, the caudal ventral root activity was relatively unaffected in that there was no apparent change before and after 5HT application. This effect of 5HT only on the rostral ventral root activity is more clearly illustrated when the first ten cycles of swimming activity under each of the experimental regimes (control, 5HT & wash) are superimposed (fig 19 Aii, Bii, Cii). In control (Aii), the activity in both rostral and caudal roots is similar, consisting of brief predominantly biphasic impulses. Following application of 5HT, the simple biphasic impulse in the rostral root is dramatically altered, consisting instead of bursts of activity of more variable duration and intensity. By contrast, the caudal activity is essentially unaltered. After wash, the discharge in the rostral root resumes a form similar to that of control, comprising brief biphasic impulses (Cii).

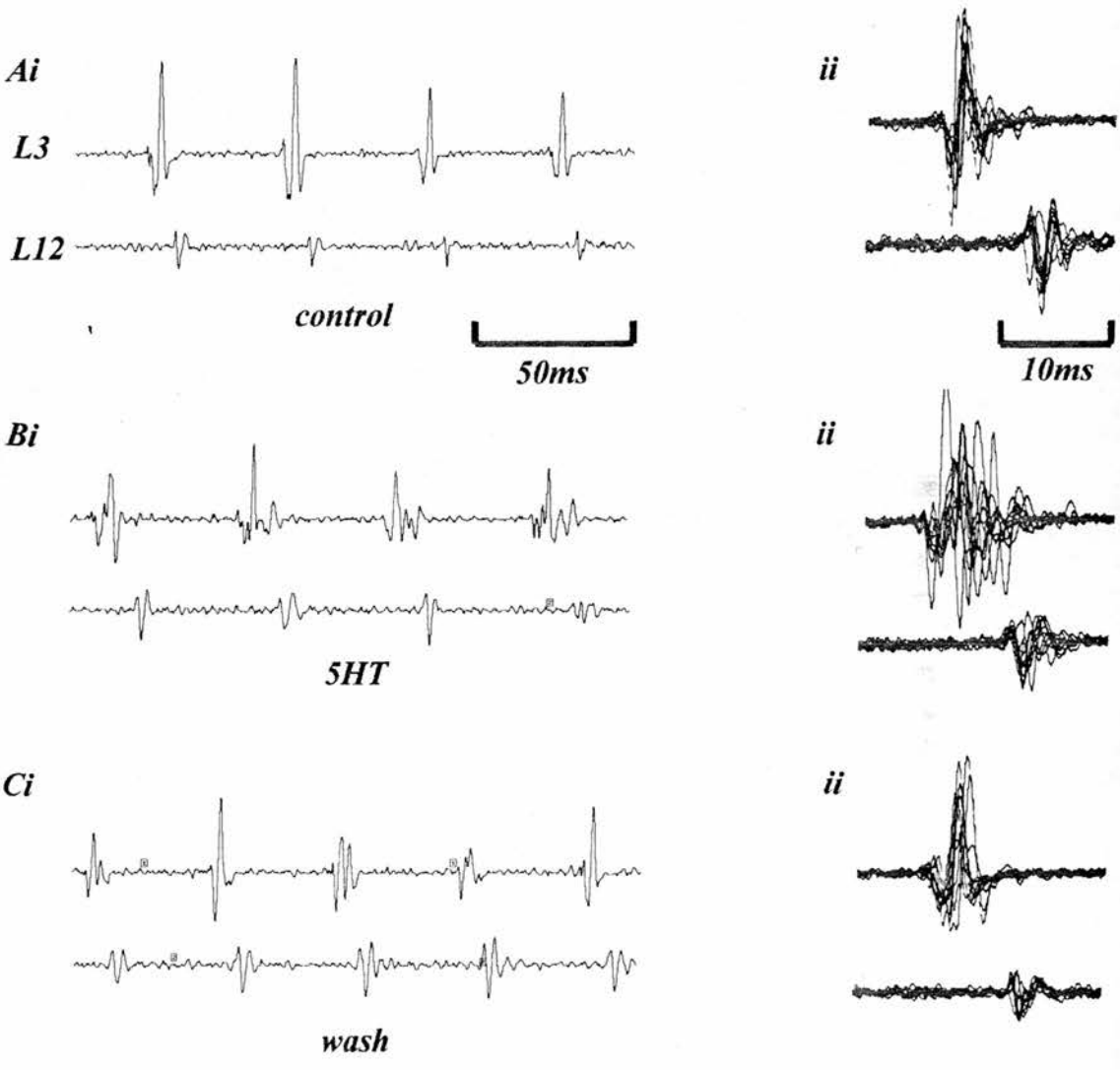
In stage 40 larvae, twelve hours post-hatching, similar applications of 5HT significantly increased the durations of rostral ventral root activity in seven preparations tested. Of these, four

fig 19. The effect of 5HT on the stage 37/38 swimming pattern

Fictive swimming in the stage 37/38 embryo was recorded from rostral (L3) and caudal (L12) ventral roots. (A) In control, the activity in both roots is similar in that it consists of brief biphasic impulses (i). This is more clearly illustrated when ten cycles of activity are superimposed (ii). (B) After application of 2μM 5HT, rostral ventral root activity was enhanced, but the caudal was unaffected (i). Ten cycles of superimposed activity (ii). The effects were reversed by 20 minutes wash (Ci & ii). Histograms show the average durations of rostral (Di) and caudal (Dii) ventral root bursts (n=20) in control, 5HT and wash.



stage 37/38


Ai
L3
L12
control
50ms
ii
10ms
Bi
5HT
ii
Ci
wash
ii

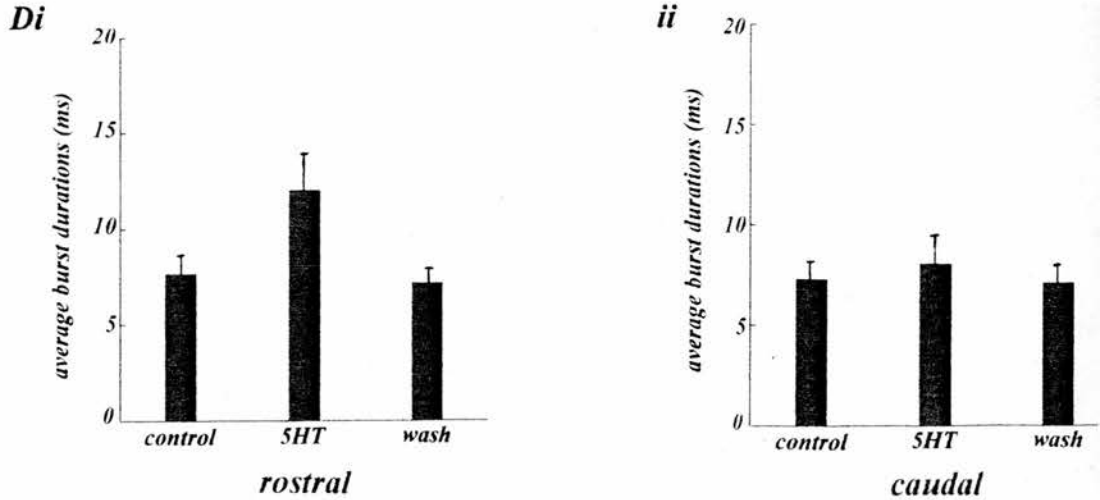
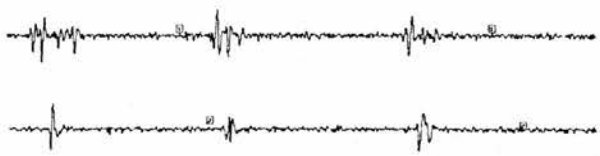

Di
average burst durations (ms)
20
15
10
5
0
control
5HT
wash
rostral
ii
average burst durations (ms)
20
15
10
5
0
control
5HT
wash
caudal

fig 20. The effect of 5HT on the stage 40 swimming pattern

(Ai) Fictive swimming in a stage 40 in control saline. The rostral (L3) ventral root activity is 'burstier' than the caudal (L10). (ii) Ten cycles of activity superimposed. (B) Both rostral and caudal ventral root activity is enhanced by bath applied 2μM 5HT (i & ii), and the effects are reversed by washing (Ci & ii). (D) Histograms of average durations of rostral (i) and caudal (i) ventral root activity (n=20) under the three experimental regimes.

stage 40

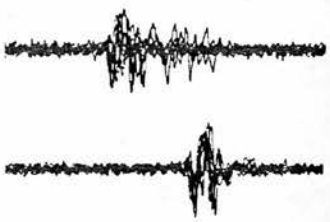
Ai



control

50ms

ii

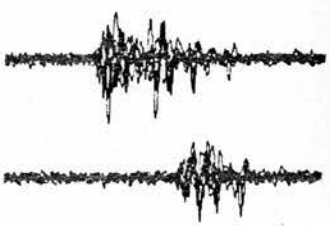


10ms

Bi

5HT

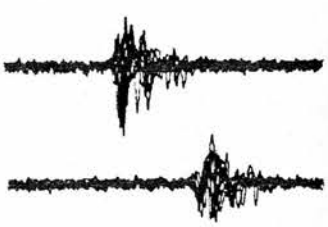
ii



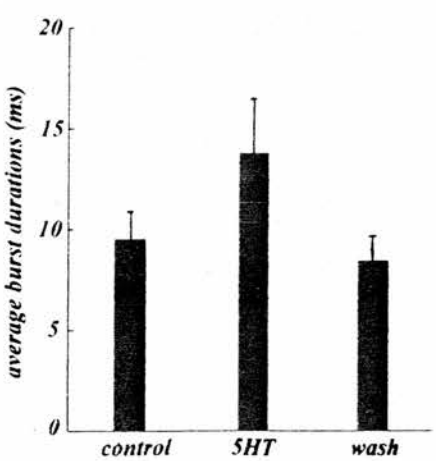
Ci

wash

ii

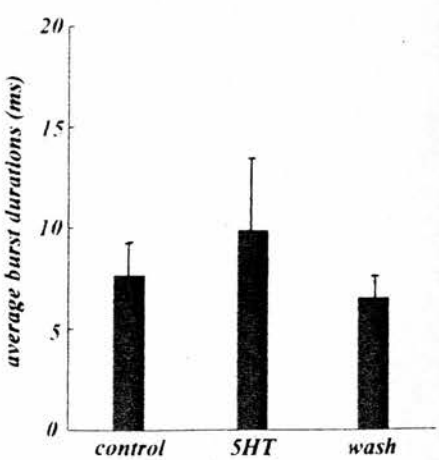


Di



rostral

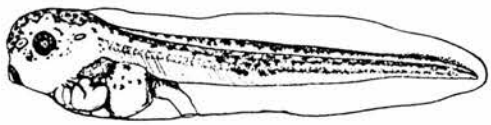
ii



caudal

fig 21. The effect of 5HT on the stage 42 swimming pattern

(Ai) Fictive swimming in the stage 42 larva in control. Both rostral (L4) and caudal (L11) ventral root activity comprise bursts of discharge lasting between 10 and 15ms. (B) Following application of 2μM 5HT, burst discharge is further enhanced down the entire length of the animal (i & ii). (C) Histograms showing the effects of 5HT on average absolute burst durations in rostral and caudal ventral roots (i), and on relative burst durations expressed as a percentage of cycle period (ii) (n=20).



stage 42

Ai

ii

L4

L11

control

50ms

10ms

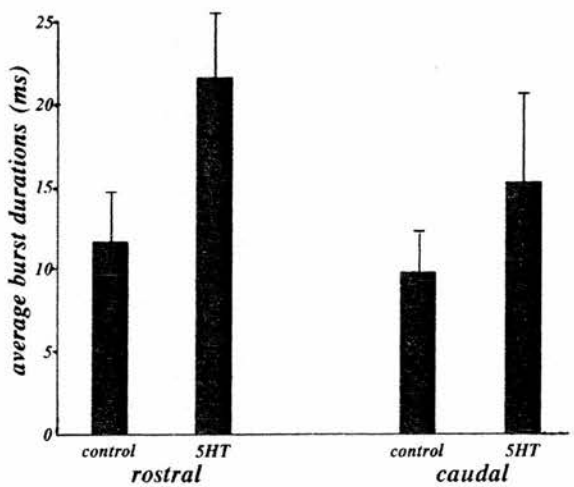
Bi

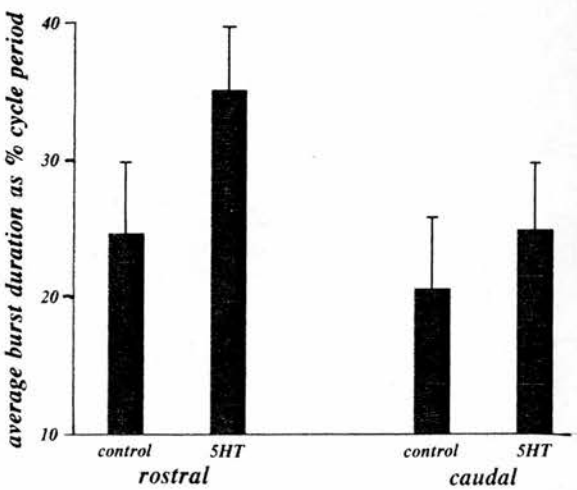
ii

5HT

Ci

ii





also demonstrated a significant increase in the duration of caudal ventral root discharge. In the illustrated example (fig 20), the bath application of 2μM 5HT caused an increase in the mean durations of both rostral and caudal ventral root discharge, the rostral increasing from 9.50 +/- 2.2 ms (about 20% of cycle period) to 13.7 +/- 3.8 ms (about 30%), and the caudal from 7.62 +/- 2.1 ms (15%) to 9.86 +/- 3.6ms (20%). Therefore, although 5HT enhances caudal ventral root activity, unlike in the embryo, it has a disproportionally greater effect on the rostral root. Ten cycles of superimposed activity clearly illustrates these differences. In control (Aii), the rostral ventral root demonstrates longer and more variable burst durations than the caudal, which remains consistently 'embryonic-like'. After application of 5HT (Bii), the activity in both roots is enhanced, but more so in the rostral. The effects are reversed by washing (Ciii).

In stage 42 larvae, 5HT had a significant effect on ventral root bursts along the entire length of the animal. In the illustrated example (fig 21), following application of 5HT, the average duration of rostral ventral root bursts increased from 11.67 +/- 2.42 ms (24%) to 21.59 +/- 3.66 ms (35%), and the caudal from 9.83 +/- 2.99 (21%) ms to 15.27 +/- 3.86 ms (25%). Also apparent from these data is that following application of 5HT, the relative increase in ventral root activity, expressed as a percentage of cycle period, is less than the absolute increase. In the stage 42, it appears, therefore, that 5HT may be also increasing the durations of cycle periods, as occurs in the lamprey. Over the same twenty consecutive cycles measured close to the onset of swim episodes, average cycle periods increased from 57.4 +/- 9.5ms to 68.9 +/- 11.9ms following application of 5HT. Although it must be added

that this was an effect only occasionally observed in the stage 42, and not examined rigorously. More importantly, however, is that burst durations relative to cycle periods markedly increase following 5HT application. In all three stages, the effects of bath applied 5HT were readily reversed after about a twenty minute wash in physiological saline, so that ventral root activity resumed its characteristic pattern seen under control conditions (figs.19 Ci & ii; 20 Ci & ii).

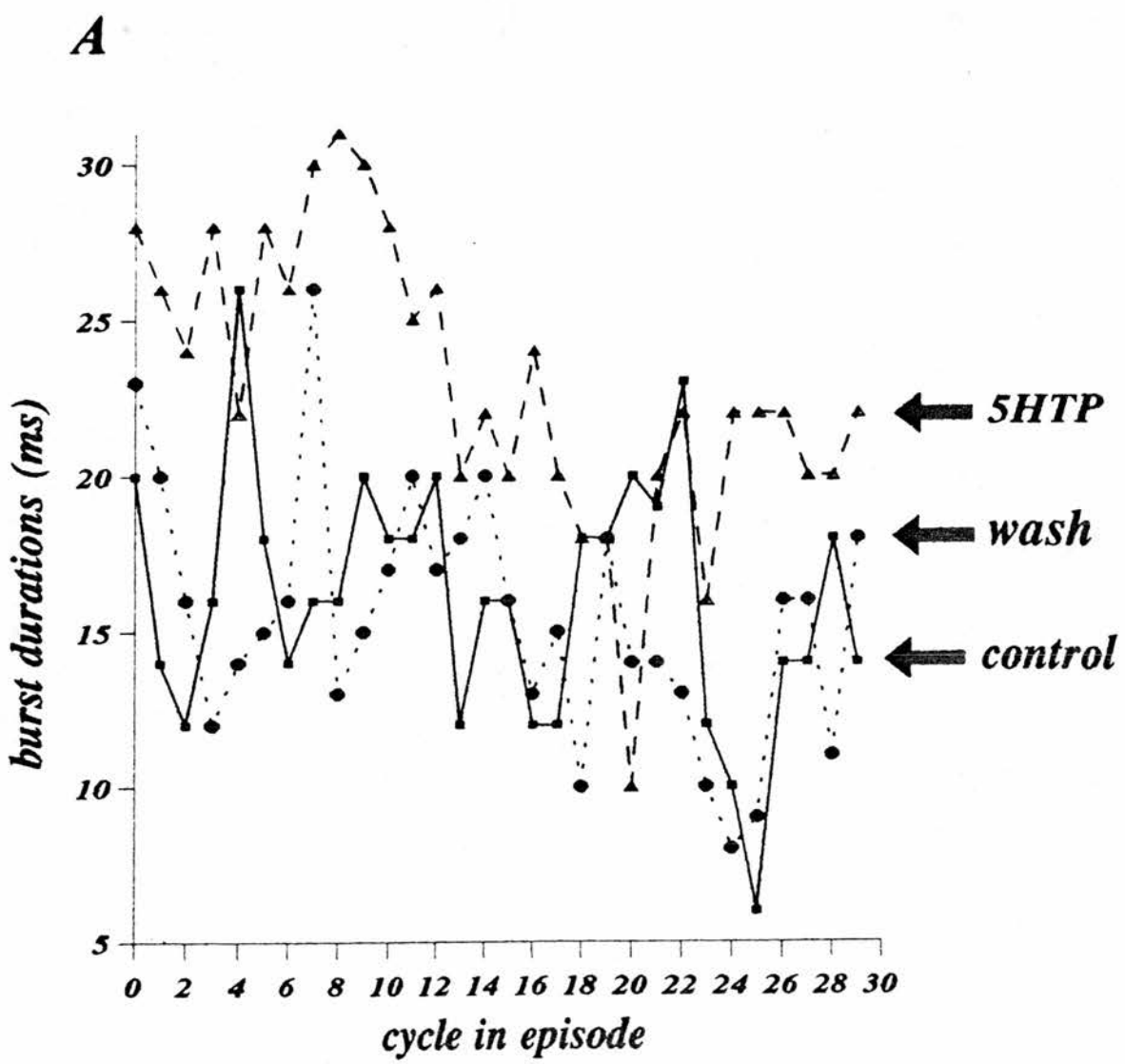
These results clearly demonstrate that the sensitivity of the swimming circuit to 5HT occurs rostro-caudally during development, as does the normal development of locomotor ventral root bursts (see Chapter 3). The stage 37/38 embryo rhythm is sensitive to 5HT only in the most rostral spinal cord. In the stage 40, sensitivity is on occasions expressed down the length of the cord but there is also a clear gradient in its degree, in that the rostral roots are more sensitive than the caudal. At stage 42, bath applied 5HT affects the locomotor network down the entire length of the animal and, although it still exerts a disproportionate effect on rostral roots compared to caudal, this is less apparent than in the stage 40. Since these data demonstrate that sensitivity to 5HT occurs first in rostral segments of hatchlings, and then proceeds rostro-caudally during post-embryonic development, further experiments were carried out to establish the onset of sensitivity to 5HT in the rostral spinal cord. The youngest embryo examined was at stage 32, about thirteen hours prior to hatching, but most experiments were carried out on stage 35/36 embryos, only a few hours before normal hatching. The swimming activity in these pre-hatching embryos appeared the same as stage 37/38 embryos, in

that it consisted of brief biphasic ventral root activity which alternated and passed down the body with a brief intersegmental delay. The bath application of 5HT caused no significant change in the durations of ventral root discharge in any of these prehatching embryos. This suggests that the locomotor circuit in the rostral spinal cord begins to acquire sensitivity to 5HT during a brief period immediately before hatching.

ii) The effects of enhanced endogenous 5HT

5-hydroxytryptophan (5HTP) is the metabolic precursor of 5HT. Its application is thought to increase the 5HT content of serotonergic neurons and thereby the availability of the transmitter for endogenous release (*cf* Barbeau & Rossignol, 1991; Viala & Buser, 1969). 5HTP is therefore a useful tool with which to examine the role of 5HT by enhancing endogenous release of the amine. The precursor was bath applied to the three developmental stages (37/38, 40 & 42), to investigate whether the resulting increase in 5HT release from raphe spinal projections (these are the only 5HT containing processes in the *Xenopus* tadpole cns; see introduction), could affect locomotor activity in a way similar to the exogenous bath application of the amine.

Applications of 5-20μM 5HTP to stage 42 larvae (n=8) caused a dramatic increase in the durations of both rostral and caudal ventral root activity and had qualitatively very similar effects to exogenous 5HT. In five stage 40 larvae, a similar application resulted in an increase in the durations of rostral ventral root bursts relative to control conditions. There appeared, however, to be little


A
burst durations (ms)
30
25
20
15
10
5
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
cycle in episode
5HTP
wash
control

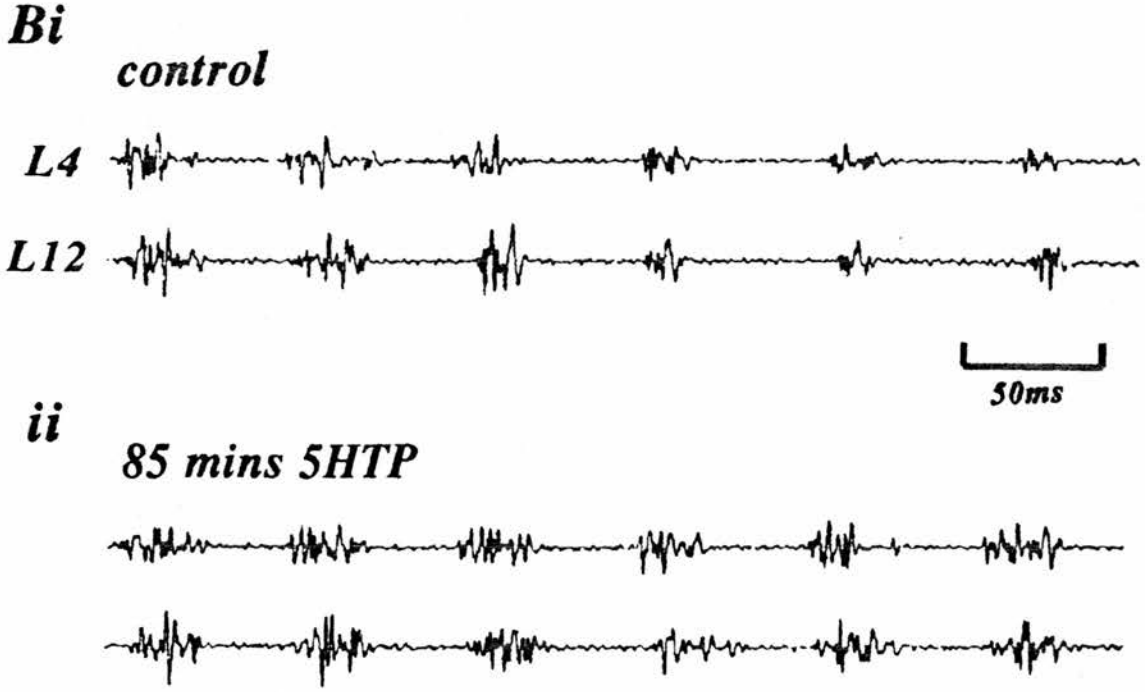

Bi
control
L4
L12
50ms
ii
85 mins 5HTP

fig 22. Effects of 5HTP on swimming activity in the stage 42 larva

(A) Rostral ventral root burst durations plotted against cycle in episode. Application of 10μM 5HTP enhanced burst durations (triangles and dotted lines). Reversed after 120mins wash in physiological saline. (B) Excerpt of swimming activity in control (i) and after 85 mins 5HTP (ii).

effect on the brief caudal ventral root discharge. When applied to stage 37/38 embryos, 5HTP appeared to have no effect on burst durations at either rostral or caudal levels (n=6). In all of the above experiments, it took substantially longer for 5HTP to exert its effect on the locomotor rhythm than it did for 5HT (1 hour or more as opposed to about five minutes). It seems likely that the considerably longer time course can be explained by the involvement of biochemical pathways in the uptake of 5HTP by serotonergic interneurons and its conversion to 5HT. Also in contrast to 5HT, recovery from 5HTP applications took considerably longer (see fig 22).

It seems therefore that the application of 5HTP only has an effect where bursts of ventral root activity have already been established during the course of normal postembryonic development. There was no effect in the stage 37/38, only in the rostral ventral roots of the stage 40 and in both rostral and caudal ventral roots of the stage 42. These effects are subtly different from those of bath applied 5HT, which induces bursts of activity at levels in the cord where ventral root activity still comprises brief biphasic impulses, but where the acquisition of 'burstiness' is imminent, namely in the rostral ventral roots of stage 37/38 and the caudal roots of stage 40.

iii) Effects of neurotoxic ablation of 5HT fibres

In order to test further the hypothesis that the post-embryonic acquisition of a more flexible and adult-like swimming rhythm might be causally linked to the ingrowth of serotonergic

axons from descending raphe interneurons, prehatching embryos at around stage 25 were treated with the specific serotonergic neurotoxin 5, 7 dihydroxytryptamine (in collaboration with A-M Woolston). At around this developmental stage, about 28 hours after fertilisation, the first serotonergic neurons in the brain stem become apparent (van Mier *et al*, 1986a). In order to disrupt normal development of brain stem serotonergic interneurons, six stage 25 embryos were maintained in neurotoxin until developmental stage 42. After neurophysiological experiments had been carried out, anatomical analysis using immunohistochemical techniques confirmed that normal development of raphe interneurons had been disrupted (A-M Woolston, unpublished observations).

The ventral root activity of toxin-treated stage 42 larvae closely resembled that of stage 37/38 embryos (fig 23 Bi; 24 Bi). It comprised mainly brief biphasic impulses with none of the features normally associated with the post-embryonic swimming rhythm. By contrast the control animals demonstrated typical stage 42 activity; ventral root activity consisting of bursts of activity of between 10 and 20ms (figs 23 Ai, Bi; 24 Ai, Bi). Superimposed traces of ten cycles of ventral root activity in the control and treated animals illustrate the differences very clearly (fig 23 Aii, Bii). Figure 24 illustrates camera lucida drawings of spinal cords of control (Aiii) and toxin-treated (Biii) animals which have been subjected to immunocytochemical techniques using antibodies raised against 5HT. In the control animal, serotonergic projections are clearly visible, while in the toxin-treated animal there is no evidence of any serotonergic innervation (drawings supplied by A-M Woolston).

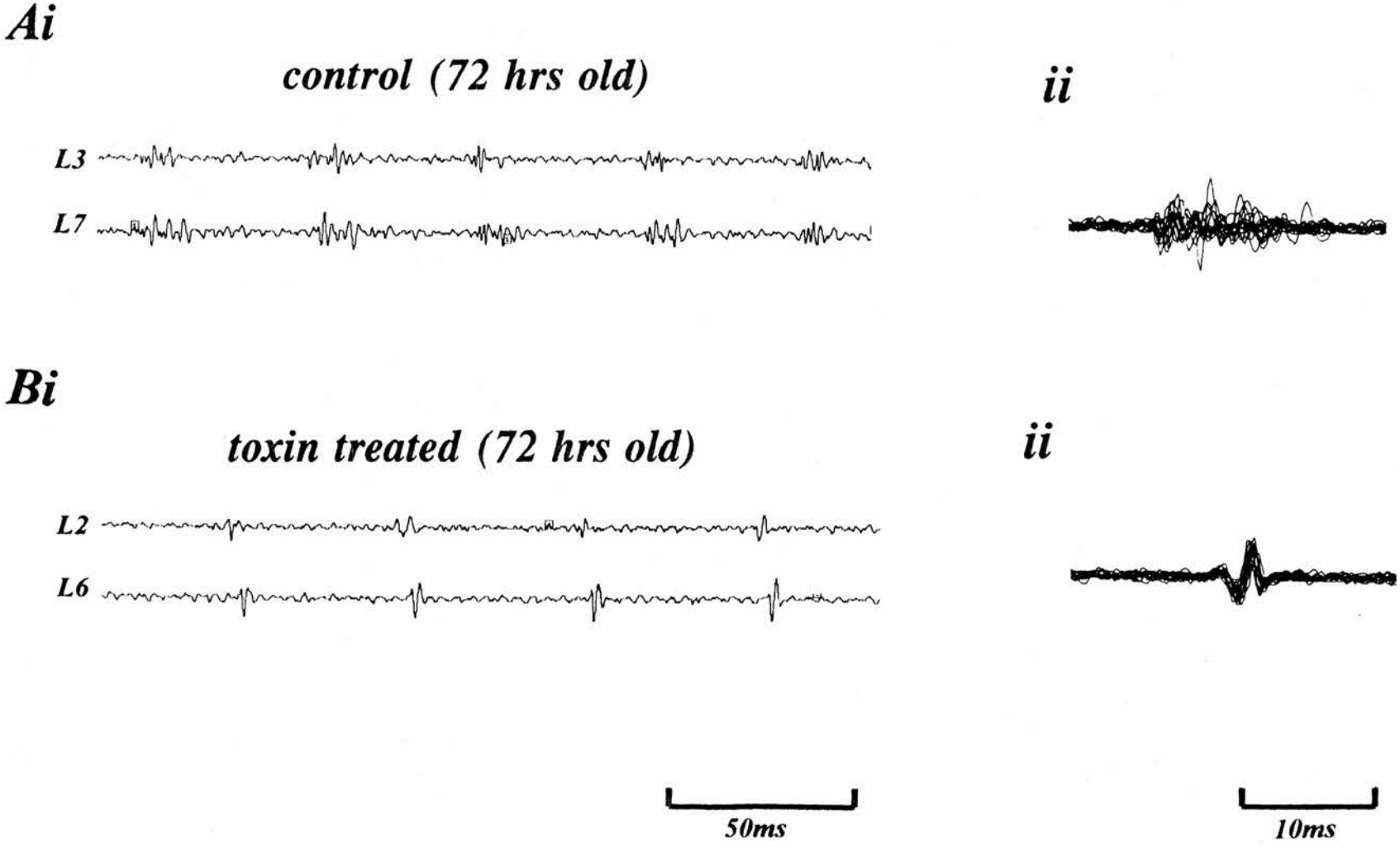

Ai
control (72 hrs old)
L3
L7
ii
Bi
toxin treated (72 hrs old)
L2
L6
ii
50ms
10ms

fig 23. Effects of selective neurotoxic ablation of 5HT fibres (a)

5,7 dihydroxtryptamine (5,7-DHT) is a selective serotonergic neurotoxin. When prehatching embryos from about developmental stage 25 are raised in the neurotoxin until stage 42 (72 hrs old), normal development of locomotor rhythmicity fails to occur. (Ai) Fictive swimming activity of a stage 42 control animal. Ventral root activity consists of bursts of discharge lasting typically between 10 and 20ms. (Aii) Ten superimposed cycles of activity in a single ventral root (L7). (Bi) Fictive swimming activity of a stage 42 which has been reared in the neurotoxin. The activity is brief and 'embryonic-like'. (Bii) Ten superimposed cycles of the same activity (L6).

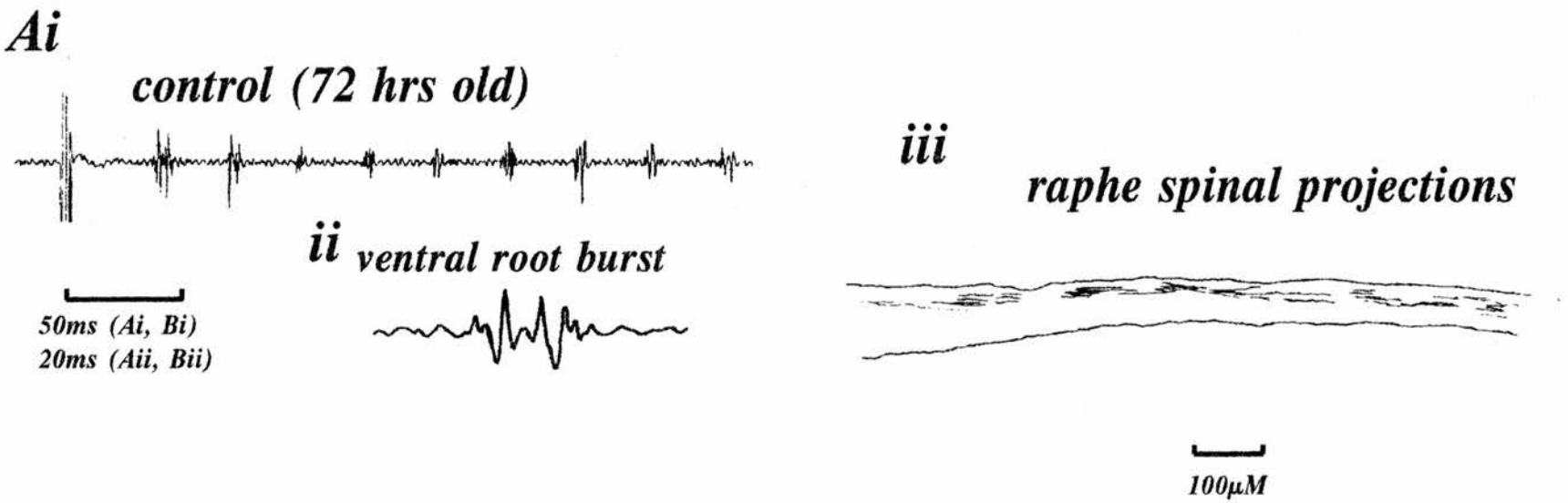

Ai
control (72 hrs old)
ii ventral root burst
50ms (Ai, Bi)
20ms (Aii, Bii)
iii
raphe spinal projections
100μM
Bi
toxin treated (72 hrs old)
ii ventral root spike
iii
no raphe spinal projections

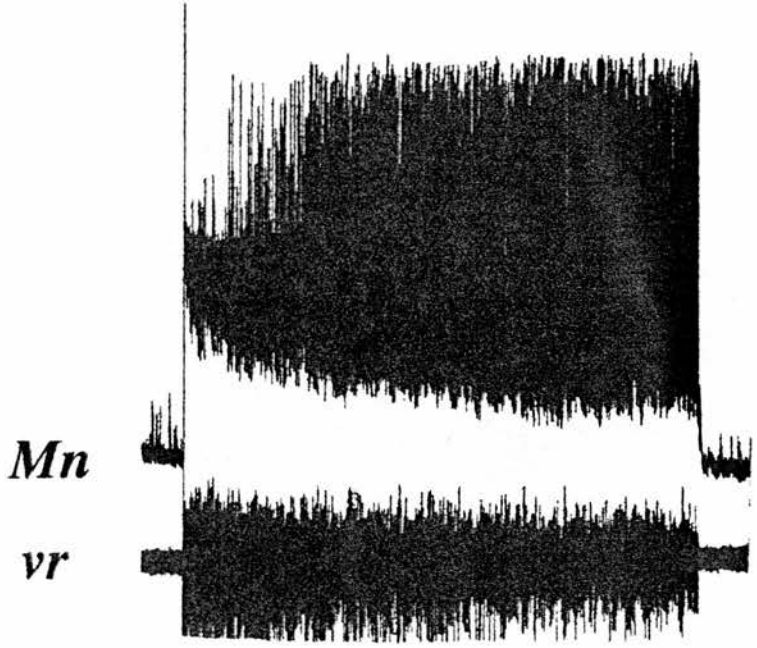
fig 24. Effects of selective neurotoxic ablation of 5HT fibres (b)

(A) Control. (Ai) ventral root activity underlying swimming in a stage 42 larva (72 hrs old) reared under control conditions. (ii) A single ventral root burst on a faster time base. (iii) Camera lucida drawing of raphe projections revealed by immunocytochemistry. (B) Toxin treated larva. (i) The ventral root activity underlying swimming is brief and 'embryonic-like'. (ii) A single ventral root spike. (iii) Immunocytochemistry failed to reveal any evidence of raphe spinal projections in toxin treated animals.
Anatomical drawings kindly supplied by A-M Woolston.

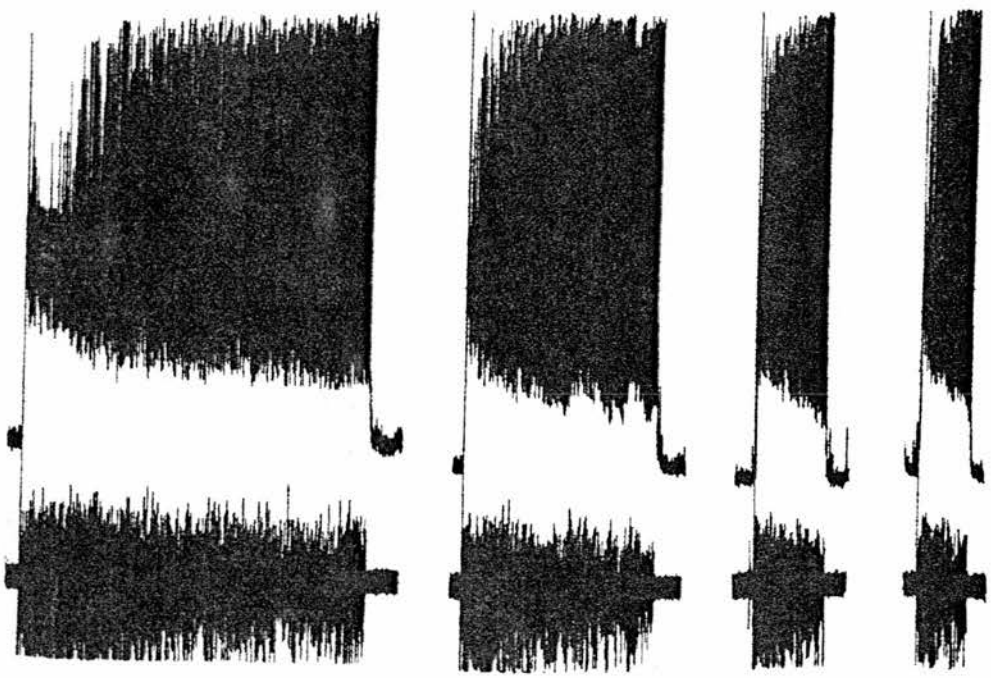
iv) Additional effects of 5HT on the swimming system

In the course of these experiments, a number of additional 5HT effects were observed. For example, the bath application of 5HT caused a dramatic reduction in the duration of swim episodes. The effect appeared to be time dependent, in that following 5HT applications episode durations progressively decreased until a new plateau of relatively consistent shorter durations was reached (fig 25). This may have been a reflection of either the time taken for the concentration of the amine to build up in the bath, or simply the time course of the physiological processes involved. When higher concentrations (5-10μM) of the amine were applied, episodes would often comprise less than five cycles, albeit with long bursts of ventral root activity.

5HT also had profound inhibitory effects on each of three separate sensory pathways which initiate swimming (see introduction). Fictive swimming activity can be initiated either by dimming the illumination or by applying a brief current pulse to the tail skin (which activates both the R-B and skin cell pathways) via a stimulating electrode ('Materials & Methods'). Following application of the amine, the stimulus voltage required for activation of swimming always increased, often by two orders of magnitude. Presumably 5HT was simultaneously suppressing both the primary mechanosensory pathway mediated by R-B cells, and also the skin cell pathway, since such a dramatic effect would not have been observed if only one of the two pathways had been affected. This was confirmed by experiments on two stage 42 larvae and one stage 37/38 embryo, in which the cord was transected about two-thirds down the length of the animal (fig 26).



control



2 mins 5HT

3 mins 5HT

4 mins 5HT

5 mins 5HT

30 secs

fig 25. 5HT decreases episode lengths

Application of 5HT reduces episode lengths. In this example, fictive swimming activity was recorded intracellularly from a motorneuron (Mn) and extracellularly from a ventral root (vr). Following bath application of 5μM 5HT, there was a progressive reduction in episode duration until a new plateau of relatively constant durations was reached after 5 minutes.

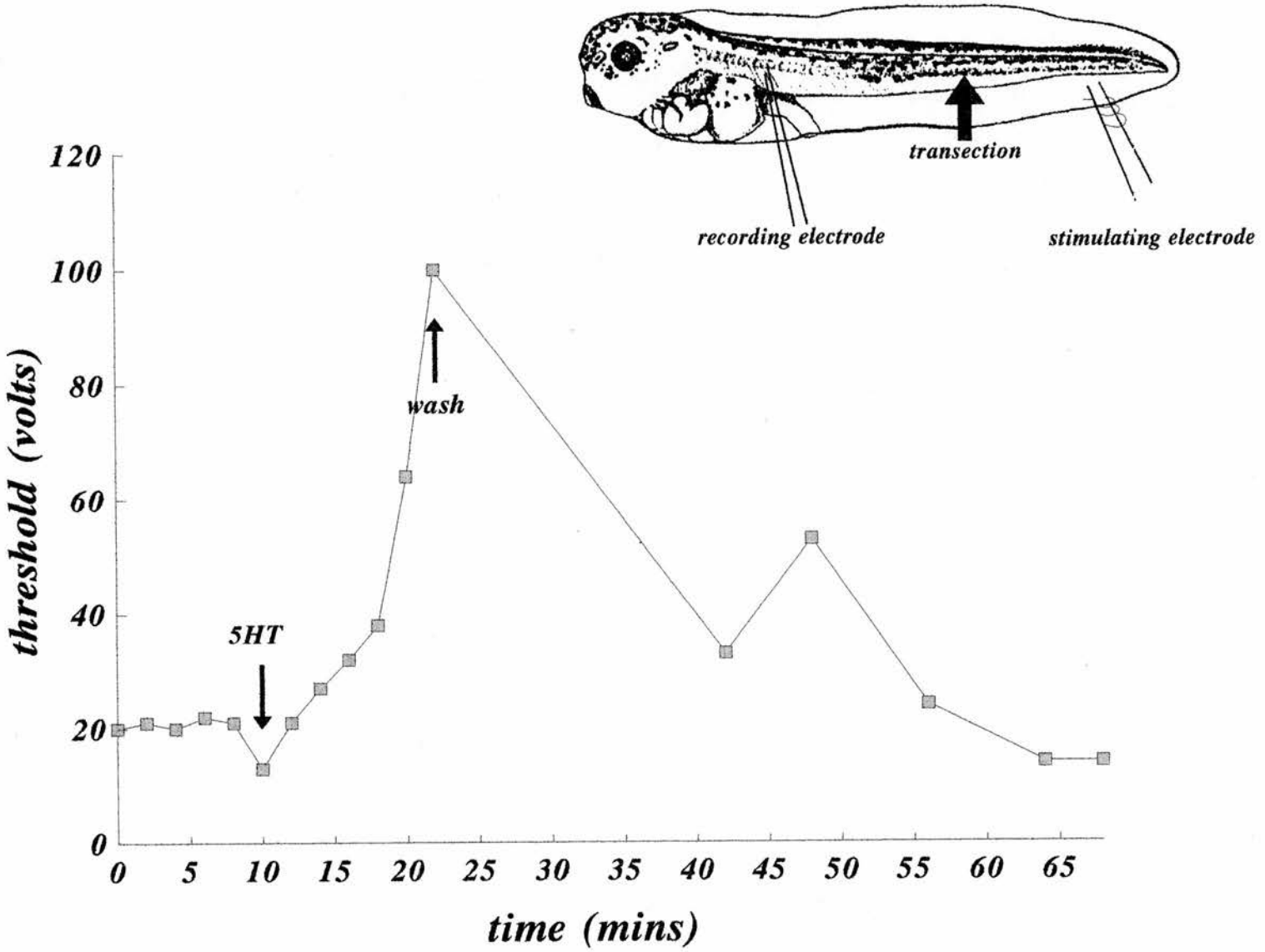

transection
recording electrode
stimulating electrode
120
100
80
60
40
20
0
threshold (volts)
wash
5HT
0
5
10
15
20
25
30
35
40
45
50
55
60
65
time (mins)

fig 26. 5HT suppresses the skin cell pathway

The skin cell pathway was isolated from the R-B cell pathway by transecting the cord about two thirds down the animal (at arrow; see insert). The stimulating electrode was positioned some distance caudal to the transection. A current pulse to the tail skin would therefore only excite the receptive fields of R-B cells caudal to the lesion, and consequently they could not excite the rostral locomotor circuit. Swimming was therefore only elicited via the skin cell pathway which is thought to have access to the cns via the trigeminal nerve. A plot of threshold against time illustrates that, following application of 2μM 5HT, there was a marked rise in threshold, which was reversed with washing in control saline.

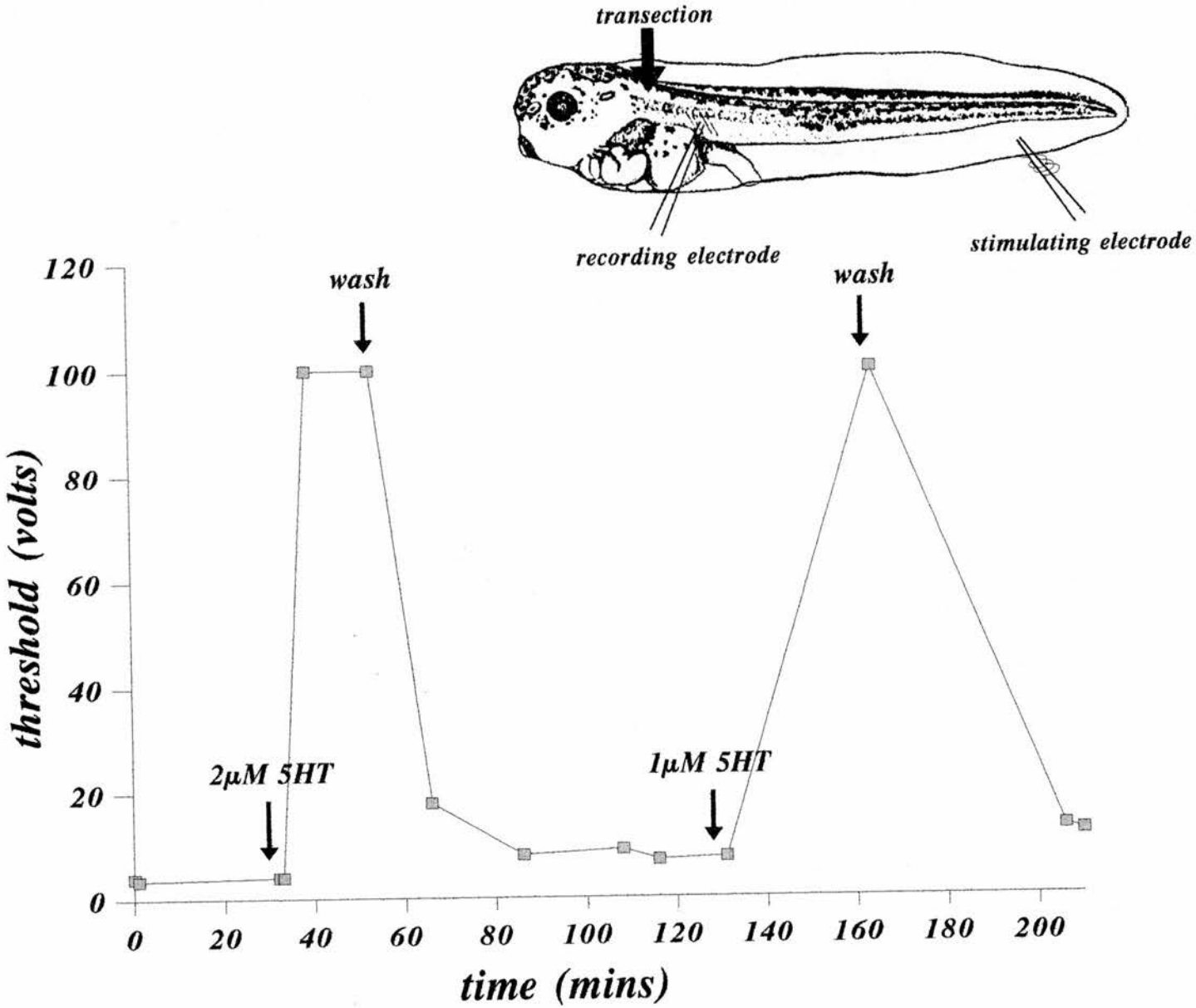

transection
recording electrode
stimulating electrode
wash
wash
2μM 5HT
1μM 5HT
threshold (volts)
time (mins)
0
20
40
60
80
100
120
140
160
180
200

fig 27. 5HT suppresses the R-B cell pathway

In order to isolate the R-B cell pathway from the skin cell pathway, the cord was cut at about the level of the 1st post-otic myotome (at arrow) to ensure that skin impulses could not excite the locomotor network caudal to the cut. A plot of threshold against time shows that 2μM 5HT rapidly and dramatically suppressed the R-B cell pathway. This effect was reversed by washing, and subsequent application of 1μM had a similar and reversible effect.

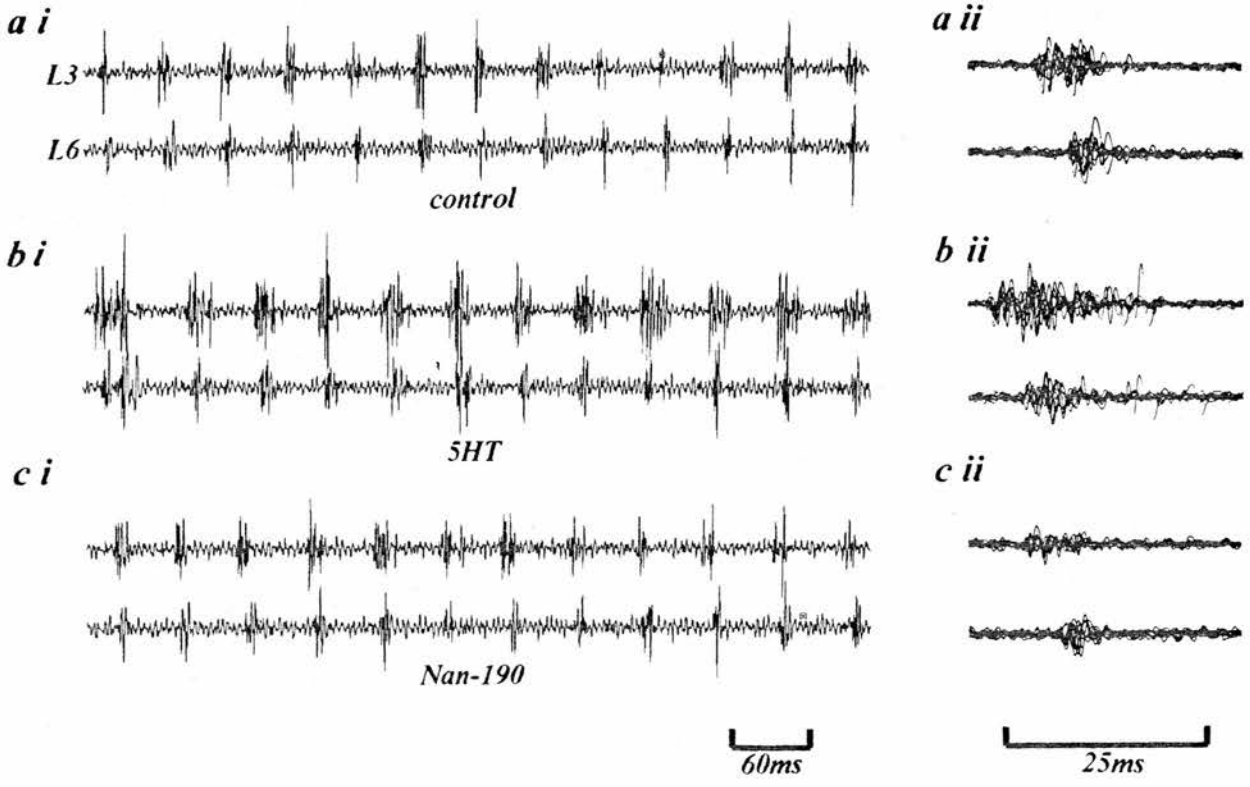
Recording electrodes were placed on clefts rostral to the level of the transection, and the stimulating electrode placed on the skin some distance caudal to it. This ensured that the R-B cells whose receptive fields would be stimulated by the current pulse, could not excite the locomotor circuit in the cord rostral to the cut (fig 26). The stimulus could, however, reliably evoke fictive swimming activity via the skin cell pathway at relatively low thresholds. For example (fig 26), in control, stimulus threshold was about 20 volts. After application of 2μM 5HT, this steadily increased until after 10 minutes, stimulus threshold was greater than 100 volts (the maximum stimulus available). It returned to control levels after return to physiological saline. Since 5HT also dramatically suppresses sensory activation in the intact preparation, it is clear that it must similarly act on the R-B cell pathway (see also Sillar *et al*, 1991; Sillar & Simmers, 1994a). Notwithstanding this, experiments were also carried out in which sensory input via the skin cell pathway was removed. Since the skin cell pathway is thought to have access to the cns via one of the trigeminal nerves (A. Roberts, unpublished observations), transecting the cord at about the level of the 1st post-otic myotome should block access of this pathway to the locomotor network. This procedure was carried out in 3 stage 42 larvae, 1 stage 40 and 1 stage 37/38 embryo. On each occasion 5HT inhibited the R-B cell pathway and caused a dramatic increase in the stimulus voltage required for activation of swimming, and as in the previous experiments, this was reversed by washing (fig 27).

5HT also reliably blocked the dimming response in the three developmental stages. For example, in embryos the dimming response is usually particularly consistent, and it was consistently

abolished by exogenous 5HT, often within a minute of exposure. Washing in control saline usually restored the dimming response.

v) Evidence for $5HT_{1a}$ receptor involvement

To investigate the identity of the 5HT receptors involved in the serotonergic modulation of swimming in post-embryonic *Xenopus*, a range of serotonergic agents were bath applied to test their effects on swimming activity. Initial experiments were carried out in the presence of exogenous 5HT, and a range of antagonists to pharmacologically distinct 5HT receptor subtypes added to test their effects on the 5HT-enhanced rhythm. Antagonists which act at the $5HT_2$ and/or $5HT_3$ families of receptors were all ineffective even at relatively high concentrations. These included the $5HT_2$ receptor antagonists mesulergine (n=11), cyproheptadine (n=5) ketanserin (n=19) and methysergide (n=11), and the $5HT_3$ receptor antagonists MDL 7222 (n=7) and ondansetron (n=2). From these experiments, I concluded that neither $5HT_2$ nor $5HT_3$ receptors play a significant role in serotonergic modulation of swimming. Attention was consequently focused on the possible role of $5HT_1$ receptors. The first circumstantial evidence for the involvement of a '$5HT_1$-like' receptor was also suggested by the effects of methysergide on the 5HT-enhanced rhythm. Although this serotonergic agent is generally regarded as a $5HT_2$ antagonist, it also has some affinity for $5HT_1$ subtypes, where it can sometimes have a partial agonist effect (Bradley *et al*, 1986; see also Peroutka, 1991). On no occasion did methysergide reverse the effects of 5HT, and in three experiments it appeared to potentiate


a i
L3
L6
control
a ii
b i
5HT
b ii
c i
Nan-190
c ii
60ms
25ms

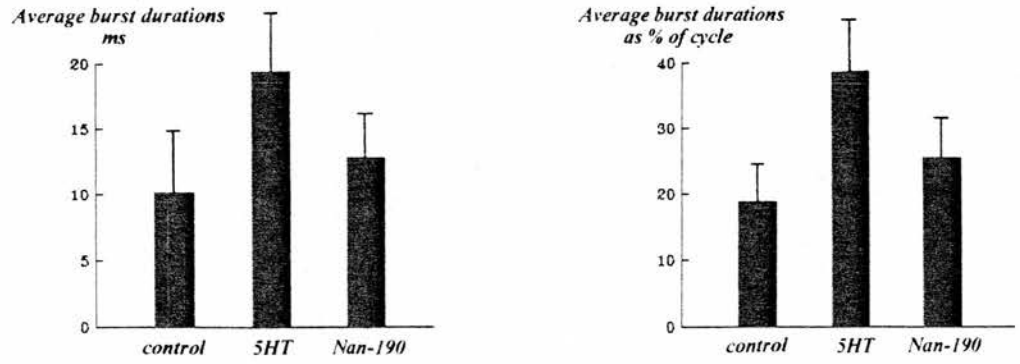
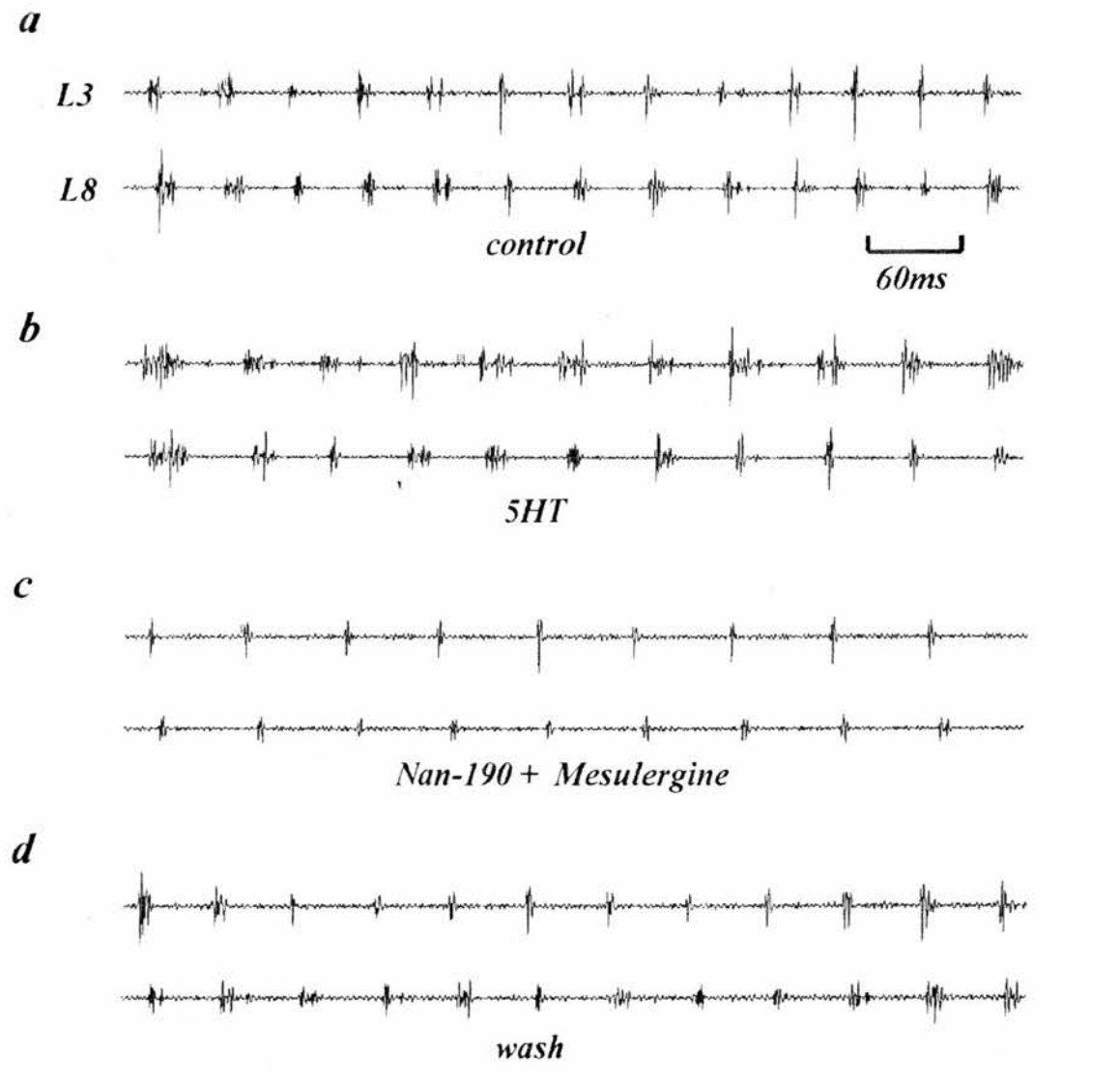

d i
Average burst durations
ms
20
15
10
5
0
control
5HT
Nan-190
ii
Average burst durations
as % of cycle
40
30
20
10
0
control
5HT
Nan-190

fig 28. Nan-190 reverses the effects of 5HT on swimming

Effects of Nan-190, a $5HT_{1a}$ receptor antagonist, on the 5HT-enhanced swimming pattern of a stage 40 larva. (ai) Excerpt of swimming in control saline. (bi) Fictive swimming in the presence of 2μM 5HT. (ci) Swimming following addition of 100μM Nan-190 to the 5HT containing saline. (aii, bii, cii) Superimposed traces of 15 cycles of ventral root activity under the three experimental regimes. (di) Histogram of the average ventral root burst durations (n=20) taken from L3 in control, 5HT and Nan-190. 5HT caused average burst durations to increase from 10.1 +/_ 2.7ms (sd) to 19.4 +/_ 4.2ms. Addition of Nan-190 almost completely reversed this effect so the average burst durations more closely resembled control (12.8 +/_ 3.3ms). (dii) Histograms illustrating average burst durations expressed as a percentage of cycle period.


a
L3
L8
control
60ms
b
5HT
c
Nan-190 + Mesulergine
d
wash

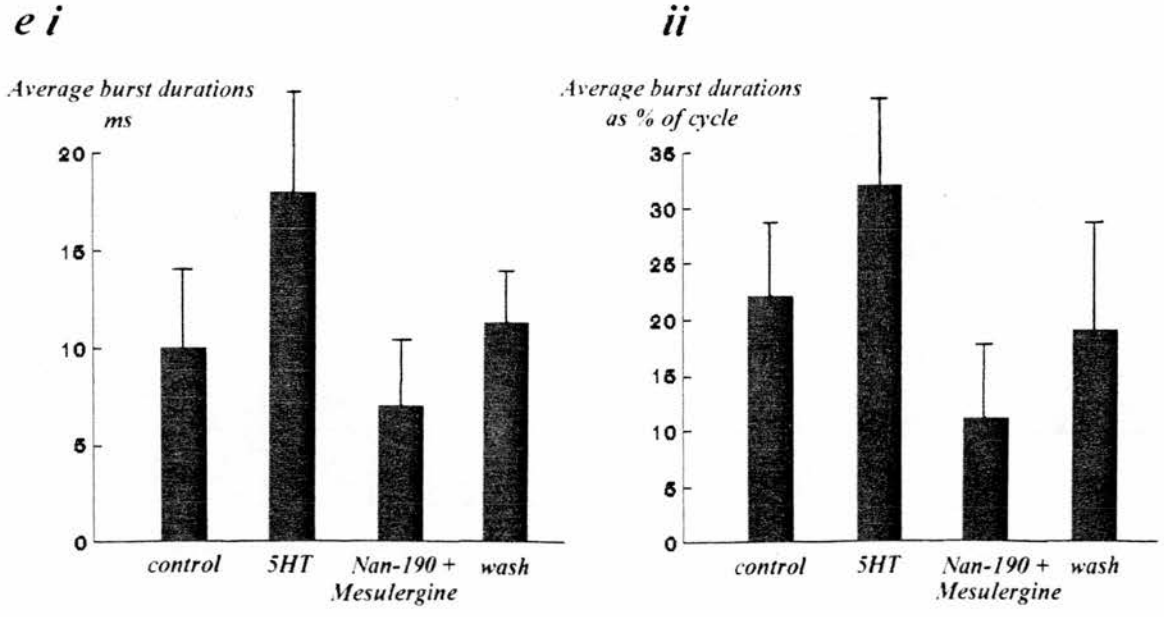

e i
Average burst durations
ms
20
15
10
5
0
control
5HT
Nan-190 +
Mesulergine
wash
ii
Average burst durations
as % of cycle
35
30
25
20
15
10
5
0
control
5HT
Nan-190 +
Mesulergine
wash

fig 29. Effects of Nan-190 on 5HT-enhanced swimming

Effects of Nan-190 on the 5HT-enhanced swimming pattern of a stage 42 larva. (a) Control swimming. (b) Fictive swimming in the presence of 5μM 5HT. (c) Swimming activity following addition of 10μM Nan-190+10μM mesulergine. Since the $5HT_2$ antagonist mesulergine consistently failed to have any effect on 5HT-enhanced swimming (n=11), it is likely that the reversal of the effects of 5HT shown here is attributable to Nan-190 alone. Note that Nan-190 appears to have **more** than reversed the effects of 5HT. (d) After return to wash, the activity resembles that of control. (e) Histogram showing average burst durations (n=20) under the four experimental regimes, expressed as absolute burst durations (i) and as a percentage of cycle period. In control these were 10 $^{+}/_{-}$ 3.3 ms (22%), in 5HT 18 $^{+}/_{-}$ 4.8ms (32%), in Nan-190 7 $^{+}/_{-}$ 3.7ms (19%) and in wash 9.4 $^{+}/_{-}$ 2.7ms (19%).

them by further increasing the durations of ventral root bursts. It is possible that, in these cases, methysergide exerted a partial agonist effect at $5HT_1$-like receptors. Also, the selective $5HT_{1a}$ antagonist Nan-190 (n=17) was effective at reversing the effects of 5HT on swimming. In the illustrated example (fig 28), the bath application of 2μM 5HT to a stage 40 larva increased the average burst durations of the rostral ventral root activity (L3) from 10.1 +/- 2.7ms (sd) to 19.4 +/- 4.2ms. This effect was reversed almost completely by addition of 100μM Nan-190 to the 5HT-containing bath solution, so that burst durations were reduced back towards control levels with average durations of 12.8ms +/- 3.3ms. Figure 29 also illustrates the effects of Nan-190 on 5HT-enhanced rhythm in a stage 42 larva. After 5 minutes, bath applied 5HT (5μM) enhanced ventral root bursts from 10 +/- 3.3ms (sd) on average (22% of cycle period) to about 18 +/- 4.8ms (32%). On addition of 10μM Nan-190 (in this case together with the $5HT_2$ antagonist mesulergine at 10μM), burst durations rapidly declined to an average of 7 +/- 3.7ms (11%). Since experiments with the $5HT_2$ antagonist mesulergine alone (n=11) failed to reveal any noticeable effect on 5HT-enhanced swimming activity, it is likely that the antagonism of 5HT was solely attributable to Nan-190. In this example, the antagonist **more** than reversed the effects of 5HT so that the resulting activity closely resembled the embryonic swimming pattern. This effect was reversed by washing to resume a pattern of activity very similar to control with burst durations of 9.4 +/- 2.7ms (19%) (in each case, 20 cycles close to the beginnings of swim episodes were used for analysis). Since Nan-190 on occasions more than reversed the effects of exogenous 5HT on swimming activity, experiments were carried out to test the

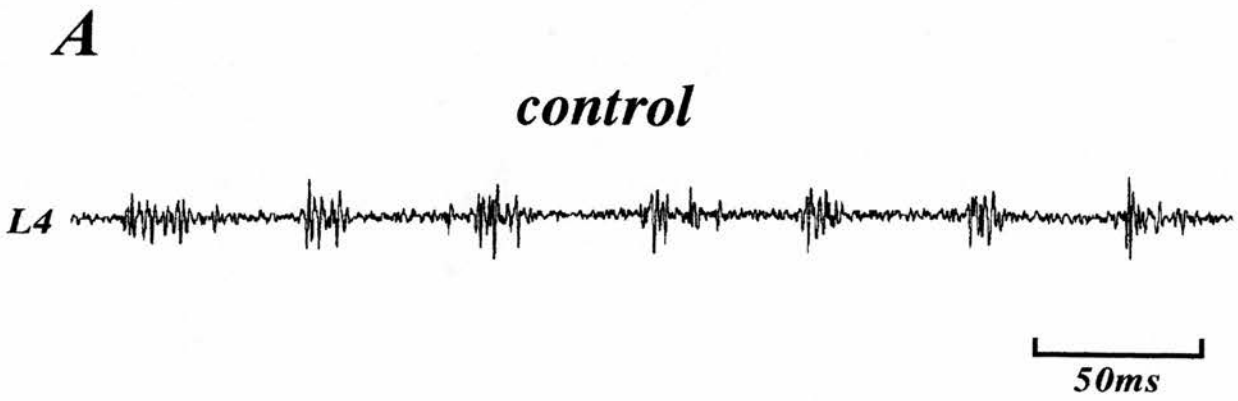

A
control
L4
50ms
B
20 mins Nan-190
C
25 mins wash

fig 30. Effects of Nan-190 on larval swimming

(A) A single ventral root (L4) recording of swimming activity in a stage 42 larva under control conditions. (B) Following application of Nan-190, the durations of ventral root bursts are reduced. (C) Recovery.

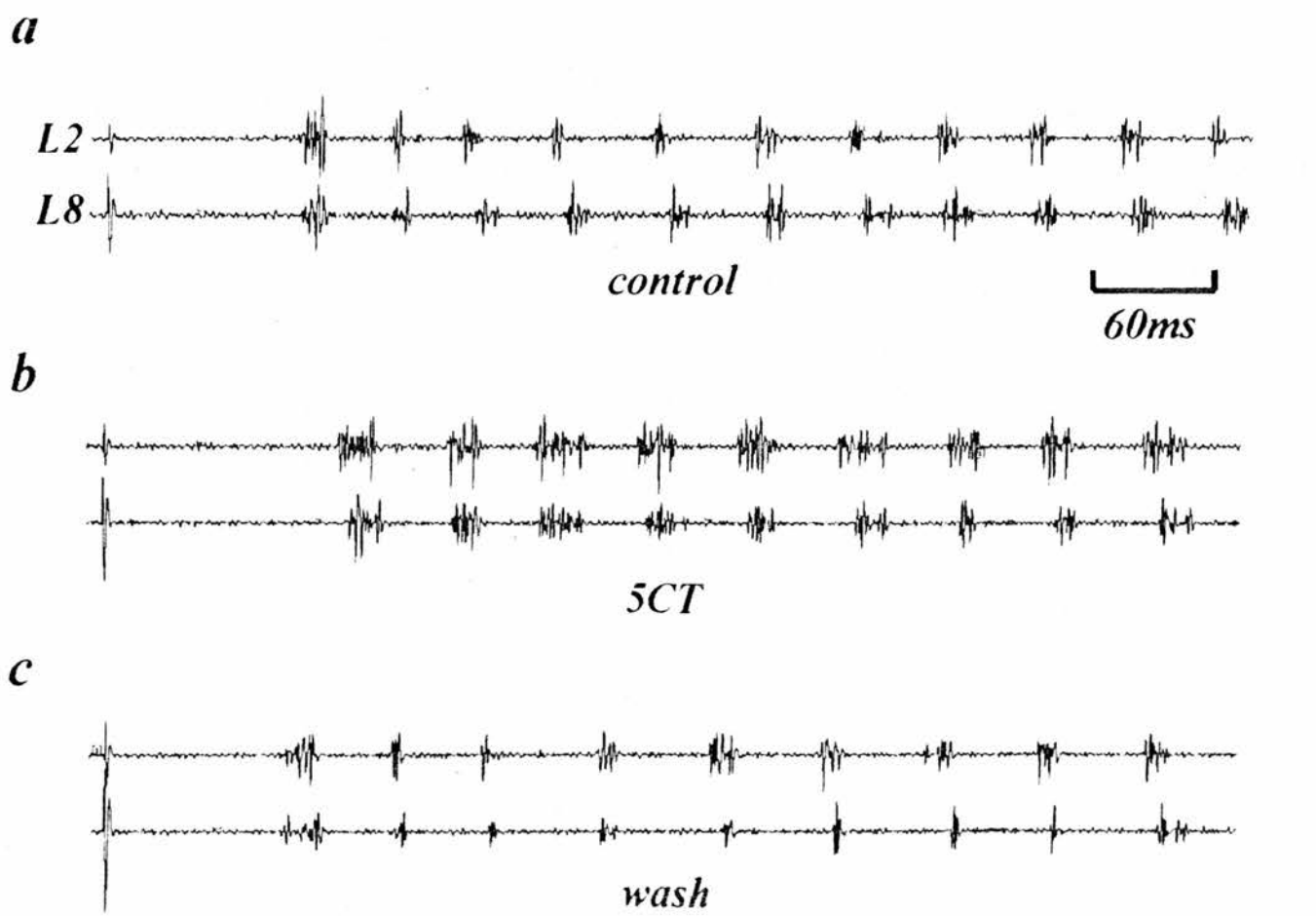
effects of Nan-190 on the 'normal' stage 42 larval swimming rhythm (n=2). Figure 30 illustrates the effects of bath applied Nan-190 (10μM) on the ventral root activity underlying larval swimming. It causes a dramatic reduction in the durations of ventral root bursts, and this effect is reversed by washing.

On the basis of these results, the non-specific $5HT_1$ receptor agonist 5-carboxamidotryptamine (5CT) was bath applied to test whether its effects on swimming activity mimicked those of 5HT. 5CT is a high affinity agonist and is an important tool in the classification and identification of the $5HT_1$ family of receptors. 5CT exerted effects which closely resembled those of 5HT, even at concentrations an order of magnitude lower (0.1μM). In a stage 42 larva, for example (fig 31), the effects of 0.1μM 5CT were similar to those of 2μM 5HT in that it dramatically increased ventral root burst durations. The average durations of the rostral activity increased from 12 +/- 3.1ms to 22.3 +/- 4.6ms (di). Also, as with 5HT, the effects were readily reversed by washing so that after twenty minutes the average burst durations resembled those of control (14 +/- 3.8ms; di). When average burst durations are expressed as a percentage of cycle period, these were 26 +/- 6.3% in control, 53 +/- 2.95% in 5CT and 25.5 +/- 6.7% in wash (dii). Like 5HT, bath applied 5CT also decreased episode lengths. In figure 32, application of 0.1μM 5CT, not only caused an increase in the ventral root activity underlying swimming (b), but also dramatically reduced episode duration (*cf* di & dii). Following application of 100μM Nan-190, the 5CT effects on burst duration and episode length were both reversed (c, diii). This example also clearly shows that Nan-190 **more** than reverses both these 5CT-induced effects on locomotor rhythm, since following its

application, burst durations are briefer than those of control (c) and the episode length is longer (diii).

The full $5HT_{1a}$ agonist R(+)-8-OH-DPAT, although not as effective as 5CT, also increased burst durations and decreased episode duration. Figure 33 illustrates the effects of 10μM R(+)-8-OH-DPAT on a stage 40 larva. Five minutes following application, average burst durations more than doubled from 7.2 +/- 1.7ms (sd) to 14.8 +/- 5ms. Its less potent isomer (+)-OH-DPAT had only a marginal effect, and buspirone, also a 5HT1a agonist when bath applied at a wide range of concentrations (5-100μM), had no noticeable effect on rhythm.

In the course of these experiments, it was also possible to observe effects of various 5HT receptor ligands on the sensory pathways whose sensitivity is modulated by 5HT. As previously described, bath applied 5HT raises the threshold for activation of swimming via the skin sensory pathways (R-B cell & skin cell) and abolishes the light dimming response. Like 5HT, the non-specific, high affinity $5HT_1$ agonist, 5CT, similarly abolished this latter response (n=7). In contrast to 5HT, however, it did not appear to have any effect on the skin sensory pathways. This suggested that a $5HT_1$-like receptor was involved in the serotonergic modulation of the dimming response, but not of the skin sensory pathways. However, experiments using specific 5HT receptor antagonists provided some evidence for the involvement of $5HT_3$ receptors in the modulation of skin sensory pathways. In two out of seven experiments in which the stimulus threshold for activation of swimming had been dramatically increased following 5HT application, the addition of the $5HT_3$ receptor antagonist


a
L2
L8
control
60ms
b
5CT
c
wash

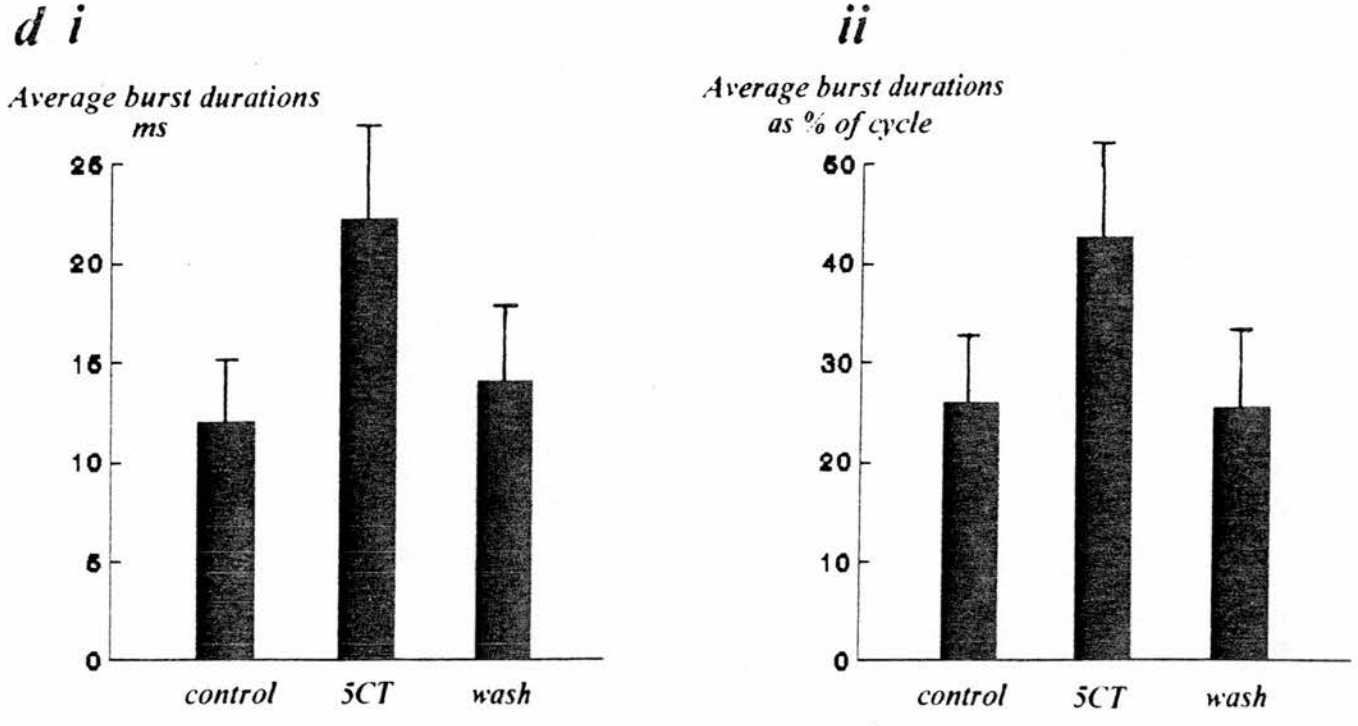

d i
Average burst durations
ms
25
20
15
10
5
0
control
5CT
wash
ii
Average burst durations
as % of cycle
50
40
30
20
10
0
control
5CT
wash

fig 31. Effects of 5-carboxamidotryptamine (5CT) swimming

(a) Control swimming in a stage 42 larva. (b) Application of 0.1μM 5CT dramatically enhances ventral root activity. (c) The effects are reversed by washing in physiological saline. (d) Histograms of average absolute (i) and relative (ii) burst durations under the three experimental regimes.

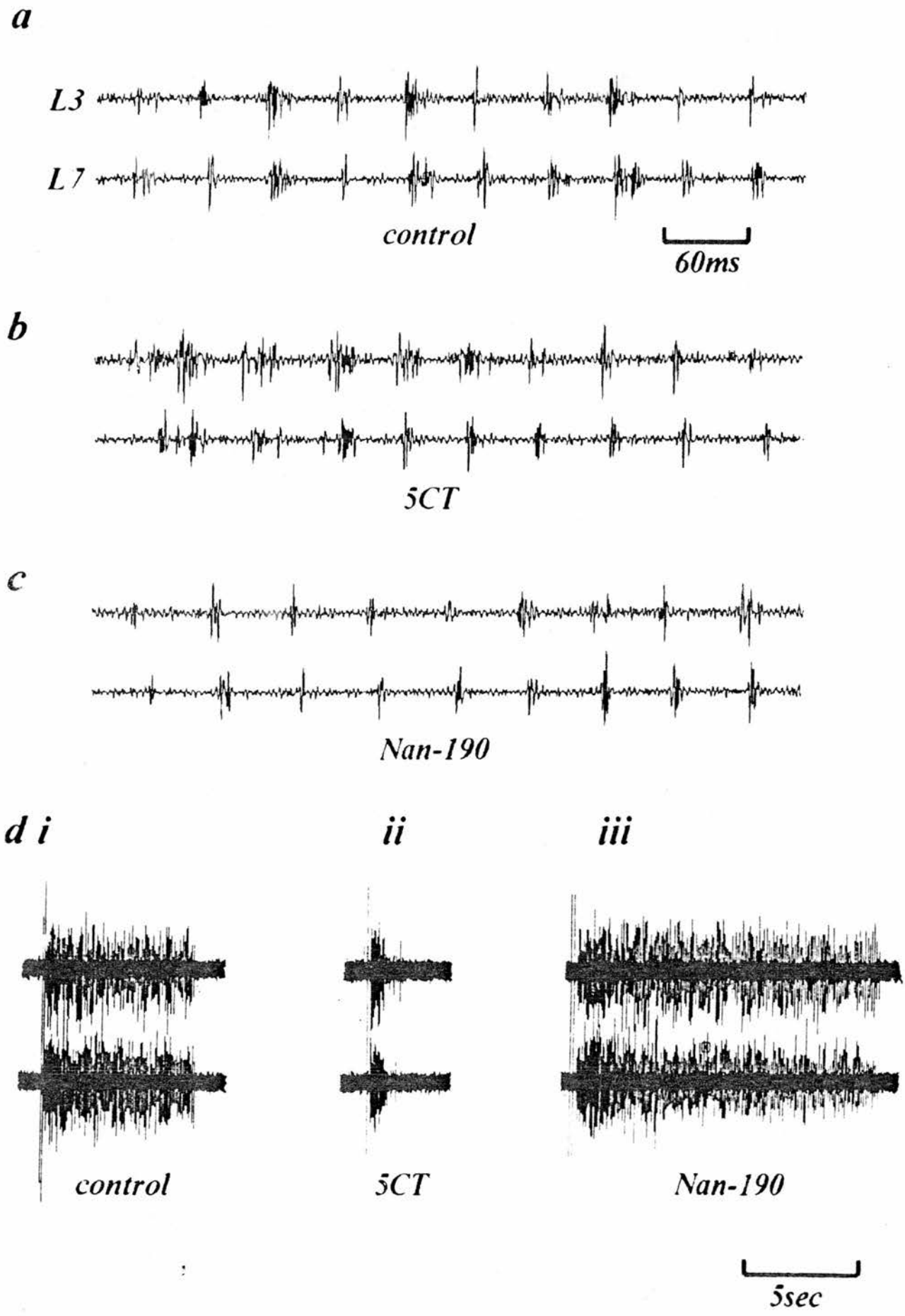
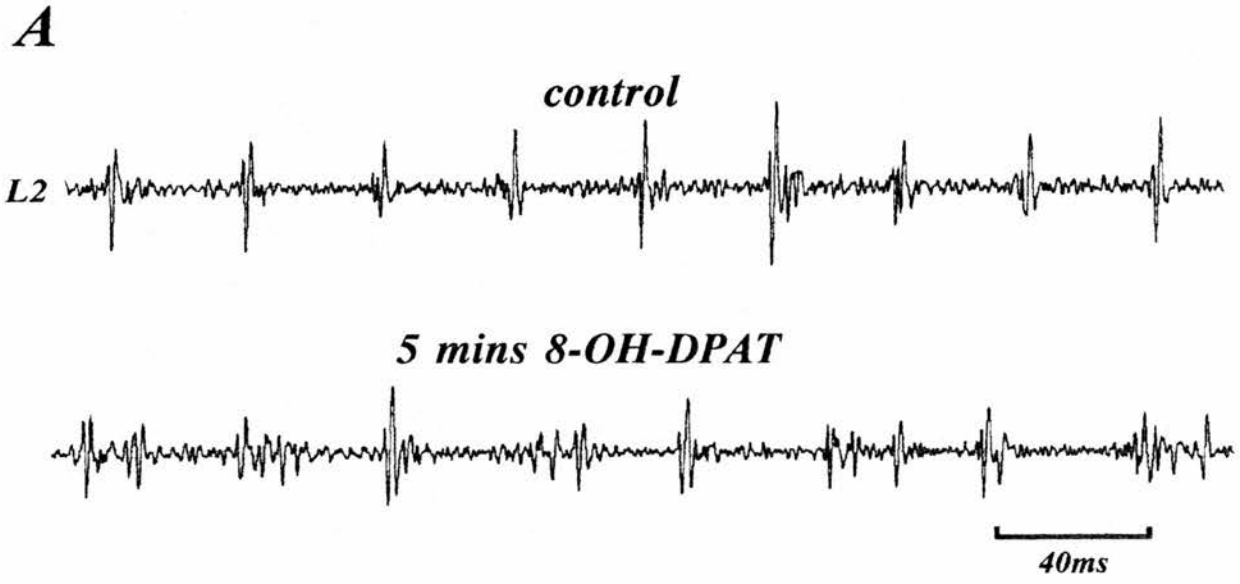

a
L3
L7
control
60ms
b
5CT
c
Nan-190
d i
ii
iii
control
5CT
Nan-190
5sec

fig 32. Nan-190 reverses the effects of 5CT

5CT has two noticeable effects on swimming activity of stage 42 larvae, it increases burst durations and decreases episode lengths. Both these effects are reversed by Nan-190. (a) Ventral root activity in control saline. (b) Activity following application of 5CT (entire episode). (c) Following addition of 100μ M Nan-190. (d) Entire episodes on a slow time base. (i) control. (ii) Following application of 5CT episode length is dramatically reduced. (iii) This effect is reversed with addition of Nan-190.


A
control
L2
5 mins 8-OH-DPAT
40ms

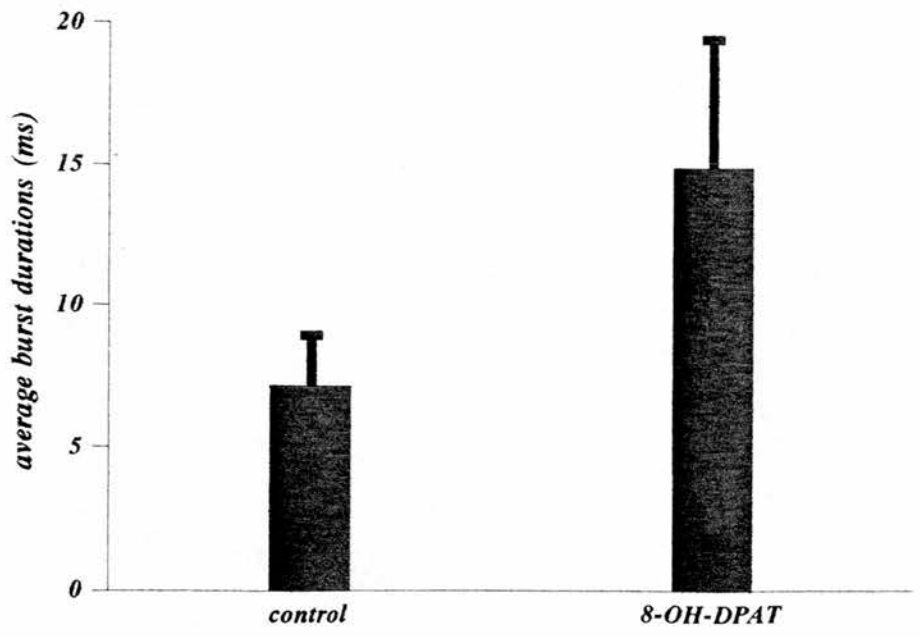

B
20
15
10
5
0
average burst durations (ms)
control
8-OH-DPAT

fig 33. The effects of R(+)-8-OH-DPAT on the fictive swimming activity of a stage 42 larva

Bath application of 10μM R(+)-8-OH-DPAT to a stage 40 larva increased the durations of the ventral root activity underlying swimming. (A) In control, burst durations in the recorded ventral root (L2) were relatively brief (7.2 $^{+}/_{-}$ 1.7ms). Five minutes following application of 8-OH-DPAT, average burst durations more than doubled (14.8 $^{+}/_{-}$ 5ms) (n=20). (B) Histogram of average burst durations in L2 before and after drug application.

MDL72222 appeared to partially reverse this effect (not illustrated; see also Sillar *et al*, 1991).

The involvement of a $5HT_1$-like receptor at some stage in the pineal eye to motor system pathway which mediates the dimming response, was suggested by the effects of 5CT. Moreover, this sensory pathway was likewise blocked by the $5HT_{1a}$ receptor agonist buspirone which did not noticeably effect swimming activity (see above). Conversely, both isomers of 8-OH-DPAT, while having an effect on swimming, had no effect on the dimming response. These results seem to indicate that there are two different subtypes of $5HT_{1a}$ receptor, one mediating serotonergic modulation of the locomotor network (sensitive to 8-OH-DPAT), and the other of the pineal pathway (sensitive to buspirone). Also, the $5HT_{1a}$ receptor antagonist Nan-190 appeared to reverse the effects of both buspirone (n=2) and 5CT (n=2) on the dimming response, but not the effects of 5HT. Although speculative, this might reflect the presence of another 5HT receptor type in the pineal eye to motor system pathway.

Pharmacological profile					***Physiological effect***			
					Locomotor rhythm		***Sensory activation***	
Drug (conc μM)	***Receptor***	***Agonist***	***Antagonist***	***N***	***Burst duration***	***Episode duration***	***Dimming response***	***Skin activation***
5HT (1-10)	All	**		160	Increase	Decrease	Abolish	Increase
5CT (0.05-1)	$5HT_1$	**		13	Increase	Decrease	Abolish	———
R(+)-8-OH-DPAT (2-10)	$5HT_{1a}$	**		3	Increase	———	———	———
(+)-8-OH-DPAT (10-50)	$5HT_{1a}$	*		5	Increase	———	———	———
Buspirone (10-50)	$5HT_{1a}$	*		9	———	———	Abolish	———
CGS maleate (10-50)	$5HT_{1b/1d}$	*		3	———	———	———	———
TFMPP (20)	$5HT_{1b/1d}$	*		1	———	———	———	———
Mianserin (50-100)	$5HT_2$>$5HT_1$		*	3	———	———	———	———
Cyproheptadine (20-80)	$5HT_2$		*	5	———	———	———	———
Ketanserin (10-100)	$5HT_2$		*	19	———	———	———	———
Nan-190 (10-100)	$5HT_{1a}$		*	17	Decrease	Increase	Restore	———
Methysergide (2-20)	$5HT_2$		*	11	———	———	———	———
Mesulergine (10-50)	$5HT_{1c}$/$5HT_2$		*	11	———	———	———	———
MDL 72222 (10-100)	$5HT_3$		*	7	———	———	———	Decrease
Ondansetron (10-20)	$5HT_3$		*	2	———	———	———	———

DISCUSSION

The development of a more complex motor output during swimming in *Xenopus* larvae is characterised by the acquisition of longer and more variable ventral root activity, which occurs rostro-caudally during a brief period after hatching (about 24 hrs) (see fig 10). In addition to the acquisition of longer and more variable ventral root activity, the normal post-embryonic development of swimming also involves a scaling of segmental delay with cycle period, and a greater variability and range of swimming frequencies (figs 13 & 8). Since the effect of bath applied 5HT is to effectively mimic the development of these facets of swimming activity in animals some twelve hours younger, it suggests that release of the amine from the terminals of descending serotonergic interneurons plays a causal role in the development and modulation of the swimming rhythm.

The evidence in support of this notion is outlined below. **Firstly**, the sensitivity of the locomotor network to 5HT appears to precede its functional innervation by descending serotonergic fibres. This is especially apparent in the stage 37/38 embryo whose ventral root activity is normally brief consisting of single biphasic impulses. Following bath application of 5HT, the rostral ventral root activity is enhanced, but the caudal is unaffected so that the pattern of activity now resembles that of a stage 40 larva. Similarly, fictive swimming at stage 40 in the presence of 5HT, closely resembles that of a stage 42 larva in physiological saline. It seems, therefore, that bath applied 5HT has the effect of inducing swimming activity normally associated with animals some twelve hours older. This implies that rhythmic spinal neurons not only

begin to express receptors for 5HT in a rostro-caudal sequence, but that they do so just prior to their functional innervation by descending serotonergic fibres. **Secondly**, direct evidence for the involvement of 5HT receptors in the rostro-caudal acquisition of serotonergic sensitivity was provided by artificially raising the levels of endogenous transmitter available for release from the terminals of serotonergic neurons by the bath application of 5HTP (fig 22). The effects of 5HTP on locomotor activity were subtly different from those of bath applied 5HT in that enhancement of ventral root activity only occurred where bursts of discharge had already developed. For example, in the stage 37/38, bath applied 5HT increased the duration of rostral ventral root activity, whereas 5HTP had no noticeable effect. This is consistent with the notion that spinal neurons of the locomotor network first express receptors for 5HT, and then await their functional innervation by descending serotonergic fibres some twelve hours later. **Thirdly**, removal of supraspinal inputs following spinalisation at the level of the otic capsule, resulted in the swimming rhythm resuming embryonic features (fig 14). This suggested that descending inputs are not only responsible for rhythm development, but also are required for the maintenance of the developed larval pattern. **Fourthly**, specific neurotoxic ablation of serotonergic neurons by 5,7-DHT, resulted in the larval swimming pattern failing to develop. Swimming in stage 42 larvae which had been reared in neurotoxin from about stage 25 (about 28 hours after fertilisation), was reminiscent of the embryonic rhythm with very brief and predominantly single spike ventral root activity (figs 23 & 24). These ablation studies provide direct evidence on the nature of the supraspinal input responsible for rhythm development, namely serotonergic raphe spinal

interneurons. **Finally**, pharmacological manipulations using the non-specific $5HT_1$ receptor agonist 5CT and the specific $5HT_{1a}$ antagonist Nan-190 can mimic and block, respectively, the effects of bath applied 5HT on the swimming rhythm. Moreover, Nan-190 more than reverses the effects of 5HT and 5CT on swimming, and when bath applied on its own to stage 42 larvae, causes the bursty rhythm to resume a more 'embryonic-like' form. This provides good evidence that 5HT released from the terminals of descending serotonergic fibres plays a central role in the development and modulation of swimming activity via a '$5HT_1$-like' receptor (without excluding the possibility that other receptor types might also be involved, but to a lesser degree).

Taken together, these lines of enquiry amount to very strong evidence that the development of the descending serotonergic system in post-embryonic *Xenopus* larvae plays a central role in the development, modulation and maintenance of a more 'adult-like' swimming pattern. In view of the flexibility of the larval swimming pattern as evidenced by the local modulation of burst durations within episodes (for example, see fig 12), it is tempting to view the spinal serotonergic system as a 'gain control' mechanism: one which can act differentially on both sides of the cord and at various levels to regulate the excitability of different 'segments' of the spinal locomotor network.

With regard to the identity of the 5HT receptor subtypes, the results described above implicate an involvement of a $5HT_{1a}$ receptor in the serotonergic modulation of locomotion in *Xenopus* larvae. This is both in part agreement and in conflict with findings in other vertebrates systems. In the lamprey, for example, the

effects of 5HT on the swimming frequency, intersegmental co-ordination and ventral root burst durations are thought to be brought about solely by a direct action on Ca^{++} dependent K^+ (K_{Ca}) channels (van Dongen *et al,* 1986; Wallén *et al,* 1989; for recent review see also Grillner *et al,* 1991). These authors searched for, but were unable to establish an involvement of second messenger pathways. They speculated, therefore, that the $5HT_3$ receptor type might be involved, since the amine appeared to act directly via a ligand-gated channel (see introduction; for recent reviews see, Cornfield & Nelson, 1991; Fozard, 1992; see also, Peroutka, 1988). More recently, however, in other vertebrates there is growing evidence for the involvement of $5HT_1$ receptors in the modulation of locomotor function. In the neonatal rat, for example, both $5HT_{1a}$ and $5HT_2$ receptors are involved in the serotonergic modulation of spinal locomotor circuits. Also, when 5HT is bath applied to the isolated brainstem-spinal cord preparation, it induces fictive locomotion and increases the duration of ventral root burst durations in a dose dependent manner, and this effect is blocked by both $5HT_1$ and $5HT_2$ receptor antagonists (Cazalets *et al,* 1992). Moreover, a detailed study of 5HT responses in rat motorneurons reveals that $5HT_{1a}$ and $5HT_2$ receptors mediate different 5HT responses, one hyperpolarising and the other depolarising (Wang & Dun, 1990). A similar finding has also been described in adult frog motorneurons in which membrane hyperpolarisations were mediated by $5HT_{1a}$ receptors and depolarisations by $5HT_2$ receptors (Holohean *et al,* 1990)

In the lamprey, 5HT increases spike discharge frequency in rhythmic spinal neurons by depressing the K_{Ca} conductance (see

above) which mediates the late phase of the after hyperpolarisation (AHP) following an action potential (Wallén *et al,* 1989), thereby reducing spike accommodation. In the *Xenopus* embryo, rhythmic neurons are limited to a single action potential in each cycle of activity. Moreover, the AHP does not appear to exhibit a similar late slow component (Soffe, 1990). However, as previously described, the most important developmental change during the first day of larval life is the acquisition of a multiple spiking capability in rhythmic neurons (see also Sillar *et al,* 1992a). Although there is strong evidence of a causal link between the development of the descending serotonergic system and the acquisition of novel membrane properties, it may be that 5HT does not play a direct developmental role and that there is simply parallel development of membrane properties and the spinal serotonergic system. However, sensitivity to 5HT occurs rostro-caudally prior to innervation from serotonergic raphe interneurons (see also Sillar *et al,* 1992b; Wedderburn & Sillar, 1993). Thereafter, endogenous 5HT released from the terminals appears to modulate the spike properties of rhythmic neurons which is reflected in the longer and more variable ventral root activity associated with larval swimming. Although the underlying mechanisms are unclear, the situation may be similar to that in the adult lamprey, whereby 5HT acts directly on a K_{Ca} conductance which is incorporated into spinal neurons at some stage after hatching. Alternatively, it may be that 5HT acts by suppressing the voltage dependent K^+ conductance which in the hatchling, limits rhythmic neurons to a single action potential in each cycle (Soffe, 1990). Unlike the lamprey, however, the results described above, offer strong evidence that the $5HT_3$ receptor type is not involved in

the modulation of locomotor activity.

In addition, it has to be considered that early post-embryonic *Xenopus laevis* tadpoles are immature and possess a rapidly developing central nervous system. The role of neurotransmitters must therefore be viewed from a developmental perspective since many, including 5HT, have different or additional roles in the developing cns than in the mature one (see introduction). It also important to add that these experiments on *Xenopus* larvae were carried out on intact animals. Therefore, the possibility can not be excluded that serotonergic agents may have modulated swimming activity, at least in part, by acting at auto-receptors on raphe neurons. However, assuming that these auto-receptors are involved in negative feedback control of 5HT release, their activation by 5CT or blockade by Nan-190 might be expected to have opposite effects to the ones described here.

These results also suggest that there may be more than one subtype of $5HT_{1a}$ receptor in this system. For example, it appeared that while the $5HT_{1a}$ receptor agonist (8-OH-DPAT) mimicked the effects of 5HT on locomotor activity, it had no effect on the light dimming response. Conversely, the agonist buspirone, while mimicking the effects of 5HT on the dimming response, did not noticeably effect the locomotor pattern. Furthermore the specific $5HT_{1a}$ antagonist Nan-190 appeared effective at reversing the blocking effects of both buspirone and 5CT on the dimming response, but not those of 5HT. This could also indicate the presence of two pharmacologically distinct types of 5HT binding site in the pineal eye to motor system pathway; 5HT would presumably act at all binding sites, and consequently an antagonist acting only at one, would be ineffective at reversing the effects of

the amine on the remaining site. More specifically, these results suggest that the additional binding site may not be a $5HT_1$-like receptor, since Nan-190 reversed the effects of 5CT (a high affinity **non**-specific $5HT_1$ receptor agonist), but not the effects of 5HT. Unfortunately, during the course of early experiments using $5HT_2$ and $5HT_3$ receptor antagonists to examine effects on locomotor activity, their possible effects on the dimming response were not observed. It is not, however, without precedent that there is more than one subtype of $5HT_{1a}$ receptor. For example, it has been demonstrated that in the rat brain, there is one presynaptic (autoreceptor) subtype of $5HT_{1a}$ receptor, and two postsynaptic subtypes located in the CA_3 region of the hippocampus (Bleier *et al*, 1993a & b).

Although these results provide good evidence for serotonergic modulation of sensory pathways, they do not furnish any evidence regarding their mechanisms of action. However, it has been shown that one important site of serotonergic modulation of the R-B cell pathway is at the synapse between the primary (R-B cells) and secondary (dla's and dlc's) sensory neurons. Moreover, it appears to involve a presynaptic inhibition of synaptic transmission via activation of a $5HT_3$ receptor type (Sillar *et al*, 1991; Sillar & Simmers, 1994a).

Bath applied 5HT exerts reciprocal effects on the locomotor network (excitatory) and on mechanosensory pathways (inhibitory) in early post-embryonic *Xenopus laevis*. Considering that at these developmental stages, there is a single population of serotonergic interneurons with two anatomically distinct descending fibre tracts innervating the dorsal and ventral spinal cord respectively (van

Mier *et al*, 1986a), endogenous 5HT release could have simultaneous and reciprocal effects on sensory and locomotor pathways. Intuitively one might expect such reciprocal modulation to occur, since especially vigorous swimming movements and the action of the surrounding water might otherwise result in inappropriate mechanosensory stimulation. Such reciprocity has been shown to occur in other vertebrate species whereby descending serotonergic projections play a role in simultaneously suppressing cutaneous pathways, especially nociceptive (Jessel & Kelly, 1991), and enhancing locomotor activity (Hultborn & Illert, 1991; Jacobs & Fornal, 1993).

With regard to the dimming response, the pineal eye is known to act as a luminance detector, and it may be important for there to be a tonic modulation of its sensitivity. The acute sensitivity that this pathway displays to bath applied 5HT suggests serotonergic modulation plays a central role. Although this pathway is poorly understood, serotonergic fibres also project rostrally from the raphe nucleus into the mid and fore-brains (van Mier *et al*, 1986a), and in a closely related amphibian species *Rana temporaria* the pineal eye has been shown to have dense serotonergic innervation (A-M Woolston, unpublished observations).

I have previously argued that the development of swimming activity is more likely to be a consequence of altered cell properties rather than any fundamental restructuring of the network. These results on the effects of 5HT on swimming suggest, therefore, that the release of endogenous 5HT plays an important role in the acquisition of novel cell properties of rhythmic neurons. As in the

lamprey, it seems likely that the amine regulates spike discharge frequency, although it is as yet unclear if a similar mechanism is involved. For example, the sustained voltage-dependent K^+ conductance following an action potential (Soffe, 1990) might be down regulated by 5HT, but it may also be that there are a number of additional mechanisms, perhaps mediated by more than one receptor subtype. In the following chapter, I investigate the effects of 5HT at a cellular level, and explore the possible mechanisms of its action.

CHAPTER 5

Cellular and synaptic mechanisms underlying serotonergic modulation of swimming

INTRODUCTION

The effects of 5HT on the cell properties of spinal cord motorneurons has now been studied in a range of vertebrates, such as the neonatal mouse and rat, and adult cat, turtle, frog and lamprey. In the lamprey, 5HT is thought to directly suppress the $K_{(Ca)}$ conductance which mediates the late phase of the AHP following an action potential in spinal motor- and interneurons (Wallén *et al,* 1989; see 'General Introduction'). This causes a reduction in spike 'accommodation' (accommodation is a decrease in spike frequency, and sometimes cessation of spikes, which occurs during prolonged membrane depolarisations), and consequently an enhancement of ventral root activity during fictive swimming. Indeed, it has been argued that in the lamprey, all of the 5HT-induced effects on swimming can be accounted for by its action on this single conductance (Grillner *et al,* 1991). This view, however, is not without controversy, since a selective blockade of this $K_{(Ca)}$ conductance by apamin has little or no effect on the fictive swimming pattern (Meer & Buchanan, 1992; see, however, Hill *et al,* 1992). A suppression of spike accommodation has also been described in cat (Hounsgaard *et al,* 1988) and turtle (Hounsgaard & Kiehn, 1989). In the acutely spinalised cat, a sustained increase in the excitability of α-motorneurons has been shown following administration of the precursor 5HTP. Similarly in turtle motorneurons, the application of 5HT also leads to an increase in firing frequency in response to a depolarising current pulse. In both of these studies, another 5HT-induced phenomenon was observed, namely membrane bistability. In response to a brief depolarising current pulse there was a sustained membrane

depolarisation or plateau potential which could be terminated with a brief hyperpolarising current pulse. This bistability was evident in intact and decerebrate cats, but not acutely spinalised ones. In the latter, however, bistability could be restored following intravenous injection of 5HTP. In turtle motorneurons, the plateau potentials induced by 5HT were TTX-resistant and calcium-dependent. As in the lamprey, 5HT reduced the $K_{(Ca)}$ conductance which mediates the late phase of the AHP. The plateau potentials also demonstrate a dependence on time and magnitude of the initiating depolarisation which might suggest that the degree of excitatory synaptic drive is important in dictating their induction and time course (see Hounsgaard & Kiehn, 1989; Kiehn 1991).

In the lamprey, TTX-resistant bistability has been described in some detail, but in contrast to turtle and cat, it is expressed in a more dynamic way. Under an appropriate experimental regime, the membrane potential oscillates continuously between two relatively stable states (Sigvardt *et al,* 1985; Wallén & Grillner, 1987). These oscillations rely upon activation of NMDA receptors in the presence of extracellular Mg^{2+} ions and may be important in contributing to locomotor rhythmicity. They are induced by similar concentrations of bath applied NMDA as elicits fictive swimming activity, and also share similar amplitudes and frequencies with the rhythmic synaptic drive for swimming. Furthermore, the frequency and timing of the intrinsic oscillations are influenced by hyper- and depolarising current pulses in a way which suggests that during real swimming, tonic synaptic input could set the appropriate frequency while phasic input could control the coupling and timing between oscillating neurons (Wallén & Grillner, 1985). However,

it should be added that this presupposes their existence in the absence of TTX, which has not been proven. The oscillations are due in large part to the voltage-dependency of the NMDA receptor channel in the presence of Mg^{++} which confers bistability on the membrane potential (see General Introduction). Unlike the turtle and cat neurons, however, the expression of bistability in lamprey motorneurons does not appear to be 5HT-dependent, although the amine does effect the time course of the oscillations by prolonging their depolarised plateau phase (Wallén *et al*, 1989).

5HT also has a role in setting neuronal excitability, and can cause both membrane hyperpolarisations and depolarisations in motorneurons. For example, in both neonatal rat (2-3 weeks old) and adult frog (*Rana pipiens)* motorneurons, membrane hyperpolarisations have been shown to be mediated by a $5HT_{1a}$ receptor subtype while depolarisations are mediated via $5HT_2$ receptors (Wang & Dun, 1990; Holohean *et al*, 1990). Interestingly, however, in younger neonate rats (3-4 days old), activation of $5HT_{1a}$ receptors excites motorneurons (Takahashi & Berger, 1990), suggesting that the role of 5HT receptor types can change during the course of development (see also chapter 4, introduction). Similar $5HT_{1a}$ receptor-mediated hyperpolarisations have also been described in hippocampal neurons (Colino & Halliwell, 1987).

There is also evidence that 5HT regulates synaptic transmission often at a presynaptic site of action. For example, in the lamprey, it depresses excitatory synaptic drive onto motorneurons from reticulospinal Müller cells (Buchanan & Grillner, 1991). It is thought that this might have a function in intersegmental co-ordination which is affected by 5HT. In the

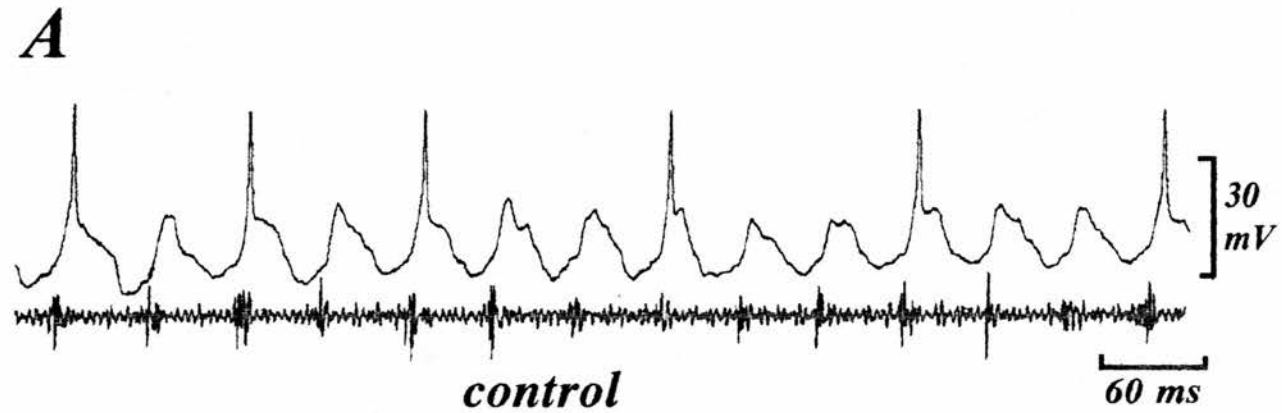
adult frog, 5HT has also been shown to facilitate glutamate mediated responses (Cardona & Rudomin, 1983), and in the adult cat, to depress afferent synaptic transmission (Bras *et al,* 1990). Modulation of inhibitory neurotransmitter release by 5HT has also been demonstrated in the vertebrate cns. Voltage clamp recordings of teleost Mauthner cells reveal an increase in both evoked and spontaneous inhibitory post-synaptic currents (ipsc's) following application of 5HT, via a mechanism involving presynaptic facilitation of transmitter release (Mintz *et al,* 1989).

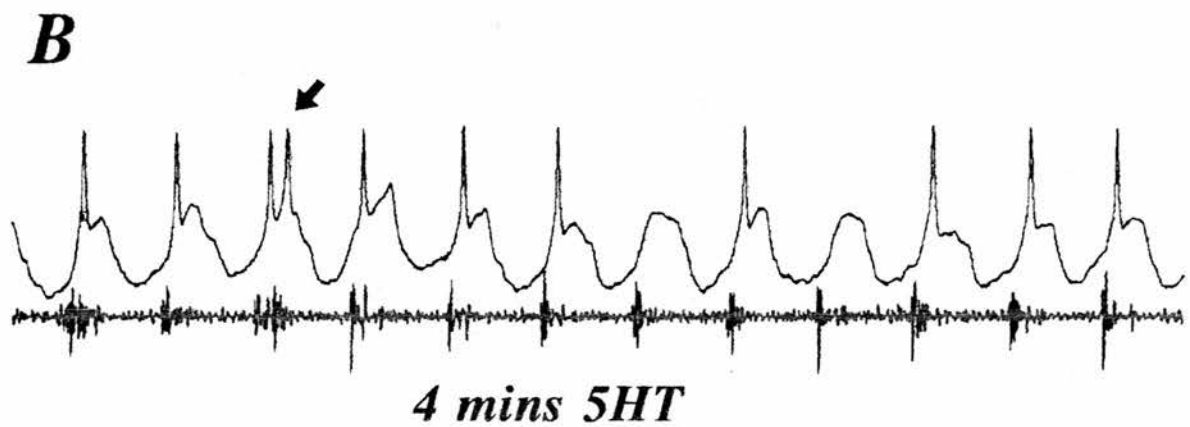
These observations suggest that 5HT can influence spinal motor circuitry in a number of ways, including regulation of spike frequency, setting the excitability of neurons, inducing bistable membrane properties and modulating excitatory and inhibitory synaptic transmission. In this chapter, I have investigated whether similar mechanisms underlie the 5HT-induced effects on swimming in post-embryonic *Xenopus*. Intracellular recordings were made from ventrally located neurons in the spinal cord (presumed motorneurons; see 'Materials and Methods'). Following 5HT application, I found evidence for, i) an increase in spike discharge capability, ii) a membrane hyperpolarisation, iii) an induction of intrinsic membrane potential oscillations, and iv) a depression of evoked and spontaneous glycinergic inhibition. These results imply that in contrast to the lamprey swimming system (see however, Meer and Buchanan, 1992) the overall effects of 5HT on circuit function are achieved not through a single action on specific K^+ channels, but via several cellular and synaptic mechanisms operating in parallel.

RESULTS

i) 5HT increases the probability of multiple firing in rhythmic neurons during fictive swimming

To investigate the serotonergic modulation of cell properties, 1-10μM 5HT was bath applied following stable penetrations of ventrally positioned rhythmic spinal neurons (see Materials & Methods). As has been previously described, the most notable change in locomotor activity in post-embryonic *Xenopus* larvae is the acquisition of a multiple spike capability in rhythmic neurons which is reflected in longer and more variable ventral root activity. Evidence based on extracellular ventral root recordings strongly suggest that the development of locomotor rhythmicity and its subsequent modulation is causally linked to the development of the descending serotonergic raphespinal system. It seems probable therefore that 5HT released from the terminals of descending serotonergic interneurons increases the spike discharge frequency of rhythmic spinal neurons. Some direct evidence has been obtained in that bath applied 5HT confers upon locomotor neurons during fictive swimming an increased tendency for multiple firing. In fig 34, for example, a ventral root recording has been made from the 5th post-otic inter-myotomal cleft in a stage 40 larva. The intracellular recording, using a KAc electrode, is from a motorneuron at about the level of the 7th myotome. As before, each passage of illustrated activity is taken from near the beginning of the episode (500ms after the stimulus), thereby excluding the initial few cycles of activity which could be contaminated with sensory inputs. In control saline (A), the motorneuron fires only a


A
30
mV
control
60 ms


B
4 mins 5HT

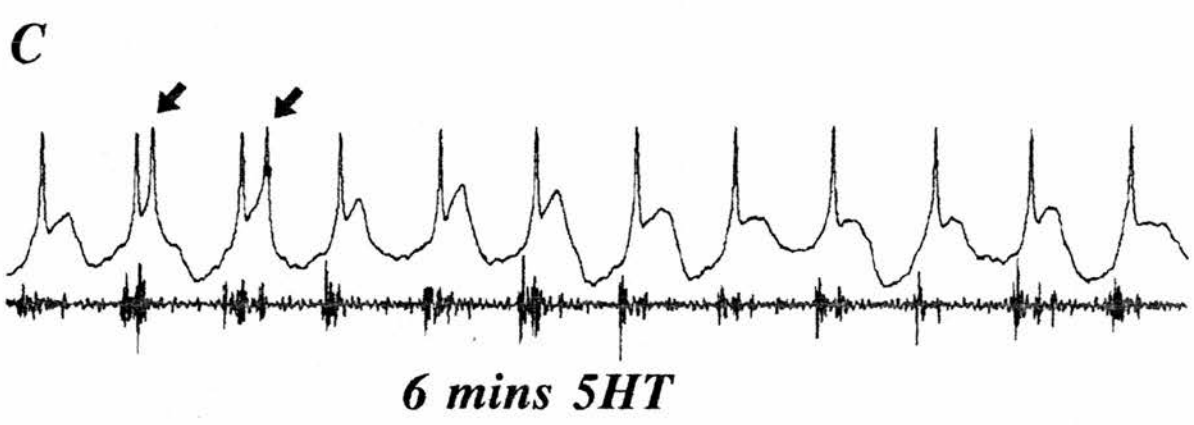

C
6 mins 5HT

fig 34. 5HT increases the probability of multiple firing in rhythmic neurons during fictive swimming

(A) Fictive swimming activity in a stage 40 larva recorded from a motorneuron with KAc electrode and an accompanying extracellular ventral root record (L5). (A) In control saline the motorneuron fires either a single action potential per cycle, or does not fire at all. (B) 4 minutes following application of 2μM 5HT, the neuron occasionally fires multiply (arrow) and also the reliability of firing is increased. (C) After 6 minutes 5HT, there is a greater incidence of multiple firing (arrows), and reliability of spiking is further enhanced. Note that ventral root activity is also enhanced.

single action potential, but not on every cycle (unlike the embryo) and on no occasion does it fire multiply. Following application of 5HT, after 4 minutes (B), the neuron begins to fire multiply (arrow) and there is an increased tendency to fire in each cycle. After 6 minutes exposure to 5HT (C), the neuron spikes reliably on every cycle, and also fires multiply on some cycles (arrowed). Also noticeable is the increase in durations and intensity of the ventral root activity. This, in combination with the effects observed in the motorneuron, is consistent with the notion that 5HT can both recruit motorneurons into the circuit and enhance their spike discharge capability (see Chapter 3, Discussion). These effects also occurred despite a membrane hyperpolarisation from -74mv to -78mv following the application of 5HT (not illustrated). The quality of the intracellular recording then deteriorated so that it was not possible to reverse the effects by washing in control saline.

ii) Effects of 5HT on the resting membrane

In the absence of swimming, 5HT has two immediately apparent effects on intracellularly recorded neurons; it reduces the rate of spontaneous inhibitory potentials and it hyperpolarises neurons usually by between 5 and 10mV. Figure 35 illustrates an experiment in which a recording was made with a KCl electrode from a rostral motorneuron in a stage 37/38 embryo (such neurons have begun to express receptors for 5HT at this stage of development; see chapter 4). Following application of 1μM TTX to block spike-mediated synaptic transmission, and 30μM bicuculline to abolish spontaneous GABAergic potentials,

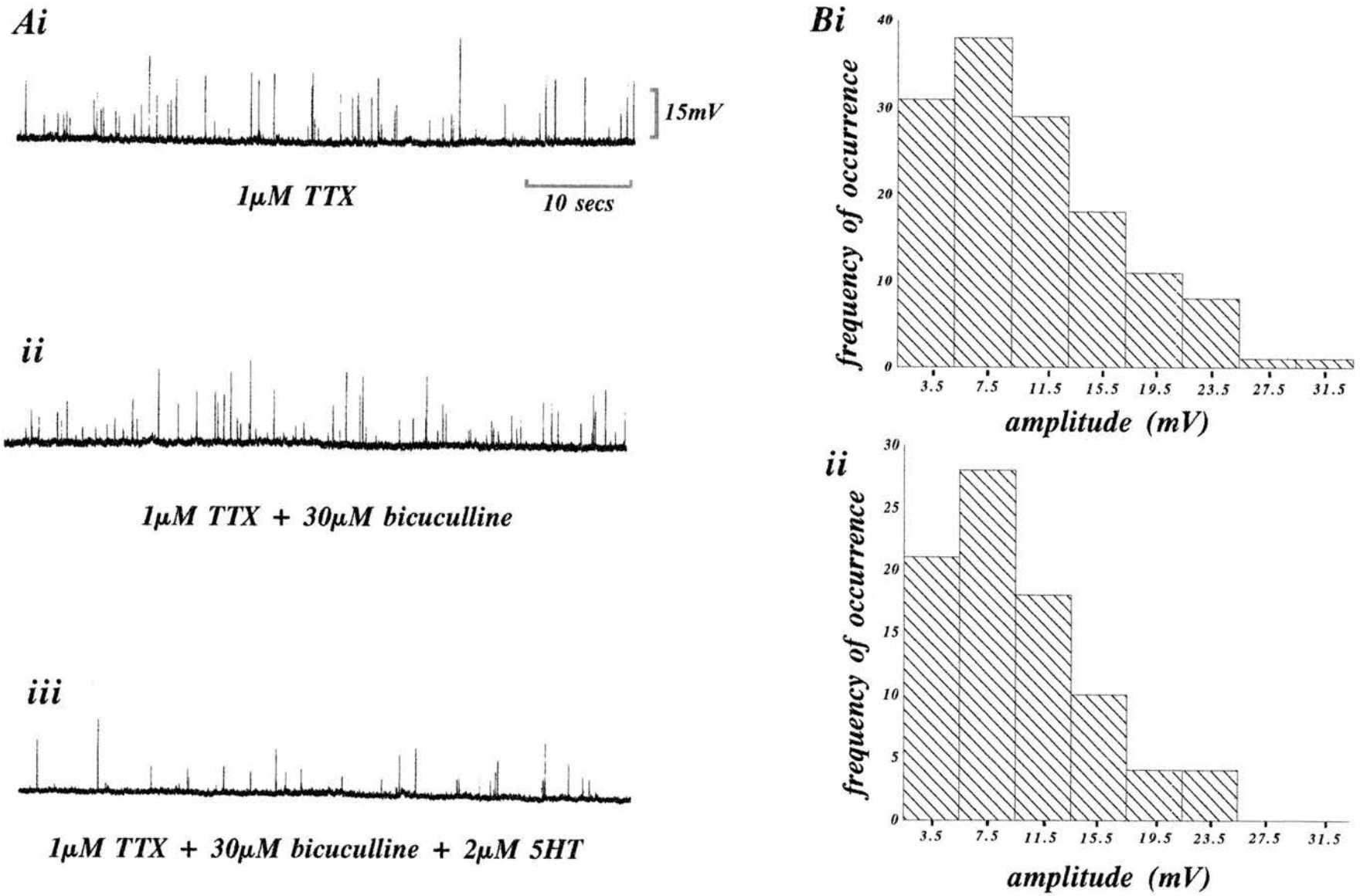

Ai
15mV
1μM TTX
10 secs
ii
1μM TTX + 30μM bicuculline
iii
1μM TTX + 30μM bicuculline + 2μM 5HT
Bi
frequency of occurrence
0
10
20
30
40
3.5
7.5
11.5
15.5
19.5
23.5
27.5
31.5
amplitude (mV)
ii
frequency of occurrence
0
5
10
15
20
25
30
3.5
7.5
11.5
15.5
19.5
23.5
27.5
31.5
amplitude (mV)

fig 35. 5HT reduces the rate of spontaneous glycinergic inhibitory potentials

(A) Recording of a rostral stage 37/38 motorneuron with a KCl electrode. (i & ii) Following application of 1μM TTX and 30μM bicuculline to block evoked and GABAergic potentials respectively, a high rate of spontaneous release remained and was presumed to be glycinergic. After application of 2μM 5HT the rate of spontaneous glycinergic ipsp's dramatically declined. Measured over two minute periods the rate declined from 138 to 86, about a 40% reduction. (B) Amplitude versus frequency of occurrence histograms before (i) and after (ii) 5HT. The distributions appear very similar suggesting a presynaptic site of action.

numerous depolarising synaptic events remained. These spontaneous psp's were presumed to be depolarising glycinergic inhibitory potentials (fig 35 Ai, ii), since spontaneous epsp's have not been reported, but similar ipsp's to those recorded here have been studied previously (Sillar & Soffe, 1987; Wall & Dale 1993). Bath application of 2μM 5HT, resulted in a rapid reduction in the rate of spontaneous presumed glycinergic ipsp's (Aiii). Analysis of the activity two minutes before and two minutes following 5HT application, revealed a 40% reduction in the frequency of spontaneous inhibitory potentials, without any significant change in the average amplitudes (10.6 $^{+}/_{-}$ 5.7mV (sd) in control, and 10.4 $^{+}/_{-}$ 5.6mV in 5HT) (see also fig 35 Bi & ii). This reduction in the rate of transmitter release without apparent effect on the post-synaptic response is suggestive of a presynaptic site of action.

Figure 36 also illustrates the dramatic effects of 5HT on reversed inhibitory potentials. As in the previous example, the recording was made with a KCl-filled electrode from a rostral motorneuron in a stage 37/38 embryo. This experiment was conducted without application of TTX and bicuculline. However, in the absence of swimming, it is probable that the psp's were predominantly spontaneous glycinergic inhibitory potentials, since the addition of TTX and bicuculline little affects rates and amplitudes (see above). In addition to the effect on spontaneous ipsp's, however, there was a concurrent membrane hyperpolarisation of 8mV. This is best illustrated on a high gain and slow time base plot (fig 36 C), and appeared to be a consistent effect of 5HT. It was observed in six cells tested and had an average magnitude of 6.3 $^{+}/_{-}$ 2.2mV (sd). Figure 36D is a graphical illustration of the same excerpt of activity, where interval

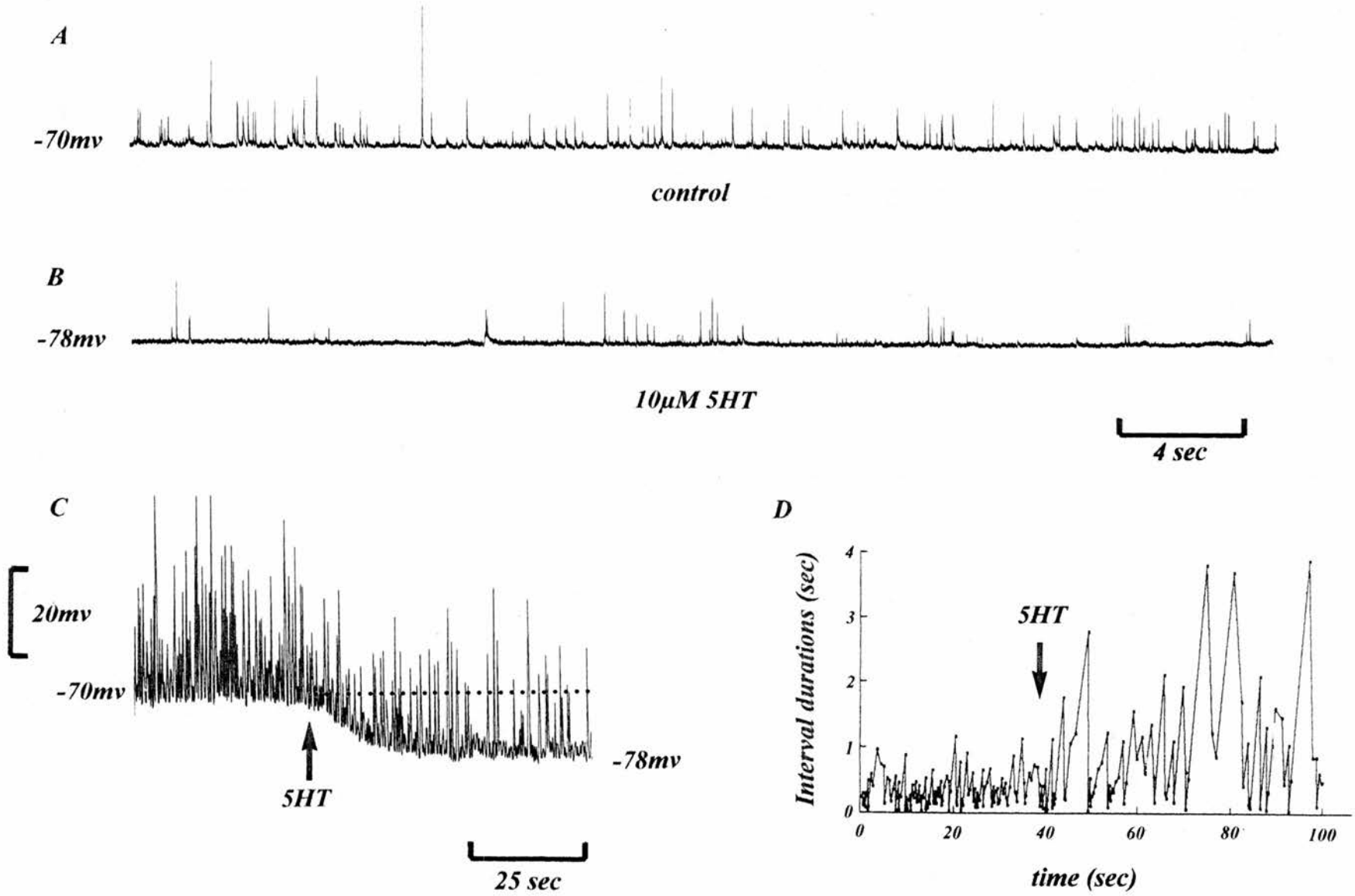
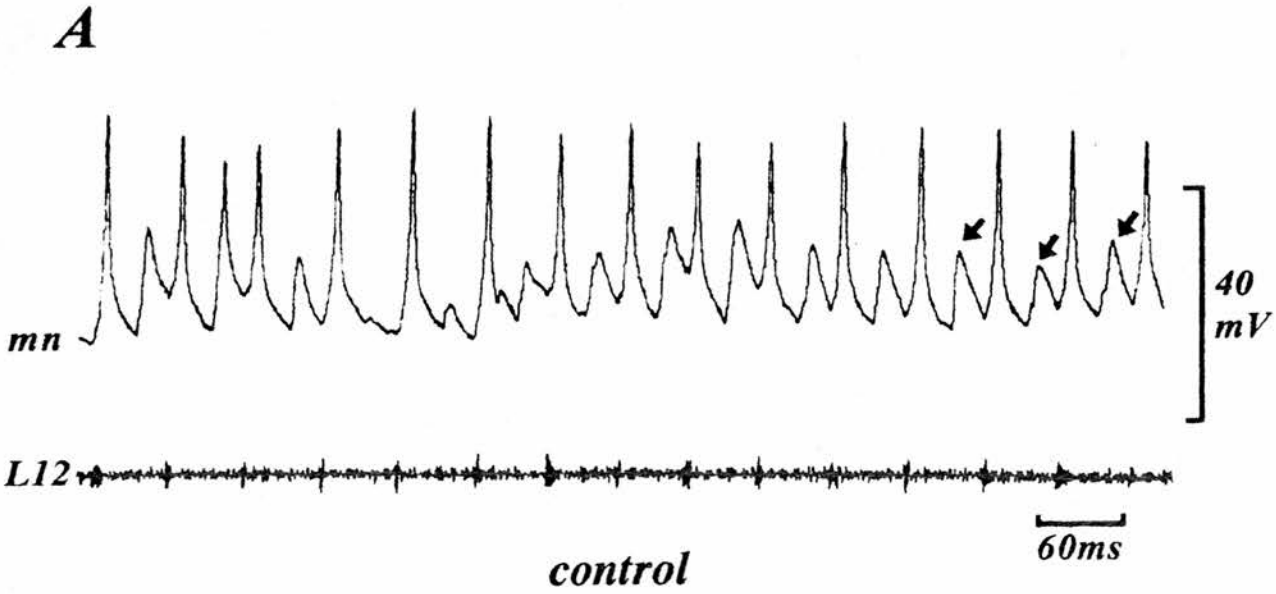

A
-70mv
control
B
-78mv
10μM 5HT
4 sec
C
20mv
-70mv
5HT
-78mv
25 sec
D
Interval durations (sec)
5HT
0
1
2
3
4
0
20
40
60
80
100
time (sec)

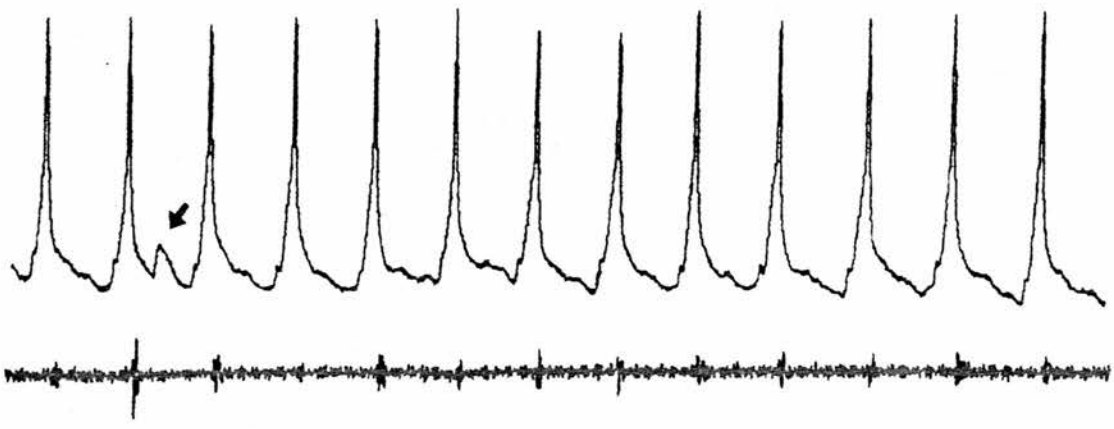
fig 36. 5HT reduces the rate of spontaneous ipsp's and hyperpolarises the membrane

Intracellular recording from a rostrally positioned motorneuron made with a KCl electrode in the quiescent stage 37/38 embryo (resting potential -70mV). (A) In control saline, there is a high rate of reversed chloride-mediated ipsp's. (B) Following application of 10μM 5HT, the rate dramatically declined. In addition to decreasing the rate of spontaneous ipsp's, the amine also caused the membrane potential to hyperpolarise by 8mV. (C) 100 second excerpt on a slow time base. The arrow shows the point at which 5HT washes on, and dotted line indicates normal resting potential. The dual effect of the amine on the resting potential and the rate of ipsp's is readily apparent. (D) When the durations of the intervals between inhibitory potentials is plotted against time (same 100 sec excerpt as C), the increase in interval durations is very marked following application of 5HT (arrowed).

durations between inhibitory potentials is plotted against time and it illustrates very clearly the immediate and dramatic effect of 5HT on spontaneous potentials.

Since 5HT appears to reduce the spontaneous release of glycine, presumably from the presynaptic terminals of commissural interneurons, the only known class of spinal glycinergic neuron (Dale *et al,* 1986), it might be expected that rhythmic evoked release of glycine such as that occurring mid-cycle during swimming (*ie.* reciprocal inhibition) would be likewise down-regulated. Evidence for this has been obtained and is presented in figure 37. It illustrates a recording with a KCl electrode of a rostral stage 37/38 motorneuron. In control saline the resting membrane potential was -71mV and during swimming activity, the reversed midcycle inhibition was readily apparent on almost every cycle throughout the episode (arrowed in fig 37A; sample taken 1 second from onset of swimming). After application of 5μM 5HT, the membrane hyperpolarised to -76mV and the rate of presumed spontaneous ipsp's declined (*cf* figs 35 & 36). At the onset of swimming activity, reversed midcycle inhibition was only apparent in about the first ten cycles, and thereafter consistently absent for the remainder of the episode (except for one ipsp in the 2nd cycle, arrowed in B). This is illustrated in figure 36B, which shows a sample of swimming taken one second from the onset of the episode. After wash in physiological saline, midcycle ipsp's were again apparent throughout the episode despite a deterioration of the recording (C).


A
mn
40
mV
L12
60ms
control


B
5HT

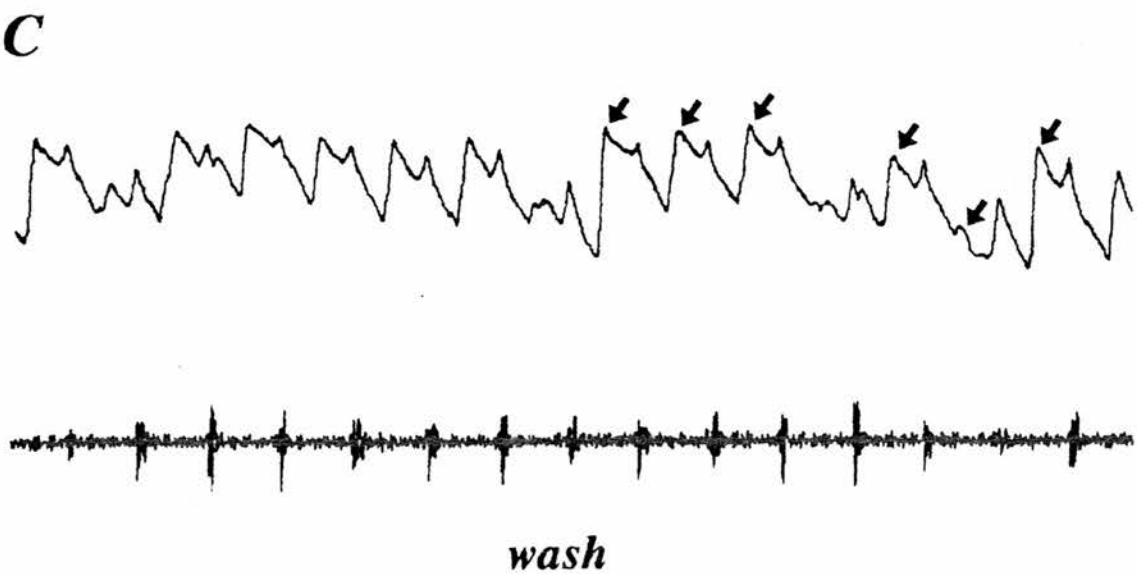

C
wash

fig 37. 5HT reduces evoked midcycle ipsp's during swimming activity

Fictive swimming in a stage 37/38 embryo showing the activity of one ventral root (L12) and a rostral motorneuron recorded with a KCl electrode (arrows indicate examples of midcycle inhibition). (A) In control saline, there is pronounced midcycle inhibition on nearly every cycle throughout the episode. (B) Following application of 5μM 5HT, midcycle inhibition failed shortly after the onset of swimming and was consistently absent throughout the rest of the episode. (C) Midcycle inhibition was restored with wash in control saline.

iii) 5HT induces TTX-resistant membrane potential oscillations

Rhythmically active spinal cord neurons of the stage 37/38 *Xenopus* embryo do not appear to exhibit membrane potential oscillations under a similar experimental regime (*ie* in the presence of TTX & NMDA) as elicits them in lamprey motorneurons (K.T.S. & J.F.S-W, unpublished observations; see also Soffe & Roberts, 1989). Experiments were consequently carried out in early larval stages to test if spinal cord neurons of older animals had acquired this more 'adult-like' feature during postembryonic development. After application of 1μM TTX, 100μM NMDA depolarised spinal neurons by at least 20mV, but no evidence of membrane potential oscillations was found (n=7) (see fig 38). Even injection of hyperpolarising current to bring the membrane potential back into the optimal region to permit regenerative blocking and unblocking of the NMDA channel by magnesium ions, failed to elicit oscillations. However, the addition of 5μM 5HT in the presence of TTX and NMDA led to membrane hyperpolarisation by about 5mV (as occurs in the resting membrane). The subsequent injection of hyperpolarising current revealed rhythmic bistability and the membrane potential oscillated in a way similar to that seen in lamprey rhythmic neurons. On no occasion did membrane potential oscillations occur unless 5HT was present, strongly suggesting that the amine was playing a critical role in their induction. Moreover, they occurred not only in larval motorneurons (n=3), but also rostral embryonic motorneurons (n=1). Figure 39 illustrates another experiment in which 100μM curare and 10μM strychnine were also bath applied to abolish spontaneous GABAergic and glycinergic ipsp's. Slow, high

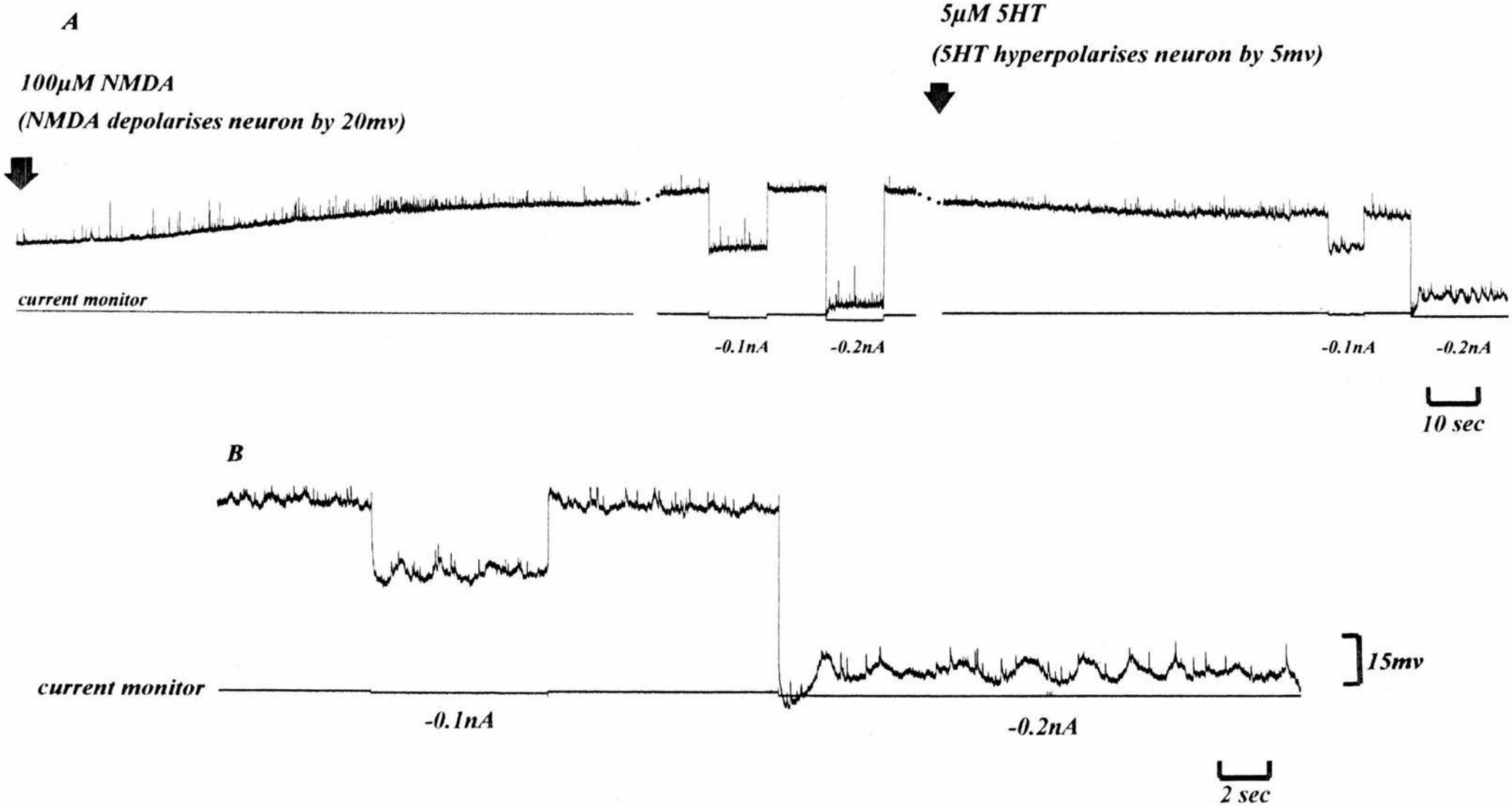

A
100μM NMDA
(NMDA depolarises neuron by 20mv)
5μM 5HT
(5HT hyperpolarises neuron by 5mv)
current monitor
-0.1nA
-0.2nA
-0.1nA
-0.2nA
10 sec
B
current monitor
-0.1nA
-0.2nA
15mv
2 sec

fig 38. 5HT induces TTX-resistant membrane potential oscillations in the presence of NMDA

(A) Intracellular recording of a motorneuron with a KCl-filled electrode. In the presence of 1μM TTX, bath applied 100μM NMDA (at arrow) depolarised the neuron by 20mv. The membrane potential showed no evidence of bistability either with or without hyperpolarising current injection. Following application of 5μM 5HT (arrow), the neuron hyperpolarised by 5mv and the membrane potential appeared less stable. Similar hyperpolarising current injection now revealed bistability and membrane potential began to oscillate. (B) Oscillations on an expanded time base. They have a frequency of about 0.5 Hz and an amplitude of about 10mv. Note that hyperpolarising current injection at both levels initiates the onset of the falling phase of a membrane potential oscillation.

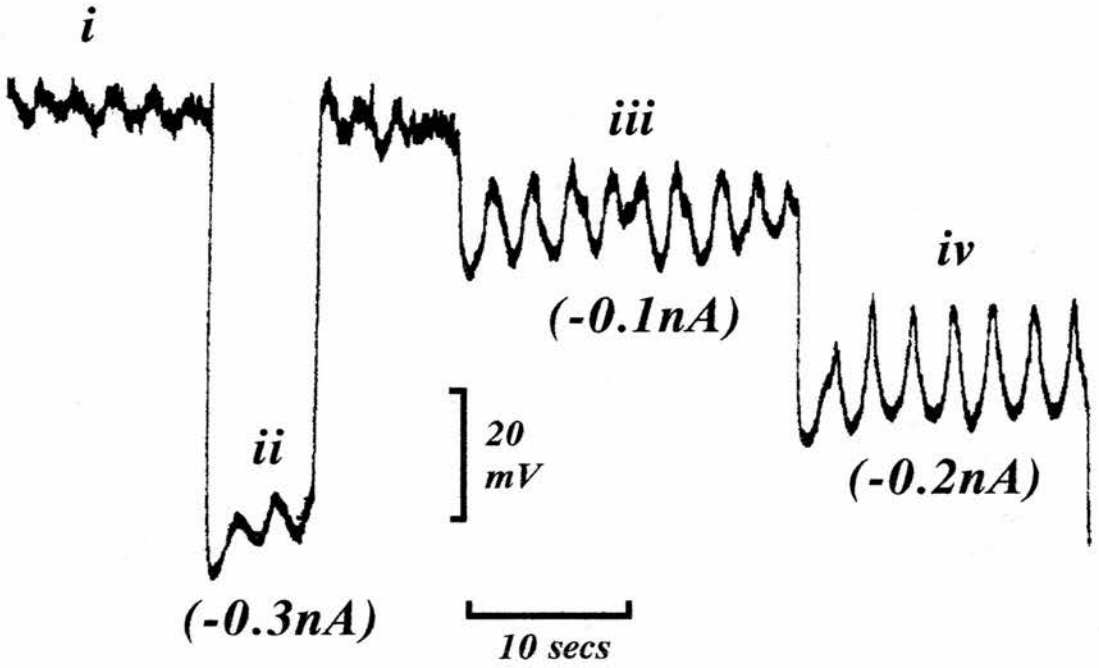

i
ii
iii
iv
(-0.3nA)
(-0.1nA)
(-0.2nA)
20
mV
10 secs

fig 39. Membrane potential oscillations are voltage dependent

1μM TTX, 100μM curare, 10μM strychnine and 5μM 5HT were initially bath applied. The subsequent addition of 100μM NMDA caused the membrane potential to depolarise from -70mv to -50mv, and in the absence of injected hyperpolarising current, very low amplitude membrane potential oscillations were apparent (i). Membrane potential oscillations demonstrate a voltage dependency. Highest amplitude oscillations occurred in response to -0.2nA hyperpolarising current injection. Smaller (iii) and greater (ii) amplitude of current injection induced lower amplitude oscillations. Note that the frequency of the oscillations is apparently unaffected by differing degrees of current injection, and as in figure 38, the onset of the hyperpolarising current injection coincides with the falling phase of an oscillation.

amplitude oscillations, like the previous example occurred at a frequency of about 0.5 Hz, similar to those in the lamprey (Wallén & Grillner, 1987). This experiment suggests that the oscillations do not involve the activation of glycine or GABA receptors. It also illustrates that membrane potential oscillations are voltage dependent in that their amplitude is related to the degree of hyperpolarising current injection. Highest amplitude oscillations occurred in response to -0.2nA and current injection less than (iii) and greater than (ii) -0.2nA induced lower amplitude oscillations. In another example (fig 40), 5μM 5HT was first bath applied to a stage 40 larva and 100μM NMDA was subsequently added in the absence of TTX. 5HT alone had no obvious effect, but the addition of NMDA led to rhythmic ventral root bursts and motorneuronal activity which closely resembled that of normal fictive swimming (ai, aii). Following the addition of 1μM TTX, spiking in the recorded neuron began to fail after about 1 minute (bi), and was abolished altogether after 3 minutes exposure to TTX (bii). The synaptic input to the neuron also progressively failed, so that after 5 minutes in TTX it appeared to have been completely abolished. It must be added, however, that it is difficult to be sure at which point TTX fully blocks activity in presynaptic neurons. What this experiment illustrates is that as synaptic drive progressively fails, there emerges an underlying low frequency oscillation in the membrane potential (di, dii). After 5 minutes in TTX, these oscillations are still apparent, but then it appears that the membrane potential begins to 'lock up' at a more depolarised level and only to 'flip' spontaneously to a more hyperpolarised state without continuously oscillating between the two voltage levels. Injection of negative current (-0.1nA), however, enables the

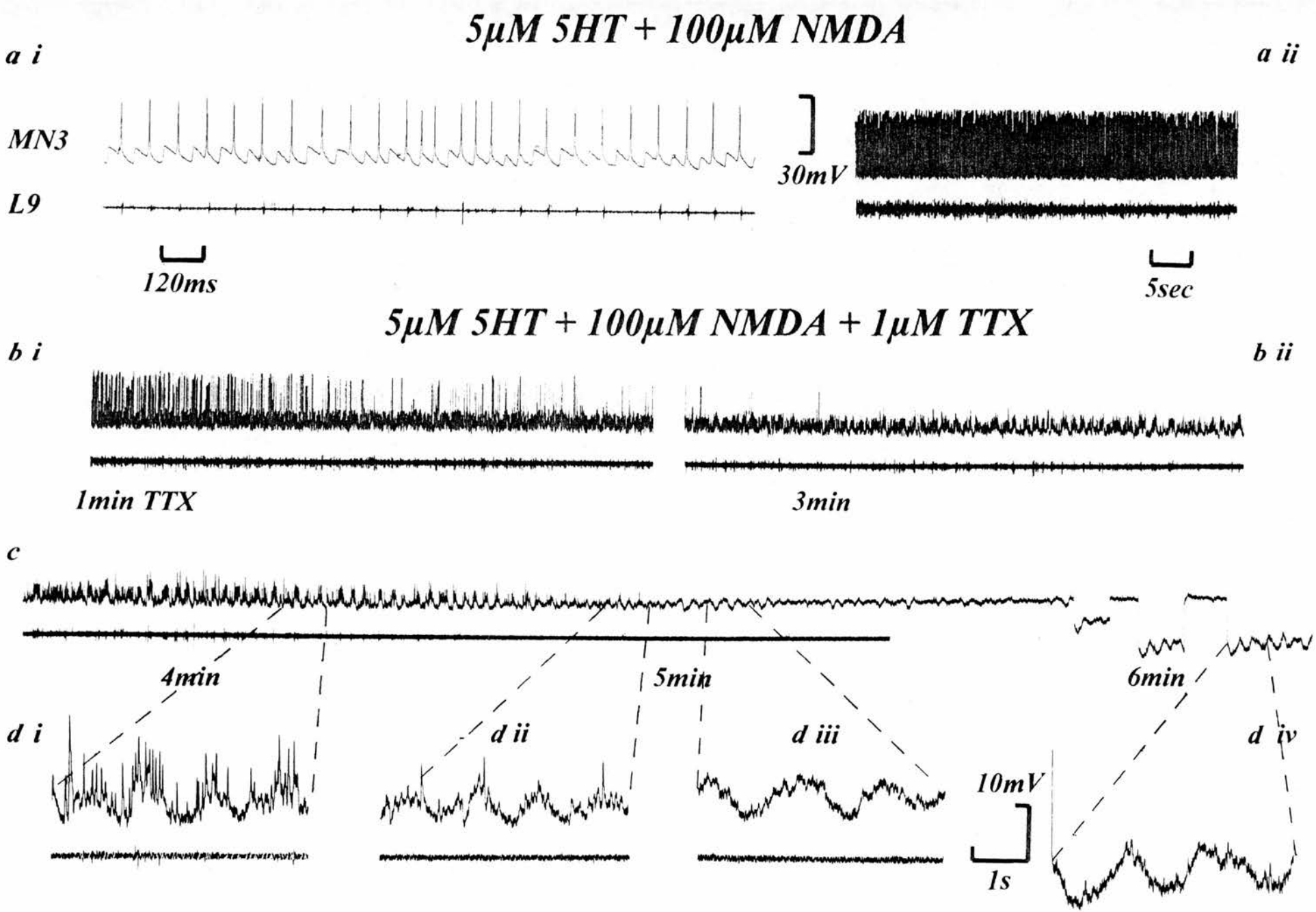

5μM 5HT + 100μM NMDA
a i
a ii
MN3
L9
30mV
120ms
5sec
5μM 5HT + 100μM NMDA + 1μM TTX
b i
b ii
1min TTX
3min
c
4min
5min
6min
d i
d ii
d iii
d iv
10mV
1s

fig 40. Membrane potential oscillations

(ai) NMDA induced fictive swimming activity in the presence of 5μM 5HT in a stage 40 larva recorded intracellularly from a motorneuron at about the level of the 3rd post-otic myotome (MN3) with an accompanying extracellular ventral root recording (L9). (aii) On a slower time base. Following addition of 1μM TTX, spiking begins to fail after about a minute (bi), and fails completely after about 3 minutes (bii). The synaptic drive also progressively fails, and after about 5 minutes appears to have been completely abolished (c). Note also the failure of ventral root activity (c). (di, ii, iii & iv) Brief excerpts of activity on an expanded time base showing more clearly evoked synaptic input to the neuron progressively failing. Also apparent is an underlying membrane potential oscillation. After 5 minutes, the membrane potential tends to 'lock' up at a more depolarised level after synaptic drive appears to have ceased. Stable oscillations, however, again occur with hyperpolarising current injection (div). Again, as in figures 38 & 39, the onset of the current pulse coincides with the falling phase of an oscillation.

membrane potential to again oscillate regularly (fig c, div).

Figure 41 illustrates an experiment carried out in the presence of extracellular Mg^{2+}, in which the input resistance of the neuron was monitored by injecting trains of 8 hyperpolarising current pulses (duration 200ms) at a frequency of 1 Hz every 30 seconds. 100μM NMDA depolarised the neuron by 25mV (Ai, Aii) and there was an apparent increase in input resistance (Aii, Bii). This effect has been reported previously and is thought to be due to the hyperpolarisation bringing the membrane potential into a region were Mg^{2+} blocks the ionophore and hence decreasing channel conductance (Soffe & Roberts, 1989). After washing, the input resistance returned to that of control (Aiii, Biii) and the membrane potential repolarised back to -70mV. 100μM NMDA was then re-applied together with 5μM 5HT, which caused the membrane to depolarise by 20mV, rather less than the 25mV depolarisation when NMDA was applied on its own, consistent with the hyperpolarising effect of 5HT. The apparent input resistance of the membrane was relatively greater in NMDA and 5HT than in NMDA alone, since there was an increase in the magnitude of the voltage deflections in response to constant amplitude current injection. When measured as ratios of NMDA+5HT/wash and NMDA/control, these measurements translate into a 22% apparent increase in membrane resistance in NMDA alone, compared to a 33% increase in NMDA+5HT. Since the apparent conductance decrease is due to voltage-dependent Mg^{2+} block of the NMDA ionophore, a possible explanation is that 5HT enhances the voltage-dependent Mg^{2+} block. In support of this idea, voltage responses in 5HT (fig 41B) reveal an added inflection (arrowed in Biv) which could be the hyperpolarising

phase of an oscillation. Related experimental evidence supports this notion, since as noted in figures 38-40, at the onset of each hyperpolarising current pulse, the induced oscillations always begin at the same point in the duty cycle, namely at the onset of the falling phase. This suggests that the membrane has been brought into a region were the voltage dependent regenerative blocking of the NMDA ionophore can occur. Since this does not occur in the absence of 5HT, the amine may play an important central role in enabling the regenerative blocking by Mg^{2+} of the NMDA receptor ionophore.

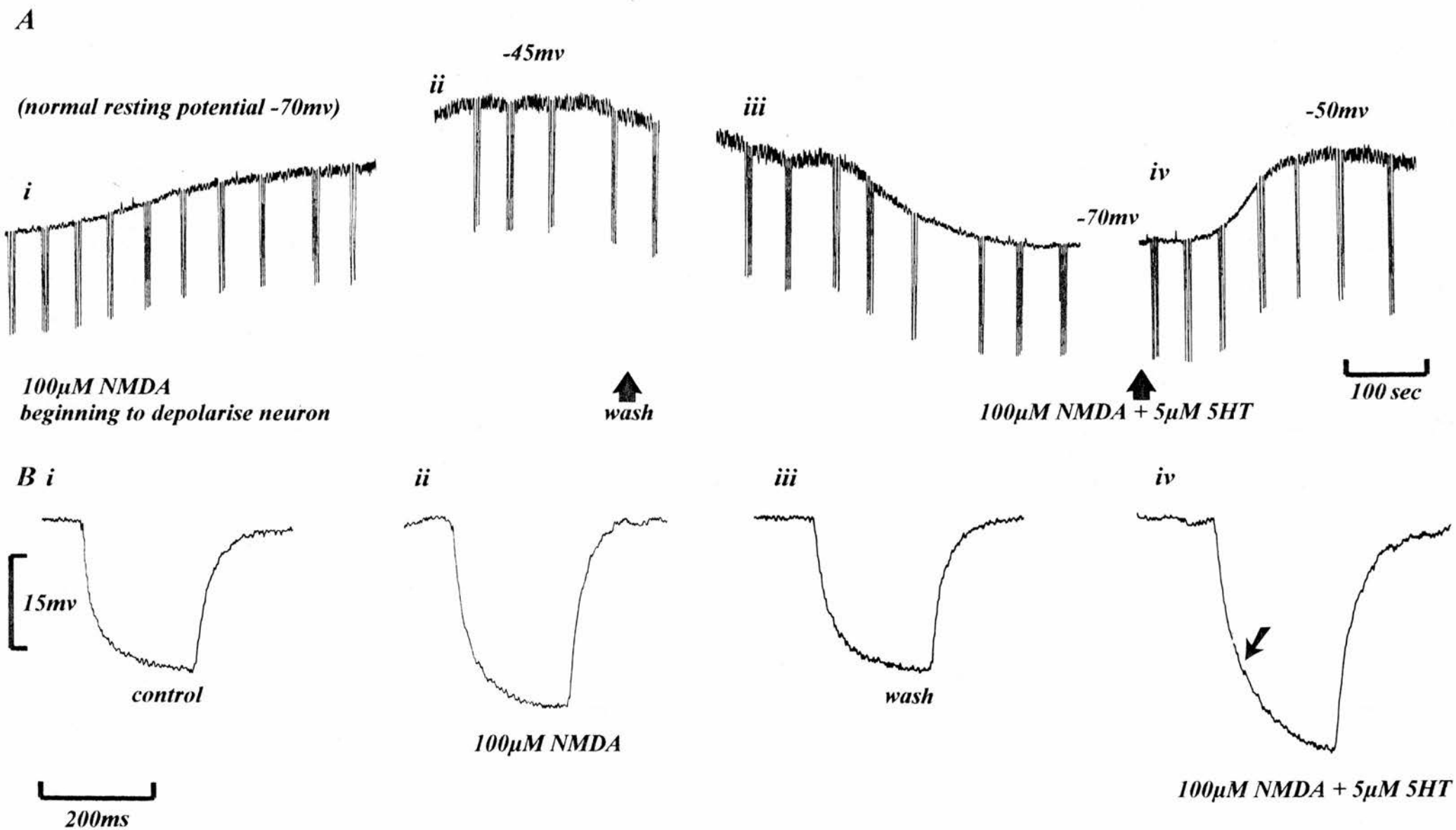

A
-45mv
ii
(normal resting potential -70mv)
iii
-50mv
i
iv
-70mv
100µM NMDA
beginning to depolarise neuron
wash
100µM NMDA + 5µM 5HT
100 sec
B i
ii
iii
iv
15mv
control
wash
100µM NMDA
100µM NMDA + 5µM 5HT
200ms

fig 41. Membrane resistance is apparently greater in NMDA+5HT than in NMDA alone

Every 30 seconds throughout this experiment, conducted in the presence of 1μM TTX, trains of 8 hyperpolarising current pulses at a frequency of 1Hz were delivered to monitor changes in membrane resistance. (Ai) 100μM NMDA depolarises the membrane by 25mV. (ii) Increased amplitude voltage deflections in response to current injection suggest an apparent increase in membrane resistance. (iii) Wash in TTX-containing saline causes membrane to repolarise back to normal resting potential (-70mV) and is accompanied by an apparent decrease in membrane resistance. (iv) 100μM NMDA is re-applied in the presence of 5μM 5HT, and the membrane depolarises to -50mV. There is also a larger increase in membrane resistance than when in NMDA alone. (b) Voltage deflections in response to current pulses during the four experimental regimes showing more clearly the relative changes in membrane resistance.

DISCUSSION

The results described above, some of which are of a preliminary nature, provide evidence that the gross effects of 5HT on locomotor rhythm generation in *Xenopus* tadpoles are mediated via a number of parallel cellular and synaptic mechanisms. These include modulation of active and passive membrane properties of spinal cord neurons, as well as of inhibitory synaptic transmission in the spinal cord. This diversity of mechanisms is in apparent contrast to the traditionally accepted scheme in the lamprey, in which the actions of 5HT on locomotor activity have been studied in detail. Notwithstanding the multiple effects on fictive locomotor activity, such as enhanced and prolonged ventral root bursts, increased cycle period and altered intersegmental co-ordination, it is thought that the amine's effects can be accounted for by a single mechanism involving a suppression of the late phase of the spike AHP (Harris-Warrick & Cohen, 1985; Wallén *et al,* 1989; for review see Grillner *et al,* 1991). Recently, however, the view that this single action of 5HT can account for its multiple effects on the locomotor network, has been questioned. For example, like 5HT, apamin is a selective blocker of $K_{(Ca)}$ channels, and its application to the isolated lamprey spinal cord results in a suppression of the late phase of the spike AHP in motorneurons and enhanced spike discharge frequencies in response to depolarising current pulses. However, it has little effect on those facets of fictive swimming which are profoundly altered by 5HT, such as cycle period and burst durations (Meer & Buchanan, 1992). This suggests, therefore, that 5HT acts via additional mechanisms to modulate swimming activity, rather than via a single action on the late AHP.

In a similar study, however, apamin did appear to affect cycle period and ventral root burst durations, although its effects were less than would be expected of 5HT (Hill *et al*, 1992). There is, therefore, some emerging controversy surrounding the mechanisms of action of 5HT on lamprey swimming, and in particular, the importance of its effect on the late spike AHP.

It is clear that in post-embryonic *Xenopus* larvae, 5HT acts via multiple and parallel mechanisms to modulate locomotor activity. For example, the bath application of 5HT increases the ventral root burst durations underlying swimming activity, suggesting an enhancement of spike discharge in rhythmic spinal neurons. It is probable that the amine exerts this effect via a reduction of voltage and/or calcium dependent K^+ conductances. Although, the effects of 5HT on spike properties were difficult to quantify in the present study, some direct evidence of enhanced firing in rhythmic neurons during fictive swimming following application of 5HT was obtained (fig 34). In addition, to this effect on spike discharge frequency, the amine also acts presynaptically to modulate evoked and spontaneous glycinergic synaptic transmission. During fictive swimming, the midcycle reciprocal inhibition plays an important role in timing the onset and termination of excitatory drive on the opposite side of the cord. For example, enhanced crossed cord inhibition would result in excitatory drive to the opposite 'half centre' being terminated sooner, and the onset on the following cycle being delayed. Since application of 5HT enhances **relative** ventral root burst durations without a marked increase in cycle periods, a concomitant down regulation of cross-cord reciprocal inhibitory drive would seem necessary. There is good experimental evidence that such a

mechanism exists for modulation of cross-cord inhibitory transmission (fig 37), and it is probable that it acts in parallel with serotonergic modulation of spike discharge frequency in rhythmic neurons during swimming. Furthermore, 5HT hyperpolarises spinal neurons by up to 10mV, and therefore appears to play an important role in setting their excitability. One of the most interesting observations, however, was the dependence on 5HT for the induction of membrane potential oscillations in the presence of NMDA. It is conceivable that the amine might act via the modulation of a K^+ conductance important for the repolarisation phase of the oscillation, or alternatively by modulating the voltage dependency of the NMDA receptor ionophore. In the lamprey, 5HT is thought to slow down the frequency of membrane oscillations by a direct inhibitory action on the $K_{(Ca)}$ conductance (Wallén *et al*, 1989). If a mechanism operated in *Xenopus* which similarly reduced K^+ conductances, this would tend to suppress rather than induce TTX-resistant membrane potential oscillations. Although it can not be ruled out that in contrast to the lamprey, in *Xenopus* tadpoles 5HT facilitates rather than inhibits K^+ conductances, it is more likely that the amine interacts directly with the NMDA channel to facilitate membrane potential oscillations. For example, there is good evidence that increased intracellular protein kinase C (PKC) potentiates NMDA responses by increasing the probability of channel openings and also by reducing the voltage dependent Mg^{2+} block (Chen & Huang, 1992). This has two important consequences; firstly there is an increase in the peak current which occurs at about -50mV, and secondly, the region of negative slope conductance is steeper. Although speculative, it is not inconceivable that 5HT might act via a G-protein coupled to a

$5HT_{1a}$ receptor to regulate intracellular levels of PKC and thereby modulate the properties of the NMDA channel. The current-voltage relationships of cultured mouse mesencephalic (Nowak, *et al*, 1984) and spinal (Mayer & Westbrook, 1984) neurons in the presence of NMDA, appear to differ in comparison with *Xenopus* embryo motorneurons (Soffe & Roberts, 1989). In the presence of 0.5mM Mg^{2+}, neurons in both species show peak current at around -40mv and negative slope conductance at more negative potentials. The steepest part of the slope occurs within the range normally induced by post synaptic potentials (-50 to -70mv). However, it may be significant that in the immature *Xenopus* embryo motorneuron, this same region of negative slope appears markedly less steep than in the mature mouse neuron. Since the relative steepness of the slope effectively determines the ability of the membrane to 'flip' between two relatively stable potentials, this may explain why membrane potential oscillations in the presence of NMDA have not been observed in *Xenopus* embryo motorneurons. It is also conceivable that there exists a mechanism which is more developed in adult animals which modulates and/or increases the relative steepness of the slope. Certainly in adult lamprey motorneurons, a marked negative slope conductance has been described which is similar to those of other adult vertebrates (Moore *et al*, 1987), and which is likely to account for membrane potential bistability. Since 5HT may enhance the region of negative slope conductance, possibly by increasing intracellular PKC (see above), this could account for the requirement of 5HT for induction of membrane potential oscillations in early post-embryonic *Xenopus*. Also, it has been suggested that the nature of Mg^{2+} blockade of the NMDA receptor channel changes during

development (see Hestrin, 1992), and this too might explain the absence of membrane potential oscillations in *Xenopus* embryo motorneurons. Interestingly, similar 5HT-dependent, TTX-resistant membrane potential oscillations have recently been described in a closely related species, *Rana temporaria*, at the equivalent stage of development, around hatching (Sillar & Simmers, 1994). In contrast to hatchling *Xenopus* embryos, the swimming pattern of *Rana*, is much more 'adult-like' with long bursts of ventral root activity in each cycle and a multiple firing capability in rhythmic spinal neurons (see fig 4 B; see also Soffe & Sillar, 1990). Notwithstanding the additional complexities of the *Rana* swimming pattern which more closely resembles lamprey swimming than that of the *Xenopus* embryo, intrinsic membrane potentials could not be elicited unless 5HT was also present (Sillar & Simmers, 1994). This in turn might suggest that lamprey oscillations are also dependent on 5HT for their induction, and that this dependence is normally masked by release of 5HT from serotonergic neurons intrinsic to the spinal cord (Franck *et al*, 1992), and despite the presence of TTX, there may be sufficient levels of the amine to enable oscillations to occur.

The membrane potential oscillations in *Xenopus* embryonic and larval spinal neurons demonstrate a strong voltage-dependency. Application of 100μM NMDA induces a membrane depolarisation of typically about 25mV. Although the subsequent addition of 5HT appears to increase the instability of the membrane potential, it does not oscillate in a regular fashion. However, injection of hyperpolarising current reveals stable oscillations, presumably by bringing the membrane potential into a region where the voltage-dependent regenerative blocking and unblocking

of the NMDA receptor by Mg^{2+} can optimally occur. Also, for the same reason, the amplitude of the oscillations appears to depend directly upon the magnitude of the injected current (fig 39), which is in keeping with findings in the lamprey. However, in contrast to the lamprey, their frequency appears to be independent of membrane potential (see fig 39). This anomaly could perhaps be explained by electrical coupling between neighbouring motorneurons (see Perrins, 1993) which oscillate in phase with the recorded neuron and therefore act as pacemakers. Thus, injected hyperpolarising current, while eliciting higher amplitude oscillations from the recorded neuron, would be insufficient to influence the combined pacemaker properties of a number of electrically coupled neighbours. Perhaps significantly, there is no reported evidence for similar electrical coupling between lamprey motorneurons.

CHAPTER 7

General Discussion

GENERAL DISCUSSION

Swimming activity in the stage 37/38 *Xenopus* embryo, although vigorous and well co-ordinated, is also unusually simple and stereotyped in comparison with more adult systems. There is now extensive knowledge of the spinal cord circuitry and mechanisms underlying the generation of this basic rhythm (see General Introduction & Roberts, 1990). The prime objective of this study was to utilise this knowledge as a base from which to explore the development of a simple and stereotyped locomotor rhythm. An important initial finding was that some of the complexities associated with more adult systems are acquired in a brief period post-hatching. Moreover, they occurred in a rostro-caudal sequence, coincident with rapid changes in the cns involving the invasion of the spinal cord by axons of brain stem interneurons (Norlander, 1984; van Mier & ten Donkelaar, 1984), suggesting a causal link between the two. This is in agreement with the widely held view that basic spinal circuits for locomotor rhythm generation are formed very early in embryogenesis, and that they are increasingly influenced by higher brain centres during development, so that they acquire they precision and flexibility necessary for adult life (Bekoff, 1976; Forssberg *et al*, 1991). Moreover, a rostro-caudal acquisition in locomotor competence has also been demonstrated in other vertebrates. For example, in the rat immediately following birth, which is at a relatively early developmental stage, propulsion is due mainly to the activity of the forelimbs. During the next two weeks, the participation of hind limbs progressively increases until they assume the dominant role

(Westerga & Gramsbergen, 1993). Given these broad similarities, the post-embryonic development of locomotor rhythmicity in *Xenopus laevis* therefore provides a rare opportunity to investigate in detail the underlying mechanisms as complexity is being added sequentially onto a relatively simple and well understood locomotor circuit.

A notable feature of the results of this study is the apparent multiplicity of mechanisms by which 5HT plays its developmental and modulatory roles. My results also raise a host of questions surrounding the further detailed exploration of 5HT-induced effects at cellular and synaptic level. For example, the mechanisms by which 5HT imparts a multiple spiking capability on rhythmic neurons are still unclear. The most plausible explanations are either a down regulation of the voltage-dependent K^+ conductance which holds the membrane refractory after a single impulse in embryonic motorneurons (Soffe, 1990), or a modulation of the slow afterhyperpolarisation via a suppression of a $K_{(Ca)}$ conductance, absent in embryos, but which might have developed subsequent to hatching. This latter possibility could be explored with the use of apamin, which like 5HT has been shown to selectively block $K_{(Ca)}$ channels in lamprey rhythmic spinal neurons (Meer & Buchanan, 1992; Hill *et al*, 1992). Also, recording with a caesium chloride-filled microelectrode blocks the voltage-dependent K^+ conductance described by Soffe and permits multiple firing in embryonic motorneurons. This technique could be exploited in larval motorneurons, and then 5HT applied to test if the amine further increases the rate of firing in response to depolarising current pulses. If so, this would suggest an alternative

mechanism, but if not might indicate that the amine imparts a variable multiple spike capability by modulating this K^+ conductance. Also, there are questions surrounding the membrane hyperpolarisation of rhythmic neurons by 5HT. Does it involve, for example, activation of a K^+ current? This could be tested by estimation of the reversal potential, and by application of K^+ blockers such as tetraethylammonium (TEA) and caesium. The pharmacological basis of the response could also be explored. Is it mediated by a $5HT_{1a}$ receptor, which not only plays a central role in the serotonergic modulation of swimming, but also has been shown to mediate a similar response in other vertebrate systems (see chapter 4, section v & discussion)? This could be tested by application of 5HT receptor agonists and antagonists to both mimic and block the 5HT-induced hyperpolarisations. With regard to presynaptic modulation of glycine release, the effects of enhanced endogenous levels of 5HT either via application of 5HTP or a 5HT uptake blocker could be tested. As before, the identity of the 5HT receptor subtype could also be pursued by use of specific 5HT receptor antagonists.

One of the major findings of this study is that 5HT induces intrinsic TTX-resistant membrane potential oscillations in the presence of NMDA and physiological levels of Mg^{2+}. In the preceding section, I have speculated in some depth that 5HT might enable membrane potential oscillations to occur via activation of a G-protein-coupled receptor leading to increased levels of PKC, which in turn results in an increased negative slope conductance. This notion could be explored by use of antagonists to disrupt this G-protein coupled second messenger pathway. Although it is difficult to envisage the behavioural significance of these slow

TTX-resistant membrane potential oscillations with regard to the generation and maintenance of the much faster *Xenopus* larval swimming rhythm, it is probable that the serotonergic modulation of the NMDA receptor channel may be of wider significance, given that NMDA receptor channels are involved in a myriad of other neural functions.

Since it is clear that 5HT exerts its influence on the locomotor circuit at multiple levels, it is also appropriate to explore other possible sites of action. For example, there is evidence from other systems that 5HT can modulate excitatory transmission. For example, in the lamprey motorneurons, 5HT depresses reticulospinal postsynaptic potentials (Buchanan & Grillner, 1991). Is excitatory synaptic transmission also modulated in post-embryonic *Xenopus*? If so, does it involve a presynaptic modulation of transmitter release, or a post-synaptic mechanism which increases activation of the NMDA receptor-ionophore complex, as has been described in other systems (Reynolds *et al*, 1988)?

On a final note, one of the important aspects of this study is the successful use of the early post-embryonic stages of *Xenopus* as a developmental model system. As the late embryo (stage 37/38) has been and continues to be used as a model system for investigating the basic mechanisms underlying vertebrate locomotor rhythm generation, the rapidly developing early larval stages can be exploited to investigate the underlying developmental mechanisms. A whole new field of endeavour is consequently available and it promises to be extremely fruitful. For example, the rostro-caudal acquisition of locomotor complexity, the progressive

invasion of spinal cord circuits by axons of brain stem interneurons and the sequential expression of receptors (see, Walton *et al,* 1993) are features which have been observed in other vertebrates. This new model system offers an unparalleled opportunity to investigate further the underlying mechanisms.

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Appendix

Publications arising from this work

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