The role of metabotropic glutamate receptors in the control of swimming in hatchling *Xenopus laevis* tadpoles

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

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Rebecca Jayne Chapman

School of Biology
Bute Medical Buildings
University of St.Andrews
St.Andrews
Fife
KY16 9TS

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Declaration

I, Rebecca Jayne Chapman, hereby certify that this thesis, which is approximately 48,000 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

Date 28/9/05 Signature of candidate .

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Finally, thank you to the Biotechnology and Biological Sciences Research Council for funding this work.

"Theories come and theories go, the frog remains."

J.Rostand 1960

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Abbreviations

(1S,3R)-ACPD	(1S,3R)-1-aminocyclopentane-cis-1,3-dicarboxylic acid
(2R,4R)-APDC	(2R,4R)-aminopyrrolidine-2,4-dicarboxylic acid
(RS)-MCPG	(RS)-α-methyl-4-carboxyphenylglycine
(s)EPSP	(spontaneous) excitatory post-synaptic potential
(s)IPSP	(spontaneous) inhibitory post-synaptic potential
5-HT	5-hydroxytryptophan
AC	Adenylyl cyclase
Ach	Acetylcholine
AIDA	1-aminoindan-1,5-dicarboxylic acid
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
BHK	Baby hamster kidney
BICUC	Bicuculline
CHO	Chinese hamster ovary
CNS	Central nervous system
CPCCOEt	cyclopropan[b]chromen-1a-carboxylate
CPG	Central pattern generator
DCG-IV	(2S,1'R,2'R,3'R)-2-(2,3-dicarboxycyclopropyl)-glycine
DHPG	(S)-3,5-dihyroxyphenylglycine
dla	Dorsolateral ascending
dlc	Dorsolateral commissural
DLR	Diencephalic locomotor region
DmGluA	*Drosophila* mGlu receptor A
EGLU	S-α-4-ethyl glutamic acid
GABA	Gamma-aminobutyric acid
GAD	Glutamic acid decarboxylase
GPCR	G-protein coupled receptor
HCG	Human chorionic gonadotrophin
HVc	High vocal centre
iGluR	Ionotropic glutamate receptor
IP_3	inositol-(1,4,5)-triphosphate
KA	Kainate
KAc	Potassium acetate

KCl	Potassium chloride
L-AHM	L-β-aspartic acid hydroxymate
LAOBP	Lysine, arginine, ornithine binding protein
L-AP4	L-2-amino-4-phosphonobutanoate
L-CCG-I	(2S,3S,4S)-α-(carboxycyclopropyl)-glycine
LIVBP	Leucine, isoleucine, valine binding protein
MAP4	(S)-2-amino-2-methyl-4-phosphonobutanoate
mGluR	Metabotropic glutamate receptor
mhr	Midhindbrain reticulospinal
MLR	Mesopontine locomotor region
MN	Motorneuron
MPEP	S-α-4-methylcarboxyphenylglycine
NA	Noradrenaline
NMDA(R)	*N*-methyl-D-aspartate (receptor)
NO	Nitric oxide
PAG	Periacqueductal grey
PBP	Periplasmic binding protein
PI	Phosphoinositide
PIP_2	L-3-phosphatidyl-D-myo-inositol-4,5-biphosphate
PLC	Phospholipase C
PTX	Pertussis toxin
R-B	Rohon-beard
R-C delay	Rostrocaudal delay
RS	Reticulospinal
SNr	Substantia nigra pars reticulata
STRY	Strychnine
TM	Transmembrane
TTX	Tetrodotoxin
VR	Ventral root

Abstract

1. I have examined the possibility that the ubiquitous amino acid neurotransmitter, glutamate, may act as an intrinsic neuromodulator in the nervous system of *Xenopus laevis* tadpoles through its actions at metabotropic glutamate receptors (mGluRs).

2. Activation of group I mGluRs increased both swimming frequency and the number of spontaneously occurring swimming episodes. Conversely, activation of group II and/or III mGluRs slowed swimming frequency and affected swimming episodes. Additionally, group II/III receptor activation reduced the size of both ventral root burst durations and amplitudes.

3. Group I, II and III receptor-mediated effects were blocked by prior applications of strychnine. Prior bicuculline applications did not affect group II/III receptor-mediated effects on motor activity, but subsequent group I receptor activation caused irregular swimming activity. Therefore, all three groups may potentially utilise the fast inhibitory pathways to modulate swimming frequency.

4. Group I mGluR activation reduced the spontaneous release of glycine and GABA in the quiescent periods between swimming episodes. Spontaneous IPSP amplitudes were also reduced suggesting a parallel pre- and post-synaptic locus of action by group I mGluRs to increase network excitability.

5. Group II mGluRs have no effect on inhibitory transmission or on resting membrane properties. Whilst the mechanism behind the group II receptor-mediated inhibition of network activity proved inconclusive, the reduced motor burst durations and amplitudes suggest a potential modulation of excitatory transmission by these receptors.

6. Group III mGluR activation pre-synaptically reduced inhibitory transmission, and, in some neurons, increased sIPSP amplitudes implicating a post-synaptic event. A significant membrane potential hyperpolarisation occurred in a proportion of neurons which could account for reduced synaptic activity, thus slowing swimming frequency. However, multiple mechanisms may be utilised by this group to reduce network activity.

7. Group III receptor activation also inhibited the sensory pathways required to initiate swimming, as swim-initiation threshold increased and the embryonic dimming response was abolished.

1 | General Introduction: The involvement of metabotropic glutamate receptors in locomotor pattern generation

1 | 1 Summary

G-protein coupled metabotropic glutamate receptors (mGluRs) are a relatively new area of excitatory amino acid research. Whilst mGluRs have been studied in the context of locomotor rhythm generation previously, notably in the lamprey, they have yet to be studied in the relatively simple nervous system of the anuran amphibian, *Xenopus laevis*, whose spinal network comprises only eight recognised classes of neuron and does not require exogenous pharmacological agents to initiate and maintain swimming. My project has focused on neurotransmission mediated through metabotropic glutamate receptors and their role in shaping the motor pattern during swimming in tadpoles of *Xenopus laevis*. Before investigating the physiological roles that these receptors might play in motor pattern generation in the tadpole, it is necessary first to review all aspects of mGlu receptor structure, function and pharmacology. A brief overview of these aspects is presented in this chapter along with an introduction to the model within which these receptors are being investigated for their contribution to motor pattern generation.

1 | 2 Background

As indicated by the abundance of glutamatergic pathways and the distribution of associated receptors, the amino acid glutamate is the major excitatory neurotransmitter in the vertebrate central nervous system (CNS) and plays vital roles in many brain functions, including nociception, motor control, neuronal cell death and synaptic plasticity (Nakanishi, 1992; Pin and Duvoisin, 1995; Pin and Bockaert, 1995; Conn and Pin, 1997). Until 1985, glutamate was thought to act exclusively at ionotropic glutamate receptors (AMPA, kainate and NMDA receptors). However the discovery that glutamate stimulates phosphoinositide (PI) turnover in cultured striatal neurons (Sladeczek *et al*, 1985) and hippocampal slices (Nicoletti *et al*, 1986), via

receptors other than the ionotropic subtypes broadened this view. The binding of a neurotransmitter to both ligand-gated channels and to G-protein coupled receptors (GPCRs) was not a new concept as it had already been documented for GABA and acetylcholine. The presence of metabotropic glutamate receptors (mGluRs) was subsequently confirmed through pharmacological manipulations and the cloning of their cDNAs (Sugiyama *et al*, 1987).

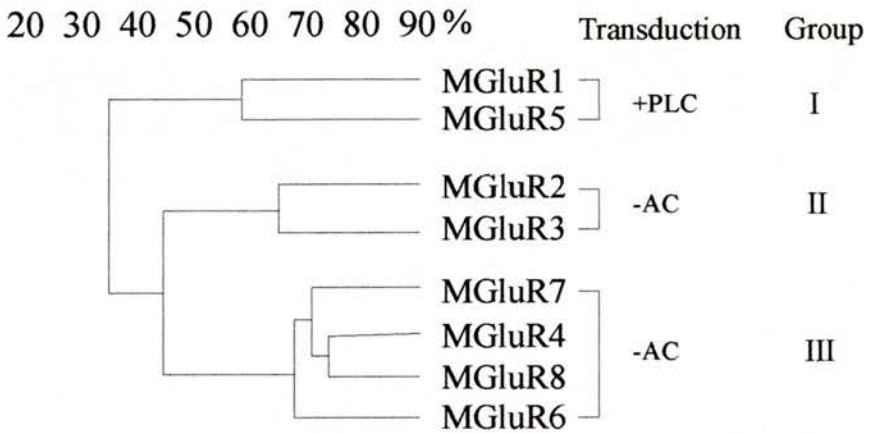
The development of specific mGluR agonists and antagonists facilitated the characterisation of various receptor subtypes within the mGluR family and helped to identify numerous roles for these receptors in brain function. For example, they are known to contribute to the induction of synaptic plasticity, including long-term potentiation and long-term depression, which are believed to lie at the root of learning and memory (Schoepp and Conn, 1993; for a review: Pin and Duvoisin, 1995). mGluRs may also play a role in glutamate-induced neurotoxicity making them a potential target for the development of therapeutic agents (McDonald and Schoepp, 1992; McDonald *et al*, 1993).

Currently, eight mGluR subtypes have been recognised. These have been classified into three groups based upon their sequence homology, signal transduction mechanisms and agonist selectivity (Figure 1.1A; Pin and Duvoisin, 1995; Pin and Bockaert, 1995). MGluRs have been located at most glutamatergic synapses and some GABAergic synapses, where they are localised pre- and/or post-synaptically. Group I receptors are thought to localise predominantly in post-synaptic membranes, but there is also some evidence for pre-synaptic activity of these receptors (Gereau and Conn, 1995; Manzoni *et al*, 1995). Group I receptors, consisting of $mGluR_1$ and $mGluR_5$, have been reported to both increase and decrease cell excitability through

Figure 1.1 | General representation of mGluRs

(A) Phylogenetic tree classification of the metabotropic glutamate receptor groups and subtypes. Scale represents sequence homology with respective groups. + = activation; - = inhibition. (B) Schematic representation of an mGluR. The small purple coloured region on the second intracellular loop is important for G-protein coupling specificity. EXTRA = extracellular, INTRA = intracellular. Modified from Pin and Duvoisin, 1995.

A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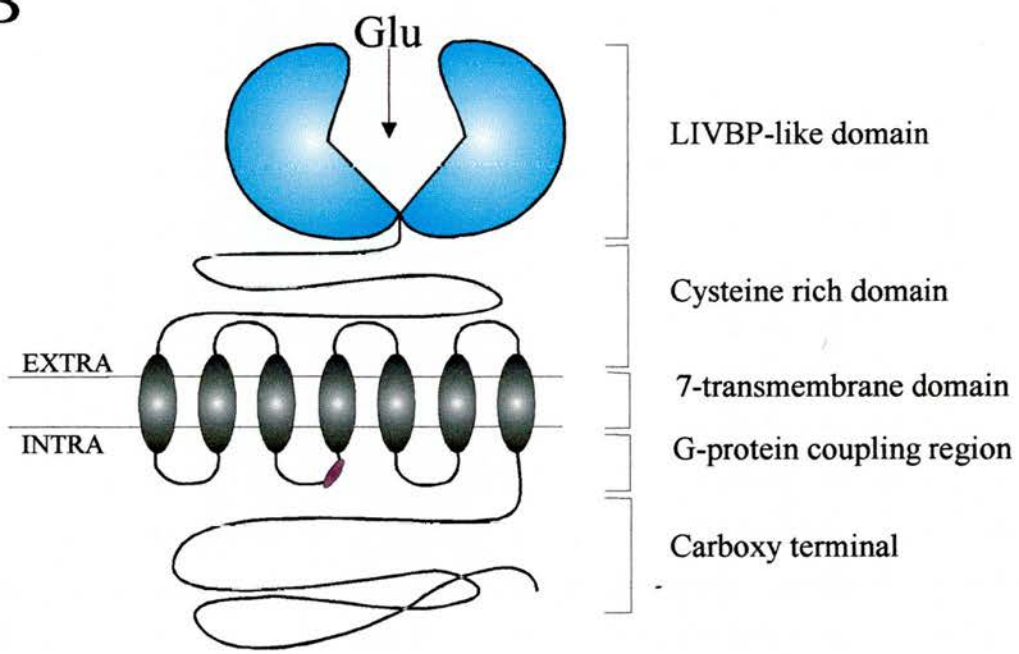
Transduction
Group
MGluR1
MGluR5
+PLC
I
MGluR2
MGluR3
-AC
II
MGluR7
MGluR4
MGluR8
MGluR6
-AC
III

B


Glu
EXTRA
INTRA
LIVBP-like domain
Cysteine rich domain
7-transmembrane domain
G-protein coupling region
Carboxy terminal

the inhibition of K^+ channels and activation of Ca^{2+}-dependent K^+ channels, respectively (Conn and Pin, 1997; Anwyl, 1999). Groups II ($mGluR_2$ & $_3$) and III ($mGluR_{4,6,7}$ & $_8$) are primarily found pre-synaptically, where they predominantly act to inhibit neurotransmitter release (Tamaru *et al*, 2001).

1 | 3 mGluRs: Cloning and Structure

Conclusive evidence for the existence of mGluRs was provided by a cloning approach which combined the *Xenopus* oocyte expression system with electrophysiological techniques (Sugiyama *et al*, 1987; Houamed *et al*, 1991). The procedure was based on an endogenous second messenger system that links G-protein activation with Cl^- currents (Houamed *et al*, 1991; Masu *et al*, 1991). Oocytes containing synthesised RNA from various rat cerebellar cDNA clones were monitored electrophysiologically to examine any oscillatory Cl^- currents in response to the application of glutamate. From this work, two laboratories were able to screen successfully the cDNA encoding the splice variant, $mGluR_{1a}$ (Houamed *et al*, 1991; Masu *et al*, 1991). Once this was established, $mGluR_{1a}$ was used to isolate and identify the other, related mGluRs.

So far, seven other genes encoding mGluRs have been isolated and characterised, along with the existence of splice variants from which even more receptors may be generated (Tanabe *et al*, 1992; Pin and Duvoisin, 1995; Conn and Pin, 1997). Within a group, the mGluRs share up to 70% sequence identity, whereas between groups this similarity drops to a maximum of 45% (Nakanishi, 1992). Splice variants have been found for only three of the mGluRs so far: $mGluR_{1a\text{-}c}$, and $_{1d}$; $mGluR_{4a}$ and $_{4b}$; and $mGluR_{5a}$ and $_{5b}$ (Conn and Pin, 1997).

The mGluR family has an unusual structural architecture when compared to other GPCRs (Pin *et al*, 1999). Simplified, the structure consists of a large extracellular N-terminal domain, a seven hydrophobic transmembrane (TM) region, and a variable length intracellular carboxy terminal domain (Figure 1.1B). Unlike other GPCRs, the transmembrane region is not the site of ligand binding in the mGluRs. The large extracellular domain and intracellular loops contain highly conserved sequences that likely correspond to the glutamate binding and G-protein coupling sites, respectively (Pin and Bockaert, 1995). Interestingly, analysis of the highly conserved regions revealed that all mGluRs share the same 21 cysteine residues in the areas of predicted binding and coupling sites (Pin and Duvoisin, 1995).

Most GPCRs have well documented binding site characteristics. However, little was known of mGluRs until two very different studies confirmed the involvement of the large extracellular domain in the selective recognition of ligands (O'Hara *et al*, 1993; Takahashi *et al*, 1993). Takahashi *et al* (1993) analysed $mGluR_1$ and $mGluR_2$ chimeras both functionally and pharmacologically, and showed that exchanging N-terminal domains changed the pharmacological profile of one receptor type into that of another. These results demonstrated the significance of the extracellular domain in ligand recognition in these receptors, and subsequently then with all the mGluR subtypes (Tones *et al*, 1995) including chimeras of mGluRs from other species (for example, *Drosophila* $DmGlu_A$ receptors (Bockaert and Pin, 1999)).

The second study by O'Hara *et al* (1993) exploited the sequence similarity between the extracellular domains of mGluRs and bacterial periplasmic binding proteins (PBP) to construct a computational model of the mGluR extracellular domain. The most homologous PBP, the leucine, isoleucine, valine binding protein (LIVBP) was used in

the model to reconstruct $mGluR_1$. Chimera receptor production through the exchanging of one LIVBP domain for another altered pharmacological profiles, providing evidence for the involvement of this domain in ligand selectivity (O'Hara *et al*, 1993). The structure of the extracellular domain proposed by the model is similar to a clamshell with two lobed domains joined by a hinged region (Figure 1.1B; Pin and Bockaert, 1995) suggesting the idea that glutamate is attracted into the structure and the two lobes close around it. Two specific residues in the structure, S165 and T188, are known to be vital for glutamate affinity to this area as mutagenesis experiments have been shown to eliminate the affinity of glutamate for this structure (O'Hara *et al*, 1993). These residues are highly conserved within the mGluR family, including mGluRs of *Drosophila* and *C. elegans* (Pin, 1999). The conclusive evidence for this model finally came from work done on the iGluR channel subunits. These subunits were shown to possess two domains homologous to PBP, one similar to LIVBP and one similar to lysine, arginine, ornithine binding protein (LAOBP) (O'Hara *et al*, 1993). These domains were suggested to be the potential glutamate recognition sites within the subunits and subsequent work with mutated $NMDAR_1$ subunits has confirmed this (Pin and Bockaert, 1995).

The site of G-protein activation has been widely studied in other GPCRs. Most GPCRs have a specific tripeptide sequence, comprising aspartic acid, arginine and tyrosine (DRY), at the amino terminal end of the second intracellular loop that is important in the coupling and activation of G-proteins (Conn and Pin, 1997). The third intracellular loop is the most critical region for G-protein specificity and it is thought that the second and third loops form a cavity into which the C-terminus of the G-protein α-subunit binds (Bluml *et al*, 1994). Unusually, DRY is not present in any of the mGluR intracellular loops. Chimeras constructed from two mGluRs of

different groups, revealed that the second intracellular loop is vital to mGluRs in G-protein coupling specificity (Pin *et al*, 1994). The other mGluR intracellular loops play a more dominant role in the control of ligand efficacy. Interestingly, this second loop has a region rich in basic residues that are most likely to form amphipathic α-helices that extend TM3 and 4 into the cytoplasm for direct interaction with the carboxyl terminus of G-protein α-subunits (Pin *et al*, 1994). Therefore, even though the amino acid sequences for G-protein coupling are not conserved for all GPCRs, the same structural features are involved. Little is known about the function of the mGluR C-terminal domain. Splicing of this large domain alters the receptors' activity rates thus indicating a possible role in signal transduction (Anwyl, 1999). Additionally, there is an abundance of phosphorylation sites on the C-terminus which could potentially be the target of many kinases that regulate receptor activity (Cai *et al*, 2001).

1 | 4 Transduction Mechanisms

MGluRs are not classified into three groups based exclusively on their amino acid sequence; their pharmacology and transduction mechanisms also support the classification (Figure 1.1A). For instance, group I can be distinguished from groups II and III through its transduction mechanisms. Investigations with cloned receptors have shown that only group I receptors ($mGluR_1$ and $mGluR_5$) and their splice variants stimulate phospholipase C (PLC) resulting in an increase in PI turnover and Ca^{2+} release from internal stores (Sugiyama *et a*l, 1987). Groups II and III inhibit adenylyl cyclase (AC) and can only be differentiated through differences in their strength of inhibition and pharmacology (next section).

Receptor affinity for G-proteins varies between subtypes and splice variants. The identification of similar G-proteins involved in PLC activation by group I mGluRs is incomplete, although initial work using cloned mGluRs implicated the G_i-G_o family of proteins. A number of studies have shown a partial sensitivity of the $mGluR_{1a}$ to pertussis toxin (PTX) (Houamed *et al*, 1991; Masu *et al*, 1991; Pickering *et al*, 1993) implying that a member of the PLC activating PTX-insensitive, G_q family is associated with these receptors. Whilst some receptor types showed no sensitivity to PTX, and thus are coupled to G_q proteins, other receptors (mainly in *Xenopus* oocytes) are PTX sensitive, and could be coupled to G_o-G_i and G_q proteins (Pin *et al*, 1992). G_s proteins may also play an as yet unconfirmed role in transduction responses as $mGluR_{1a}$ receptors expressed in CHO or BHK cells increase AC production (Conn and Pin, 1997).

The strength of response varies between group II and III mGluRs, yet it is not always enough to differentiate between the two groups. Group II strongly inhibits forskolin stimulated cAMP production in brain slices and neuronal cultures (Tanabe *et al*, 1992), whereas group III receptors only weakly inhibit the same response. Unlike group I, complete inhibition with PTX is observed confirming G_i involvement (Tanabe *et al*, 1993). Of these subtypes, $mGluR_6$ has a most unusual transduction mechanism, coupling to both cAMP inhibition and to cGMP inhibition (Pin and Bockaert, 1995). There is evidence for the reduction of cGMP accumulation through $mGluR_6$ activation in retinal ON-bipolar cells (Sheills and Falk, 1992). This response was initially thought to result from the coupling of $mGluR_6$ and a cGMP phosphodiesterase through the G-protein, transducin, which subsequently inhibits cGMP-induced cation channel activation (Sheills and Falk, 1992; Thoreson and Miller, 1994). More recent evidence revealed that $mGluR_6$ activates the G-protein,

G_o, rather than transducin, which does not interact with phosphodiesterase but possibly alters cationic channel activity through an unclear membrane delimited pathway (Nawy, 1999; Gerber, 2003; Shepard, 2004). In addition, $mGluR_6$ is postulated as being the first identified metabotropic receptor responsible for fast excitatory transmission within the central nervous system. The range of effects controlled by mGluRs throws doubt onto the traditional amino acid transmitter classification as 'excitatory' as the response from glutamate release at the synapse depends entirely on the type of mGluR stimulated, and can be either excitatory or inhibitory (Schoepp and Conn, 1993).

1 | 5 Pharmacology

The classification of mGluR subtypes into three groups has been highly beneficial in categorising pharmacological properties, initially due to the paucity of available subtype-selective pharmacological agents and the sparse understanding of the localisation and function of each subtype (Schoepp *et al*, 1999). The pharmacological profile of each mGluR group is constantly evolving with the production of newer, more selective agonists and antagonists. But even with the discovery of new synthetic derivatives, much neurochemical data is still lacking. Cloned mGluRs, expressed in heterologous systems, have been the only method of characterising the response of specific mGluR subtype activation following drug application (Bordi and Ugolini, 1999). Whilst the potencies measured in expression systems may not necessarily reflect those in native systems, at least the certainty of a specific receptor subtype being linked to a particular response is higher (Conn and Pin, 1997). The potential number of mGluR subtypes in the brain may be even more numerous as the development of new clones continues. Without the characterisation of an individual

receptors pharmacological profile, the identification and understanding of the mGluRs physiological effects cannot be undertaken fully.

Our knowledge of the current pharmacological profiles is not as clear for some subtypes as it is for others. Agonists termed 'non-selective' have shown activity at all three mGluR groups, and all of them appear to be restricted to the mGluRs with the exception of quisqualate, which also has an efficacy at the ionotropic AMPA type glutamate receptor (Cartmell and Schoepp, 2000). Unless a pharmacological agent has been characterised across all three mGluR groups it should not be labelled 'group selective' (Schoepp *et al*, 1999). There are compounds available that act as high affinity agonists and antagonists for specific mGluR groups and subtypes, which is necessary for determining the receptors physiological characteristics. However, whilst certain derivatives are specific to particular mGluR subtypes, it is not true of all the compounds commercially available. Not all agonists/antagonists have been characterised with regards to their effect on every mGluR clone produced and as a result caution must be taken in the interpretation of such data.

It has been argued that the pharmacological profile of PI-linked group I mGluRs was primarily determined using $mGluR_{1a}$ and $_{5a}$ splice variants (Conn and Pin, 1997). The agonist, 3,5-DHPG has been characterised in rat brain slices (Schoepp *et al*, 1994) and is highly group I specific. This specificity makes DHPG preferable to quisqualate, which is not only a potent stimulant of ionotropic AMPA receptors (Watkins *et al*, 1990), but has also been reported to stimulate some group III receptors at high concentrations (Schoepp *et al*, 1999). The development of antagonists for group I has proved to be challenging since most phenylglycine analogues appear to be preferentially selective for $mGluR_{1a}$ over $mGluR_{5a}$ receptors (Brabet *et al*, 1995;

Kingston *et al*, 1995; Moroni *et al*, 1997). The discovery of two novel non-competitive antagonists, to $mGluR_1$ and $mGluR_5$, in the rat aided in the pharmacological separation of the group I subtypes (Van Wagenen *et al*, 1998). The selective non-competitive $mGluR_1$ antagonist, CPCCOEt, and the selective non-competitive $mGluR_5$ antagonist, MPEP, when used in combination are useful pharmacological tools for the assessment of group I physiological function (Schoepp *et al*, 1999).

Currently, there is only one highly specific agonist at group II mGluRs, namely 2*R*,4*R*-APDC. This agent is potent at both native and recombinant human $mGluR_2$ and $mGluR_3$ receptors (Schoepp *et al*, 1995, 1996), and more importantly does not produce any agonist or antagonist effects with natively expressed PI-coupled mGluRs or recombinant group III receptors (Schoepp *et al*, 1996). The first antagonist characterised for group II receptors was MCPG, but this compound was found to be non-selective, and additional effects on the other groups became evident (Conn and Pin, 1997). Other phenylglycine derived competitive antagonists of this receptor include MPPG and MCCG-I. As the variety of potent and selective group II antagonists grows, the challenge remains now to produce new agents to antagonise each subtype more specifically.

The greater diversity of receptor subtypes within group III has made the establishment of pharmacological agents for this group more tricky. But this group does appear to be distinctly sensitive to the agonists L-AP4 and L-SOP, which are not active at any other group. Fewer selective antagonists have been reported for this group of all the three. Of these, MAP4 and MSOP, have been shown to be the most potent antagonists in the reversal of group III-mediated depression of synaptic transmission

in the neonatal rat spinal cord (Jane *et al*, 1996). Again, caution must be taken in the interpretation of the data, as certain compounds appear to affect different areas of the CNS, and there is little or no consistency between species.

1 | 6 Neurophysiological Roles and Regulations of mGluRs

Collated evidence over the last decade from electrophysiological research on mGluRs has revealed numerous and diverse cellular effects resulting from mGluR activation. These include: inhibition of K^+ and Ca^{2+} currents; activation of K^+ and Ca^{2+} currents; potentiation of AMPA and NMDA receptor-mediated responses and their involvement in oscillatory membrane properties; presynaptic inhibition of neurotransmitter release; and a potential calcium-sensing function in high external calcium environments (Bleakman *et al*, 1992; Gerber and Gahwiler, 1994; Glaum and Miller, 1994; Kubo *et al*, 1998; for a review: Anwyl, 1999). The most frequently observed physiological effect with mGluR agonist applications throughout the whole CNS is the depression of synaptic transmission at glutamatergic synapses (Baskys and Malenka, 1991; Mitchell and Silver, 2000). The presynaptic mGluRs which mediate this effect act as negative-feedback autoreceptors and many mGluR subtypes exhibit this behaviour (Cartmell and Schoepp, 2000). Whether or not the mGluRs serve as autoreceptors depends on various factors, including location on the neuron and the developmental stage of the tissue (Schoepp and Conn, 1993). For example, in the CA1 region of the adult rat hippocampus, group II mGluRs do not function as autoreceptors, yet there is evidence for them being autoreceptors in the neonatal CA1 region (Baskys and Malenka, 1991). This implies possible developmental changes in the role of this class of receptor.

The mechanism behind the observed decrease in presynaptic glutamate release following mGluR activation is not fully understood. Nevertheless, possibilities include the reduction of voltage-gated Ca^{2+} currents or activation of presynaptic K^+ currents (Anwyl, 1999). In the lamprey spinal cord, there is electrophysiological evidence that presynaptic mGluRs modulate glutamate transmitter release in an autoreceptor-mediated fashion from reticulospinal neurons through two separate mechanisms: firstly, application of the group III selective agonist, L-AP4, activates a presynaptic K^+ current that depresses the release of transmitter; secondly, activation of group I receptors by DHPG can increase transmitter release via the rapid release of Ca^{2+} from intracellular stores. This acts in a Ca^{2+}-dependent manner to amplify the action potential-evoked presynaptic calcium signal (Cochilla and Alford, 1998). Various conditions control the balance of effect that glutamate will therefore have over the firing frequency of reticulospinal neurons. These include the location of mGlu receptor subtypes with regard to the synaptic terminal, the affinity of these receptors to glutamate, presynaptic Ca^{2+} concentration during stimulation and the rate and duration of stimulation (Cochilla and Alford, 1998). In addition, there is also evidence suggesting direct mGluR-mediated modulation of the molecular events responsible for vesicle docking and fusion, causing a reduction in transmitter release (Anwyl, 1999; Cartmell and Schoepp, 2000). For example, mGluR agonists have been shown to reduce the frequency of miniature EPSPs, an effect which could not be enhanced by activation of pre-synaptic Ca^{2+} channels (Sladeczek *et al*, 1993; Burke and Hablitz, 1994; Tyler and Lovinger, 1995).

In addition to mGluRs having a clear effect on excitatory transmission, evidence is emerging that mGluR activation also affects inhibitory amino acid transmission, in that mGluRs can modulate release of glycine and GABA. For example, mGluR

activation in dissociated rat spinal cord neurons induces a significant reduction in intracellular cAMP levels, causing a reduction in glycinergic transmission (Katsurabayashi *et al*, 2001). There is recent evidence that mGluR activation also reduces glycinergic transmission in the lamprey spinal cord via release of endocannabinoids (Kettunen *et al*, 2005). Similarly, mGluRs acting as presynaptic heteroceptors at GABA synapses in many brain regions can modulate inhibitory transmission (Hayashi *et al*, 1993; Jouvenceau *et al*, 1995; Schoepp, 2001). For instance, in the SNr, one of the primary output nuclei of the basal ganglia, group I receptor activation reduces GABAergic transmission (Marino *et al*, 2001). Conversely, this class of receptor also facilitates GABAergic transmission in the periaqueductal grey (PAG) of rats (de Novellis *et al*, 2003; Drew *et al*, 2004).

In addition to mGluR-mediated modulation of excitatory and inhibitory transmission, mGluRs can also modulate ligand gated ion channels, thus regulating synaptic integration at the post-synaptic membrane (Bleakman *et al*, 1992; Fitzjohn *et al*, 1996). For example, mGluR activation in spinal cord dorsal horn neurons potentiates both NMDA and non-NMDA currents (Bleakman *et al*, 1992), and in striatal neurons, depresses NMDA-evoked depolarisations (Colwell *et al*, 1994). In the lamprey spinal cord, activation of group I receptors potentiates the amplitude of NMDA-induced currents in both motorneurons and interneurons, thereby regulating locomotor burst frequency (Krieger *et al*, 1998, 2000). Therefore, mGluR activation of ligand-gated ion channels is highly variable between neuronal cell populations and possibly plays very different modulatory roles in synaptic transmission in various parts of the brain (Conn and Pin, 1997).

1 | 7 mGluRs and central pattern generation during locomotion

An important aim of neuroscience is to fully understand how a nervous system produces motor behaviour, be it walking, flying or swimming. In vertebrates, neuronal circuits known as central pattern generators (CPGs), contained predominantly within the spinal cord, produce the rhythmic motor patterns underlying locomotion even in the absence of sensory feedback or inputs from the brain (for a recent review: Grillner, 2003). The anatomical organisation of these intrinsic neuronal circuits varies between species and even throughout the development of any one species. However, there appear to be recurrent fundamental features in both the CPG organisation and how it generates locomotion in all vertebrate species. Pioneering work by Graham Brown at the beginning of the twentieth century led him to propose a 'half-centre' model to explain the reciprocal activation of extensor and flexor muscles within and between the limbs of vertebrates (Brown, 1911, 1914). The core feature of Browns model involves two locomotor half-centres being reciprocally coupled by inhibition so that when one half-centre is active, the other is inhibited. In a revised view of spinal cord organisation several half-centre unit burst generators have been proposed to exist along the spinal cord to account for the high degree of flexibility in the output of the spinal networks and in both inter- and intra-limb co-ordination (Grillner, 1981). Evidence of this reciprocal interconnectivity between antagonistic half-centres can be recorded within each cycle of motor activity as alternating synaptic excitation and inhibition of the motorneurons. I will now provide a brief overview of the origins and nature of these two forms of synaptic input during locomotion.

1 | 7 | 1 Inhibition in the spinal motor system

The circuitry required for rhythm generation is present in both spinal half-centres and it is the reciprocal inhibition between them that allows each half-centre to be active in alternation. Thus, experiments involving surgical division of the spinal cord into left and right halves removes left/right alternation providing evidence of this reciprocal organisation of the CPG (e.g. neonatal rat (Kjaerulff *et al*, 1996), lamprey (Cangiano and Grillner, 2003), *Xenopus* (Soffe and Roberts, 1982)). The main neurotransmitter responsible for mediating the reciprocal inhibition between half-centres in a range of vertebrates is the amino acid glycine. Evidence from numerous studies shows that following application of the glycine receptor antagonist, strychnine, left/right alternation is disrupted, and in legged animals, left/right and flexor/extensor alternation is converted into synchronous activity (e.g. cat (Noga *et al*, 1993), neonatal rat (Cowley and Schmidt, 1995), lamprey (Cohen and Harris-Warrick, 1984), tadpole (Soffe, 1987, 1989)).

In the *Xenopus* embryo and lamprey spinal networks, reciprocal inhibition during swimming activity and during walking activity in the neonatal rat is mediated by glycine which activates a post-synaptic (strychnine-sensitive) receptor which gates a chloride channel (Hamill *et al*, 1983; Cohen and Harris-Warrick, 1984; Soffe, 1989). Intracellular recordings from spinal neurons in each of these species has shown that interneurons crossing the spinal cord midline elicit strychnine-sensitive IPSPs in phase with contralateral motorneuron firing (Buchanan, 1982; Dale, 1985; Buchanan and Grillner, 1987; for a review on the neonatal rat: Kiehn and Butt, 2003). These interneurons are the most likely candidates for mediating the reciprocal inhibition in these vertebrates and are known as crossed-caudal interneurons in the lamprey, and commissural interneurons in the neonatal rat and *Xenopus*. Additionally, in *Xenopus*,

immunocytochemical detection of glycine revealed a population of labelled neurons corresponding to the commissural interneurons supporting the evidence provided above for the glycinergic nature of reciprocal inhibition (Dale *et al*, 1986).

The other fast inhibitory transmitter system, utilising GABA, does not appear to be involved in rhythm generation during swimming and there is no evidence for it contributing to the mid-cycle inhibition in the lamprey (Grillner and Wallen, 1980), or the tadpole (Soffe, 1987). However, applications of the $GABA_A$ receptor antagonist, bicuculline, increased locomotor cycle frequency in neonatal rat, lamprey and tadpole (Grillner and Wallen, 1980; Soffe, 1987; Cazalets *et al*, 1994) suggesting some role for this inhibitory transmitter in the shaping of the motor output. Interestingly, however, another study by Cowley and Schmidt (1995), showed that bicuculline could disrupt reciprocal inhibition in neonatal rat.

Despite GABA being present within the locomotor CPG of many vertebrates, it may not have a single function in any one species, or indeed throughout the development of any one species. For example, GABA responses are initially depolarising and excitatory in embryonic rats but switch to become inhibitory later in development (for a review: Nishimaru and Kudo, 2000; Clarac *et al*, 2004). In the post-natal rat, the locomotor network is strongly suppressed by activity in GABAergic neurons in the lumbar region of the spinal cord (Cazalets *et al*, 1994). Applications of antagonists to $GABA_{A\text{-}B}$ receptors were found to increase the frequency and amplitude of motor output but did not modify phase relationships between left/right and flexor/extensor units at this stage of development (Cazalets *et al*, 1994). Thus, whilst GABA can shape the on-going motor activity, it does not appear to be involved in mediating reciprocal inhibition between the two locomotor half-centres. Conversely, during

early embryonic life, GABA has been shown to mediate left-right alternation which is subsequently replaced by the traditional core template of reciprocal glycinergic inhibition and glutamatergic excitation (Nakayama *et al*, 2002).

There is evidence in other locomotor systems that glycine and GABA are co-localised in spinal cord axon terminals (e.g. cat (Destombes *et al*, 1992; Ornung *et al*, 1994, 1996); rat (Bohlhalter *et al*, 1994; Todd *et al*, 1996); mouse (Raiteri *et al*, 2001)) and that these two amino acids can be co-released from the same terminal (Jonas *et al*, 1998; Katsurabayashi *et al*, 2004). It is possible that GABA interacts with glycine during the generation and modulation of locomotion, although this possibility has yet to be fully explored. For example, synapses known to release both glycine and GABA are located on the motorneurons in fetal and neonatal rats (Jonas *et al*, 1998; Gao *et al*, 2001). Strychnine applications partially blocked GABAergic synapses in these preparations, thus implicating GABA receptor involvement with the commissural inputs at these stages of development (Jonas *et al*, 1998). Moreover, glutamic acid decarboxylase (GAD) immunoreactivity was found in a substantial number of commissural neurons at early fetal stages which decreased in number towards birth (Phelps *et al*, 1999). Simultaneously, during the late fetal stages, the relative number of GABAergic synaptic inputs to lumbar motorneurons decreased, whilst the number of functional glycinergic synaptic inputs increased (Gao *et al*, 2001). Therefore, it appears that the network shifts during development, favouring glycinergic transmission as the dominant neurotransmitter involved in left/right alternation shortly before birth (Nakayama *et al*, 2002).

In *Xenopus* tadpoles however, co-release of GABA and glycine is unlikely to be occurring as a study by Reith and Sillar (1997) showed that TTX-resistant

spontaneous IPSPs exhibit two over-lapping populations which can be selectively blocked by the glycine and GABA receptor antagonists, strychnine and bicuculline respectively. It has been postulated that GABA may act to slow and prematurely terminate motor activity in the *Xenopus* locomotor network. Thus, GABA plays a major role in the control of swimming duration in both hatchling *Xenopus* embryos and larvae, via related but different mechanisms (Boothby and Roberts, 1992a, 1992b; Reith and Sillar, 1999).

Immunocytochemical staining studies in young *Xenopus* tadpoles show that GABA is extensively distributed throughout the CNS (Roberts *et al*, 1987). In embryos, primary afferents from the rostral 'cement' gland activate GABAergic midhindbrain reticulospinal (mhr) interneurons via a glutamatergic pathway that triggers the release of GABA onto the CPG for swimming, terminating activity when the animal encounters an obstacle (e.g. the meniscus of water (Roberts, 1990; Boothby and Roberts, 1992a)). The cement gland secures the animal temporarily motionless to a surface during its early development, and operates until the animal becomes free-swimming. In the period between hatching and the onset of free-swimming (around larval stage 46), the cement gland degenerates completely and is replaced by an intrinsic stopping mechanism whereby swimming episodes often terminate with a barrage of $GABA_A$-receptor mediated IPSPs onto the spinal network. These IPSPs closely resemble those evoked in stage 37/38 embryos through cement gland stimulation (Reith and Sillar, 1999). As immunocytochemical studies show that mhr neurons are still present in larval stages, it has been suggested that a re-wiring of the circuitry occurs in order to accommodate developmental changes and larval lifestyle (Reith, 1995); the trigger for activation of mhr neurons switches from cement gland

afferents to another, as yet unidentified central pathway, during early larval development.

However, the situation is further complicated because GABA is not exclusive in controlling the duration of swimming episodes in these animals; there are two other proposed mechanisms that cause the slowing and ultimately the termination of swimming in *Xenopus* tadpoles. One mechanism involves the breakdown product of ATP, adenosine, that builds up over the course of a swimming episode. Adenosine depresses voltage-gated Ca^{2+} currents, causing a decrease in motor network excitability, slowing down swimming until its eventual cessation (Dale and Gilday, 1996). A second proposed mechanism comes from the neuromodulation of Ca^{2+}-dependent K^+ currents that shape the firing patterns of individual neurons. A build-up of calcium during swimming from action potentials and NMDA receptor activation gradually increases the activation of these K_{Ca} currents, leading to an increase in the firing threshold of individual neurons which eventually stops swimming (Wall *et al*, 1995).

1 | 7 | 2 Excitation in the spinal motor system

Early work on the lamprey spinal cord suggested that the excitatory drive to each locomotor half-centre involved an excitatory amino acid, presumably glutamate (Cohen and Wallen, 1980; Poon, 1980), which was then found to activate the CPGs via NMDA and non-NMDA glutamate receptors (Alford and Grillner, 1990; for a review: Grillner and Matsushima, 1991). These excitatory amino acid receptors are found to be ubiquitously expressed throughout vertebrates with glutamate being the major excitatory neurotransmitter in the vertebrate nervous system. The activation of

these iGluRs is a recurrent feature of CPGs in many vertebrates (e.g. tadpole (Dale and Roberts, 1984); cat (Brownstone *et al*, 1994); neonatal rat (Cazalets *et al*, 1996)).

In *Xenopus* embryos, glutamate released from spinal premotor excitatory interneurons provides approximately 30% of the excitation which generates the rhythmic motor pattern underlying swimming (Zhao and Roberts, 1998). Equivalent excitatory interneurons provide similar excitatory synaptic drive in the lamprey locomotor network (Dale and Grillner, 1986; Buchanan and Grillner, 1987; Buchanan *et al*, 1989). In both species, the resulting EPSPs are termed 'dual-component' due to the simultaneous activation by glutamate of both NMDA and non-NMDA receptors (Dale and Roberts, 1985; Dale and Grillner, 1986) which leads to both slow rise-and-fall and fast rise-and-fall EPSPs respectively. In *Xenopus*, the NMDA receptor-mediated EPSPs underlie the tonic excitatory drive which persists throughout swimming episodes. These EPSPs are slow rise-and-fall and have a long duration (~200ms) compared to the average length of a swimming cycle (50-100ms). Consequently they summate over several cycle periods to produce a tonic depolarisation of interneurons and motorneurons during swimming (Dale and Roberts, 1985). The non-NMDA receptor-mediated fast rise-and-fall EPSPs, on the other hand, contribute to the phasic excitatory component of the synaptic drive for swimming which triggers the action potentials in each cycle of activity. In the lamprey locomotor network, it has been postulated that the entire range of swimming frequencies can be accounted for by the activation of both NMDA receptors, producing frequencies of 0.1-3Hz, and non-NMDA (kainate) receptors, producing frequencies between 1-8Hz (Grillner and Matsushima, 1991).

It should be noted that in *Xenopus* embryos glutamate cannot account for a significant proportion of the phasic excitatory synaptic drive during swimming, which derives from two additional sources: cholinergic excitation, from the motorneurons (Perrins and Roberts, 1995a, 1995b); and electrotonic coupling, between motorneurons (Perrins and Roberts, 1995a). The majority of excitatory input comes from the electrical coupling between motorneurones, which is thought to contribute up to about 50% of the synaptic drive. These connections are characteristically bi-directional and local (within 70μm of each other; Perrins and Roberts, 1995a), and presumably occur between homonymous motorneurons and with some pre-motor interneurons. The second source also originates directly from the motorneurons which make local cholinergic synaptic connections with each other, and contribute about 20% of the synaptic drive (Perrins and Roberts, 1995a). Thus, in combination, these motorneuron output synapses contribute approximately 70% of the fast excitation underlying motor activity.

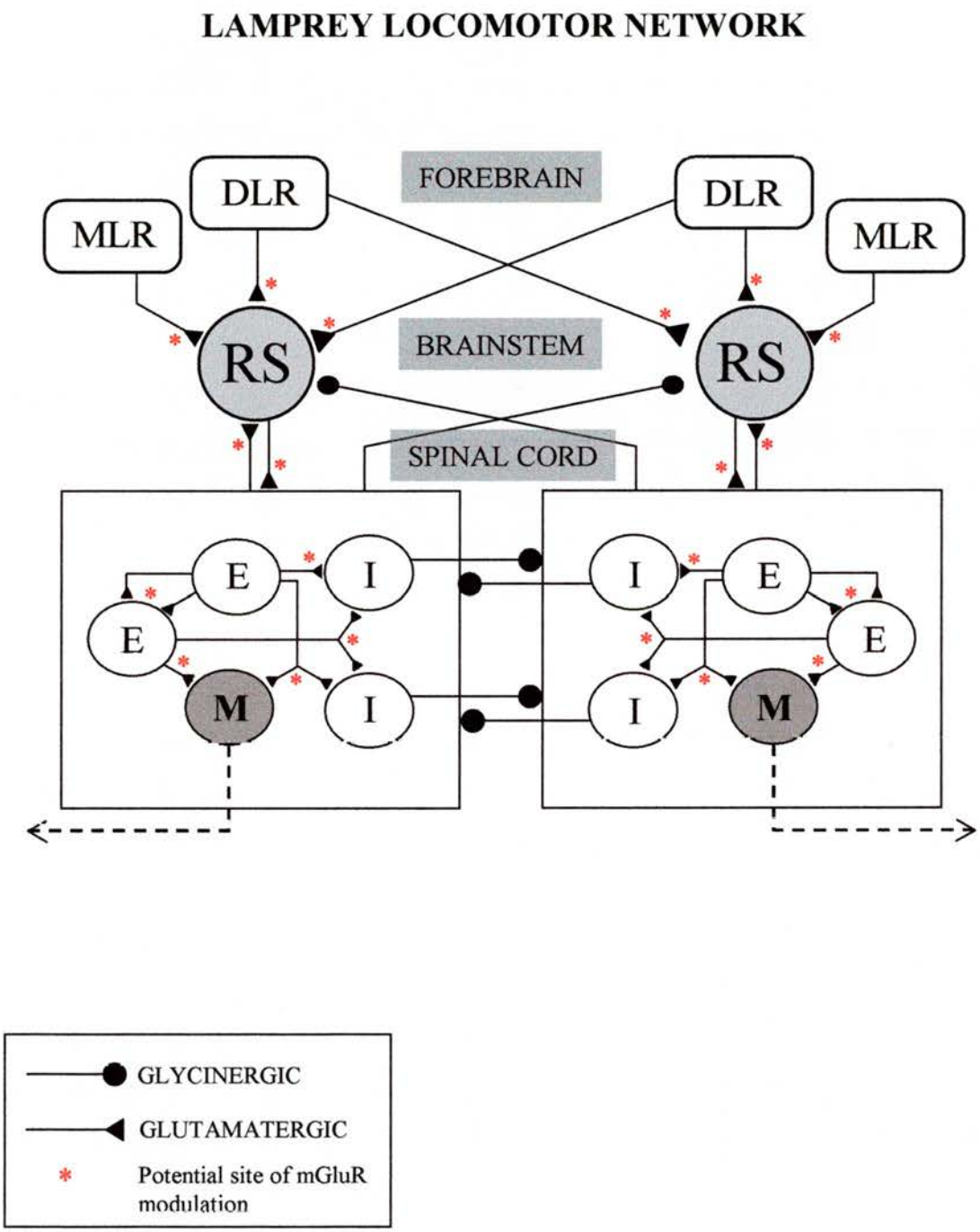
In higher vertebrates, such as the neonatal rat, the exact identity of excitatory interneurons contributing to the excitatory drive has not been fully established, but more recent experiments using genetic markers may aid in the identification of projecting glutamatergic CPG neurons in future (Kiehn and Butt, 2003; Kiehn and Kullander, 2004; Lanuza *et al*, 2004). Nevertheless, it is clear that iGluR activation is the key to activating locomotor CPGs in a range of vertebrates and applications of NMDA are often used to activate motor pattern generation experimentally. In summary, CPG networks underlying locomotion are based upon glutamatergic excitatory and glycinergic inhibitory neurons which provide on-cycle excitation and reciprocal mid-cycle inhibition, respectively, of the motorneurons. Both contributing

sets of neurons, as well as the motorneurons themselves, are potential targets for modulation to shape the motor output (see below).

Classically, glutamate has always been considered a neurotransmitter acting through iGluRs to generate the excitatory drive to initiate and maintain locomotion in vertebrates. However, more recently, the idea of glutamate solely being a neurotransmitter has changed due to the identification and characterisation of mGluRs. The physiological roles of mGluR activation within the complex neuronal systems underlying motor behaviour have only recently begun to be investigated. To date, mGluR-mediated modulation of motor behaviour has been most extensively studied in the lamprey. The synaptic connectivity and membrane properties of the neurons within the lamprey locomotor network have already been well characterised (Buchanan, 1982; Buchanan and Grillner, 1987; for a review: Grillner *et al*, 1998). Locomotion is initiated by the diencephalic and mesopontine locomotor (DLR and MLR) regions of the brain, which make glutamatergic connections onto reticulospinal neurons (RS) (Sirota *et al*, 2000). These RS neurons, in turn, activate the spinal network underlying locomotion via activation of iGluRs located on excitatory and inhibitory interneurons (Figure 1.2; Grillner *et al*, 1997; Orlovsky *et al*, 1999). The excitatory interneurons within the spinal cord similarly make glutamatergic connections onto each other, crossed-caudal inhibitory interneurons and the motorneurons themselves. The numerous glutamatergic connections within the lamprey locomotor network are all potential sources of modulation by mGluRs. Therefore, the lamprey has proven to be an important model system in which to investigate the modulatory effects of mGluR activation on both locomotor network output as well as at the cellular and synaptic levels (Krieger *et al*, 1994).

Figure 1.2 | The lamprey locomotor network

Schematic diagram of the neural circuitry underlying rhythmic swimming activity in the lamprey. The symbols represent populations rather than individual cells. Note that the spinal locomotor CPGs are represented as half-centres. The principle classes of neurons in the circuit: diencephalic and mesopontine (DLR & MLR) locomotor regions receive synaptic inputs from the basal ganglia. These regions excite the reticulospinal neurons (RS) that make glutamatergic excitatory outputs to all spinal interneurons and motor neurons. Reticulospinal neurons are responsible for the initiation of swimming. Excitatory interneurons (E) within the spinal cord excite all types of spinal neuron (equivalent to the dorsolateral interneurons in *Xenopus*; see figures 1.3 and 1.4), including the reciprocal inhibitory interneurons (I) that inhibit the contralateral side, and motorneurons (M). Diagram adapted from Grillner, 2003.


LAMPREY LOCOMOTOR NETWORK
FOREBRAIN
DLR
DLR
MLR
MLR
RS
RS
BRAINSTEM
SPINAL CORD
E
I
I
E
E
E
M
I
I
M
GLYCINERGIC
GLUTAMATERGIC
Potential site of mGluR modulation

Group I mGluR activation following applications of either 3,5-DHPG or 1S,3R-ACPD depolarised spinal neurons inducing an increase in both the burst frequency and amplitude of NMDA-induced depolarisations (Krieger *et al*, 1998). Moreover, recent evidence has implicated the group I subtype $mGluR_1$ as the primary contributor to the observed increase in burst frequency through an inhibition of a leak current probably carried by K^+ (Kettunen *et al*, 2003). Interestingly, cannabinoid receptor antagonists were found to reverse group I effects on lamprey network activity thus implicating their involvement in mGluR-mediated modulation of the spinal motor pattern (Grillner, 2003). Further work revealed that group I receptor activation induces the release of endocannabinoids from post-synaptic neurons in the motor network that act as a retrograde signal to cause a depression of inhibitory transmission onto motorneurons and crossed-caudal interneurons, thereby increasing network excitability (Kettunen *et al*, 2005). Therefore, group I receptors mediate their effect on the lamprey locomotor network through multiple mechanisms which converge to increase excitability.

Conversely, the activation of presynaptic group II and III mGluRs in the lamprey spinal cord reduces transmitter release from descending RS neurons and causes a decrease in network excitability (Herrero *et al*, 1992; Krieger *et al*, 1996). RS neurons are responsible for the initiation of locomotion via the activation of both NMDA and AMPA/KA receptors on (i) excitatory interneurons, (ii) crossed caudal inhibitory interneurons and (iii) motorneurons (Ohta and Grillner, 1989). There is evidence for group III receptors acting as autoreceptors, causing a reduction in excitatory synaptic transmission and, consequently, burst frequency during fictive swimming (Krieger *et al*, 1998, 2000). It has been postulated that the group I receptors drive the locomotor network whilst group III receptors regulate glutamate

release through autoinhibition, yet how the activation of different subtypes is regulated remains to be elucidated. Therefore, it appears that glutamate can act through fast (AMPA/KA), intermediate (NMDA) and slow (mGluR) receptor systems in the modulation of lamprey locomotor behaviour.

The diversity of mGluRs and their anatomical distribution within the lamprey locomotor network provides a wide range of potential modulatory mechanisms by which glutamate can control swimming activity. By comparison, the effects of mGluR activation in the neonatal rat are less well characterised (Taccola *et al*, 2003, 2004a). Group I mGluR activation induces mixed effects on the rat locomotor network, where activation through endogenously released glutamate facilitates activity, whilst activation via exogenous application of DHPG inhibits activity (Marchetti *et al*, 2003; Taccola *et al*, 2004a). Groups II and III, on the other hand, do not appear to be endogenously activated in the rat spinal cord, but do slow locomotor rhythm frequency when activated by exogenous pharmacological agents, potentially through a reduction in synaptic transmission (Taccola *et al*, 2004b).

Until now, the roles of mGluRs in young *Xenopus laevis* tadpoles have, surprisingly, not yet been investigated. Focus turns now to arguably the best understood neuro-behavioural model system for vertebrate locomotion.

1 | 8 *Xenopus laevis*: A model for behavioural studies

The relatively simple nervous system controlling movements in tadpoles of the South African clawed frog, *Xenopus laevis*, has made it an ideal model for studying the neural mechanisms involved in the generation and development of vertebrate locomotion. There are many attractive features of using *Xenopus* as a model in

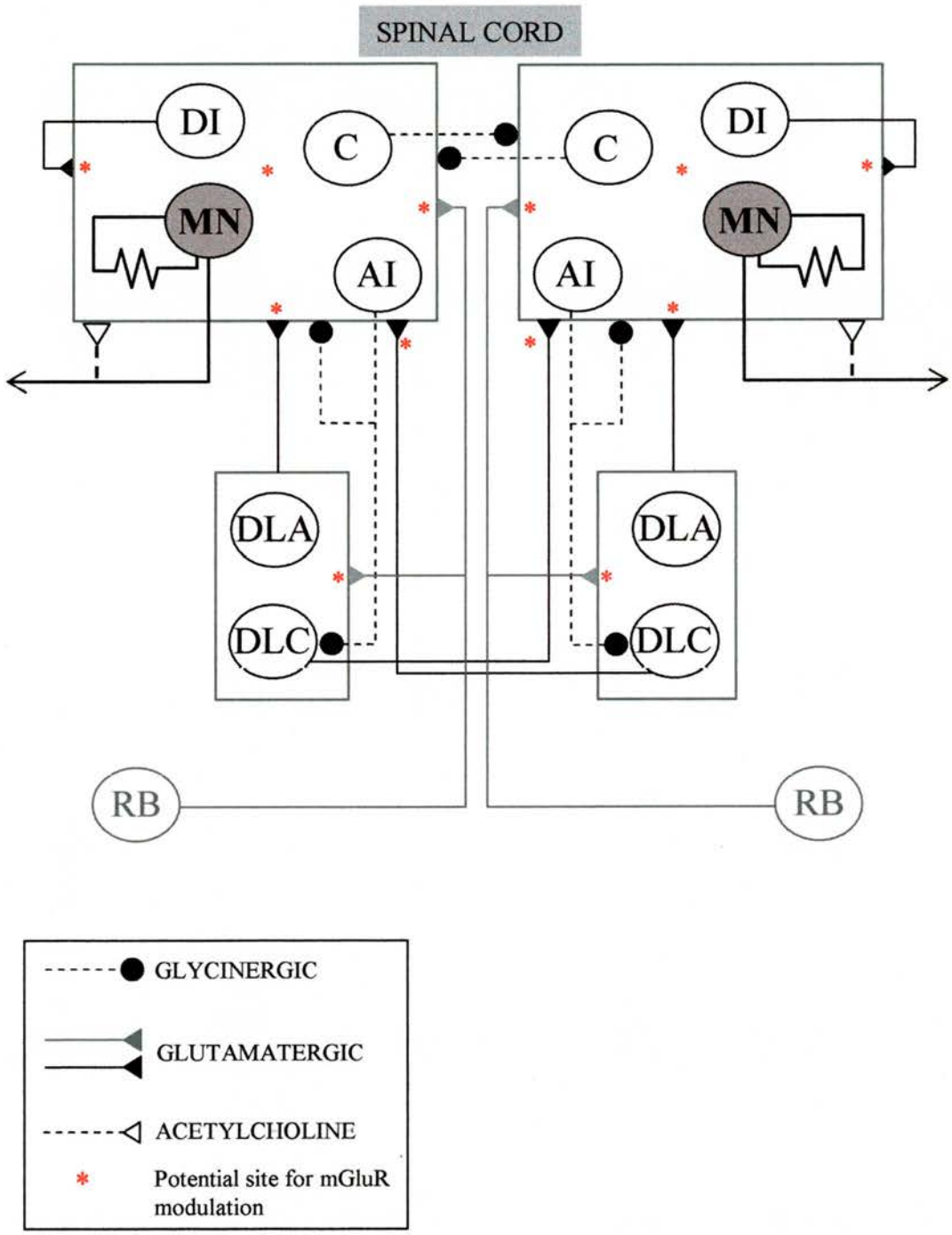
neuroethological studies. Firstly, at the time of hatching (stage 37/38; Nieuwkoop and Faber, 1956) these *Xenopus* embryos possesses only eight classes of differentiated spinal neuron (Figure 1.3 and 1.4; Roberts and Clarke, 1982). They also display a restricted behavioural repertoire, being capable of only three easily identifiable and distinct forms of behaviour: a body flexion/avoidance reflex, struggling, and swimming (Figure 1.5; Kahn *et al*, 1982; Kahn and Roberts, 1982b). The avoidance reflex is the simplest behaviour, elicited when the body is touched and characterised by a bending of the body in the opposite direction. Struggling can be triggered by a prolonged sensory stimulus and is characterised by intense rhythmic coiling of the body with a caudo-rostral delay. Briefer sensory stimuli, on the other hand, induce swimming which exhibits alternating left-right bends in the body that propagate along the length of the spinal cord with a rostrocaudal delay (R-C delay).

Secondly, these behaviours can be easily studied using electrophysiological recordings of neural activity in intact animals that have been immobilised in a neuromuscular blocker and "fictive" activity can then be elicited by sensory stimulation (see next section). Recordings taken during the avoidance reflex show a short-latency ventral root burst in motorneurons opposite to the side of touch, which is then usually followed by the onset of swimming (Figure 1.5; Li *et al*, 2003). On the other hand, struggling is characterised by intense bursts of alternating left/right ventral root activity, which propagates with a caudo-rostral delay. By comparison, swimming exhibits alternating brief ventral root impulses, which propagate with a rostro-caudal delay (Figure 1.5). This model has major advantages over the lamprey or the neonatal rat as it does not require either excision of the spinal cord or bath application of pharmacological excitants to initiate and maintain locomotor activity.

Figure 1.3 | The *Xenopus* locomotor network

Schematic diagram of the neural circuitry underlying rhythmic swimming activity in *Xenopus laevis*. Note that the symbols represent populations rather than individual cells and the spinal locomotor CPGs are represented as half-centres. The principle classes of neuron involved in the circuit: swimming can be initiated following brief electrical stimulation of the skin and subsequent activation of the mechanosensory Rohon-Beard (RB) neurons. These in turn excite dorsolateral commissural (DLC) and ascending interneurons (DLA), which excite contralateral and ipsilateral motor half-centres respectively. The motor half-centres consist of descending (DI) and ascending (AI) interneurons, commissural interneurons (C), and the motorneurons (MN). Glutamatergic excitation within each locomotor half-centre is complimented by motorneuron-to-motorneuron electrical coupling and cholinergic excitation (see main text). Diagram adapted from Roberts *et al*, 1998.

Xenopus LOCOMOTOR NETWORK



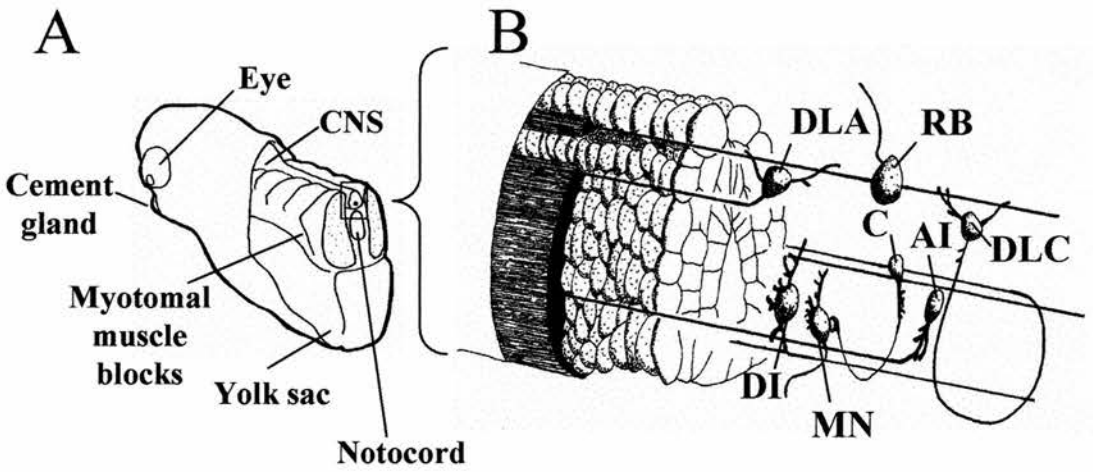
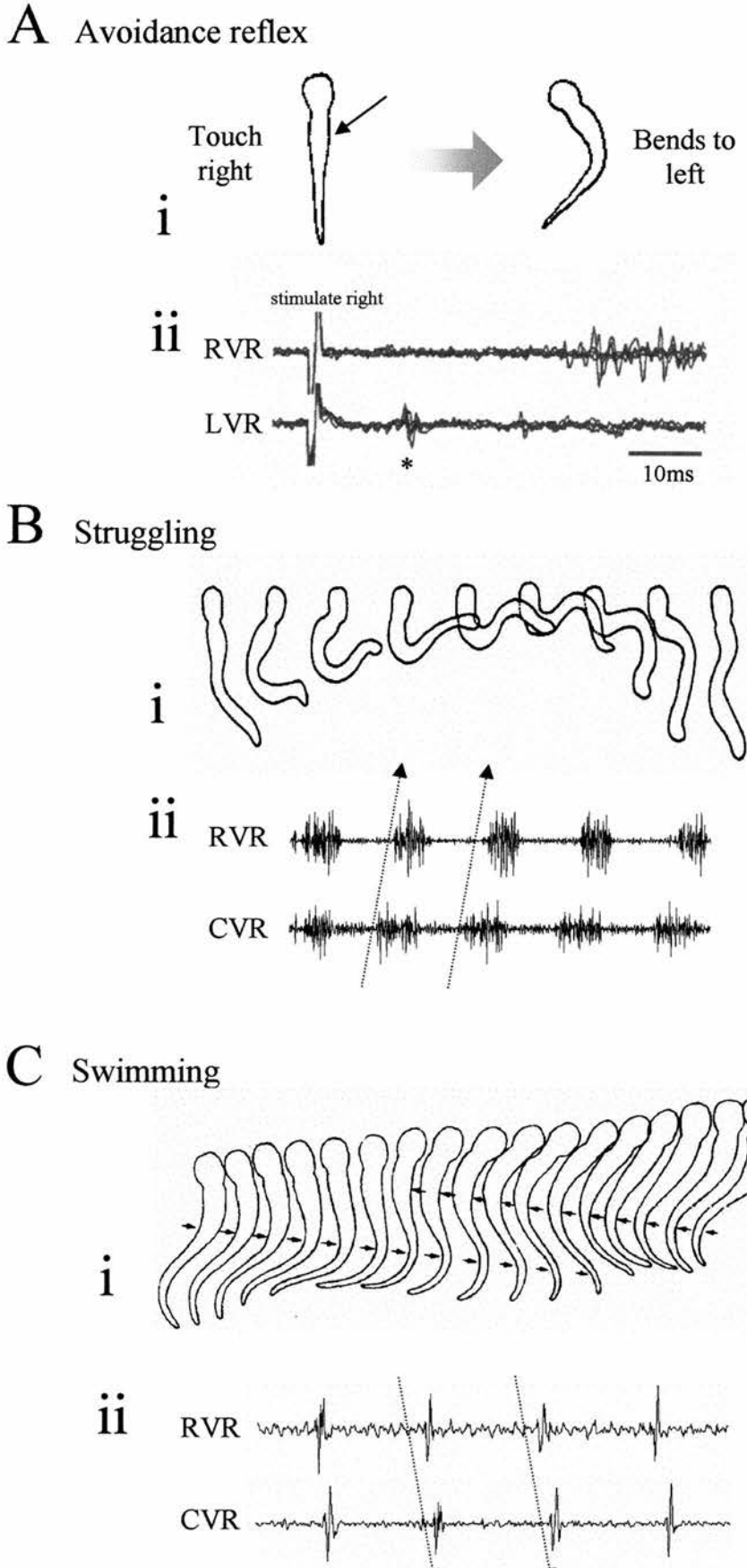


Figure 1.4 | Anatomy of the *Xenopus* spinal cord at stage 37/38

(A) Schematic diagram illustrating the principle anatomical features of the tadpole. (B) Close up illustration showing in detail the principle classes of neurons and their projections within the spinal cord. The sensory neurons: Rohon-Beard (RB), dorsolateral ascending (DLA), and dorsolateral commissural (DLC) interneurons. The neurons of the locomotor network: commissural (C), ascending interneurons (AI), descending interneurons (DI), and motorneurons (MN). The eighth class of neuron not illustrated here are the Kolmer-Agdhur cells. Diagram in (A) taken from McLean *et al*, 2000; Diagram in (B) adapted from original drawing by S.R.Soffe.

Figure 1.5 | *Xenopus* displays distinct behavioural repertoires

The simplest behaviour exhibited by *Xenopus*, the avoidance reflex (Ai), is caused by a touch (solid arrow) to the side of a quiescent animal producing a bending of the body in the opposite direction. (Aii) When stimulated on the right hand side of the body, there is a short-latency ventral root burst (asterisk) in motorneurons on the opposite side of the body that precedes the onset of swimming activity. Struggling behaviour (Bi), is triggered by a more prolonged sensory stimulus involving a characteristically intense ventral root activity (Bii) and a caudo-rostral delay (Bii box). Swimming (Ci) can be initiated by a briefer sensory stimulus than struggling or through the dimming response (see main text), and is characterised by an alternating pattern of ventral root activity propagating down the length of the spinal cord with a brief rostrocaudal delay (Cii & box). RVR, right ventral root; LVR, left ventral root. Schematic diagrams adapted from Sillar and Roberts, 1988 (A) and Kahn & Roberts, 1982 (B,C). Ventral root data in (A) adapted from Li *et al*, 2003; Ventral root data in (B) and (C) courtesy of S.D. Merrywest.


A Avoidance reflex
Touch right
Bends to left
i
ii
stimulate right
RVR
LVR
*
10ms
B Struggling
i
ii
RVR
CVR
C Swimming
i
ii
RVR
CVR

1 | 8 | 1 Sensory pathways involved in the initiation of swimming

Fictive swimming can be elicited by either applying a brief electrical current pulse to the trunk skin or by a dimming of the illumination. A current pulse applied to the skin excites the mechanosensory Rohon-Beard (R-B) neurons which innervate the skin. These R-B neurons excite both dorsolateral commissural (dlc) and dorsolateral ascending (dla) sensory interneurons (Roberts and Clarke, 1982; Roberts, 1990), which in turn activate the motor network. The synaptic connections between R-B sensory neurons and dorsolateral sensory interneurons involved in the activation of swimming are glutamatergic in nature (Sillar and Roberts, 1988; Roberts and Sillar, 1990). The dimming response can also initiate swimming activity and involves a glutamatergic descending pathway from the light-sensitive pineal gland (Roberts, 1978; Foster and Roberts, 1982; Jamieson, 1997). This response is usually more effective in embryos (stage 37/38), compared to larvae (stage 42) where the response becomes unreliable, such that by stage 44 the response has disappeared completely (Boothby and Roberts, 1992b). Changes in illumination cause an excitation of pineal photoreceptors, which activate pineal ganglion cells, which excite the diencephalic/mesencaphalic descending interneurons that in turn activate spinal locomotor networks (Roberts, 1978; Foster and Roberts, 1982; Jamieson, 1997). It should be noted that each of these pathways required for the initiation of locomotion involves a glutamatergic connection which are, therefore, potential targets for modulation by mGluRs.

1 | 8 | 2 Developmental changes in swimming

Xenopus tadpoles are also interesting from a developmental perspective as profound changes in the rhythmic motor output for swimming occur within the first 24 hours after hatching. The motor network in stage 37/38 embryos produces a distinctive

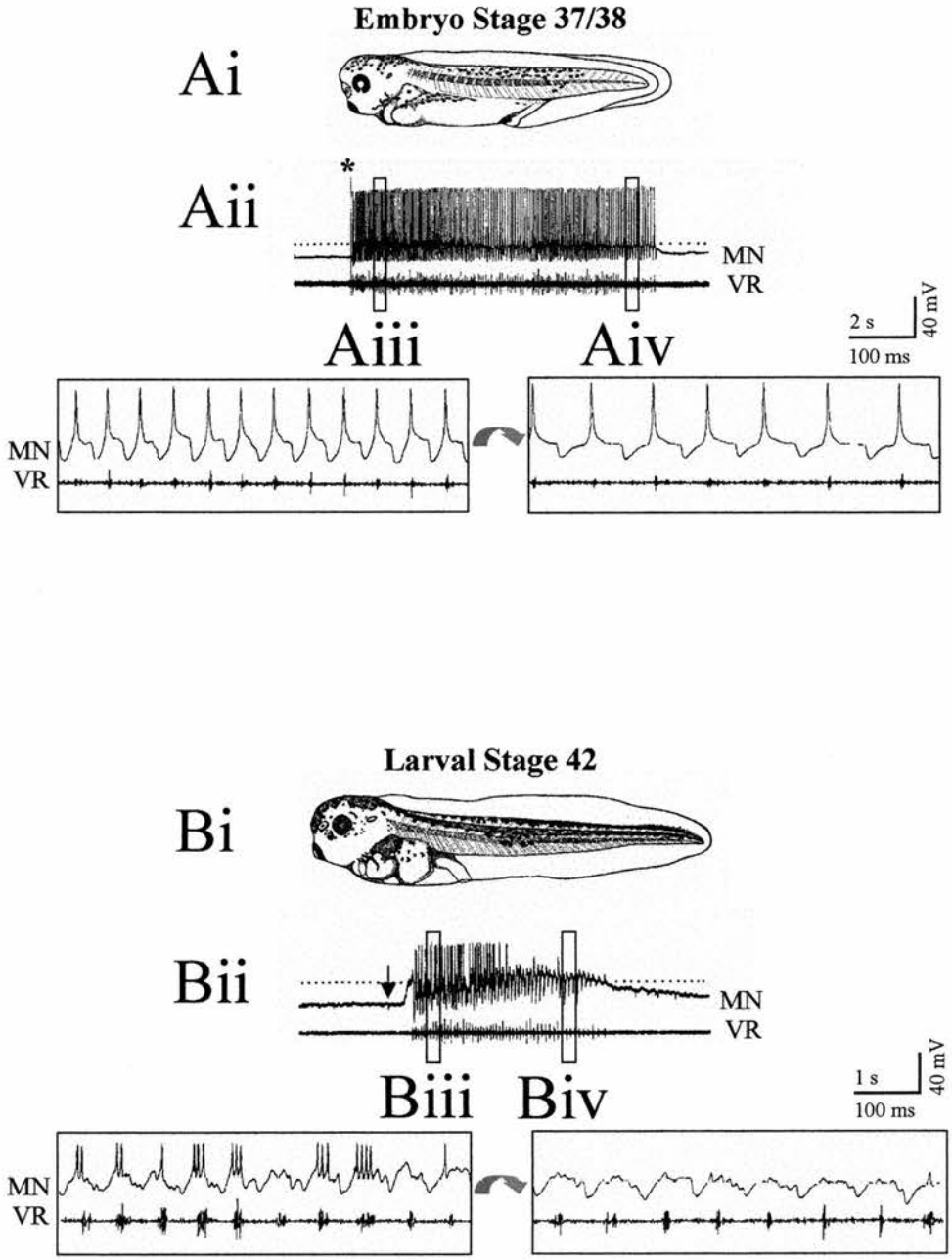
swimming rhythm. Motorneuron firing alternates across the spinal cord and propagates from head to tail, with a brief rostrocaudal delay, at frequencies of 10-20Hz (Figure 1.6; for a review see Roberts *et al*, 1998). The embryonic ventral root burst durations during swimming usually consist of compound impulses of about 7ms duration which comprise activity recorded from a population of ~10 motorneurons exiting any one ventral root and firing only once per swim cycle (Kahn and Roberts, 1982a). Approximately 24-hours later however, at stage 42, larval swimming constitutes ventral root bursts that last ~20ms per swim cycle, but which vary in duration within each swimming episode (Figure 1.6; Sillar and Roberts, 1992) thus affording the animal a greater degree of flexibility in the motor output for swimming compared to embryo.

Developmental changes in the balance of ionic conductances may be responsible for this increased flexibility in neuronal firing properties observed in the larval stage. During the transition period from embryo to larvae the delayed rectifier K^+ currents are down-regulated and the number of K^+ currents that are Ca^{2+}-dependent increases. This both permits repetitive firing properties of larval neurons and aids in the termination of bursts during swimming (Sun and Dale, 1998). However, it is likely that this switch in the balance of ionic currents is not the only contributing factor towards increasing neuronal flexibility. For example, *Xenopus* neural plate neurons *in vitro* were able to express Ca^{2+}-sensitive K^+ currents within hours of dissociation, compared to a few days in the intact animal, suggesting some extrinsic source may contribute to the timing of expression of these currents (Blair *et al*, 1985).

It is interesting that the maturation of the motor system mirrors the development of serotonergic projections from the raphe region of the brain into the spinal cord during

Figure 1.6 | Developmental changes in synaptic output

Stage 37/38 embryo (Ai) and stage 42 larva (Bi) of *Xenopus* showing the characteristic swimming patterns observed at each stage recorded intracellularly from presumed motorneurons using potassium acetate microelectrodes and extracellularly using suction electrodes (A-Bi-iv). (Aii) Stimulus to the skin (asterisk marks stimulus artefact) initiates swimming episodes which terminates after ~5s. Throughout the episode a tonic depolarisation can be observed (dotted line). Excerpts of swimming from the beginning (Aiii) and end (Aiv) of the episode are expanded to show single impulse per cycle in time with the ipsilateral VR discharge and a clear mid-cycle IPSP. (Bii) In stage 42 larva, dimming the lights (arrow) initiates swimming and a sustained tonic depolarisation can be observed over the episode. However, in contrast to stage 37/38, up to four impulses per cycle can be observed towards the start of the episode (Biii), and towards the end of the episode, no impulses are recorded per cycle (Biv). MN, motorneuron; VR ventral root. Adapted from Sillar *et al*, 1992.


Embryo Stage 37/38
Ai
Aii
*
MN
VR
2 s
40 mV
Aiii
Aiv
100 ms
MN
VR
Larval Stage 42
Bi
Bii
MN
VR
1 s
40 mV
Biii
Biv
100 ms
MN
VR

early larval development (Sillar and Roberts, 1992; Wedderburn and Sillar, 1994; Sillar *et al*, 1995). Larvae at about 12 hours post-hatching show an intermediate type of motor rhythm with 'bursty' stage 42 rostral ventral root activity and briefer, embryonic-like ventral root activity more caudally down the spinal cord (Sillar *et al*, 1991). This 'bursty' larval swimming can also be reverted back to a more embryonic-like pattern if 5-HT receptors are blocked, underlining the networks dependence on this amine to sustain a more mature and flexible swimming output (Sillar *et al*, 1995).

It has long been recognised that motor neurons do not just show passive membrane response properties and that motor activity can be shaped by more than just synaptic connectivity alone (for a review: Kiehn, 1991). Complex and voltage-dependent, non-linear intrinsic membrane properties contribute significantly to the shaping and timing of the motor output. In the lamprey spinal cord, for example, bistable membrane behaviour of spinal neurons in the presence of NMDA results from a region of negative slope resistance in the current-voltage relation of the neuron which permits individual neurons to generate self-sustaining membrane potential oscillations under certain experimental conditions (Wallen *et al*, 1987). This 'conditional' oscillatory membrane behaviour is mainly attributable to the voltage-dependent properties of the NMDA receptor; the underlying mechanism revolves around the cyclical voltage-dependent blocking and unblocking of the NMDA receptor ionophore by magnesium. Firstly, depolarisation of the neuron enables a gradual release from the magnesium block allowing regenerative depolarisation; secondly, activation of voltage-dependent K^+ channels and Ca^{2+} entry through the NMDA receptor activating Ca^{2+}-dependent K^+ channels slowly repolarises the neuron towards a voltage region where the regenerative magnesium block can occur and the membrane hyperpolarises beginning the cycle again (Wallen *et al*, 1987).

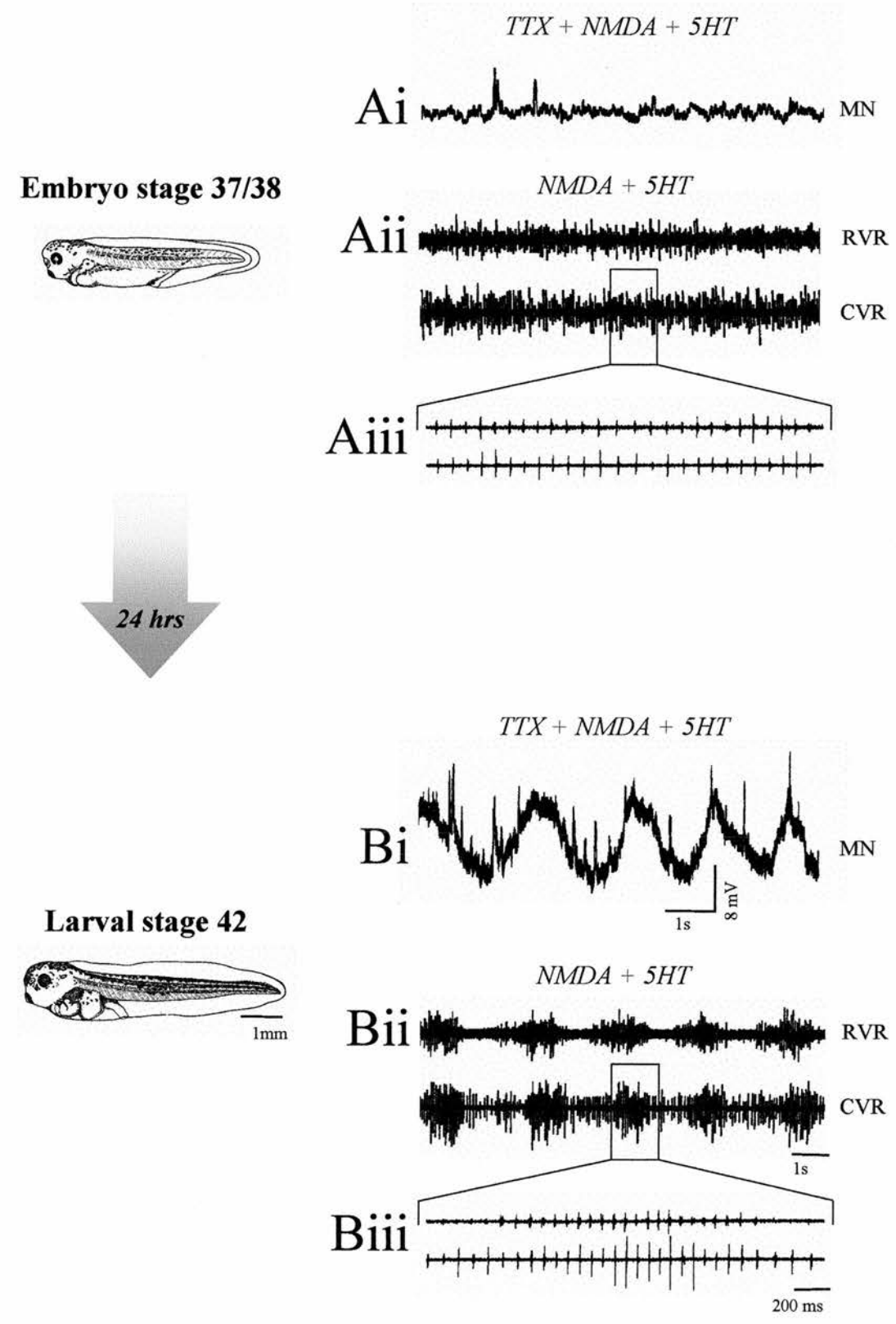
In *Xenopus* embryo spinal neurons, such properties appear to be absent despite the presence of NMDA receptors, which are known to be blocked by magnesium ions in a voltage-dependent manner (Soffe and Roberts, 1989). However, in stage 42 larvae, oscillatory behaviour can be induced by the co-application of 5-HT and NMDA (Figure 1.7; Scrymgeour-Wedderburn *et al*, 1997). This co-dependence on NMDA and 5-HT for the expression of voltage oscillations has also been reported in the neonatal rat (Cazalets *et al*, 1992; MacLean *et al*, 1997, 1998; Schmidt *et al*, 1998), and in the related anuran species, *Rana temporaria* (Sillar and Simmers, 1994a). Therefore, this developmental emergence of membrane potential oscillations may depend upon the post-embryonic in-growth of functional serotonergic projections from the brainstem into the cord during early larval life (Sillar *et al*, 1995). The physiological role these oscillations play in normal behaviour has yet to be determined. Potentially, the release of 5-HT onto the swimming network could induce a temporary but immediate transition to fast intense bursts of neural activity, which may serve as a boosting mechanism during escape responses. Long-lasting plateau potentials recorded in cat and turtle motorneurons are similarly dependent upon 5-HT (Hounsgaard *et al*, 1988; Hounsgaard and Kiehn, 1989), though in these instances they may well be involved in postural control.

1 | 8 | 3 Neuromodulation of motor output

For an organism to survive in a perpetually changing environment it must be able to adjust its behavioural output and this requires an inherent flexibility of underlying locomotor neural circuits. Flexibility in locomotor output can be accomplished through the neuromodulation of the motor networks (Harris-Warrick, 1991; Pearson, 1993), which goes against the traditional view that neuronal networks are 'hard-

Figure 1.7 | 5-HT-induced NMDA receptor-mediated voltage oscillations in *Xenopus laevis*

(Ai) TTX-resistant intrinsic voltage oscillations in motorneurons are absent in embryonic (stage 37/38) *Xenopus* following application of NMDA and 5-HT. (Aii) In the absence of TTX, NMDA and 5-HT trigger ventral root activity which remains a relatively constant amplitude and frequency (expanded out in Aiii). (Bi) Application of NMDA and 5-HT in larval (stage 42) *Xenopus* reveals pronounced TTX-resistant membrane voltage oscillations. (Bii) In the absence of TTX, NMDA and 5-HT applications trigger fluctuations in the amplitude and frequency of ventral root activity which correspond temporally with the underlying intrinsic oscillations (expanded out in Biii). MN, motorneuron; RVR, rostral ventral root; CVR, caudal ventral root. Adapted from Reith and Sillar, 1998; Scrymgeour-Wedderburn *et al*, 1997.

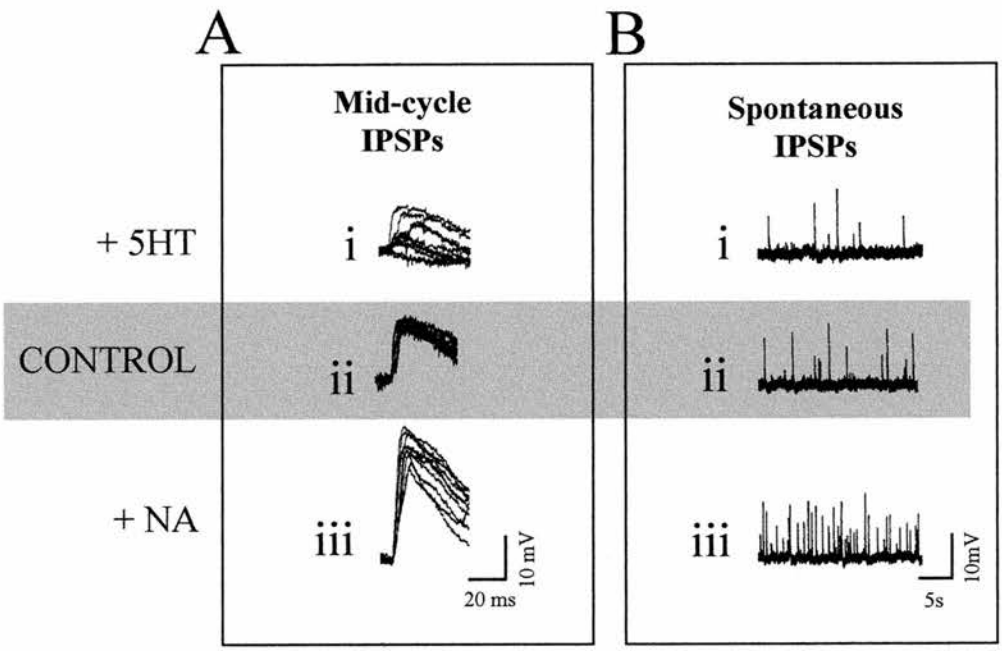

TTX + NMDA + 5HT
Ai
MN
Embryo stage 37/38
NMDA + 5HT
Aii
RVR
CVR
Aiii
24 hrs
TTX + NMDA + 5HT
Bi
MN
8 mV
1s
Larval stage 42
1mm
NMDA + 5HT
Bii
RVR
CVR
1s
Biii
200 ms

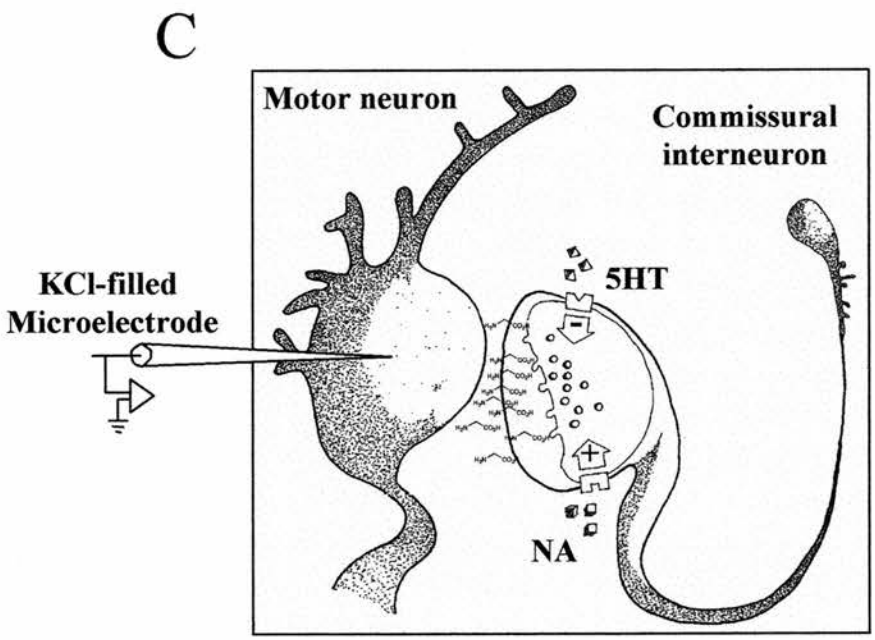
wired' circuits. In principle, neuromodulation can be described as: extrinsic, where the circuit is influenced from sources not themselves part of the CPG; or intrinsic, where the circuit is influenced by components of the CPG as a result of CPG activity (for a review: Katz, 1995).

Several neurochemicals have been shown to be important extrinsic neuromodulators of motor activity in *Xenopus*, including the monoamines 5-hydroxytryptophan (5-HT) and noradrenaline (NA), and more recently, the free radical gas nitric oxide (NO) (Sillar and Simmers, 1994b; McDearmid *et al*, 1997; Sillar *et al*, 1998; McLean and Sillar, 2000). As discussed above, 5-HT imparts greater flexibility to the motor pattern during development, but it can also actively shape the on-going motor output, for instance, through the induction of oscillatory membrane properties. In addition, both 5-HT and NA appear to modulate swimming by differentially regulating glycine release from commissural interneurons via a pre-synaptic mechanism (McDearmid *et al*, 1997; Figure 1.8). In this way, these two biogenic amines control the intensity of motor output and swimming frequency through their actions on the mid-cycle inhibitory component of the synaptic drive. NA increases the mid-cycle IPSP amplitude, resulting in an increase in cycle periods and hence a reduction in swimming frequency. 5-HT has the opposite effect of reducing the mid-cycle IPSP amplitude, contributing to swimming becoming more intense (McDearmid *et al*, 1997; Figure 1.8). More recent evidence suggests that NA reduces the duration of swimming episodes through the potentiation of GABAergic transmission, as observed by an increase in the number of $GABA_A$-receptor mediated IPSPs coinciding with the termination of swimming (Fischer *et al*, 2001; Merrywest *et al*, 2002). Moreover, NA may also control swim frequency through modulating GABAergic transmission, as cycle periods increase following NA application in the presence of strychnine. This

Figure 1.8 | Differential modulation of glycinergic transmission by the monoamines, 5-HT and NA

The biogenic amines 5-HT and NA differentially modulate glycinergic inhibition in the *Xenopus* spinal cord. (Ai-ii,Bi-ii) 5-HT depresses the mid-cycle IPSP amplitude during swimming and reduces the rate of TTX-resistant spontaneous inhibitory transmitter release. (Aii-iii,Bii-iii) NA has the opposite effect, it facilitates the amplitude of mid-cycle IPSPs and increases spontaneous transmitter release rate. The use of KCl-filled microelectrodes during intracellular recordings make these IPSPs strongly depolarising due to chloride leakage into the cell. (C) Schematic diagram (taken from McLean *et al*, 2000), showing the singular synaptic contact between a commissural interneuron and motorneuron, illustrating the opposing effects of 5-HT and NA on pre-synaptic glycinergic release. Diagram in (A) and (B) adapted from McDearmid *et al.*, 1997.


A
B
Mid-cycle
IPSPs
Spontaneous
IPSPs
+ 5HT
CONTROL
+ NA
i
ii
iii
10 mV
20 ms
10mV
5s


C
Motor neuron
Commissural
interneuron
KCl-filled
Microelectrode
5HT
NA

effect on cycle periods can be blocked by the $GABA_A$ antagonist, bicuculline (Merrywest *et al*, 2002).

In addition to the monoamines modulating swimming, several recent publications suggest NO exerts similar actions to NA, inducing a net inhibitory effect on the spinal locomotor network and acts as a "brake" to slow down swimming and prematurely terminate swimming episodes (McLean and Sillar, 2000, 2002; McLean, 2001). NO is also thought to act presynaptically to facilitate transmission at glycinergic and GABAergic synapses with little or no effect on excitatory transmission (McLean and Sillar, 2000). It has also been proposed that NO could be acting as a "metamodulator", by modulating the activity of aminergic neurons which then subsequently modulate glycinergic synapses (McLean and Sillar, 2004). NO also appears to work postsynaptically, as applications of NO donors such as SNAP revealed a pronounced membrane potential depolarisation and conductance decrease in motorneurons which would be consistent with the closure of a positively charged outward current, such as a K^+ conductance (McLean and Sillar, 2002). These combined pre- and post-synaptic effects of NO provide a mechanism to fine-tune inhibitory synapses in the modulation of the locomotor pattern in *Xenopus* (McLean and Sillar, 2002).

The most likely sources of 5-HT, NA and NO are located in clusters of neurons in the brainstem, indicating that these substances act as extrinsic neuromodulators of the spinal motor network. By comparison, intrinsic neuromodulation of the motor circuit involves the release of neuromodulators as a direct result of network activity. In *Xenopus*, intrinsic neuromodulation by purinergic neurotransmitters is a well documented example (Dale and Gilday, 1996; Dale, 1998, 2002; Brown and Dale,

2000, 2002). As mentioned previously, the purinergic transmitters ATP and adenosine, can control the duration of swimming episodes. ATP is thought to be released from neurons that form part of the network and is then broken down over the course of a swimming episode by ectonucleotidases, producing adenosine as a by-product. Adenosine then activates P1 receptors which cause a reduction in voltage-gated Ca^{2+} currents, lowering excitability of the motor circuit, opposing the actions of ATP, and thus slowing swimming until it eventually stops (Dale and Gilday, 1996). Therefore a neuromodulator produced by the motor network is able to self-terminate its own motor output.

Given the knowledge that excitatory interneurons of the spinal CPG activate iGluRs, it is possible that glutamate may also act as an 'intrinsic' neuromodulator in the *Xenopus* spinal cord via activation of mGluRs. It should be noted however that the classification of 'intrinsic neuromodulator' can only be used loosely in this instance, as glutamate could also function as an 'extrinsic neuromodulator'. For example, the glutamatergic RS neurons in the lamprey locomotor network initiate swimming activity and can be modulated by mGluR activation, although strictly speaking they are not part of the spinal locomotor CPG. In *Xenopus*, mGluRs could also function at multiple locations given the likely widespread distribution of the neurotransmitter (See figure 1.3 red asterisks). MGluRs could act intrinsically at the level of either the excitatory or inhibitory interneurons within the locomotor network, enhancing or suppressing transmission to directly modulate motor output. Similarly, these receptors could act extrinsically, modulating excitation or inhibition within the brainstem, to indirectly affect the locomotor pattern.

1 | 9 Scope of the present study

In the following, chapters I investigate whether the main excitatory neurotransmitter, glutamate, through its actions at metabotropic glutamate receptors is able to act as a neuromodulator of the neural network underlying swimming. I will provide: (1) a description of the effects on rhythmic motor activity of all 3 mGluR groups; (2) more specifically, evidence that group I mGluRs potentiate swimming frequency in part through a reduction in inhibitory transmission; (3) evidence that group II receptors depress motor activity without influencing the fast inhibitory pathways; (4) data revealing that group III mGluRs produce a strong inhibitory effect on both motor and sensory pathways, possibly through multiple mechanisms; (5) a general discussion exploring these results in light of recent findings in other species and proposed future directions for work in the *Xenopus* model.

2 | Materials and Methods

2 | 1 Animals

Experiments were performed on stage 37/38 embryos and stage 42 larvae of the South African Clawed frog, *Xenopus laevis* (staged according to Nieuwkoop and Faber, 1956). *Xenopus* tadpoles were obtained by induced breeding following injection of human chorionic gonadotrophin (HCG; 1000 U/ml, Sigma) into pairs of adults from a laboratory colony. Male and female frogs were injected in the afternoon with 0.3ml and 0.7ml HCG, respectively, by personnel holding current Home Office Personal Licences. Eggs were collected the following day and raised in dechlorinated/oxygenated tap water at 17-23°C until the preferred developmental stages were reached. Tadpoles usually develop to stage 37/38 in ~53.5hrs, and stage 42 in ~87hrs at 23°C (Nieuwkoop and Faber, 1956). By placing trays of eggs at different temperatures their rates of development were controlled to ensure a daily supply of experimental animals at the desired stages. The two developmental stages used can be easily distinguished by the size of their body and yolk sac alone. Embryos are ~5-6mm in length and have a yolk sac that is about half the length of their body (Figure 2.1A). Larvae, on the other hand, are ~7-8mm in length, and the yolk sac is about 1mm in length.

2 | 2 Experimental preparation and electrophysiology

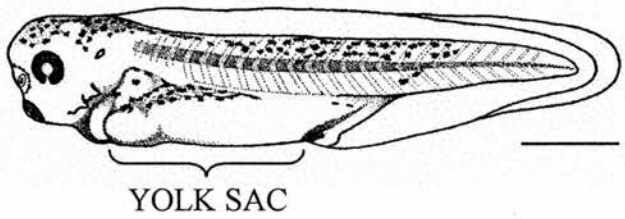
Animals were immobilized by having their tail skin slashed along the dorsal fin with tungsten needles (to aid drug access) and then placed in a chamber containing 2mls of

Figure 2.1 | The *Xenopus* tadpole and experimental preparation

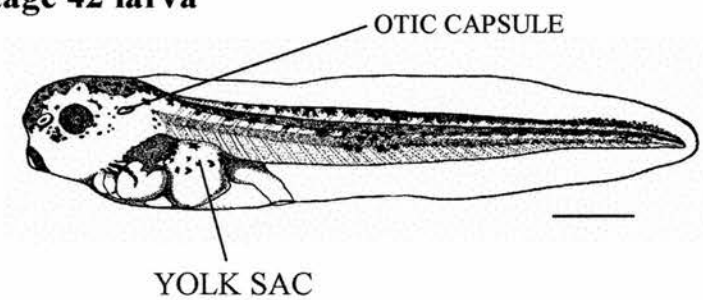
(A) The two developmental stages of *Xenopus* tadpole used in this study. Stage 37/38 embryos are ~5-6mm in length with a large yolk sac, and stage 42 larvae are ~7-8mm in length with a smaller yolk sac compared to embryos. (B) The *Xenopus* tadpole experimental preparation showing placement of the stimulating electrode on the tail skin; two extracellular suction electrodes placed rostrally and caudally over the myotomal clefts, and a sharp microelectrode positioned ventrolaterally over the spinal cord for intracellular recordings. VR = ventral root. Scale bars represent 1mm.

A

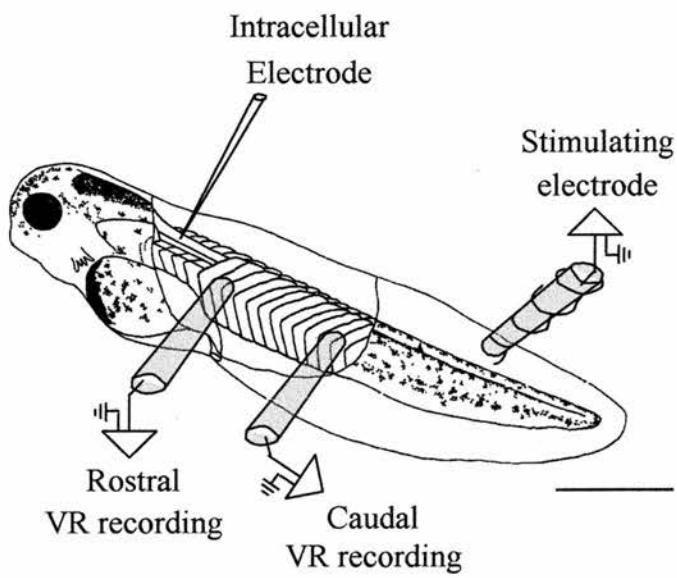
Stage 37/38 embryo



Stage 42 larva



B



α-bungarotoxin ($10\mu moll^{-1}$). Complete immobilisation took approximately 1hr. Animals were then removed from the α-bungarotoxin and transferred to an experimental chamber (~2ml volume). The chamber was perfused with recirculating frog Ringer (ionic composition in $mmoll^{-1}$: NaCl, 120; KCl, 2.5; $CaCl_2$, 2 (for extracellular experiments) or 4 (for intracellular experiments); $MgCl_2$, 1; $NaHCO_3$, 2.5; Hepes, 10; pH 7.4) that was gravity-fed from a 10ml header tank via a stock bottle containing 100ml of saline. Animals were secured on their side with two finely etched tungsten pins (through the notochord) to the Sylgard-coated surface of a rotatable Perspex platform within the chamber. The pins were positioned rostrally near the otic capsule, and caudally about midway along the length of the body. Next, the flank skin was removed using tungsten needles to reveal the myotomal swimming muscles underneath. Extracellular recordings of ventral root activity were made by placing glass suction electrodes (~50μm tip opening) over the exposed intermyotomal clefts wherein lie the axons of the spinal motorneurons (Figure 2.1B). The extracellular suction electrodes were hand-pulled over a microbunsen flame using 1mm-diameter fibreless borosilicate glass (Harvard Apparatus), which was then cut to the desired tip opening size with a diamond knife.

For intracellular recordings of individual neuron activity, tungsten needles were first used to remove a block of myotomes so as to expose the underlying spinal cord. This was achieved by first scoring rostrocaudally along the myotomes with a tungsten needle at the level of the notocord, from the region just caudal to the otic capsule down to approximately the 7^{th} or 8^{th} myotome, and then gently inserting the needle between the myotomes and spinal cord, thus teasing the muscle block away from the cord. Recordings were then made with glass microelectrodes pulled on a Campden (model 753) or a Sutter (P2000) microelectrode puller (Figure 2.1B). All recordings

were made from rhythmically active cells in the ventrolateral quarter of the spinal cord where motorneurons are known to predominate (Roberts and Clarke, 1982). These cells were, therefore, presumed to be motorneurons. Neurons were penetrated using capacity overcompensation and intracellular signals were amplified (x10) using a purpose built amplifier (courtesy of S.R. Soffe, University of Bristol). Neurons were considered to be successfully penetrated when the membrane potential stabilised below -50mV for ~5mins and the membrane potential did not fluctuate during this time. Microelectrodes were filled with 2M potassium chloride (KCl) and had DC resistances of 100-150MΩ. Chloride dependent IPSPs are reversed in sign to become strongly depolarising when recording with these electrodes, presumably due to chloride leakage into the cell. This causes the intracellular chloride concentration to increase to the extent that it exceeds the extracellular concentration. Therefore, when transmitter-gated chloride channels open, Cl^- leaves the cell to produce a depolarisation as Cl^- ions are driven towards their new equilibrium potential. An injection of depolarising current into the cell can reverse these depolarising IPSPs.

Fictive swimming episodes were evoked either by a 1ms current pulse applied with a Digitimer DS2 isolated stimulator to the tail skin via a glass suction electrode (c.f. Clarke, 1984) or by dimming the illumination (Roberts, 1978). The stimulating electrode was hand-pulled in the same manner as the extracellular recording electrodes described above. Current pulses were transmitted via a copper wire, which was coiled around the length of the electrode shaft down to the tip opening. By minimising the distance between the electrode tip and the cut end of the copper wire, stimulus artefacts were also minimised.

Known concentrations of pharmacological agents were added to the stock bottle (100ml) and agitated to achieve the desired final concentration. The following table details the drugs used in this study.

DRUG	CONCENTRATION (μM)	DESCRIPTION	SOURCE
DHPG	10	Group I specific agonist	TOCRIS
AIDA	100	Group I general antagonist	TOCRIS
L-AHM	100	Glutamate uptake inhibitor	TOCRIS
CPCCOEt	50	$mGluR_1$ specific antagonist	TOCRIS
MPEP	100	$mGluR_5$ specific antagonist	TOCRIS
APDC	100	Group II specific agonist	TOCRIS
EGLU	100	Group II specific antagonist	TOCRIS
L-AP4	1-50	Group III specific agonist	TOCRIS
MAP4	100	Group III specific antagonist	TOCRIS
Strychnine	1	Glycine receptor antagonist	Sigma
Bicuculline	40	$GABA_A$ receptor antagonist	Sigma
TTX	1	Na^+ channel blocker	Sigma

2 | 3 Data collection and analysis

All electrophysiological data was recorded via a Digidata 1322A analogue to digital converter (Axon Instruments) and analysed using Dataview software (courtesy of W.J. Heitler, University of St.Andrews; version 1.2h). The first 500ms of each episode of swimming activity was excluded from analysis to rule out any contribution from sensory stimulus evoked potentials (which last ~200ms in duration; Sillar and

Roberts, 1988). Unless stated otherwise, all samples of swimming analysed are taken 500ms from the onset of swimming and measurements comprised thirty consecutive cycles of motor activity from at least 4 episodes of swimming. Where stated, data for experiments was pooled and some figures show 'representative' individual experiments where ventral root data or intracellular recordings are shown.

For statistical analyses, comparisons with equal variances were carried out using One-way Analysis of Variance (ANOVA) and where data was not normally distributed according to Anderson-Darling tests, Kruskal-Wallis tests were used. Normally distributed parametric data are presented as means and standard error of the mean. Non-parametric data are presented as medians and the standard errors bars represent the difference between lower and upper quartile ranges (Inter-quartile range, I.Q.R.). Statistical analysis was computer aided (Minitab, version 13). Statistics were considered to be significant at $P<0.05$. n equals the number of animals.

Extracellular measurements:

Measurements were normally taken 15-20mins after drug applications to allow maximum drug penetration. The following parameters of the fictive swimming rhythm (Figure 2.2) were measured: (a) *burst durations (ms)* - the duration of the discrete burst of ventral root activity within each cycle period; (b) *burst amplitudes (mV)* - measured from the peak to the trough of the ventral root burst; (c) *cycle periods (ms)* - measured as the interval from the start of the burst in one cycle to the start of the burst in the next cycle; (d) *rostrocaudal delays (ms)* - measured as the interval between ipsilateral ventral root bursts, one recorded rostrally (5-6 clefts in from the otic capsule) and another recorded at least 5 clefts more caudally; and (e) *episode durations (s)* - measured from the first to the last motor burst observed during

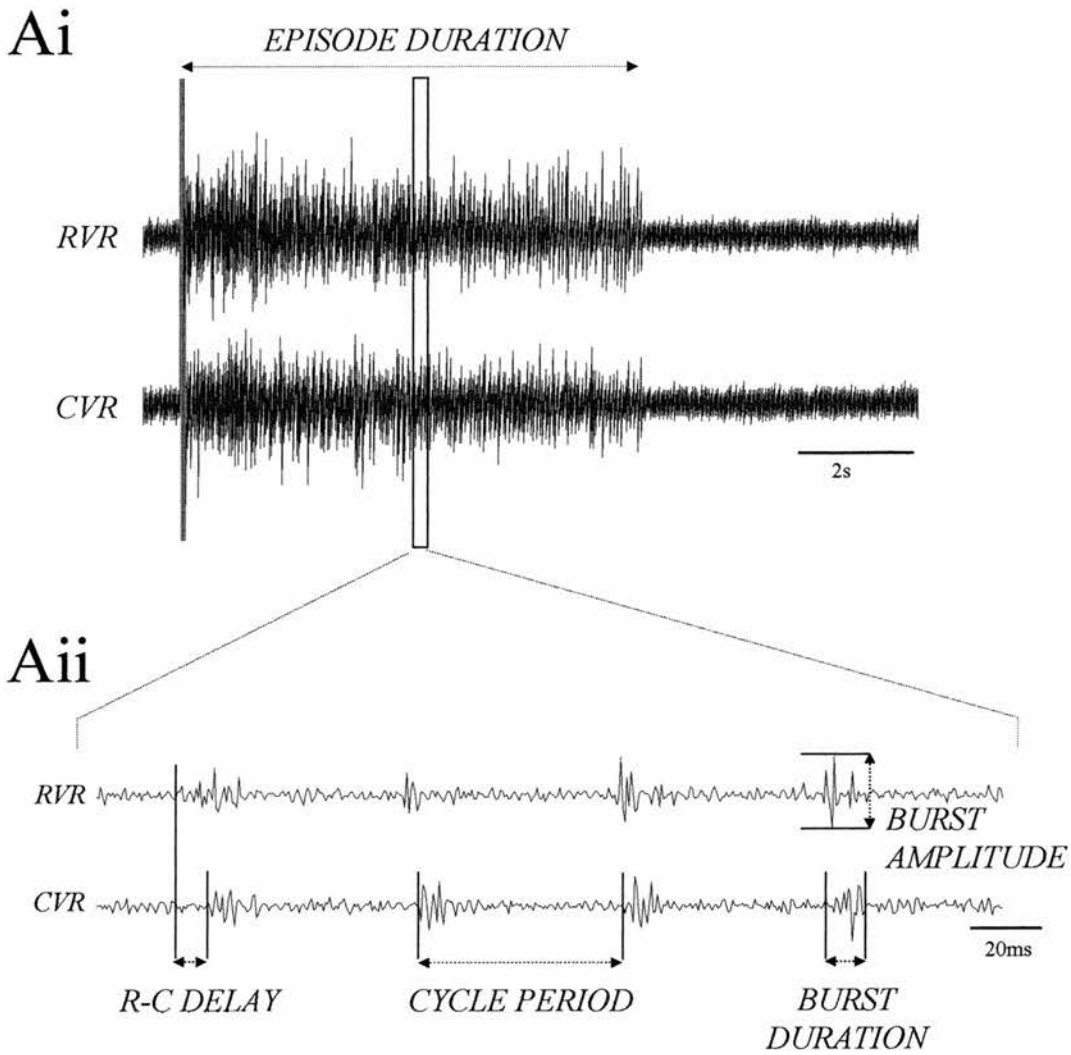


Figure 2.2 | Extracellular measurements of fictive swimming parameters

(Ai) Extracellular recordings showing a whole episode of swimming from a rostral ventral root (RVR) and a more caudal ventral root (CVR). (Aii) Trace expanded out from (Ai) showing ventral root activity in detail. Parameters measured include the cycle period (ms), rostrocaudal delay (R-C delay; ms), burst duration (ms) and burst amplitude (mV). See text for fuller description.

an episode. Possible drug effects on the sensitivity of sensory pathways were assessed in the following manner: (a) *skin threshold* - stepwise 1V increments in the voltage of constant current pulses were delivered to the skin until swimming was evoked; (b) *dimming response* - effects of drug applications on the response to a change in illumination were monitored throughout all experiments by turning off the light source to the Perspex chamber during quiescent periods.

Intracellular measurements:

During intracellular recordings (Figure 2.3) the following measurements were taken, again 15-20mins after drug applications: (a) *Conductance pulses* - 100ms hyperpolarising test current pulses, administered once every second, were applied to assess changes in ionic conductance under each condition. Means of the voltage responses of the neuron were calculated from 5 measurements within each condition. (b) *Calculating changes in mid-cycle IPSP amplitude* - the amplitude was calculated by subtracting the resting membrane potential from the peak amplitude. Swimming excerpts were selected in these instances so that the frequency of swimming was approximately the same under each condition, i.e. the excerpts were frequency matched. This permits a more accurate comparison of changes in the inhibitory drive which should otherwise be roughly the same at similar frequencies. Mean IPSP amplitudes were then calculated from 30 measurements (10 consecutive cycles from each of 3 episodes) under each condition and expressed as a percentage of control. (c) *Calculating the frequency and amplitude of spontaneous IPSPs* - the frequency was calculated by measuring the number of sIPSPs within 5 selected 20s periods under each condition, pooled and converted to hertz. The percentage occurrence of sIPSP amplitudes categorised into 1mV bins from the 5 selected 20s periods were collated and graphed so any changes in distribution can be monitored. The sIPSP frequencies

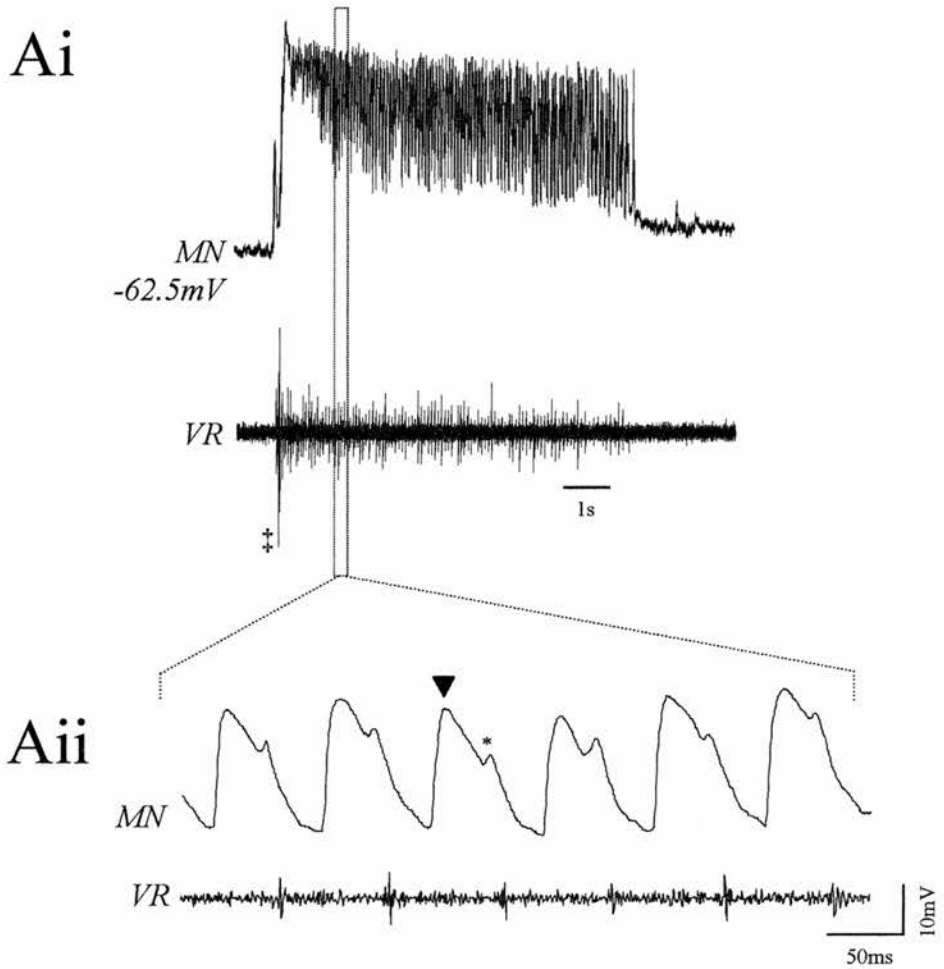


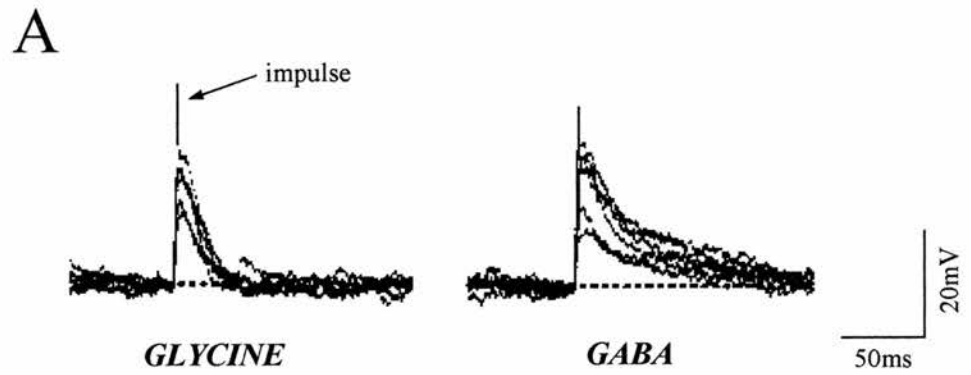
Figure 2.3 | Intracellular measurements of fictive swimming parameters

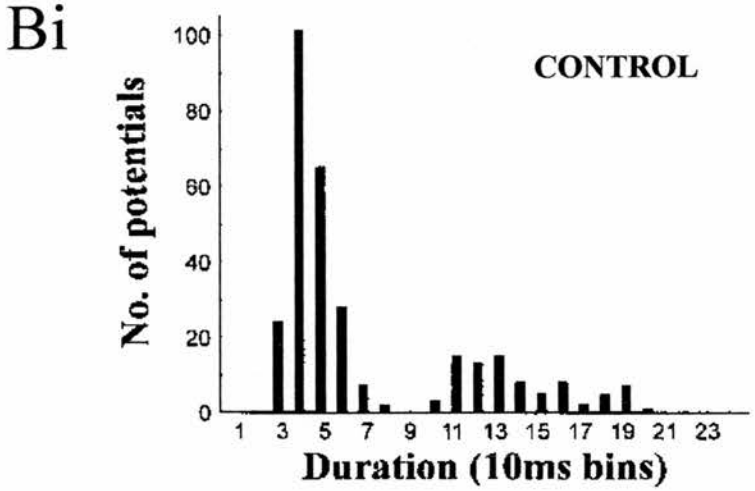
(Ai) Intracellular recording from presumed motorneuron using KCl microelectrode with corresponding ventral root recording measured extracellularly. (Aii) Trace expanded out from (Ai) showing intracellularly the synaptic components of swimming. MN = motorneuron, VR = ventral root. Inhibitory (arrowhead) and excitatory (asterisk) components of the synaptic drive underlying swimming is marked. The excitatory drive corresponds to the ventral root burst on the extracellular trace. ‡ represents stimulation artefact.

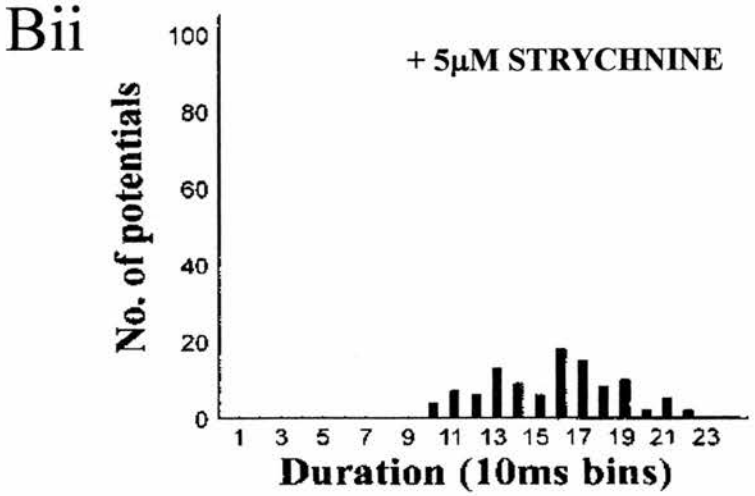
and amplitude distributions allow insight into pre- versus post-synaptic events induced by drug applications. Effects on sIPSP frequency are presumed to reflect a modulation of the probability of transmitter release pre-synaptically, whilst effects on the sIPSP amplitude distributions are indicative of modulation of post-synaptic receptors (McDearmid *et al*, 1997). This type of analysis can be applied to both glycinergic and GABAergic sIPSPs, because the two types of sIPSP can be distinguished by their distinct differences in duration (Figure 2.4A; 20-80ms for glycine and 90-200ms for GABA; Reith and Sillar, 1997), and more definatively, by their selective pharmacological blockade through the application of strychnine or bicuculline, respectively (Figure 2.4B).

Figure 2.4 | Spontaneous GABAergic and glycinergic IPSPs have different time courses

During quiescent periods between episodes of swimming, two types of depolarising inhibitory potential are recorded in motorneurons which can be separated on the basis of their duration. Six over-lapped traces of glycine and GABA potentials are shown in (A). Shorter duration, glycinergic, potentials have durations which range from 20 to 80ms, whilst the longer duration, GABAergic, potentials range from 90 to 200ms. In some cases sodium-dependent impulses can be observed (arrow). (Bi) Graph of sIPSP durations ranging from 30 to 200ms under control conditions. (Bii) Application of the glycine receptor antagonist strychnine (5μM) selectively blocks the population of shorter duration, glycinergic potentials. Figure adapted from Reith and Sillar, 1997.


A
impulse
GLYCINE
GABA
20mV
50ms


Bi
CONTROL
No. of potentials
0
20
40
60
80
100
1 3 5 7 9 11 13 15 17 19 21 23
Duration (10ms bins)


Bii
+ 5μM STRYCHNINE
No. of potentials
0
20
40
60
80
100
1 3 5 7 9 11 13 15 17 19 21 23
Duration (10ms bins)

3 | Group I mGluR-mediated modulation of a spinal locomotor network

3 | 1 SUMMARY

Using young tadpoles of the anuran amphibian *Xenopus laevis*, the involvement of mGluRs in modulating spinal locomotor networks has been investigated using a pharmacological approach. In this chapter, focus on group I mGluRs has revealed a functional role for this class of receptor in the modulation of swimming activity. The group I general agonist DHPG significantly increased both the frequency of swimming and the number of spontaneous swimming episodes, as monitored by extracellular recordings from ventral roots. This effect could be significantly reversed both with the group I antagonist, AIDA, and with washout. Applications of the glycine and $GABA_A$ receptor antagonists, strychnine and bicuculline, respectively, before and after the activation of group I receptors provided preliminary evidence that DHPG may be influencing the motor network for swimming through a reduction of glycinergic and GABAergic inhibitory transmission. This evidence was supported further with intracellular recordings from presumed motorneurons. During evoked swimming, one of the major components underlying the synaptic drive for swimming, the glycinergic mid-cycle IPSP, is depressed by the actions of DHPG. It was also observed during quiescent periods, that the frequency of spontaneous 'miniature' IPSPs mediated by glycine and GABA is reduced by DHPG. This suggests a pre-synaptic locus of modulation by group I mGluRs. There was no detectable change in membrane potential or input resistance following group I receptor activation. Taken together, this reduction in fast synaptic inhibition provides a plausible explanation for the increased excitability of the locomotor network, although other contributory mechanisms activated by group I receptors in parallel cannot be ruled out.

3 | 2 INTRODUCTION

Rhythmic forms of vertebrate locomotion such as walking, swimming or flying, results from intrinsic spinal cord circuits, CPGs, which can produce patterns of motorneuronal discharge even in the absence of sensory feedback or descending inputs from higher brain centres (Delcomyn, 1980; Grillner, 2003). These basic locomotor patterns involve the reciprocal activation of antagonistic muscle groups to produce co-ordinated cyclical contractions of power and return stroke muscles. It is

vital to an organism's survival that it is able to quickly alter its behavioural output in a perpetually changing environment, which ultimately requires an inherent ability of the neural networks underlying locomotion to adapt their output. Precise changes both in the cellular properties of individual neurons and in the synaptic interconnections between them confers the flexibility a network needs to modify its output.

Rhythmic motor patterns are subjected to neuromodulation as one means of accomplishing this flexibility in behaviourally relevant motor output. Neuromodulators can influence the integrative electrical properties of neurons and/or the strength of synaptic interconnections within the spinal network to produce a 'desired' output that is appropriate to the animals prevailing behavioural requirements (Kiehn and Katz, 1999). In the relatively simple nervous system of young tadpoles of *Xenopus laevis*, neuromodulators which affect fast inhibitory pathways that utilise the amino acids glycine and GABA have been extensively examined (for a review: Sillar, 2002). Glycinergic transmission mediates the reciprocal, mid-cycle inhibition that is important for the alternating activity between the two locomotor half-centres during both rhythmic swimming (Dale *et al*, 1986; Soffe, 1987) and struggling (Soffe, 1993), whilst GABA modulates the duration of swimming episodes and contributes to the termination of swimming (see chapter 1; Boothby and Roberts, 1992a, 1992b).

The biogenic amines, NA and 5-HT, are two such neuromodulators of the fast inhibitory pathways. They have been shown to differentially modulate reciprocal glycinergic transmission producing a profound increase and decrease, respectively, of the amplitude of mid-cycle inhibition (McDearmid *et al*, 1997). Any enhancement of the mid-cycle IPSP amplitude will delay the onset of the next cycle and consequently slow swimming. On the other hand, a reduction in mid-cycle IPSP amplitude will

release neurons from inhibition early and allow them to fire more on the next cycle, thus increasing the intensity and/or frequency of swimming. There is evidence from analysis of TTX-resistant spontaneous IPSPs that these amines can increase, through NA, and decrease, through 5-HT, the probability of quantal glycine release suggesting a pre-synaptic locus for the observed effects on mid-cycle inhibition (McDearmid *et al*, 1997). At a neurobehavioural level, 5-HT increases the intensity and duration of motor bursts making swimming activity more intense, whilst NA causes a lengthening of cycle periods and hence produces a reduction of fictive swimming frequency. The opposing effects of the two amines on glycinergic inhibition are presumed to be important in mediating these changes in the fictive swimming rhythm which represent two extremes in the motor output pattern.

The excitatory synaptic drive for swimming in *Xenopus* has been well documented and reviewed (see chapter 1). The excitatory neurotransmitter responsible for generating a significant proportion of the excitatory drive for swimming is glutamate, although cholinergic and electrotonic components have also been described (Perrins and Roberts, 1995a, 1995b). Glutamate activates both NMDA receptors, which contribute to the sustained tonic excitation throughout each episode, and non-NMDA receptors, which are responsible for the phasic excitatory component underlying spike production within each cycle of activity (Dale and Roberts, 1985). Given that glutamate's action at these iGluRs plays such a pivotal role in the generation of swimming in *Xenopus* tadpoles, is it possible that mGluRs also allow glutamate to play neuromodulatory roles within the locomotor network?

With three known classes, and eight different subtypes of mGluR already cloned (see chapter 1), the mGluRs appear to have evolved to fulfil roles that are either additional

to the functions of the iGluRs or complement and control the effects of the iGluRs. Collectively, the three mGluR groups produce a diverse range of inhibitory and excitatory CNS effects, which varies within species, between species, during development and under normal and abnormal conditions (for an extensive review: Anwyl, 1999). Such effects include: inhibition and activation of K^+, Ca^{2+} and non-specific cationic currents; suppression and facilitation of neurotransmitter release; and the potentiation of NMDA and non-NMDA responses and their involvement in oscillatory and epileptiform activity.

Group I mGluRs comprise $mGluR_1$ and $mGluR_5$ subtypes, which are predominantly coupled to the activation of PLC, leading to the formation of IP_3. Both subtypes have been shown to be present in many CNS structures including the hippocampus, cerebral cortex, thalamus and cerebellum (for a review: Pin and Duvoisin, 1995; Conn and Pin, 1997). In the neonatal mammalian spinal cord, group I mGluRs, specifically the $mGluR_5$ subtype, have been shown to be the most prevalent group, although this changes during development through a down-regulation of the mRNA encoding the $mGluR_5$ protein (Valerio *et al*, 1997).

The involvement of mGluR activation in locomotor behaviour was first studied in the lamprey where a specific role for each group I subtype has since been established (Krieger *et al*, 1996, 1998; Kettunen *et al*, 2002, 2003). In adult lampreys, group I receptors are located postsynaptically on neurons within the spinal locomotor network and are able to regulate neuronal excitability; activation of group I receptors produces membrane potential oscillations and enhanced ventral root bursting, thus increasing locomotor frequency (Krieger *et al*, 1996, 1998). Studies of group I receptor-mediated effects on the larval lamprey locomotor network when compared with those

in adults, have revealed developmental differences, as in these younger animals group I receptor activation causes an increase in swimming frequency through a pre-synaptic enhancement of glutamate transmitter release (Cochilla and Alford, 1998). There is evidence that blocking group I receptors can completely abolish fictive swimming activity in larval lampreys raising the possible requirement for a tonic activation of group I receptors to sustain the release of glutamate which in turn activates NMDA receptors that are necessary to maintain swimming (Takahashi and Alford, 2002). In addition, there has been some more recent evidence for group I mediated modulation of fictive locomotion and disinhibited bursting in the rat spinal cord (Taccola *et al*, 2003, 2004a).

In this chapter I have used hatchling *Xenopus laevis* as a model for investigating the control of rhythmic locomotor behaviour by group I mGluRs. In contrast to many other model systems it has a relatively simple nervous system that is capable of generating a self-sustaining swimming rhythm without the need for exogenous application of pharmacological stimulants. This provides an opportunity to study the role of group I mGluRs during more normal CPG activity. Using a specific group I receptor agonist and non-specific antagonists, I have first asked what modulatory effects on the fictive swimming rhythm are produced following group I mGluR activation. I have then studied the extent to which these receptors are activated endogenously during swimming by applying group I receptor antagonists on their own. Thirdly, I have examined whether the observed effects are due specifically to either one of the group subtypes, $mGluR_1$ or $mGluR_5$. Fourthly, using glycine and $GABA_A$ antagonists, the possibility that group I receptors mediate their effects through modulation of fast inhibitory pathways has been tested. Finally, a more

detailed analysis of the underlying synaptic or cellular changes mediated by group I mGluRs has been undertaken using intracellular recordings from spinal motorneurons.

3 | 3 RESULTS

3 | 3 | 1 Group I receptor activation influences swimming frequency

It should be emphasised from the onset that during the investigations of group I mGluR activation on the locomotor network, no substantial developmental differences were observed during the pharmacological manipulations, aside from the well documented increase in burst duration and complexity that occurs within the first 24hrs after hatching (see chapter 1). The effects described are therefore applicable to both embryonic (stage 37/38) and larval (stage 42) *Xenopus*, but the numbers of experiments performed in each stage will be made clear along with any quantitative differences observed.

Initial experiments involved the application of the general agonist, DHPG, in order to selectively activate only group I mGluRs and thereby to investigate what influence this group of glutamate receptors has on the motor network in *Xenopus* tadpoles. Dose-response experiments were carried out (data not shown) to first determine what concentration of DHPG produced an effect. The optimum concentration was established as being between 5 and 10μM; higher concentrations produced episodes of swimming that were either too short to analyse accurately or that became highly irregular. Therefore, all of the experiments used 10μM DHPG, giving maximum effect without compromising my ability to accurately analyse the data. This concentration of agonist is similar to that used in other motor systems (e.g. neonatal rat, 5-50μM (Taccola *et al*, 2004a); lamprey, 20-100μM (Krieger *et al*, 1996, 1998, 2000; Cochilla and Alford, 1998; Kettunen *et al*, 2002)).

In data pooled from 17 animals (10 stage 37/8 embryos and 7 stage 42 larvae), bath application of 10μM DHPG significantly reduced cycle periods, from 64.42±0.35ms in control saline to 59.26±0.28ms (One-way ANOVA: O-A, $F_{3,2295}$=79.48, P<0.001, n=17; Figures 3.1Ai-ii and 3.2Ai-ii). A significant reversal of this facilitatory effect on swimming frequency was achieved following washout in 9 out of 9 animals (62.53±0.46ms; 0-A, $F_{3,2295}$=79.48, P<0.001, n=9; Figure 3.1Ai-ii) and in 8 out of 8 animals following application of the general group I mGluR antagonist AIDA (100μM; 67.32±0.51ms; K-W, P<0.05, n=8; Figure 3.2Ai-ii). The shorter cycle periods following activation of group I mGluRs persisted throughout episodes of swimming (*t*-test, P<0.05, n=17; Figure 3.1Aiii). Ventral root burst durations and amplitudes were not significantly affected following application of DHPG in either stage 37/38 embryos or stage 42 larvae (O-A, P>0.05, n=17; Figures 3.1Bi-ii and 3.2Bi-ii). Subsequent washout or application of AIDA similarly did not affect burst durations or amplitudes at either developmental stage (O-A, P<0.05, n=9 and n=8 respectively; Figures 3.1Bi-ii and 3.2Bi-ii). R-C delays decreased, but not significantly, in the presence of DHPG (3.36±0.21ms in control saline to 2.9±0.18ms; O-A, P>0.05, n=17; Figures 3.1C and 3.2C) and did not change with application of AIDA (3.19±0.16ms; O-A, P>0.05, n=8; Figure 3.2C). However, R-C delays did significantly increase during washout (3.8±0.18ms; O-A, $F_{3,2295}$=3.8, P<0.001, n=9; Figure 3.1C) suggesting that although the decrease in R-C delays under DHPG was insignificant, the trend was in the appropriate direction, and such a decrease would be hypothesised to accompany the decrease in cycle periods (cf. Tunstall and Sillar, 1993). Lastly, episode durations were not obviously affected with application of

Figure 3.1 | Effects of DHPG on fictive swimming

Ventral root recording (Ai; from a stage 42 animal) and graphical representation (Aii) showing reduced cycle periods following application of the specific group I receptor agonist DHPG (10μM; O-A, $P<0.001$, n=17). The effect on swimming frequency was reversed during washout in 9 animals (O-A, $P<0.001$, n=9). (Aiii) Graph illustrating on a cycle-by-cycle basis the decrease in cycle periods induced by DHPG over the first 100 cycles of an episode of swimming (average of 4 episodes per condition). Burst durations (Bi) and amplitudes (Bii) were not significantly altered in either stage 37/38 embryos or stage 42 larvae after activation of group I receptors (O-A, $P>0.05$, n=10 and n=7 respectively) or during washout (O-A, $P>0.05$, n=5 and n=4 respectively). (C) R-C delays did not significantly change following application of DHPG (O-A, $P>0.05$, n=17) or during washout (O-A, $P>0.05$, n=9). (D) Episode durations were similarly unaffected following agonist application (O-A, $P>0.05$, n=17) or during washout (O-A, $P>0.05$, n=9). * = significant, ns = not significant. Values are means ± S.E.M.

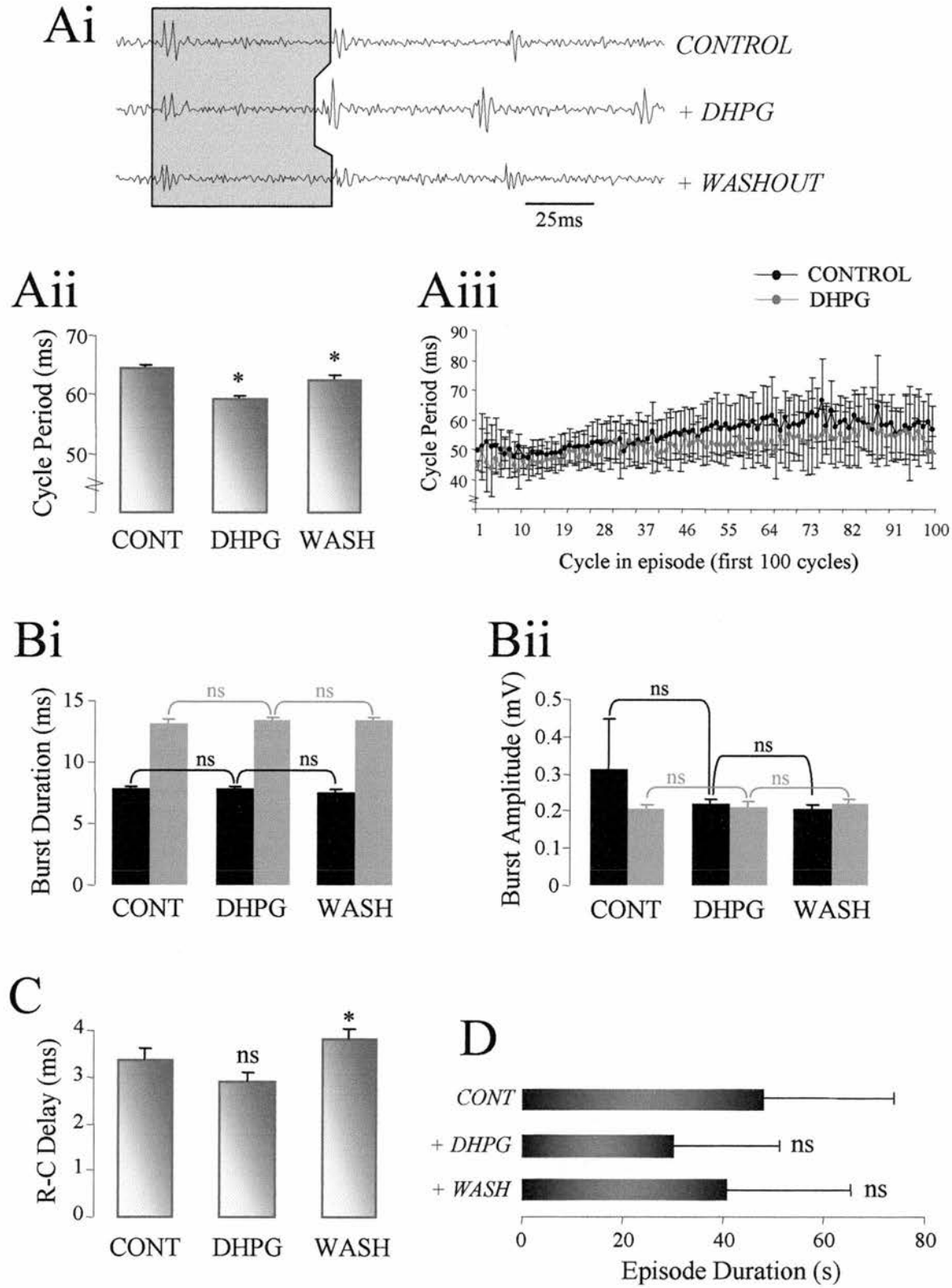
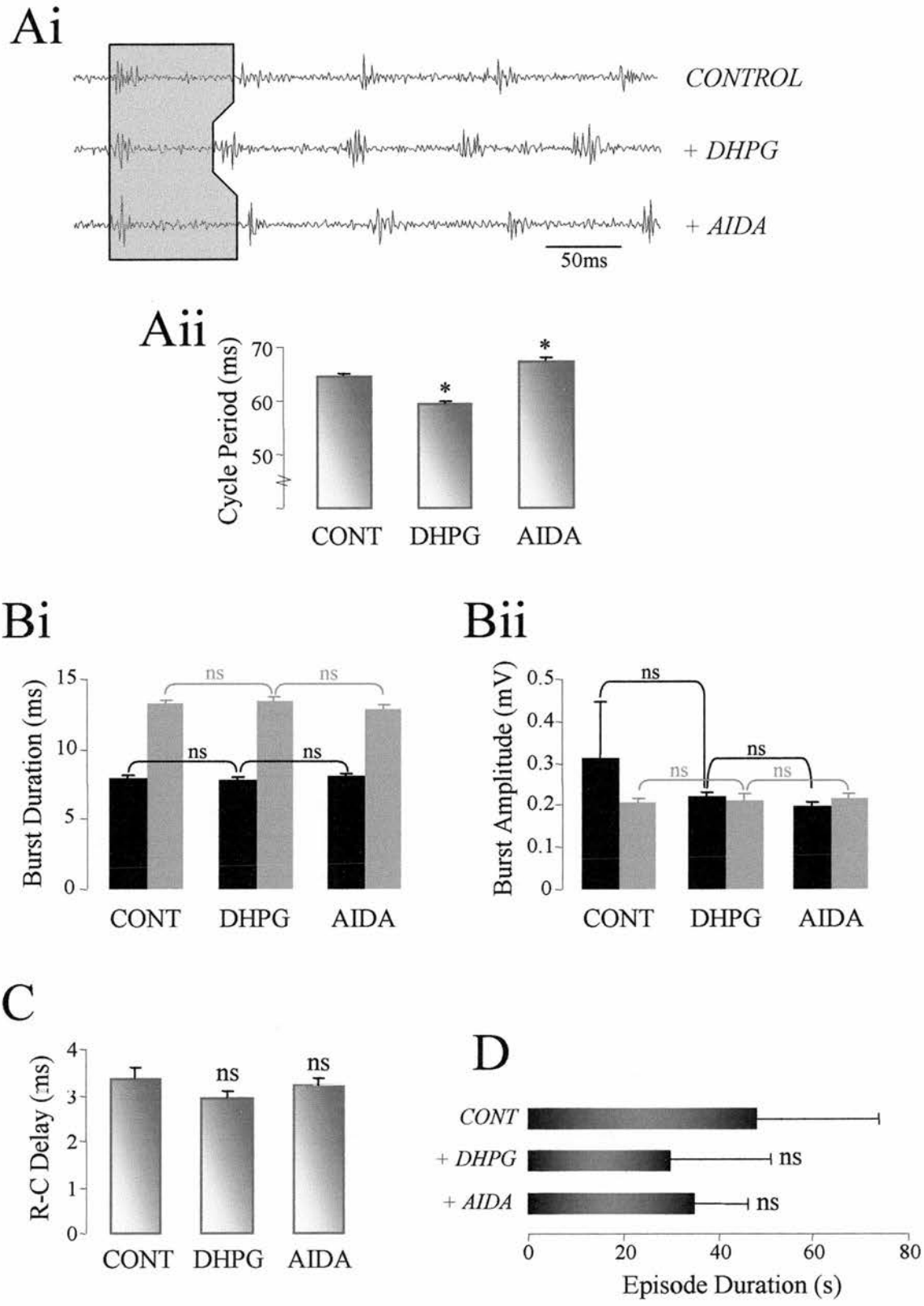

Ai
CONTROL
+ DHPG
+ WASHOUT
25ms
Aii
Cycle Period (ms)
70
60
50
CONT
DHPG
WASH
Aiii
CONTROL
DHPG
Cycle Period (ms)
90
80
70
60
50
40
1
10
19
28
37
46
55
64
73
82
91
100
Cycle in episode (first 100 cycles)
Bi
Burst Duration (ms)
15
10
5
0
ns
CONT
DHPG
WASH
Bii
Burst Amplitude (mV)
0.5
0.4
0.3
0.2
0.1
0
ns
CONT
DHPG
WASH
C
R-C Delay (ms)
4
3
2
1
0
ns
CONT
DHPG
WASH
D
CONT
+ DHPG
+ WASH
ns
0
20
40
60
80
Episode Duration (s)

Figure 3.2 | Effects of DHPG are reversed by the general group I antagonist AIDA

Ventral root recording (Ai; from a stage 42 animal) and graphical representation (Aii) showing reduced cycle periods following application of DHPG (O-A, $P<0.01$, n=17). Subsequent application of the general group I antagonist, AIDA (100μM) in 8 animals, reversed this effect on swimming frequency (O-A, $P>0.05$, n=8). (Bi-ii) Burst durations and amplitudes did not change in either stage 37/38 embryos or stage 42 larvae following application of DHPG (O-A, $P>0.05$, n=10 and n=7 respectively) or AIDA (O-A, $P>0.05$, n=5 and n=3 respectively). R-C delays (C) and episode durations (D) are unaffected by DHPG (O-A, $P>0.05$, n=17) and following AIDA application (O-A, $P>0.05$, n=8). * = significant, ns = not significant. Values are means ± S.E.M.


Ai
CONTROL
+ DHPG
+ AIDA
50ms
Aii
Cycle Period (ms)
70
60
50
*
*
CONT
DHPG
AIDA
Bi
Burst Duration (ms)
15
10
5
0
ns
ns
ns
ns
CONT
DHPG
AIDA
Bii
Burst Amplitude (mV)
0.5
0.4
0.3
0.2
0.1
0
ns
ns
ns
ns
CONT
DHPG
AIDA
C
R-C Delay (ms)
4
3
2
1
0
ns
ns
CONT
DHPG
AIDA
D
CONT
+ DHPG
+ AIDA
ns
ns
0
20
40
60
80
Episode Duration (s)

DHPG (O-A, P>0.05, n=17; Figures 3.1D and 3.2D) or AIDA, or during washout (O-A, P>0.05, n=8 and n=9; Figures 3.2D and 3.1D).

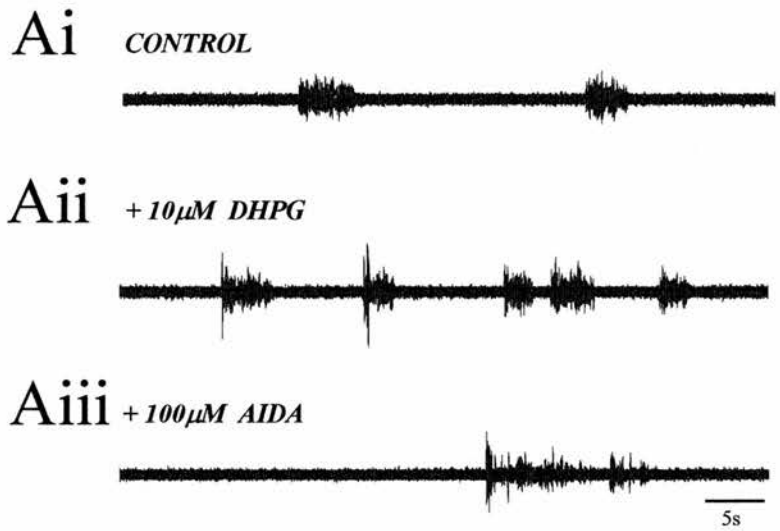
These initial experiments suggest that the selective activation of group I receptors leads to an increase in swimming frequency. Another obvious and consistent effect observed with bath application of DHPG (10μM; Figure 3.3Ai-ii) is an increase in the number of spontaneously occurring swimming episodes, an effect which reversed with subsequent application of AIDA (100μM; Aiii). In 8 animals, during the periods between experimentally evoked swimming, the number of spontaneous swim episodes significantly increased from an average of 0.9±0.29 episodes per min in control saline to 2.6±0.49 episodes per min following DHPG application (B; O-A, $F_{2,72}$=6.21, P<0.05, n=8). This effect was significantly reversed following application of AIDA (to a mean of 0.87±0.39 episodes per min; O-A, $F_{2,72}$=6.21, P<0.05, n=8). A reversible increase in spontaneous activity was also consistently observed in the other experiments conducted with DHPG application, although this was not quantified. These data indicate that activating group I mGluRs produces an increase in the excitability of the motor network for swimming in *Xenopus*, as reflected in the increase in spontaneous swim episodes and the consistent and significant increase in swimming frequency.

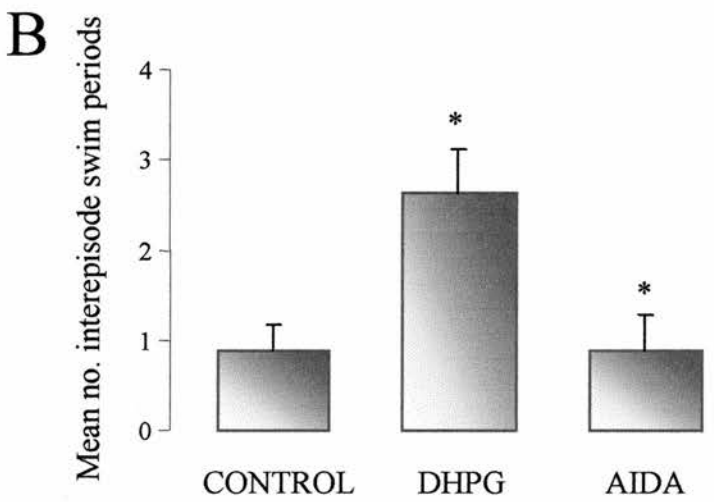
3 | 3 | 2 Evidence that group I receptor-mediated increase in swim frequency involves modulation of glycinergic transmission

An increase in swimming frequency could, in principle, be explained by a shift in the balance between synaptic excitation and inhibition in the spinal network, and in *Xenopus* tadpoles small changes in inhibitory synaptic strengths are known to have dramatic effects on swim frequency (McDearmid *et al*, 1997). To explore whether

Figure 3.3 | Activation of group I mGluRs increases the frequency of spontaneous swimming episodes

(Ai-ii) Ventral root recording showing increased episodes of spontaneous swimming between episodes of evoked swimming following application of 10μM DHPG compared to control. (Aiii,B) The mean number of spontaneously occurring episodes (per minute) of swimming increased significantly under DHPG (O-A, $P<0.05$, n=8) and decreased following application of the antagonist, AIDA (100μM; O-A, $P<0.05$, n=8) . * = significant, ns = not significant. Values are means ± S.E.M.


Ai
CONTROL
Aii
+10μM DHPG
Aiii
+100μM AIDA
5s


B
Mean no. interepisode swim periods
0
1
2
3
4
*
*
CONTROL
DHPG
AIDA

the group I receptor-mediated modulation of swimming frequency involved effects on inhibitory transmission, my next set of exploratory experiments were performed using the specific glycine receptor antagonist, strychnine.

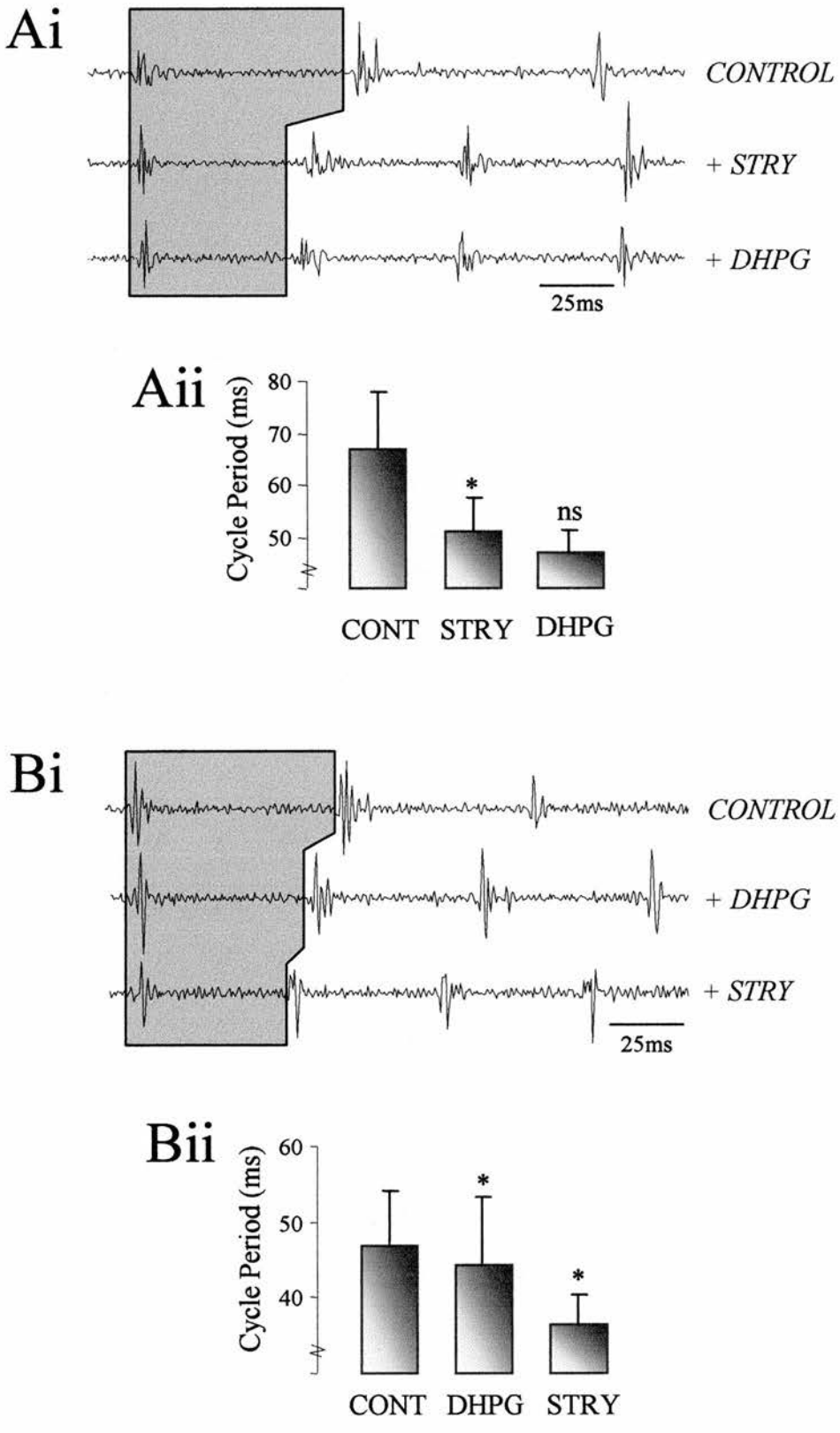
In the presence of 1μM strychnine the effects of 10μM DHPG on cycle periods in 4 embryos and 2 larvae are largely negated (significant decrease from 66.75±11.12ms in control saline to 51±6.37ms with strychnine (K-W, $P<0.05$, n=6), and a statistically insignificant decrease to 47±4ms with DHPG; K-W, $P>0.05$, n=6; Figure 3.4Ai-ii). Application of strychnine after activating group I mGluRs in 4 embryos and 2 larvae produced a further, significant decrease in cycle periods from 44.25±9ms in the presence of DHPG, to 36.5±4ms (K-W, $P<0.05$, n=6; Figure 3.4Bi-ii). This further small reduction observed with strychnine could be explained either if DHPG only partially reduces glycinergic inhibition and/or if strychnine has other additional effects that will influence the swimming frequency (e.g. blocking K^+ channels; Dale and Kuenzi, 1997). On the other hand, the occlusion of DHPG's effects in the presence of strychnine implies that group I receptor activation may specifically involve a reduction of glycinergic inhibition, at least at this stage of development. Certainly, the data presented here are consistent with the idea that group I receptor activation depresses cycle periods in part by reducing glycinergic transmission.

3 | 3 | 3 GABAergic transmission as a possible target for modulation by group I mGluRs

The other fast inhibitory neurotransmitter, GABA, is known to play a role in controlling the duration of swimming episodes in both *Xenopus* embryos and larvae (Boothby and Roberts, 1992a, 1992b), but, unlike glycine, there is no phase-locked GABAergic component to the swim cycle that could account for the observed changes

Figure 3.4 | Activation of group I mGluRs before and after the blockade of glycine receptors

Ventral root recording (Ai) and graph (Aii) showing reduced cycle periods following strychnine application (1μm; K-W, $P<0.05$, n=6), which are not significantly affected by subsequent application of DHPG (10μm; K-W $P>0.05$, n=6). (Bi-ii) Cycle periods significantly decreased following application of DHPG and decreased further in the presence of strychnine (K-W $P<0.05$, n=6). * = significant, ns = not significant. Data are median values ± I.Q.R.

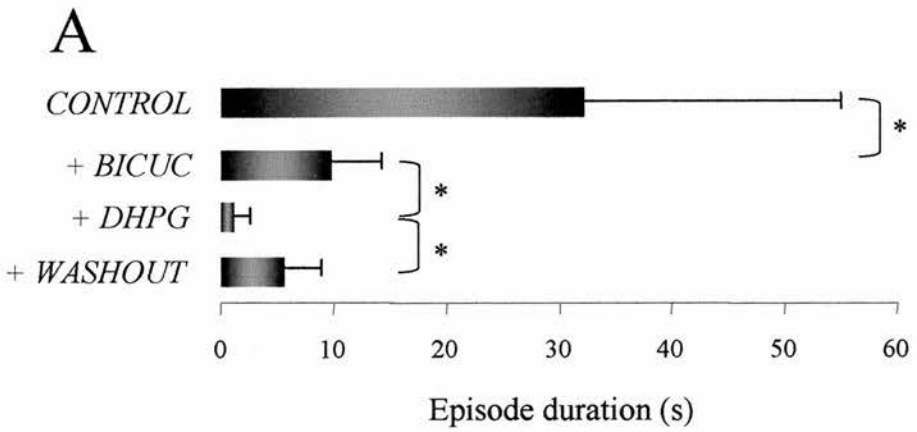

Ai
CONTROL
+ STRY
+ DHPG
25ms
Aii
Cycle Period (ms)
80
70
60
50
*
ns
CONT
STRY
DHPG
Bi
CONTROL
+ DHPG
+ STRY
25ms
Bii
Cycle Period (ms)
60
50
40
*
*
CONT
DHPG
STRY

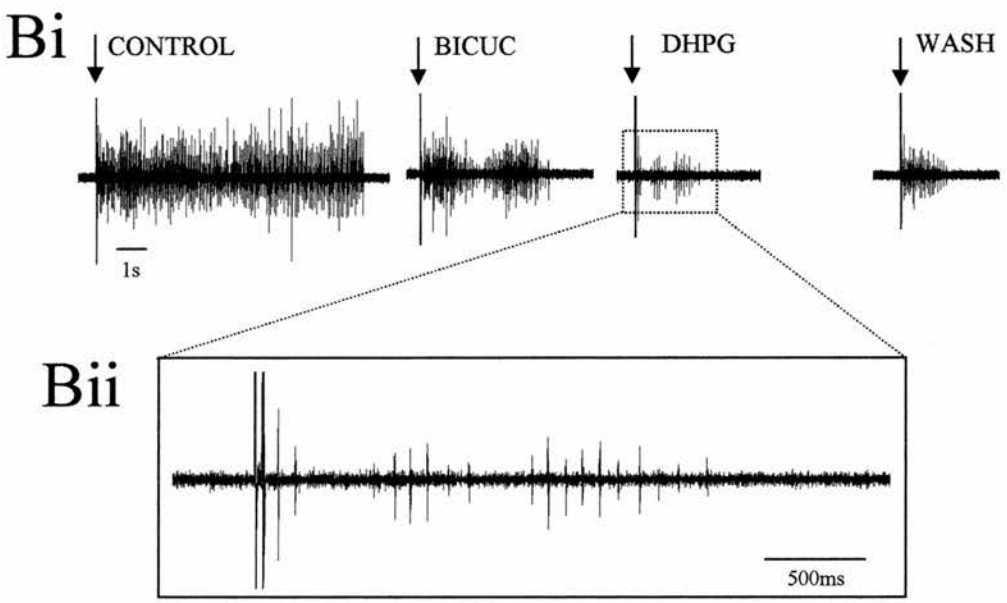
in swimming frequency in the presence of DHPG. However, since GABA neurotransmission is a common target for neuromodulation in *Xenopus* (for a review: Sillar, 2002), and given that there is precedent for mGluR-mediated modulation of GABA transmission in various brain regions (including the PAG (Drew *et al*, 2004), hippocampus (Jouvenceau *et al*, 1995), and SNr (Marino *et al*, 2001)), a preliminary investigation of the influence of group I mGluR activation on GABAergic transmission in the *Xenopus* system was conducted.

The $GABA_A$ receptor antagonist, bicuculline, was bath applied both before and after DHPG application to assess the extent to which group I mGluRs might influence GABAergic inhibitory transmission. Characteristically, application of bicuculline produces short intense episodes of swimming, with increased burst durations, reduced cycle periods and reduced R-C delays (Reith and Sillar, 1999). In 4 of 6 animals (all stage 42 larvae), application of 40μM bicuculline significantly reduced the duration of fictive swimming episodes (32.16±16.5s in control to 9.74±4.22s with bicuculline; K-W, $P<0.05$, n=4; Figure 3.5A). Subsequent application of DHPG (10μM) also significantly reduced episode durations following bicuculline treatment (1.13±1.05s; K-W, $P<0.05$, n=4; Figure 3.5A), which could be reversed during washout (to 5.55±2.97s; K-W, $P<0.05$, n=4; Figure 3.5A). The remaining 2 animals showed no obvious change in episode durations under DHPG. The shortening of swimming episodes in the presence DHPG was also associated with some irregularity of the swimming rhythm as can be seen in an example of a swimming episode expanded in Figure 3.5Bi-ii. This variable nature of swimming prevented reliable and accurate measurements of cycle periods to determine what influence prior application of bicuculline has on swim frequency with subsequent application of DHPG. However, it should be noted that bicuculline was not present in the saline during washout and as

Figure 3.5 | Activation of group I mGluRs after the blockade of GABA receptors

(A) Graph shows significantly reduced episode durations following application of bicuculline, which decrease further in the presence of DHPG (K-W, $P<0.05$, n=4 out of 6). (Bi) Ventral root recording on a slow time scale showing the effect of the $GABA_A$ receptor antagonist, bicuculline (40μM; BICUC), and subsequent DHPG (10μM) application on episode durations. Arrows indicate stimulus artefact. (Bii) Box shows enlarged ventral root recording of swimming induced in the presence of DHPG on a faster time scale. * = significant, ns = not significant. Data are median values ± I.Q.R.


A
CONTROL
+ BICUC
+ DHPG
+ WASHOUT
*
*
*
0
10
20
30
40
50
60
Episode duration (s)


Bi
CONTROL
BICUC
DHPG
WASH
1s
Bii
500ms

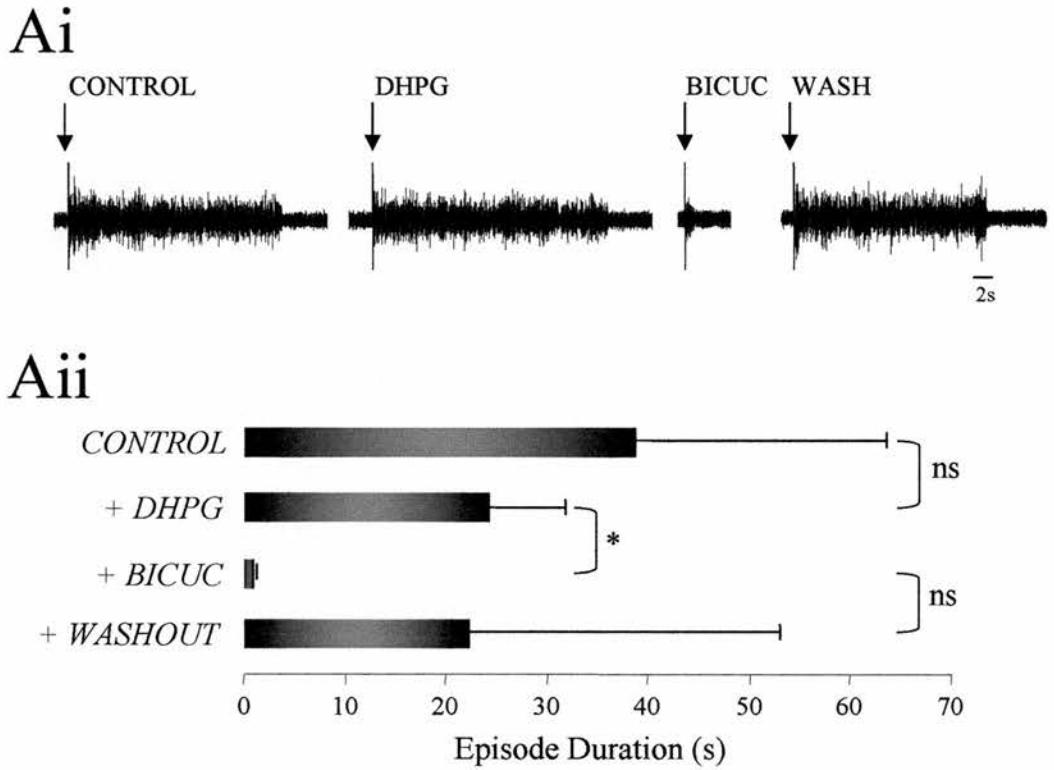
a consequence it is possible that the observed irregularity of swimming under DHPG may be a continued effect of bicuculline.

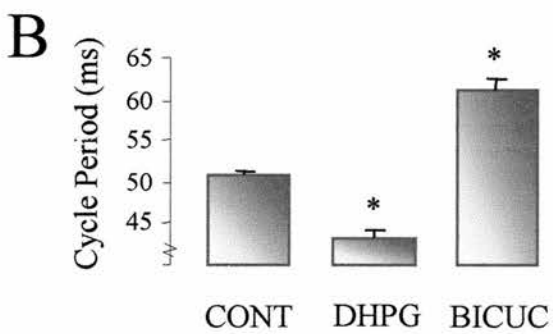
10μM DHPG application has already been shown not to significantly affect the duration of fictive swimming episodes (Figure 3.1 and 3.2). In 5 out of 7 animals, when bicuculline is subsequently applied in the presence of DHPG there is a significant reduction in episode durations from 24.2±7.4s under DHPG, to 0.9±0.27s following application of bicuculline (K-W, $P<0.05$, n=5; Figure 3.6Ai-ii). The 2 other animals showed no change in episode durations. From these results it seems most likely that the previously observed irregular swimming with prior application of bicuculline is due to a continued effect of bicuculline, rather than the effects of DHPG, which does not on its own influence episodes.

Although much shorter, swimming episodes were still long enough to accurately measure cycle periods in these experiments using the same analysis procedure as elsewhere (see chapter 2: Materials and Methods). As observed previously, DHPG application reduced cycle periods (50.85±0.5ms in control saline to 42.95±0.78ms; O-A, $F_{2,243}=125.79$, $P<0.001$, n=7; Figure 3.6B). In 5 of 7 animals (all stage 42), subsequent application of 40μM bicuculline produced a significant *increase* in cycle periods (to 61.23±1.12ms; O-A, $F_{2,243}=125.79$, $P<0.001$, n=5; Figure 3.6Biii) reversing the effects induced by DHPG. However, this could be an artefact of shortened swimming episodes under bicuculline. As cycle periods increase normally towards the end of a swimming episode, the region of measured cycle periods under bicuculline (>500ms, see chapter 2 on measurement of parameters) may include those longer cycle periods commonly found towards the end of an episode of swimming. Consequently, the measured cycle periods may be longer compared to the same

Figure 3.6 | Activation of group I mGluRs before the blockade of GABA receptors

(Ai) Ventral root recording on a slow time scale showing the effect of blocking GABA receptors on episode durations after the application of DHPG. (Aii) Episode durations are not significantly altered in the presence of DHPG, but subsequent application of bicuculline (40μM; BICUC) caused a significant decrease (K-W, $P<0.05$, n=5 out of 7). (B) DHPG application reduced cycle periods which reversed following application of bicuculline (O-A, $P<0.001$, n=5). * = significant, ns = not significant. Data are median values ± I.Q.R. (Aii) or means ± S.E.M. (Bii).


Ai
CONTROL
DHPG
BICUC
WASH
2s
Aii
CONTROL
+ DHPG
+ BICUC
+ WASHOUT
ns
*
ns
0
10
20
30
40
50
60
70
Episode Duration (s)


B
Cycle Period (ms)
65
60
55
50
45
*
*
CONT
DHPG
BICUC

region measured under control conditions and following application of DHPG. Taken together, from these results it seems unlikely that group I receptors are modulating GABAergic transmission, at least to the extent that this significantly affects swimming.

From these extracellular experiments so far it can be suggested that group I mGluRs mediate their effects on swimming frequency in part through the modulation of glycinergic transmission. To test this hypothesis further, a closer examination of the effects of DHPG on the cellular and synaptic properties of individual motorneurons is now needed using intracellular recordings.

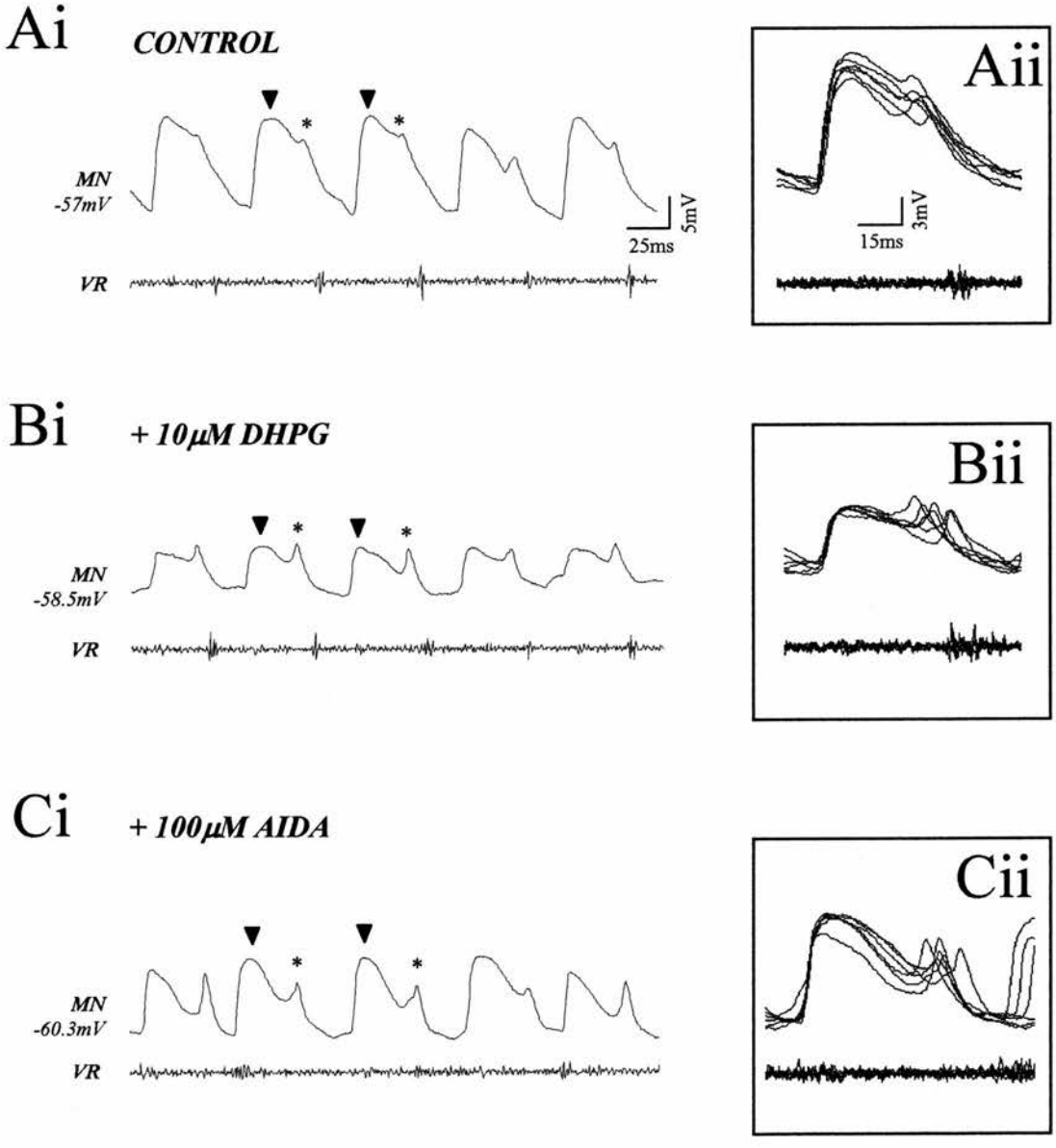
3 | 3 | 4 Group I receptor activation reduces the mid-cycle IPSP

As mentioned previously, the synaptic drive underlying swimming in *Xenopus* tadpoles consists of a tonic depolarisation, superimposed upon which is fast on-cycle excitation, underlying spike production in each cycle, and glycinergic mid-cycle inhibition (see chapter 1: figure 2.1). Using KCl-filled microelectrodes to record intracellularly from ventral neurons, presumed to be motorneurons, I initially investigated the effects of DHPG on inhibition during evoked swimming to test for changes in the mid-cycle IPSP as a possible mechanism by which group I receptors affect swim frequency.

DHPG (10μM) application produced a significant 61.2±7.1% decrease in the amplitude of the glycinergic mid-cycle IPSP during swimming in 4 out of 6 animals (O-A, $P<0.05$, $n=4$; Figure 3.7A-Bi-ii,D). The remaining two animals showed no clear alteration in either the amplitude of the mid-cycle IPSP or, importantly, in the frequency of evoked swimming (data not shown). For the 4 preparations in which

Figure 3.7 | Activation of group I mGluRs reduces mid-cycle inhibition

A significant reduction in the mid-cycle inhibitory component of the synaptic drive for swimming is observed following DHPG application (Ai-Bi; 10μM; arrowheads represent the inhibitory component, stars represent the excitatory component). This effect is partially reversed by application of the general group I antagonist, AIDA (Ci; 100μM). (A-Cii boxes) Excerpts of ~6 cycles of activity are amplified and overlaid showing a clear and reliable reduction of the mid-cycle IPSP. mV = Resting membrane potential prior to the illustrated swimming episode. MN = motorneuron, VR = ventral root. (D) Graph showing percentage change in IPSP amplitude height (significant 61.2±7.1% reduction in IPSP amplitude under DHPG, that increases by 34.9±6.7% when the antagonist is applied; O-A, $P<0.05$, $n=4$). * = significant, ns = not significant. Values are means ± S.E.M.


Ai
CONTROL
MN
-57mV
VR
25ms
5mV
Aii
3mV
15ms
Bi
+ 10μM DHPG
MN
-58.5mV
VR
Bii
Ci
+ 100μM AIDA
MN
-60.3mV
VR
Cii
D
IPSP Amplitude (%)
100
80
60
40
20
0
CONT
DHPG
AIDA

DHPG depressed mid-cycle IPSP amplitudes, subsequent application of AIDA (100μM) reversed this effect, increasing the mid-cycle IPSP amplitude by 34.9±6.7% (O-A, $P<0.05$, n=4; Figure 3.7Ci-ii,D). This depression of the mid-cycle IPSP could allow the onset of excitation in the next cycle to occur sooner, thus contributing to the observed increase in swimming frequency following group I receptor activation.

3 | 3 | 5 sIPSP frequency is reduced following activation of group I receptors

The reduction in mid-cycle IPSP amplitude indicates that activation of group I receptors is causing a reduction of glycinergic transmission. In principle, this modulation of synaptic transmission could be occurring either pre-synaptically, involving a reduction in the amount of transmitter released, or post-synaptically, involving a reduction in the effects of the transmitter on the motorneurons, or there may be parallel pre- and post-synaptic effects working in tandem. To try to distinguish between these possibilities, I next investigated what effect DHPG application has during quiescent periods, where spontaneous depolarising IPSPs (sIPSPs) can be recorded from presumed motorneurons using KCl-filled microelectrodes. These sIPSPs are thought to represent the quantal release of neurotransmitter, in this case, glycine and GABA from inhibitory interneurons (McDearmid *et al*, 1997). The two types of sIPSP can be distinguished either pharmacologically by selective blockade through the application of strychnine or bicuculline, respectively, or by their distinct differences in duration (20-80ms for glycine and 90-200ms for GABA; Reith and Sillar, 1997); see chapter 2: Materials and Methods).

Bath application of DHPG (10μM) produced a 42% decrease in the rate of occurrence of sIPSPs in 5 out of 7 recordings (from 1.75±0.14Hz in control saline to

1.01±0.06Hz; O-A, $F_{2,75}$=7.63, P<0.05, n=5; Figure 3.8Ai-ii,Bi). The two remaining experiments showed no significant change in sIPSP frequency; these two experiments were the same as those discussed above in which DHPG produced no change in mid-cycle IPSP amplitude or swim frequency. This effect on the rate of occurrence of sIPSPs following DHPG application was successfully reversed by AIDA (100μM) to 1.63±0.05Hz (O-A, $F_{2,75}$=7.63, P<0.05, n=5; Figure 3.8Aiii,Bi). The reduction in sIPSP frequency was observed for both the shorter duration, presumed glycinergic sIPSPs (from 1.38±0.06Hz in control saline to 0.76±0.05Hz; O-A, $F_{2,75}$=14.13, P<0.05, n=5; Figure 3.8Bii) and the longer duration, but less frequent, presumed GABAergic sIPSPs (from 0.18±0.01Hz in control saline to 0.11±0.01Hz; O-A, $F_{2,75}$=10.5, P<0.05, n=5; Figure 3.8Bii). This effect on glycinergic and GABAergic sIPSP frequency was reversed with application of AIDA (O-A, P<0.05, n=5; Figure 3.8Bii). From these results it can be suggested that group I receptors are located pre-synaptically where they modulate the release of inhibitory transmitter.

To determine whether DHPG also induces any post-synaptic effects, sIPSP amplitude distribution histograms were constructed, as shifts in both the range and mean of sIPSP amplitudes could provide evidence for an additional post-synaptic locus of modulation. Figure 3.9 shows that in 5 experiments, DHPG induced a significant reduction in mean sIPSP amplitude. The sIPSP amplitude histograms (from one representative experiment), show that DHPG application caused a significant shift in the range of amplitudes produced. In control conditions the range of sIPSP amplitudes is between 3 and 34mV, with a mean of 12.53±0.67mV (Figure 3.9Ai). DHPG application significantly decreased the range of amplitudes to between 3 and 17mV, and reduced the mean to 9.44±0.37mV (O-A, $F_{2,347}$=10.1, P<0.05; Figure

Figure 3.8 | Group I mGluR activation reduces the frequency spontaneous IPSPs

(Ai-iii) Spontaneous IPSPs during inter-episode quiescent periods decreased following DHPG (10μM) application and reversed with the application of the general antagonist, AIDA (100μM). (Bi) Graph showing the reduced rate of occurrence of sIPSPs with group I receptor activation (from 1.75±0.14Hz in control saline to 1.01±0.06Hz; O-A, $P<0.05$, n=5), which reversed in the presence of AIDA (to 1.63±0.05Hz; O-A, $P<0.05$, n=5). (Bii) The reduction in sIPSP frequency included both glycinergic (from 1.38±0.06Hz in control saline, to 0.76±0.05Hz; O-A, $P<0.05$, n=5) and GABAergic sIPSPs (from 0.18±0.01Hz in control saline, to 0.11±0.01Hz; O-A, $P<0.05$, n=5). The rate of glycinergic and GABAergic sIPSP occurrence reversed following AIDA application (O-A, $P<0.05$, n=5). * = significant, ns = not significant. Values are means ± S.E.M.

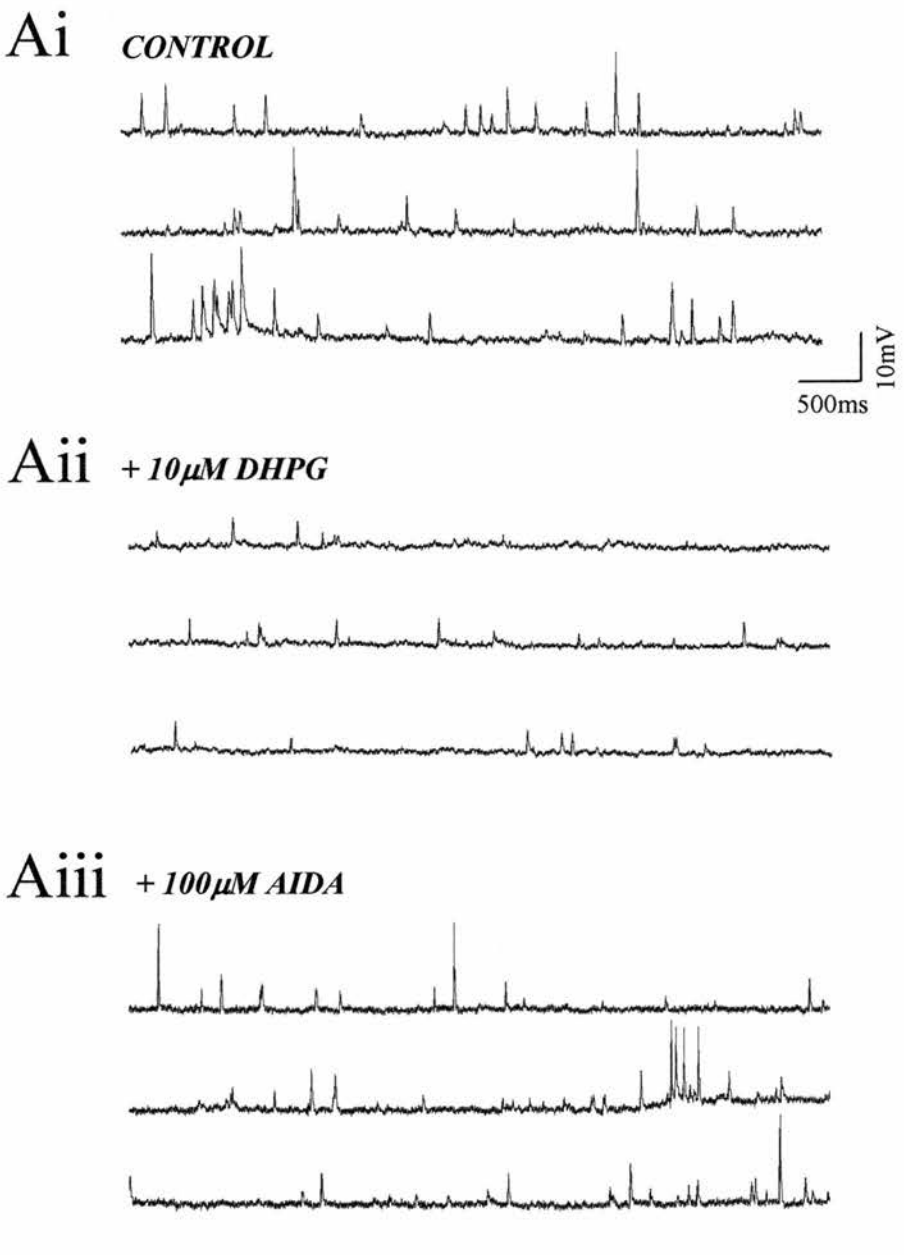
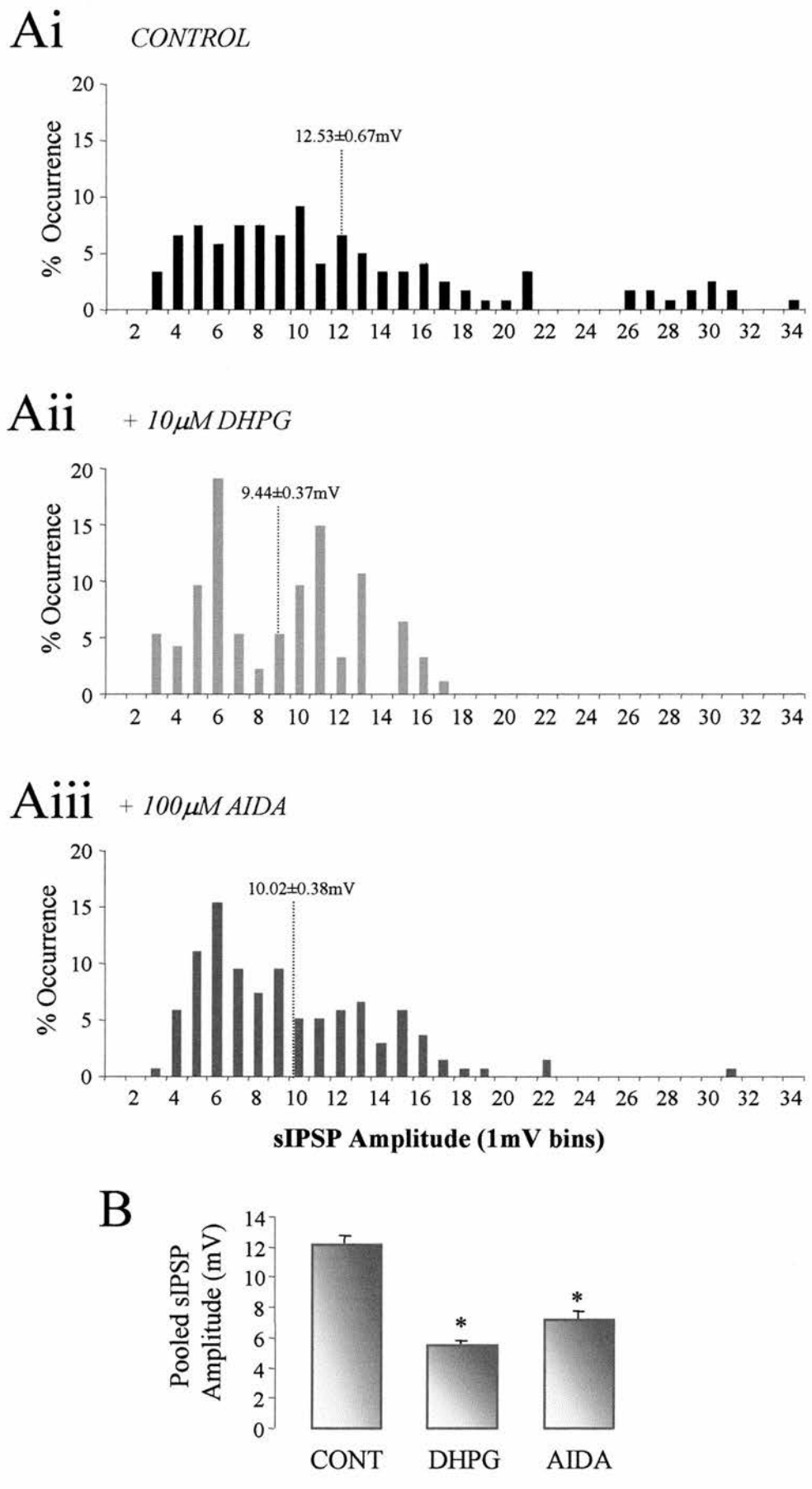

Ai
CONTROL
10mV
500ms
Aii
+ 10μM DHPG
Aiii
+ 100μM AIDA
Bi
sIPSP Frequency (Hz)
2
1.5
1
0.5
0
CONT
DHPG
AIDA
Bii
GLYCINE
GABA
sIPSP Frequency (Hz)
1.5
1
0.5
0
CONT
DHPG
AIDA

Figure 3.9 | Group I mGluR activation affects the amplitude of sIPSPs

In 5 out of 7 experiments, the mean sIPSP amplitude significantly decreased following group I receptor activation. (A) Histogram showing the distribution of sIPSP amplitudes under control conditions, ranging from 3 to 34mV. The calculated mean in control was 12.53±0.67mV (from this representative experiment). (Aii) After application of 10μM DHPG, the range of sIPSP amplitudes shifted to the left of the histogram, now ranging from 3 to 17mV. This is a significant shift as the mean sIPSP amplitude decreased to 9.44±0.37mV under DHPG (O-A, $P<0.05$). (Aiii) This effect is reversed following application of the antagonist, AIDA, as the range of amplitudes increased, from 3 to 31mV, and the mean amplitude significantly increased, to 10.02±0.38mV (O-A, $P<0.05$). (B) sIPSP amplitudes pooled from the 5 experiments showed a decrease in mean sIPSP amplitude following group I receptor activation (from 12.12±0.49mV in control saline, to 5.49±0.21mV under DHPG; O-A, $P<0.001$, $n=5$), which reversed following AIDA application (to 7.19±0.45mV; O-A, $P<0.001$, $n=5$). Dotted lines represent the mean amplitude under each condition. Values are means ± S.E.M.

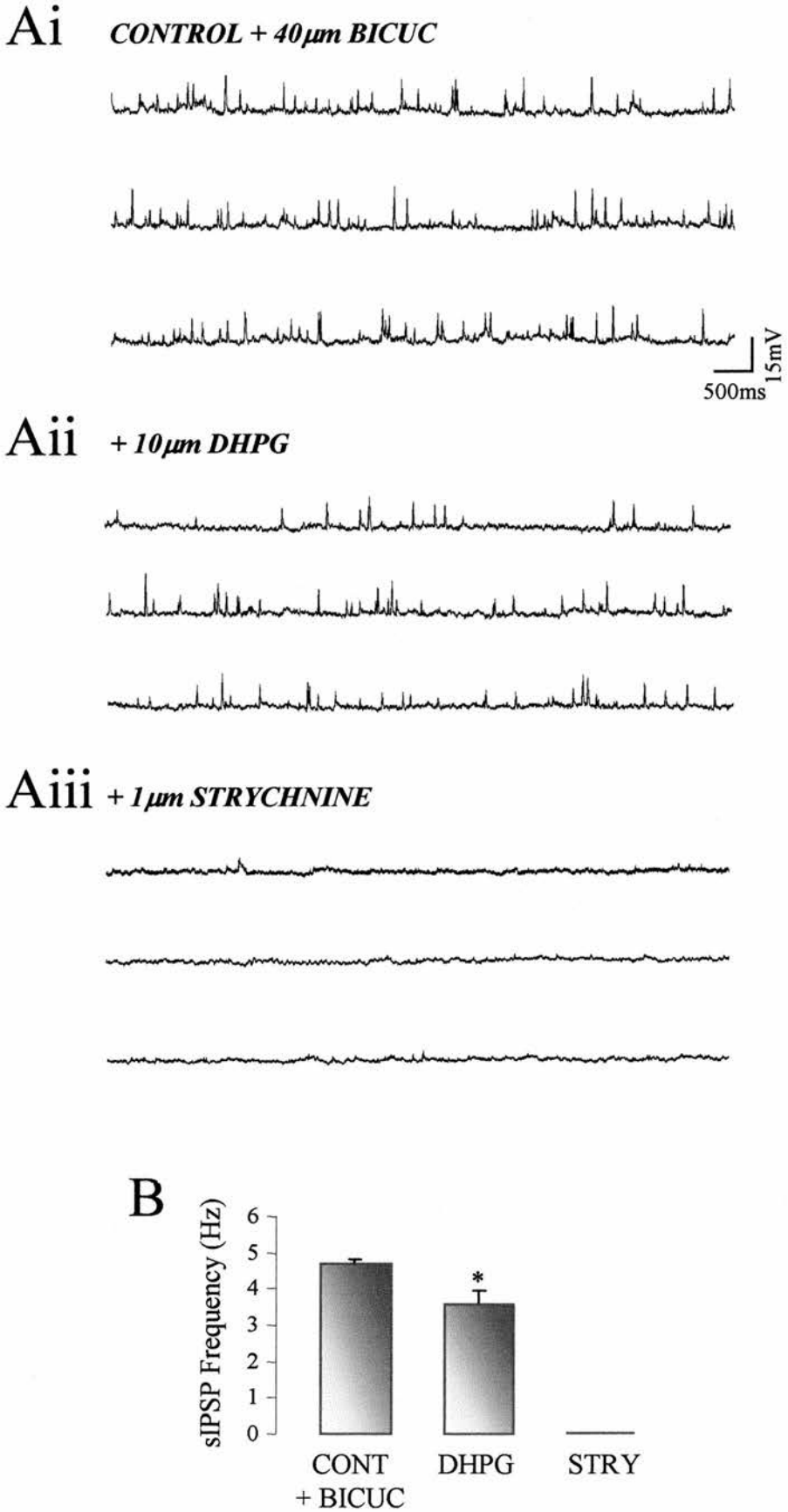

Ai
CONTROL
% Occurrence
12.53±0.67mV
Aii
+ 10μM DHPG
% Occurrence
9.44±0.37mV
Aiii
+ 100μM AIDA
% Occurrence
10.02±0.38mV
sIPSP Amplitude (1mV bins)
B
Pooled sIPSP Amplitude (mV)
CONT
DHPG
AIDA

3.9Aii). This effect was partially but significantly reversed with application of 100μM AIDA as the range increased up to 31mV and the mean increased to 10.02±0.38mV (O-A, $F_{2,347}$=10.1, P<0.05; Figure 3.9Aiii). In addition, when data are pooled from the 5 experiments, a significant decrease in mean sIPSP amplitude is observed following activation of group I receptors (from 12.12±0.49mV in control saline, to 5.49±0.21mV under DHPG; O-A, $F_{2,261}$=56.48, P<0.001, n=5; Figure 3.9B). This effect on pooled mean sIPSP amplitude was reversed following antagonist application, to 7.19±0.45mV (O-A, $F_{2,261}$=56.48, P<0.05, n=5; Figure 3.9B). In summary, the observed effects on sIPSP amplitude indicate that this group may also be located post-synaptically and thus, group I mGluRs may have parallel pre- and post-synaptic effects on the motor circuit (see Discussion).

From the above results, it is clear that group I receptor activation reduces the frequency and amplitude of both glycinergic and GABAergic sIPSPs. To confirm specifically that group I receptors are targeting glycinergic transmission, a pharmacological block of GABAergic sIPSPs was carried out. Pre-application of 40μM bicuculline to selectively eliminate GABAergic sIPSPs, revealed that subsequent DHPG treatment reduced the rate of occurrence of remaining, presumably glycinergic, sIPSPs by ~20% (from 4.68±0.12Hz in control saline to 3.58±0.33Hz; O-A, $F_{4,120}$=8.94, P<0.05, n=6; Figure 3.10Ai-ii,B). Confirmation that the sIPSPs were indeed glycinergic was obtained by subsequent application of 1μM strychnine which completely abolished all remaining sIPSPs (Figure 3.10Aiii,B). In 50% of these experiments (n=3 out of 6), DHPG also caused a reduction in the range of glycinergic sIPSP amplitudes. However, in 2 experiments there was no significant change in mean sIPSP amplitude, and in a further 1 experiment, there was an observed increase

Figure 3.10 | Group I mGluR activation reduces the frequency of glycinergic IPSPs

(Ai-ii,B) In the presence of bicuculline (40mM; BICUC) to block GABAergic transmission, application of DHPG decreased glycinergic sIPSP frequency by ~20% (from 4.68±0.12Hz in control saline/BICUC, to 3.58±0.33Hz; O-A, $P<0.05$, $n=6$). (Aiii,B) Subsequent application of the glycine receptor antagonist, strychnine, caused an abolition of all remaining sIPSPs. * = significant, ns = not significant. Values are means ± S.E.M.


Ai
CONTROL + 40μm BICUC
15mV
500ms
Aii
+ 10μm DHPG
Aiii
+ 1μm STRYCHNINE
B
sIPSP Frequency (Hz)
0
1
2
3
4
5
6
*
CONT
+ BICUC
DHPG
STRY

(O-A, $P<0.05$, data not shown). Figure 3.11 shows sIPSP amplitude distribution histograms from a representative experiment in the presence of bicuculline. In control conditions amplitudes ranged from 2 to 14mV with a mean of 6.35±0.11mV in the presence of bicuculline. Subsequent application of DHPG did not alter the range of amplitudes, which remained between 2 and 14mV, but the mean sIPSP amplitude decreased significantly to 5.91±0.12mV (O-A, $F_{3,550}=7.81$, $P<0.05$; Figure 3.11Ai-ii). In data pooled from the 3 experiments, the mean sIPSP amplitude significantly decreased (from 6.64±0.1mV in control saline/BICUC, to 6.22±0.1mV under DHPG; O-A, $F_{3,550}=7.81$, $P<0.05$, n=3 out of 6; Figure 3.11B).

So far, the reduction in the frequency of sIPSPs, and specifically glycinergic sIPSPs, provides evidence for a pre-synaptic locus of modulation by group I receptors. To confirm that these effects truly reflected pre-synaptic modulation of the probability of release, rather than changes in the spontaneous firing of presynaptic neurons, tetrodotoxin (TTX; 1μM) was applied to block sodium spikes and thereby synaptically isolate the cell prior to the application of agonist. In data pooled from 3 experiments, DHPG applied in the presence of TTX caused a 37% significant reduction in the frequency of sIPSPs (1.27±0.8Hz in control saline to 0.8±0.05Hz following DHPG application; K-W, $P<0.05$, n=3; Figure 3.12Ai-ii,Bi). This reduction occurred for both glycinergic (1.5±0.1Hz in control saline to 0.85±0.07Hz; O-A, $F_{2,15}=13.85$, $P<0.05$, n=3; Figure 3.12Bii) and GABAergic sIPSPs (0.28±0.03Hz in control saline to 0.08±0.01Hz; O-A, $F_{2,15}=17.14$, $P<0.05$, n=3; Figure 3.12Bii) identified on the basis of their durations. The effect on glycinergic sIPSP frequency reversed with application of the antagonist (1.05±0.1Hz; O-A, $F_{2,15}=13.85$, $P<0.05$, n=3; Figure 3.12Bii), but the rate of occurrence of GABAergic sIPSPs did not

Figure 3.11 | The effect of DHPG application on sIPSP amplitude after the blockade of $GABA_A$ receptors

In 3 out of 6 experiments, DHPG caused a decrease in sIPSP amplitudes following prior blockade of $GABA_A$ receptors. (Ai) Histogram showing the distribution of sIPSP amplitudes in the presence of 40μM bicuculline. The sIPSP amplitudes ranged from 2 to 14mV, and the calculated mean was 6.35±0.11mV (from this representative experiment). (Aii) Subsequent application of 10μM DHPG did not show a shift in the range of amplitudes (remaining between 2 and 14mV), but the mean amplitude showed a significant reduction to 5.91±0.12mV (O-A, $P<0.05$). (B) Mean sIPSP amplitudes pooled from the 3 experiments showed a small but significant decrease following agonist application (from 6.64±0.1mV in control saline/bicuculline, to 6.22±0.1mV under DHPG; O-A. $P<0.05$, n=3 out of 6). Dotted lines represent mean sIPSP amplitude. Values are means ± S.E.M.

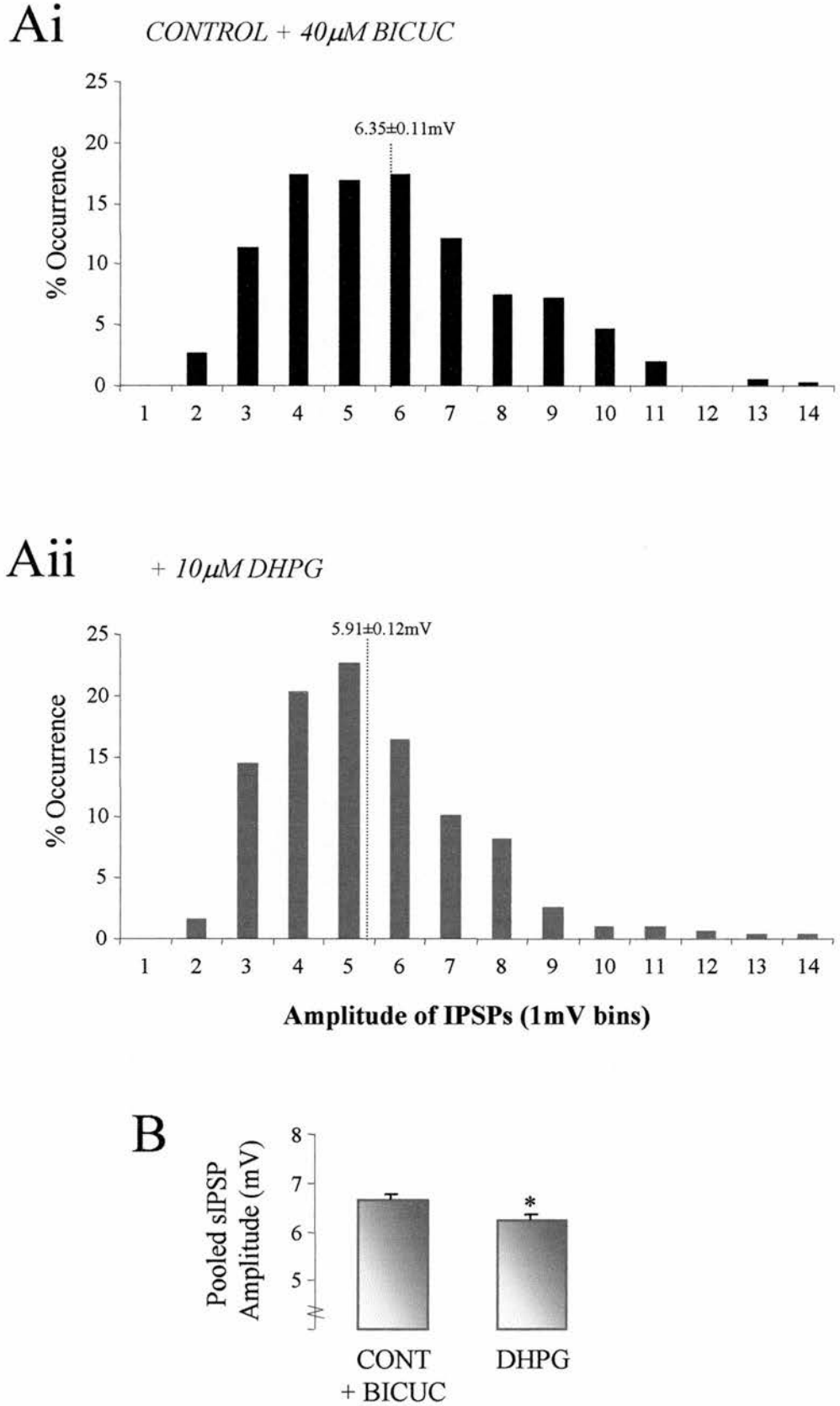
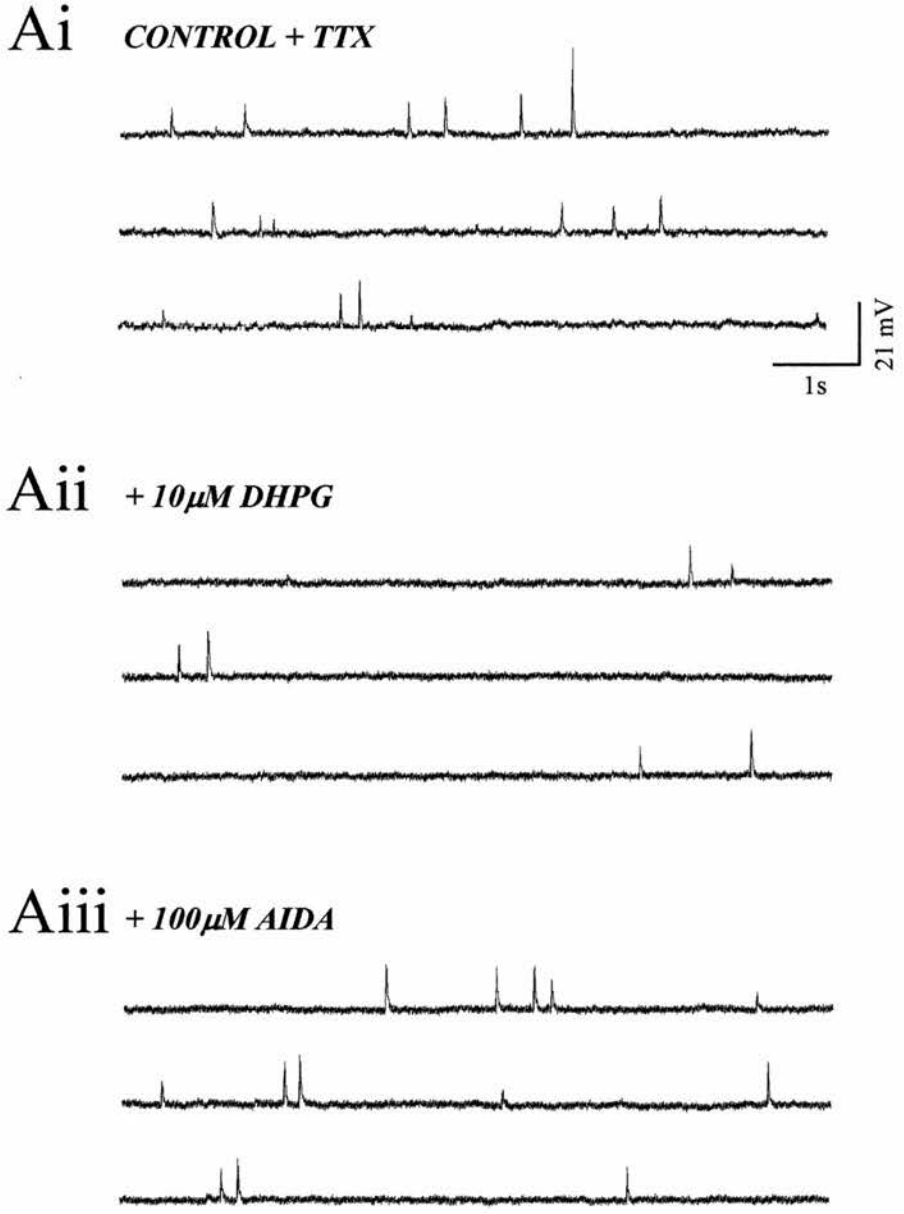

Ai
CONTROL + 40μM BICUC
6.35±0.11mV
% Occurrence
25
20
15
10
5
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Aii
+ 10μM DHPG
5.91±0.12mV
% Occurrence
25
20
15
10
5
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Amplitude of IPSPs (1mV bins)
B
Pooled sIPSP Amplitude (mV)
8
7
6
5
*
CONT + BICUC
DHPG

Figure 3.12 | The rate of TTX-resistant sIPSPs decreased after the activation of group I mGluRs

(Ai-iii) In the presence of 1μM TTX, spontaneous glycine and GABA transmitter release during inter-episode quiescent periods reduced following DHPG (10μM) application. This effect was reversed following the application of the general antagonist, AIDA (100μM). (B) Graph showing the reduced rate of occurrence of sIPSPs in the presence of TTX following the activation of group I mGluRs (from 1.27±0.8Hz in control saline to 0.8±0.05Hz; K-W, $P<0.05$, n=3). Subsequent application of AIDA reversed this effect on sIPSP frequency (to 1.2±0.25Hz; K-W, $P<0.05$, n=3). (C) The DHPG-induced decrease in rate of sIPSP occurrence included both glycinergic (from 1.5±0.1Hz in control saline to 0.85±0.07Hz; O-A, $P<0.05$, n=3) and GABAergic sIPSPs (from 0.28±0.03Hz in control saline to 0.08±0.01Hz; O-A, $P<0.05$, n=3). Antagonist application reversed the effect on glycinergic sIPSP frequency (O-A, $P<0.05$, n=3), but did not reverse GABAergic sIPSP frequency (O-A, $P>0.05$, n=3). It should be noted that the frequency of GABAergic sIPSPs was lower in the first instance, therefore a statistically significant reversal by AIDA may yield to more n numbers. * = significant, ns = not significant. Values are medians ± I.Q.R. or means ± S.E.M.


Ai
CONTROL + TTX
21 mV
1s
Aii
+ 10μM DHPG
Aiii
+ 100μM AIDA
Bi
sIPSP Frequency (Hz)
2.5
2
1.5
1
0.5
0
*
*
CONT + TTX
DHPG + TTX
AIDA + TTX
Bii
GLYCINE
GABA
sIPSP Frequency (Hz)
2
1.5
1
0.5
0
*
ns
*
ns
CONT + TTX
DHPG + TTX
AIDA +TTX

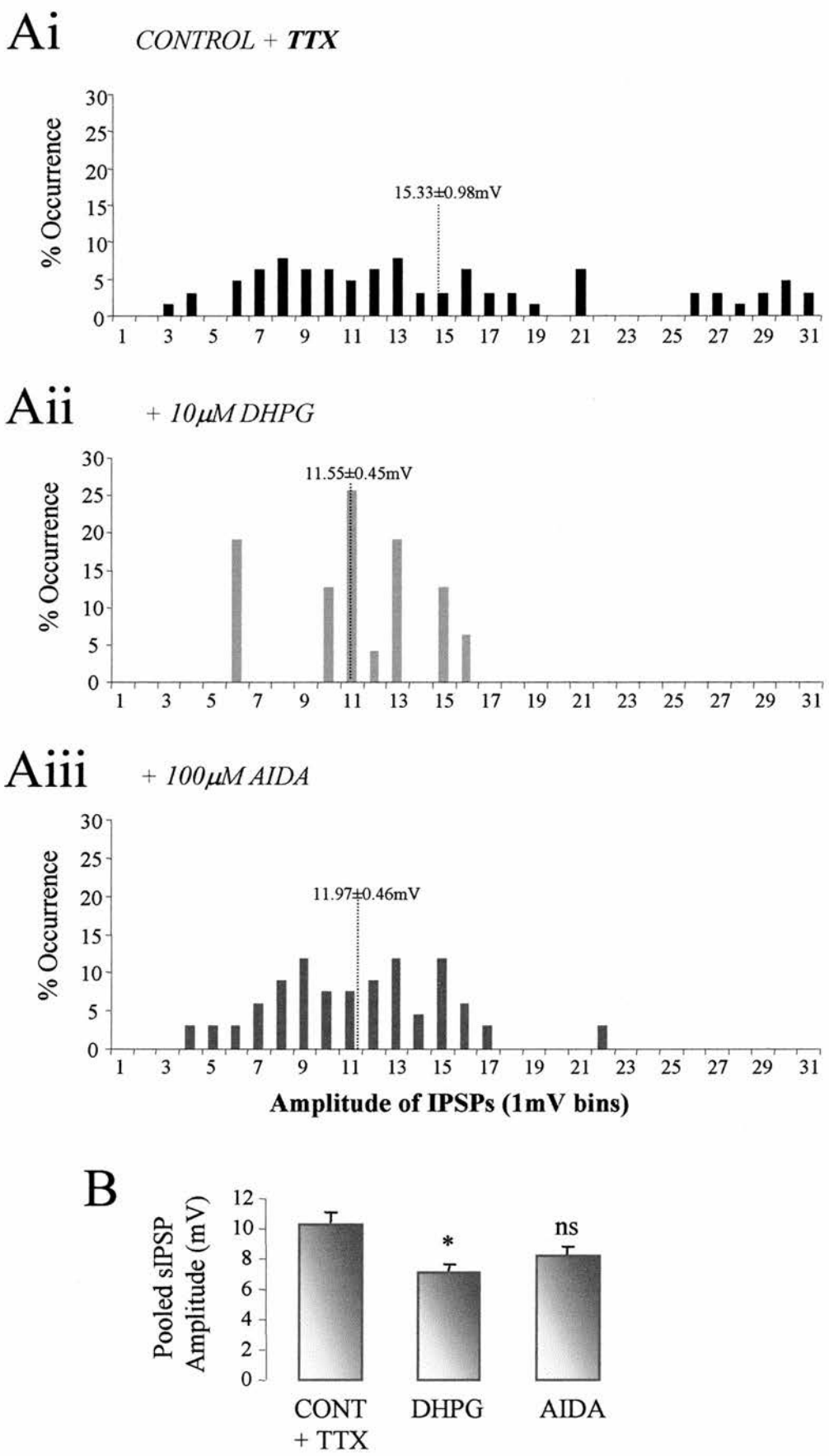
(0.08±0.04Hz; O-A, $F_{2,15}$=17.14, P<0.05, n=3; Figure 3.12Bii). However, the frequency of GABAergic sIPSPs in the first instance was low, and given the small n numbers, a statistically significant reversal of this effect by AIDA may yield to more experiments.

DHPG application in the presence of TTX also revealed a significant reduction in the range and mean sIPSP amplitudes. The sIPSP amplitude distribution histograms in figure 3.13 are from one representative experiment showing the reduction in both the mean and range of sIPSP amplitudes following group I receptor activation in the presence of TTX. sIPSP amplitudes ranged from 3 to 31mV in control conditions, with a mean of 15.33±0.98mV (Figure 3.13Ai). This distribution of amplitudes includes both glycinergic and GABAergic sIPSPs. After activation of group I receptors, the histogram shifts to the left, now ranging between 6 and 16mV and with a significantly reduced mean of 11.55±0.45mV (O-A, $F_{2,176}$=8.55, P<0.05; Figure 3.13Aii), although there was not a significant reversal with AIDA (11.97±0.45mV; O-A, P>0.05; Figure 3.13Aiii). When data from all 3 experiments are pooled, the mean sIPSP amplitude significantly decreased from 10.28±0.48mV in control conditions to 7.09±0.32mV following DHPG application (O-A, $F_{2,447}$=15.5, P<0.05, n=3; Figure 3.13B), but this effect was not significantly reversed by AIDA (8.02±0.4mV; O-A, P>0.05, n=3; Figure 3.13B). However, these data suggest that group I receptors induce a post-synaptic modulation of inhibitory transmission.

The preceding results indicate that the activation of group I mGluRs modulates both the glycinergic and GABAergic inhibitory pathways. The continued effect of group I receptor activation on sIPSP frequency in the presence of TTX suggests that these

Figure 3.13 | DHPG affects the distribution of sIPSP amplitudes in the presence of TTX

In 3 experiments, DHPG application in the presence of TTX decreased mean sIPSP amplitude. (Ai) Histogram showing the variation in sIPSP amplitudes in control conditions (from this representative experiment), in the presence of TTX. sIPSP amplitudes ranged from 3 to 31mV with a mean amplitude of 15.33±0.98mV (represented by the dotted line). (Aii) DHPG application induced a shift to the left of the histogram as the range of sIPSP amplitudes decreased, ranging between 6 and 16mV. The mean amplitude was significantly reduced under DHPG, to 11.55±0.45mV (O-A, $P<0.05$). (Aiii) Application of AIDA increased the range of amplitudes (ranging between 4 and 22mV), but did not significantly reverse the effects on mean amplitude (11.97±0.45mV; O-A, $P>0.05$). (B) sIPSP amplitudes pooled from the 3 experiments similarly decreased following group I receptor activation (from 10.28±0.48mV in control saline/TTX, to 7.09±0.32mV under DHPG; O-A, $P<0.05$, $n=3$), but could not be reversed following application of AIDA (8.2±0.4mV; O-A, $P>0.05$, $n=3$). Values are means ± S.E.M.


Ai
CONTROL + TTX
% Occurrence
15.33±0.98mV
Aii
+ 10μM DHPG
11.55±0.45mV
Aiii
+ 100μM AIDA
11.97±0.46mV
Amplitude of IPSPs (1mV bins)
B
Pooled sIPSP Amplitude (mV)
*
ns
CONT + TTX
DHPG
AIDA

receptors are located pre-synaptically on the terminals of both glycinergic and GABAergic neurons. Pharmacological blockade of $GABA_A$ receptors to specifically isolate glycinergic sIPSPs revealed in a further 5 experiments that the decreased rate of occurrence of glycinergic sIPSPs by DHPG is also TTX-resistant (1.76±0.08Hz in control saline to 1.25±0.11Hz; O-A, $F_{4,100}$=122.07, P<0.05, n=5; Figure 3.14Ai-ii,B) and this effect could be reversed on washout (4.9±0.1Hz; O-A, $F_{4,100}$=122.07, P<0.05, n=5; Figure 3.14Aiii,B). The increased sIPSP frequency following washout was significantly higher than control, which could be due to a time-dependent increase in the rate of sIPSPs over the course of the recordings. This in turn suggests that the DHPG-induced reduction of glycinergic sIPSP frequency in these experiments may be more dramatic than it first appears.

The sIPSP amplitude distribution histograms represented in figure 3.15 show that group I receptor activation can simultaneously modulate glycinergic transmission pre- and post-synaptically. However, this effect only occurred in 3 out of 5 experiments suggesting that this post-synaptic locus of modulation by group I receptors may only occur in a proportion of cells. In these cases, glycinergic sIPSP amplitudes ranged between 1 and 10mV with a mean of 4.96±0.18mV (Figure 3.15Ai). Following group I receptor activation, even in the presence of TTX, there is a small but significant decrease in the amplitude range, now between 1 and 7mV, and in the mean amplitude, to 3.56±0.14mV following application of DHPG (O-A, $F_{2,464}$=29.02, P<0.05; Figure 3.15Aii). This effect could be reversed on washout as the range of amplitudes increased to between 2 and 11mV, and the mean increased to 5.34±0.13mV (O-A, $F_{2,464}$=29.02, P<0.05; Figure 3.15Aiii). In data pooled from 3 experiments, the decrease in mean sIPSP amplitude following group I receptor activation is significant (from 5.26±0.1mV in control saline, to 4.5±0.12mV under DHPG; O-A, $F_{2,876}$=11.34,

Figure 3.14 | Group I mGluR-induced increase in glycinergic sIPSP rate of occurrence is TTX-resistant

(Ai-ii,B) In the presence of both TTX, to block spike-mediated transmission, and bicuculline, to block GABAergic transmission, activation of group I mGluRs reduced the frequency of the remaining, presumably glycinergic, sIPSPs by ~30% (from 1.76±0.08Hz in control saline to 1.25±0.11Hz; O-A, $P<0.05$, $n=5$). (Aiii,B) The rate of occurrence of these glycinergic sIPSPs subsequently increased during washout, reversing the effect of DHPG (to 4.9±0.1Hz; O-A, $P<0.05$, $n=5$). * = significant, ns = not significant. Values are means ± S.E.M.

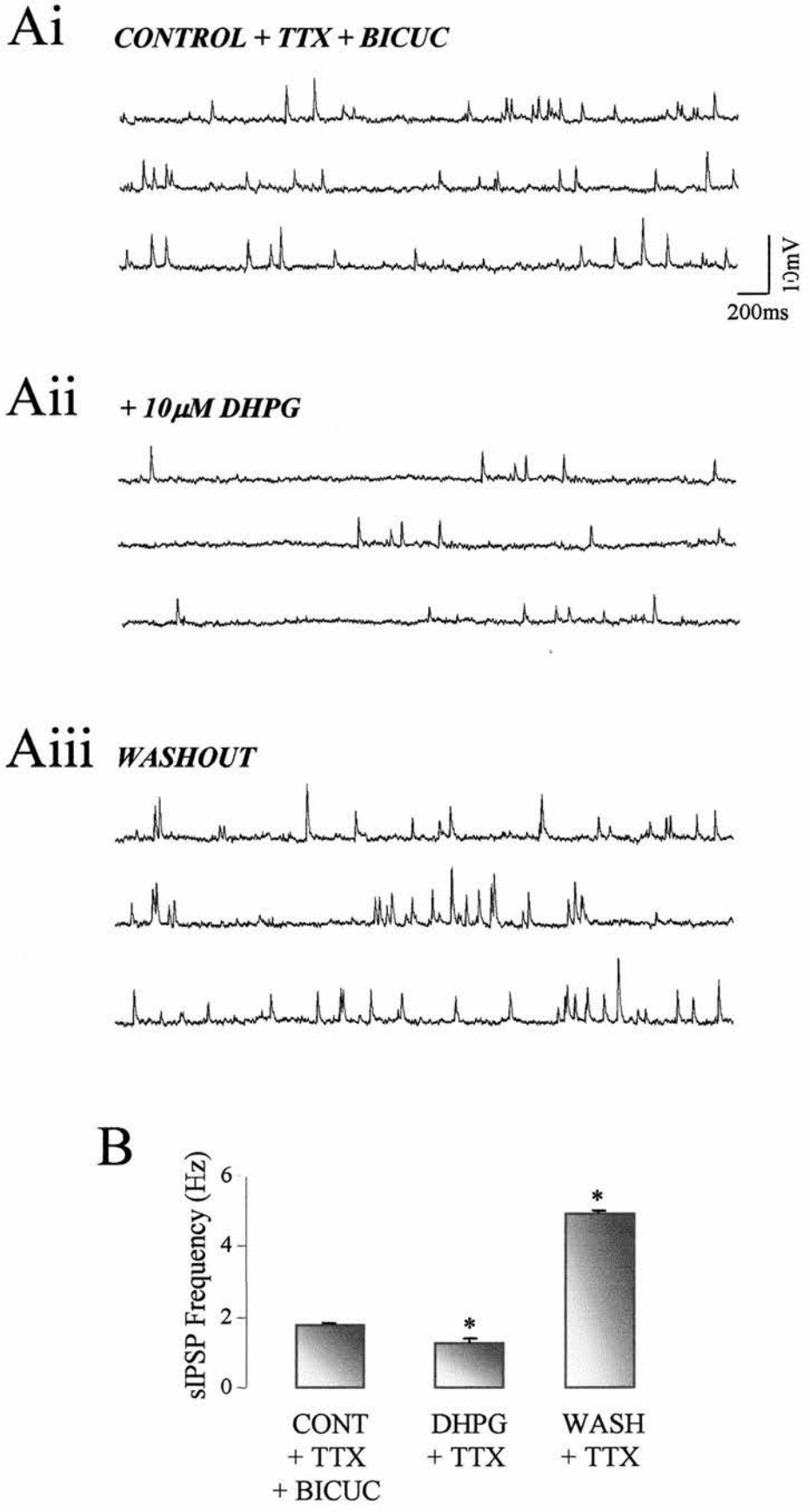
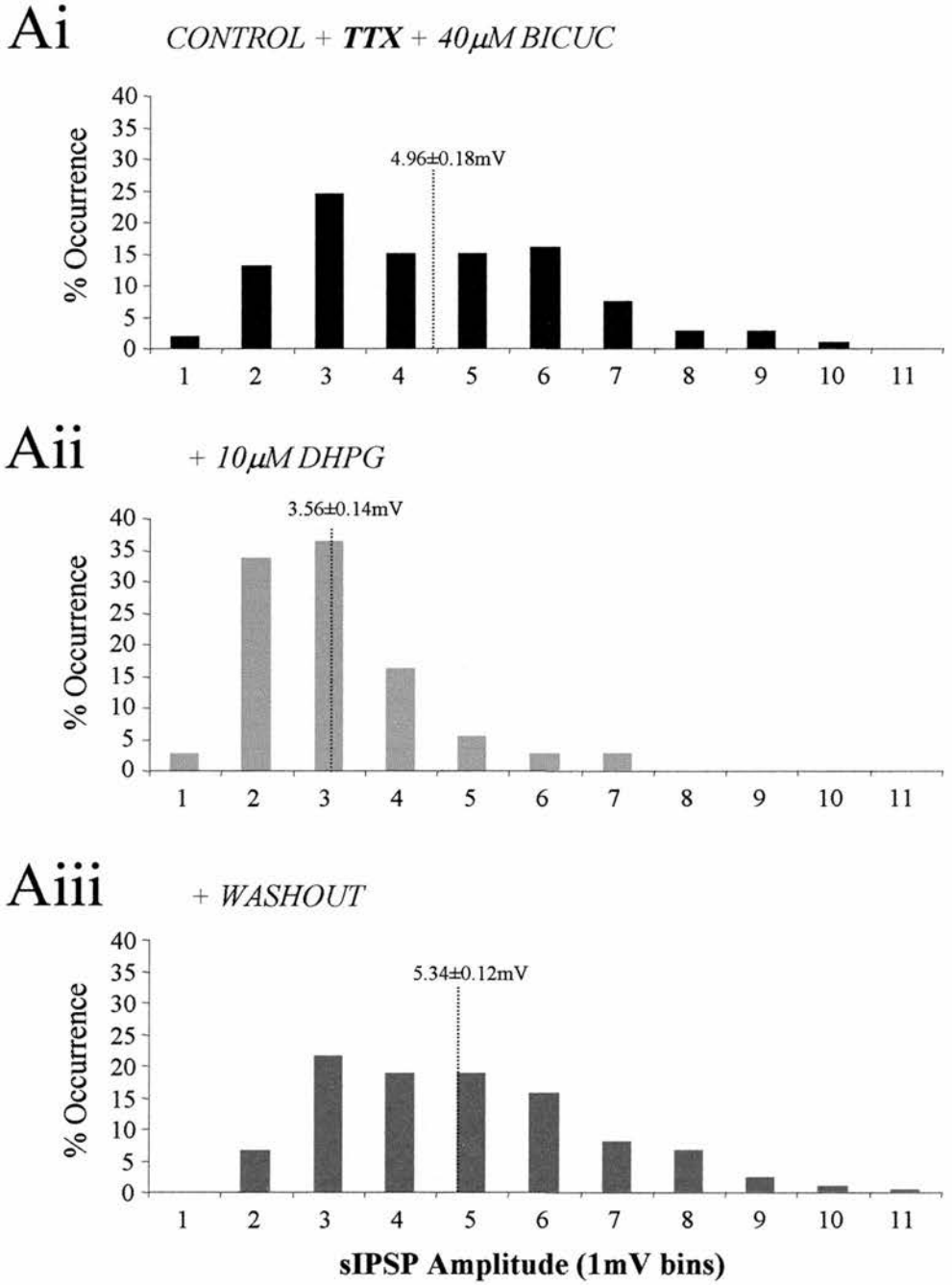

Ai
CONTROL + TTX + BICUC
10mV
200ms
Aii
+ 10μM DHPG
Aiii
WASHOUT
B
sIPSP Frequency (Hz)
0
2
4
6
*
*
CONT
+ TTX
+ BICUC
DHPG
+ TTX
WASH
+ TTX

Figure 3.15 | DHPG application affects sIPSP amplitudes after the blockade of $GABA_A$ receptors and in the presence of TTX

In 3 out of 5 experiments, DHPG induced a reduction in the mean amplitude of TTX-resistant glycinergic sIPSPs. (Ai) Histogram showing that sIPSPs varied in amplitude in the presence of both TTX and bicuculline (BICUC), ranging between 1 to 10mV. The mean amplitude in this control condition was 4.96±0.18mV (from this representative experiment). (Aii) Subsequent application of DHPG caused a significant shift to the left of the histogram where the range of amplitudes decreased, to between 1 and 7mV, and the mean amplitude significantly decreased, to 3.56±0.14mV (O-A, $P<0.05$). (Aiii) This effect was reversed during washout as the range of amplitudes increased to between 2 and 11mV, and the mean amplitude significantly increased to 5.34±0.13mV (O-A, $P>0.05$). (B) Pooled mean sIPSP amplitudes from the 3 experiments show a decrease in mean amplitude following group I receptor activation (from 5.26±0.1mV in control saline, to 4.5±0.12mV under DHPG; O-A, $P<0.05$, $n=3$). This effect on mean sIPSP amplitude was reversed with washout (to 5.3±0.11mV; O-A, $P<0.05$, $n=3$). Dotted lines represent the mean amplitude under each condition. Values are means ± S.E.M.


Ai
CONTROL + TTX + 40μM BICUC
% Occurrence
4.96±0.18mV
Aii
+ 10μM DHPG
3.56±0.14mV
Aiii
+ WASHOUT
5.34±0.12mV
sIPSP Amplitude (1mV bins)
B
Pooled sIPSP Amplitude (mV)
CONT + TTX + BICUC
DHPG + BICUC
WASH + BICUC

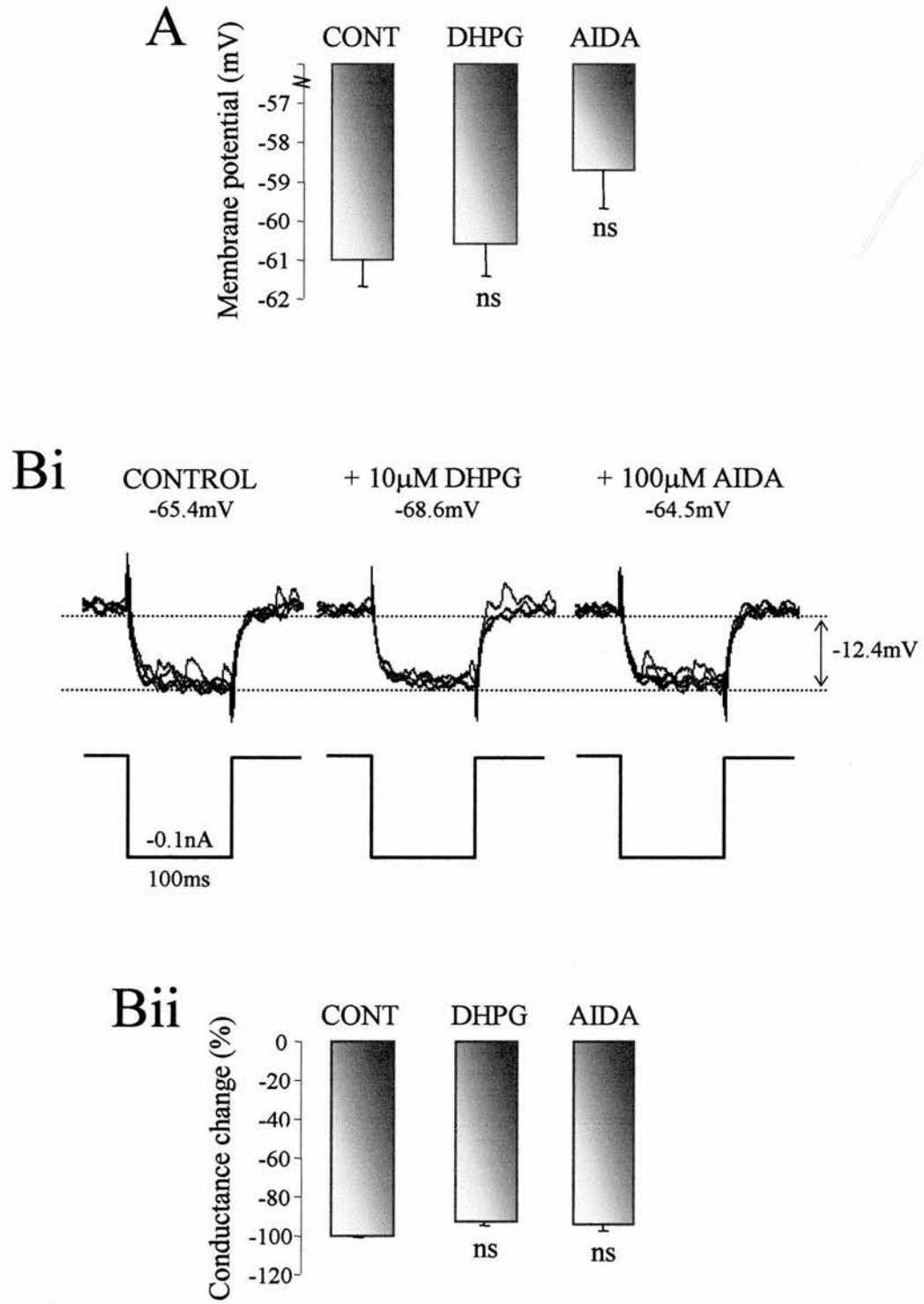
$P<0.05$, n=3; Figure 3.15B) and can be reversed with washout (to 5.3±0.11mV; $F_{2,876}=11.34$, $P<0.05$, n=3; Figure 3.15B).

There were no obvious changes in the resting membrane properties that might account for the changes in sIPSP amplitudes. For example, DHPG did not affect the resting membrane potential (O-A, $P>0.05$, n=7; Figure 3.16A), and subsequent AIDA application similarly had no influence (O-A, $P>0.05$, n=5 out of 7; Figure 3.16A). Furthermore, hyperpolarizing conductance pulses injected before, during and after application of DHPG and AIDA produced no detectable effect on input resistance (-12.4±0.21mV in control saline, to –11.52±0.16mV under DHPG; O-A, $P>0.05$, n=7; Figure 3.16Bi-ii). AIDA similarly had no effect on conductance (-11.68±0.28mV; O-A, $P>0.05$, n=7; Figure 3.16Bi-ii). It is possible that changes in conductance are occurring but are not accounted for because the conductance pulses were not large enough or because the change is occurring too remote from the recording site. From these and the preceding results, group I receptors appear to act pre-synaptically to modulate glycine release. However, the potential post-synaptic mechanisms behind the group I receptor-mediated reduction in mean sIPSP amplitude remain unclear.

To summarise, the above findings suggest that group I mGluRs are acting both pre-synaptically, to modulate the probability of quantal inhibitory transmitter release, and post-synaptically, involving the reduction of transmitters' effects on the motorneurons. The persistence of the effect of DHPG in the presence of TTX confirms a pre-synaptic locus of modulation by these receptors. However, the DHPG-induced post-synaptic effects on sIPSP amplitude distribution did not consistently occur in all recordings, implying that only a proportion of cells respond in this manner to the activation of group I receptors. Nevertheless, group I receptor activation causes

Figure 3.16 | Activation of group I mGluRs produces no change in conductance or membrane potential

(A) There is no change in membrane potential following application of DHPG or AIDA (O-A, $P>0.05$, n=5 out of 7). (Bi) Hyperpolarising conductance test pulses, (5 x 100ms), are overlapped and show no detectable change in conductance following application of 10μM DHPG. Dotted line for ease of comparison of membrane potential. (Bii) Measurements are shown as percentage changes from control, pooled from 7 experiments and show no change with either DHPG or AIDA application (O-A, $P>0.05$, n=7). Data are means ± S.E.M.


A
CONT
DHPG
AIDA
Membrane potential (mV)
-57
-58
-59
-60
-61
-62
ns
ns
Bi
CONTROL
-65.4mV
+ 10μM DHPG
-68.6mV
+ 100μM AIDA
-64.5mV
-12.4mV
-0.1nA
100ms
Bii
CONT
DHPG
AIDA
Conductance change (%)
0
-20
-40
-60
-80
-100
-120
ns
ns

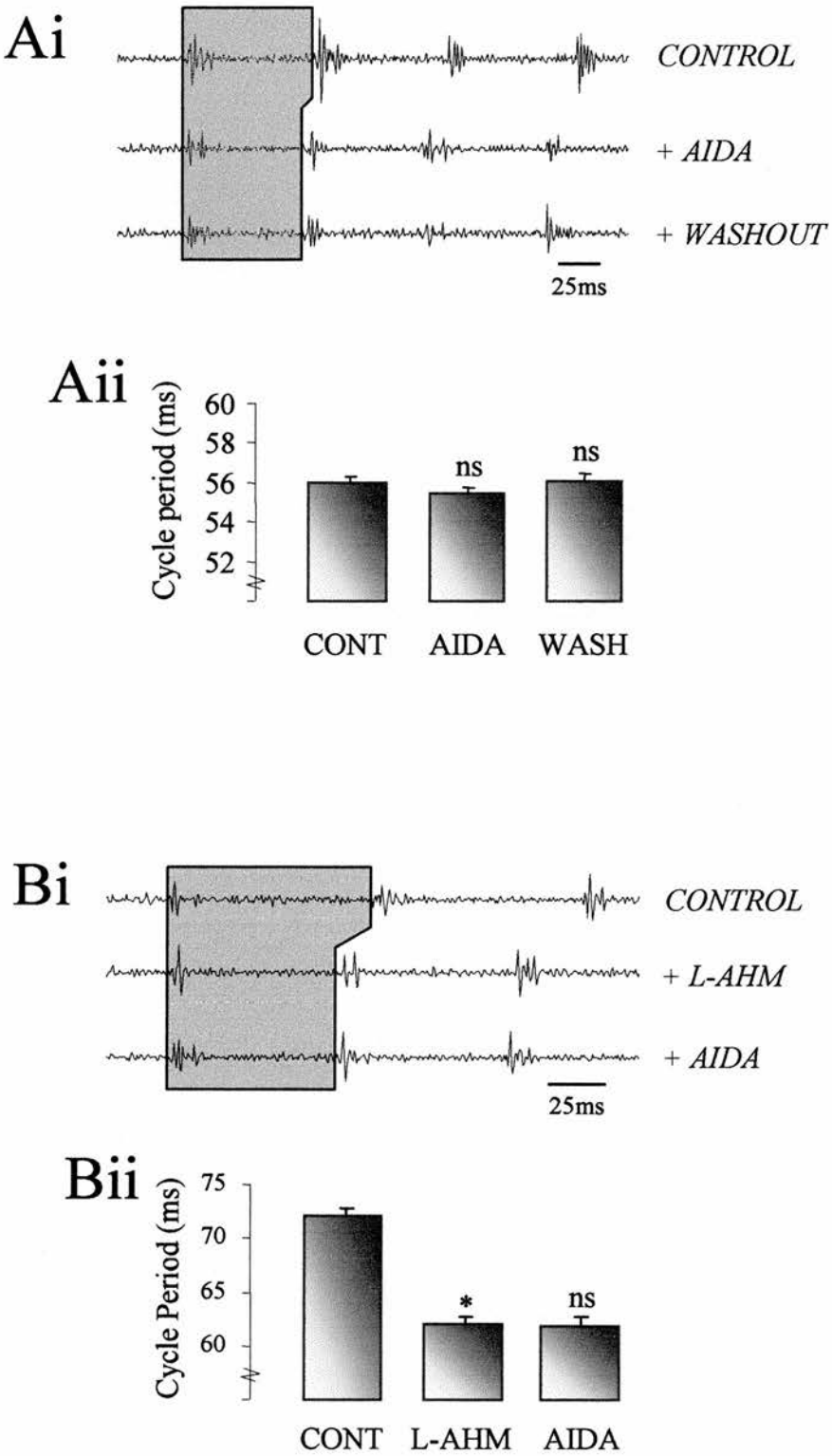
a direct modulation of inhibitory, and specifically glycinergic transmitter release from pre-synaptic terminals which supports the idea that these receptors are reducing inhibitory transmission to increase network excitability.

3 | 3 | 6 Endogenous and subtype specific activation of group I receptors

To assess the potential contribution of endogenous activation of group I receptors within the locomotor network during swimming activity, the general antagonist AIDA (100μM) was bath applied alone. However, no significant changes in cycle periods were observed in any of the 7 animals tested (3 embryos and 4 larvae; 55.96±0.35ms in control to 55.4±0.32ms with AIDA; O-A, $P>0.05$, n=7; Figure 3.17Ai-ii) and all other parameters of swimming were similarly unaffected (data not shown). This lack of effect of AIDA suggests that group I receptors are not normally activated during swimming. Attempts to enhance endogenous activation of group I mGluRs by increasing the extracellular levels of glutamate using the glutamate uptake inhibitor, L-AHM (100μM) produced the expected increase in swim frequency with cycle periods decreasing from 71±11ms in control saline to 61.5±6.125ms (K-W, $P<0.05$, n=7; Figure 3.17Bi-ii) in the presence of the uptake inhibitor. However, the application of AIDA in the presence of L-AHM still caused no change in cycle periods (K-W, $P>0.05$, n=7; Figure 3.17Bi-ii). The resultant rise in extracellular glutamate due to L-AHM will not only cause an activation of all mGluRs subtypes, but also ionotropic glutamate receptors and other glutamate transporters not blocked with L-AHM. Therefore, the effects of AIDA on group I receptors may be masked by the additive effects of glutamate acting simultaneously on other glutamate receptors and transporters.

Figure 3.17 | Effects of the general group I antagonist, AIDA, on ventral root activity

Ventral root recording (Ai) and graphical representation (Aii) showing no significant change in cycle periods following application of the general group I mGluR antagonist AIDA (100μM; O-A, P>0.05, n=7). (Bi-ii) AIDA did not affect cycle periods even with prior application of the glutamate uptake inhibitor L-AHM (100μm; K-W, P>0.05, n=7). All other parameters of swimming activity were unaffected in the presence of AIDA (data not shown). * = significant, ns = not significant. Values are means ± S.E.M. or medians ± I.Q.R.

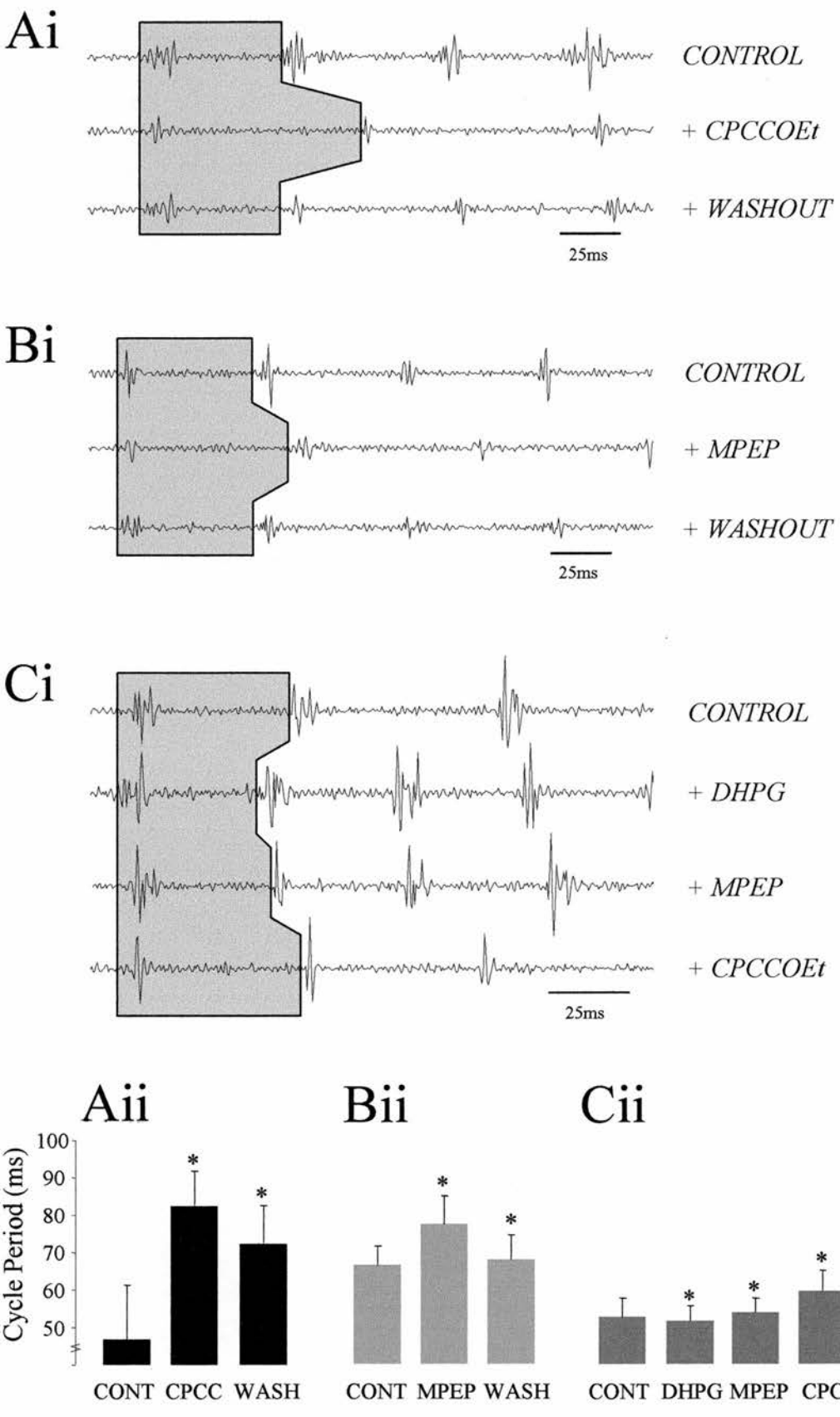

Ai
CONTROL
+ AIDA
+ WASHOUT
25ms
Aii
Cycle period (ms)
60
58
56
54
52
ns
ns
CONT
AIDA
WASH
Bi
CONTROL
+ L-AHM
+ AIDA
25ms
Bii
Cycle Period (ms)
75
70
65
60
*
ns
CONT
L-AHM
AIDA

Another possibility is that AIDA is not as 'general' an antagonist at the group I mGluRs as previously assumed. There is evidence that AIDA may be more selective for the group I subtype, $mGluR_1$, over $mGluR_5$ (Moroni *et al*, 1997). If this is the case in *Xenopus* then either each subtype has equal and opposing effects, which cancel out the expected increase in cycle periods, or group I mGluRs have no endogenous modulatory control on motor activity under normal experimental conditions.

To assess each of these possibilities, the subtype specific antagonists CPCCOEt ($mGluR_1$; 50μM) and MPEP ($mGluR_5$; 100μM) were bath applied both individually and sequentially following DHPG applications. 50μM CPCCOEt application profoundly affected swimming frequency, increasing cycle periods by ~46% from 45.75±16.62ms in control saline to 81±11ms (in 3 embryos and 4 larvae; K-W, $P<0.05$, n=7; Figure 3.18Ai-ii), an effect which was partially but significantly reversed after washout (to 72±10.25ms; K-W, $P<0.05$, n=7). The $mGluR_5$ subtype specific antagonist, MPEP (100μM) also increased cycle periods, but only by ~14% from 65.5±5ms in control saline to 77.5±2.75ms (in 3 embryos and 3 larvae; K-W, $P<0.05$, n=6; Figure 3.18Bi-ii), which was also reversed successfully after washout (to 68±6.5ms; K-W, $P<0.05$, n=6). Whilst it appears from these data that both group I subtypes are present and can be activated endogenously, there do not appear to be differential roles for the two subtypes on motor activity (cf. Lamprey; Kettunen *et al*, 2002, 2003; see Discussion). The extent to which $mGluR_5$ increases cycle periods appears to be less than $mGluR_1$, which can be observed when antagonists to each receptor are applied sequentially after activating the group I receptors with DHPG (Figure 3.18Ci-ii). In 3 animals, DHPG application increased swimming frequency,

Figure 3.18 | Group I subtype specific antagonists, CPCCOEt and MPEP, both decreased swimming frequency

Ventral root recording (Ai) and graph (Aii) showing increased cycle periods following application of the $mGluR_1$ specific antagonist, CPCCOEt (50μm, CPCC: K-W, $P<0.05$, $n=7$). (Bi-ii) Similarly, application of the $mGluR_5$ antagonist, MPEP, also increased cycle periods (100μm; K-W, $P<0.05$, $n=6$). The effects induced by these two antagonists were reversed during washout (K-W, $P<0.05$, $n=7$ and $n=6$). (Ci-ii) Cycle periods reduced following DHPG application were significantly increased in the presence of MPEP (K-W, $P<0.05$ $n=3$), which increased further following application of CPCCOEt (K-W, $P<0.05$, $n=3$). * = significant, ns = not significant. Values are medians ± I.Q.R.


Ai
CONTROL
+ CPCCOEt
+ WASHOUT
25ms
Bi
CONTROL
+ MPEP
+ WASHOUT
25ms
Ci
CONTROL
+ DHPG
+ MPEP
+ CPCCOEt
25ms
Aii
Bii
Cii
Cycle Period (ms)
100
90
80
70
60
50
CONT CPCC WASH
CONT MPEP WASH
CONT DHPG MPEP CPCC

reducing cycle periods (from 52.75±5ms in control to 51.75±4.125ms; K-W, $P<0.05$, n=3; Figure 3.18Ci-ii), and subsequent application of MPEP increased cycle periods significantly by ~4.5% to 53.75±4ms (K-W, $P<0.05$, n=3), which increased another 10.7% with application of CPCCOEt to 59.5±5.5ms (K-W, $P<0.05$, n=3). In summary, it appears that group I mGluRs can be activated by endogenously released glutamate, yet there does not appear to be differential roles for each group I subtype, as has been shown in the lamprey.

3 | 4 DISCUSSION

The main aim in this series of electrophysiological experiments was to determine a functional role in swimming activity of the group I mGluRs in young tadpoles of *Xenopus laevis*. Using a pharmacological approach, it is evident from the results that group I mGluRs are indeed capable of modulating the CPG for swimming in *Xenopus*. Group I receptor activation with the specific agonist, DHPG, caused a significant increase in swimming frequency and an associated increase in the number of spontaneously occurring episodes, effects which could be reversed with the general group I antagonist, AIDA (Figures 3.1-3.3). These excitatory effects of group I mGluRs could potentially be accounted for by a shift in the balance between synaptic excitation and inhibition in the spinal network. As DHPG application does not affect motor burst durations or amplitudes (Figures 3.1 and 3.2), it can be suggested that the balance involves a shift in favour of reduced inhibition rather than increased excitation. Indeed, initial experiments with the glycine and GABA antagonists, strychnine and bicuculline, respectively (Figures 3.4, 3.5, and 3.6), indicated that group I receptors may be utilising the fast inhibitory pathways as a mechanism to shape the motor output. Furthermore, the lack of significant effect of DHPG in the

presence of strychnine supports the proposal that glutamatergic excitation is not affected by group I mGluRs.

Fast inhibitory pathways have been shown to be common targets for neuromodulators regulating motor pattern generation in *Xenopus* (for a review: Sillar, 2002). For example, the biogenic amines, NA and 5-HT, are known to facilitate and depress glycinergic transmission, respectively, effects which have been shown to occur pre-synaptically via amine receptors located on the terminals of the inhibitory interneurons (McDearmid *et al*, 1997; Merrywest *et al*, 2002; Sillar, 2002). There is also evidence that the free radical gas, nitric oxide (NO), can potentiate both GABAergic and glycinergic synaptic inhibition causing swimming to slow down and stop prematurely (McLean and Sillar, 2000; McLean *et al*, 2001). In other species, there is also evidence for group I mGluRs modulating these inhibitory transmitters. For example, in dissociated rat spinal cord neurons, glycinergic transmission is reduced through a decrease in intracellular cAMP levels following group I mGluR activation (Katsurabayashi *et al*, 2001). Similarly, group I mGluRs reduce GABAergic transmission in the SNr, one of the primary output nucleii of the basal ganglia (Marino *et al*, 2001), but this class of receptor can facilitate GABAergic transmission in the periaqueductal grey of rats (de Novellis *et al*, 2003; Drew *et al*, 2004).

Glycine is responsible for mediating the mid-cycle reciprocal inhibition between locomotor half-centres in most vertebrates (Grillner *et al*, 1995; Sillar, 2002). There is well documented experimental and modelling evidence indicating that the strength

of the mid-cycle inhibition is an important determinant of the frequency of swimming. Small changes to this inhibitory component of the synaptic drive can produce profound changes in swimming frequency. For instance, a reduction in glycinergic transmission through the application of strychnine, will increase swimming frequency (Dale, 1995), whilst a facilitation by the application of noradrenaline (NA) or nitric oxide (NO) will decrease swimming frequency (McDearmid *et al*, 1997; McLean and Sillar, 2000). These effects have also been simulated in computer models of the swimming network in both *Xenopus* and lamprey, where artificially changing the levels of reciprocal inhibition can alter the frequency of swimming (Dale, 1995; Hellgren *et al*, 1992). For example, increasing inhibitory conductances in the simulated network slows the rhythmic output from the simulated network, and conversely, decreasing an inhibitory conductance increases output frequency. In this chapter it was found that the effects of DHPG on the motor network were occluded by the prior application of strychnine (Figure 3.4A), consistent with the idea that group I mGluRs affect swimming, at least in part, by modulating the strength of glycinergic inhibition. Blocking glycine receptors after the activation of group I receptors produced a further increase in swimming frequency. There are two potential explanations for this finding: either, DHPG, at the concentration applied, does not depress all glycinergic transmission; or strychnine may have other effects such as closing voltage-dependent K^+ channels to cause an increase in frequency.

Similarly, at least in principle, GABA could also be modulating swimming frequency despite there being no GABAergic inhibitory component phase-locked to each swim cycle. GABAergic IPSPs have a long duration (90-200ms), making it possible for them to span more than one cycle of swimming (~50-70ms long). Consequently, over

the course of an episode sporadic GABA IPSPs could summate to produce a background level of tonic inhibition that ultimately slows swimming (Reith and Sillar, 1999). Interestingly, during these experiments, DHPG caused the duration of swimming episodes to become irregular when applied after the blocking of $GABA_A$ receptors (Figure 3.5). Although the resulting irregular pattern of swimming did not allow for reliable and accurate measurement of cycle periods, any reduction in inhibition will cause the balance between inhibition and excitation to shift in favour of higher excitability within the network causing an increase in the frequency at which the animal swims. DHPG, through suppression of glycinergic transmission and combined with reduced GABAergic transmission may cause irregular activity, by removing the majority of the inhibitory drive underlying swimming. There is evidence that blocking both glycine and GABA receptors with strychnine and bicuculline, respectively, will completely abolish all swimming activity in larval *Xenopus*, leaving only intense non-rhythmic motor bursts (Reith and Sillar, 1999). This perhaps highlights the requirement of a baseline level of inhibition to maintain rhythm generation. As mentioned previously, DHPG does not fully depress all glycinergic transmission and as a consequence sufficient inhibition may be present within the motor network to permit a certain amount of albeit irregular rhythmic activity.

Conversely, DHPG application *before* the blocking of $GABA_A$ receptors does not induce the same loss of rhythmicity (Figure 3.6). Rhythmic swimming activity persists and despite episodes being shortened, there are sufficient cycles per episode to analyse any effects on the frequency of swimming. Bicuculline characteristically produces an increase in swimming frequency in both embryos and larvae, along with reduced R-C delays, and in larvae only, an increase in the duration of bursts (Reith

and Sillar, 1999). Paradoxically however, from this set of experiments an *increase* in cycle periods is seen with bicuculline application after group I mGluR activation. One explanation may lie in the method of analysis. Towards the end of an episode of swimming, cycle periods become longer as the animal slows down and eventually stops altogether. Bicuculline is known to reduce episode durations (Figure 3.5 and 3.6), making it is possible that the region in which cycle periods were measured and included in the analysis fell towards the end of an episode where the cycles are longer. Therefore, direct comparisons of cycle periods cannot be accurately made in these experiments.

Evidence for group I receptor-mediated modulation of inhibitory transmission was supported further by intracellular recordings from presumed motorneurons. DHPG caused a depression of glycinergic transmission which was reflected by a reduction in the amplitude of the mid-cycle IPSP and in the rate of spontaneous IPSPs during quiescent periods between swimming episodes (Figures 3.7 and 3.8). In addition, GABA sIPSP rate was inhibited by DHPG suggesting GABA transmission is also impaired following group I receptor activation. As mentioned previously, changing the strength of glycinergic synaptic connections has a dramatic influence on swimming frequency (Dale, 1995; McDearmid *et al*, 1997) so the effects of group I mGluRs on mid-cycle inhibition could explain the acceleration of swimming following their activation. The reduction in sIPSPs suggests that group I mGluR activation reduces the glycinergic mid-cycle IPSP (Figure 3.7) in part by reducing the probability of glycine release (Figure 3.8), which in turn results in the observed increase in swimming frequency. A concomitant reduction in GABAergic transmission would also contribute towards increased excitability within the motor network.

The reduction in the rate of 'miniature' glycinergic and GABAergic sIPSPs by DHPG remained in the presence of TTX (Figure 3.12), which synaptically isolates the cell. Therefore, these data strongly indicate a pre-synaptic site of action of group I mGluRs on the terminals of inhibitory interneurons. A single population of glycinergic interneurons, commissural interneurons, mediate the reciprocal inhibition during swimming in *Xenopus* and are therefore a likely candidate for group I receptor modulation.

The exact mechanism targeted by DHPG to reduce transmitter release cannot be deduced from the present experiments. Group I receptor activation could act indirectly, via a second messenger pathway, or by directly coupling to the vesicular release machinery to decrease the transmitter discharged into the synaptic cleft. Suppression and enhancement of both excitatory and inhibitory synaptic transmission through mGluR activation is well documented although it is thought that multiple mechanisms may be responsible (Poncer *et al*, 1995; Glitsch *et al*, 1996; Anwyl, 1999). For example, in larval lampreys two separate mechanisms have been shown to be responsible for facilitating and depressing glutamate transmission: group I receptors function to facilitate release of transmitter by releasing Ca^{2+} from internal stores, whilst group III receptors suppress transmitter release through the activation of a pre-synaptic K^+ current (Cochilla and Alford, 1998).

In addition to these group I receptor-induced effects on sIPSP frequency, DHPG also caused a reduction in the mean amplitude of sIPSPs, suggesting a parallel post-synaptic locus of action for this class of receptor. There was a shift to the left of the

amplitude distribution histograms after the activation of group I mGluRs (Figure 3.9) providing some evidence for some post-synaptic modulation. This finding is similar to studies conducted in various brain regions (including hippocampal CA1 cells, frontal cortex neurons and auditory cortex neurons (Desai and Conn, 1991; Burke and Hablitz, 1994)) where group I mGluR activation has been shown to reduce both the frequency and the amplitude of GABAergic sIPSPs. However, I did not observe any changes in membrane potential or conductance following the activation of group I receptors suggesting that DHPG does not directly affect a post-synaptic conductance. It is possible that conductance changes did occur, but were too far from the recording site to be detected, or that the conductance pulses were not large enough to detect the change. It is more likely that DHPG acts indirectly on the post-synaptic membrane to alter sIPSP amplitudes via a second messenger system which may alter either the glycine receptor or its ability to bind glycine. Nevertheless, whilst the shift in sIPSP amplitudes is indicative of a post-synaptic effect, it is also possible that this effect on sIPSP amplitudes is partly due to DHPG acting pre-synaptically but differentially on certain contacts. For example, it is possible that the glycinergic and GABAergic synapses onto the motorneurons show a maturational gradient from the cell body out towards more distal regions that might account for the distribution of amplitudes. Synaptic maturation is accompanied by an increase in the reliability and amount of vesicular neurotransmitter released. Those synapses further from the motorneuron cell body may release more vesicles but natural attenuation of the sIPSP as the signal progresses may cause them to appear smaller than those fewer vesicles released onto the neuron closer to the cell body. DHPG causes a decrease in the probability of glycinergic and GABAergic vesicular release which may indirectly remove all the large amplitude sIPSPs, which, by this argument would already have a low probability of release, and leave only those sIPSPs being generated at more distal synapses, hence

an appearance of the mean amplitude being reduced. This line of argument implies a pre-synaptic site of action of DHPG, compared to, for example, the agonist causing a reduction of the opening kinetics of the glycinergic and GABAergic receptor ion channels or causing a desensitisation of the receptors post-synaptically.

Recent evidence from the lamprey has revealed that $mGluR_1$ activation induces the release of endocannabinoids that act in a retrograde fashion to cause a depression of glycinergic inhibitory transmission onto motorneurons and crossed-caudal interneurons increasing network excitability (Kettunen *et al*, 2005). This finding in the lamprey compliments the results presented in this chapter and provides an elegant mechanism by which group I mGluRs could act post-synaptically to reduce inhibitory transmission. The involvement of endocannabinoids as a means by which group I receptors execute their effect on the *Xenopus* motor network has not been investigated in this thesis, but should certainly be addressed in the future. In summary, it appears that group I mGluRs are probably working both pre- and post-synaptically to reduce inhibitory transmission.

This investigation has also provided evidence that group I mGluRs are endogenously activated when the *Xenopus* swim motor network is cycling. Initially, experiments with the general group I antagonist AIDA showed no alteration in swim frequency (Figure 3.17). However, AIDA may not be as general an antagonist for group I receptors as was first thought (Krieger *et al*, 1998), and evidence exists for it being more specific for $mGluR_1$ than $mGluR_5$ (Moroni *et al*, 1997). Therefore, experiments using the specific subtype non-competitive antagonists, CPCCOEt ($mGluR_1$) and MPEP ($mGluR_5$) were conducted. Swimming frequency decreased with both these

specific antagonists suggesting that the two receptor subtypes are activated during swimming by endogenously released glutamate (Figure 3.18). It is generally thought that the mGluRs may be acting to mop-up spillover of glutamate in the synaptic cleft, as would be indicated by the fact that most mGluRs are located extrasynaptically. Further intracellular experiments with these subtype-specific antagonists are now important to assess whether blocking group I receptors increases inhibitory transmission leading to a reduction in swim frequency in *Xenopus*.

These two group I mGluR subtypes produce very distinct endogenous cellular effects on the lamprey locomotor network (for a review: El Manira *et al*, 2002): $mGluR_1$ causes a potentiation of NMDA receptors leading to an increase in locomotor frequency (Krieger *et al*, 1998, 2000), whilst $mGluR_5$ induces intracellular Ca^{2+} oscillations producing a decrease in swim frequency (Kettunen *et al*, 2002). The mechanisms underlying the modulation of neuronal excitability within the lamprey locomotor network are also different for each subtype, in that $mGluR_1$, but not $mGluR_5$, inhibits a leak current boosting depolarisation and permitting ventral root bursting and, when combined with the potentiation of NMDA receptors, produces a profound increase in locomotor frequency (Kettunen *et al*, 2003). Certainly from my extracellular data with group I specific antagonists there do not seem to be opposing effects on the swim frequency mediated by the two group I receptor subtypes, as both cause a profound decrease in swim frequency (Figure 3.18). Whether this is due to the developmental stage of the animals or phylogenetic differences compared to the lamprey remains to be determined.

Group I receptors induce different effects through a various mechanisms in a variety species and developmental stages. In comparison to the reported effects in adult

lamprey mentioned above, larval lamprey locomotor activity is modulated by activation of group I mGluRs located pre-synaptically on reticulospinal axons causing facilitation of synaptic transmission by release of Ca^{2+} from ryanodine-sensitive internal stores (Cochilla and Alford, 1998). In another species of frog, *Rana pipiens*, it has been shown in adult animals that group I mGluRs potentiate NMDA-depolarisation of motorneurons via multiple mechanisms, including: activation of an as yet unknown, G-protein; a rise in intracellular Ca^{2+} levels; the binding of Ca^{2+} to calmodulin; and a decrease of the NMDA channel block by Mg^{2+} ions (Holohean *et al*, 1999). In the *Xenopus* model, at least during these early stages of development, group I mGluR activation apparently mediates a facilitation of swim frequency primarily through a reduction of glycinergic inhibitory transmission causing an increase in network excitability. There is no indication from my results that group I mGluRs mediate any modulation of excitatory transmission. For example, there is no effect on either ventral root burst durations or amplitudes, and more convincingly, in the presence of strychnine, the effects of DHPG are occluded. Nevertheless, the exact mechanisms underlying this effect remain subject to further investigation.

4 | Group II mGluR-mediated modulation of a spinal locomotor network

4 | 1 SUMMARY

In this chapter, pharmacological manipulations of group II mGluRs have revealed profound influences of this group of receptor upon the swimming activity in young *Xenopus laevis* tadpoles. Bath applications of the specific group II agonist, APDC, produce a significant inhibitory effect on many of the parameters of fictive swimming including a reduction in swim frequency and episode duration, and motor burst amplitude and duration. In experiments conducted in the presence of the $GABA_A$ antagonist, bicuculline, APDC was still able to exert its effects on the network suggesting that group II mGluRs probably do not facilitate this pathway to reduce swim episode duration. The glycine receptor antagonist, strychnine, appeared to occlude the effects of APDC on cycle period suggesting that glycinergic transmission is a potential target of these mGlu receptors to mediate their effects. However, during intracellular recordings from presumed motorneurons using KCl-filled microelectrodes, no evidence was found to support the idea that group II receptors mediate their effect through the fast inhibitory pathways. There was no clear alteration of the mid-cycle IPSP amplitude during evoked swimming and similarly no effect of APDC on the frequency of spontaneous glycinergic or GABAergic IPSPs. There was also no detectable change in the membrane potential or input resistance following activation of group II receptors. The reduction in motor burst durations and amplitudes suggest that group II receptors may inhibit excitatory transmission by acting as negative feedback autoreceptors at glutamatergic synapses.

4 | 2 INTRODUCTION

Nearly all rhythmic motor systems can vary their motor output to produce a wide range of frequencies and intensities, but relatively little is known of the neural mechanisms utilised to impart such flexibility. In principle, flexibility in the output of any network can be achieved through alterations to the cellular and synaptic properties of its constituent neurons and such alterations are often mediated by neuromodulators acting on metabotropic receptors (for a review: Pearson, 1993; Kiehn and Katz, 1999). At one level of organisation neuromodulation can be

categorised into two types: extrinsic neuromodulation, in which the network output is altered by modulatory neurons that are not themselves components of the rhythm generating machinery; and intrinsic neuromodulation, where members of a given network exert modulatory effects on the network output (Kiehn and Katz, 1999). Two extensively studied extrinsic neuromodulators in various vertebrate motor systems are the biogenic amines, NA and 5-HT; for example - cat (Barbeau and Rossignol, 1990, 1991), rat (Kiehn *et al*, 1999) and amphibians (Sillar *et al*, 1998). These are produced by brainstem interneurons and released within the spinal cord where various effects on the properties of the network are exerted. Less is known about intrinsic neuromodulation, but in the tadpole, purinergic transmission within the spinal cord has been shown to regulate the duration of swim episodes by modulating voltage dependent channels (Dale and Gilday, 1996). In addition, the lamprey spinal cord contains a ventral neuromodulatory plexus which releases a range of modulators including 5-HT (here acting as an intrinsic neuromodulator) and substance P.

The excitatory neurotransmitter glutamate mediates its actions within the CNS through the activation of both ionotropic and metabotropic glutamate receptors. In the tadpole, the iGluRs, NMDA and non-NMDA (AMPA), are known to be important in the initiation and maintenance of the synaptic drive underlying swimming in *Xenopus* (see chapter 1). They are activated by a population of excitatory glutamatergic interneurons located within the spinal cord (Dale and Roberts, 1984, 1985). However, nothing is known in the *Xenopus* model about the presence or role that mGluRs might play in the generation or modulation of motor activity.

mGluRs comprise eight subtypes, $mGluR_{1\text{-}8}$, contained within three groups (see chapter 1). Group II consists of subtypes $mGluR_2$ and $mGluR_3$, and these subtypes

are negatively coupled to AC. Various labelling studies indicate that group II mGluRs are not always closely associated with glutamatergic synapses and often have a perisynaptic localisation (Lujan *et al*, 1997; Shigemoto *et al*, 1997). Agonists of group II mGluRs characteristically reduce excitatory transmission in a variety of CNS structures, including spinal cord motorneurons (Ishida *et al*, 1993), the olfactory bulb (Hayashi *et al*, 1993), the striatum (Lovinger *et al*, 1995), and the dentate gyrus (Bushell *et al*, 1996; Kilbride *et al*, 1998). The $mGluR_3$ subtype is also highly expressed in glial cells providing a novel form of glial-neuronal communication that can potentially reduce glutamatergic transmission when co-activated with β-adrenergic receptors in adult hippocampal neurons (Ohishi *et al*, 1993; Conn and Pin, 1997). There is evidence that increased cAMP levels following activation of β-adrenergic receptors, following isoproterenol application, is enhanced via activation of group II mGluRs located on glia. The resultant rise in cAMP levels induces a release of either cAMP or adenosine, which acts pre-synaptically at A_1 adenosine receptors to reduce excitatory transmission (Gereau and Conn, 1994a, 1994b, 1995; Winder and Conn, 1996).

Evidence for group II mGluR involvement in the modulation of locomotor behaviour was first shown in the lamprey (Krieger *et al*, 1994, 1996), and then more recently in mouse (O'Neill *et al*, 2003), and neonatal rat (Taccola *et al*, 2004b). Studies on the lamprey locomotor network have revealed that activation of presynaptic group II mGluRs causes a reduction in excitatory transmitter release from descending RS neurons onto the motor network (Krieger *et al*, 1996). However, the mechanisms behind this group II receptor-mediated reduction in excitatory transmission in lamprey spinal neurons remains to be elucidated. This depression of excitation by group II receptors also holds true in the spinal networks of the mouse and rat where group II

antagonist applications consistently enhance locomotor activity (O'Neill *et al*, 2003; Taccola *et al*, 2004b). It is possible to achieve a similar overall effect on network output by modulating fast synaptic inhibition and a variety of neuromodulators in the *Xenopus* system have been shown to target these synapses, including 5-HT, NA and NO (McDearmid *et al*, 1997; McLean and Sillar, 2000; Fischer *et al*, 2001; McLean, 2001; Merrywest *et al*, 2002). Moreover, there is precedence for mGlu receptors to affect inhibitory transmission not only in the tadpole (see chapter 3), but also in the lamprey spinal cord (Kettunen *et al*, 2005).

In this chapter, I have investigated: firstly, whether activation of group II mGluRs using general agonists and antagonists produced any modulatory effect on the rhythm generating network for swimming in *Xenopus* and whether these receptors might be activated endogenously; secondly, if any of the observed effects are due to the modulation of the fast inhibitory pathways as I have shown possible for group I receptors (see chapter 3); and thirdly, using KCl-filled microelectrodes, I have investigated any synaptic and cellular changes that might underlie the group II mGluR-mediated modulation of swimming.

4 | 3 RESULTS

4 | 3 | 1 General influences of group II receptor activation on network activity

The data presented in this chapter are derived from both embryonic (stage 37/38) and larval (stage 42) *Xenopus*. Any differences attributable to stage of development observed during pharmacological investigation are clearly stated, but in general the findings apply to both stages of development.

To assess the influence of group II mGluR activation on the locomotor network, the general agonist, APDC, was initially bath applied. In 11 animals, 100μM APDC produced a profound effect on many of the measured parameters of swimming. This concentration of APDC was established as being most effective during extracellular dose-response experiments (data not shown). Cycle periods were significantly increased, from 56.56±0.4ms in control saline to 67.54±0.4ms with APDC (One-way ANOVA; O-A, $F_{3,1603}$=139.73, P<0.001, n=11; Figure 4.1Ai-ii and 4.2Ai-ii). This effect was significantly reversed in 4 animals following washout (to 60.8±0.5; O-A, $F_{3,1603}$=139.73, P<0.001, n=4; Figure 4.1Ai-ii) and in 7 animals following application of the group II specific antagonist EGLU (100μM; 60.8±0.5ms with EGLU; O-A, $F_{3,1603}$=139.73, P<0.001, n=7; Figure 4.2Ai-ii). The increase in cycle periods persisted throughout episodes of swimming following APDC application compared to control (First 100 cycles: *t*-test, P<0.05, n=7; Figure 4.1Aiii). However, the range of cycle periods did not significantly differ from the beginning to the end of an episode of swimming; cycle periods ranged from 44-94ms in control conditions, and between 47-98ms under APDC, suggesting that the cycle periods may have increased due to a shortening of swimming episode durations.

Figure 4.1 | Effects of group II mGluR activation are reversed with washout

Ventral root recording (Ai; stage 37/38 embryo) and graph (Aii) to show increased cycle periods following application of the group II agonist APDC (100μM; O-A, $P<0.001$, $n=11$). This effect on swimming frequency was reversed during washout (O-A, $P<0.001$, $n=4$). (Aiii) Graph illustrating on a cycle-by-cycle basis the increase in cycle periods induced by APDC (grey circles) over the first 100 cycles in a representative episode of swimming, compared to control (black circles). Burst durations (Bi) were significantly reduced under APDC in stage 42 larvae (O-A, $P<0.001$, $n=8$), but not in stage 37/38 embryos (O-A, $P>0.05$, $n=3$), and significantly decreased further in larval animals during washout (O-A, $P<0.05$, $n=3$). (Bii) Burst amplitudes were significantly reduced in both larvae (O-A, $P<0.001$, $n=8$) and embryos (O-A, $P<0.05$, $n=3$), but could only be reversed in larval animals during washout (O-A, $P<0.05$, $n=3$). (C) R-C delays increased significantly following APDC application (O-A, $P>0.05$, $n=11$) and reversed during washout (O-A, $P<0.05$, $n=4$). (D) Episode durations decreased in the presence of APDC (O-A, $P<0.05$, $n=11$) and did not reverse during washout (O-A, $P>0.05$, $n=4$). * = significant, ns = not significant. Values are means ± S.E.M.

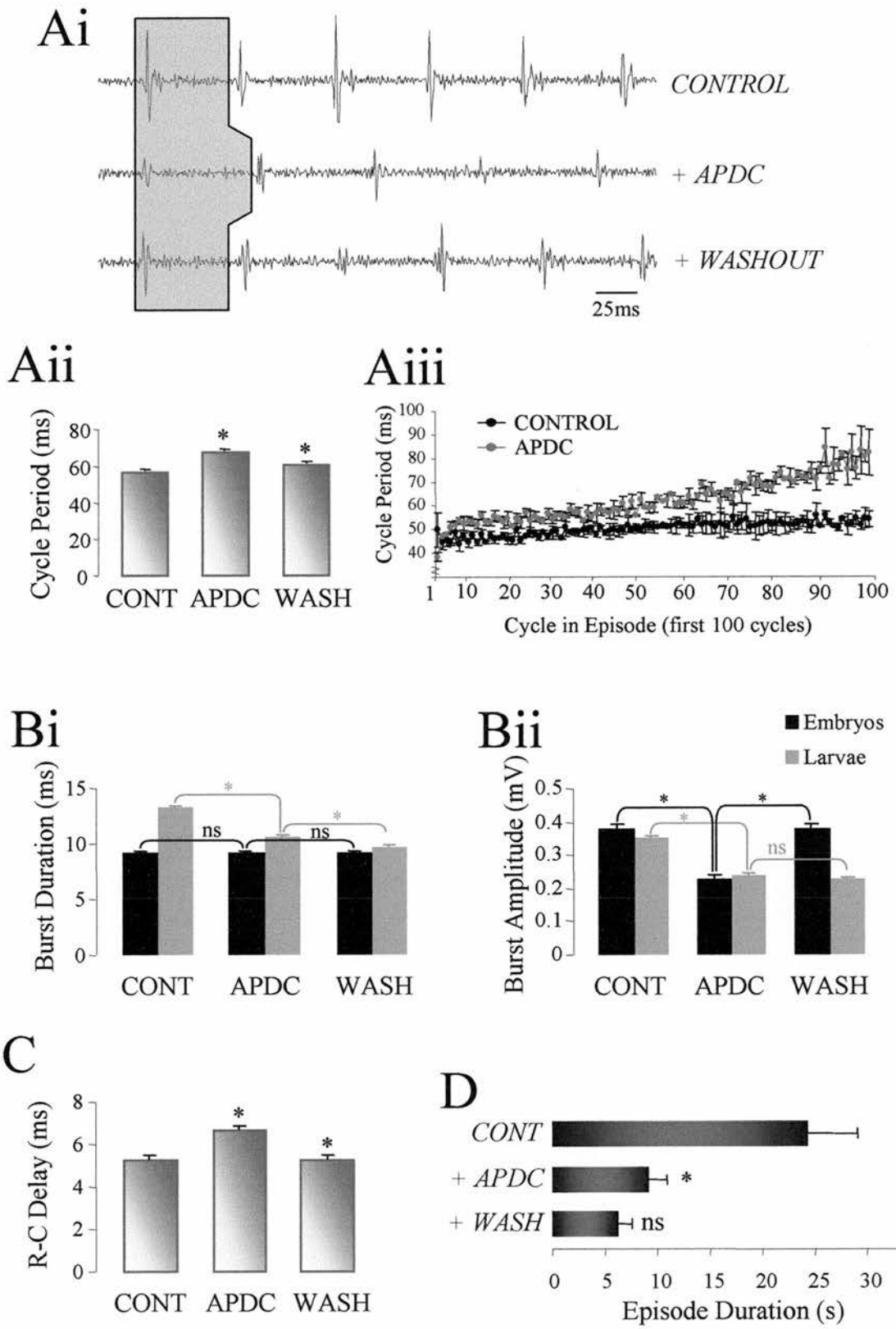
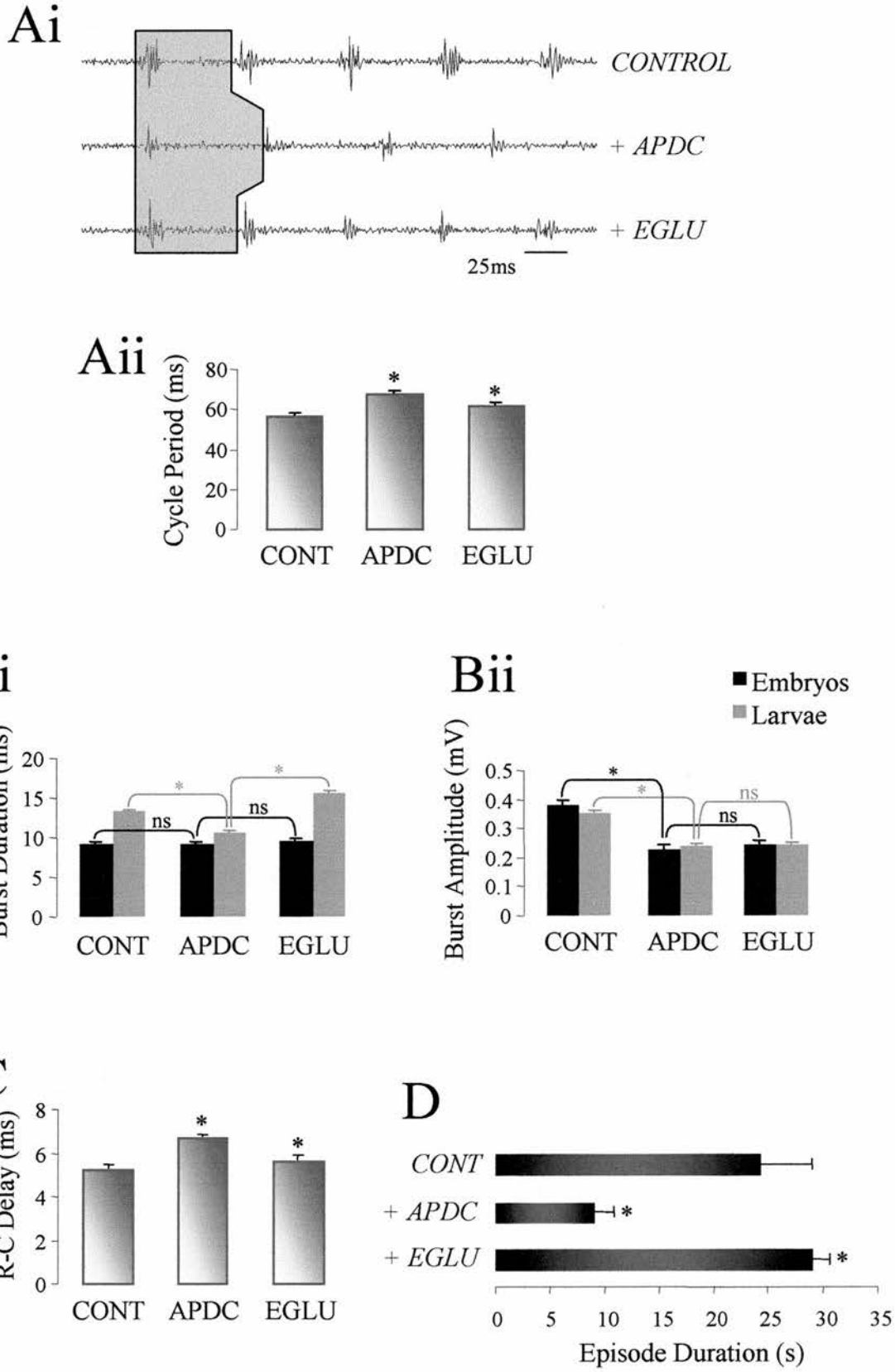

Ai
CONTROL
+ APDC
+ WASHOUT
25ms
Aii
Cycle Period (ms)
CONT APDC WASH
Aiii
CONTROL
APDC
Cycle Period (ms)
Cycle in Episode (first 100 cycles)
Bi
Burst Duration (ms)
ns
CONT APDC WASH
Bii
Embryos
Larvae
Burst Amplitude (mV)
ns
CONT APDC WASH
C
R-C Delay (ms)
CONT APDC WASH
D
CONT
+ APDC
+ WASH
ns
Episode Duration (s)

Figure 4.2 | Effects of APDC are partially reversed with the group II antagonist EGLU

Ventral root recording (Ai; stage 42 larvae) and graphical representation (Aii) showing increased cycle periods following the application of the specific group II agonist APDC (100μM; O-A, $P<0.001$, n=11). This effect was reversed with application of the antagonist, EGLU (100μM; to O-A, $P<0.001$, n=7). (Bi) APDC reduced ventral root burst durations in stage 42 larvae (O-A, $P<0.001$, n=8), but not in stage 37/38 embryos (O-A, $P>0.05$, n=3), as presented previously in Figure 4.1. This effect on burst durations in larvae could be reversed by EGLU (O-A, $P<0.05$, n=5). (Bii) Burst amplitudes were also reduced following APDC application in both larvae (O-A, $P<0.001$, n=8) and embryos (O-A, $P<0.05$, n=3). EGLU did not antagonise the observed reduction in burst amplitudes in either embryos or larvae (O-A, $P>0.05$, n=2 and n=5). (C) R-C delays increased following agonist application (O-A, $P>0.05$, n=11) and were significantly reversed by EGLU (O-A, $P<0.05$, n=7). Episode durations were significantly decreased by APDC (O-A, $P<0.05$, n=11) and were reversed by EGLU (O-A, $P<0.05$, n=7). * = significant, ns = not significant. Values are means ± S.E.M.


Ai
CONTROL
+ APDC
+ EGLU
25ms
Aii
Cycle Period (ms)
80
60
40
20
0
*
*
CONT
APDC
EGLU
Bi
Burst Duration (ms)
20
15
10
5
0
ns
ns
*
*
CONT
APDC
EGLU
Bii
Embryos
Larvae
Burst Amplitude (mV)
0.5
0.4
0.3
0.2
0.1
0
*
*
ns
ns
CONT
APDC
EGLU
C
R-C Delay (ms)
8
6
4
2
0
*
*
CONT
APDC
EGLU
D
CONT
+ APDC
+ EGLU
*
*
0
5
10
15
20
25
30
35
Episode Duration (s)

APDC produced a developmental stage-specific decrease in burst durations. Thus, in 3 stage 37/38 embryos, ventral root burst durations were not affected with APDC application (9.23±0.16ms in control and 9.16±0.13ms under APDC; O-A, $P>0.05$, n=3; Figures 4.1Bi and 4.2Bi). Similarly, there was no change in burst durations during washout in 1 embryo (9.23±0.16ms; O-A, $P>0.05$, n=1; Figure 4.1Bi) or following the application of EGLU in 2 embryos (9.23±0.13ms; O-A, $P>0.05$, n=2; Figure 4.2Bi). Burst amplitudes decreased in embryos with application of APDC (from 0.38±0.01mV in control saline to 0.23±0.01mV; O-A, $F_{3,267}=103.23$, $P<0.05$, n=3; Figure 4.1Bii and 4.2Bii), and could be reversed during washout (0.37±0.01mV; O-A, $F_{3,267}=105.36$, $P<0.001$, n=1). However, the effect on embryonic burst amplitudes did not reverse with EGLU application (0.24±0.01mV; O-A, $P>0.05$, n=2). The reversibility of APDC's effect on burst amplitudes and durations in embryos may become clearer with larger numbers of embryos included in the study.

In contrast, by larval stage 42, APDC application caused a significant decrease in burst durations (from 13.23±0.11ms in control saline to 10.59±0.14ms; O-A, $F_{3,1529}=213.84$, $P<0.05$, n=8; Figure 4.1Bi and 4.2Bi) that could be reversed during washout (to 15.52±0.24ms; O-A, $F_{3,1529}=205.7$, $P<0.05$, n=3). However, larval burst durations decreased further after the application of EGLU (to 9.63±0.1ms; O-A, $F_{3,1529}=213.84$, $P<0.05$, n=5; Figure 4.2Bi), suggesting a continued effect of APDC. Similar to embryos, larval burst amplitudes also decreased after group II receptor activation (from 0.35±0.004mV in control saline to 0.24±0.004mV; O-A, $F_{3,1529}=197.03$, $P<0.05$, n=8; Figure 4.1Bii and 4.2Bii) but this effect was not reversed during washout (0.24±0.004mV; O-A, $P>0.05$, n=3) or following EGLU application (0.23±0.004mV; O-A, $P>0.05$, n=5). As EGLU was bath applied 15-20mins before

measurements were taken (see chapter 2: Materials and methods), it is possible that a longer exposure time is required to fully antagonise all of the effects of APDC.

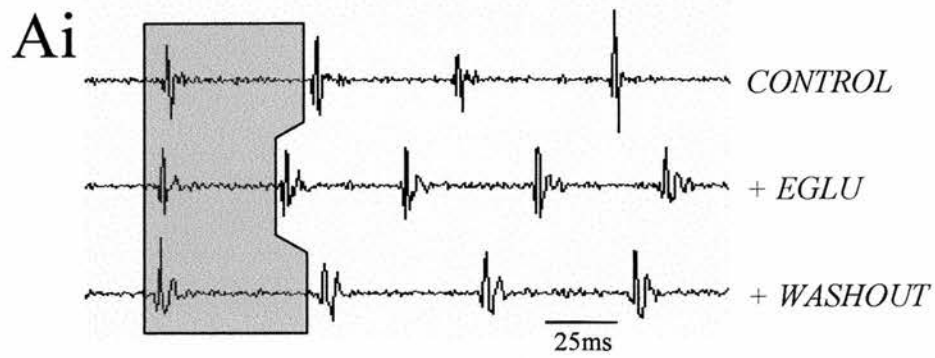
R-C delays increased following the activation of group II mGlu receptors (from 5.27±0.14ms in control saline to 6.65±0.13ms; O-A, $F_{3,1529}$=20.89, P<0.05, n=11; Figure 4.1C and 4.2C) and this effect was reversed during washout in 4 animals (to 5.27±0.14ms; O-A, $F_{3,267}$=31.16, P<0.05, n=4) and with EGLU application in 7 animals (to 5.62±0.18; O-A, $F_{3,267}$=20.89, P<0.05, n=7). Lastly, episode durations decreased in the presence of APDC (from 24.37±4.67s in control saline to 9.13±1.72s with APDC; O-A, $F_{3,79}$=7.15, P<0.001, n=11; Figure 4.1D and 4.2D) but did not reverse during washout (6.28±1.27s; O-A, P>0.05, n=4; Figure 4.1D). However, the reduction in episode durations was fully reversed following the application of EGLU (29.15±1.42s with EGLU; O-A, $F_{3,79}$=7.15, P<0.001, n=7; Figure 4.2D) and sometimes increased beyond measurements taken in control suggesting endogenous activation of group II receptors. From this set of experiments, it is clear that activating group II mGluRs with exogenously applied APDC causes a depression of motor activity within the spinal cord of *Xenopus*.

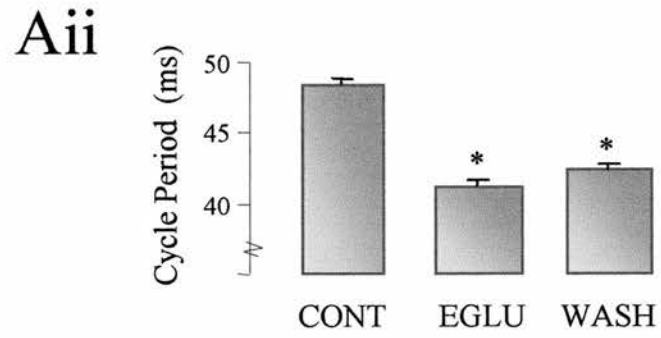
4 | 3 | 2 Evidence for endogenous activation of group II mGluRs

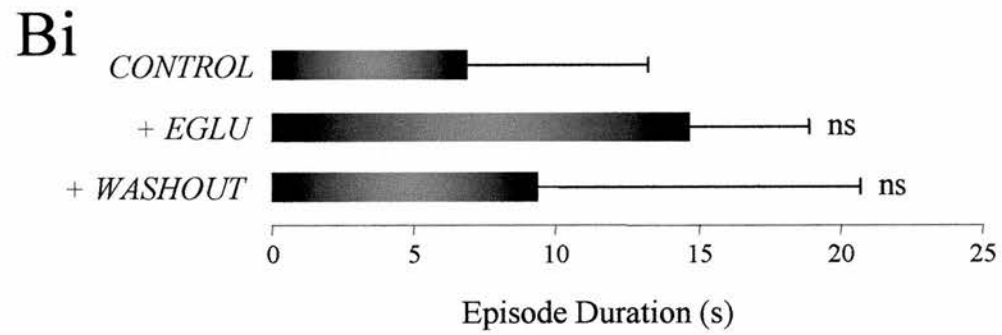
To explore further the extent to which these receptors are activated endogenously during swimming, the general group II antagonist EGLU (100μM) was bath applied. In 5 animals EGLU application caused a decrease in cycle periods (from 48.24±0.49ms in control saline to 41.1±0.5ms under EGLU; O-A, $F_{2,267}$=46.79, P<0.001, n=5; Figure 4.3Ai-ii), although all other parameters of swimming remained unaffected. Although episode durations were not significantly affected, in contrast to

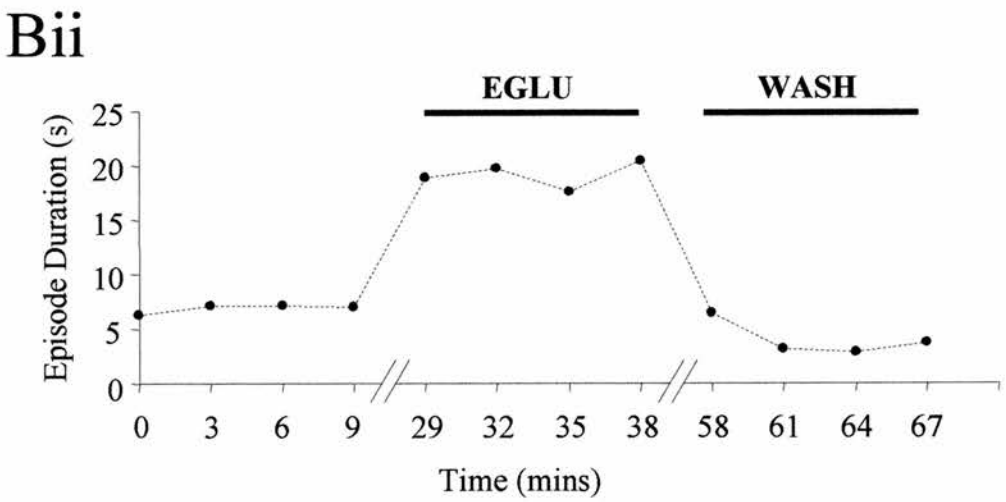
Figure 4.3 | Effects of the group II mGluR antagonist, EGLU, on ventral root activity

Ventral root recording (Ai) and graphical representation (Aii) showing reduced cycle periods with application of the group II antagonist, EGLU (100μM; O-A, $P<0.001$, n=5). This effect on swimming frequency only partially reversed during washout. (Bi) Pooled data showing EGLU applications did not significantly affect episode durations but the trend was in the appropriate direction (c.f. effect with prior application of agonist, APDC); O-A, $P>0.05$, n=5. However, following removal of outlying data, the graph in Bii shows an increase in episode durations following EGLU application over the course of an experiment (note the break in the time line as drug has 15-20mins wash-on; see chapter 2: Materials and methods) * = significant, ns = not significant. Values are means ± S.E.M.


Ai
CONTROL
+ EGLU
+ WASHOUT
25ms


Aii
Cycle Period (ms)
50
45
40
*
*
CONT
EGLU
WASH


Bi
CONTROL
+ EGLU
+ WASHOUT
ns
ns
0
5
10
15
20
25
Episode Duration (s)


Bii
EGLU
WASH
Episode Duration (s)
25
20
15
10
5
0
0
3
6
9
29
32
35
38
58
61
64
67
Time (mins)

the effective reversal EGLU produces after activation of the receptors with APDC, figure 4.3Bi shows a trend towards increased episode durations in the presence of EGLU. The lack of significance may be a consequence of small numbers of episodes measured and a significant difference may yield to further investigation. Therefore, when episode durations are plotted individually over the course of an experiment (Figure 4.3Bii), removing outlying data, it is clear that EGLU causes an increase in episode durations. These results suggest that group II receptors are indeed activated by endogenously released glutamate during swimming and that the major effect is to cause the frequency to slow and perhaps also to reduce swim episode durations.

4 | 3 | 3 Does group II mGluR-mediated reduction of swim frequency involve modulation of fast inhibitory pathways?

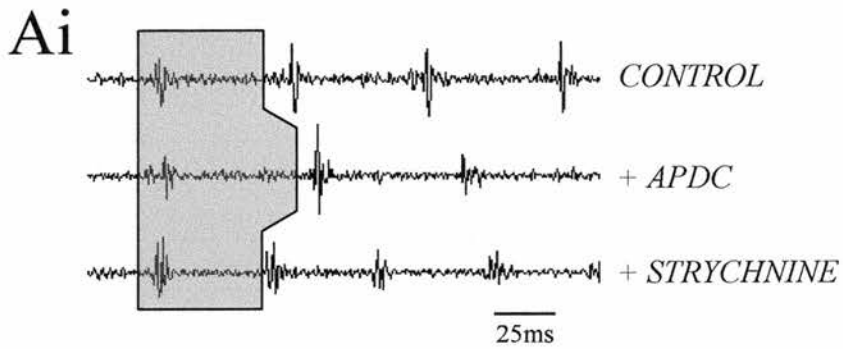
In the previous chapter, group I mGluRs were found to modulate the fast inhibitory pathways as a mechanism to increase swim frequency and network excitability. Although group II receptor-induced effects may well involve modulation of excitatory transmission, the *Xenopus* motor network allows easy examination of effects on inhibitory transmission, not least because intracellular recordings with KCl-filled microelectrodes produce IPSPs that are strongly depolarising. Therefore, using the glycine receptor antagonist, strychnine, and the $GABA_A$ receptor antagonist, bicuculline, initial extracellular experiments were conducted to assess whether the reduction in swimming frequency observed above with group II receptor activation involves modulation of these fast inhibitory pathways. An increase in inhibitory synaptic strength is a potential contributing factor for the observed slowing in swimming frequency.

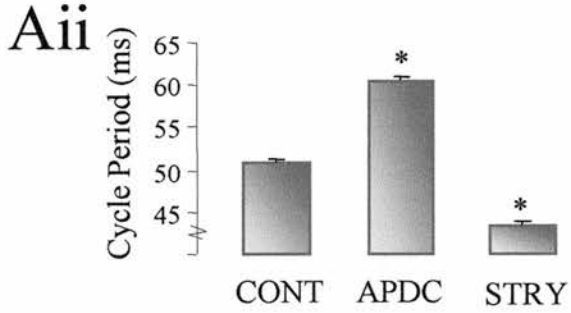
Initially the effects of applying 1μM strychnine to block glycinergic transmission after the activation of group II receptors with APDC were investigated. APDC produced the same increase in cycle periods and reduced larval burst durations, burst amplitudes and episode durations as observed previously (O-A, $P<0.05$, n=6; Figure 4.4). Subsequent application of strychnine caused a significant reduction in cycle periods (from 60.48±0.41ms with APDC to 43.3±0.24ms with strychnine; O-A, $F_{2,1267}=741.32$, $P<0.001$, n=6; Figure 4.4Ai-ii). Surprisingly a further reduction in larval burst durations was also observed (from 10.84±0.3ms under APDC to 8.84±0.17ms with strychnine; O-A, $F_{2,1267}=212.15$, $P<0.001$, n=3; Figure 4.4Bi). This is most likely due to continuing effects of APDC, rather than strychnine, which would be expected to cause an increase in burst durations. If the decrease in burst durations with APDC is better explained by group II receptor activation reducing excitatory transmission, then strychnine may have little effect because the excitatory drive underlying swimming is already depressed. There was also no change in embryonic burst amplitudes (O-A, $P>0.05$, n=3; Figure 4.4Bii) in the presence of APDC (c.f. Figure 4.1Bii and 4.2Bii), but a reduction was observed with strychnine application (O-A, $P<0.05$, n=3). The decrease in burst amplitudes in larval animals following APDC application did not change in the presence of strychnine (O-A, $P>0.05$, n=3). Episode durations similarly did not change after application of strychnine (O-A, $P>0.05$, n=6; Figure 4.4Ci-ii). As it is already known that strychnine increases swim frequency (Reith and Sillar, 1997), the next approach was to apply strychnine prior to the activation of group II receptors.

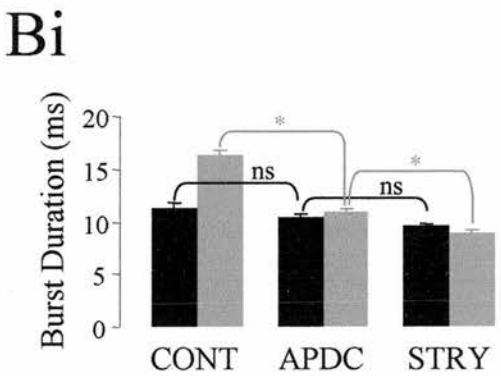
In data pooled from 5 experiments (all stage 42 larvae), 100μM APDC produced no change in cycle periods in the presence of strychnine (significant decrease from 55.8±0.48ms in control saline to 49.9±0.33ms with strychnine, remaining similar at

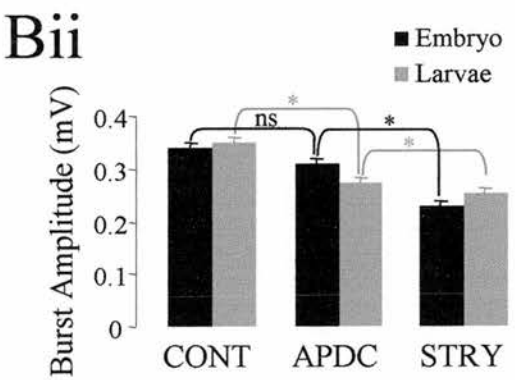
Figure 4.4 | Activation of group II mGluRs prior to the blockade of glycine receptors

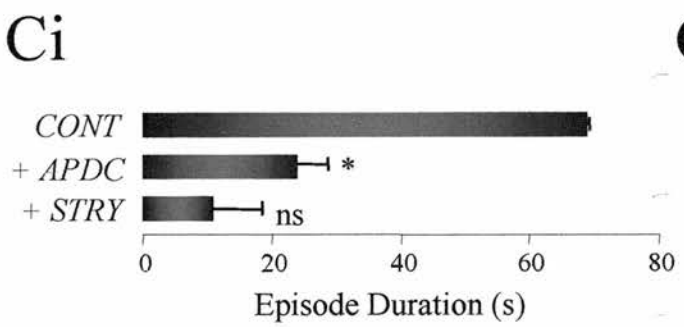
Ventral root recording (Ai) and graph (Aii) showing increased cycle periods following the application of APDC (100μM; O-A, $P<0.001$, n=6). This effect on swimming frequency significantly decreased following application of strychnine (1μM; O-A, $P<0.001$, n=6). (Bi) Burst durations were reduced by APDC in stage 42 larvae only (O-A, $P<0.001$, n=3) and further decreased under strychnine suggesting a continued effect of APDC (O-A, $P<0.001$, n=3). (Bii) Burst amplitudes decreased following agonist application in larval animals (O-A, $P<0.001$, n=3), but were not affected by application of strychnine. In 3 stage 37/38 embryos, burst amplitudes decreased under strychnine (O-A, $P<0.05$, n=3). (Ci-ii) A decrease in episode durations following bath application of APDC (O-A, $P<0.001$, n=6) did not change with application of strychnine. Downward arrows represent the stimulation artefact. * = significant, ns = not significant. Values are means ± S.E.M.

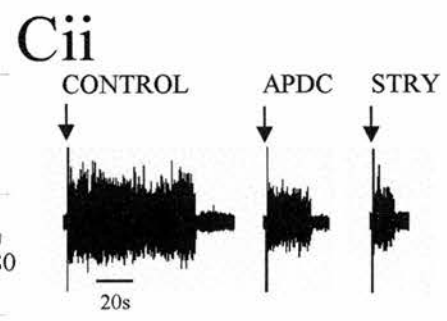

Ai
CONTROL
+ APDC
+ STRYCHNINE
25ms


Aii
Cycle Period (ms)
65
60
55
50
45
*
*
CONT
APDC
STRY


Bi
Burst Duration (ms)
20
15
10
5
0
ns
ns
*
*
CONT
APDC
STRY


Bii
Embryo
Larvae
Burst Amplitude (mV)
0.4
0.3
0.2
0.1
0
ns
*
*
*
CONT
APDC
STRY


Ci
CONT
+ APDC
+ STRY
*
ns
0
20
40
60
80
Episode Duration (s)


Cii
CONTROL
APDC
STRY
20s

48.9±0.33ms with APDC; O-A, $F_{2,1077}$=90.13, P<0.001, n=5). There was an observed reduction with bath applied APDC in both burst durations (from 12.2±0.27ms in control saline, increasing to 13.9±0.29ms with strychnine, then decreasing to 11.2±0.27ms with APDC; O-A, $F_{2,1077}$=23.17, P<0.001, n=5; Figure 4.5Bi) and burst amplitudes (from 0.52±0.02mV in control saline to 0.6±0.02mV with strychnine, decreasing to 0.47±0.01mV with APDC; O-A, $F_{2,1077}$=9.77, P<0.001, n=5; Figure 4.5Bii). However, both R-C delays and episode durations remained unaffected in these 5 animals. From these results it is unclear whether group II receptors are utilising the glycinergic pathways to influence swimming frequency. Certainly, strychnine's occlusion of the effects of APDC (Figure 4.5Aii) suggests that this pathway is a potential target. However, the continued effect of APDC on burst durations and amplitudes implicates, albeit indirectly, a modulation of excitatory pathways. Strychnine is known to exert effects other than blocking glycine transmission, for example, it can cause the closing of voltage-dependent K^+ channels, which could mask the effect of APDC on swim frequency if group II receptors do indeed directly modulate excitatory transmission.

Next, APDC was applied before and after the blocking of GABA receptors with bicuculline to assess whether group II mGluRs might influence GABAergic inhibitory transmission. As observed previously, APDC produced an increase in cycle periods (from 59.81±0.4ms in control saline to 65.79±0.33ms with APDC; O-A, $F_{2,1562}$=109.35, P<0.001, n=7; Figure 4.6Ai-ii) that was significantly reduced following application of 40μM bicuculline (to 59.8±0.4ms; O-A, $F_{2,1562}$=109.35, P<0.001, n=7; Figure 4.6Ai-ii). Bicuculline application also causes a reversal of the APDC-induced decrease in larval burst durations (from 12.41±0.2ms in control saline

Figure 4.5 | Blockade of glycine receptors prior to the activation of group II mGluRs

Ventral root recording (Ai) and graphical (Aii) representation of reduced cycle periods following strychnine application that are subsequently unaffected by APDC (100μM; O-A, $P>0.05$, n=5). (Bi) Burst durations increased by strychnine application and reduced under APDC (O-A, $P<0.001$, n=5). (Bii) Burst amplitudes increased following strychnine application and decreased in the presence APDC (O-A, $P<0.001$, n=5). R-C delays (C) and episode durations (D) were not significantly affected. * = significant, ns = not significant. Data are means ± S.E.M.

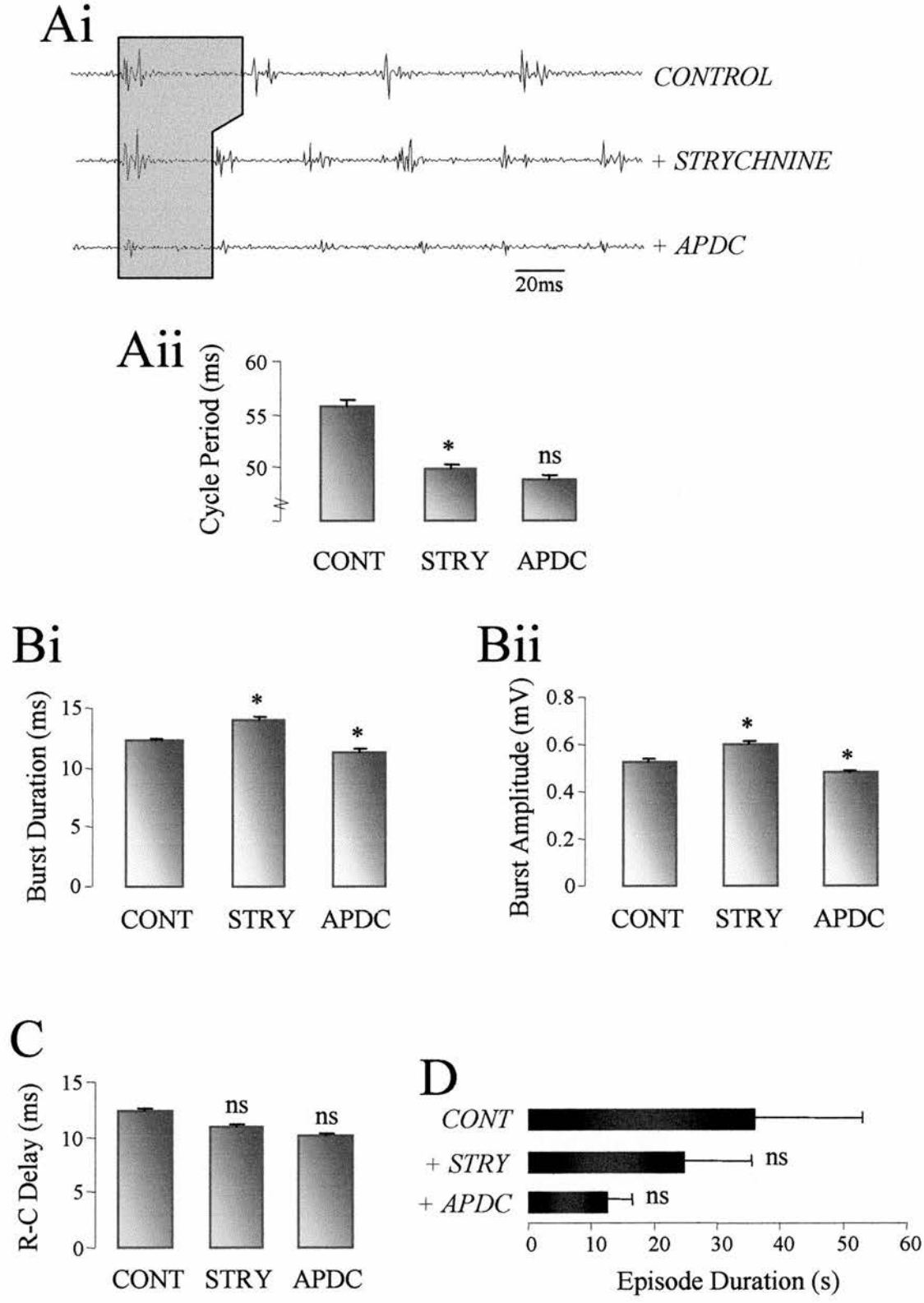
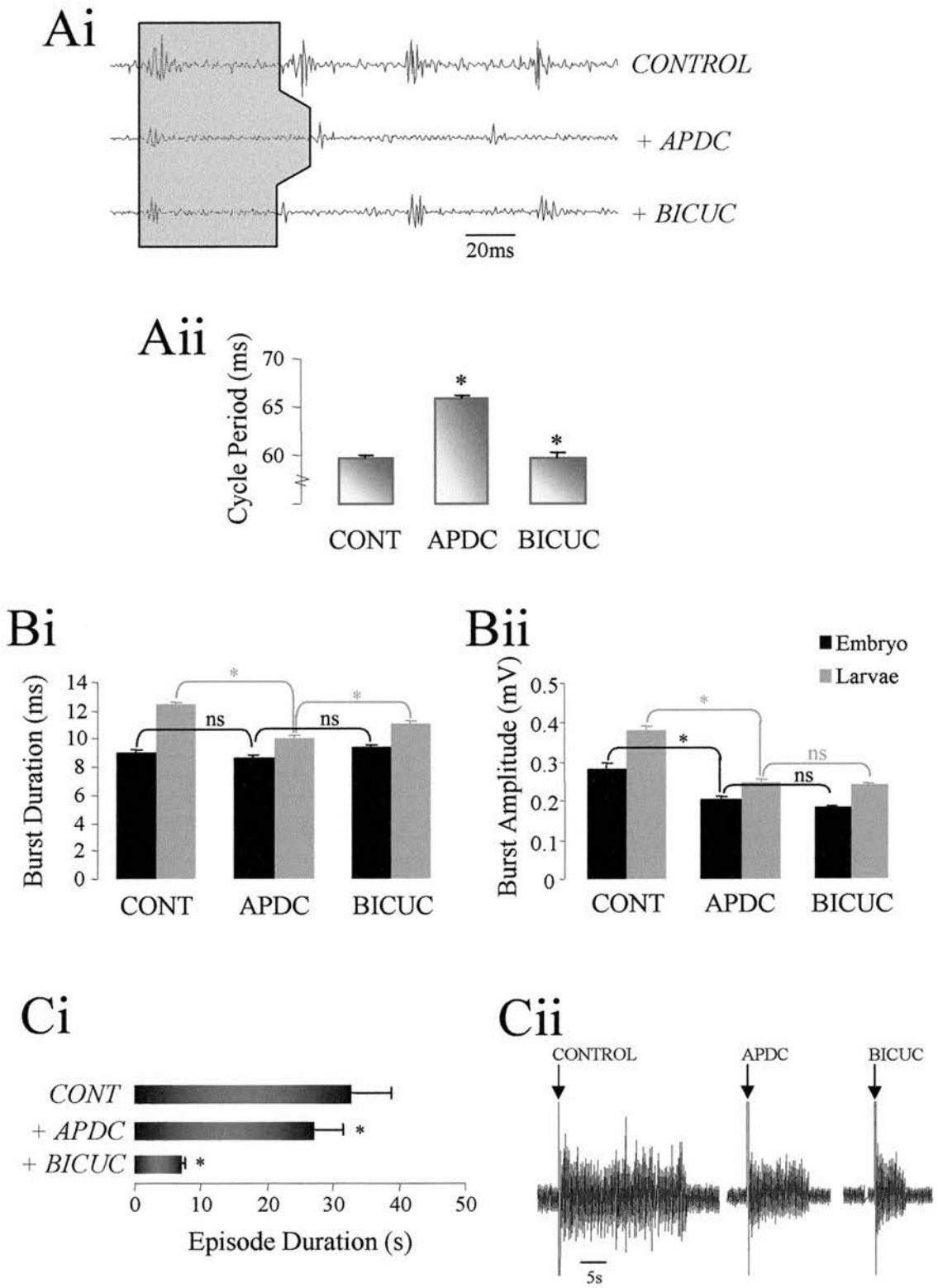

Ai
CONTROL
+ STRYCHNINE
+ APDC
20ms
Aii
Cycle Period (ms)
60
55
50
*
ns
CONT
STRY
APDC
Bi
Burst Duration (ms)
15
10
5
0
*
*
CONT
STRY
APDC
Bii
Burst Amplitude (mV)
0.8
0.6
0.4
0.2
0
*
*
CONT
STRY
APDC
C
R-C Delay (ms)
15
10
5
0
ns
ns
CONT
STRY
APDC
D
CONT
+ STRY
+ APDC
ns
ns
0
10
20
30
40
50
60
Episode Duration (s)

Figure 4.6 | Group II mGluR activation prior to blockade of GABA receptors

Ventral root recording (Ai) and graphical representation (Aii) showing increased cycle periods under APDC that are subsequently reduced with application of bicuculline (BICUC 40μM; O-A, $P<0.001$, $n=7$). (Bi) Following application of APDC, burst durations decreased in stage 42 larvae only, and significantly increased under bicuculline (O-A, $P<0.001$, $n=4$). (Bii) Burst amplitudes decreased under APDC in both stage 42 larvae (O-A, $P<0.001$, $n=4$) and stage 37/38 embryos (O-A, $P<0.001$, $n=3$), and did not alter with application of bicuculline. (Ci-ii) Episode durations decreased after the application of APDC and reduced further under bicuculline (O-A, $P<0.001$, $n=7$). Arrows represent stimulation artefacts. * = significant, ns = not significant. Data are means ± S.E.M.


Ai
CONTROL
+ APDC
+ BICUC
20ms
Aii
Cycle Period (ms)
70
65
60
*
*
CONT
APDC
BICUC
Bi
Burst Duration (ms)
14
12
10
8
6
4
2
0
ns
ns
*
*
CONT
APDC
BICUC
Bii
Embryo
Larvae
Burst Amplitude (mV)
0.5
0.4
0.3
0.2
0.1
0
*
*
ns
ns
CONT
APDC
BICUC
Ci
CONT
+ APDC
+ BICUC
*
*
0
10
20
30
40
50
Episode Duration (s)
Cii
CONTROL
APDC
BICUC
5s

to 10.07±0.12ms with APDC, increasing to 11.1±0.12ms with bicuculline; O-A, $F_{2,807}$=57.71, P<0.001, n=4; Figure 4.6Bi). The decrease in both embryonic and larval burst amplitudes following APDC application did not change with subsequent application of bicuculline (embryo: 0.2±0.004mV under APDC to 0.19±0.004mV; O-A, $F_{2,807}$=89.89, P<0.001, n=3; larvae: 0.24±0.01mV under APDC to 0.25±0.004mV; O-A, $F_{2,807}$=229.78, P<0.001, n=4; Figure 4.6Bii).

The shortened episode durations with APDC further decreased with bicuculline (from 32.7±6.1s in control saline to 27.2±4.16s with APDC, reducing to 6.89±0.54s with bicuculline; O-A, $F_{2,48}$=8.35, P<0.001, n=7; Figure 4.6Ci-ii). Further experiments were conducted to assess whether APDC produced the same effect on the network in the presence of bicuculline as, like strychnine, it is also known to increase swimming frequency (Reith and Sillar, 1997). In data pooled from 7 experiments, cycle periods decreased under bicuculline (from 58.7±0.21ms in control saline to 55.2±0.29ms; O-A, $F_{2,951}$=197.97, P<0.001, n=7; Figure 4.7Ai-ii). Larval burst durations increased (from 12.15±0.14ms in control saline to 13.9±0.18ms with bicuculline; O-A, $F_{2,681}$=42.02, P<0.001, n=4; Figure 4.7Bi), larval burst amplitudes increased (from 0.24±0.005mV in control saline to 0.313±0.01mV under bicuculline; O-A, $F_{2,681}$=21.24, P<0.001, n=4; Figure 4.7Bii), and episode durations reduced (from 39.69±11.2s in control saline to 15.37+3.; O-A, $F_{2,951}$=2.06, P<0.001, n=7; Figure 4.7Ci-ii). Subsequent application of APDC caused an increase in cycle periods (to 64.49±0.44ms; O-A, $F_{2,951}$=197.97, P<0.001, n=7; Figure 4.7Ai-ii), a decrease in larval burst durations (to 12.12±0.14mV; O-A, $F_{2,681}$=42.02, P<0.001, n=4), an increase in larval burst amplitudes (to 0.27±0.01mV; O-A, $F_{2,681}$=21.24, P<0.001, n=4; Figure 4.7Bii), and no obvious change in episode durations (O-A, P>0.05, n=7;

Figure 4.7 | Effects of group II mGluR activation following blockade of GABA receptors

Ventral root recording (Ai) and graphical representation (Aii) showing decreased cycle periods after application of the $GABA_A$ receptor antagonist, bicuculline (40μM; O-A, $P<0.001$, n=7). Subsequent application of APDC produced a significant increase in cycle periods (O-A, $P<0.001$, n=7). (Bi) Burst durations increased in the presence of bicuculline in stage 42 larvae only (O-A, $P<0.001$, n=4), and significantly decreased following application of APDC (O-A, $P<0.001$, n=4). Burst durations also significantly decreased under APDC in stage 37/38 embryos (O-A, $P<0.001$, n=3). (Bii) Burst amplitudes increased following bicuculline application in larval animals, which reduced with APDC application (O-A, $P<0.001$, n=4). APDC also caused burst amplitudes to decrease in embryonic tadpoles (O-A, $P<0.001$, n=3). (Ci-ii) Episode durations decreased under bicuculline and did not change after APDC application (O-A, $P>0.05$, n=7). Arrows indicate stimulation artefact. * = significant, ns = not significant. Data are means ± S.E.M.

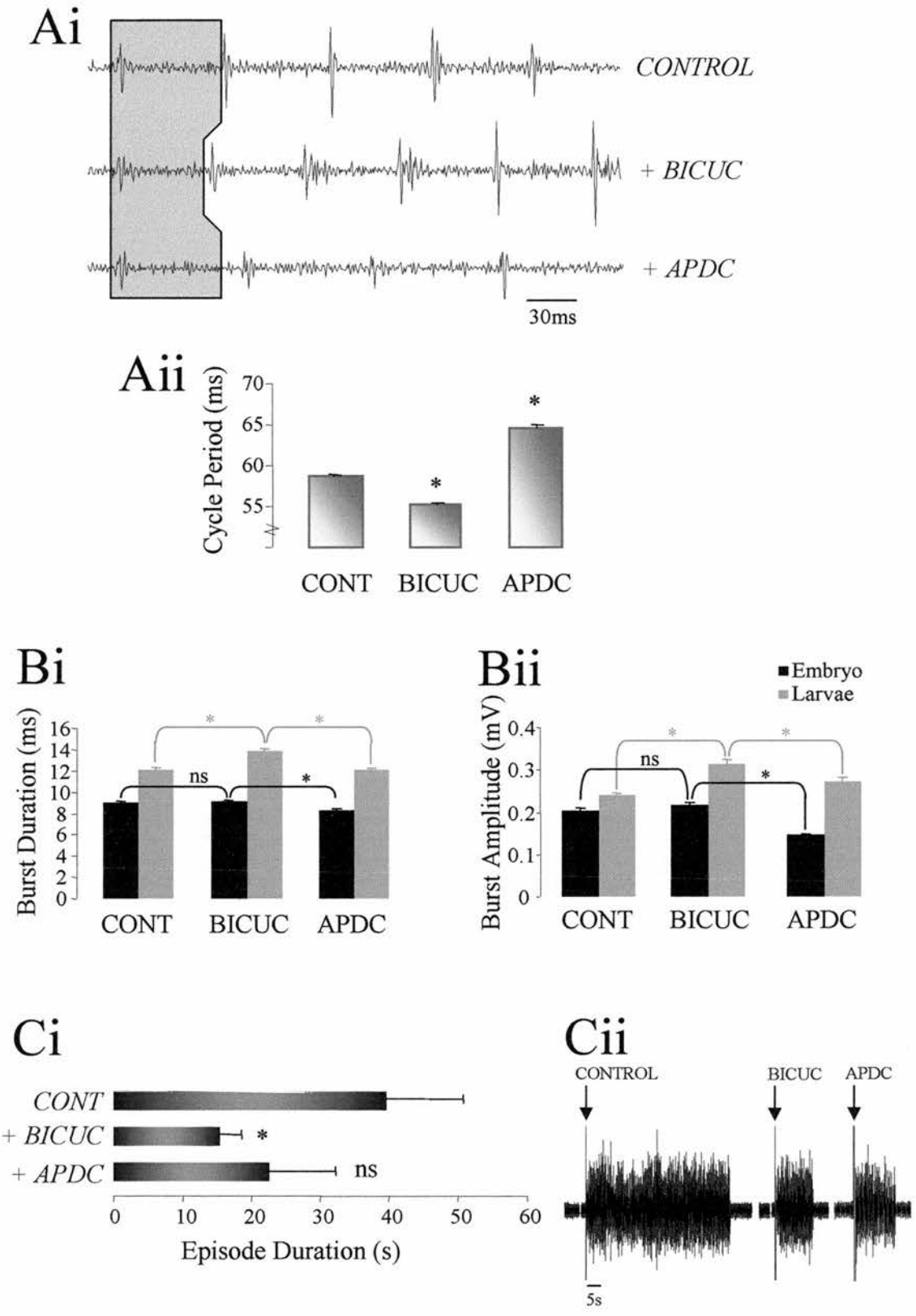

Ai
CONTROL
+ BICUC
+ APDC
30ms
Aii
Cycle Period (ms)
70
65
60
55
*
*
CONT
BICUC
APDC
Bi
Burst Duration (ms)
16
14
12
10
8
6
4
2
0
ns
*
*
CONT
BICUC
APDC
Bii
Embryo
Larvae
Burst Amplitude (mV)
0.4
0.3
0.2
0.1
0
ns
*
*
CONT
BICUC
APDC
Ci
CONT
+ BICUC
+ APDC
*
ns
0
10
20
30
40
50
60
Episode Duration (s)
Cii
CONTROL
BICUC
APDC
5s

Figure 4.7Ci-ii). Embryonic burst amplitudes did not change with bicuculline application, but decreased in the presence of APDC (O-A, $F_{2,681}$=68.11, P<0.001, n=3; Figure 4.7Bii). These results suggest that group II mGluRs do not utilise the GABAergic pathways to modulate either swimming frequency or the duration of swimming episodes.

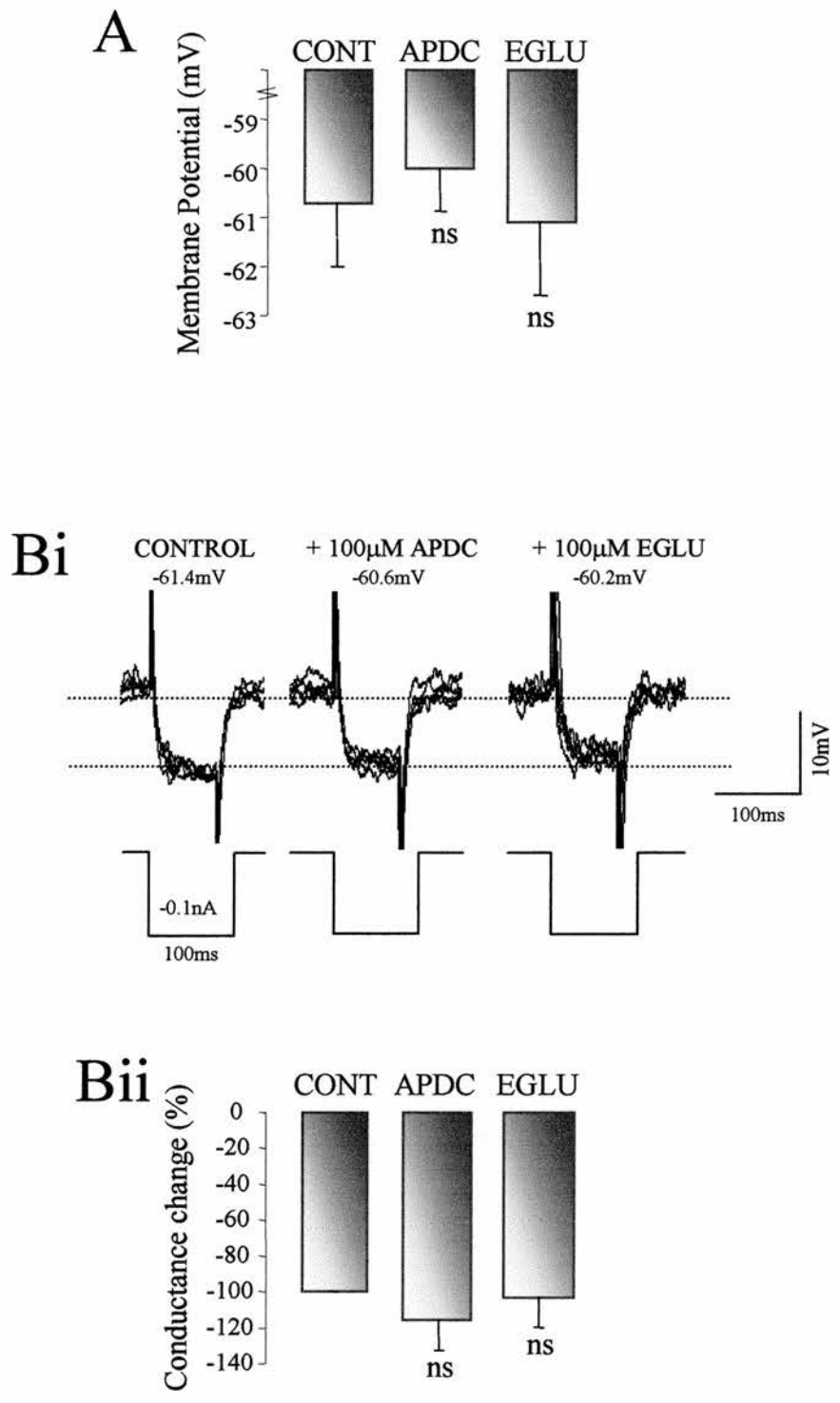
4 | 3 | 4 Effects of group II receptor activation on the cellular and synaptic properties neurons rhythmically active during swimming

Further experiments involving intracellular recordings using KCl-filled microelectrodes were undertaken to examine more closely any APDC-induced effects on neuronal properties and on the synaptic drive underlying swimming. During these experiments, no significant change was observed in the membrane potential (-60.7±1.29mV in control saline, –59.99±0.88mV under APDC; O-A, P>0.05, n=5, Figure 4.8A). Hyperpolarizing conductance pulses were applied before, during and after application of APDC to assess whether activation of group II mGluRs changes the motorneurons' input resistance. In 5 pooled experiments there was no significant or detectable change in conductance with APDC application (O-A, P>0.05, n=5; Figure 4.8Bi-ii). These data suggest that APDC has no direct effect on the post-synaptic resting membrane properties of spinal motorneurons.

As already mentioned, there are three components to the synaptic drive underlying swimming; a tonic depolarisation, on-cycle fast excitation underlying spike production in each cycle, and reciprocal glycinergic mid-cycle inhibition (see chapter 1; figure 2.1). Alterations to the strength of the mid-cycle IPSP are known to cause significant changes in swimming frequency (Dale, 1995; McDearmid *et al*, 1997). Moreover, strychnine appears to occlude the effects of APDC (Figure 4.5). Therefore,

Figure 4.8 | APDC has no effect on membrane potential or conductance

(A) There was also no change in membrane potential under APDC (from -60.7±1.29mV in control saline, to –59.99±0.88mV with APDC; O-A, P>0.05). There was also no significant alteration of the membrane potential in the presence of EGLU (O-A, P>0.05, n=5). (Bi-ii) Hyperpolarising conductance test pulses show no change in conductance with the application of APDC or subsequent application of the antagonist EGLU (O-A, P>0.05, n=5). Measurements are shown as percentage changes from control, pooled from 5 experiments. * = significant, ns = not significant. Data values are means ± S.E.M.


A
CONT
APDC
EGLU
Membrane Potential (mV)
-59
-60
-61
-62
-63
ns
ns
Bi
CONTROL
-61.4mV
+ 100μM APDC
-60.6mV
+ 100μM EGLU
-60.2mV
10mV
100ms
-0.1nA
100ms
Bii
CONT
APDC
EGLU
Conductance change (%)
0
-20
-40
-60
-80
-100
-120
-140
ns
ns

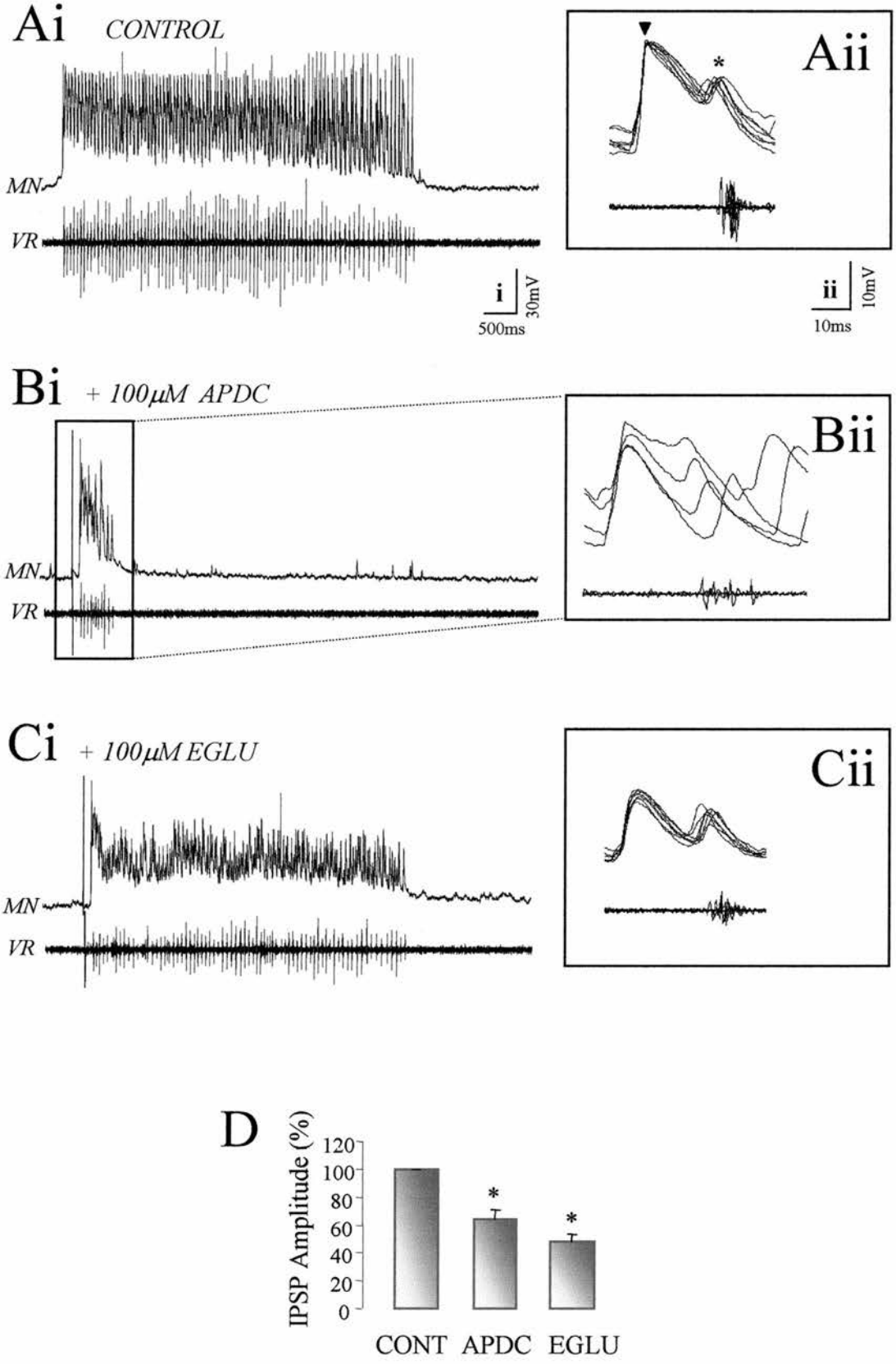
the initial aim was to test the hypothesis that APDC increases the amplitude of the mid-cycle IPSPs as a contributory mechanism through which this group of receptor reduces swimming frequency.

With the spinal cord exposed for intracellular recording, 100μM APDC produces a much more pronounced reduction of episode duration, such that only a few cycles of swimming occur per episode (Figure 4.9Bi-ii). The protocol used in similar experiments in chapter 3 was to calculate average mid-cycle IPSP amplitudes by subtracting the resting membrane potential from the peak amplitude of the IPSP from a total of 30 cycles of swimming (10 consecutive cycles from each of 3 episodes). The excerpts of swimming were chosen so that the frequency of swimming was approximately the same in each condition (i.e. frequency matched; see chapter 2: Materials and Methods). However, it proved necessary to modify this protocol when using APDC because many of the episodes were so brief. As a consequence, all cycles under APDC were included in the analysis and several episodes were used to obtain 30 cycles for comparison with control activity. Using this modified protocol, the mean mid-cycle IPSP amplitude decreased following group II receptor activation (63.6±7% of control IPSP amplitude; O-A, $P<0.05$, $n=5$; Figure 4.9Ai-ii,D) and decreased further with the application of EGLU (to 9.5±5.5% of the control; O-A, $P<0.05$, $n=5$; Figure 4.9Ci-ii,D). Whilst EGLU was unable to fully reverse the effect on this calculated mid-cycle IPSP amplitude, it did counteract the effect on episode duration (Figure 4.9Ci-ii).

The drastic reduction in episode durations under APDC meant that cycles from the start, middle and end of each episode were included in the analysis, in contrast to control episodes. Because the frequency of swimming (and presumably the

Figure 4.9 | APDC produces little effect on the synaptic drive for swimming

Episodes of evoked swimming shown on a slow (i) and fast (ii) time scale revealed little alteration to the components of the synaptic drive for swimming following the application of 100μM APDC (A-B). Reduced episode durations are reversed with the general group II antagonist EGLU (C; 100μM). (Aii and Cii boxes) Excerpts of ~7 cycles of activity are expanded and overlaid. (Bii box) The reduced number of cycles per episode of swimming under APDC causes the amplitude of the mid-cycle IPSP to appear smaller (D; fell to ~63.6±7% of the control under APDC) as frequency could not be matched (see main text). Arrowhead represents the inhibitory component; asterisk represents the excitatory component. MN = motorneuron, VR = ventral root. Data are means ± S.E.M.

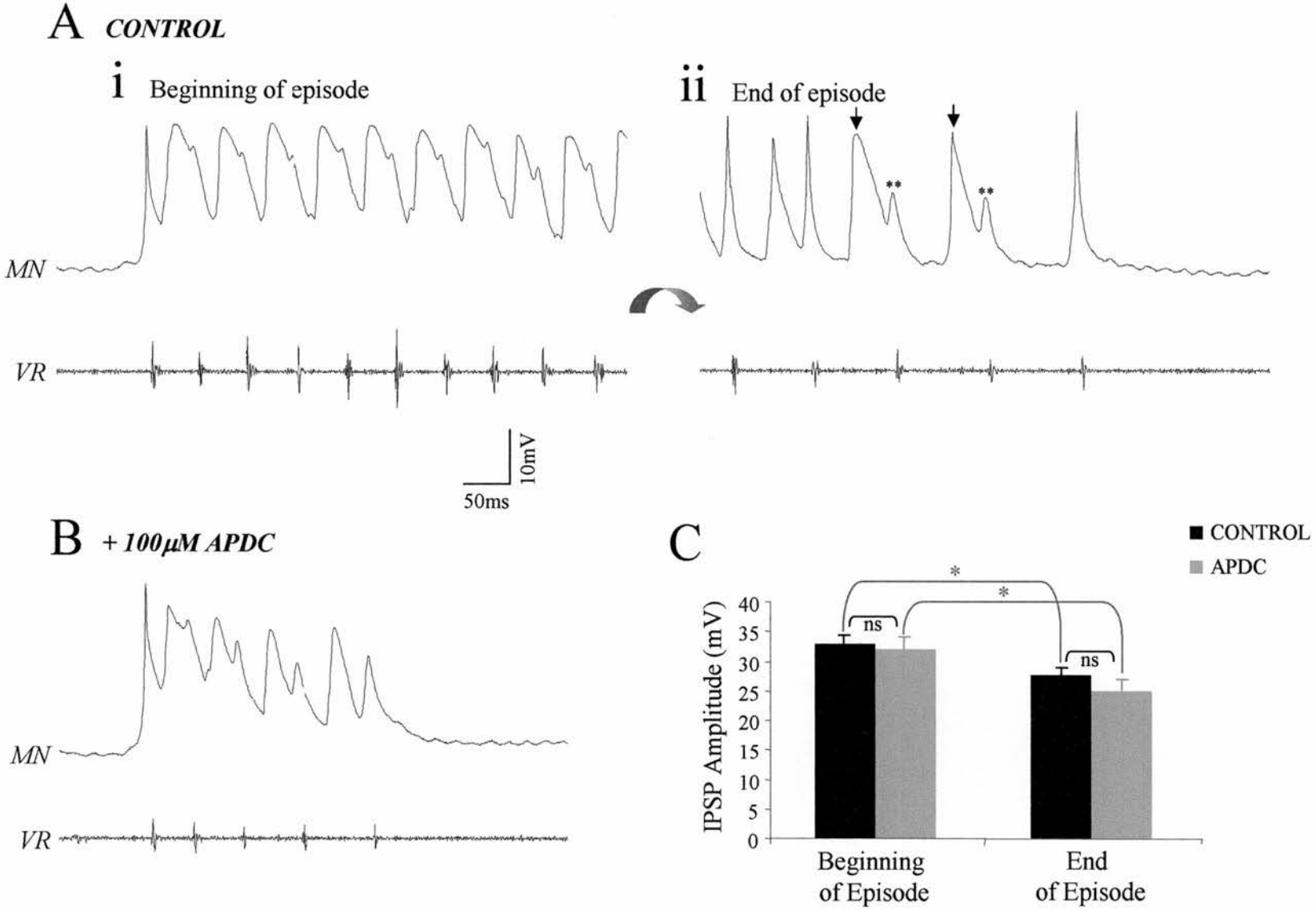

Ai
CONTROL
MN
VR
i
30mV
500ms
Aii
*
ii
10mV
10ms
Bi
+ 100μM APDC
MN
VR
Bii
Ci
+ 100μM EGLU
MN
VR
Cii
D
IPSP Amplitude (%)
120
100
80
60
40
20
0
*
*
CONT
APDC
EGLU

underlying excitatory drive) starts high but gradually slows down towards the end of an episode as the drive weakens, for example as interneurons fall below threshold and drop out of the network (c.f. Sillar and Roberts, 1993). Thus, one might expect that the amplitude of the mid-cycle IPSP will depend in part upon where in the episode the measurements are taken. Therefore, to allow a better comparison between control and APDC, I compared amplitudes at representative stages within each episode. In Figure 4.10, excerpts from the beginning (Ai) and end (Aii) of an episode of swimming in control conditions (represented fully in Figure 4.9A) can be compared to a whole episode of swimming under APDC. The IPSP amplitudes within the first and last two cycles of each episode of swimming were measured under each condition and pooled from 5 animals. Note that the last 2 cycles under control conditions are longer than those under APDC. Therefore, the last 2 cycles under APDC were frequency matched to 2 swimming cycles towards the end of the swimming episode in control conditions. The mean mid-cycle IPSP amplitude at the beginning or at the end of an episode of swimming was not significantly different under each condition (O-A, $P>0.05$, $n=5$; Figure 4.10C). However, the amplitude of mid-cycle IPSPs did decrease significantly towards the end of an episode in control and under APDC (*t*-test, $P<0.001$, $n=5$; Figure 4.10C). In summary, these experiments suggest that the amplitudes of the mid-cycle IPSPs are not significantly affected, although the ability of the network to sustain rhythmic activity is very clearly impaired, an effect which is reversed by EGLU (see Discussion).

It should be noted that it proved more difficult to assess the effects of APDC on the excitatory phase of swimming. The on-cycle synaptic drive led to an impulse, albeit one that had been shunted by the preceding IPSP (asterisk in figure 4.9Aii), and this suprathreshold event persisted on every cycle in control and in the presence of APDC.

Figure 4.10 | A closer examination of the synaptic drive for swimming following APDC application

Excerpts from the beginning (Ai) and end (Aii) of the episode of swimming seen in Figure 4.9 in control conditions, expanded on a faster time scale to show differences in inhibitory synaptic drive at these points during swimming. (B) Following APDC application, only a few cycles of activity are recorded per episode. A comparison of the mid-cycle IPSP amplitude at the beginning (i) and end (ii) of an episode of swimming in both control conditions and in the presence of APDC can be made. (C) The first and last 2 cycles of activity per episode of swimming were measured after being frequency matched, pooled from 5 experiments and compared under each condition. At the beginning and end of an episode there is no significant difference between mean mid-cycle IPSP amplitude under each condition (O-A, $P>0.05$, $n=5$). The mean mid-cycle IPSP amplitude decreased at the end of an episode of swimming in control condition and following the application of APDC (*t*-test, $P<0.05$, $n=5$). Arrowhead represents the inhibitory component and the double-asterisk represents the excitatory component underlying the synaptic drive. MN = motorneuron, VR = ventral root. * = significant, ns = not significant.


A CONTROL
i Beginning of episode
ii End of episode
MN
VR
**
**
10mV
50ms
B + 100μM APDC
MN
VR
C
CONTROL
APDC
IPSP Amplitude (mV)
40
35
30
25
20
15
10
5
0
ns
ns
*
*
Beginning of Episode
End of Episode

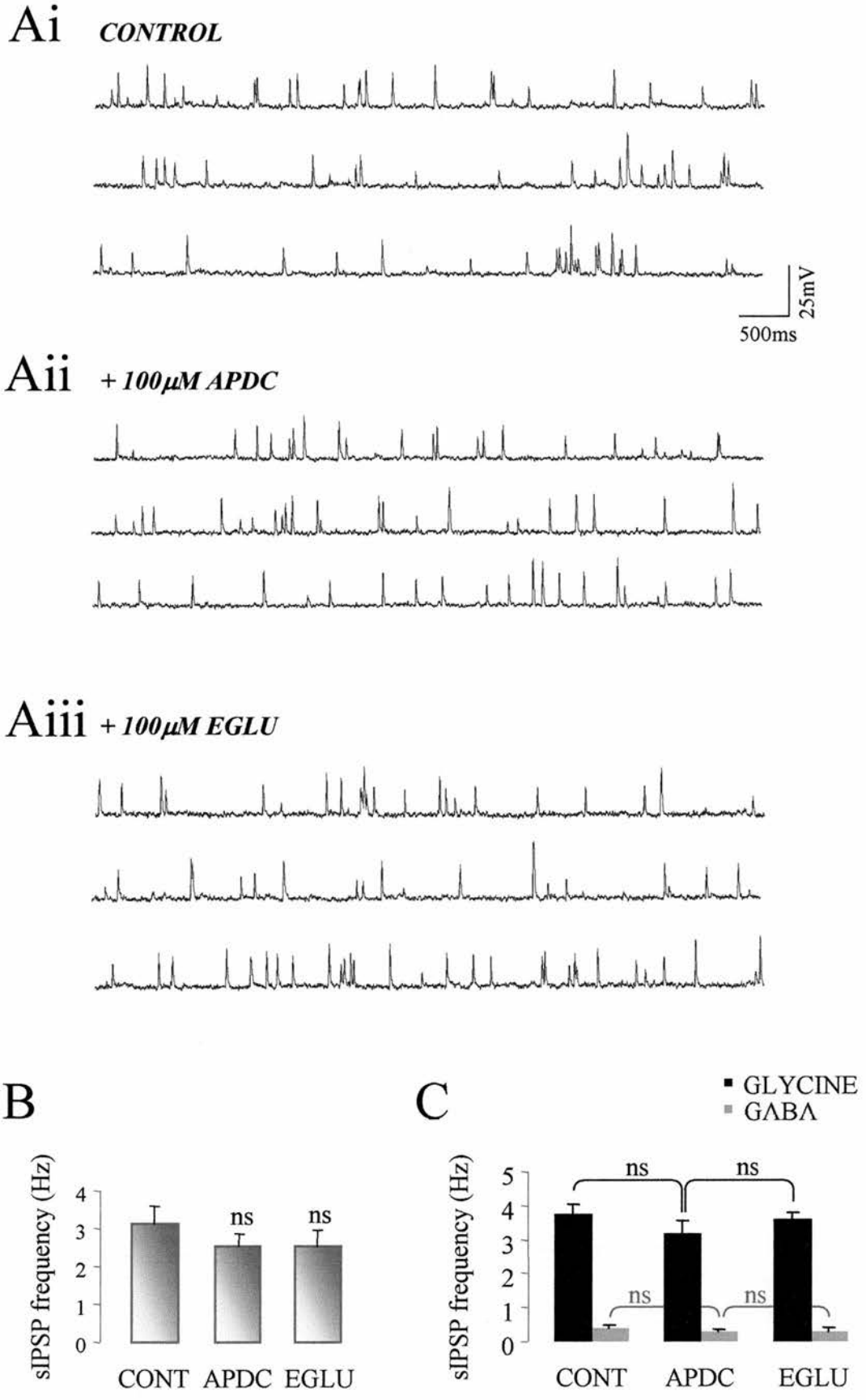
4 | 3 | 5 APDC has no effect on spontaneous glycinergic and GABAergic IPSPs

The preceding experiments have clearly revealed a pronounced reduction of swim frequency and episode duration after the activation of group II mGluRs by bath application of 100μM APDC. However, a preliminary investigation into whether group II receptors target fast inhibitory pathways as a means to modulate motor output proved inconclusive, although this seems unlikely (see above). Another approach to test this hypothesis was to examine effects of APDC on spontaneous IPSPs during quiescent periods between episodes of swimming. This approach allows pre- and/or post-synaptic effects induced by group II receptor activation to be established even in the absence of any effect on evoked transmission.

In data pooled from 5 experiments, APDC produced no significant change in the rate of occurrence of sIPSPs recorded (measurements taken 15-20mins after application of the agonist; 3.12±0.48Hz in control saline, to 2.55±0.33Hz under APDC; O-A, $P>0.05$, n=5; Figure 4.11Ai-ii,B). The frequency of sIPSPs also did not alter with subsequent application of the antagonist EGLU (to 2.53±0.44Hz; O-A, $P>0.05$, n=5; Figure 4.11Aiii,B). When the shorter duration glycinergic sIPSPs and longer duration GABAergic sIPSPs were analysed separately, no change in the rate of occurrence of either type of IPSP with group II receptor activation could be detected (glycine: 3.73±0.31Hz in control saline, to 3.16±0.14Hz with APDC; O-A, $P>0.05$, n=5; GABA: 0.38±0.11Hz in control saline, to 0.3±0.05Hz under APDC; O-A, $P>0.05$, n=5; Figure 4.11C). Again, application of the antagonist EGLU did not significantly influence the frequency of glycinergic or GABAergic sIPSPs (glycine: 3.6±0.21Hz; GABA: 0.31±0.09Hz; O-A, $P>0.05$, n=5; Figure 4.11C).

Figure 4.11 | Group II mGluR activation does not affect the frequency of spontaneous IPSPs

(Ai-iii) During inter-episode quiescent periods, spontaneous glycine and GABA transmitter release is unaffected following the application of 100μM APDC (ii) or EGLU (iii). (B) No change in the rate of occurrence of sIPSPs was observed under APDC (from 3.12±0.48Hz in control saline, to 2.55±0.33Hz; O-A, $P>0.05$, n=5). (C) There was no change in the rate of either glycinergic (from 3.73±0.31Hz in control, to 3.16±0.4Hz with APDC; O-A, $P>0.05$, n=5) or GABAergic sIPSPs (from 0.38±0.11Hz in control to 0.3±0.05Hz with APDC; O-A, $P>0.05$, n=5). In all cases, subsequent application of the antagonist ELGU had no effect on sIPSP frequency (Aiii,B,C). * = significant, ns = not significant. Values are means ± S.E.M.

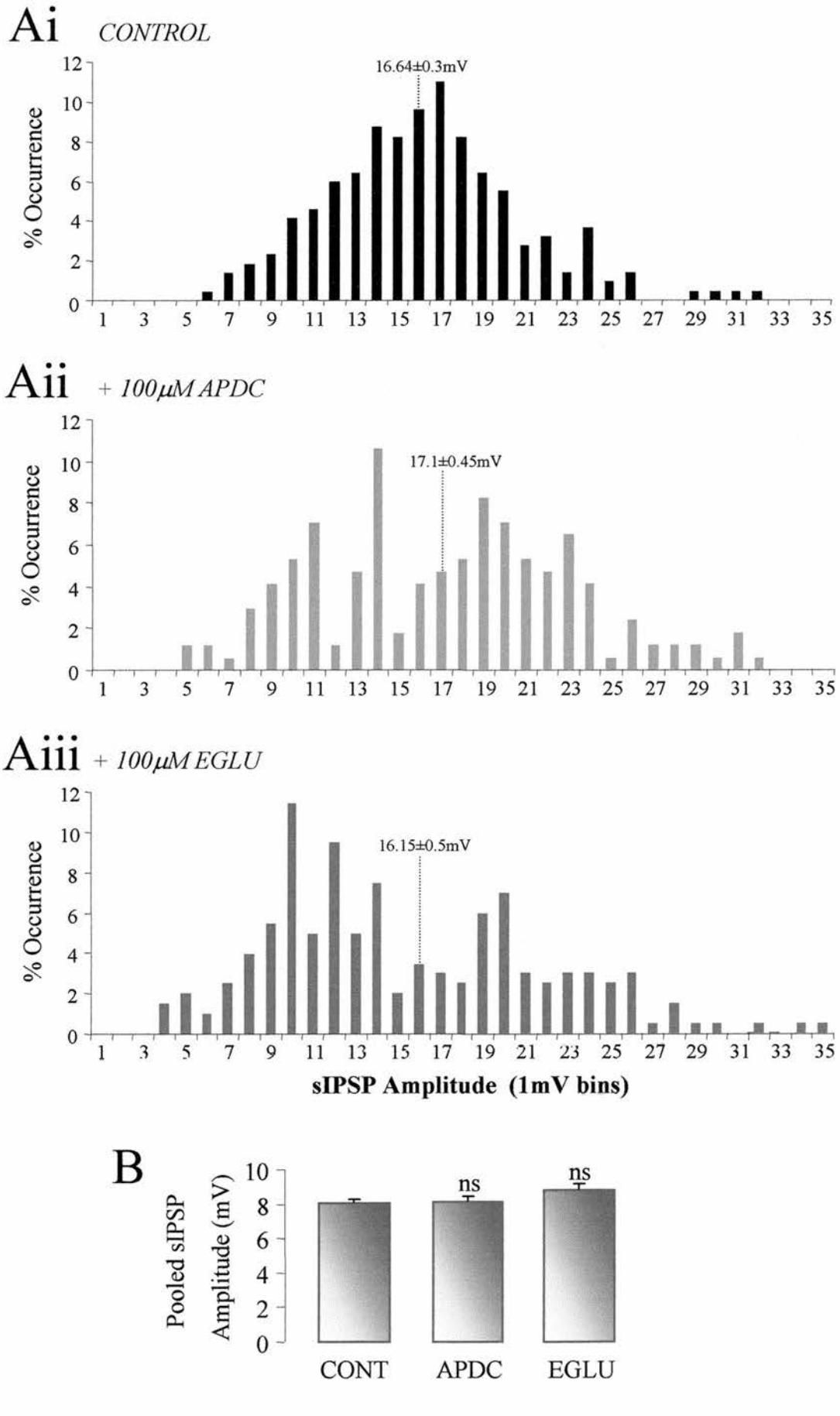

Ai
CONTROL
25mV
500ms
Aii
+ 100μM APDC
Aiii
+ 100μM EGLU
B
sIPSP frequency (Hz)
0
1
2
3
4
ns
ns
CONT
APDC
EGLU
C
GLYCINE
GABA
sIPSP frequency (Hz)
0
1
2
3
4
5
ns
ns
ns
ns
CONT
APDC
EGLU

The lack of influence on sIPSP frequency suggests that group II receptors do not act pre-synaptically to modulate the probability of either glycine or GABA release. To determine if APDC had any post-synaptic effects, the range of spontaneous glycinergic and GABAergic IPSP amplitudes was examined under each condition and compared. Figure 4.12 shows the sIPSP amplitude distribution histograms (from the same experiment represented in figure 4.11) under control (Ai), APDC (Aii) and EGLU (Aiii). Application of APDC showed little change in the range of sIPSP amplitudes, ranging from 6 to 32mV, and the mean amplitude did not significantly change from control (from 16.64±0.3mV under control saline, to 17.1±0.45mV with APDC; O-A, $P>0.05$, n=5; Figure 4.12Ai-ii). After EGLU application the range of sIPSPs remained similar, between 4 and 35mV and the mean amplitude also did not significantly change (16.15±0.5mV; O-A, $P>0.05$, n=5; Figure 4.12Aiii). The mean sIPSP amplitudes pooled from 5 experiments also revealed no significant change following group II receptor activation (from 8.02±0.18mV under control saline, to 8.11±0.28mV with APDC, insignificantly increasing to 8.82±0.29mV with EGLU; O-A, $P>0.05$, n=5; Figure 4.12B). These results suggest that group II mGluR activation does not affect the responsiveness of the post-synaptic neuron to GABA or glycine.

The data on sIPSP frequency and amplitude distribution taken together with the probable lack of effect on mid-cycle IPSP amplitudes, suggests that it is unlikely that group II receptors are influencing the fast inhibitory pathways as a means of modulating the motor output for swimming. Moreover, when this is combined with the lack of effect of APDC on resting membrane properties, a plausible explanation is that group II receptors exert their effects on the motor network by reducing excitatory glutamatergic transmission either directly or by affecting voltage-dependent currents.

Figure 4.12 | APDC does not affect the amplitude of sIPSPs

The effect of group II receptor activation on sIPSP amplitude distribution. Histograms in Ai-iii correspond to the example shown in figure 4.11A. (Ai) Histogram showing that spontaneous IPSPs varied in amplitude, ranging from 6 to 32mV, under control conditions. The mean amplitude measured under control was 16.64±0.3mV. (Aii) After 15mins in the presence of APDC, the range of sIPSP amplitudes remained similar, between 5 and 32mV. The measured mean under APDC was calculated to be 17.1±0.45mV and was not significantly different from control (O-A, $P>0.05$, $n=5$). (Aiii) After application of the antagonist, EGLU, sIPSP amplitudes ranged between 4 and 35mV. The measured mean amplitude following EGLU was 16.15±0.5mV and was not significantly different from the mean amplitudes under control or APDC (O-A, $P>0.05$, $n=5$). (B) sIPSP amplitudes pooled from 5 experiments similarly showed no change in their mean amplitude (8.02±0.18mV in control conditions, 8.11±0.28mV under APDC, and 8.82±0.29mV under EGLU; O-A, $P>0.05$, $n=5$). Dotted lines represent the mean amplitude under each condition. The represented data is from 5 pooled values. Values are means ± S.E.M.


Ai
CONTROL
16.64±0.3mV
% Occurrence
Aii
+ 100μM APDC
17.1±0.45mV
% Occurrence
Aiii
+ 100μM EGLU
16.15±0.5mV
% Occurrence
sIPSP Amplitude (1mV bins)
B
Pooled sIPSP Amplitude (mV)
ns
ns
CONT
APDC
EGLU

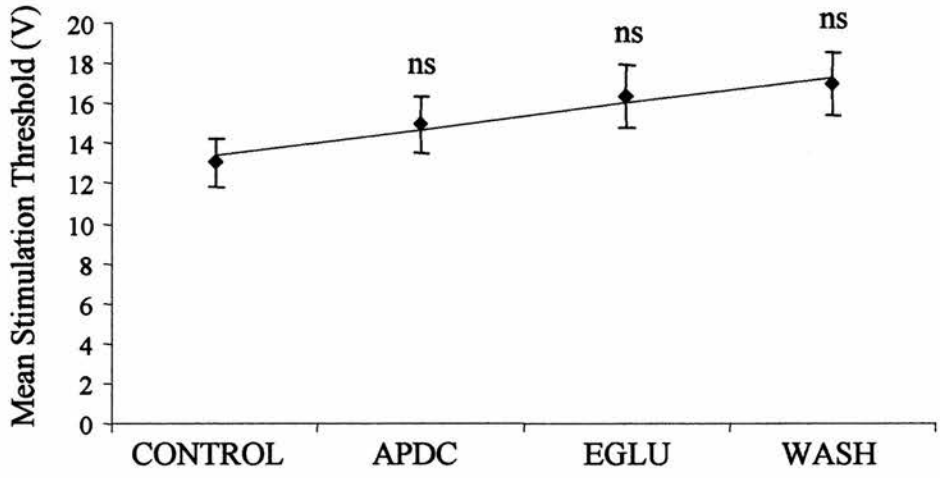
4 | 3 | 6 Influences of APDC on spinal sensory transmission?

The Rohon-Beard skin sensory pathway involved in the activation of motor activity is known to involve glutamatergic neurotransmission (Clarke and Roberts, 1984; Sillar and Roberts, 1988; Roberts and Sillar, 1990), and given that APDC may influence glutamatergic transmission, it is possible that APDC affects the threshold voltage required to initiate swimming and the level of activation of the swim network. This threshold is ascertained by stepwise increments of 1V current pulses until swimming is evoked (see chapter 2: Materials and Methods). In data pooled from 6 animals no significant change in the mean voltage to initiate swimming was observed under APDC (13.08±1.2V in control saline, to 14.9±1.46V under APDC; O-A, $P>0.05$, $n=6$; Figure 4.13A), and subsequent application of EGLU had no effect (16.3±1.56V; O-A, $P>0.05$, $n=6$; Figure 4.13A). Application of the antagonist, EGLU, alone incurred no change in the mean voltage required to initiate swimming either (11.67±0.8V in control saline, to 11.7±0.7V with EGLU; O-A, $P>0.05$, $n=6$; Figure 4.13B). In conclusion it seems unlikely that APDC is causing a depression of the skin sensory pathways as the voltage threshold required to initiate swimming does not change after the activation of group II mGluRs. This in turn suggests that group II receptors are not located in the skin sensory pathway.

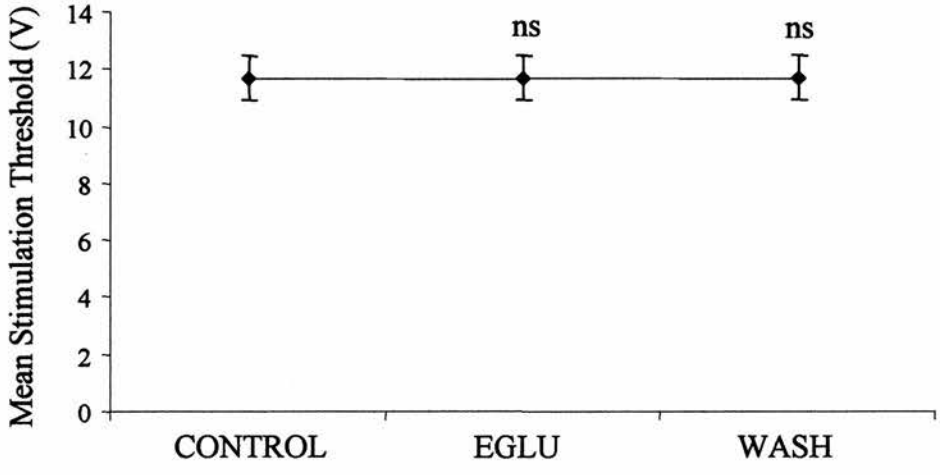
Figure 4.13 | Stimulation threshold to initiate swimming is unaffected by activating or blocking group II receptors

(A) Activating group II receptors with the application of 100μM APDC did not affect sensory transmission, as there was not change in the voltage required to stimulate swimming (O-A, $P>0.05$, $n=6$). (B) Addition of 100μM EGLU also did not affect the threshold voltage required to stimulate swimming. Grey lines represent trendlines. * = significant, ns = not significant. Values are means ± S.E.M.

A


Mean Stimulation Threshold (V)
20
18
16
14
12
10
8
6
4
2
0
ns
ns
ns
CONTROL
APDC
EGLU
WASH

B


Mean Stimulation Threshold (V)
14
12
10
8
6
4
2
0
ns
ns
CONTROL
EGLU
WASH

4 | 4 DISCUSSION

In this chapter, a series of studies using both extracellular and intracellular recordings in tandem with bath applications of group II specific pharmacological agents was undertaken with the aim of uncovering a modulatory role for this class of mGlu receptors within the *Xenopus* spinal locomotor network. The group II specific agonist, APDC, caused a profound effect on numerous parameters of fictive swimming activity which could be reversed with the specific antagonist, EGLU. Most notably, the swimming frequency slowed and the swim episode duration decreased in the presence of APDC (Figures 4.1 and 4.2), producing a net inhibitory effect on the swimming network. Group II receptor activation had no influence on the voltage threshold required to initiate swimming suggesting that the APDC-induced effects are mediated within the motor network and that the skin sensory pathways are unaffected (Figure 4.13). Thus, my evidence indicates not only that group II receptors are present in the spinal cord and that they can be pharmacologically activated, but also that their functional distribution is restricted to components, as yet unknown, of the swimming rhythm generator.

Applications of EGLU alone significantly increased swimming frequency, providing strong evidence that group II receptors are activated endogenously during swimming and that their activation exerts a powerful inhibitory control on swimming activity (Figure 4.3). Based on previously published anatomical data on group II receptors in other systems, their perisynaptic location suggests that they are most likely to be activated by glutamate spillover from the synaptic cleft either as a result of normal network activity or induced by certain pathological conditions (for a review: Cartmell and Schoepp, 2000). In the case of the *Xenopus* swimming network it is conceivable that at the start of an episode when levels of excitation are at their highest, the

elevated synaptic release of glutamate sets an upper limit on swimming frequency. This could afford the network some protection against the possible excitotoxic damage caused by the resultant increase in calcium entry. Another scenario in which group II receptors could be activated further is during struggling behaviour (Kahn and Roberts, 1982b; Soffe, 1991, 1993), and it would be interesting in future experiments to test the effects of group II agents on the expression of this more intense motor behaviour.

At the level of the network output, the reduced swimming frequency and episode duration induced by APDC applications is similar to the inhibitory effects imposed on the *Xenopus* motor network by the neuromodulators, NA and nitric oxide (NO). There is evidence that each of these endogenous neuromodulators facilitates glycinergic transmission, which strengthens the reciprocal mid-cycle inhibition, making cycle periods longer and, presumably, causing swimming frequency to decrease (McDearmid *et al*, 1997; McLean, 2001). In addition, NA and NO concomitantly facilitate GABAergic transmission, which is thought to induce the premature termination of swimming episodes by triggering a barrage of $GABA_A$ receptor-mediated IPSPs (McLean and Sillar, 2000; Fischer *et al*, 2001; Merrywest *et al*, 2002). There is also evidence from studies on isolated tortoise spinal neurons where group II receptor activation causes a decrease in the probability of GABAergic, as well as glutamatergic release (Kozhanov *et al*, 2001). Taken together, the similarity of effects of NA and NO to APDC-induced effects, the precedent for group II receptor modulation of inhibitory transmission, and the previously documented group I receptor-mediated depression of inhibition in *Xenopus* (see chapter 3), makes the fast inhibitory pathways a potential target for group II receptors. However, during a series of extracellular experiments using the $GABA_A$ antagonist bicuculline, I was

unable to support the hypothesis that episode durations were reduced as a consequence of increased GABA transmission (Figures 4.6 and 4.7). On the other hand, preliminary evidence suggested that APDC might facilitate glycinergic transmission as a potential mechanism to lengthen cycle periods as pre-application of strychnine seemed to occlude group II receptor-mediated effects on swim frequency.

To assess the potential modulation of the fast inhibitory pathways by group II mGluRs more thoroughly, intracellular recordings were made from presumed motorneurons using KCl-filled microelectrodes. This electrolyte makes IPSPs strongly depolarising due to Cl^- leakage into the cell, aiding the identification and quantification of inhibitory transmission. Initially, evoked IPSPs during swimming were examined, but this proved difficult to quantify due to the extremely short episodes of swimming induced by 100μM APDC (Figure 4.9). This was probably due to the drug having better access to the spinal cord as the myotomes are removed during intracellular experiments. It will be important to apply lower concentrations of APDC in future to try and increase the number of cycles per episode for analysis. Despite this obstacle, a closer observation of the IPSPs at the beginning and towards the end of the episode were comparable under each condition and do not appear different (Figure 4.10). Similarly, at the end of the swimming episode there was no apparent barrage of GABA IPSPs that has been observed with NA (Merrywest, 2002) or NO (McLean and Sillar, 2002). This apparent lack of effect on inhibitory transmission was further corroborated by the examination of spontaneous IPSPs during quiescent periods between episodes of swimming. APDC, nor EGLU, were able to significantly alter the frequency of sIPSPs (Figure 4.11) or their mean amplitude (Figure 4.12). These data effectively rule out the possibility that group II

mGluRs modulate inhibitory transmission as a mechanism to depress swimming frequency or episode duration.

Other mechanisms by which group II receptors induce a net inhibitory effect on the motor network therefore require consideration. The integrative electrical properties of the constituent neurons of the motor network could be a potential target for modulation by group II mGluRs. However, measurements of the passive membrane properties in the presence of APDC during intracellular experiments did not reveal an alteration of the membrane potential or input resistance (Figure 4.8). Moreover, changes in these passive properties would also likely be reflected in a change in the mean sIPSP amplitude, with a predicted shift in the amplitude histograms, which did not occur (Figure 4.12).

However, whilst there were no obvious changes in passive membrane properties, it will be important in future to address the potential modulation of active membrane properties by these receptors. For example, alterations to the balance of ionic currents mediated through voltage-dependent channels can induce a plethora of effects at the network level. If, for example, group II receptor activation reduced Ca^{2+} currents, then synaptic transmission would be depressed and the threshold required for neuronal firing may not be reached. Indeed, the effects of the intrinsic neuromodulator, adenosine, on the *Xenopus* motor network broadly parallels those induced by APDC, and adenosine slows swimming by reducing voltage-gated Ca^{2+} currents without altering the membrane potential or input resistance, or the excitatory or inhibitory component of the synaptic drive underlying swimming (Dale and Gilday, 1996). Numerous studies in other systems suggest that mGluR activation causes

inhibition of voltage-gated Ca^{2+} channels leading to the depression of glutamatergic transmission (for a review: Anwyl, 1999).

A reduction in glutamatergic transmission would compromise the ability of neurons to reach the threshold for firing. Both motorneurons and pre-motor interneurons fire only once per cycle in the *Xenopus* embryo and the number of interneurons active per episode of swimming can greatly influence the frequency of swimming (Sillar and Roberts, 1993). Thus, if glutamatergic transmission was reduced, following the activation of group II mGluRs, then the number of excitatory interneurons firing could decrease, such that the number of neurons contributing to the synaptic drive underlying swimming fall below the level required to sustain rhythm generation. This may go some way towards explaining why the network is unable to sustain long periods of rhythm generation under the influence of APDC.

In the lamprey locomotor network, group II mGluR activation caused a reduction in excitatory transmission from descending reticulospinal neurons onto the motor network as revealed by a depression of the reticulospinal-evoked EPSP with the agonist L-CCG-I (Krieger *et al*, 1996). It was found that group II activation with ACPD (which, unlike APDC, simultaneously activates group I receptors) had no influence on Ca^{2+} currents recorded in the reticulospinal neurons (Cochilla and Alford, 1998), thus discounting modulation of Ca^{2+} channels as a mechanism to inhibit synaptic transmission. It was proposed that group II receptors act directly on vesicular release machinery at the reticulospinal synapse to cause the observed depression of synaptic transmission.

Therefore, the most likely explanation to account for the APDC-induced effects on network activity in *Xenopus*, given the lack of detectable effect on inhibitory transmission, is that group II receptors act as negative feedback autoreceptors to reduce glutamate release. The first studies showing that mGluRs could mediate presynaptic inhibition of excitatory transmitter release used the pharmacological agent *trans*-ACPD in CA1 of rat hippocampal slices (Baskys and Malenka, 1991; Desai and Conn, 1991). There is growing evidence of the widespread effects of agonists to pre-synaptic group II and III receptors on the depression of synaptic transmission and in some studies there appears to be an additive effect of group II and III receptors whereby sequential activation of both causes a progressively larger effect, for example, in the basolateral amygdala, the locus coeruleus and the striatum (for a review: Anwyl, 1999). Whilst this theory has not been addressed in this study, future work may benefit from direct analysis of excitatory interneuron to motorneuron synapses. Experiments involving paired patch recordings (c.f. Li *et al*, 2001) would allow the direct examination of APDC-induced effects on evoked glutamatergic EPSPs and on spontaneous EPSPs, which is not possible with the sharp microelectrode recordings, as used in this study.

In summary, from the results presented in this chapter, group II receptor activation induces an inhibitory effect on motor activity in *Xenopus*, reducing both swimming frequency and episode durations. The exact mechanism(s) this group utilises to mediate this inhibitory effect on the swimming network remains subject to further investigation. Nevertheless, I have shown that group II receptors are not modulating fast inhibitory pathways to exert their effects at the network level and may possibly be modulating excitatory transmission or voltage-dependent currents. Often the effects of group II and III are intertwined, possibly as they are both negatively coupled to

adenylyl cyclase and cause similar effects. As discussed in the next chapter, group III receptors share some common features with group II mGluRs but also have some significant differences.

5 | Group III mGluR-mediated modulation of a spinal locomotor network

5 | 1 SUMMARY

In this chapter, group III mGluRs were manipulated pharmacologically revealing significant effects of these receptors on the rhythm-generating network in *Xenopus*. Bath application of the group III mGluR agonist, L-AP4, significantly affected many of the parameters of swimming, producing effects similar to those induced by the activation of group II mGluRs (Chapter 4). L-AP4 application significantly reduced swimming frequency often causing swimming activity to cease completely. Group III receptor activation also caused a reduction in burst durations and amplitudes, a decrease in R-C delays and, in contrast to group II receptors, simultaneously produced an inhibitory effect on the sensory pathways used to initiate swimming. Experiments conducted in the presence of the $GABA_A$ receptor antagonist, bicuculline, revealed that L-AP4 could still exert its effects on the network effectively, ruling out facilitation of this inhibitory transmitter as a mechanism to inhibit swimming activity. However, the L-AP4-induced effect on cycle periods was occluded in the presence of the glycine receptor antagonist, strychnine, suggesting modulation of the glycinergic inhibitory pathway as a possible mechanism to slow swimming frequency. Despite this, no evidence was found during intracellular recordings to support the idea that group III receptors mediate their effects through facilitating inhibitory transmission. There was no obvious alteration in the mid-cycle IPSP amplitude during evoked swimming, and, paradoxically, the frequency of spontaneous IPSPs decreased in the presence of L-AP4. Applications of L-AP4 also did not consistently affect resting membrane potential or input resistance, although L-AP4 induced a clear hyperpolarisation of the membrane potential in a proportion of neurons. It is possible that group III receptors are simultaneously reducing excitatory transmission in a manner previously discussed for group II receptor-mediated modulation of swimming frequency. However, there are distinct differences in the cellular mechanisms used by group II and III receptors to exert their inhibitory effect on the motor network, and in the case of group III mGluRs, the sensory pathways as well.

5 | 2 INTRODUCTION

In the preceding two results chapters I investigated the roles of two classes of mGluR, group I and group II, and found that both exert very profound but opposing effects on the swim-generating network in *Xenopus*. Group I receptors appear to reduce mid-

cycle glycinergic inhibition as a means to increase excitability within the network and presumably this mechanism contributes to the increased swimming frequency observed following the activation of these mGlu receptors (see chapter 3). Group II receptors, on the other hand, do not influence inhibitory transmission but may be acting as autoreceptors on excitatory pre-motor interneurons to reduce glutamatergic transmission onto the motorneurons in order to produce the observed reduction of swimming frequency (see chapter 4). In the present chapter I consider the last group of mGluRs, group III. This class of mGluRs constitutes four subtypes, $mGluR_{4,6,7,8}$, which, like group II receptors, have been reported to be negatively coupled to AC (see chapter 1). Similar to the group II receptors, group III mGluRs are thought to be mostly located in or around presynaptic active zones (Shigemoto *et al*, 1997) where they mainly act to depress excitatory synaptic transmission (for a review: Cartmell and Schoepp, 2000).

A role for this class of mGluR in locomotor behaviour has briefly been described in two other vertebrate preparations, the lamprey and the neonatal rat. Studies involving stimulation of presynaptic RS neurons in the lamprey spinal cord revealed that application of the group III agonist, L-AP4, depresses the monosynaptic EPSPs evoked in post-synaptic spinal neurons (Krieger *et al*, 1996). The frequency of the locomotor rhythm was also reduced by L-AP4, although not as strongly as one would expect from the large reduction of synaptic transmission (for a review: El Manira *et al*, 2002). Also, in the lamprey, this group III receptor-mediated inhibitory effect at the RS synapse is mirrored by the actions of group II mGluRs, where applications of the agonist L-CCG-I, similarly reduced excitatory transmission and depressed the reticulospinal-evoked EPSP (Krieger *et al*, 1996). More recently, it was discovered that group III receptor activation also depressed sensory synaptic transmission in the

lamprey spinal cord, causing a reduction in the amplitude of the monosynaptic dorsal cell-evoked EPSP in giant interneurons (Kreiger and El Manira, 2002). Similar effects are found in the neonatal rat spinal cord where L-AP4 application depresses the cumulative depolarisation induced by repetitive dorsal root stimulation from peripheral inputs, consistent with a reduction in excitatory synaptic transmission, and reduces the frequency of fictive locomotion (Taccola *et al*, 2004b). In both of these cases the effects of L-AP4 were described on glutamate receptor-activated locomotor activity rather than on naturally generated, self-sustaining locomotor network.

A role for the group III class of receptor has not yet been described in the spinal rhythm generating network in *Xenopus* tadpoles. As mentioned previously, and extensively in chapter 1, the relatively simple nervous system of young *Xenopus* tadpoles around the time of hatching has provided an excellent and amenable model in which to study the neural mechanisms underlying the generation and development of vertebrate locomotion. Moreover, in contrast to other models in which mGluRs have been studied, self-sustaining activity can be readily generated without the need for pharmacological intervention The synaptic drive underlying swimming in *Xenopus* results from a combination of descending ipsilateral excitation superimposed upon which is reciprocal mid-cycle inhibition (for a review: Roberts, 1989). The excitatory neurotransmitter responsible for generating a significant proportion (~30%) of the excitatory drive for swimming is glutamate, although an important contribution from cholinergic and electrotonic components has also been identified (Perrins and Roberts, 1995a, 1995b). Glutamate acts through the iGluRs, NMDA and non-NMDA, to produce the tonic and phasic excitatory components underlying the synaptic drive for swimming, respectively (Dale and Roberts, 1985). When iGluRs are blocked pharmacologically, swimming cannot be generated. It has already been

seen in the previous two chapters that glutamate can act through metabotropic receptors to modulate the output of the swimming network. Does this 'neuromodulatory' role of glutamate through these metabotropic receptors extend through all three classes of receptor?

In this chapter, using agonists and antagonists for the group III mGluRs, I first investigated whether activation of this class of receptor has any modulatory effect on the spinal locomotor network and, using antagonist applications alone, whether these receptors might be activated by endogenously released glutamate. Next I studied if any of the group III receptor-mediated effects could be accounted for by the modulation of fast inhibitory pathways. Finally, I explored the synaptic and cellular changes induced by group III mGluR activation by recording from motorneurons intracellularly using KCl filled microelectrodes. The results show that as for group II receptors, activation of group III receptors slows swimming frequency and shortens episode duration. However, the data presented in this chapter suggest that the underlying cellular and synaptic mechanisms may differ. Moreover, in contrast to group II receptors, group III mGluRs inhibit sensory transmission indicating topological differences in the distribution of the two groups of receptor.

5 | 3 RESULTS

5 | 3 | 1 Effects of group III receptor activation on network activity

Both stage 37/38 embryos and stage 42 larvae were used in the following investigations and, unless stated otherwise, the data is a combination of the two stages. For the most part, no discernable developmental differences in the response to group III receptor agents were observed.

Initially, I investigated the effects of activating group III mGluRs on the neural network underlying swimming by bath applying the specific agonist, L-AP4. During these extracellular experiments it became apparent that only a narrow range of agonist concentrations was able to affect the motor network without abolishing motor activity completely. The optimum concentration of L-AP4 found to be effective during these experiments was 50μM, but even at this concentration swimming activity was completely abolished in 4 out of 15 animals. By comparison, in the lamprey model, L-AP4 was used at far higher concentrations of between 100-500μM (Krieger *et al*, 1996, 1998). Thus, the *Xenopus* tadpole swimming network seems particularly sensitive to the activation of group III receptors.

In data pooled from the remaining 11 (of 15) animals, bath application of 50μM L-AP4 caused a decrease in swimming frequency, with cycle periods increasing significantly, from 53.75±0.47ms in control saline to 59.97±0.34ms under L-AP4 (One-way ANOVA: O-A, $F_{2,619}$=55.5, P<0.001, n=11; Figure 5.1 and 5.2Ai-ii). This effect persisted through episodes; cycle periods measured on a cycle-by-cycle basis following L-AP4 application were consistently longer compared to control (First 100 cycles: *t*-test, P<0.05, n=11; Figure 5.1Aiii). The range of cycle periods over the whole episode of swimming was higher following L-AP4 application (ranging from

Figure 5.1 | Effects of group III mGluR activation on swimming frequency are reversed with washout

Ventral root recording (Ai) and graphical (Aii) representation showing increased cycle periods following application of the group III specific agonist L-AP4 (50μM; O-A, $P<0.001$, n=11). This effect on swimming frequency was significantly reversed during washout (Aii; O-A, $P<0.001$, n=3). (Aiii) Graph illustrating on a cycle-by-cycle basis the L-AP4-induced increased cycle periods over the first 100 cycles of an episode of swimming. There was a reduction in burst durations (Bi) and amplitudes (Bii) following L-AP4 application and an increase in R-C delays (C) that did not reverse during washout (O-A, $P>0.05$, n=3). All 3 animals subjected to washout were stage 42 larvae. * = significant, ns = not significant. Values are means ± S.E.M.

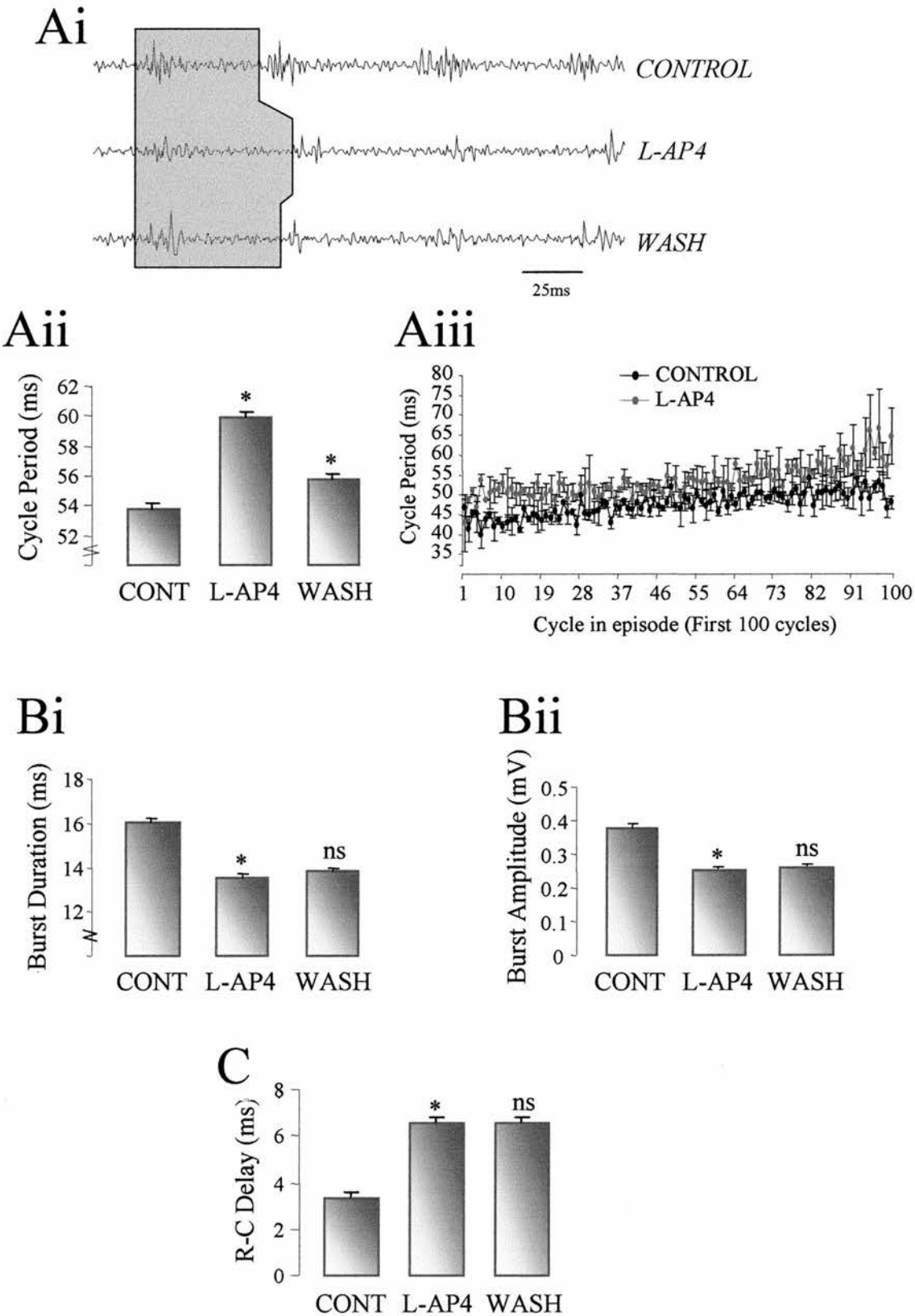
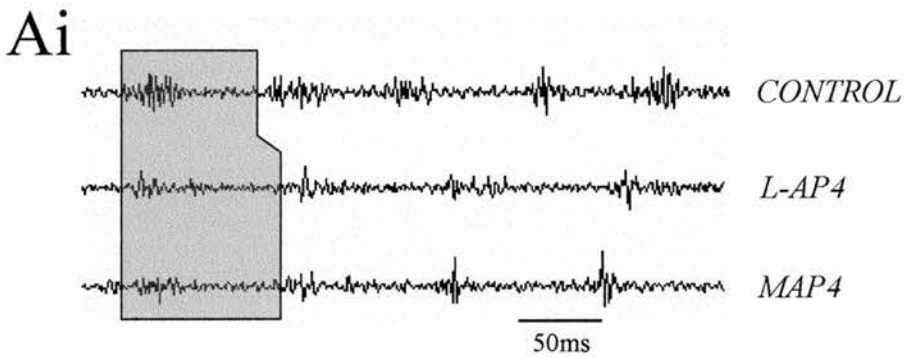
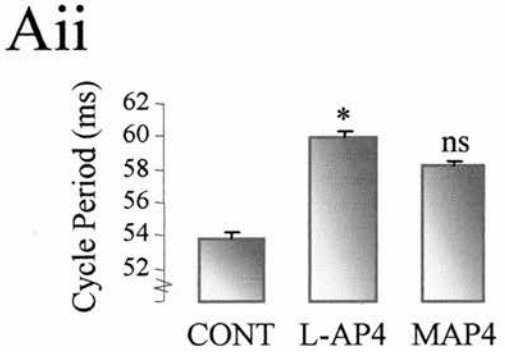
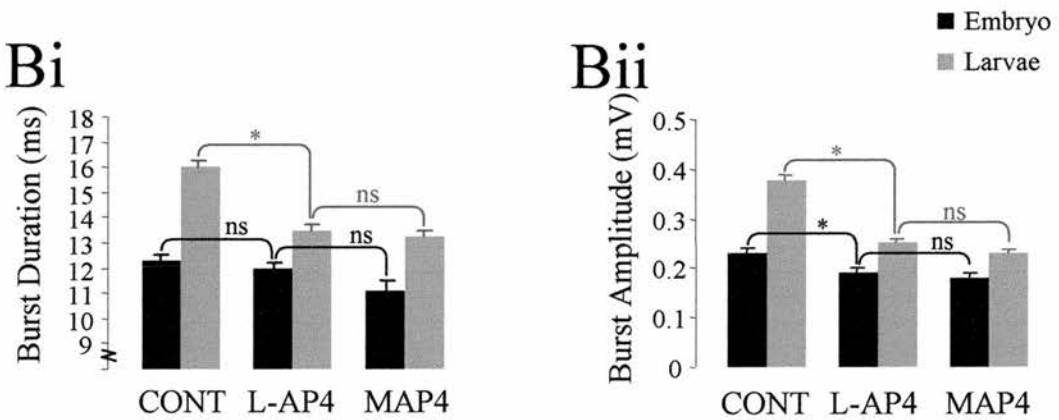

Ai
CONTROL
L-AP4
WASH
25ms
Aii
Cycle Period (ms)
62
60
58
56
54
52
*
*
CONT
L-AP4
WASH
Aiii
CONTROL
L-AP4
Cycle Period (ms)
80
75
70
65
60
55
50
45
40
35
1
10
19
28
37
46
55
64
73
82
91
100
Cycle in episode (First 100 cycles)
Bi
Burst Duration (ms)
18
16
14
12
*
ns
CONT
L-AP4
WASH
Bii
Burst Amplitude (mV)
0.5
0.4
0.3
0.2
0.1
0
*
ns
CONT
L-AP4
WASH
C
R-C Delay (ms)
8
6
4
2
0
*
ns
CONT
L-AP4
WASH

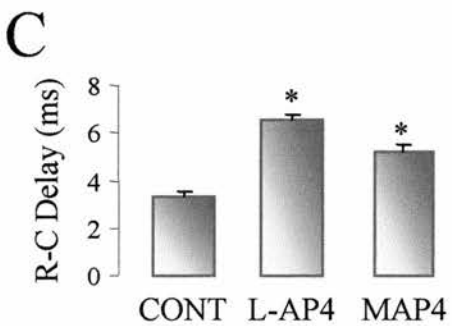
Figure 5.2 | Effects of L-AP4 are not always reversed with the general group III mGluR antagonist MAP4

Ventral root recording (Ai) and graphical (Aii) representation showing increased cycle periods with L-AP4 (O-A, $P<0.001$, $n=11$) that did not reverse in the presence of the general group III receptor antagonist MAP4 (100μM; O-A, $P>0.05$, $n=8$). In stage 42 larvae, L-AP4 application significantly decreased burst durations (Bi; O-A, $P<0.05$, $n=9$) and burst amplitudes (Bii; O-A, $P<0.05$, $n=9$), which could not be reversed by MAP4 (O-A, $P>0.05$, $n=6$). In stage 37/38 embryos, L-AP4 application did not significantly affect burst durations (O-A, $P>0.05$, $n=2$), but reduced burst amplitudes (Bi-ii; O-A, $P<0.001$, $n=2$). The reduced burst amplitudes in embryos also did not reverse following MAP4 application (O-A, $P>0.05$, $n=6$). R-C delays significantly increased in the presence of L-AP4 and were partially reversed with antagonist application (O-A, $P<0.001$, $n=8$). * = significant, ns = not significant. Values are means ± S.E.M.


Ai
CONTROL
L-AP4
MAP4
50ms


Aii
Cycle Period (ms)
62
60
58
56
54
52
*
ns
CONT
L-AP4
MAP4


Bi
Burst Duration (ms)
18
17
16
15
14
13
12
11
10
9
*
ns
ns
ns
CONT
L-AP4
MAP4
Embryo
Larvae
Bii
Burst Amplitude (mV)
0.5
0.4
0.3
0.2
0.1
0
*
*
ns
ns
CONT
L-AP4
MAP4


C
R-C Delay (ms)
8
6
4
2
0
*
*
CONT
L-AP4
MAP4

42-91ms in control and from 43-119ms under L-AP4) suggesting that L-AP4 application can affect cycle periods independently of any effects on episode duration. In 3 animals (all stage 42 larvae), this effect could be significantly reversed with washout (to 55.8±0.36ms; O-A, $F_{2,709}$=54.62, P<0.001, n=3; Figure 5.1Ai-ii). In a further 8 animals (2 stage 37/38 embryos and 6 stage 42 larvae), the increased cycle periods under L-AP4 could not be reversed with application of the general group III antagonist MAP4 (100μM; to 58.17±0.36ms with MAP4; O-A, P<0.05, n=8; Figure 5.2Ai-ii).

There is a significant decrease in burst durations following L-AP4 application, but only in stage 42 larval animals. Thus, ventral root burst durations decreased with 50μM L-AP4 in 9 larval animals from 16.03±0.22ms in control saline, to 13.5±0.2ms (O-A, $F_{2,535}$=39.05, P<0.001, n=9; Figure 5.1 and 5.2Bi), an effect which did not reverse during washout in 3 animals (13.82±0.17ms; O-A, P>0.05, n=3) or following application of MAP4 in 6 animals (13.23±0.23ms; O-A, P>0.05, n=6). In 2 stage 37/38 embryos, there was no change in burst durations with either L-AP4 or MAP4 application (O-A, P> 0.05, n=2; Figure 5.2Bi). The amplitudes of the ventral root bursts also significantly decreased after L-AP4 application not only in 9 larval animals (from 0.38±0.01mV in control saline, to 0.25±0.01mV with L-AP4; O-A, $F_{2,535}$=46.3, P<0.001, n=9; Figure 5.1 and 5.2Bii) but also in 2 embryonic animals (from 0.25±0.01mV in control saline, to 0.19±0.01mV with L-AP4; O-A, $F_{2,186}$=25.96, P<0.001, n=2; Figure 5.2Bii). The effect on burst amplitudes could not be reversed by washout (0.26±0.01mV; O-A, P>0.05, n=3) or by MAP4 application (larvae: 0.23±0.01mV; embryo: 0.18±0.01mV; O-A, P>0.05, n=6 and 2 respectively). R-C delays also significantly increased in the presence of L-AP4 in 11 animals from

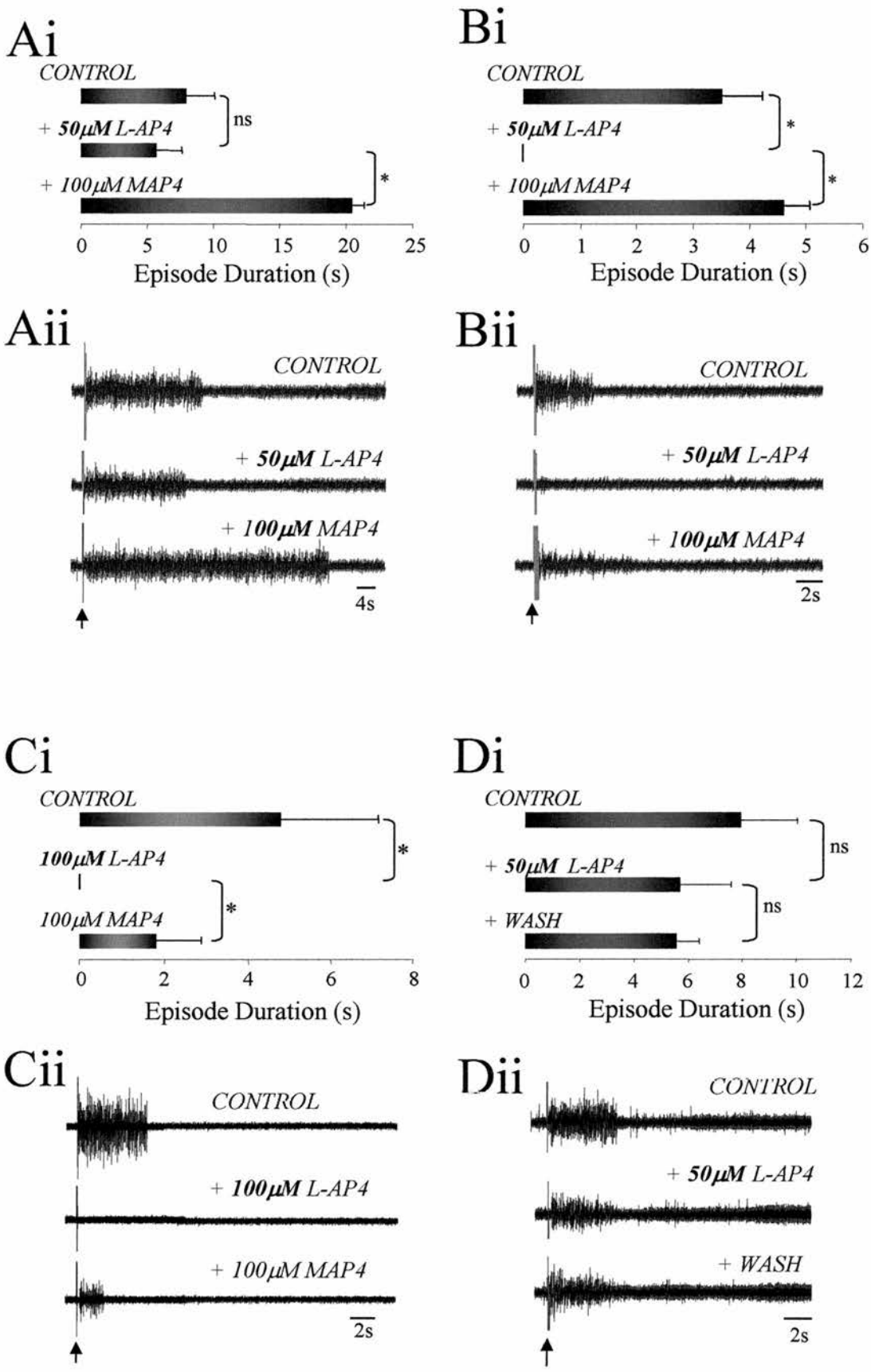
3.34±0.18ms in control saline to 6.52±0.19ms under L-AP4 (O-A, $F_{2,619}$=70.08, P<0.001, n=11; Figure 5.1 and 5.2C). Although this effect on R-C delays could not be reversed during washout, they increased with the antagonist MAP4 (to 5.23±0.31ms; O-A, $F_{2,619}$=70.08, P<0.001, n=8; Figure 5.2C).

Episode durations appeared to be reduced in an "all-or-nothing" manner following application of L-AP4 (Figure 5.3). At 50μM L-AP4, episode durations, though shorter, were not significantly affected in 11 animals (from 7.95±2.12s in control saline, to 5.73±1.86s with L-AP4; O-A, P>0.05, n=11; Figure 5.3Ai-ii, Di-ii). Subsequent application of MAP4 in 8 (of 11) animals significantly increased episode durations, even beyond the value of control condition (to 20.51±0.87s; O-A, P<0.05, n=8; Figure 5.3Ai-ii), suggesting endogenous activation of these receptors (see next section). However, washout was without effect on episode durations in 3 (of 11) animals (5.57±0.84s; O-A, P>0.05, n=3; Figure 5.3Di-ii). In 4 animals swimming activity was completely abolished by application of 50μM L-AP4, and in these cases application of MAP4 was always able to restore activity (Figure 5.3Bi-ii). At concentrations of 100μM L-AP4, swimming activity was always completely abolished very rapidly and the antagonist MAP4 (100μM) was able to partially but significantly restore activity (K-W, P<0.05, n=5; Figure 5.3Ci-ii).

From this set of experiments, it is clear that activating group III mGluRs with L-AP4 has a significant inhibitory effect on swimming activity. With such a profound depression of motor activity following exogenous activation of group III mGluRs, I next asked whether they might be activated by endogenously released glutamate within the *Xenopus* spinal cord to modulate the motor output underlying swimming.

Figure 5.3 | Effects of group III mGluR activation on the duration of swimming episodes

50μM L-AP4 (Ai-ii) does not greatly affect the duration of episodes (O-A, $P>0.05$, n=11 of 15 animals). However, in 4 animals (Bi-ii), 50μM L-AP4 caused complete abolition of swimming activity which was reversed following the application of MAP4 (100μM; O-A, $P<0.05$, n=4). (Ci-ii) 100μM L-AP4 always abolished swimming, which could be partially restored with MAP4 (O-A, $P<0.05$, n=5). (Di-ii) In a further 3 animals, L-AP4 did not affect episode durations and a subsequent washout similarly had no effect. Arrowhead donates the stimulation artefact. * = significant, ns = not significant. Values are means ± S.E.M.


Ai
CONTROL
+ 50μM L-AP4
ns
+ 100μM MAP4
*
0 5 10 15 20 25
Episode Duration (s)
Bi
CONTROL
+ 50μM L-AP4
*
+ 100μM MAP4
*
0 1 2 3 4 5 6
Episode Duration (s)
Aii
CONTROL
+ 50μM L-AP4
+ 100μM MAP4
4s
Bii
CONTROL
+ 50μM L-AP4
+ 100μM MAP4
2s
Ci
CONTROL
100μM L-AP4
*
100μM MAP4
*
0 2 4 6 8
Episode Duration (s)
Di
CONTROL
ns
+ 50μM L-AP4
ns
+ WASH
0 2 4 6 8 10 12
Episode Duration (s)
Cii
CONTROL
+ 100μM L-AP4
+ 100μM MAP4
2s
Dii
CONTROL
+ 50μM L-AP4
+ WASH
2s

5 | 3 | 2 Evidence for endogenous activation of Group III mGluRs

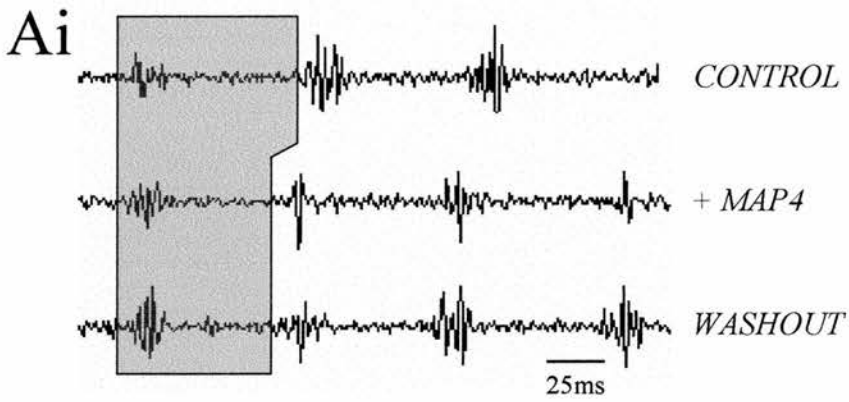
The group III receptor antagonist, MAP4 (100μM), was bath applied to explore whether these receptors are normally activated during swimming. In 8 animals MAP4 caused a significant reduction in cycle periods (from 54.84±1.28ms in control saline, to 51.55±0.55ms under MAP4; O-A, $F_{2,267}$=6.63, P<0.001, n=8; Figure 5.4Ai-ii), and thus increased swimming frequency. All other parameters of swimming were apparently unaffected by MAP4, including episode durations (from 8.1±4.99s in control saline, to 9.76±1.55s under MAP4; K-W, P>0.05, n=8; Figure 5.4Bi-ii), which was surprising in light of the effective reversal MAP4 produces after swimming activity is abolished following application of L-AP4 (Figure 5.3). It is possible, that if group III receptors utilise multiple mechanisms to affect the spinal network, including the activation of slower second messenger systems, then 20mins bath application prior to recordings may not be long enough for MAP4 to produce a maximum effect on the network. This might, in part, explain why the ability of MAP4 to reverse the effects induced on the motor network by L-AP4 is not reliable and washing is far more effective. However, the reduction in cycle periods following MAP4 application does suggest that group III receptors are activated by endogenously released glutamate to reduce swimming frequency.

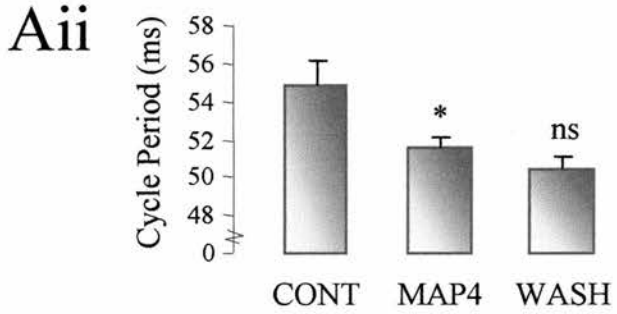
5 | 3 | 3 Group III receptor activation may modulate glycinergic transmission to reduce swim frequency

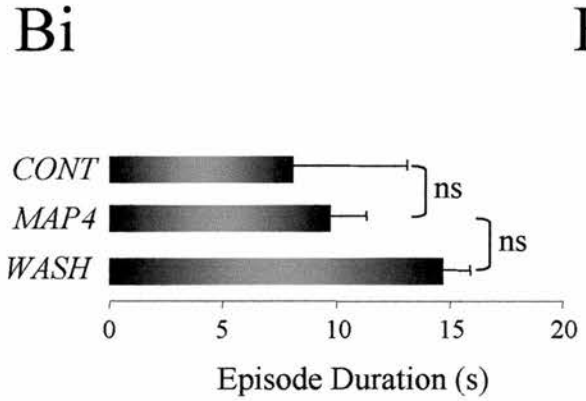
In *Xenopus*, as in many other vertebrate motor systems, glycine is the main neurotransmitter responsible for mediating the reciprocal inhibition between the two locomotor half-centres of the spinal cord. Small changes in the strength of glycinergic inhibition can have dramatic effects on the frequency of swimming and

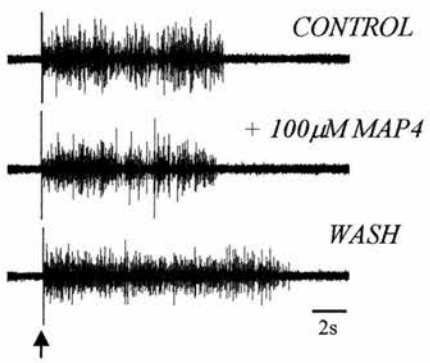
Figure 5.4 | Endogenous activation of group III mGluRs influences swimming frequency

Effects of the general group III antagonist, MAP4, on ventral root activity. Ventral root recording (Ai) and graphical representation (Aii) showing decreased cycle periods following MAP4 application (100μM; O-A, $P<0.001$ n=8). Washout for ~20 minutes did not reverse the observed effect on swimming frequency (O-A, $P>0.05$, n=8). (Bi-ii) MAP4 application did not influence the duration of swimming episodes (K-W, $P>0.05$, n=8). Arrowhead represents stimulation artefact. * = significant, ns = not significant. Values are means ± S.E.M. or medians ± I.Q.R.


Ai
CONTROL
+ MAP4
WASHOUT
25ms


Aii
Cycle Period (ms)
58
56
54
52
50
48
0
*
ns
CONT
MAP4
WASH


Bi
CONT
MAP4
WASH
ns
ns
0
5
10
15
20
Episode Duration (s)

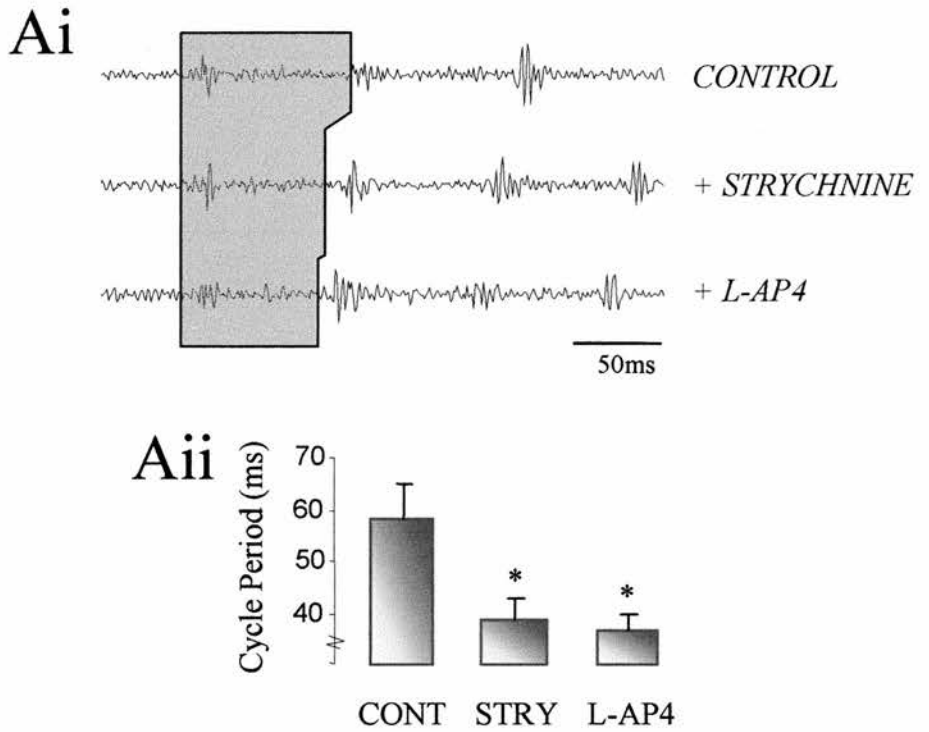

Bii
CONTROL
+ 100μM MAP4
WASH
2s

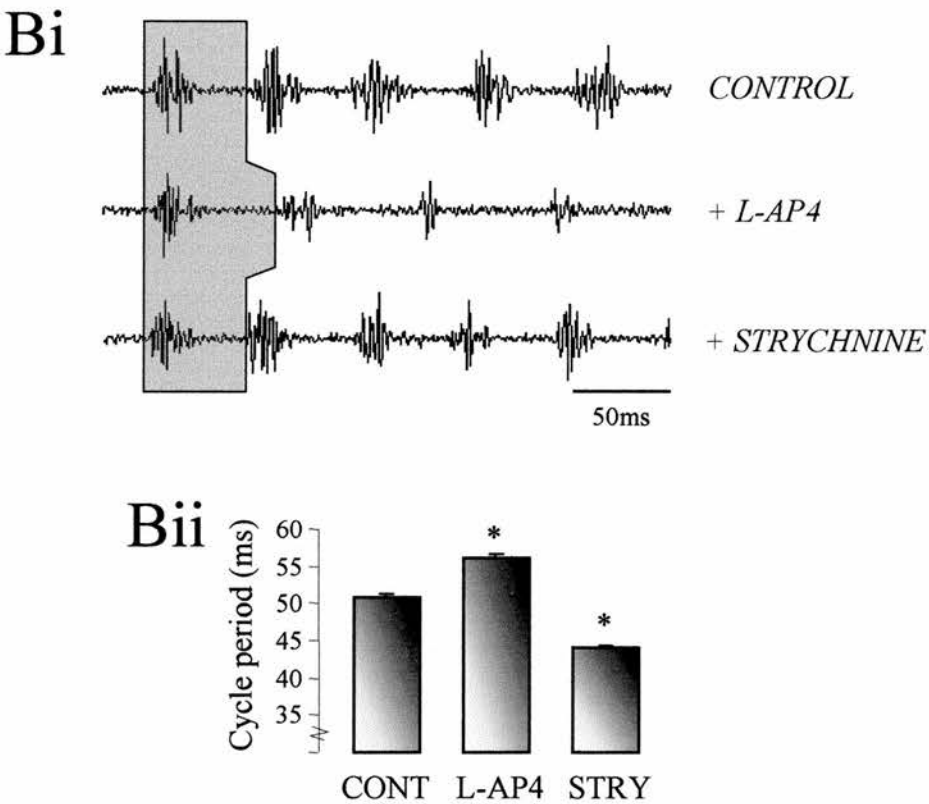
these synapses are known to be a common target for neuromodulation in *Xenopus* tadpoles. For example, the neuromodulators NA and NO both cause a reduction in swimming frequency through a facilitation of glycinergic transmission (McDearmid *et al*, 1997; McLean, 2001). The effects on the motor network induced by NA and NO are comparable in certain respects to those produced by L-AP4 application, suggesting that group III receptors could similarly potentiate glycinergic transmission as a mechanism to reduce swimming frequency. Therefore, extracellular experiments involving the glycine receptor antagonist, strychnine, were initially conducted to assess this possibility.

Strychnine (1μM), was first bath applied prior to the activation of group III mGluRs with 50μM L-AP4. Strychnine caused a characteristic and significant decrease in cycle periods that decreased further in the presence of L-AP4 (from 58.33±6.31ms in control saline to 38.67±3.94ms under strychnine, decreasing to 36.6±2.9ms with L-AP4; O-A, $F_{3,356}$=479.19, P<0.001, n=5; Figure 5.5Ai-ii). It is conceivable that the reduced cycle periods under L-AP4 is a continued effect of strychnine rather than an additive effect of the group III receptor agonist. However, the previously observed increase in cycle periods following L-AP4 application did not occur in the presence of strychnine. Application of strychnine after the activation of group III receptors caused the L-AP4-induced increase in cycle periods to be significantly reduced (increased from 50.82±0.38ms in control saline to 56.2±0.49ms under L-AP4, and reduced to 44.02±0.38ms by strychnine; O-A, $F_{3,356}$=212.69, P<0.001, n=5; Figure 5.5Bi-ii). The occlusion of L-AP4's effect on cycle periods in the presence of strychnine (Figure 5.5Aii) and the reversal of L-AP4 effects by strychnine suggests that group III receptors may indeed be influencing glycinergic transmission as a

Figure 5.5 | Activation of group III mGluRs before and after the blockade of glycine receptors

(Ai-ii) Applications of strychnine (1μM) prior to adding the group III specific agonist L-AP4 (50μM) caused a significant decrease in cycle periods (O-A, $P<0.001$, n=5), which decreased further following L-AP4 application. Ventral root recording (Bi) and graphical representation (Bii) showing increased cycle periods following L-AP4 application (O-A, $P<0.001$, n=5) and subsequent reduction of cycle periods by strychnine (O-A, $P<0.001$, n=5) . * = significant, ns = not significant. Values are means ± S.E.M.


Ai
CONTROL
+ STRYCHNINE
+ L-AP4
50ms
Aii
Cycle Period (ms)
70
60
50
40
*
*
CONT
STRY
L-AP4


Bi
CONTROL
+ L-AP4
+ STRYCHNINE
50ms
Bii
Cycle period (ms)
60
55
50
45
40
35
*
*
CONT
L-AP4
STRY

means to reduce swim frequency, but obviously this result on its own cannot rule out other possible contributory effects.

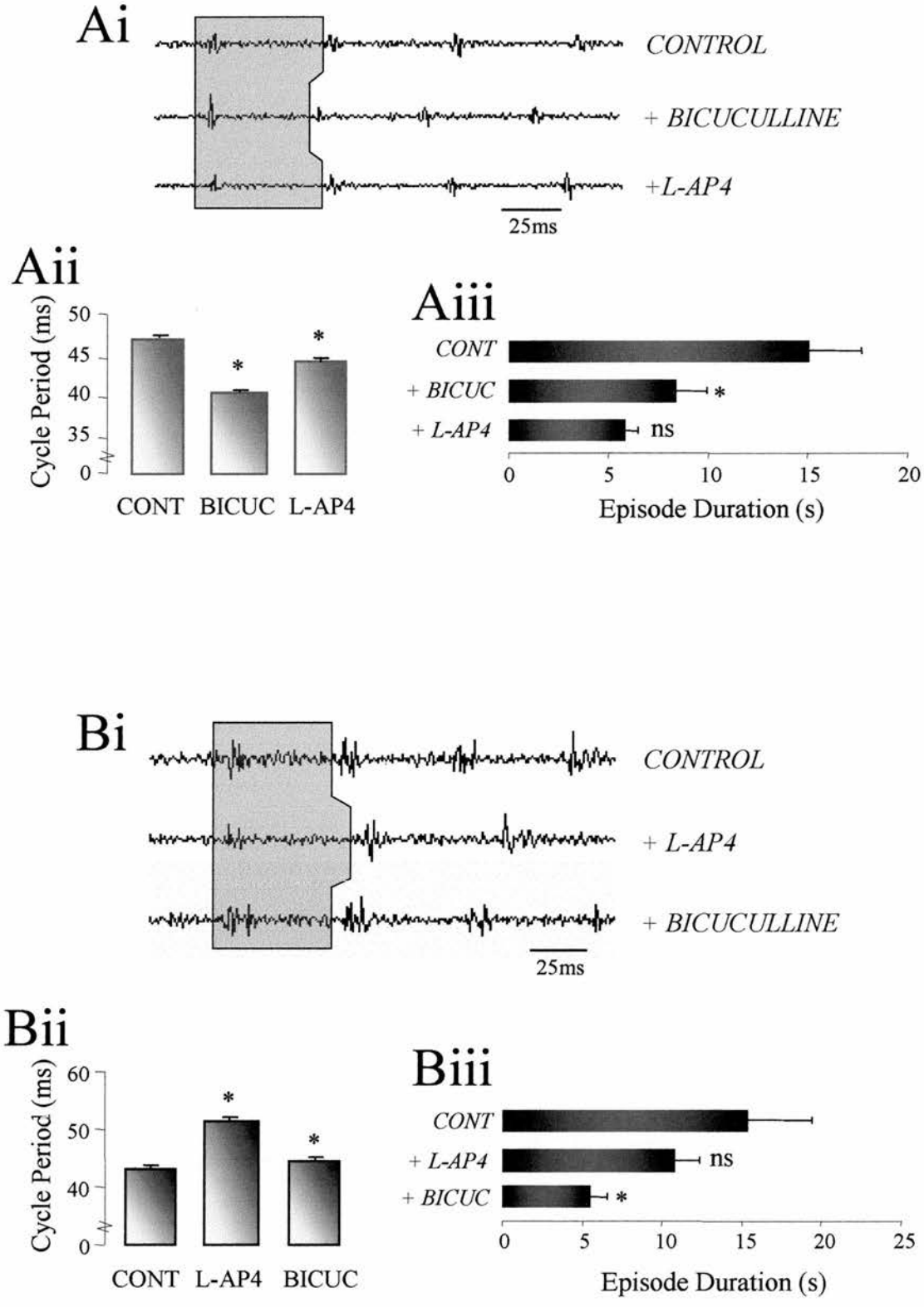
5 | 3 | 4 Modulation of GABA transmission by group III receptors as a means to prematurely terminate swimming?

The fast inhibitory neurotransmitter GABA is known to play a role in the control of swim episode duration in *Xenopus* embryos and larvae (Boothby and Roberts, 1992a; Boothby and Roberts, 1992b; Reith and Sillar, 1999). Also, whilst there is no evidence of GABAergic IPSPs being phase-locked to the swim cycle, reducing GABA transmission through the application of the $GABA_A$ receptor antagonist bicuculline, causes an increase in swimming frequency (Reith and Sillar, 1999). Therefore, it is important not to discount the potential group III receptor-mediated modulation of GABAergic transmission as a means to influence swimming frequency. In the presence of bicuculline (40μM), cycle periods decreased significantly (from 46.9±0.3ms in control saline, to 40.11±0.3ms under bicuculline; O-A, $F_{2,267}$=106.1, P<0.001, n=7; Figure 5.6Ai-ii) and subsequent application of 50μM L-AP4 caused a significant increase to 44.2±0.4ms (O-A, $F_{2,267}$=106.1, P<0.001, n=7; Figure 5.6Ai-ii). There was also the characteristic reduction in episode durations that is observed following bicuculline application (Reith and Sillar, 1999; from 15.12±3.99s in control saline, to 8.4±1.6s under bicuculline; O-A, $F_{2,60}$=6.71, P<0.001, n=7; Figure 5.6Aiii). Subsequent application of L-AP4 had no significant effect on episode durations (5.84±0.73s; O-A, P>0.05, n=7; Figure 5.6Aiii).

As observed previously, L-AP4 causes an increase in cycle periods that are subsequently reduced following application of bicuculline (from 51.23±0.48 under L-

Figure 5.6 | Activation of group III mGluRs before and after the blockade of GABA receptors

Ventral root recording (Ai) and graphical representation (Aii) showing decreased cycle periods with application of the $GABA_A$ receptor antagonist bicuculline (BICUC; 40μM; O-A, $P<0.001$, n=7). Cycle periods subsequently increased following the application of L-AP4 (50μM; O-A, $P<0.001$, n=7). (Aiii) Bicuculline also caused a significant reduction in episode durations (O-A, $P<0.001$, n=7), that did not change after application of L-AP4 (O-A, $P>0.05$, n=7). (Bi-ii) The increased cycle periods induced by group III receptor activation decreased following application of bicuculline (O-A, $P<0.001$, n=5). (Biii) L-AP4 also had no significant effect on episode durations (O-A, $P>0.05$, n=5), but subsequent application of bicuculline decreased the duration of swimming episodes (O-A, $P<0.001$, n=5). * = significant, ns = not significant. Values are means ± S.E.M.


Ai
CONTROL
+ BICUCULLINE
+L-AP4
25ms
Aii
Cycle Period (ms)
50
45
40
35
0
CONT
BICUC
L-AP4
Aiii
CONT
+ BICUC
+ L-AP4
ns
0
5
10
15
20
Episode Duration (s)
Bi
CONTROL
+ L-AP4
+ BICUCULLINE
25ms
Bii
Cycle Period (ms)
60
50
40
0
CONT
L-AP4
BICUC
Biii
CONT
+ L-AP4
ns
+ BICUC
0
5
10
15
20
25
Episode Duration (s)

AP4 to 44.32±0.9ms with bicuculline; O-A. $F_{2,233}$=58.3, P<0.001, n=5; Figure 5.6Bi-ii). In these 5 pooled experiments, 50μM L-AP4 had no significant effect on episode durations (from 15.41±3.99s in control saline, to 10.8±1.6s under L-AP4; O-A, P>0.05, n=5; Figure 5.6Biii), but subsequent bicuculline application caused a significant reduction (to 5.5±1.02s; O-A, $F_{2,33}$=3.76, P<0.05, n=5; Figure 5.6Biii). From these results its seems unlikely that group III receptors are modulating GABA transmission to prematurely terminate swimming or influence swim frequency, as the effects of L-AP4 were not occluded in the presence of bicuculline.

So far, these extracellular experiments involving pharmacological manipulations of the fast inhibitory pathways have suggested that group III mGluR activation could influence glycinergic transmission to modulate swimming frequency and that they appear not to influence GABA transmission to mediate their effects on swimming episode duration or frequency.

5 | 3 | 5 Effects of group III receptor activation on the synaptic drive underlying swimming

Experiments involving intracellular recordings from presumed motorneurons using KCl-filled microelectrodes were next undertaken to explore any L-AP4-induced effects on the individual components of the synaptic drive underlying swimming and/or on the integrative electrical properties of motorneurons. As mentioned in previous chapters, the synaptic drive underlying the swimming rhythm in *Xenopus* has three main components: fast on-cycle excitation, superimposed upon a tonic depolarisation, with activity on the two sides of the spinal cord coupled in anti-phase by reciprocal mid-cycle glycinergic inhibition (see chapter 1 and figure 2.1).

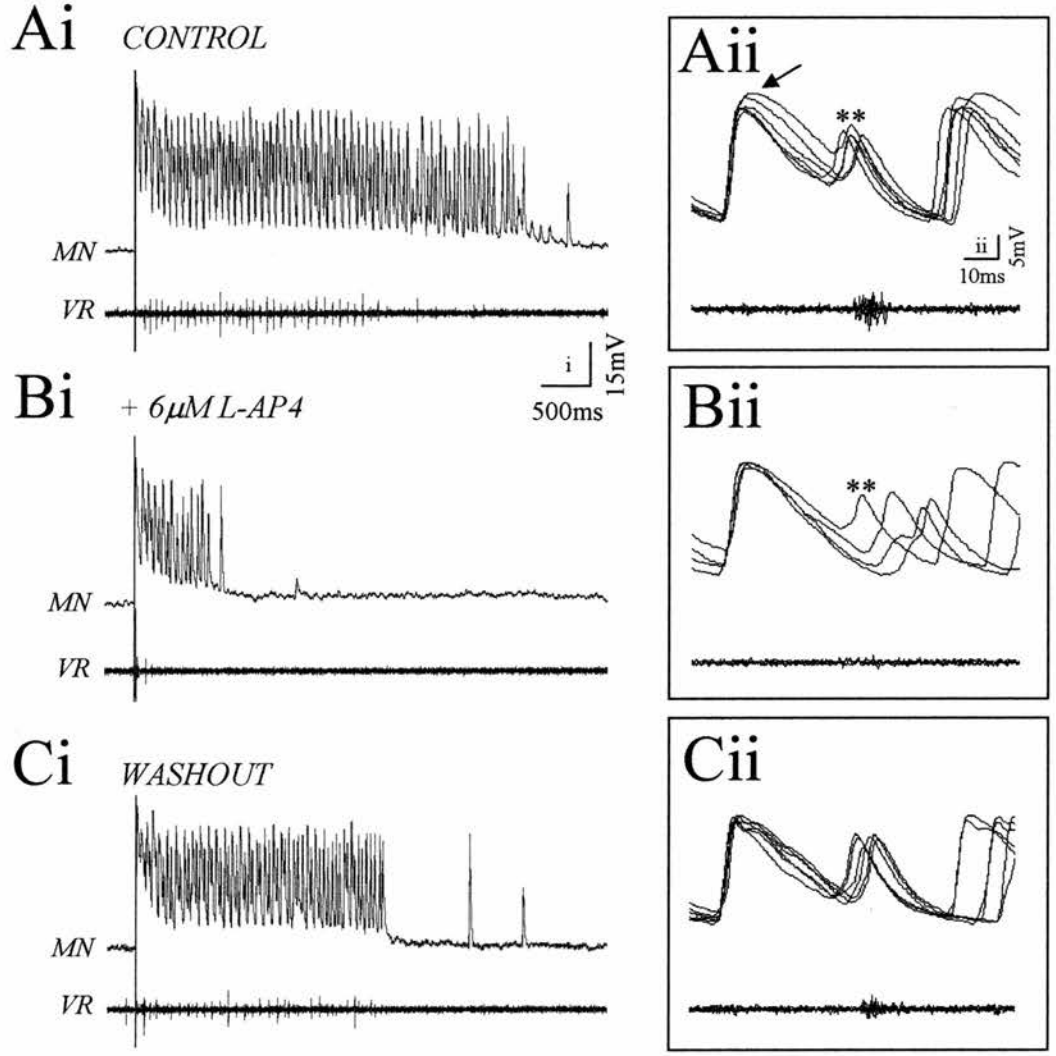
To record intracellularly the spinal cord must be exposed by removal of the overlying muscle blocks (see chapter 2: Methods and Materials) and this procedure thus allows better access to any bath applied pharmacological agents. At the concentrations of agonist used in extracellular experiments (50μM), swimming was always completely abolished by L-AP4 when the cord was exposed making it impossible to assess any effects of L-AP4 on mid-cycle inhibition. Therefore, in 4 experiments, the agonist concentration was reduced to between 1-6μM; episode durations were still profoundly reduced by L-AP4 and in one instance swimming was abolished. In only 1 of these experiments was a reversal of episode durations observed, with washout, and is illustrated in figure 5.7.

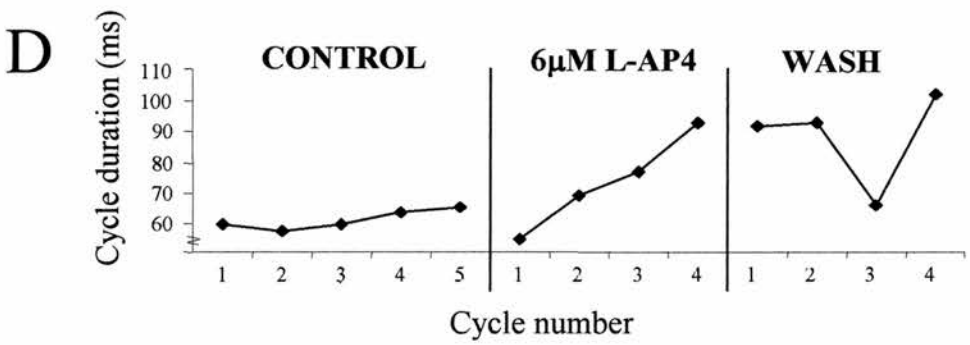
Application of L-AP4 caused a dramatic reduction in episode durations, to the extent that only ~10 cycles of activity could be recorded in each episode (Figure 5.7Bi-ii,D). Note that the ventral root activity 'appears' to vanish following L-AP4 application (Figure 5.7Bi-ii) despite the fact that several cycles of swimming activity are recorded intracellularly. The ventral root recording is measured caudally during intracellular experiments (see figure 2.1), and caudal neurons generally have a lower level of excitability than rostral neurons (Tunstall and Roberts, 1994). It is possible that group III receptor activation reduces excitability such that caudal motorneurons either produce a weaker extracellular signal that may be lost in the amplifier noise and not be detected or that they fall sub-threshold altogether. This also suggests that during the preceding extracellular experiments the motor network may have been active in the presence of L-AP4, but ventral root discharge was not recorded.

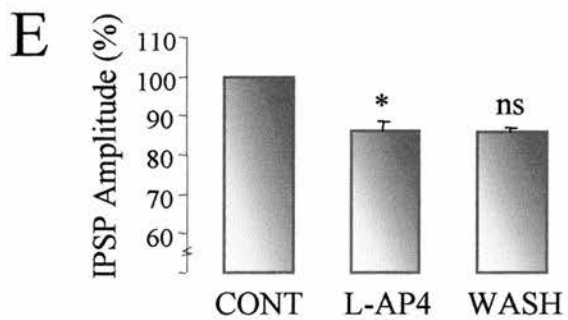
As mentioned previously in chapter 4 (section 4.3.4), the protocol normally used to calculate the mean mid-cycle IPSP amplitude could not be used accurately because

Figure 5.7 | The effect of L-AP4 application on the synaptic drive underlying swimming

Episodes of evoked swimming shown on a slow (i) and faster (ii) time scale. Application of 6μM L-AP4 caused a significant reduction in episode durations that reversed during washout (A-C). Excerpts of ~6 cycles of swimming are expanded and overlaid in Aii-Cii. (D) Under L-AP4, cycle periods increase dramatically on a cycle-by-cycle basis. Arrow represents inhibitory component and the double-asterisk represents the excitatory component of the synaptic drive underlying swimming. Under L-AP4 there were fewer cycles per episode and an apparent decrease in mid-cycle IPSP (E; note that frequency matching was not possible, see chapter 2: Materials and Methods). MN = motorneuron, VR = ventral root. * = significant, ns = not significant.


Ai
CONTROL
MN
VR
i
15mV
500ms
Aii
**
ii
5mV
10ms
Bi
+ 6μM L-AP4
MN
VR
Bii
**
Ci
WASHOUT
MN
VR
Cii


D
CONTROL
6μM L-AP4
WASH
Cycle duration (ms)
110
100
90
80
70
60
1 2 3 4 5 1 2 3 4 1 2 3 4
Cycle number

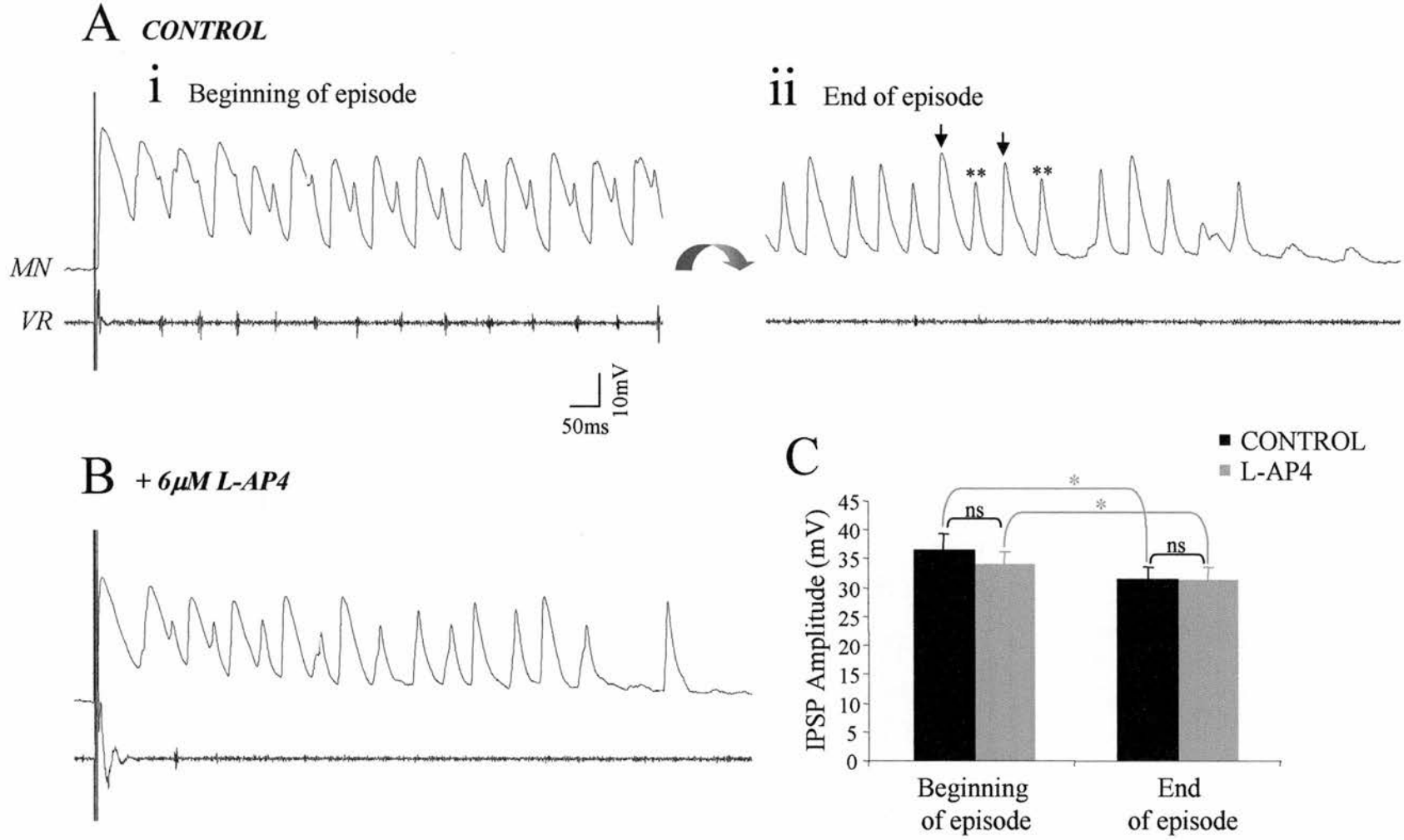

E
IPSP Amplitude (%)
110
100
90
80
70
60
*
ns
CONT
L-AP4
WASH

the episodes of swimming in the presence of L-AP4 were so brief. As the effects following application of L-AP4 on episode durations during intracellular recordings are similar to the effects induced by the group II receptor agonist, APDC, the modified protocol described in chapter 4 was also applied to the data presented in this chapter. Using this modified protocol, the mean mid-cycle IPSP amplitude decreased with agonist application (to ~86.3±2.37% of control IPSP amplitude; O-A, $F_{2,86}$=39.87, P<0.001, n=3; Figure 5.7E). The mean mid-cycle IPSP amplitude did not increase during washout (O-A, P>0.05, n=1; Figure 5.7Ci-ii,E), although the effect on episode durations did reverse (Figure 5.7Ci).

Due to the limited number of cycles per episode of swimming recorded under L-AP4, cycles from the beginning, middle and end of an episode were included in the analysis. The amplitude of the mid-cycle IPSP might depend on where in the episode measurements are taken from. As swimming frequency slows towards the end of an episode the synaptic drive will likely weaken, for example, due to interneuronal drop-out as they fall below threshold (Sillar and Roberts, 1993). Therefore, to enable a better comparison of mid-cycle IPSP amplitudes under each condition, measurements of amplitudes are taken at representative stages within each episode. In figure 5.8, excerpts from the beginning (Ai) and end (Aii) of an episode of swimming in control condition (represented fully in figure 5.7A) can be compared to a whole episode of swimming under L-AP4 (Figure 5.8B). The mean mid-cycle IPSP amplitude within the first and last two cycles of each episode of swimming were measured under each condition and compared. The IPSP amplitude at the beginning and at the end of an episode of swimming was not significantly different under each condition (O-A, P>0.05; Figure 5.8C). However, the IPSP amplitude did decrease significantly at the end of an episode in each condition compared to the beginning of the episode (*t*-test,

Figure 5.8 | A closer examination of the synaptic drive for swimming following L-AP4 application

(A) Excerpts of evoked swimming under control conditions from figure 5.7 are shown on a faster time scale to illustrate the beginning (i) and end (ii) of an episode of swimming. This enables a comparison of the mid-cycle IPSPs at equivalent points with those in the presence of L-AP4 (B). The characteristic swimming cycles at the beginning and end of the short episode under L-AP4 are similar to those in control, suggesting that L-AP4 does not significantly influence the inhibitory drive to slow swimming. (C) Amplitudes of the first and last 2 cycles of activity per episode of swimming were compared under each condition. At the beginning and end of an episode there is no significant difference between mean mid-cycle IPSP amplitude under each condition (O-A, $P>0.05$). However, the mean IPSP amplitude was smaller at the end of an episode of swimming compared to the beginning (*t*-test, $P<0.05$). Arrow represents inhibitory component and the double-asterisk represents the excitatory component of the synaptic drive underlying swimming. MN = motorneuron, VR = ventral root. * = significant, ns = not significant.


A CONTROL
i Beginning of episode
ii End of episode
MN
VR
50ms
10mV
**
**
B + 6μM L-AP4
C
CONTROL
L-AP4
IPSP Amplitude (mV)
45
40
35
30
25
20
15
10
5
0
ns
*
*
ns
Beginning of episode
End of episode

P<0.001; Figure 5.8C). From these results, the amplitudes of the mid-cycle IPSP do not appear to be significantly affected by activation of group III receptors, although swimming activity, especially episode duration, is clearly affected by L-AP4 application.

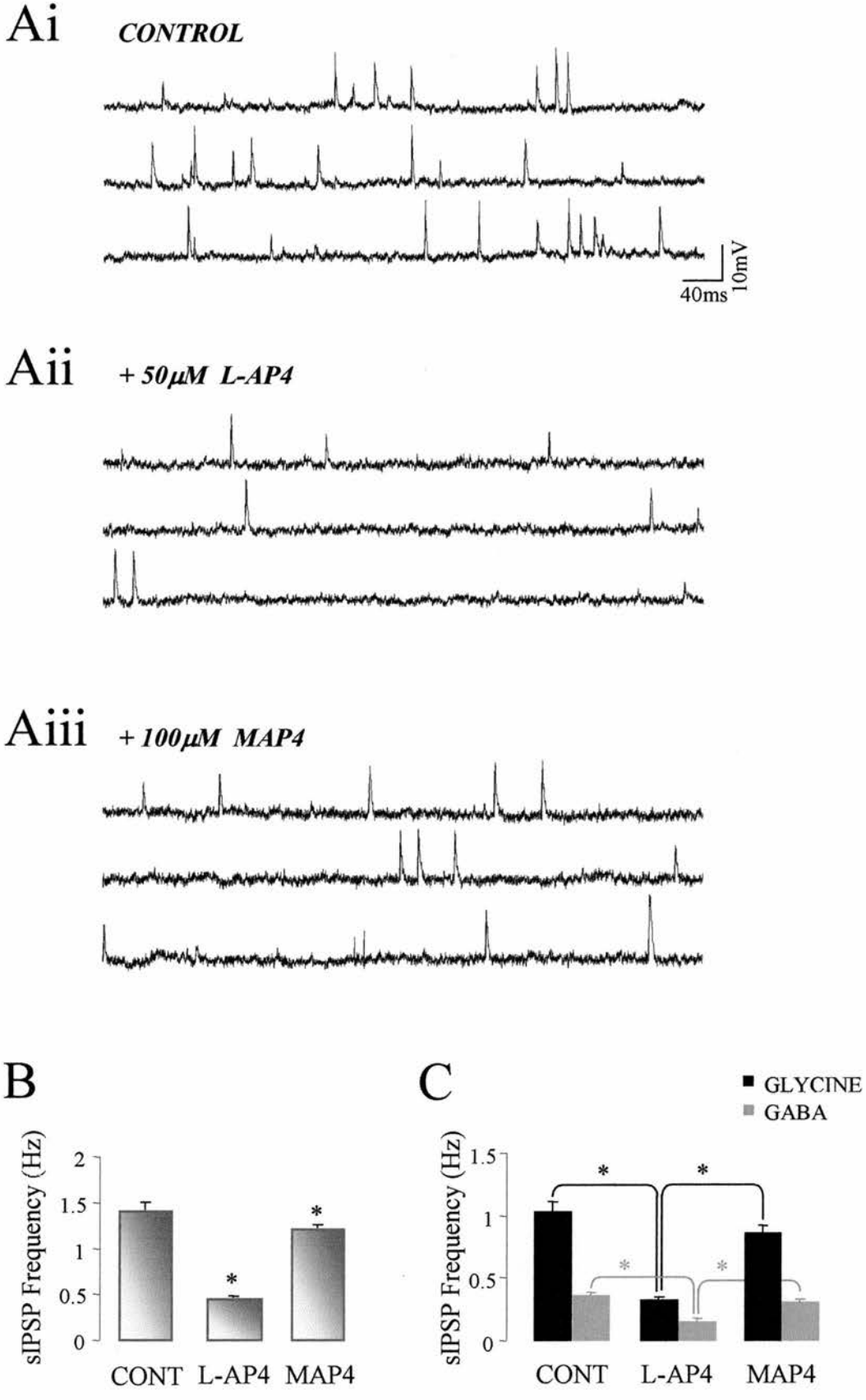
5 | 3 | 6 Effects of group III mGluR activation on glycinergic and GABAergic spontaneous IPSPs

So far my results have shown a profound reduction of swimming frequency following application of the group III receptor specific agonist L-AP4. Whilst the effects on the mid-cycle IPSP suggest that L-AP4 does not enhance glycinergic transmission (see above), despite strychnine's apparent occlusion of L-AP4s effects extracellularly, another approach to test this hypothesis was to examine spontaneous IPSPs during quiescent periods between episodes of swimming. Moreover, this approach allows any L-AP4-induced pre- and/or post-synaptic effects to be examined even in the absence of evoked transmission. If L-AP4 slows swimming through a facilitation of glycinergic transmission, an increase in spontaneous release would be hypothesised.

Data pooled from 4 cells following application of 1-6μM L-AP4, showed no significant change in the rate of occurrence of sIPSPs recorded (from 1.32±0.18Hz in control, to 1.3±0.24Hz with L-AP4; O-A, P>0.05, n=4; data not shown). When the concentration of L-AP4 was increased to 50μM, the concentration used extensively during extracellular experiments, applications of L-AP4 caused a profound *reduction* in sIPSP frequency (from 1.4±0.08Hz in control saline to 0.45±0.01Hz under L-AP4; O-A, $F_{2,120}$=82.63, P<0.001, n=8; Figure 5.9Ai-ii,B). This effect was reversed effectively with the antagonist MAP4 in 6 out of 8 experiments (to 1.2±0.04Hz; O-A, P>0.05, n=6 out of 8; Figure 5.9Aiii,B). This reduction in frequency was observed

Figure 5.9 | Group III mGluR activation reduces the frequency of spontaneous IPSPs

(Ai-ii) During inter-episode quiescent periods, spontaneous glycine and GABA transmitter release is reduced following the application of 50μM L-AP4. (B) The rate of sIPSP occurrence decreased following L-AP4 application (from 1.4±0.08Hz in control, to 0.45±0.01Hz; O-A, $P<0.001$, n=8) and application of the antagonist, MAP4 (Aiii,B) reversed this effect in 6 out of 8 experiments (to 1.2±0.04Hz; O-A, $P<0.001$, n=6 out of 8). (C) The decreased sIPSP frequency was observed for both glycinergic (from 1.03±0.08Hz in control, to 0.33±0.01Hz under L-AP4; O-A, $P<0.001$, n=8) and GABAergic sIPSPs (from 0.36±0.01Hz in control, to 0.15±0.02Hz; O-A, $P<0.001$, n=8). Both glycinergic and GABAergic sIPSP frequency was restored following MAP4 application (C; O-A, $P<0.001$, n=6 out of 8). * = significant, ns = not significant. Values are means ± S.E.M.

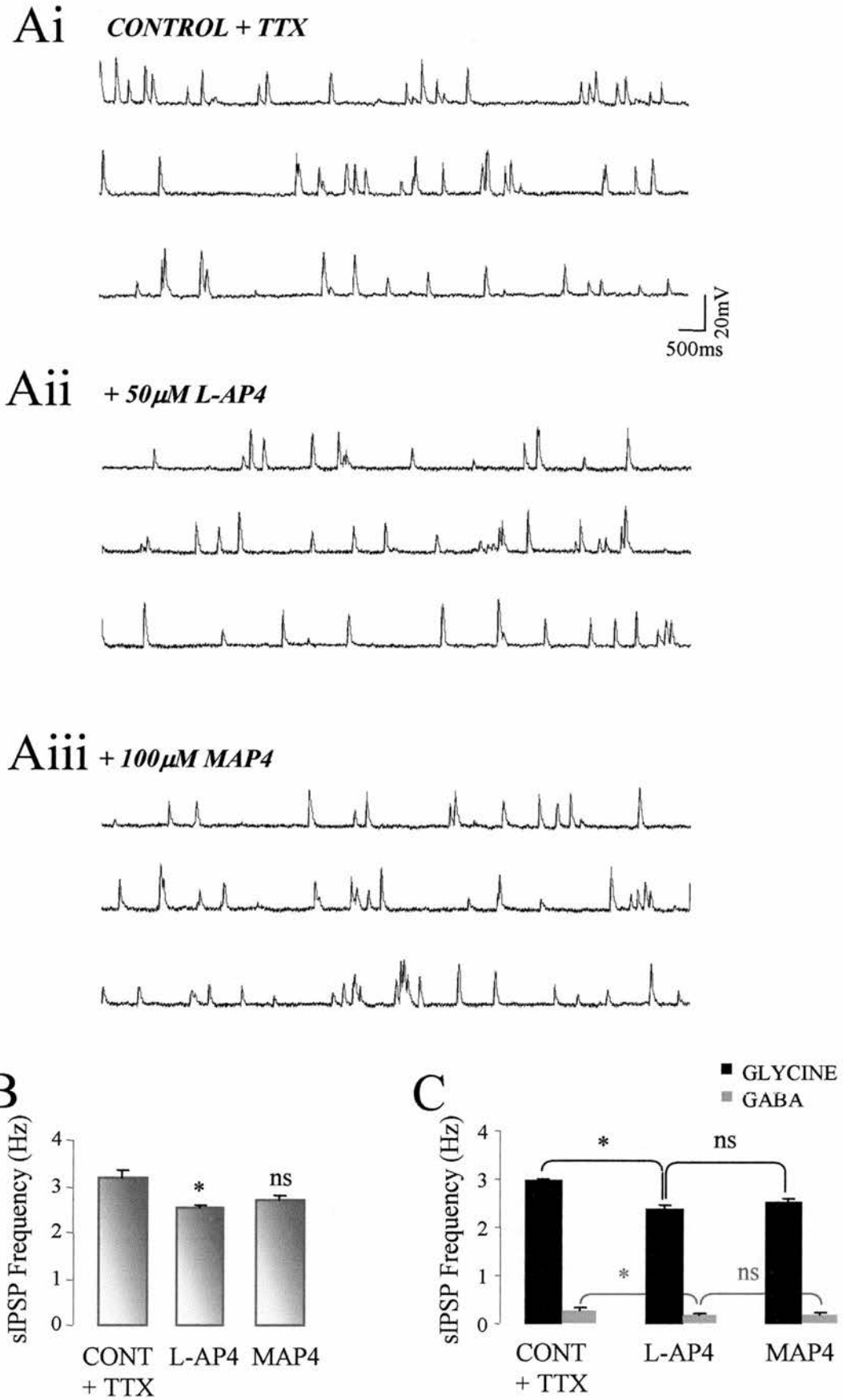

Ai
CONTROL
40ms
10mV
Aii
+ 50μM L-AP4
Aiii
+ 100μM MAP4
B
sIPSP Frequency (Hz)
2
1.5
1
0.5
0
*
*
CONT
L-AP4
MAP4
C
GLYCINE
GABA
sIPSP Frequency (Hz)
1.5
1
0.5
0
*
*
*
*
CONT
L-AP4
MAP4

for both glycinergic (from 1.03±0.08Hz in control saline, to 0.33±0.01Hz with L-AP4; O-A, $F_{2,120}$=35.39, P<0.001, n=8; Figure 5.9C) and GABAergic (from 0.36±0.01Hz in control saline, to 0.15±0.02Hz under L-AP4; O-A, $F_{2,120}$=57.19, P<0.001, n=8; Figure 5.9C) sIPSPs, identified by their distinct differences in duration (see chapter 2: Materials and Methods). Subsequent application of MAP4 in 6 out of 8 experiments significantly reversed the effects on both glycinergic and GABAergic sIPSP frequency (glycine: 0.86±0.08Hz; GABA: 0.3±0.02Hz; O-A, $F_{2,120}$=35.39 and 57.19 respectively, P<0.001, n=6 out of 8; Figure 5.9C).

These experiments were then repeated in the presence of TTX to check that the effects on sIPSP frequency could not be due to changes in the firing of pre-synaptic neurons. In the presence of TTX, application of the higher concentration of L-AP4 (50μM) caused a significant reduction in the rate of occurrence of sIPSPs (from 3.21±0.12Hz in control saline to 2.54±0.07Hz under L-AP4; O-A, $F_{2,120}$=14.21, P<0.001, n=8; Figure 5.10Ai-ii,B). This effect could not be reversed following antagonist application (O-A, P>0.05, n=6 out of 8; Figure 5.10B). Similarly, the reduced frequency of sIPSPs was observed for both glycinergic (from 2.94±0.08Hz in control saline, to 2.37±0.07Hz under L-AP4; O-A, $F_{2,120}$=11.03, P<0.001, n=8; Figure 5.10C) and GABAergic sIPSPs (from 0.27±0.03Hz under control saline, to 0.16±0.01Hz under L-AP4; O-A, $F_{2,120}$=5.58, P<0.001, n=8; Figure 5.10C). MAP4 application was, again, unable to significantly increase the rate of occurrence of either glycinergic or GABAergic sIPSPs (O-A, P>0.05, n=6 out of 8; Figure 5.10C) although the trend was in the expected direction.

Figure 5.10 | The rate of occurrence of TTX-resistant spontaneous IPSPs is reduced with the activation of group III mGluRs

(Ai-ii) In the presence of TTX (1μM), spontaneous glycine and GABA transmitter release reduced with 50μM L-AP4 application, and did not reverse with the group III antagonist MAP4 (Aiii). (B) The sIPSP rate of occurrence is significantly reduced with activation of group III mGluRs (from 3.21±0.12Hz in control, to 2.54±0.07Hz after L-AP4 application; O-A, $P<0.001$, n=8). (C) This reduced sIPSP frequency was observed for both glycinergic (from 2.94±0.08Hz in control, to 2.37±0.07Hz; O-A, $P<0.001$, n=8) and GABAergic sIPSPs (from 0.27±0.03Hz under control, to 0.16±0.03Hz; O-A, $P<0.001$, n=8), and whilst MAP4 did not significantly reverse this effect in 6 out of 8 experiments, there was a trend toward increasing sIPSP frequency. * = significant, ns = not significant. Values are means ± S.E.M.


Ai
CONTROL + TTX
20mV
500ms
Aii
+ 50μM L-AP4
Aiii
+ 100μM MAP4
B
sIPSP Frequency (Hz)
0
1
2
3
4
*
ns
CONT + TTX
L-AP4
MAP4
C
GLYCINE
GABA
sIPSP Frequency (Hz)
0
1
2
3
4
*
ns
*
ns
CONT + TTX
L-AP4
MAP4

The observed reduction in the rate of occurrence of both glycinergic and GABAergic sIPSPs following application of L-AP4 and in the presence of TTX implies that group III receptors are located pre-synaptically and can modulate inhibitory transmission by affecting the probability of transmitter release. sIPSP amplitude distributions were next assessed to check for any evidence for post-synaptic effects of L-AP4. Figure 5.11 shows that in 6 out of 8 experiments, following group III receptor activation there is no change in mean sIPSP amplitude. In the amplitude distribution histograms for one representative experiment, the range of sIPSP amplitudes shifted slightly from between 3 and 26mV in control, to between 6 and 31mV under L-AP4, although the mean amplitude did not significantly change (from 11.7±0.64mV in control saline, to 12.7±1.24mV under L-AP4; O-A, $P>0.05$; Figure 5.11Ai-ii). Subsequent MAP4 application also did not alter the range (between 6 and 39mV) or the mean sIPSP amplitude (12.07±1.6; O-A, $P>0.05$; Figure 5.11Aiii). When data is pooled from 6 experiments, the mean sIPSP amplitude similarly did not change (from 8.78±0.28mV in control saline, to 9.17±0.29mV under L-AP4; O-A, $P>0.05$, $n=6$; Figure 5.11B). Applications of the antagonist, MAP4, did not significantly alter the mean sIPSP amplitude in these instances (8.2±0.45mV; O-A, $P>0.05$, $n=6$; Figure 5.11B). However, in a further 2 experiments, L-AP4 application caused an *increase* in the range of sIPSP amplitudes. In the representative experiment shown in Figure 5.12Ai-iii, L-AP4 caused the range of amplitudes to increase, from between 4 and 26mV in control to between 5 and 30mV under L-AP4, and produced an increase in the mean sIPSP amplitude (from 14.64±0.37mV in control saline, to 16.63±0.67mV under L-AP4; O-A, $F_{2,318}=10.66$, $P<0.001$; Figure 5.12Ai-ii) that was successfully reversed with MAP4 (to 12.5±0.56mV; O-A, $F_{2,318}=10.66$, $P<0.001$; Figure 5.12Aiii).

Figure 5.11 | Group III mGluR activation does not affect the mean amplitude of sIPSPs

In 6 out of 8 experiments, the sIPSP amplitude distributions were not obviously affected following group III receptor activation. The histograms in Ai-iii correspond to the example shown in figure 5.9A. (Ai) Histogram showing the distribution of sIPSP amplitudes, ranging from 3 to 26mV, under control conditions. The measured mean amplitude in control was 11.7±0.64mV. (Aii) Variation in sIPSP amplitudes under L-AP4, ranging from 6 to 31mV, and the mean was not significantly different to control conditions (12.7±1.24mV; O-A, $P>0.05$). (Aiii) The range of amplitudes under MAP4 was between 6 and 39mV, and the mean was not significantly different to those in control conditions or L-AP4 (12.07±1.6mV; O-A, $P>0.05$). (B) sIPSP amplitudes pooled from 6 experiments similarly showed no change in mean sIPSP amplitudes following group III receptor activation (8.78±0.28mV in control saline, to 9.17±0.29mV under L-AP4, and 8.2±0.45mV under MAP4; O-A, $P>0.05$, $n=6$). Dotted lines represent mean amplitude under each condition. * = significant, ns = not significant. Values are means ± S.E.M.

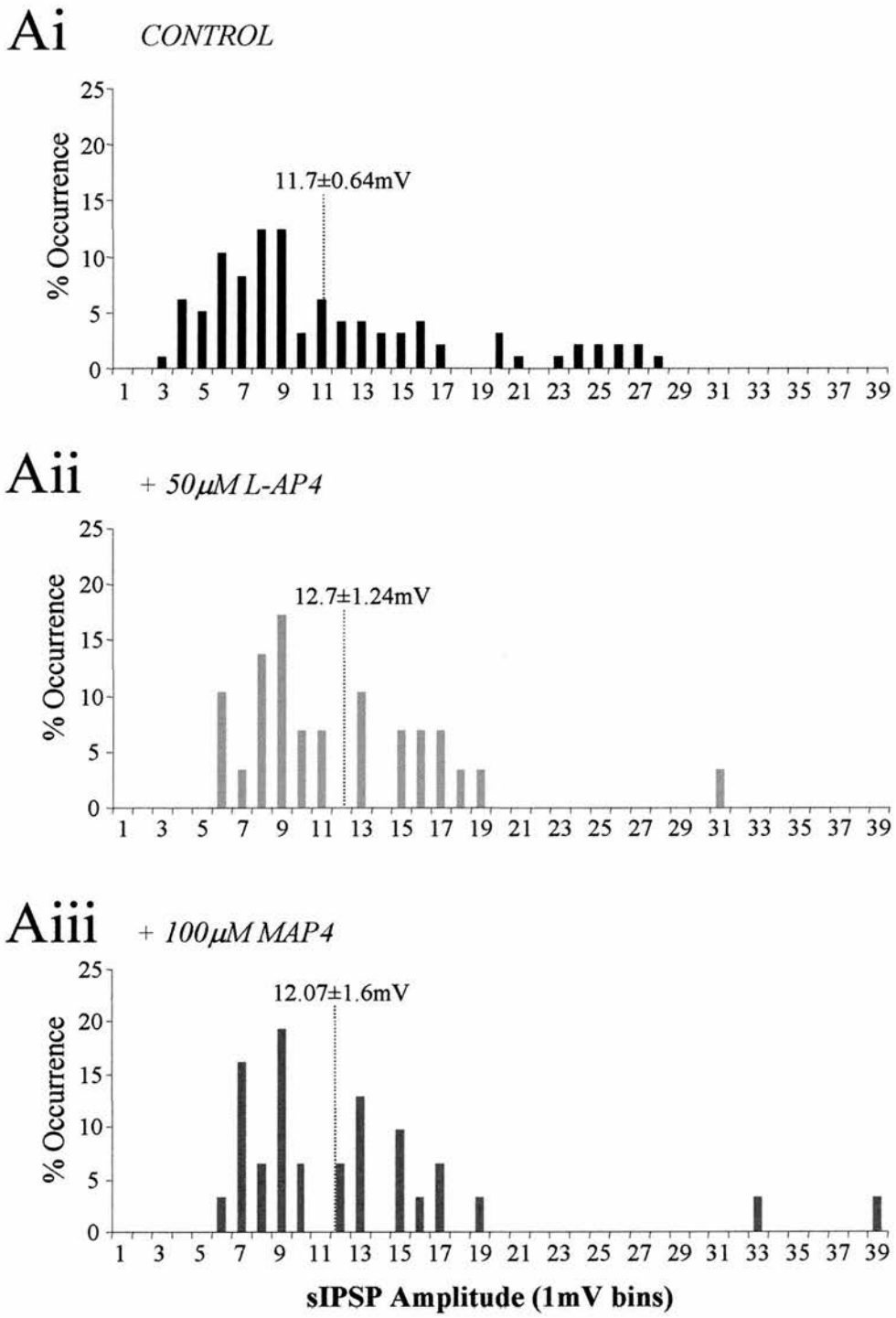
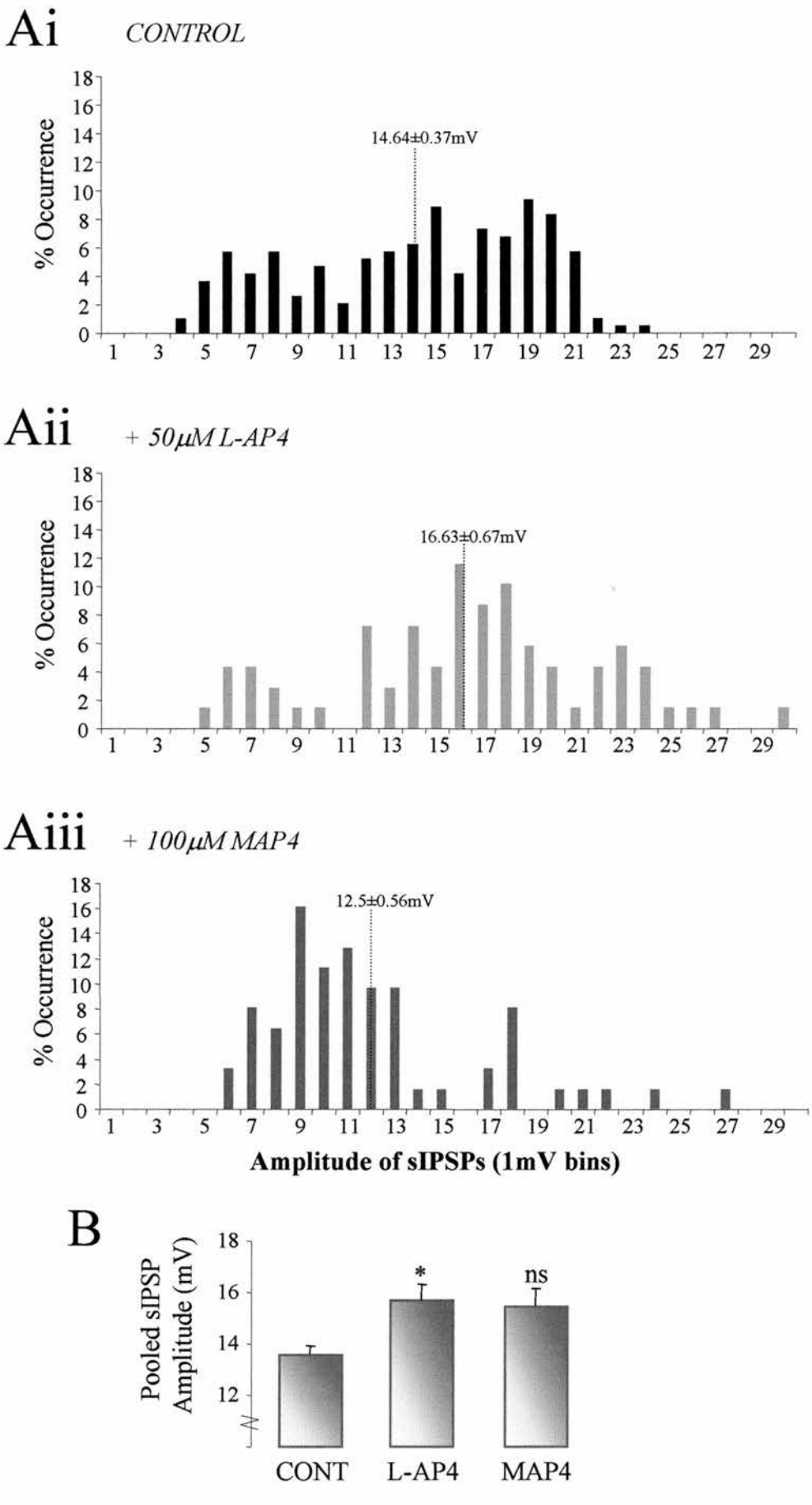

Ai
CONTROL
% Occurrence
11.7±0.64mV
Aii
+ 50μM L-AP4
% Occurrence
12.7±1.24mV
Aiii
+ 100μM MAP4
% Occurrence
12.07±1.6mV
sIPSP Amplitude (1mV bins)
B
Pooled sIPSP Amplitude (mV)
ns
ns
CONT
L-AP4
MAP4

Figure 5.12 | Group III mGluR activation increases the mean amplitude of sIPSPs

In 2 out of 8 experiments, the mean sIPSP amplitude significantly increased following group III receptor activation. (Ai) Histogram showing the distribution of sIPSP amplitudes, ranging from 4 to 26mV, under control conditions. The measured mean amplitude in control was 14.64±0.37mV (from this representative experiment). (Aii) L-AP4 induced a shift in the distribution of sIPSP amplitudes, ranging from 5 to 30mV, and the mean significantly increased to 16.63±0.67mV (O-A, $P<0.001$). (Aiii) Under MAP4, sIPSP amplitudes ranged between 6 and 27mV, and the mean significantly decreased to 12.5±0.56mV (O-A, $P<0.001$). (B) sIPSP amplitudes pooled from the 2 experiments showed an increase in mean sIPSP amplitudes following group III receptor activation (13.65±0.33mV in control saline, to 15.67±0.62mV under L-AP4; O-A, $P<0.001$, $n=2$) which did not reverse following MAP4 application (15.45±0.68mV; O-A, $P>0.05$, $n=2$). Dotted lines represent mean amplitude under each condition. * = significant, ns = not significant. Values are means ± S.E.M.


Ai
CONTROL
% Occurrence
14.64±0.37mV
Aii
+ 50μM L-AP4
% Occurrence
16.63±0.67mV
Aiii
+ 100μM MAP4
% Occurrence
12.5±0.56mV
Amplitude of sIPSPs (1mV bins)
B
Pooled sIPSP Amplitude (mV)
*
ns
CONT
L-AP4
MAP4

In addition, agonist applications in the presence of TTX did not significantly affect the range or mean amplitudes of sIPSPs in 3 out of 6 experiments. Figure 5.13Ai-iii shows a representative experiment where the amplitude distribution histograms are unaffected by application of either L-AP4 or MAP4. The range of sIPSP amplitudes varied between 3 and 25mV in control conditions, with a mean of 12.39±0.64mV. Application of L-AP4 did not significantly alter the range (between 3 and 23mV) or the mean sIPSP amplitude (12.91±0.55mV; O-A, $P>0.05$; Figure 5.13Ai-ii). Under MAP4, sIPSP amplitudes varied between 2 and 23mV, and the mean amplitude was 11.96±0.54mV which was not significantly different to those under L-AP4 (O-A, $P>0.05$; Figure 5.13Aiii). Similarly, when data is pooled from 3 experiments, the mean sIPSP amplitudes do not change in the presence of L-AP4/TTX (from 11.39±0.18mV in control saline, to 11.41±0.14mV under L-AP4/TTX; O-A, $P>0.05$, $n=3$), or following MAP4 application (10.81±0.26mV; O-A, $P>0.05$, $n=3$; Figure 5.13B).

However, in a further 3 experiments in the presence of TTX, a significant *increase* was observed in the range of sIPSP amplitudes and the mean amplitude following L-AP4 application. The experiment represented in figure 5.14Ai-iii shows an increase in the range of sIPSP amplitudes from between 2 and 15mV in control, to between 3 and 18mV under L-AP4, which decreased to between 2 and 13mV after application of MAP4. The mean sIPSP amplitude in this example increased from 6.52±0.19mV in control conditions, to 7.52±0.23mV following activation of group III receptors (O-A, $F_{2,520}=31.44$, $P<0.001$; Figure 5.14Ai-ii). This effect was successfully reversed by MAP4 (to 5.34±0.16mV; O-A, $F_{2,520}=31.44$, $P<0.001$; Figure 5.14Aiii). Data pooled from 3 experiments showed the same significant increase in mean sIPSP amplitude

Figure 5.13 | The effect of L-AP4 application on sIPSP mean amplitude in the presence of TTX

In 3 out of 8 experiments there was no change in mean sIPSP amplitude following group III receptor activation in the presence of TTX. (Ai) Histogram showing the distribution of sIPSP amplitudes from a representative experiment, ranging from 3 to 25mV, under control conditions in the presence of TTX. The measured mean amplitude in control was 12.39±0.64mV. (Aii) Subsequent application of L-AP4 did not alter the range of amplitudes (2 to 23mV), and the mean did not change (12.91±0.55mV; O-A, $P>0.05$). (Aiii) The group III antagonist MAP4 similarly had no effect on the range of amplitudes (2 to 23mV) or the calculated mean amplitude (11.91±0.54mV; O-A, $P>0.05$). (B) Graph showing sIPSP amplitudes pooled from 3 experiments in which there was no significant change in amplitudes (11.39±0.18mV in control saline, 11.41±0.14mV under L-AP4, and 10.41±0.26mV under MAP4; O-A, $P>0.05$, $n=3$). Dotted lines represent mean amplitude under each condition. * = significant, ns = not significant. Values are means ± S.E.M.

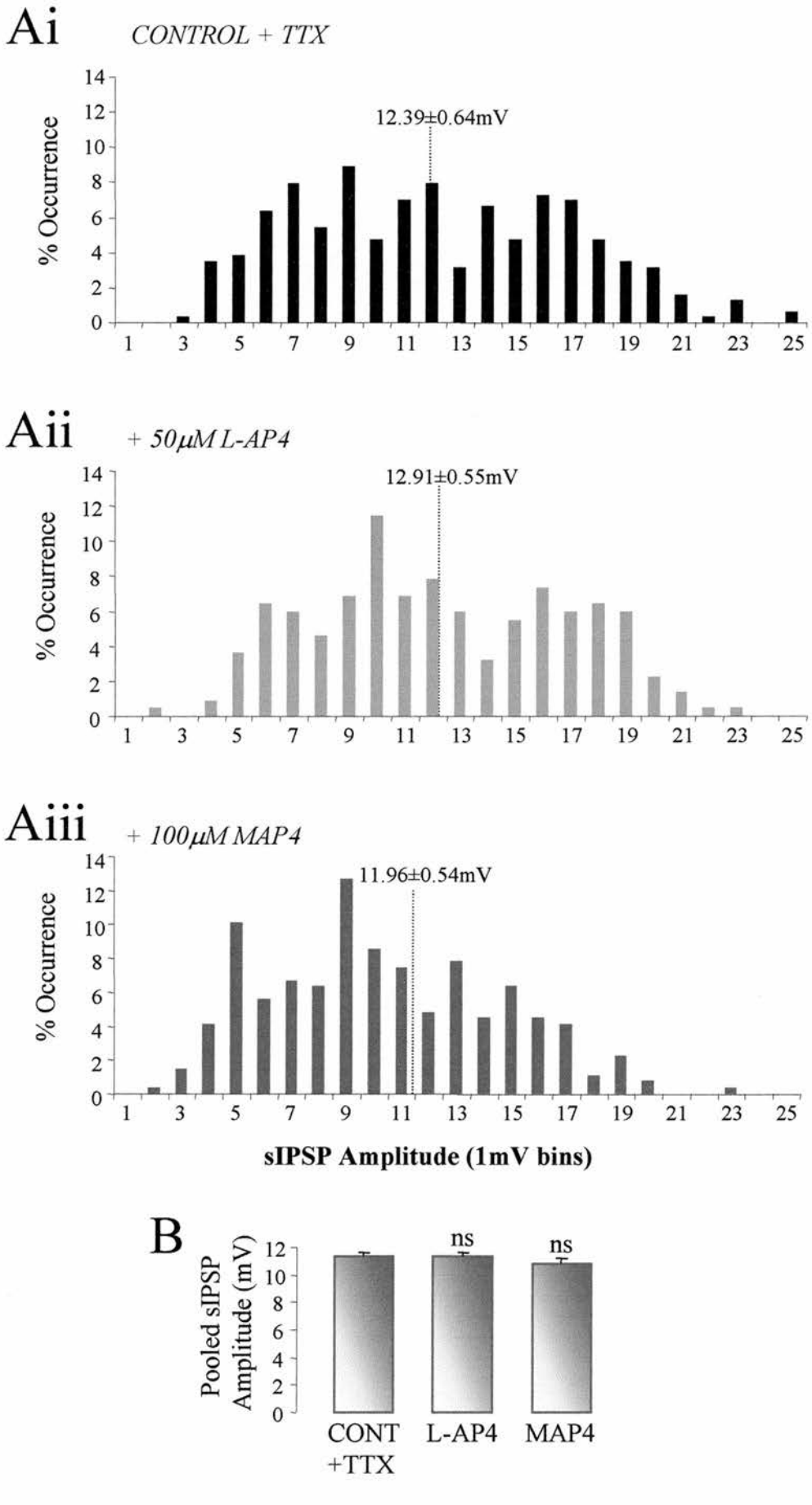
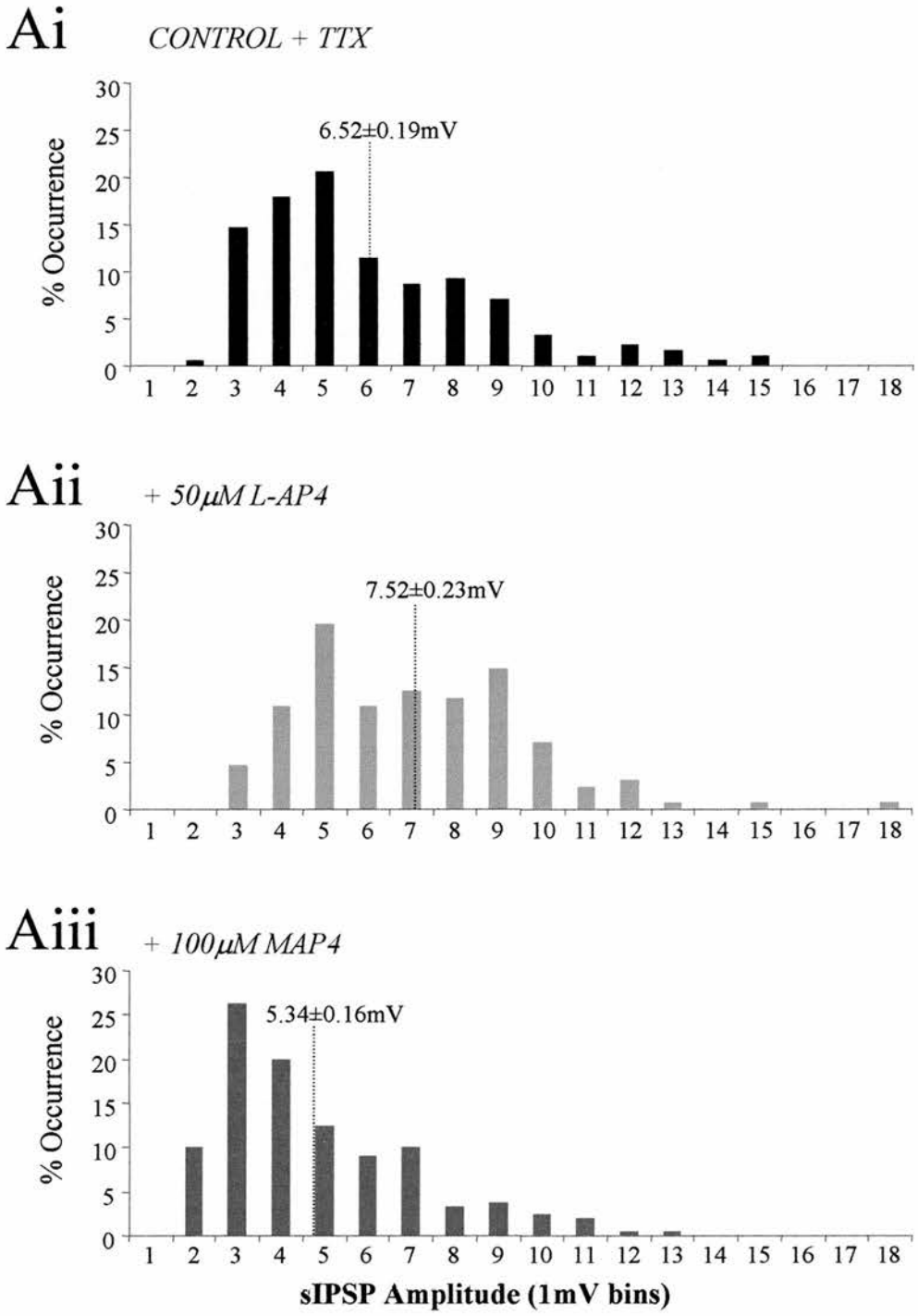
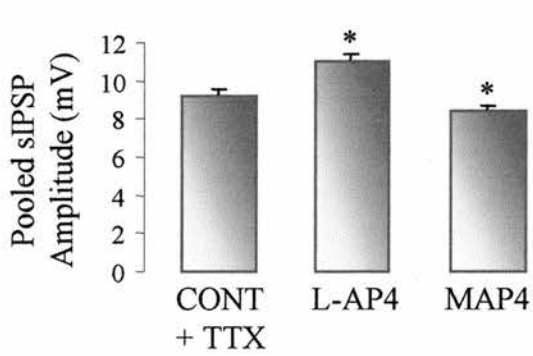

Ai
CONTROL + TTX
12.39±0.64mV
% Occurrence
Aii
+ 50μM L-AP4
12.91±0.55mV
% Occurrence
Aiii
+ 100μM MAP4
11.96±0.54mV
% Occurrence
sIPSP Amplitude (1mV bins)
B
Pooled sIPSP Amplitude (mV)
ns
ns
CONT +TTX
L-AP4
MAP4

Figure 5.14 | L-AP4 induces an increase in mean sIPSP amplitude in the presence of TTX

In 3 out of 8 experiments there was a significant increase in mean sIPSP amplitude following group III receptor activation in the presence of TTX. (Ai) Histogram showing the distribution of sIPSP amplitudes from a representative experiment, ranging from 2 to 15mV, under control conditions in the presence of TTX. The measured mean amplitude under control conditions was 6.52±0.19mV. (Aii) Application of L-AP4 caused an increase in both the range of amplitudes (3 to 18mV), and in the mean amplitude (7.52±0.23mV; O-A, $P<0.001$). (Aiii) MAP4 reversed the effect on the range of amplitudes (2 to 13mV) and the mean amplitude (5.34±0.16mV; O-A, $P<0.001$). (B) Graph showing sIPSP amplitudes pooled from 3 experiments showing a significant increase in mean amplitudes following group III receptor activation (9.23±0.21mV in control saline, to 11.04±0.26mV under L-AP4, and decreasing to 8.41±0.2mV under MAP4; O-A, $P<0.001$, $n=3$). Dotted lines represent mean amplitude under each condition. * = significant, ns = not significant. Values are means ± S.E.M.


Ai
CONTROL + TTX
% Occurrence
6.52±0.19mV
Aii
+ 50μM L-AP4
% Occurrence
7.52±0.23mV
Aiii
+ 100μM MAP4
% Occurrence
5.34±0.16mV
sIPSP Amplitude (1mV bins)


B
Pooled sIPSP Amplitude (mV)
CONT + TTX
L-AP4
MAP4

following application of L-AP4 in the presence of TTX (from 9.23±0.21mV in control saline, to 11.04±0.26mV under L-AP4/TTX; O-A, $F_{2,1466}$=30.62, P<0.001, n=3; Figure 5.14B) and a similar reversal by MAP4 (to 8.41±0.2mV; O-A, $F_{2,1466}$=30.62, P<0.001, n=3; Figure 5.14B).

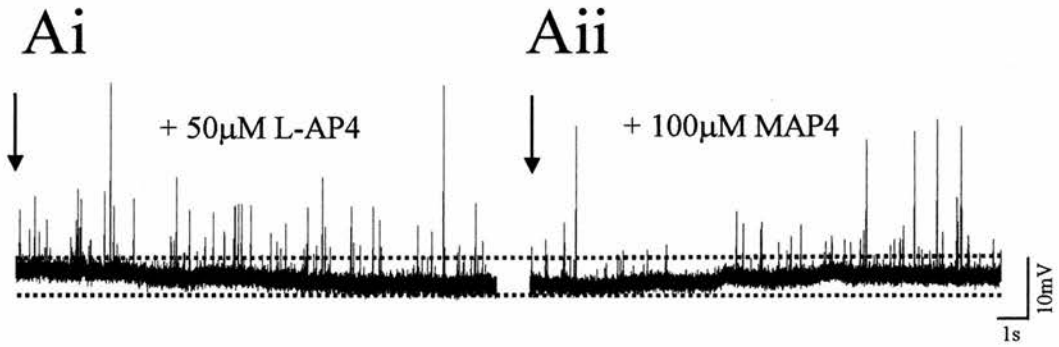
To summarise, in the majority of recordings, no significant effect on the mean or the range of sIPSP amplitudes (n=6 out of 8; n=3 out of 6 in the presence of TTX) was observed. However, in a small proportion of cells, a significant *increase* was observed in both the mean and the range of sIPSP amplitudes (n=2 out of 6; n=3 out of 6 in the presence of TTX), an effect which was reversed with the antagonist, MAP4. From these results there appear to be parallel, though somewhat contradictory, pre- and post-synaptic effects produced by L-AP4 application in a proportion of neurons, as reflected by the reduction in sIPSP frequency but an increase in mean sIPSP amplitude. The reduction of inhibitory transmitter release seems paradoxical in light of the finding that L-AP4 causes a depression of motor activity as a decrease in inhibition might be expected to favour higher excitability within the network and increase swim frequency, as observed with group I receptor activation (see chapter 3). There is quite clearly a pre-synaptic inhibition of inhibitory transmission with the activation of group III mGluRs, at least at relatively high agonist concentration, but this cannot readily explain the reduction of swimming frequency unless L-AP4 concomitantly reduces excitatory transmission.

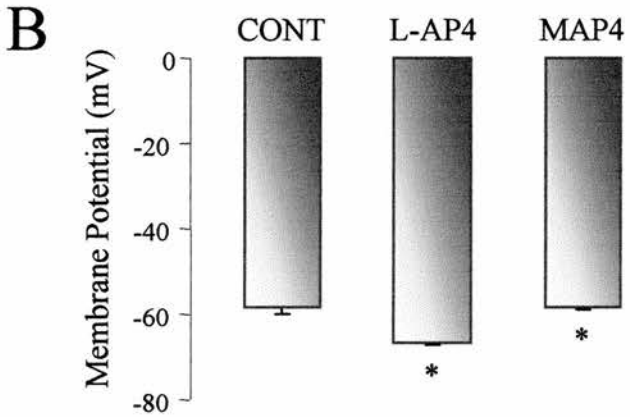
5 | 3 | 7 Group III receptor effects on motorneuron membrane properties

In 25% of the neurons exposed to 50μM L-AP4, a significant hyperpolarisation of the membrane potential was observed (from -58.56±0.78mV in control saline, to -66.46±0.48mV; O-A, $F_{2,33}$=65.85, P<0.05, n=2 out of 8; Figure 5.15A-B), that could

Figure 5.15 | L-AP4-induced hyperpolarisation of the membrane potential

(A-B) Application of 50μM L-AP4 revealed a significant hyperpolarisation of the resting membrane potential in 2 cells (from an average of –58.56±0.78mV in control saline, to –66.46±0.48mV under L-AP4; O-A, $P<0.001$, n=2). This effect occurred in ~2-3mins following bath application of L-AP4, and this reversed with application of the group III antagonist MAP4 (100μM; to –58.12±0.41mV; O-A, $P<0.001$, n=2). Solid arrows represent drug application. * = significant, ns = not significant. Values are means ± S.E.M.


Ai
Aii
+ 50μM L-AP4
+ 100μM MAP4
10mV
1s

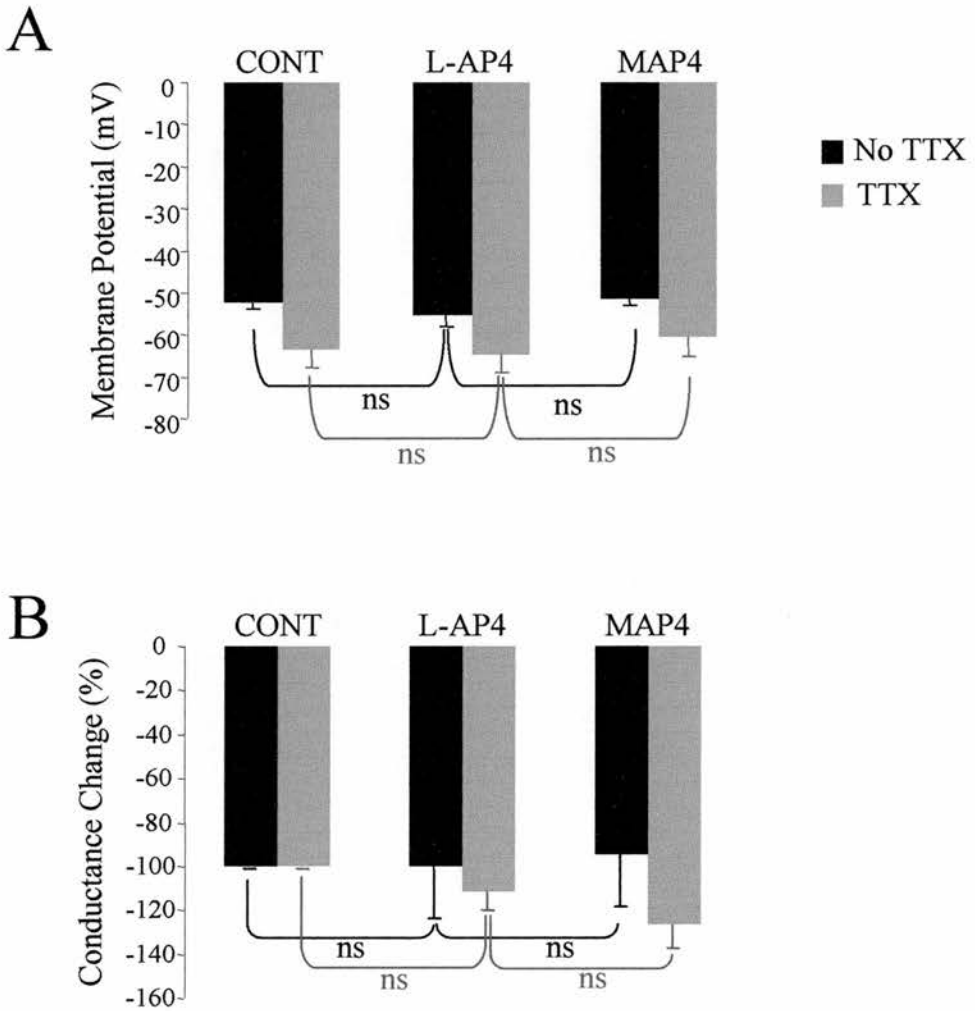

B
CONT
L-AP4
MAP4
Membrane Potential (mV)
0
-20
-40
-60
-80
*
*

be reversed by the antagonist, MAP4 (100μM; to –58.12±0.41mV; O-A, $F_{2,33}$=65.85, P<0.05, n=2; Figure 5.15A-B). In a further 25% of these cells there was no change in membrane potential after application of L-AP4 (from -52.32±1.62mV in control saline, to -55.48±2.37mV; O-A, P>0.05, n=2 out of 8; Figure 5.16A), which remained unaltered with the antagonist (at -51.5±1.77mV; O-A, P>0.05, n=2; Figure 5.16A). However, in the remaining 4 cells treated with L-AP4, the membrane potential was insufficiently stable throughout the recordings to permit accurate measurements and have consequently been removed from this analysis. Further experiments conducted in the presence of TTX (1μM) showed that L-AP4 produced a small but statistically insignificant hyperpolarising effect on membrane potential (-63.51±4.2mV in control saline, to -64.71±4.45mV with L-AP4; O-A, P>0.05, n=7 out of 8; Figure 5.16A). Subsequent MAP4 application in the presence of TTX also produced no change in membrane potential (to -60.78±4.45mV; O-A, P>0.05, n=6 out of 8; Figure 5.16A).

An increase in mean sIPSP amplitude with L-AP4 application in 2 experiments was observed previously (see figures 5.11B and 5.12B), which I interpreted as indicative of a post-synaptic event induced by the activation of group III mGluRs. The increase in the mean sIPSP amplitude accompanied a membrane hyperpolarisation in these 2 experiments, which presumably takes the membrane potential further away from the chloride equilibrium potential. Interestingly, in a further 3 experiments involving agonist application in the presence of TTX, the mean sIPSP amplitude similarly increased significantly (see figures 5.13B and 5.14B), but in these cells there was only a small and insignificant corresponding membrane potential hyperpolarisation. Nevertheless, these data suggest that L-AP4 can, in some neurons induce a

Figure 5.16 | Effects of group III mGluR activation on the resting membrane potential

(A) In the remaining 6 out of 8 cells, where no detectable hyperpolarisation was observed, only 2 cells could be accurately measured and showed no significant change in resting membrane potential (from an average of –52.32±1.62mV in control saline, to –55.48±2.37mV under L-AP4; O-A, $P>0.05$, n=2). Application of the antagonist, MAP4, similarly did not alter the membrane potential. In experiments repeated in the presence of TTX, L-AP4 application showed no significant effect on resting membrane potential (O-A, $P>0.05$, n=7 out of 8). (B) There is no alteration in conductance observed following L-AP4 application in all 8 cells including those that hyperpolarised (O-A, $P>0.05$, n=8). Similarly, in the presence of TTX, there was no significant change in conductance following L-AP4 application (O-A, $P>0.05$, n=7 out of 8). Subsequent application of MAP4, in the presence of TTX, produced no change in resting membrane potential or conductance (O-A, $P>0.05$, n=6 out of 8). * = significant, ns = not significant. Values are means ± S.E.M.


A
CONT
L-AP4
MAP4
Membrane Potential (mV)
0
-10
-20
-30
-40
-50
-60
-70
-80
ns
ns
ns
ns
No TTX
TTX
B
CONT
L-AP4
MAP4
Conductance Change (%)
0
-20
-40
-60
-80
-100
-120
-140
-160
ns
ns
ns
ns

hyperpolarisation of the membrane potential which may be one mechanism utilised by group III mGluRs to reduce swimming frequency.

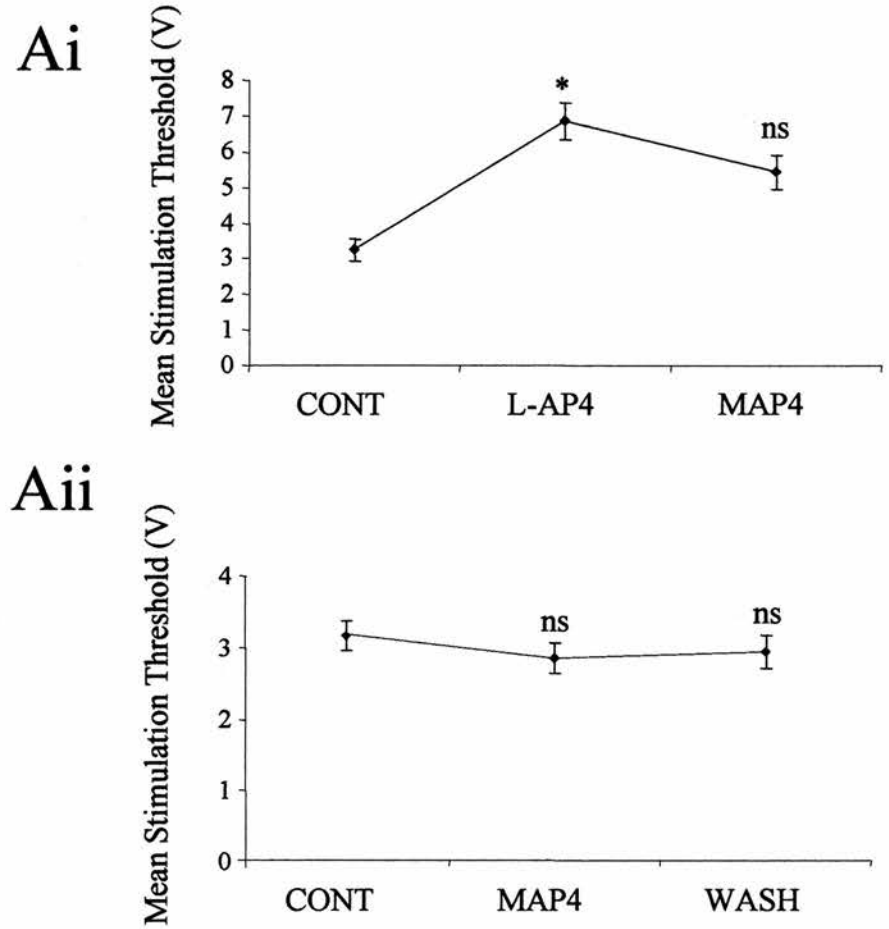
Hyperpolarising current pulses were also applied before, during and after L-AP4 application (see chapter 2: Methods and Materials) to assess any changes in ionic conductance within the motorneuron. In all 8 cells, including those that hyperpolarised, there was no obvious change in conductance with either L-AP4 application (~99.85±24.1% of control conductance; O-A, $P>0.05$, n=8; Figure 5.16B) or MAP4 application (~94.5±23.7% of control conductance; O-A, $P>0.05$, n=8; Figure 5.16B). In addition, there was no change in conductance with either L-AP4 or MAP4 application in the presence of TTX (~111.45±8.68% of control conductance; O-A, $P>0.05$, n=8; Figure 5.16B).

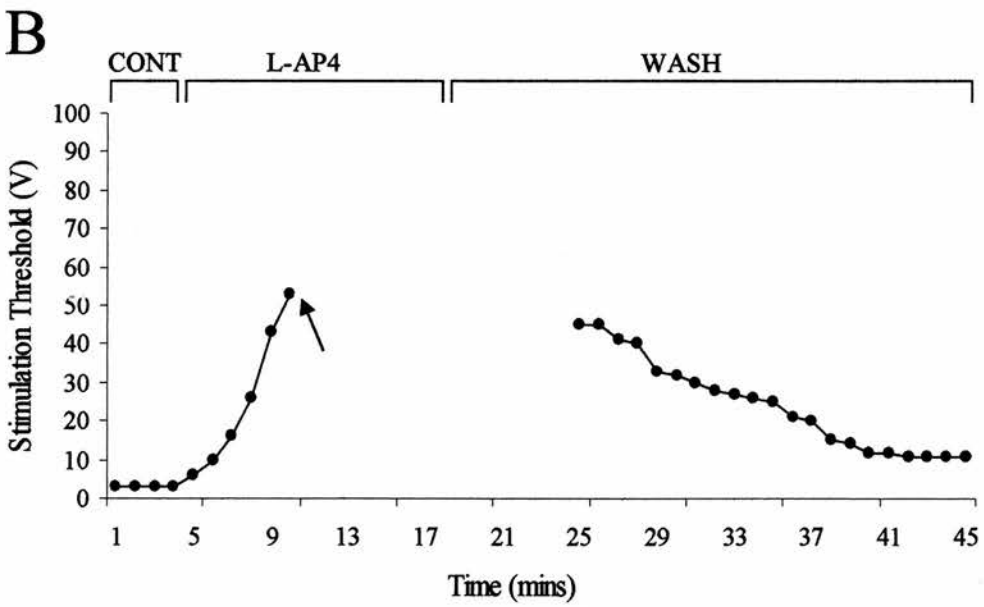
5 | 3 | 8 Group III receptor effects on sensory transmission?

In addition to the inhibitory effects of group III receptor activation on the motor network, L-AP4 application also increased the threshold voltage required to initiate swimming via stimulation of the trunk skin (from a mean of 3.24±0.3V in control saline, to 6.85±0.53V under 50μM L-AP4; O-A, $F_{2,81}=16.15$, $P<0.001$, n=12; Figure 5.17Ai). Since the Rohon-Beard skin sensory pathway involved in the activation of motor activity is known to involve glutamatergic neurotransmission (see chapter 1; Sillar and Roberts, 1988; Roberts and Sillar, 1990), increases in the threshold voltage required to initiate swimming may indicate that L-AP4 is affecting these glutamatergic synapses. MAP4 was unable to reverse the effects of the agonist on threshold voltage (100μM; O-A, $P>0.05$, n=12; Figure 5.17Ai) but it did not alter threshold voltage when applied alone (O-A, $P>0.05$, n=12; Figure 5.17Aii) suggesting that these receptors may not be endogenously active at these synapses. Similarly,

Figure 5.17 | Stimulus threshold to initiate swimming increases with group III receptor activation

(Ai) Activation of group III mGluRs with 50μM L-AP4 caused the skin stimulation voltage required to initiate swimming to increase (O-A, $P<0.001$, $n=7$), an effect which did not reverse with application of the general group III receptor antagonist MAP4 (O-A, $P>0.05$, $n=7$). MAP4 applied alone had no effect on the threshold voltage required to initiate swimming (Aii; O-A, $P>0.05$, $n=5$). However, with the spinal cord exposed for intracellular recordings, the concentration of L-AP4 was reduced, to ~1-6μM (see main text). In the example in (B), where swimming activity was abolished with L-AP4, the voltage required to initiate swimming increased rapidly until swimming ceased (indicated by arrow), and gradually returned shortly after returning to fresh saline. * = significant, ns = not significant. Values are means ± S.E.M.


Ai
Mean Stimulation Threshold (V)
8
7
6
5
4
3
2
1
0
*
ns
CONT
L-AP4
MAP4
Aii
Mean Stimulation Threshold (V)
4
3
2
1
0
ns
ns
CONT
MAP4
WASH

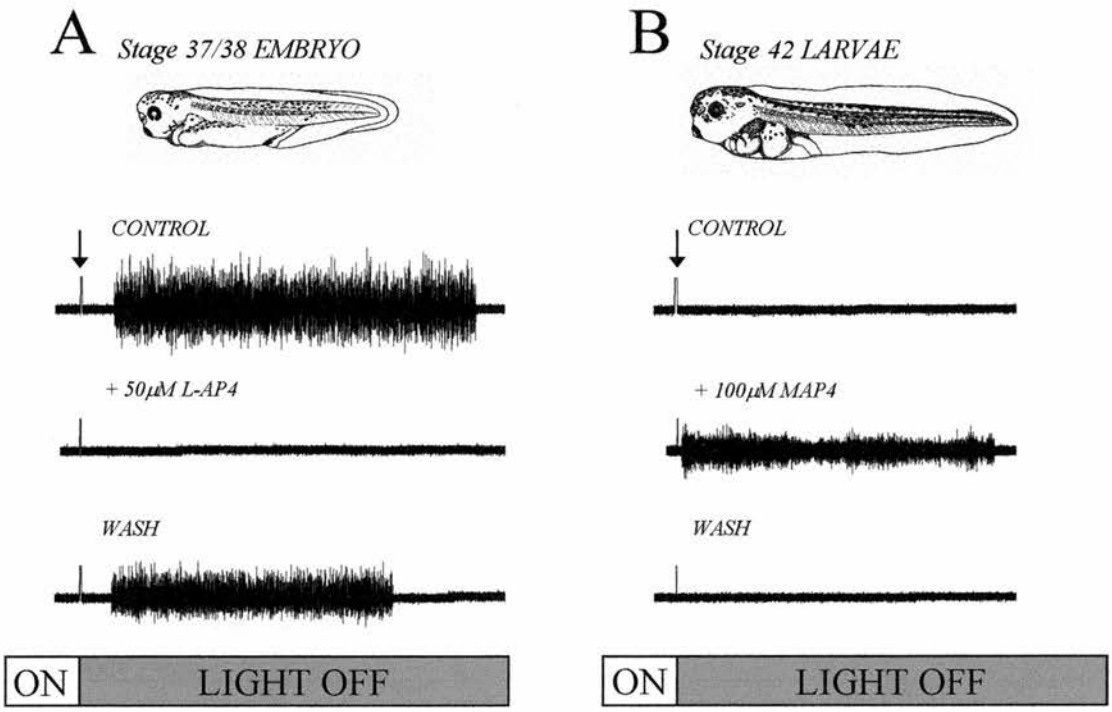

B
CONT
L-AP4
WASH
Stimulation Threshold (V)
100
90
80
70
60
50
40
30
20
10
0
1
5
9
13
17
21
25
29
33
37
41
45
Time (mins)

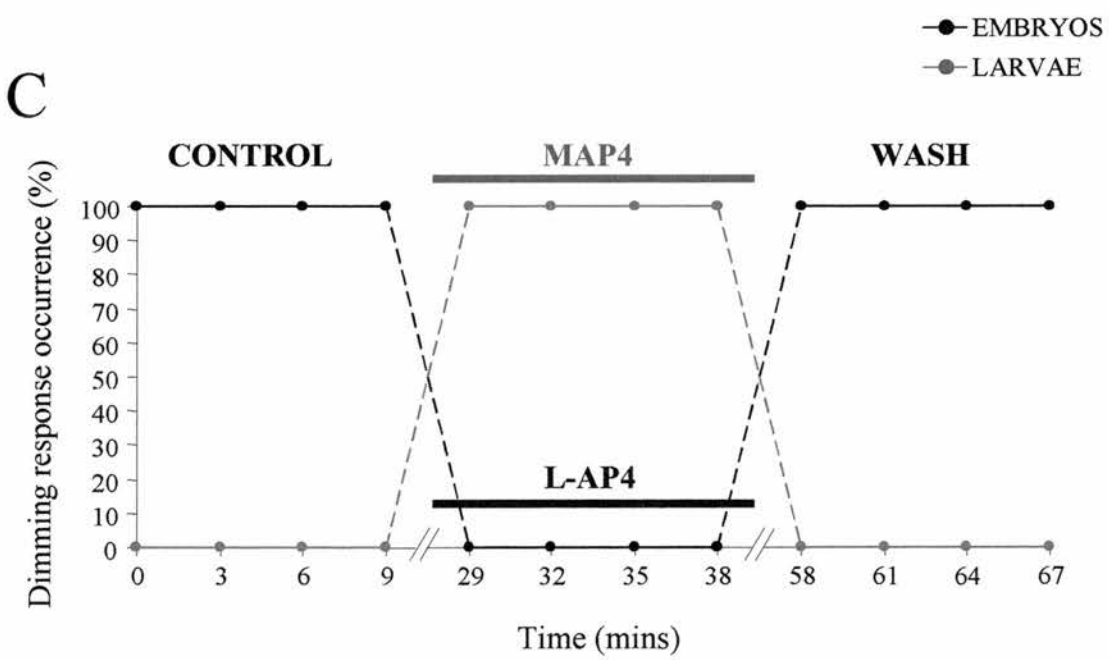
during applications of L-AP4 when the spinal cord is more exposed for intracellular recordings, much lower concentrations of L-AP4 (~1-6μM) similarly affected stimulation threshold, with very rapid results. Figure 5.15B shows that within ~5-6mins of 1μM L-AP4 application onto an exposed spinal cord, the threshold required to initiate swimming increases rapidly until swimming could not be initiated. This effect was gradually restored by washout over about 20mins. Nevertheless, this finding suggests that L-AP4 depresses the skin sensory pathways in tandem with an inhibitory effect on the motor network.

I also observed developmental differences in group III receptor-mediated effects on the dimming response, which involves a glutamatergic descending pathway from the pineal gland to initiate swimming (see chapter 1; Roberts, 1978; Foster and Roberts, 1982; Jamieson, 1997). In 5 embryos, where the dimming response was shown to occur reliably, application of 50μM L-AP4 abolished the dimming response, in much the same way L-AP4 inhibits the skin stimulation pathway (Figure 5.18A). Washout could reverse the effects of L-AP4 on the dimming response in these instances (Figure 5.18A). Curiously, however, in 4 out of 6 larvae, where the dimming response was unreliable, applications of the group III receptor antagonist MAP4 restored the dimming response such that it could now reliably evoke swimming (Figure 5.18B). Washout caused an abolition of the dimming response, reversing the effects of MAP4 (Figure 5.18B). The graph in figure 5.18C shows the effect of either activating or blocking group III receptors on the dimming response in embryos and larvae, whether the response occurred or failed. These findings suggest that the sensory pathways involved in this response are still present in larvae and are either 'masked' or inhibited by activation of group III receptors, such that MAP4 is able to disinhibit the pathway in these stage 42 larval animals.

Figure 5.18 | L-AP4 abolishes the dimming response in embryos whilst MAP4 restores the dimming response in larvae

Swimming can be initiated in stage 37/38 *Xenopus* embryos by dimming the illumination, a pathway that becomes less reliable in larvae. (A) Dimming the illumination (arrows) in embryos in the presence of L-AP4 cannot elicit swimming. This effect can be washed off and the dimming response returns. (B) In stage 42 larvae where the dimming response does not occur reliably anymore, application of the group III antagonist MAP4 restores the response to a change in illumination which now elicits swimming. This effect can also be washed off and subsequently no swimming occurs when the lights are turned off. (C) Graph showing percentage occurrence of dimming response after application of L-AP4 in embryos and MAP4 in larvae. Measurements were taken 3mins apart under each condition. Drugs are washed on for 20mins (represented by break in the graph axis and dotted lines) before measurements are taken.


A
Stage 37/38 EMBRYO
CONTROL
+ 50μM L-AP4
WASH
ON
LIGHT OFF
B
Stage 42 LARVAE
CONTROL
+ 100μM MAP4
WASH
ON
LIGHT OFF


C
EMBRYOS
LARVAE
CONTROL
MAP4
WASH
L-AP4
Dimming response occurrence (%)
100
90
80
70
60
50
40
30
20
10
0
0
3
6
9
29
32
35
38
58
61
64
67
Time (mins)

5 | 4 DISCUSSION

This aim of this chapter was to establish whether group III mGluRs play any role in the modulation of swimming in *Xenopus* tadpoles using a pharmacological approach combined with extracellular and intracellular recordings. Applications of the group III specific agonist, L-AP4, caused significant changes to many parameters of swimming. This group produces a very strong inhibitory effect on the swim network, causing a profound increase in cycle periods and often arresting activity altogether (Figures 5.1-5.3). Cycle periods and R-C delays are known to be positively correlated in stage 42 *Xenopus* larvae, creating a phase-lag along the body to maintain undulatory body movements during fictive swimming (Tunstall and Sillar, 1993) and, presumably as a result, R-C delays in the presence of L-AP4 increased in tandem with the increased cycle periods (Figures 5.1 and 5.2). In these larval animals, L-AP4 also significantly decreased ventral root burst durations and amplitudes, whilst in stage 37/38 embryos only burst amplitudes decreased (Figures 5.1 and 5.2). It should be noted that burst durations in larvae are much longer than in embryos (Sillar *et al*, 1991); in the latter, ventral root bursts reflect the synchronous firing of single spikes per motorneuron in each cycle so that there is little or no scope for any reduction under L-AP4.

Not all of these changes induced by L-AP4 could be significantly reversed following application of the group III antagonist MAP4. For example, the effects of L-AP4 on cycle periods, burst amplitudes and durations were not significantly reversed, although the trend under MAP4 was in the expected direction. In keeping, R-C delays partially but significantly reduced with MAP4 and in cases where L-AP4 completely abolished activity (both to skin stimulation and to light dimming), MAP4 was able to restore episodes of swimming. Moreover, when MAP4 was applied on its own, a

significant decrease in cycle periods occurred providing evidence for activation of this class of receptor by endogenously released glutamate (Figure 5.4). The reasons why MAP4 proved less able to reverse L-AP4 effects are not clear. It is possible that the concentrations of L-AP4 used in this study were able to saturate all group III receptors, producing a maximum effect on the network. This might go some way to explaining why MAP4 was not always able to reverse the effects induced by L-AP4, but could influence the network alone. For example, MAP4 may be able to displace glutamate from the receptor binding site more readily than L-AP4. Alternatively, as group III mGluRs comprise four subtypes ($mGluR_{4,6\text{-}8}$), it is possible that L-AP4 and MAP4 have differing affinities for the different subtypes. MAP4 is more selective for $mGluR_4$ over the other subtypes (Schoepp *et al*, 1999). Therefore, most effects on *Xenopus* network activity through group III receptor activation by L-AP4 may be a result of $mGluR_{6,7,8}$.

At the level of the network output, the overall inhibitory effects induced by L-AP4 are strikingly similar to those produced by group II receptor activation (see chapter 4), although I have obtained evidence that the two groups may mediate these effects through divergent mechanisms. In contrast to group II receptors, for example, group III receptor activation also significantly increased the threshold voltage required to initiate swimming following electrical stimulation of the skin (Figure 5.14), suggesting that L-AP4 affects the skin sensory pathways as well as the motor network. In further contrast to group II receptors, L-AP4 impaired the dimming response, an effect which could be reversed by MAP4. These data suggest that group III receptors have a different distribution to group II receptors.

The effects of group III receptor activation on swim frequency are similar not only to group II mGluRs, they are also comparable at the network level to the inhibitory effects produced by the neuromodulators NA and nitric oxide (NO). As mentioned previously, these two neuromodulators exert their effects, in part, through the facilitation of glycinergic transmission (to strengthen mid-cycle inhibition and increase cycle periods), and via facilitation of GABAergic transmission (to cause swimming to prematurely terminate with a barrage of $GABA_A$-receptor mediated IPSPs) (McDearmid *et al*, 1997; Fischer *et al*, 2001; McLean, 2001; Merrywest *et al.*, 2002). I have shown in chapter 3 that activation of group I mGluRs causes a depression of glycinergic transmission as a mechanism to increase network excitability. It was plausible, therefore, that group III mGluRs could similarly facilitate the fast inhibitory pathways as a means to exert their net inhibitory effects on the network. However, I found no evidence during initial extracellular experiments to support the idea that group III receptors are modulating GABA transmission to influence episode durations (Figure 5.6). Given that L-AP4 seems to influence episode durations in an "all-or-nothing" manner, it seems unlikely that alterations in GABA transmission alone could induce such a profound effect. For example, in a previous study, both *depressing* GABAergic transmission with 50μM bicuculline application or *facilitating* $GABA_A$ receptor function with the steroid 5β3α (1-3μM) significantly reduced episode durations in young *Xenopus* tadpoles, but a complete loss of swimming activity was never observed (Reith and Sillar, 1999).

The data presented in this chapter showed that pre-application of the glycine receptor antagonist, strychnine, seemed to occlude the L-AP4-induced lengthening of cycle periods implying that group III receptors may modulate glycinergic transmission as a mechanism to slow swimming frequency (Figure 5.5), so to test this possibility

further, intracellular recordings were made. Examination of evoked mid-cycle glycinergic inhibition during swimming proved problematic because the concentrations of L-AP4 used in extracellular experiments (50μM) caused all activity to cease shortly after application of agonist. This was presumably due to the surgical exposure of the spinal cord for intracellular recordings and the resulting improvement in drug access. Lower concentrations of L-AP4 permitted activity, but the resulting swimming episodes often comprised only a small number of cycles. Interestingly, the ventral root recording would appear to vanish whilst rhythmic synaptic drive at swimming frequency could still be observed intracellularly. Retrospectively, it is possible that some swimming activity did occur under L-AP4 during those extracellular experiments where swimming was 'abolished', but the weakened synaptic drive fell below spike threshold for motorneurons. It is also possible that caudal motorneurons are more susceptible to the agonist and drop-out of the network before more rostral neurons (the extracellular electrode is placed caudal to the intracellular one; see figure 2.1).

In the 3 experiments where swimming activity could be measured intracellularly, the extremely shortened episode durations induced by 1-6μM L-AP4 created difficulties in the accurate quantification of mid-cycle IPSP amplitude (Figure 5.7). In spite of this, it is clear upon closer examination of IPSPs towards the beginning and end of an episode that the maximum IPSP amplitude is comparable under each condition (Figure 5.8). More notably, the mid-cycle IPSP did not increase in amplitude, as would be expected if activation of group III mGluRs facilitated glycinergic transmission. It should also be noted that at the end of the swimming episode no barrage of GABA IPSPs was observed with application of L-AP4, which is in contrast to the effects of both NA and NO (McLean and Sillar, 2002; Merrywest *et al*, 2002).

At this concentration of agonist, there was no alteration in the frequency of spontaneously occurring IPSPs during inter-episode quiescent periods, which would support the above lack of effect on inhibitory transmission. However, experiments conducted with the higher concentration of L-AP4 (50μM), as used during extracellular recordings, indicated that activation of group III receptors can induce a *reduction* in the rate of spontaneous glycinergic and GABAergic IPSPs (Figures 5.9 and 5.10). This reduction in the probability of inhibitory transmitter release is paradoxical given the observed net inhibition on both the motor network and skin sensory pathways because one might expect an increase in swim frequency with a decrease in glycinergic transmission, as was observed following group I receptor activation (see chapter 3). The alternative possibility is that group III receptors cause a global depression of both inhibitory *and* excitatory synaptic transmission (see below).

Other possible mechanisms by which group III receptors mediate their depression on the motor network and sensory pathways must therefore be considered. One possible target is the integrative electrical properties of the constituent neurons of the motor network, and in 50% of stable recordings of motorneurons there was a significant and reversible hyperpolarisation of the membrane potential (Figure 5.15), which could potentially explain the depression on swimming activity. Simultaneously in these neurons, the mean sIPSP amplitudes increased with L-AP4, as would be predicted by the membrane potential being taken further from the chloride equilibrium potential (Figures 5.12). Moreover, these data suggest that at least in some neurons group III receptors are located both pre- and post-synaptically. Any hyperpolarisation will take the membrane potential further away from spike threshold and hence reduce excitability. This could cause failure of impulses in pre-motor interneurons and

motorneurons and hence a reduced synaptic drive during swimming leading in turn to a decrease in both swimming frequency and duration. This in turn would lead to a rapid decline in rhythm generation capability (see below). My evidence indicates that the L-AP4-induced hyperpolarisation is only exhibited by a subset of spinal neurons, which may in part explain why it was not observed consistently and moreover, why it was not observed in those cells pre-treated with TTX (Figure 5.16). No change in conductance could be detected during L-AP4 applications, whether or not there was a change in resting membrane potential, suggesting that L-AP4 does not directly affect an ion channel to mediate its cellular or synaptic effects.

Post-synaptic responses to group III receptor activation, such as the hyperpolarisation described here are rare. One exception is in the retina, where it has been documented that retinal ON bipolar cells are hyperpolarised by the activation of group III mGluRs, specifically the $mGluR_6$ subtype (Thoreson and Miller, 1994). More recently, an L-AP4 induced post-synaptic hyperpolarisation has also been reported in the song-control nucleus HVc of the zebra finch (Dutar *et al*, 1999), which is part of the primary motor pathway for song generation. This response was found to be mediated by activation of a G-protein coupled, inwardly rectifying K^+ (GIRK) channel (Dutar *et al*, 1999).

In this study the effects of group III receptor activation on only passive membrane properties have been monitored. Clearly, effects on the active membrane properties have not been addressed, and may represent an area of fruitful study in the future. Adjustments to ionic currents mediated through voltage-dependent channels can significantly alter output at the network level. For instance, numerous K^+ currents in *Xenopus* spinal neurons can be modulated to induce excitatory effects on the

swimming rhythm (Dale and Kuenzi, 1997). Group III receptor activation in the lamprey locomotor network causes a significant reduction of locomotor frequency, mediated by the activation of K^+ conductances on pre-synaptic membranes and causing an inhibition of synaptic transmission (Krieger *et al*, 1996, 1998; Cochilla and Alford, 1998). A reduction of Ca^{2+} currents can cause a reduction in synaptic transmission, making it less likely that neurons will reach the threshold for firing. For example, the neuromodulator adenosine slows swimming in *Xenopus* by reducing voltage-gated Ca^{2+} currents without affecting membrane potential, input resistance, or the excitatory or inhibitory component of the synaptic drive for swimming (Dale and Gilday, 1996). The effects on the motor network following application of L-AP4 are similar to the effects produced by adenosine, and indeed, group II mGluR activation (see chapter 4). L-AP4 has been shown to be effective in inhibiting Ca^{2+} currents in many cell types (olfactory bulb (Trombley and Westbrook, 1992); hippocampal neurons (Swartz and Bean, 1992; Sahara and Westbrook, 1993)), ultimately leading to a reduction in glutamate release. Group III receptor-mediated blocking of voltage-dependent Ca^{2+} currents could therefore be another potential mechanism by which this class of receptor causes a slowing of swimming frequency in *Xenopus*.

There is a growing body of evidence for group III mGluR-mediated inhibition of excitatory transmission and this can occur through a range of mechanisms. A reduction in excitatory transmission could explain why the *Xenopus* swimming network was unable to sustain rhythmic activity for long periods of time in the presence of L-AP4. Interneuronal drop-out, alluded to above, has been proposed as a mechanism for the gradual reduction in frequency and eventual termination of swimming implying that a critical number of active neurons is required for the maintenance of the rhythm. (Sillar and Roberts, 1993). Any reduction in excitatory

transmission will reduce the likelihood of threshold being reached causing neurons within the motor network to drop-out more quickly and hence, prematurely terminating swimming. In keeping with this notion, albeit indirectly, burst durations and amplitudes were significantly reduced following L-AP4 application, suggesting a reduction in the number of motorneurons reaching threshold and contributing to network excitability.

A reduction in excitatory neurotransmission would also help to explain the effects of group III receptor activation on the skin sensory pathway in which the threshold voltage required to initiate swimming increases under L-AP4 (Figure 5.17). This pathway involves activation of R-B primary sensory neurons, which excite dorsolateral sensory interneurons via the activation of iGluRs (Clarke and Roberts, 1984; Sillar and Roberts, 1988; Roberts and Sillar, 1990). It can be postulated that group III receptors act as negative feedback autoreceptors to regulate primary afferent transmission. In support, group III receptor-mediated inhibitory effects on the equivalent pathway have also been documented in the lamprey, where L-AP4 induced a depression of monosynaptic dorsal-cell evoked EPSPs in giant interneurons (Kreiger and El Manira, 2002). Interestingly, there is no evidence in either species that group II receptors are positioned in the skin sensory pathway. It is possible that the distribution of group II mGluRs is confined to the motor network, whilst group III receptors are distributed more widely and are also expressed in the membranes of sensory pathway neurons.

The dimming response in *Xenopus* (Roberts, 1978; Foster and Roberts, 1982; Jamieson, 1997), which involves a glutamatergic descending pathway from the pineal gland to initiate swimming, was also inhibited following the activation of group III

receptors (Figure 5.18). It is interesting from a developmental perspective that the dimming response was abolished following L-AP4 applications in both embryos and in the few larvae where the response was present. However, in larvae, the dimming response becomes much less reliable, and in such preparations, application of the group III receptor antagonist MAP4 was able to restore the response in most cases (Figure 5.18). It is possible that by stage 42 of larval development, group III receptors become tonically activated to inhibit the response to changes in illumination. If so, then an endogenous mechanism for setting the sensitivity of this sensory pathway is engaged by glutamate release in post-embryonic tadpoles. Group III mGluRs are known to play a role in the visual pathways of other vertebrates (Sheills and Falk, 1992; Nawy, 1999; Hirasawa *et al*, 2002), but to the best of my knowledge, my data represent the first demonstration of an endogenous role for mGluRs in the pineal pathway.

Perhaps the most likely explanation for the group III receptor-mediated effects on both the motor network and sensory pathways that initiate swimming, is their involvement as negative feedback glutamatergic autoreceptors located on the terminals of excitatory interneurons. There is precedent for group III mGluRs functioning as autoreceptors that are activated under certain physiological conditions (for reviews; Anwyl, 1999; Cartmell and Schoepp, 2000). A reduction of glutamatergic inputs onto the inhibitory interneurons in *Xenopus* would also explain why a reduction in inhibitory transmission is observed. It will be important in future to test this hypothesis, for example, through analysis of excitatory interneuron to motorneuron transmission using paired patch recordings (Li *et al*, 2001; Li *et al*, 2003). This will allow direct quantification of any L-AP4 induced effects on both

evoked glutamatergic EPSPs and sEPSPs, which cannot be detected during sharp microelectrode recordings, as used in this study.

From the results presented in this chapter, it is clear that activation of group III mGluRs exerts a profound inhibitory effect on both motor and sensory pathways in *Xenopus* tadpoles. Whilst I have not been able to identify the exact mechanism(s) that this group utilises to exert its inhibitory effect on the network, I have been able to effectively rule out the facilitation of inhibitory transmission. The effects of group II and III receptors at the network level are strikingly similar in that both significantly reduce swimming frequency. However, differences do exist in that whilst group II receptors reduce episode durations, group III receptors have a much more potent effect in abolishing activity altogether. At the cellular level I have presented evidence that activation of group III receptors can lead to membrane potential hyperpolarisation and, at higher concentrations modulate the quantal release of inhibitory transmitters. In these respects group III receptors differ markedly from group II receptors.

6 General Discussion: The involvement of metabotropic glutamate receptors in locomotor pattern generation

Tadpoles of the South African clawed toad, *Xenopus laevis*, are an extremely attractive and well established model system in which to study the neural networks underlying locomotion. At the time of hatching *Xenopus* tadpoles possess a relatively simple nervous system which comprises only eight classes of differentiated spinal neuron (Roberts and Clarke, 1982) and produces a distinctive swimming pattern that quickly matures soon after hatching (see chapter 1; Sillar *et al*, 1991). The rhythmic motor pattern underlying swimming can be initiated 'fictively' in intact immobilised animals by sensory stimulation and studied easily using electrophysiological recording methods. Once initiated, motor activity is self-sustaining without the need for exogenously applied pharmacological agents, providing this model system with an important advantage over those in which NMDA and/or 5-HT must be added to the bathing solution to trigger activity (e.g. lamprey, neonatal rat).

The CPG for swimming in *Xenopus* tadpoles can be considered as two locomotor half-centres on the left and right sides of the spinal cord. The synaptic drive underlying swimming results from a combination of descending ipsilateral excitation together with reciprocal mid-cycle inhibition which couples the two half-centres in antiphase (Roberts, 1989). Spinal excitation originates from three sources (see chapter 1): glutamatergic interneurons (Dale and Roberts, 1984); cholinergic connections from motorneurons (Perrins and Roberts, 1995a, 1995b); and electrotonic

coupling between motorneurons (Perrins and Roberts, 1995a). Of primary interest in this thesis is the neurotransmitter, glutamate. This ubiquitous excitatory amino acid generates a significant proportion, about 30%, of the excitation required for locomotor activity when released from the glutamatergic pre-motor interneurons onto the motor network in *Xenopus* (Dale and Roberts, 1984, 1985; Zhao and Roberts, 1998; Li *et al*, 2001).

It is the actions of glutamate at two ionotropic glutamate receptors, NMDA and non-NMDA, that contributes, respectively (Dale and Roberts, 1985), to the tonic and phasic excitatory synaptic components underlying the drive for swimming. However, in addition to these iGluRs, there is another class of glutamate receptors that has not been characterised in *Xenopus* until now, and it is the investigation of these metabotropic receptors that forms the focus of this thesis. MGluR-mediated modulation of locomotor pattern generation has been documented in only a handful of other vertebrates (eg. lamprey (for a review: El Manira *et al*, 2002); mouse (O'Neill *et al*, 2003); and neonatal rat (Marchetti *et al*, 2003; Taccola *et al*, 2003, 2004a, 2004b), whilst other studies have examined the roles of mGluR activation in isolated spinal neurons (e.g. turtle (Delgado-Lezama *et al*, 1997; Russo *et al*, 1997; Svirskis and Hounsgaard, 1998); tortoise (Kozhanov *et al*, 2001); *Rana pipiens* (Holohean *et al*, 1999)). However, this is the first time that mGluR-mediated effects on the motor network have been shown in hatchling *Xenopus* tadpoles. It was of considerable interest therefore to establish whether the effects on a self-sustaining CPG output were similar to effects described in other model systems and to explore the extent to which mGluRs are activated by endogenously-released glutamate during 'normal' swimming.

As glutamate is such a ubiquitous neurotransmitter within the CNS it was necessary to use specific pharmacological agonists to activate specific mGluR groups. Group I receptor activation with the general agonist, DHPG, significantly increased both swimming frequency and the number of spontaneously occurring swimming episodes. These findings broadly parallel what has been found in the lamprey, where group I receptor activation increases both the frequency of the locomotor rhythm and motorneuron excitability (Krieger *et al*, 1998, 2000). In contrast to the effects induced by group I receptor activation in *Xenopus*, applications of the group II and III agonists, APDC and L-AP4 respectively, caused a net inhibitory effect on swimming frequency. Group II and group III receptor activation also reduced episode durations. Group III mGluRs severely affected swimming episode durations in an "all-or-nothing" manner, to the extent that L-AP4 often completely abolished activity. In addition, but in contrast to group II mGluRs, group III receptors also inhibited the sensory pathways involved in the initiation of swimming. The effects of group II and III receptor activation at the network level can be directly compared to the group II and III mGluR-mediated depression of motor activity in the lamprey (Krieger *et al*, 1996), neonatal rat (Taccola *et al*, 2004b) and for group II only, mouse (O'Neill *et al*, 2003). Therefore, my work has provided evidence for the functional presence of all three groups of mGluRs in the *Xenopus* swimming system.

Moreover, applications of group specific antagonists have provided evidence that these receptors can be activated endogenously during *Xenopus* swimming. Group I mGluRs consist of two subtypes, $mGluR_1$ and R_5, and applications of subtype specific antagonists revealed that a blockade of either of these receptor subtypes slowed swimming frequency suggesting some endogenous activation of the two receptor subtypes (Figure 3.18). Interestingly, blocking each subtype individually produced a

similar decrease in swim frequency, in contrast to the effects observed in lamprey, where $mGluR_1$ activation increases burst frequency, whilst $mGluR_5$ activation causes a reduction (Krieger *et al*, 2000, 2002). The reason why both group I receptor subtypes produced the same effect in the *Xenopus* spinal network cannot be determined at this stage, but it is not entirely impossible that their expression may be developmentally regulated. By comparison, blockade of group II and III receptors with their respective group antagonists, EGLU and MAP4, increased swimming frequency in *Xenopus*, again implying that these two groups are activated endogenously, presumably as a result of glutamate released when the network is cycling. However, it should be noted that previous studies have proposed that this endogenous activation by glutamate may only occur during periods of high activity or pathological conditions when this transmitter is in excess. For example, there are many pharmacological studies reporting that group II compounds are only active in animal models under situations of anxiety, ischemia and psychosis (for a review: Schoepp *et al*, 1999). This seems consistent with studies showing group II and III receptors are located perisynaptically (Petralia *et al*, 1996; Shigemoto *et al*, 1997; Cartmell and Schoepp, 2000), and are activated when glutamate 'spills over' from the synaptic cleft.

One mechanism contributing to the group I receptor-mediated increase in swimming frequency in *Xenopus* was found to be a reduction of inhibitory transmission (Chapter 3). Applications of the group I agonist, DHPG, reduced both glycinergic and GABAergic transmission, as was reflected by a depression in the amplitude of the mid-cycle glycinergic IPSP and in the rate of spontaneously occurring glycinergic and GABAergic IPSPs during quiescent periods between swimming episodes (Figures 3.7 and 3.8). Any reduction in inhibitory inputs would logically lead to a more excitable

motor network permitting faster swimming and increasing the likelihood of spontaneously occurring swimming episodes. In particular, the strength of glycinergic synaptic connections has a dramatic influence on swimming frequency (Dale, 1995; McDearmid *et al*, 1997). Thus the effects of group I mGluRs on mid-cycle inhibition could well explain the increased swimming frequency following their activation.

These findings compliment those recently reported for group I receptor-mediated modulation of the lamprey locomotor network. However, group I mGluRs have been shown to work through multiple mechanisms in the lamprey spinal cord to increase locomotor frequency. Firstly, group I mGluRs interact with NMDA receptors post-synaptically, potentiating NMDA-induced calcium responses and currents leading to an increase in burst frequency (Krieger *et al*, 1998, 2000). In addition, activation of group I receptors reduces a leak K^+ conductance, producing a depolarisation of the membrane potential and increasing network excitability (Kettunen *et al*, 2003). Evidence has recently been provided for a third mechanism, involving the $mGluR_1$-mediated release of endocannabinoids that act retrogradely to cause a depression of glycinergic inhibitory transmission onto motorneurons and crossed-caudal interneurons, causing an increase in network excitability (Kettunen *et al*, 2005).

The evidence for group I receptor-mediated modulation of glycinergic transmission in the lamprey motor network directly compliments the findings of this thesis. Whilst my data do not rule out the possibility that group I receptors in *Xenopus* may simultaneously modulate NMDA receptor function, it is notable that strychnine effectively occludes the effects of DHPG on network output. This indicates that the most important mechanism underlying group I effects involves the direct pre-synaptic

modulation of glycinergic inhibition. Additionally, there may be other, complimentary group I receptor-mediated mechanisms in *Xenopus*, as the evidence presented in chapter 3 suggests parallel pre- and post-synaptic effects may be occurring. This is similar to studies in the lamprey where group I receptors have been shown to act post-synaptically, but release endocannabinoids which function as a retrograde signal to pre-synaptically inhibit glycine release (Kettunen *et al*, 2005). The potential involvement of endocannabinoids needs to be addressed in future work as this provides an elegant mechanism by which group I receptors could modulate inhibitory release post-synaptically in *Xenopus*.

By comparison, I found no evidence in this study that either group II or III mGluRs potentiate inhibitory transmission as a mechanism to reduce the frequency of motor activity. This possibility was worth pursuing given the above evidence presented for group I receptor-mediated modulation of glycinergic transmission in *Xenopus*, and also, given that there is precedent in other studies for group II and III modulation of inhibitory transmission (for a review: Conn and Pin, 1997; Kozhanov *et al*, 2001). Group II receptor activation did not affect inhibitory transmission at all (Figure 4.11), whilst group III receptors actually mediated a *reduction* in both glycine and GABA release (Figure 5.9). Since an increase in network excitability would be expected with a decrease in inhibition, as observed for group I receptors, the effects of group III receptor activation are paradoxical given the very profound inhibitory effect produced on both swimming activity and the sensory pathways that initiate swimming. The possibility exists that group III receptors exert a general inhibitory effect on both excitatory and inhibitory transmission, thus producing a net inhibitory effect on the motor output and distinguishing group III receptor-mediated effects from those produced by group I receptor activation.

Whilst group II and III receptors both cause a net inhibitory effect on motor activity in *Xenopus*, they may utilise very separate cellular mechanisms, such as the group III receptor-mediated reduction in inhibitory transmission which did not occur with group II (Chapters 4 and 5). The group II agonist, APDC, reduced both motor burst durations and amplitudes suggesting modulation of the excitatory drive underlying swimming (Figure 4.1). Therefore, group II mGluR activation may selectively reduce glutamatergic transmission within the motor network to cause an inhibitory effect on motor output while group III simultaneously inhibits all chemical synaptic transmission. Theoretically, even small changes in glutamatergic transmission, for instance, following the activation of group II receptors, could affect the firing of pre-motor interneurons, causing the synaptic drive for swimming to decrease (Sillar and Roberts, 1993). This drop-out of neurons in the network will cause a reduction of swimming frequency, burst durations and amplitudes, and episode durations. It is equally possible that group II receptors could be modulating Ca^{2+} currents, or acting directly on the vesicular release machinery, to act as negative-feedback autoreceptors to reduce glutamatergic transmission (for a review: Anwyl, 1999), but I have no evidence to support this hypothesis at this time.

A group II receptor-mediated modulation of excitatory transmission would tie in with findings in both the lamprey motor network, where group II receptors can regulate synaptic transmission at reticulospinal synapses (Cochilla and Alford, 1998), and in neonatal rat, where group II receptor activation depresses dorsal-root evoked reflexes consistent via a reduction in pre-synaptic transmitter release (Dong and Feldman, 1999; Taccola *et al*, 2004b). The exact mechanisms this group utilises to exert its inhibitory effect on the *Xenopus* motor network requires further investigation. It

proved difficult to quantify effects on the excitatory components of the synaptic drive for swimming using KCl microelectrodes. Further experiments either using KAc as the electrolyte in sharp microelectrode recordings or making paired patch recordings would be useful.

Similar to the effects produced by group II receptor activation, group III receptors reduce swimming frequency when activated with L-AP4 (Figure 5.1). In contrast to group II receptors however, group III mediates a reduction of inhibitory transmission and in a proportion of neurons, induces a prominent hyperpolarisation of the membrane potential (Figures 5.15 and 5.16). Whilst a reduction in inhibition will favour higher excitability causing a likely increase in swimming frequency (c.f. group I receptor activation), a hyperpolarisation of the motorneuron will take the membrane potential further away from spike threshold and hence reduce excitability within the network, potentially over-riding any effect induced by reduced inhibitory transmission. Consequently the synaptic drive for swimming could decrease as impulses fail in pre-motor interneurons and motorneurons, with swimming frequency being compromised as a result. This group III-mediated hyperpolarisation was not consistently observed in all cells recorded suggesting that only a proportion of neurons are affected in this manner. Nevertheless, control of the membrane potential appears to be unique to the activation of this group of mGluRs. Therefore, group III receptors appear to utilise multiple mechanisms to exert their inhibitory effects on the motor network.

As mentioned above, another plausible explanation for the observed effects induced by group III receptors is a reduction in excitatory synaptic transmission, similar to that proposed for group II receptors. Group III mGluR-induced effects are more

pronounced than group II as swimming activity could be completely abolished following L-AP4 application. This could be a consequence of a global reduction in both excitatory and inhibitory synaptic transmission. The effects of group III receptor activation in this study compare and contrast with those produced in the lamprey and neonatal rat locomotor network. In the lamprey, L-AP4 acts pre-synaptically, activating a K^+ conductance and thus reducing synaptic transmission (Krieger *et al*, 1996; Cochilla and Alford, 1998; Krieger *et al*, 1998), whilst in the neonatal rat, L-AP4 strongly depresses synaptic transmission by reducing the cumulative depolarisation induced by repetitive dorsal root stimulation from peripheral inputs (Taccola *et al*, 2004b). However, in these instances the profound reduction in synaptic transmission only led to a moderate reduction in locomotor frequency (El Manira *et al*, 2002; Taccola *et al*, 2004b), which contrasts with the very potent effect of L-AP4 on the *Xenopus* motor network. Perhaps in the lamprey the continuous exposure to NMDA keeps the network in an activated state, whilst in *Xenopus* tadpoles the CPG is cycling normally but is more susceptible to small changes in levels of excitation as a result.

Interestingly, I found that the net inhibitory effect of group III mGluRs on the motor network in *Xenopus* also extended to the sensory pathways involved in the initiation of swimming (Figures 5.17 and 5.18). Thus, the voltage threshold required to initiate swimming quickly increased after the activation of group III receptors. This effect did not occur with group II receptor agonists suggesting that group III receptors are selectively expressed at some stage in the skin sensory pathway. A similar effect on an equivalent sensory pathway has been documented in the lamprey, where L-AP4 induced a depression of monosynaptic dorsal-cell evoked EPSPs in giant interneurons (Kreiger and El Manira, 2002). Certainly, a reduction in excitatory transmission

could potentially be responsible for this observed effect as the skin sensory pathway involved in the initiation of swimming in *Xenopus* utilises glutamate (Sillar and Roberts, 1988; Roberts and Sillar, 1990). In addition, the dimming response in *Xenopus* embryos also initiates swimming and similarly involves glutamatergic transmission (Jamieson, 1997). I discovered that group III receptor activation blocks this response in embryos, but conversely, blocking these receptors restored the dimming response in larvae. This effect is interesting from a developmental perspective as it could be postulated that by stage 42 of development, group III receptors are tonically activated by glutamate to inhibit the dimming response in order to accommodate the larval lifestyle. These receptors are, however, present in embryonic *Xenopus* as agonist applications abolish the dimming response. It is conceivable that the expression and/or location of group III receptors changes developmentally such that they are more likely to be activated by endogenously released glutamate. This effect on the dimming response appears to be unique to this class of mGluR, as neither group I nor group II receptor agents affected this pathway (data not shown).

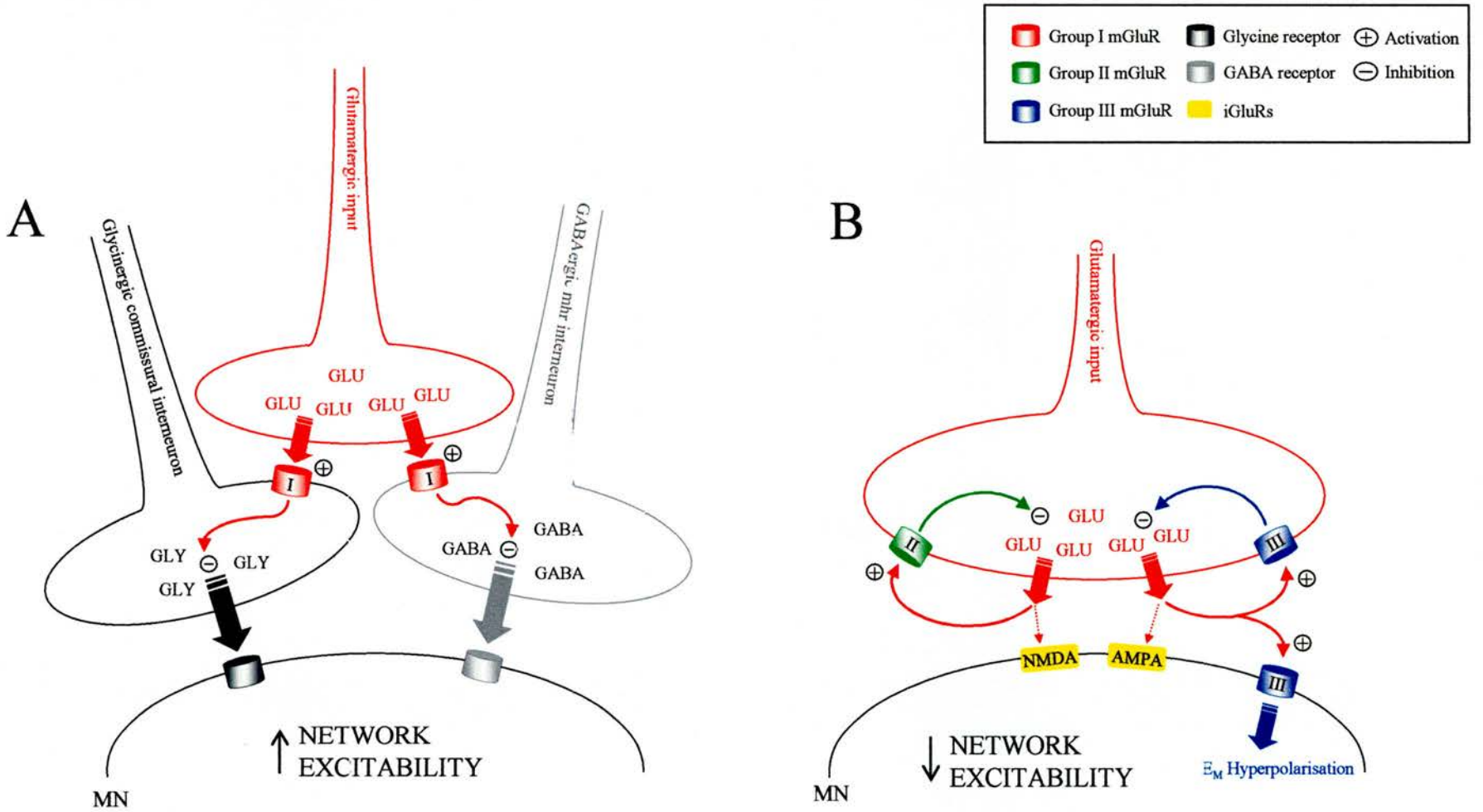
From the results presented in this thesis, it is clear that activation of each group of mGlu receptors exerts a significant modulatory influence on motor activity, which in the case of group III, extends to include inhibition of the sensory pathways normally used to initiate swimming. Precisely how each receptor group functions in relation to one another remains unclear at this time. Furthermore, neither ionotropic nor metabotropic glutamate receptors are likely to be exclusive to this system and it would be naïve to assume that they could not interact with other neurotransmitter systems. For example, it has already been shown in chapter 3 that group I receptors interact with the fast inhibitory pathways. In other studies, there is evidence for

mGluR-mediated modulation of aspartate, 5-HT, Ach, purine, and substance P transmission (for a review: Cartmell and Schoepp, 2000). 5-HT has been well documented as a modulator of the locomotor network in *Xenopus*, causing a reduction in inhibition and producing a more intense motor output (see chapter 1; McDearmid *et al*, 1997). Interestingly, studies in the medial prefrontal cortex have shown co-localisation of group II and 5-HT_{2A} receptors (Marek *et al*, 2000), and antagonists to group II receptors increase 5-HT-induced EPSPs. It could be hypothesised that group II receptor activation modulates 5-HT in the raphe region of the *Xenopus* brainstem to modulate swimming activity. In the same vein, interactions between mGluRs and other well documented neuromodulators of the *Xenopus* motor pattern (e.g. NA and NO) also require consideration. NA and NO interact with the fast inhibitory pathways to mediate, in part, their inhibitory effect on network activity (for a review: Sillar, 2002), therefore, it is conceivable that group I receptors could potentially modulate one of these neuromodulators as a mechanism to reduce inhibitory transmission. If so, glutamate would function simultaneously as a "metamodulator", as well as a modulator, and as a conventional fast chemical transmitter.

In figure 6.1 I have proposed a model of where each group may be located and functioning based on their effects on the output of the network. Immunocytochemical studies labelling different receptors should allow better insight as to the location of the mGluRs. Combined with physiological evidence using antagonists for other neurotransmitter receptors, a complete picture of neuromodulation within the *Xenopus* CNS will emerge. Further physiological experiments will clearly be necessary to clarify more precisely the exact mechanism(s) of the group II and III receptor-mediated inhibition of the motor and sensory pathways, including in particular the possibility that these receptors act as negative-feedback autoreceptors to reduce

Figure 6.1 | Hypothesised mechanisms behind the mGluR-mediated modulation of swimming in *Xenopus*

(A) Group I mGluRs located on the terminals of both glycinergic commissural and GABAergic mhr interneurons are activated by projections of glutamatergic interneurons. Activation of these receptors reduces the release of both glycine and GABA onto motorneurons, causing an increase in swimming frequency and network excitability. (B) Group II and III mGluRs, located on the terminals of descending glutamatergic interneurons are likely to be activated by glutamate, acting as negative feedback autoreceptors to reduce glutamate release. A decrease in glutamatergic transmission (represented by dotted red arrow) will reduce the number of iGluRs (NMDA and AMPA) activated, reducing the drive to motorneurons and potentially causing them to drop-out of the network. This will weaken the synaptic drive for swimming and hence both swimming frequency and episode durations will decrease. In addition, activation of post-synaptic group III receptors hyperpolarises the resting membrane potential, similarly causing a reduction in network excitability. MN = motorneuron, GLY = glycine, GLU = glutamate.


Group I mGluR
Group II mGluR
Group III mGluR
Glycine receptor
GABA receptor
iGluRs
Activation
Inhibition
A
Glutamatergic input
Glycinergic commissural interneuron
GABAergic mhr interneuron
GLU
GLU GLU GLU GLU
I
I
GLY
GLY
GLY
GABA
GABA
GABA
NETWORK
EXCITABILITY
MN
B
Glutamatergic input
II
III
GLU
GLU GLU GLU GLU
NMDA
AMPA
III
NETWORK
EXCITABILITY
E_M Hyperpolarisation
MN

excitatory transmission. Similarly, as group I mGluRs are known to increase intracellular Ca^{2+} levels through PLC formation, and both groups II/III negatively couple to AC (for reviews: Pin and Duvoisin, 1995; Conn and Pin, 1997), it will be important in future to pharmacologically manipulate the underlying second messenger systems to either elucidate or eliminate additional mechanisms utilised by each group to exert their effect on the motor network. In addition, experiments on spinalised preparations will aid in the separation of mGluR effects on motor output originating in the brainstem compared to those originating from within the spinal cord. I have provided evidence that each group can potentially be activated by endogenously released glutamate, but these findings must now be complimented with further intracellular recordings to provide a clearer understanding of the cellular and synaptic mechanisms underlying each mGluR groups effect.

The sheer variety of mGluRs, each with differing cellular mechanisms between species, cell type and developmental stage, offers a wealth of intricately intertwined modulatory mechanisms. mGluRs appear to be able to 'fine-tune' the effects of glutamate (and other neurotransmitters) in the motor network, and are probably distributed throughout the whole nervous system. Whilst some of the mechanisms behind the observed effects remain unclear and in need of further experimental scrutiny, what has become apparent from my work, is that glutamate not only acts as a neurotransmitter, but also as an intrinsic neuromodulator within the *Xenopus* spinal cord.

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