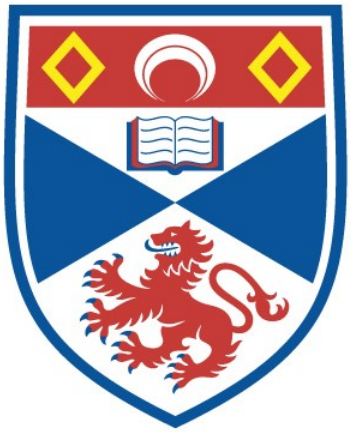
A MODULATORY ROLE FOR THE NMDA RECEPTOR GLYCINE BINDING SITE DURING FICTIVE LOCOMOTION IN *XENOPUS LAEVIS* LARVAE

Eva Rebecka Björnfors

A Thesis Submitted for the Degree of MPhil
at the
University of St Andrews



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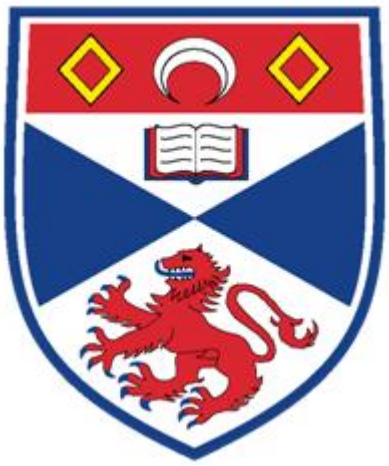

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Abbreviations

5-HT: serotonin

Aβ: amyloid beta

AD: Alzheimer's disease

aIN: ascending interneuron

ALX 5407: (GlyT1b antagonist)

AMPA: α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate

APV/D-AP5: NMDA receptor antagonist

ASC-1: alanine-serine-cysteine-1 receptor

ATP: adenosine tri-phosphate

cIN: commissural interneuron

CNS: central nervous system

CPG: central pattern generator

DAAO: D-amino acid oxidase

DAB:3.3'-Diaminobenzidine tetra-hydrochloride

dIN: descending interneuron

GABA: Gamma aminobutyric acid

GlyR: glycine receptors

GlyT1: glycine transporter 1

GlyT2: glycine transporter 2

GlyT1b: glycine transporter 1, subtype b

GRIP: glutamate-receptor-interacting protein

EPSP: excitatory post-synaptic potential

L-NAME:N_{ω}-Nitro-L-arginine methyl ester hydrochloride

LTP: long term potentiation

mEPSP: mini excitatory post-synaptic potential

MN: motoneuron

NA: noradrenaline

NFPS: (GlyT1 antagonist)

NMDA: N-methyl-D-Aspartate

NO: nitric oxide

NOS: nitric oxide synthase

PB: phosphate buffer

PBS: phosphate-buffered saline

PSC: post synaptic current

PLP: pyridoxal-5-phosphate

PTIO: 2-Phenyl-4,4,5,5-tetramethylidazoline-1-oxyl 3-oxide

SNAP: S-Nitroso-N-acetyl-DL-penicillamine

SR: serine racemase

TTX: tetrodotoxin

Abstract

This thesis follows up previous work carried out investigating the role of the NMDA receptor glycine site in fictive swimming in *Xenopus laevis* tadpole larvae. Extracellular glycine has been assumed to be present in the synaptic cleft at saturating levels, leaving no vacant NMDA glycine binding sites. D-serine, a ligand at the NMDA glycine binding site, has been found to increase the level of excitation in the CPG, increasing episode duration, increasing the occurrence of spontaneously occurring episodes, and producing more variation in burst frequency and amplitude within swim episodes leading to a waxing and waning pattern of swimming. These effects are also seen when glycine is co-applied with strychnine, and when glycinergic uptake is inhibited by the transporter blocker ALX. In this work, these findings have been confirmed and examined further by means of whole cell patch clamp recordings of spinal neurons of the CPG. D-serine causes a reversible increase in tonic depolarisation of spinal neurons along with a reversible decrease in spike height and an increase in spike frequency. D-serine also produces a paradoxical decrease in the membrane conductance and produces the above mentioned waxing and waning of swim episodes during which the neurons fire more or less intensely along with the waxing and waning. Furthermore, in the presence of TTX, bicuculline, and strychnine, D-serine increases the occurrence of miniature excitatory postsynaptic potentials. All of the above mentioned effects could be partially or fully reversed by either the NMDA receptor antagonist D-AP5 or the NMDA glycine binding site antagonist L-689560. In addition, D-serine could enhance NMDA-dependent intrinsic membrane potential oscillations, and in some experiments produce oscillations of a frequency reminiscent of oscillations dually dependent on NMDA and 5-HT. In conclusion, this work has shown that there are vacant glycine binding sites on NMDA receptors that can be bound by D-serine leading to an increase in overall excitation in the CPG. This is most likely achieved by tonic activation of NMDA receptors that is masked until the glycine site is occupied. Furthermore, D-serine has a modulatory effect on the fictive swimming rhythm, possibly by modulating the

1.Introduction

1.1 Locomotion in vertebrates is generated and maintained by central pattern generators

1.1.1 Basic organisation of vertebrate central pattern generators

Rhythmic locomotor activity in the mammalian central nervous system (CNS) was proposed as early as 1911 by T. Graham Brown to be generated and maintained by neuronal networks in the spinal cord and brainstem that do not require input from higher brain centres. His proposed model was composed of two lumbar centres, (or four in antagonistic pairs) in opposite sides of the spinal cord (Brown, 1911). These self-sustaining networks have been found to control a range of rhythmic behaviours in many other vertebrates and have been named central pattern generators (CPGs) (Brown, 1911, reviewed in McCrea and Rybak, 2008).

The precise output of locomotor CPGs result from a fine balance between excitatory and inhibitory inputs to motoneurons. The pattern of activation of motoneurons is produced by amino acid neurotransmitters, the most common excitatory transmitter being glutamate, which acts on ionotropic N-methyl-D-aspartate receptors (NMDAR), α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate receptors (AMPAR), and kainate receptors. The main neurotransmitters mediating inhibition of motoneurons are glycine, acting on ionotropic glycine receptors (GlyR) and gamma aminobutyric acid (GABA) acting on $GABA_A$ receptors (reviewed in Liu et al., 2010).

1.1.2 Basic outline of CPGs of different vertebrate species

The basic plan of the CPG is phylogenetically conserved across vertebrate species. In the rodent, AMPA, kainate and NMDA receptors are all involved in locomotor rhythm generation. Inhibition is mediated by both GABA and glycine which are responsible for the alternation between flexors and extensors and left-right coordination via complex inhibitory systems that involve both direct and indirect pathways. Direct excitatory pathways responsible for synchronous activity are provided by glutamatergic commissural interneurons (for review see Kiehn and Dougherty, 2010).

Another widely used model for locomotion is swimming in the lamprey. The spinal cord of the lamprey contains a CPG designed for left-right alternations of the body during swimming, but whose organisation is reminiscent of that of the rodent. The lamprey CPG contains pools of excitatory glutamatergic interneurons that receive glutamatergic input from the brain stem. The major inhibitory transmitters are glycine and GABA, the former being the key transmitter involved in the alternation between left and right muscle segments, and the latter in fine tuning the output by contributing to intersegmental coordination (for review see Grillner and Wallén, 2010).

A more recent and genetically tractable animal model system for locomotion is the zebrafish, a teleost. Electrophysiological studies in this species have shown that glutamate acts on NMDA- and AMPA receptors to produce fictive swimming. In addition, glycine has been shown to be important for left-right alternation of the muscle segments, similar to the case in the lamprey (Gabriel et al., 2008; Kyriakatos et al., 2011).

1.2 The CPG controlling swimming in tadpoles of the African clawed frog *Xenopus laevis*

1.2.1 Basic outline of the tadpole CPG

In this study, I have used the *Xenopus laevis* tadpole as a model organism to study the neural control of locomotor rhythm generation. The *Xenopus* tadpole CPG has been extensively researched and its constituent neurons and their interconnections mapped (Reviewed in Roberts et al., 2010, fig. 1). The system is well understood, particularly at the hatchling stage 37/38 (fig.2 Ai) and also the larval stage 42 (fig.2 Aii). In *Xenopus,* fictive swimming can be recorded from the ventral roots located in the inter-myotomal clefts and, in addition, whole cell patch clamp recordings of spinal neurons can be obtained (Li et al., 2002). Swim episodes recorded from ventral roots (fig.2 Bi) are made up of bursts of axonal impulse activity (fig.2 Bii). If swimming is recorded from both sides of the animal simultaneously, these bursts can be seen to alternate between left and right sides at 10 to 20 Hz (fig.2 Ci). Furthermore, if recordings are made from a rostral and a more caudal location on the same side, a rostro-caudal delay can be observed between the bursts of the two recording sites, showing how locomotion activity is generated by a rostro-caudal wave of activity (fig.2 Cii).

An important advantage of the tadpole model system is that little or no part of the nervous system has been removed and fictive swimming can be evoked by electrical stimulation of the tail skin activating the mechanosensory pathways. Therefore, no addition of NMDA and/or 5-HT is necessary to generate locomotor rhythm as is the case, for example, in spinal cord preparations of rodent (Smith and Feldman, 1987) and lamprey (Grillner et al., 1981). This means that the CPG is operating under near-natural conditions and generates a self-sustaining output without the need for pharmacological intervention.

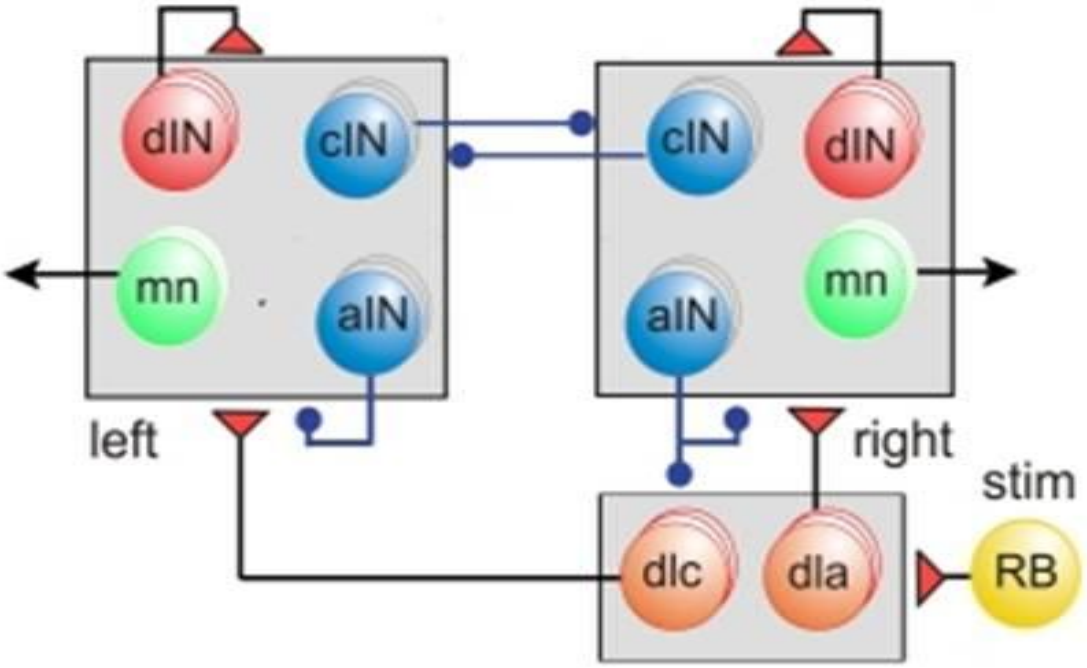

Figure 1. Diagram of the tadpole swim circuit. The CPG gets excitatory glutamatergic input from descending interneurons (dINs) which also have a feedback function. Left-right coordination is made possible by inhibitory glycinergic commissural interneurons (cINs) and further inhibition is provided to the network by the ascending interneurons (aINs). (Figure from Roberts et al., 2010).

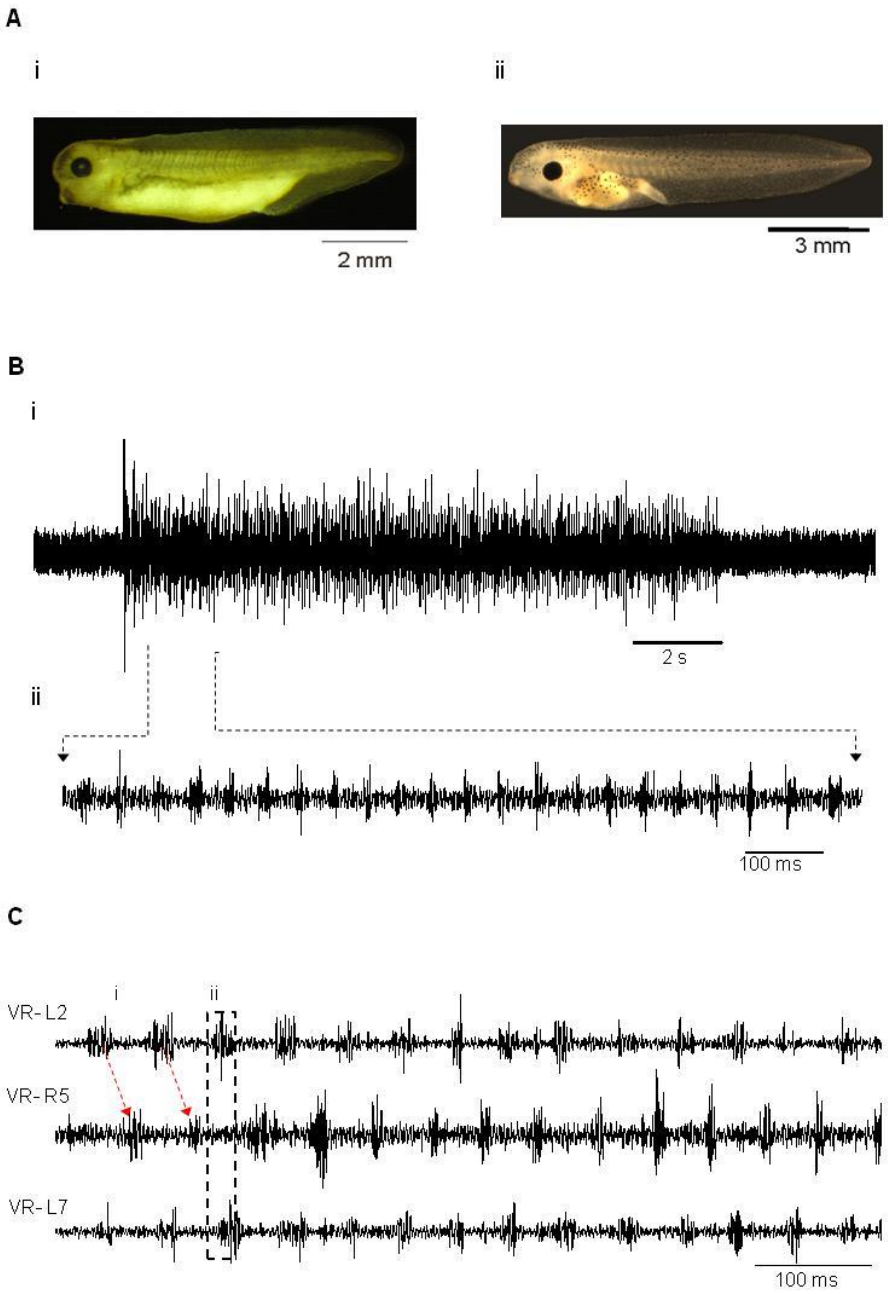

Figure 2. Fictive swimming in *Xenopus laevis*. A, photographs of Xenopus laevis tadpoles at stage 37/38 (Ai) and stage 42 (Aii). B, fictive swimming episodes can be recorded from the ventral roots in the intermyotomal clefts of the tadpoles (Bi). These episodes comprise bursts of neuronal activity (Bii). C, if swimming is recorded from three ventral roots simultaneously, the bursts can be seen to alternate between the left (L2) and the right (R5) sides (Ci, red arrows) with a simultaneous rostro-caudal delay between the rostral (L2) and the more caudal (L7) roots (Cii, black box). This indicates that normal fictive swimming propagates in a rostro-caudal direction. Y-scale of the ventral root recordings is arbitrary and varies due to variable signal.

1.2.2 Major transmitters

The tadpole CPG organisation at the embryonic stage 37/38 is similar to that of other vertebrate systems. Glutamate is the major excitatory neurotransmitter acting on NMDA receptors, while glycine is the main inhibitory transmitter (Li et al., 2004; reviewed in Sillar and Li, 2010). The glutamate is released from the reticulospinal descending interneurons (dINs) that have been found responsible for providing the excitatory synaptic drive to the spinal neurons via activation of NMDA receptors (Li et al., 2006; Soffe et al., 2009). In their turn, these excitatory glutamatergic dINs receive input from sensory pathways via the Rohon Beard (RB) neurons, an input which has been shown to be mainly mediated by AMPA (Roberts et al., 2010). Glycine is not necessary for rhythm generation which persists in the presence of the glycinergic antagonist strychnine; however glycinergic commissural interneurons in the *Xenopus* spinal cord are thought to be responsible for mid-cycle inhibition during swimming, which is abolished by strychnine. Altering the strength of glycinergic inhibition changes the frequency of the swimming rhythm (Dale, 1985; reviewed in McLean and Sillar, 2002). GABA does not play a significant role in *Xenopus* embryonic swimming; however, it is released from mid-hindbrain reticulospinal (mhr) neurons where it inhibits swimming when mhr neurons are activated by input from the rostrally located cement gland (Roberts and Blight, 1975; Roberts, 1980). Thus GABA is important for the termination of swim episodes in the *Xenopus* embryo when it contacts obstacles in the environment (Boothby and Roberts, 1992a, b; Perrins et al., 2002; Li et al., 2003).

1.2.3 The role of amines and their importance in the development of the CPG

The basic mechanisms of the tadpole swimming circuit remain essentially unchanged during development. However at larval stage 42 there is more variability in swim frequency and intensity, and with development, possibilities for modulation of the circuitry are added. Serotonin (5-HT) has been suggested to be of great importance in the development of the swim pattern seen in larval tadpoles (Sillar et al., 1992). Raising tadpoles until stage 42 in the neurotoxin 5,7-dihydroxytryptamine blocks serotonergic projections from the raphe nucleus in the brainstem and prevents normal development of the larval swim pattern (Sillar et al., 1995). Both 5-HT and noradrenaline (NA) are important modulators of locomotor output in the *Xenopus* spinal cord. Serotonin has a boosting effect on swimming, increasing the intensity and duration of ventral root bursts but without much change in cycle periods, whereas NA produces the opposite effect of decreasing the swimming frequency (McDearmid et al., 1997). These effects are thought to be due to depression and facilitation of glycinergic mid-cycle inhibition respectively, and strychnine has similar effects on locomotion to serotonin. Furthermore, serotonin reduces the amplitude of midcycle IPSPs recorded from motoneurons whereas NA increases them. A decrease in the frequency of mini IPSPs recorded in the presence of tetrodotoxin (TTX) and serotonin suggests a presynaptic modulation of the mid-cycle glycinergic inhibition (McDearmid et al., 1997).

1.2.4 The role of nitric oxide in modulation of the CPG

Another important neuromodulator in the *Xenopus* CPG is the free radical gas nitric oxide (NO). Three clusters of NO-producing cells have been found in the brainstem of *Xenopus* tadpoles at stage 42, some of which are thought to be reticulospinal. An increase in levels of NO by the addition of NO donors such as S-Nitroso-N-acetyl-DL-penicillamine (SNAP) leads

to a decrease in swim episode duration and an increase in cycle period within these episodes (McLean and Sillar, 2000). NO has been suggested to exert its effects on cycle period in a metamodulatory fashion by acting via NA to enhance glycinergic inhibition, whereas its effects on the duration of swim episodes involve a more direct effect on the release of GABA (McLean and Sillar, 2004).

1.3 The role and modulation of the NMDA receptor during fictive swimming

1.3.1 The NMDA receptor –pharmacology and structure

The NMDA receptor is commonly pentameric and can be composed of the N1, N2A, N2B, N2C, N2D, N3A, and N3B subunits, some of which can have different isoforms generated from alternative splicing. The pentameric structure forms a channel permeable to Na^+, K^+, and Ca^{2+}. NMDA receptors that contain the N1 and the N2 subunits require glycine to bind to the S1 and S2 regions of the N1 subunit and glutamate to the S1 and S2 regions of the N2 subunit. In addition, there are important modulatory sites for Mg^{2+} and Zn^{2+}, Mg^{2+} blocking the channel at resting membrane potential (Alexander et al., 2011). The subunit composition of the NMDA receptors found in the tadpole is not known, however due to pharmacological manipulations carried out previously (Reith and Sillar, 1998; Issberner and Sillar, 2007) and during the work for this thesis it is safe to presume that they at least contain the N1 and N2 subunits.

1.3.2 Effects of NMDA on fictive swimming

In *Xenopus* tadpoles, bath application of NMDA produces tonic excitation of neurones leading to activation of the CPG and continuous fictive swimming. Furthermore, the membrane depolarisation induced by NMDA is associated with an increased membrane

conductance of motoneurons due to NMDA channels opening which leads to a tonic excitation of neurones (Dale and Roberts, 1984). Paradoxically, a membrane conductance decrease has been reported in subsequent studies (Soffe and Roberts, 1989; Scrymgeour-Wedderburn et al., 1997). The explanation for these conflicting results is that the negative current pulses used to measure the conductance take the membrane down to a level where Mg^{2+} can block the NMDA ionophore (Soffe and Roberts, 1989).

The fictive swimming rhythm produced by NMDA differs between the embryonic and larval preparations in that the embryo shows a swimming rhythm of relatively constant ventral root burst amplitude and frequency in response to NMDA whereas the larval rhythm frequency is more variable and the ventral root rhythm slowly waxes and wanes over many consecutive cycles. This larval NMDA rhythm is accentuated by blocking inhibitory transmission with strychnine and the GABAR antagonist bicuculline, supporting the idea that the excitatory drive underlying this rhythmic pattern of locomotion is modulated (Reith and Sillar, 1998). Furthermore, in the presence of TTX, NMDA can induce intrinsic oscillations of the neuronal membranes in isolated spinal cord preparations from embryonic tadpoles (Prime et al., 1998). These TTX-resistant membrane oscillations have also more specifically been found to occur in the descending interneurons of embryonic tadpoles and have been proposed to underlie rhythm generation and additionally work as a pacemaker for swimming (Li et al., 2010). Evidence of these NMDA-dependent TTX-resistant oscillations at the larval stage 42 has not yet been reported, however, in combination with 5-HT NMDA produces slower, intrinsic oscillations of the membrane in both embryonic and larval preparations. Due to the slower frequency of these oscillations, rather than underlying normal cycle-by-cycle swimming they have been suggested to have a role in modulating the swimming pattern over several

consecutive swim cycles (Reith and Sillar, 1998). Both the NMDA-dependent and the combined NMDA- and 5-HT-dependent oscillations are described in more detail below.

1.3.2 Glycine, an indirect excitatory component in the spinal circuitry

Apart from being the major fast inhibitory neurotransmitter in the spinal cord, glycine also plays an excitatory role via a binding site on the glutamatergic NMDA receptor (called the glycine binding site), as for example described in cultured mouse brain neurons (Johnson and Ascher 1987) and rat hippocampal slices (Greene et al., 1998). This glycine site needs to be occupied alongside the glutamate site for activation of the receptor, as has been shown in voltage clamped *Xenopus* oocytes injected with rat brain messenger RNA (Kleckner and Dingledine, 1988). Hence it is essential for NMDA receptor activation and works to potentiate the excitatory response of glutamatergic cells.

It has previously been assumed that extracellular glycine concentrations are sufficiently high for the NMDAR glycine site to be fully saturated at all times. However, recent research has pointed to the contrary. The NMDAR glycine site agonist D-serine, or glycine applied in the presence of strychnine, can potentiate the activation of the NMDA receptors during fictive swimming in *Xenopus* tadpoles. It increases swim episode duration, probability of occurrence of spontaneous episodes, and increases the variability in frequency, amplitude and burst duration. All of these effects are reversed by applying the NMDA glycine site antagonist L-689560 (Issberner and Sillar, 2007). Hence, it would seem that the NMDA receptor glycine site is not fully saturated and thus available for modulation.

The re-uptake of glycine is mediated by glycine transporter 2 (GlyT2) located on the terminals of inhibitory glycinergic interneurons, and by glycine transporter 1 (GlyT1) located on glial cells surrounding NMDA receptors (Greene et al., 1998). In *Xenopus* tadpoles,

blocking glycine uptake by inhibiting the GlyT1b transporter with sarcosine co-applied with strychnine (to remove the inhibitory effects of glycine at the postsynaptic site), or by applying the selective GlyT1 inhibitor ALX 5407, results in the same effects as application of D-serine, i.e. increasing episode duration and the number of spontaneously occurring episodes and increasing the variance in burst frequency. This suggests that D-serine/glycine is important for modulation of NMDA receptor-mediated effects in the tadpole swimming circuit and that these modulations can occur also as a response to raised endogenous levels of glycine (Issberner and Sillar, 2007). Furthermore, in a study by Greene et al. (1998), the GlyT1 antagonist NFPS greatly enhances the postsynaptic currents (PSCs) recorded from rodent hippocampal pyramidal neurons, a response that can be blocked by the NMDA receptor antagonist APV. Additionally, blocking the glycine site at the NMDA receptor blocks the effect of the GlyT1 antagonist. These are strong indications that glycine may not always be present at concentrations high enough for all NMDA receptor glycine sites to be fully occupied. Furthermore, these findings support the theory that glycine transporter GlyT1b could modulate the NMDA response by regulating glycine concentrations in the synaptic cleft. Also, addition of exogenous glycine in the presence of the GlyT1 antagonist enhances the effect on the PSCs, showing that application of the transporter antagonist alone does not lead to saturating levels at the NMDA receptor glycine site (Greene et al., 1998).

1.3.3 D-serine as an intrinsic modulator at the NMDA glycine site

In the CNS, the glycine-site agonist D-serine is produced primarily by astrocytes, and is therefore a 'gliotransmitter' (Schell et al., 1995). However, it has also been found within neurons. It is synthesised from L-serine by the enzyme glial pyriodoxal 5'phosphate (PLP)-dependent serine racemase (SR) which co-occurs with D-serine in the CNS. SR activity can be regulated by Mg^{2+} which is needed for ATP to de-activate SR. SR has been found to

interact with the AMPA-associated protein glutamate-receptor-interacting protein (GRIP). The activation of AMPA receptors appears to strongly correlate with an increase in the activity of SR, which has led to speculations of whether this might be a mechanism by which to activate the NMDA receptors. However, there is no evidence for co-expression of AMPA receptors and SR to adequately support this theory (Kim et al., 2005, reviewed in Martineau et al., 2006).

D-serine has been proposed by Mothet et al. (2000) to be an endogenous agonist at the NMDA glycine site in rat hippocampal neurons since selective degradation of it by D-amino acid oxidase (DAAO), which leaves endogenous glycine levels unchanged, greatly attenuated the NMDA receptor response. These effects were reversed by the addition of exogenous D-serine, however in this study exogenously applied D-serine could not itself potentiate the NMDA receptor response (Snyder et al., 2000).

Furthermore, DAAO deficient mice show a great increase in levels of D-serine in the brainstem and the spinal cord, and the NMDA receptor response is accentuated in spinal cord dorsal horn neurons from these mice (Wake et al., 2001 reviewed in Mothet et al., 2006). Moreover, knock out of the neuronal transporter Asc-1 leads to hyperexcitability mediated by NMDA receptors, probably due to heightened levels of extracellular D-serine (Xie et al., 2005, reviewed in Mothet et al., 2006). In addition, when exogenous D-serine is co-applied with NMDA it potentiates the NMDA response in the initiation of locomotion in the mudpuppy (Cheng et al. 1998).

Interestingly, DAAO diminishes levels of nitric oxide synthase (NOS) and thus the production of NO in cerebellar slices from immature rats. From these observations it was suggested that D-serine can have an up-regulating function on NOS, leading to an increase in NO production (Mothet et al., 2000). In addition, NO has been reported via ATP to S-nitrosylate SR and thus

diminish intrinsic levels of D-serine (Snyder et al., 2006). These are discoveries that are worth keeping in mind when studying either of these two neuromodulators.

As already described above, exogenous application of D-serine in *Xenopus laevis* tadpoles at stage 42 leads to an increase in fictive swim episode duration and a larger variation in burst frequency, amplitude and duration in comparison to control. These effects could be reversed by the glycine site antagonist L-689560, suggesting that the NMDA glycine binding site is not fully saturated but available for modulation (Issberner and Sillar, 2007).

1.4 Membrane potential oscillations that underlie swimming

In many species, the application of NMDA leads to oscillations in the membrane potential. These oscillations are still present when synaptic transmission is blocked with TTX suggesting that they are intrinsic properties of the neurons involved.

1.4.1 NMDA-dependent oscillations in various species

NMDA-dependent TTX-resistant oscillations were first described in the lamprey (Sigvardt and Grillner, 1981; Grillner and Wallén, 1985). They are voltage-dependent; changing the membrane with injection of hyperpolarising current leads to an increase in the oscillation amplitude. Furthermore, they are abolished in Mg^{2+} -free saline. The underlying mechanism relies upon a slow initial depolarisation that takes the membrane potential into the voltage region at which the Mg^{2+} block of the ionophore is alleviated to produce a rapid and regenerative rising phase of the oscillation. The opening of voltage-dependent K^{+} channels limits the peak of the oscillation; meanwhile calcium entry through the now open NMDA channels activates Ca^{2+} -dependent K^{+} channels, hyperpolarising the membrane potential back towards the region where Mg^{2+} can re-block the channels and trigger the repolarising phase of

the oscillation. These features of the NMDA receptors confer a regime of negative slope conductance in the I/V relationship which causes the membrane potential to be unstable and consequently flip between two voltage levels when NMDA receptors are activated. In the lamprey, these NMDA-dependent oscillations are slow (~1 Hz) and have been suggested to contribute to the generation of locomotion during fictive swimming as the oscillation frequency and the burst frequency during fictive swimming span a similar frequency range (Sigvardt and Grillner, 1981; Grillner and Wallén, 1985).

NMDA-induced, TTX-resistant membrane oscillations have also been observed in mammalian motoneurons. In motoneurons of neonatal rat, NMDA produces a tonic depolarisation of about 11 mV followed by membrane oscillations of 0.5-3.0 Hz (reviewed in Schmidt et at., 1998). The amplitude of these oscillations is, as in the lamprey, dependent upon the membrane potential, however, the frequency remains unchanged at different voltage levels. This type of oscillation has also been observed in mammalian interneurons, however these oscillations span a different frequency range (2-26 Hz) than their motoneuron counterparts and the frequency appears to change with the membrane voltage level. In addition, 5-HT can induce membrane oscillations in preparations where NMDA has failed to do so and 5-HT antagonists can block the NMDA-induced oscillations (reviewed in Schmidt et al., 1998).

Newly hatched tadpoles of *Rana temporaria* also display membrane potential oscillations, however in addition to NMDA these oscillations are, like in the mammal, also dependent on 5-HT. In the presence of NMDA, application of 5-HT hyperpolarises the membrane potential and the appearance of oscillations follows. These oscillations do not occur in the presence of 5-HT alone and are abolished in Mg^{2+} free saline. Furthermore, they can be blocked by the NMDA antagonist APV. They have a frequency of 0.2-0.5 Hz and hyperpolarisation of the

membrane by negative current injection increases the oscillation amplitude but not frequency (Sillar and Simmers, 1994).

1.4.2 Intrinsic membrane potential oscillations in Xenopus laevis

In *Xenopus* tadpole neurons, 100 μM NMDA produces a TTX-resistant depolarisation of about 20mV (Scrymgeour-Wedderburn et al 1997). It also produces a paradoxical conductance decrease explained by injected negative current pulses taking the cell to a level where Mg^{2+} can block the ion channel (Soffe and Roberts, 1989). However, in neither embryonic nor larval stages does NMDA produce membrane oscillations like those reported in lamprey, or rodent (Scrymgeour-Wedderburn et al 1997). However, reminiscent of what has been reported in *Rana temporaria*, membrane oscillations become apparent when NMDA is co-applied with 5-HT in the presence of TTX. Like NMDA, 5-HT applied on its own does not produce any oscillations but rather causes a small hyperpolarisation of the membrane potential. The occurrence of these NMDA- and 5-HT -dependent oscillations becomes more prominent with development, occurring in only 12% of embryonic cells but as many as 70% of larval cells. Removal of 5-HT from the experimental preparation terminates the oscillations as does application of the NMDAR antagonist APV or removal of extracellular Mg^{2+}, further strengthening the notion that both NMDA and 5-HT are essential for these oscillations to occur (Scrymgeour-Wedderburn et al, 1997).

Nevertheless, TTX-resistant NMDA oscillations have been reported to occur in isolated *Xenopus* spinal cord preparations from stage 32-37/38 (Prime et al., 1999). These oscillations were present in 5/14 preparations and grouped into either low frequency (~0.6 Hz) or a high frequency (2.3 Hz) oscillations. Furthermore, oscillations could also occur in the presence of 5-HT at a frequency of 0.6 Hz or 4.6 Hz. Oscillations were also present when glycine was applied with NMDA and had frequencies of 0.4 and 7.5 Hz. From these experiments it was

concluded that 5-HT and glycine accentuate NMDA-dependent oscillations but are not necessary for these to occur. The frequency of the slow type of oscillations was reported to decrease with hyperpolarising current whereas the amplitude increased. The frequency of the fast oscillations, however, was insensitive to injected negative current, but their amplitude decreased. This difference in voltage-dependence of the oscillation frequency differs from what was reported by Scrymgeour-Wedderburn et al. (1997), a difference that was accounted for by enzymatic treatment of the isolated spinal cord prep possibly disrupting these cell-cell connections (Prime et al., 1999).

Moreover, TTX-resistant NMDA-dependent oscillations have recently been reported to occur in *Xenopus* tadpoles using whole cell recordings, however, they occur more commonly in descending interneurons (dINs) than other types of neurons at stage 37/38. Furthermore, oscillation frequency varies depending on neuron type, dINs producing oscillations of ~10 Hz, within the normal swim frequency for tadpoles, whereas other neuron types had lower frequencies of ~2-7 Hz. The dINs have been suggested to make up a pacemaker network, and were in this study shown to do so by producing NMDA-dependent membrane oscillations that underlie the generation of the swimming rhythm, a theory which was given further support by the finding that this rhythm still occurs in an isolated hemi CNS. Thus swimming is produced by a neuronal network of dINs with intrinsic pacemaker properties (Li et al., 2010).

1.4.3 Possible roles for these intrinsic oscillatory membrane properties

The oscillation frequencies reported by Li et al. (2010) fall into the frequency range of normal swimming. However, some of the oscillation frequencies reported by Prime et al. (1999), and additionally the frequencies of the NMDA- and 5-HT -dependent oscillations reported by Scrymgeour-Wedderburn et al. (1997) and Reith and Sillar (1998) are too slow (~0.5 Hz) to contribute to the normal cycle-by-cycle swimming rhythm (~10-20 Hz). There are a few

possible explanations regarding what role these oscillations might play during tadpole locomotion. It is possible that these slow oscillations enhance the responsiveness of the membrane potential to synaptic drive, facilitating motor output. Another possibility is that they are a mechanism helping motor neurons to fine-tune and time their firing-threshold. Finally, they could be working to enhance the gain of swimming over several consecutive cycles. (Scrymgeour-Wedderburn et al., 1997). It is clear that whatever the role it probably differs from that in other systems, like the lamprey and rodent, where the oscillation frequency is a closely matched to the CPG output frequency.

A possible explanation for the involvement of 5-HT in these oscillations is that it could work to boost activity at the NMDA receptor. In principle, this could be done via inhibition of glycine uptake by GlyT1b, thus enhancing the occupancy of the NMDAR glycine site and increasing the current flow following receptor activation by glutamate (Issberner and Sillar, 2007). However, the function of these slow oscillations remains open to debate.

1.5 Project outline

1.5.1 The effects of D-serine on swimming

This project is divided into two parts. The first part is based upon the findings by Issberner and Sillar (2007) that D-serine can modulate fictive swimming in tadpoles at stage 42 by occupying vacant glycine sites. It aims firstly to further investigate the cellular and synaptic consequences of modulation of the NMDA glycine site by D-serine during fictive swimming in the larval stage. In addition, by means of whole cell patch clamp recordings from spinal neurons of the CPG, it aims to determine how the neuronal properties change in response to D-serine and other modulators of the glycine site and how this might contribute to the changes observed in the swimming rhythm.

1.5.2 A possible role for the glycine site in intrinsic membrane potential oscillations

The second part of the project builds on the work on the NMDA- and 5-HT-dependent membrane oscillations in spinal neurons by Scrymgeour-Wedderburn et al. (1997) and Reith and Sillar (1998). It aims to investigate further the possible involvement of the glycine transporter GlyT1b for the dependence upon 5-HT for the occurrence of these oscillations. It aims to explore the influence that D-serine might have on the intrinsic membrane potential oscillations and to deduce what might be the role of these slow frequency oscillations when translated into fictive swimming and actual locomotion of the animal.

2. Materials and Methods

2.1 Animal rearing

Experiments were performed on *Xenopus laevis* frog tadpoles at larval stage 42 (Nieuwkoop & Faber, 1956). Tadpoles were obtained by induced mating of pairs of adults from an in-house colony by injection with human chorionic gonadotropin (1000 Uml−1; Sigma). Collected eggs were reared in aerated trays at temperatures between 17-23 °C to stagger their development until they had reached the desired stage. All experiments were in compliance with the UK Home Office regulations and have been approved by the University of St Andrews Animal Welfare Ethics Committee (AWEC).

2.2 Dissection and electrophysiology

Tadpoles were selected and immobilised in α-bungarotroxin (12.5 μM; Sigma). Access of the toxin to the muscles was facilitated by making fine gashes in the trunk skin prior to immobilisation. Tadpoles were then pinned using fine-tipped tungsten needles through the notochord on their sides to a rotatable stage covered with Sylgard, in a bath of saline (in mM: 115 NaCl, 3 KCl, 2 $CaCl_2$, 2.4 $NaHCO_3$, 1 $MgCl_2$, 10 Hepes, adjusted with 4 M NaOH to pH 7.4). The trunk skin was removed from both flanks using finely etched tungsten dissecting needles, leaving some skin on the tail for subsequent electrical stimulation of the sensory pathway during experiments. The yolk sac was removed to optimise the view of the spinal cord, and the myotomes overlying the spinal cord were carefully removed. The melanophores were cleared away from the spinal cord and the cord opened dorsoventrally to the neurocoel from approximately the obex to the 8^{th} myotome. Glial cells were carefully cleared away down the midline of the spinal cord to allow better access and visualisation of spinal neurons

for patching. The animal was moved to a recording bath in the experimental setup (for diagram of dissection and placement of recording electrodes see figure 3). Saline was gravity fed into the experimental chamber and pumped out using a Gilson volumetric minipulse2 pump. Extracellular recordings were made with suction electrodes from ventral roots in approximately the 2-4th first myotomal clefts (fig.3 Ai and Bi). Extracellular signals were amplified using differential AC amplifiers (Model 1700, A-M Systems Inc., Carlsborg, WA, USA), and digitized using a CED micro 1401 and stored and processed on a personal computer using Spike2 software (v. 3.21, Cambridge Electronic Design, Cambridge, UK). Swimming was initiated by a 1 ms electrical current pulse delivered via a stimulation electrode made from a borosilicate glass capillary with an insulated copper wire wrapped around it placed on the tail skin to stimulate the mechanosensory pathways (fig.3 Aii and Bii). Stimulation threshold was determined for each experiment and stimulation then set to 1.5 times threshold value. In experiments where drug effects on sensory threshold were assessed, the threshold was established for every individual swim episode. Whole cell patch clamp recordings were made in current clamp mode from presumed CPG neurons (fig.3 Aiii and Biii) (anatomy determined by staining, see below) using electrodes (10-20 MΩ) pulled from borosilicate glass capillaries containing a fine filament (Harvard Apparatus Ltd) on a Narishige PP830 pipette puller. Electrodes were filled with intracellular solution (in mM: 100 potassium gluconate, 2 $MgCl_2$, 10 EGTA, 10 Hepes, 3 Na_2 ATP, 0.5 NaGTP adjusted to pH 7.3 with KOH) containing 0.1% neurobiotin. Patch clamp signals were amplified with an Axoclamp 2B amplifier, displayed on a Gould digital oscilloscope (20MHz (DSO) 1604) and digitized using a CED micro 1401 and recorded using Spike2 software.

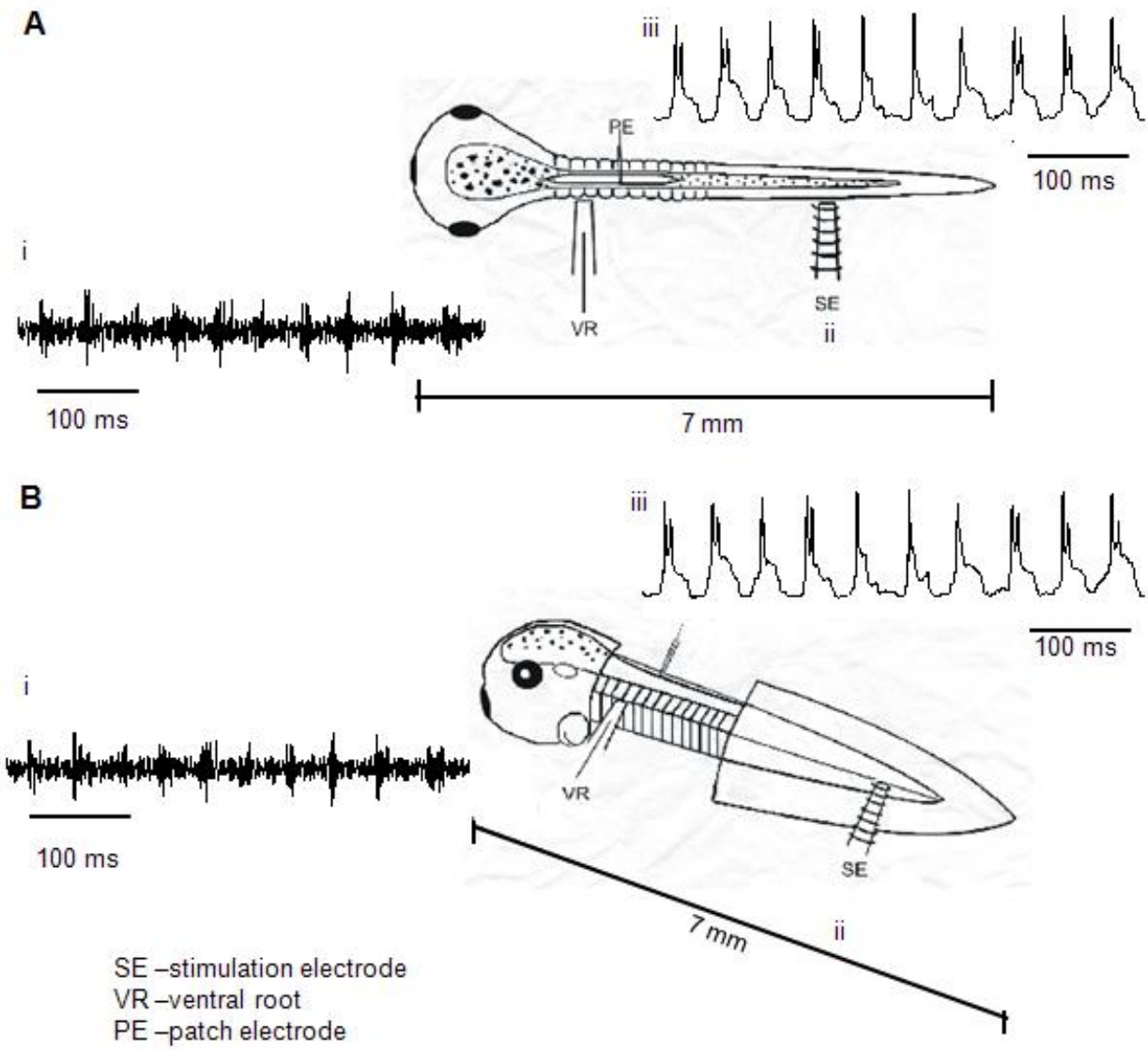

Figure 3. **Patch dissection diagrams**. A, dorsal and B, lateral views of the patch dissection employed in this study. Some skin has been left on the tail for electrical stimulation of the sensory pathway (Aii and Bii). A ventral root recording was obtained from a root approximately between the 2-6th myotome (Ai and Bi) and likewise a patch recording of a spinal cell was obtained in approximately the same region (Aiii and Biii). For more detail of the dissection see the main text.

2.3 Obtaining a GΩ seal and a patch recording

The patch electrode with applied positive pressure was carefully positioned close to a spinal neuron. In order to patch the neuron the positive pressure was released to attract the cell membrane to the electrode and paired negative and positive (±100 pA, 10 ms pulse dur., 20 ms interval) square wave current pulses were passed through the electrode. The neuron's response to these pulses was carefully monitored by looking at their amplitude. When a GΩ seal had been formed between the electrode and the cell membrane, shown by an increase in the amplitude of the neuron's response to the injected current pulses, the membrane was broken using another program of strong current pulses, each pulse composed of ten consecutive pulses (5 nA, 2-5 ms) from the electrode. The zero resistance for the neuron was then set by injecting small paired negative and positive square pulses of current (±30 pA, 2 ms pulse dur., 25 ms interval) into the neuron and while observing the neuron's response, adjusting the capacitance neutralisation and the bridge appropriately in order to compensate for the resistance of the patch electrode before experiments were started.

2.4 Pharmacology

Drugs were made up from powder to aliquots of 50-100 µL and stored in a -20 °C freezer. During experiments aliquots were made up to a volume of 50 mL in saline (the obtained concentration corresponding to the desired final concentration) and bath applied via a gravitation system. Concentrations and suppliers were as follows: D-serine (25-300 µM; Sigma/Tocris Biosciences), L-689560 (10 µM; Tocris biosciences), L-NAME (0.5 mM; Sigma), PTIO (200- 400 µM; Sigma), bicuculline (20 µM; Sigma), strychnine (5 µM; Sigma), TTX (1 µM; Sigma), glycine (100 µM; Tocris Biosciences), NMDA (50-100 µM; Sigma), 5-

HT (2 µM; Sigma), ALX 5407 (10-20 µM; Tocris Biosciences), sarcosine (1 mM; Sigma), D-AP5 (25-50 µM; Ascent Scientific).

2.5 Staining

Neurons were stained with neurobiotin (0.1 %) in the patch pipette, which diffused passively into the cells and its processes during the course of the experiment. After recordings, the animals were fixed in a mixture of glutaraldehyde (0.2%; VWR International Ltd.) and phosphate buffer (0.1 M; pH 7.2; Sigma). The staining procedure was performed according to the following protocol. Animals were rinsed with four changes of 0.1 M phosphate-buffered saline (PBS) (120 mM NaCl in 0.1 M PB pH 7.2; Sigma). Then washed in two changes of 1 % Triton X -100 (Sigma) in PBS. Animals were incubated in a 1:3000 dilution of extravidin peroxidase conjugate (Sigma) in PBS containing 0.5% Triton X-100 for 2-3 h. Animals were then rinsed again in four changes of PBS as described above. A DAB staining kit was used mixed with PBS and H_2O_2 (Sigma). The staining reaction was terminated by washing in four changes of running dH_2O. Animals were then dehydrated through an alcohol series of (in percentages) 57, 70, 82, 91, 97, and 2x100 made up from ethanol (Sigma), Butan-1-OL (Sigma) and H_2O for 3-4 minutes in each percentage. Then animals were cleared in histoclear (Raymond A Lamb Laboratory Supplies) 2x30 minutes and mounted between two coverslips using histomount (Kimberly Research) in a stainless steel slide.

2.6 Drawing

Neurons were traced by hand using a Zeiss microscope with a *camera lucida* attachment, courtesy of Dr. Bill Heitler, University of St Andrews.

2.7 Analysis

Analysis of collected data was carried out using DataView software, courtesy of Dr. Bill Heitler. Graphs and figures were created in Excel and constructed and modified aesthetically in CorelDraw and Powerpoint. Statistical analysis was carried out using Graphpad InStat®3. For experiments containing only two groups, a student t-test was run, whereas experiments with more than three experimental conditions were analysed using a one-way analysis of variance (ANOVA). Where applicable, a Tukey's post hoc test was run.

3. Results

3.1 Effects of D-serine and L-689560 on fictive swimming

To confirm the previous demonstration that the NMDA receptor glycine site in stage 42 *Xenopus* larvae is not fully saturated and hence available for modulation during fictive locomotion (Issberner and Sillar, 2007) the effects of D-serine on the duration of fictive swimming episodes were assessed. Ventral root recordings were made from the inter-myotomal clefts and fictive swimming was initiated by a 1 ms electrical pulse applied to the tail skin. In order to make episode duration as regular as possible and thus make any drug effects clearly distinguishable, the inter-episode interval was kept constant at 20 seconds.

3.1.1 Effects on fictive swim episodes

D-serine (100 µM) increased episode duration by 81% (±12%), (one-way ANOVA, $P<0.005$, n=5, fig.4 A and B) consistent with earlier reports. This effect could be reversed by the potent NMDA glycine site antagonist L-689560. L689560 (10 µM) caused a dramatic reversal in episode duration decreasing it by17% (±2.6) of control episode duration ($P<0.005$, n=5, fig.4 Aiii and B). In addition, when applied prior to D-serine, L-689560 caused a decrease in episode duration by 69% (±18%). This effect was not statistically significant. However, since this effect was approaching significance, it is most likely due to a low n-number ($P=0.0842$, n=3, fig.4 C). Furthermore, D-serine had no significant effect on episode duration when applied in the presence of L-689560. The episode duration varied a lot between experiments, however the effects of D-serine and L-689560 were consistent (fig.3 D).

Within these longer swim episodes the bursts became more variable in frequency, amplitude and duration. In particular, the frequency and intensity of swimming often waxed and waned over many tens of consecutive cycles, producing a slow irregular oscillation of the faster

underlying swimming rhythm (e.g. fig.5 A). This effect is reminiscent of the effect of exogenous NMDA on the swimming rhythm, causing a slow rhythm with a cycle period of around 2 seconds (Reith and Sillar, 1998). The antagonist L-689560 reversed the increase in episode duration, curtailing this oscillatory behaviour although some variability of cycle period and burst durations within the episodes still persisted (fig.5 Aiii). Furthermore, D-serine increased the probability of occurrence of the alternative motor programme called fictive struggling (fig.5 B). This locomotor behaviour propagates caudo-rostrally, the opposite to swimming (Soffe et al. 2007). All of the above are effects which have been reported and quantified previously (Issberner and Sillar, 2007).

3.1.2 Effects on spontaneously occurring activity and system threshold

In addition to the swim episodes that were initiated by an electrical stimulus, many stage 42 tadpoles occasionally swam in the absence of this stimulation, a phenomenon which has been termed spontaneous swimming. The probability of such spontaneous episodes of swimming occurring in control conditions varied from preparation to preparation. However, these spontaneous episodes occurred significantly more frequently in D-serine, particularly in higher concentrations (>100 µM). D-serine (25-500 µM) increased the occurrence of spontaneous episodes from 2.6 episodes per ten minutes to 6.6 episodes per ten minutes, or 154% (±45%) ($P<0.01$, n=10, fig.5 C). This increase in spontaneous episodes was reversed by L-689560 to 1.4 episodes per ten minutes or by 54% (±5%) of control ($P<0.001$, n=10, fig.5 C). This finding is in agreement with the previously reported effects of modulation at the NMDA glycine site on fictive swimming in *Xenopus* stage 42 tadpoles (Issberner and Sillar, 2007).

Furthermore, previous observations that L-689560 increased the threshold for the electrical stimulus needed to initiate swimming, an effect which was reversed upon the addition of D-serine, was observed but not quantified. This finding is in agreement with reports that the NMDA receptors are involved in the skin sensory pathways (Sillar and Roberts, 1988; Issberner and Sillar, 2007). Work to ascertain that the antagonistic effects of L-689560 are acting on the CPG, rather than on the sensory pathways has been carried out by means of activating swimming by the application of exogenous NMDA. The swimming rhythm initiated by NMDA could be curtailed by L-689560, an effect that could be partially reversible by wash and was thus independent of any external sensory stimulus (Issberner and Sillar, 2007).

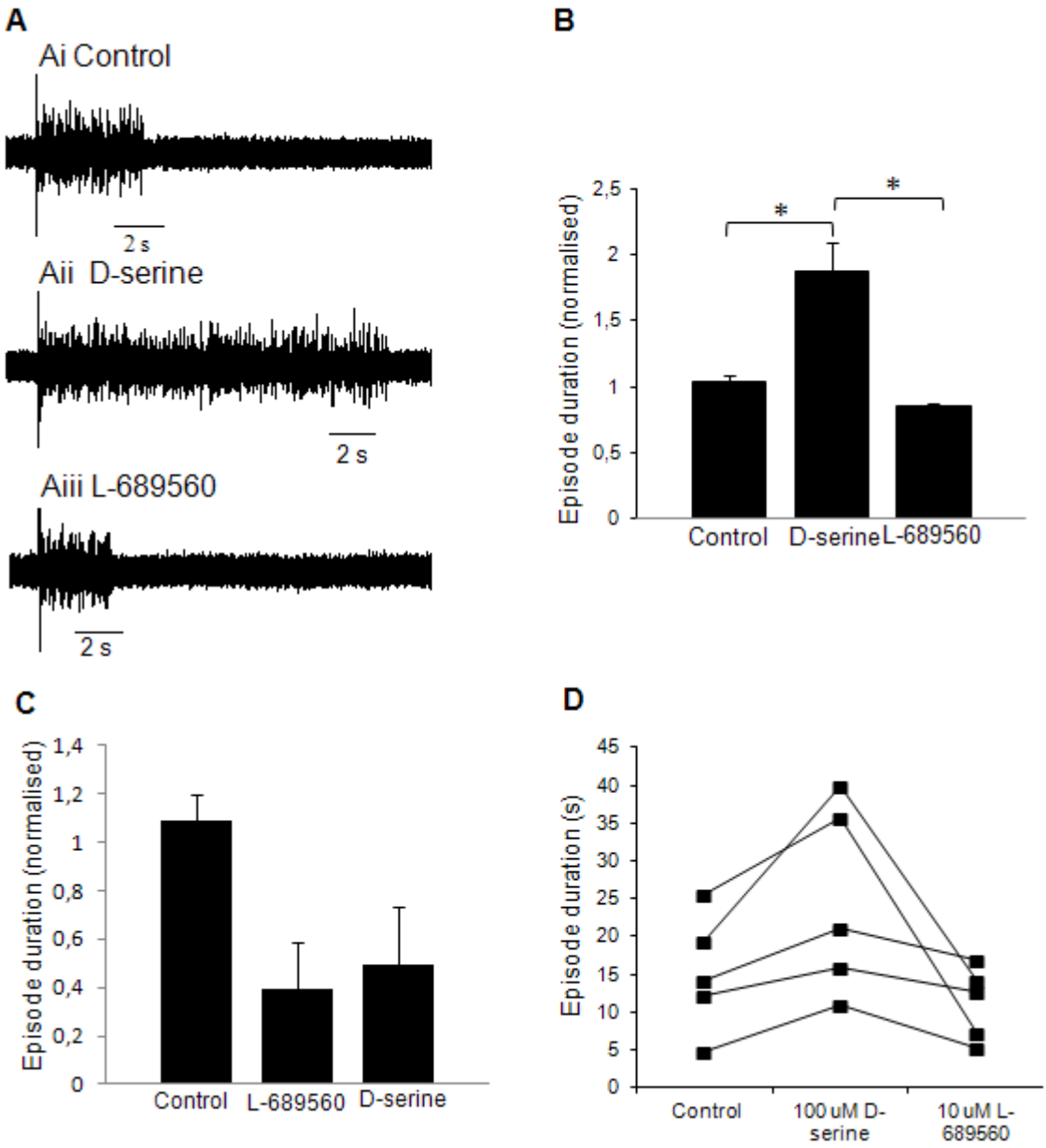

Figure 4. **Effects of D-serine and L-689560 on fictive swimming**. A, raw traces from a ventral root recording showing that D-serine (100µM) increased episode duration (Ai), an effect that was reversible by the NMDA glycine site antagonist L-689560 (10µM) (Aiii). B, D-serine increased episode duration by 81% (±12%), ($P<0.005$, n=5) and L-689560 decreased it by 17% (± 2.6%) of control episode duration ($P<0.005$, n=5). C, Episode duration varied greatly between preparations. D, L-689560 decreased episode duration by 69% (±18%) when applied prior to D-serine however due to the low number of experiments this result was not statistically significant ($P=0.0842$, n=3). Illustrated values are means ±SEM.

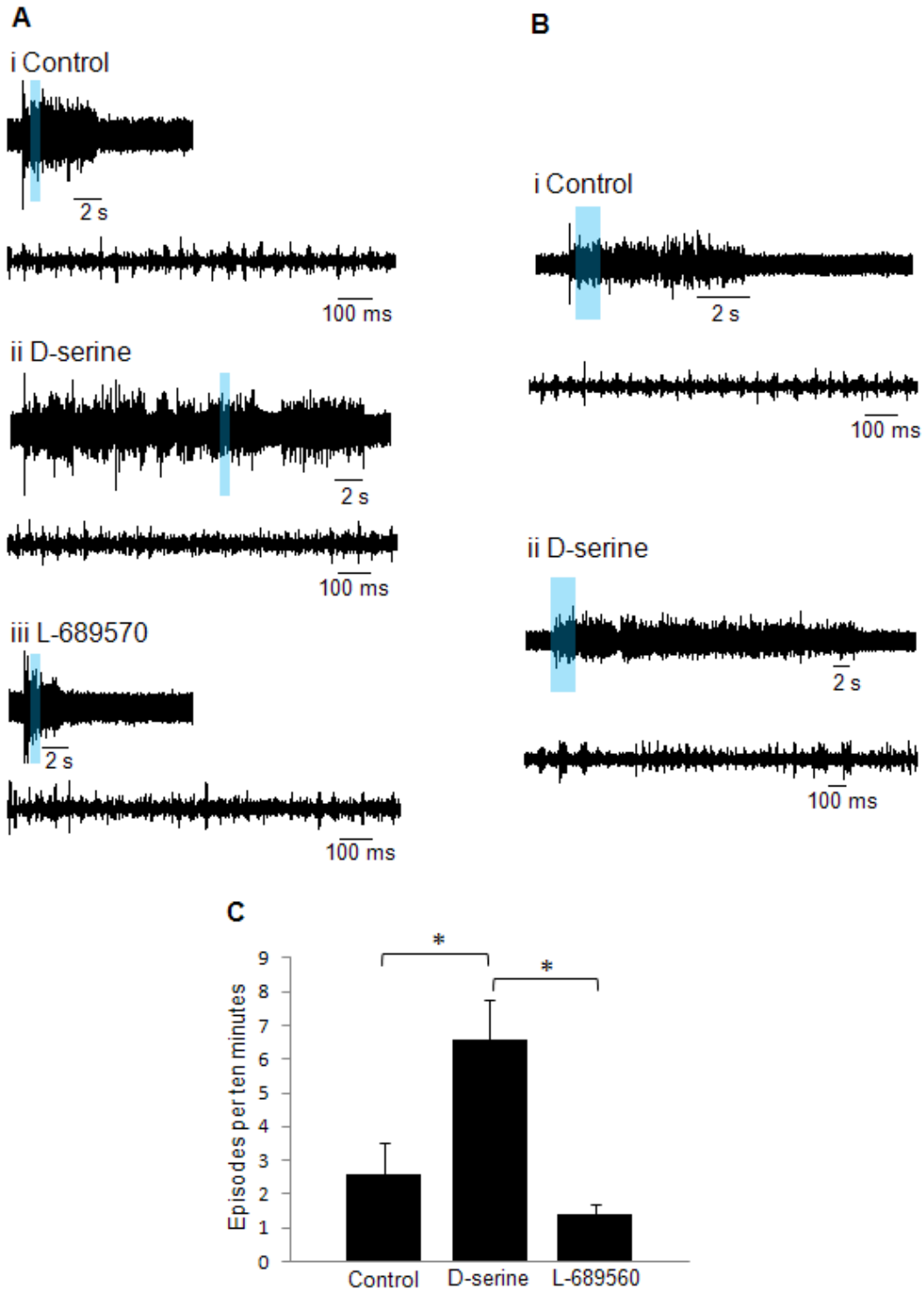

Figure 5. **Effects of D-serine on swimming pattern and spontaneous activity**. A, D-serine (100µM) (Ai) caused the burst amplitude and frequency to become more variable compared to the control (Aii) (blue squares indicate parts of the traces that have been expanded), an effect that was difficult to reverse (Aiii). B, D-serine (<100µM) also increased the occurrence of the alternative motor programme called struggling (Bii). C, the amount of spontaneous swim episodes increased in D-serine (<100µM) by 154% (±45%) ($P<0.01$, n=10) and decreased again by L-689560 (10µM) to 54% (±5%) of the control condition ($P<0.001$, n=10). Values are means ± SEM.

3.1.3 Effects of D-serine in the presence of NOS inhibitors

Despite the results above, initial observations of the effects that D-serine had on fictive swimming were inconsistent and did not replicate previously published data, for unknown reasons. However, it led to speculation that D-serine might be acting on something in the system with an inhibitory effect, thus counteracting the effects that D-serine had on fictive swimming.

The dissection used in this study differed from that used by Issberner and Sillar (2007) in that the opening of the CNS for patch clamp recordings allows greater drug access to the brainstem and the entire spinal cord. In the rodent, it has been suggested that D-serine can have an up-regulating function on nitric oxide synthase (NOS) positive neurons which would mean an increase in the production of nitric oxide (NO) (Martineau et al., 2006). In the tadpole, NOS positive neurons can be found in the brainstem and an up-regulation of NOS would lead to increased levels of NO in the swim network. NO is an inhibitory modulator of fictive swimming, decreasing episode duration while increasing cycle period (McLean and Sillar, 2000; see introduction). Although, D-serine has been found to increase episode duration and decrease cycle period (Issberner and Sillar, 2007), it is possible that D-serine effects on swimming are reduced by NO, an effect that is exaggerated in my preparations where drug access to the nervous system has been improved by the more extensive dissection.

Hence, to investigate any confounding effects that D-serine might indirectly exert on the NO system in the tadpole CPG, an inhibitor of NOS, L-NAME (0.5 mM) was applied before the addition of D-serine. In 7 out of 9 experiments, D-serine produced an increase in episode duration. This increase was greater than when D-serine was applied in the absence of L-NAME. D-serine increased episode duration by 101.5% (±60%) of control (fig.6 C, P=0,4636, n=7). Variance was large between experiments but, when analysed individually, D-serine

caused a statistically significant increase in every experiment (fig.6 D $P<0.0001$ or $P<0.05$, n=6 (tested individually)). In 4 out of 7 experiments changes in episode duration reversed partially when switched back to L-NAME only. Wash off was estimated to 11% (±44%) of the D-serine treated condition and did thus not reverse back to control levels ($P=0.4636$, n=7).

Considering that in these experiments D-serine produced a greater effect on episode duration than when applied with the NO system intact, it was decided that NO should be depleted from the system while the effects of D-serine on spinal cord neurons were investigated using patch clamp recordings. I chose to scavenge NO with 2-Phenyl-4,4,5,5-tetramethylidazoline-1-oxyl 3-oxide (PTIO), which is reported to be membrane permeable and so can scavenge intracellular as well as extracellular NO.

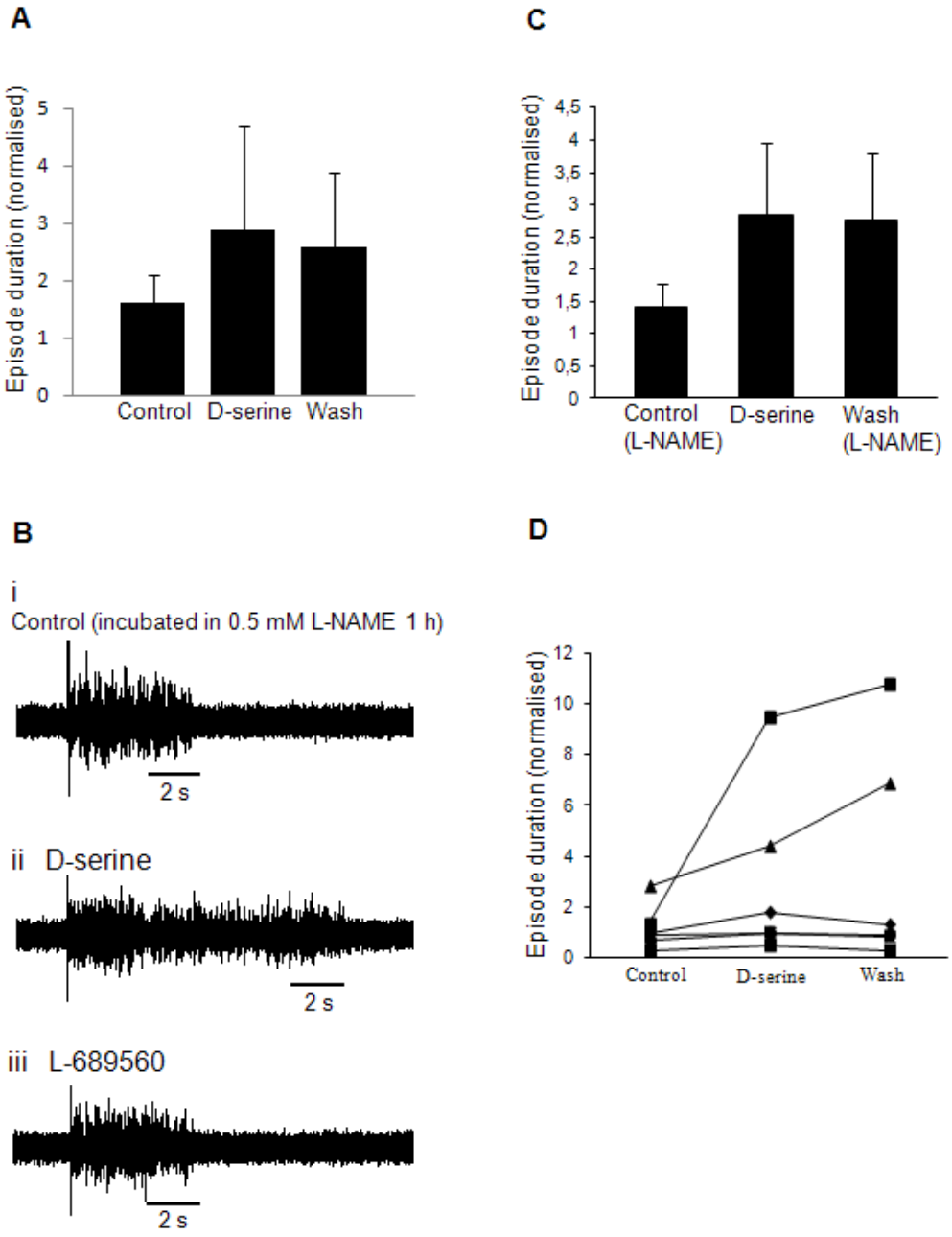

Figure 6. **Effects of D-serine on ficitive swimming after pre-treatment with L-NAME**. A, initial analysis of episode duration was inconclusive. D-serine (100µM) increased episode duration by 35% (±36%) of control and wash off was small or absent (P=0.6496, n=5). B, incubation in 0.5 mM L-NAME lead to a marked increase in episode duration when D-serine was added (Bii). This effect could be reversed by the antagonist L-689560 (10µM) in 4/7 experiments (Biii). C, D-serine increased episode duration by 101.5% (±60%) of control. D, there was a large variation in episode durations and drug effects between the individual experiments. Although when pooled, the effect of D-serine on episode duration was insignificant, 6/7 experiments showed a significant increase in episode duration when analysis was done on individual experiments (P<0.0001 or P<0.05, n=6). Values are means ± SEM, L-NAME is present in all conditions in B, C, and D..

3.2 Effects of D-serine and L-689560 on neuronal firing properties during fictive swimming

Since my observations based on extracellular recordings suggest that bath applied D-serine modulates the CPG output by binding to vacant NMDA receptor glycine sites, I decided to investigate in more detail the cellular consequences of this modulation on spinal cord neurons that are active during fictive swimming. Whole cell patch clamp recordings were made from neurons within the spinal cord which were later identified by neurobiotin staining as motoneurons, with projections emanating from the spinal cord to innervate the myotomal muscles, (nine cells in total) (fig.7 Ai-Aiii) or interneurons, with processes restricted to the spinal cord out of which 24 were ascending interneurons (aINs) (fig.8 B) ten were commissural interneurons (cINs) (fig.8 A), 13 were descending interneurons (dINs) (fig.8 C); and 25 unidentified interneurons. Drugs were bath applied, thus able to affect receptors throughout the CNS. The intracellular NO-scavenger PTIO (100-200 μM) was applied for 15-20 minutes before adding the NMDA glycine site modulators to reduce any confounding effects that an up-regulation of NOS-positive neurons might have on the system (described in detail above). As before, episodes of fictive swimming were evoked by a 1 ms stimulus which was kept at 1.5 times threshold from a stimulating electrode attached to the tail skin.

The firing patterns of the neurons recorded during fictive swim episodes were variable. For example the firing threshold, defined by the level of the injected depolarising current needed for the cell to start firing action potentials, varied. Some neurons had a low threshold for firing (fig.9 Di), while others had intermediate thresholds (fig.9 Dii), and yet others had much higher thresholds (fig.9 Diii). This range of firing properties appeared to be a feature of all neuron types, and was observed in both motoneurons (fig.7 B-C) and interneurons (fig.8 A-C). Furthermore, firing threshold appeared to correlate with how active a given neuron was

during fictive swim episodes; lower current threshold neurons would fire action potentials on nearly every cycle (fig.9 Aii) while higher current threshold neurons would fire more rarely during swimming (fig.9 Ci-Cii). This variability in firing properties would allow for the neurons to contribute differently to the CPG network output, with some being recruited only during fast swimming and others being active more reliably during swimming across a range of frequencies. This property has been described in detail for motoneurons in *Xenopus* at stage 42 (Zhang et al., 2011), however, less is known about the larval interneurons.

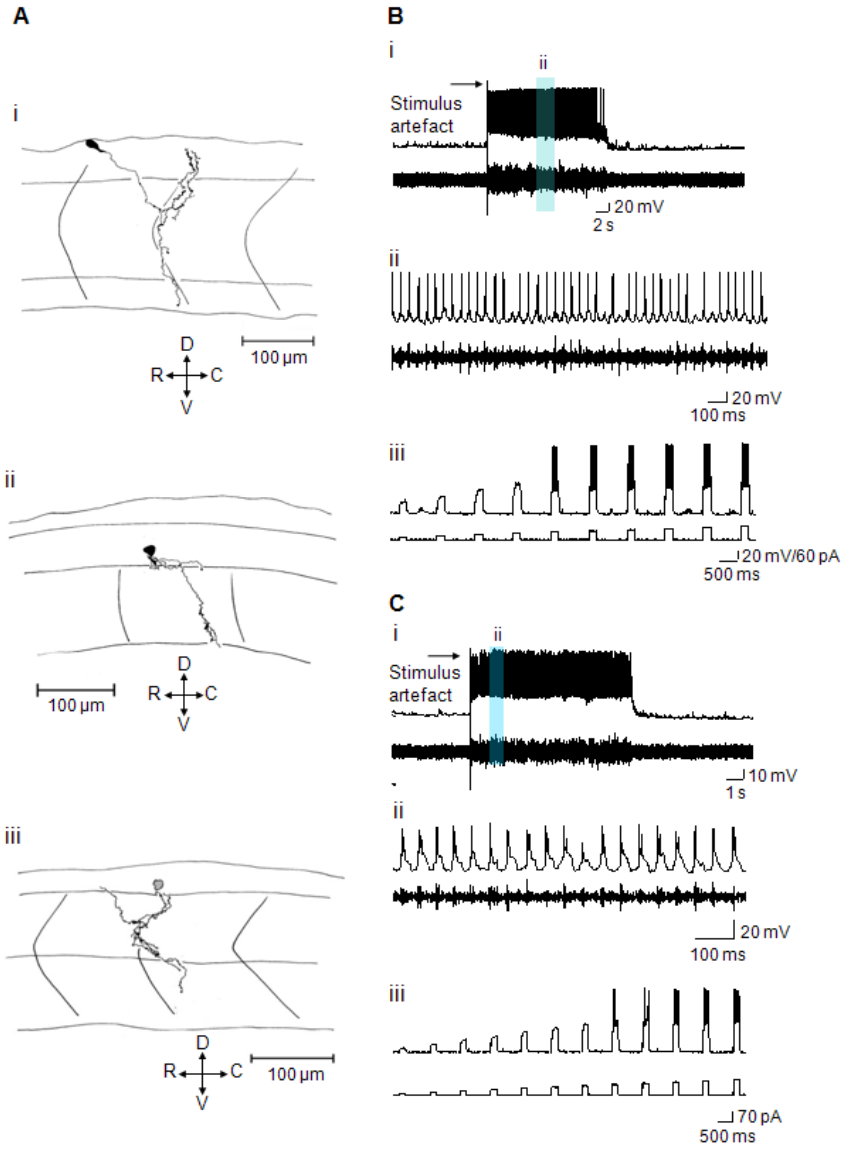

Figure 7. **Morphology and firing patterns of spinal motoneurons** A, lateral views of motoneurons labeled with neurobiotin during patch clamp experiments and subsequently traced using a camera lucida. Vertical lines indicate the divisions between the myotomes where the ventral roots are located. Grey colour indicates a post-experimentally lost cell body. The small directional diagrams indicate dorsal, ventral, rostral and caudal directions in the traces. The motoneurons have various innervation fields, Ai extending prominently to the dorsal muscle sector, Aii to the ventral and Aiii the medial. B and C, two examples of different firing patterns in motoneurons. Bi, a typical swim episode with paired recordings from a motoneuron (upper trace) and a ventral root (lower trace). Bii, expanded trace showing the ventral root bursts and the corresponding firing of the neuron. This cell fires one spike per cycle as opposed to that illustrated in C which fires multiple spikes per cycle (Cii). Biii and Ciii show the neurons' response to gradually increasing current steps. The first step is on 10 pA magnitude. Steps increase with 10 pA at the time. Both neurons have a low/medium firing threshold judged by the amount of injected current needed for the neuron to start firing. (for more detail on the firing properties of motoneurons of stage 42 tadpoles see Zhang et al., 2011).

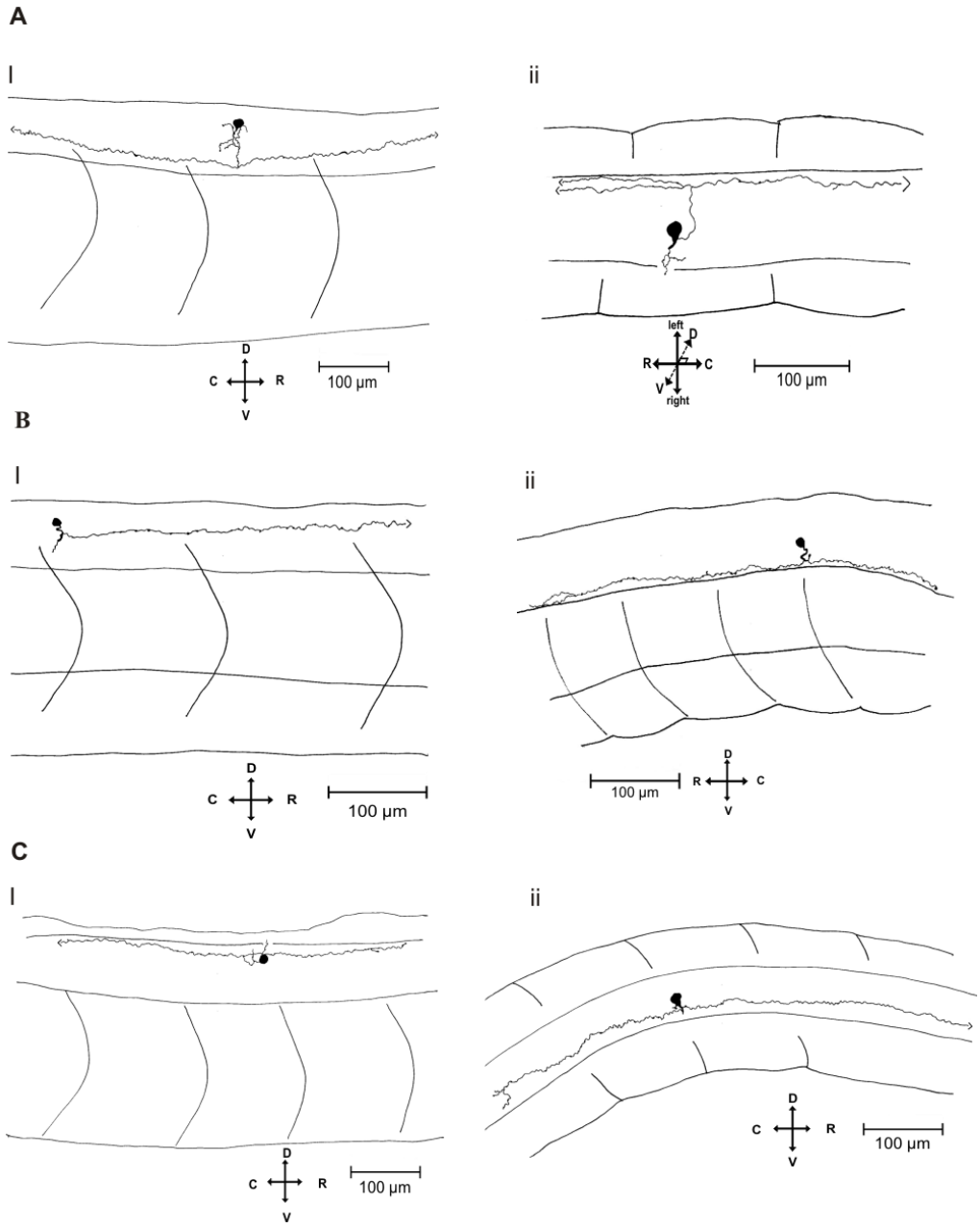

Figure 8. Interneuron morphology. Neurons labelled with neurobiotin during patch clamp experiments and subsequently traced using a *camera lucida*. Vertical lines indicated the divisions between myotomes where the ventral roots can be found. The small directional diagrams indicate dorsal, ventral, rostral ,and caudal directions in the figures. A., commissural interneurons (cIN). Ai, lateral view showing the neuron extending both descending and ascending processes in the spinal cord. Aii, dorsal view showing another neuron crossing the spinal cord to innervate the contralateral side. B., lateral views of ascending interneurons (aIN) showing the variation in axon length and number. Bi, having only one ascending axon and no descending and Bii having a short descending axon. C., one lateral and one dorsal view of descending interneurons (dIN). Both have ascending processes to some degree in addition to the descending. Cii illustrates how the dIN extends axons to innervate the ipsilateral side of the spinal cord as opposed to the cIN in Aii.

D-serine enhances the swimming CPG output by leading to more spontaneous activity and longer swimming episodes. In addition, D-serine (25-500 µM) causes swimming activity and burst intensities to wax and wane in an almost oscillatory fashion within an episode (fig.10 B). In the presence of D-serine the firing patterns of the neurons varies within episodes, in parallel with the waxing and waning of burst frequency and amplitude in the extracellular recordings. Rather than firing reliably on each cycle, neurons could be seen to drop out during a trough (fig.10 Bii red arrows) and fire multiple spikes during a more intense period of activity on the peak of an oscillation (fig.10 Biii, red arrows) in contrast to control conditions (fig.10 Aii). D-serine also increases the number of spikes per cycle, leading to neuron recruitment compared to control conditions (fig.10 Aii vs Biii). These effects could be achieved by D-serine modulating either the electrical properties of neurons and/or the strength of the synaptic connections. However the addition of D-serine (25-500 µM) had no obvious effect on the firing thresholds of spinal neurons (fig.11 Ai-Aii), and neither did the antagonist L-689560 (10 µM) irrespective of whether it was added before (fig.11 B) or after (fig.11 Aiii) D-serine (n=29). The fact that D-serine did not cause any detectable change in the firing threshold of the recorded neurons suggests that it exerts its excitatory effects on the system through another, presumably synaptic mechanism.

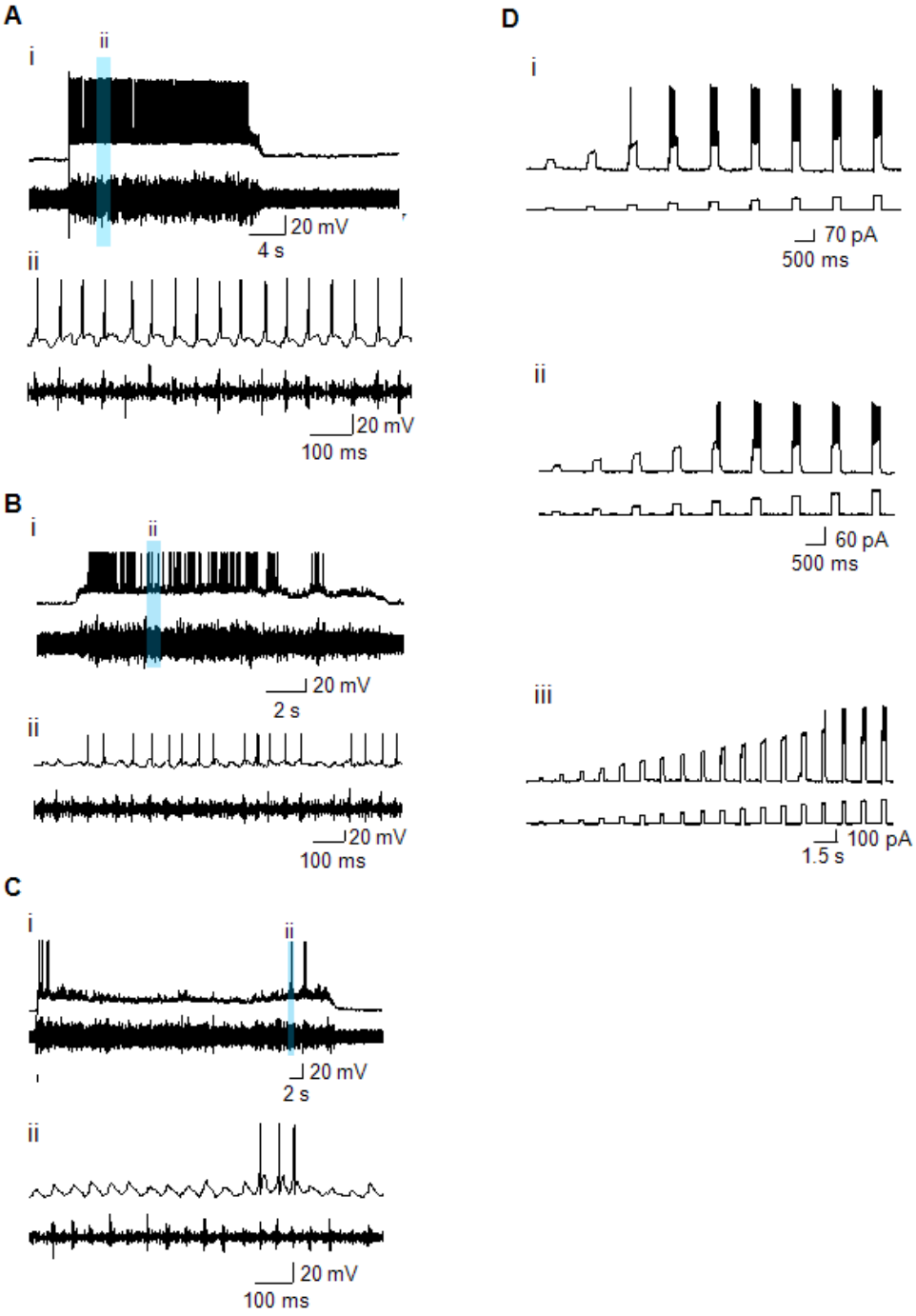

Figure 9. Firing pattern is dependent upon the firing threshold. The firing patterns of neurons vary with firing threshold defined by the amount of injected current needed for the neuron to fire. A, is a low threshold neuron. In the expanded trace Aii it can be seen to fire regularly on every cycle. B is a medium threshold neuron. In the expanded trace Bii it can be seen to fire on most cycles but missing out on a few, and C shows a high threshold neuron spiking only very rarely during swimming which can be seen in (Cii). D, Examples of firing thresholds illustrated by steps of increasing current (lower trace) and the neuron's response (upper trace). Di, low firing threshold, in this case 30 pA. Dii, medium threshold (50 pA), and Diii, high firing threshold (150 pA).

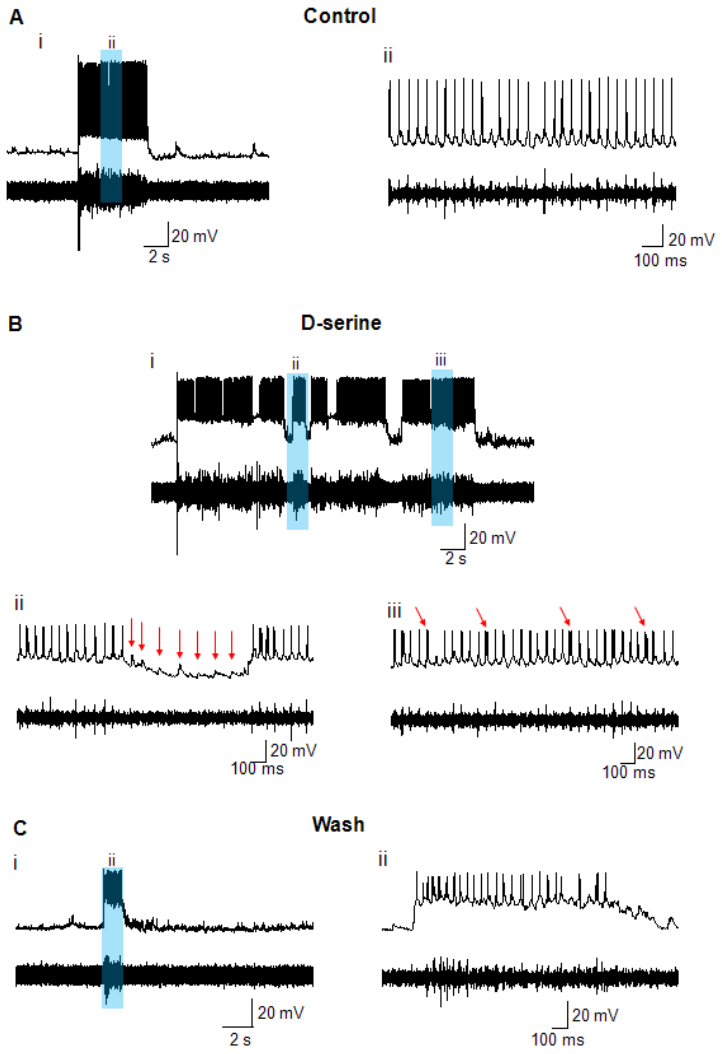

Figure 10.**D-serine changes the firing pattern of spinal neurons**. Ventral root recordings paired with patch clamp recordings from a neuron, in this case a motoneuron. A, shows a control episode of swimming, Ai a whole episode and Aii shows an expanded trace illustrating how the cell fired reliably, one spike per cycle. B, illustrates both how episode duration increased in D-serine (200 µM) and how the frequency waxed and waned more in comparison to control. Bii and Biii are expanded traces at different points in the episode in Bi showing that the neuron no longer fired on every cycle but rather followed the waxing and waning pattern seen in the ventral root recording. The spiking from the neuron dropped out completely when the intensity of the bursts decreased, however, it is evident that excitatory input is still present (red arrows) (Bii) and fired multiple spikes per burst when the bursts were more intense (red arrows) (Biii). C shows the effect of L-689560 on swim episodes. Episode duration decreased (Ci) and the neuron no longer fired as intensely (Cii), however some properties observed in D-serine remained such as the firing of multiple spikes per burst and a vague waxing and waning in activity. Furthermore, D-serine reduces spike height (compare Ai and Bi) however, in this example this effect is not washed off.

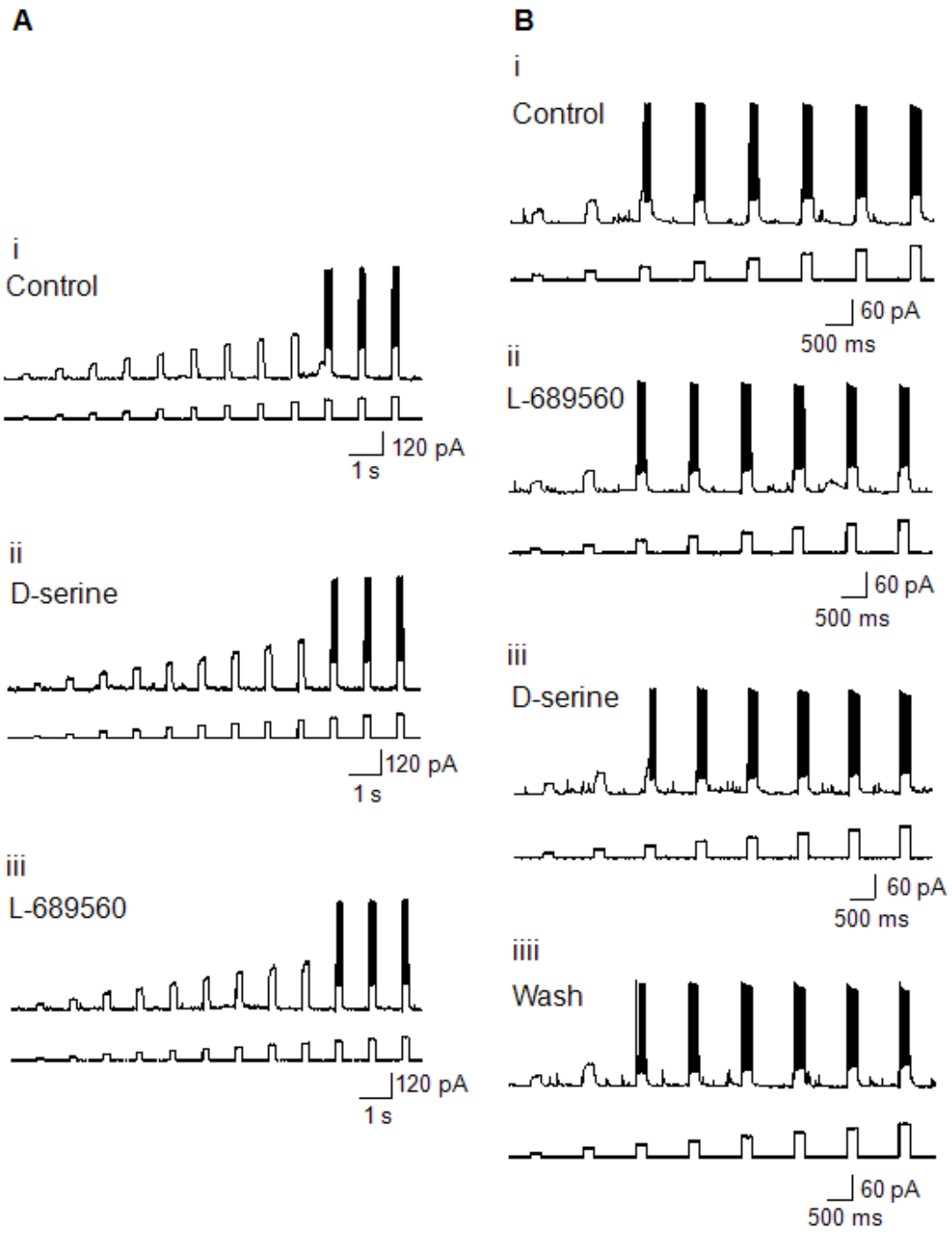

Figure 11. **D-serine has no effect on the firing threshold of spinal neurons**. A, the firing threshold of a neuron here defined as the amount of current needed for a cell to fire remains unchanged after addition of D-serine (Aii) and after subsequent addition of the NMDA glycine site antagonist L-689560 (Aiii). B, also when applied in the reverse order the threshold of the neurons remains unaffected.

Given that D-serine is an agonist at the NMDA receptor glycine site, it is likely that it causes these excitatory effects on the system by means of enhancing the NMDA receptor-mediated excitatory drive. In keeping with this idea, D-serine significantly increased the amplitude of the tonic depolarisation during swim episodes. In association with this, the spike height of the action potentials was significantly reduced, consistent with them being shunted by the increased excitation. 11 out of 20 experiments, in which the recording was sufficiently stable, were analysed for effects on tonic depolarisation and spike height. Addition of D-serine (25-500 µM) increased the tonic depolarisation to 117% (±4.3%) of control (fig.12 Aii, Bii and C, $P<0.05$, n=11). This increase could be reversed by the L-689560 (10 µM) or the NMDA antagonist D-AP5 (50 µM), to 98% (±8.3%) of control (fig.12 Aiii, and C, $P<0.05$, n=11). Furthermore, D-serine (25-500 µM) reduced spike height to 85% (±4.7%) of control (fig.12 Aii, Bii, and C, $P<0.05$, n=11), however this effect was only reversed by L-689560 (10 µM) in two experiments. The absence of a wash off of the effect on spike height may be due to deterioration of the neuron or of the seal in these long recordings. Further strengthening the idea that D-serine exerts its effects via a synaptic mechanism is the fact that there is little or no change in spike height between drug conditions when the neurons respond to injected current as opposed to the obvious effect on spike height during swimming episodes (compare fig.12 B and fig.13 A).

In addition, I investigated the relationship between spike frequency and injected current. D-serine caused a small, statistically insignificant increase in spike frequency in response to increasing current injections (fig.13 A). This effect was large during the first few increasing current steps and then appeared to get smaller and cease when the injected current reached above 50-60 pA above the neurons' firing threshold (fig.13 B, n=5). As has been shown above (fig.11), neurons have varying firing thresholds, some responding to low amounts of

current (10 pA) whereas others require current above 100 pA to fire action potentials. Furthermore, neurons vary in how strongly they respond to injected current, some firing only a few spikes in contrast to others which have a higher firing frequency. Hence firing frequency varied greatly from neuron to neuron, however, D-serine had a tendency to increase the firing frequency, in response to injected current just over firing threshold, in all neurons.

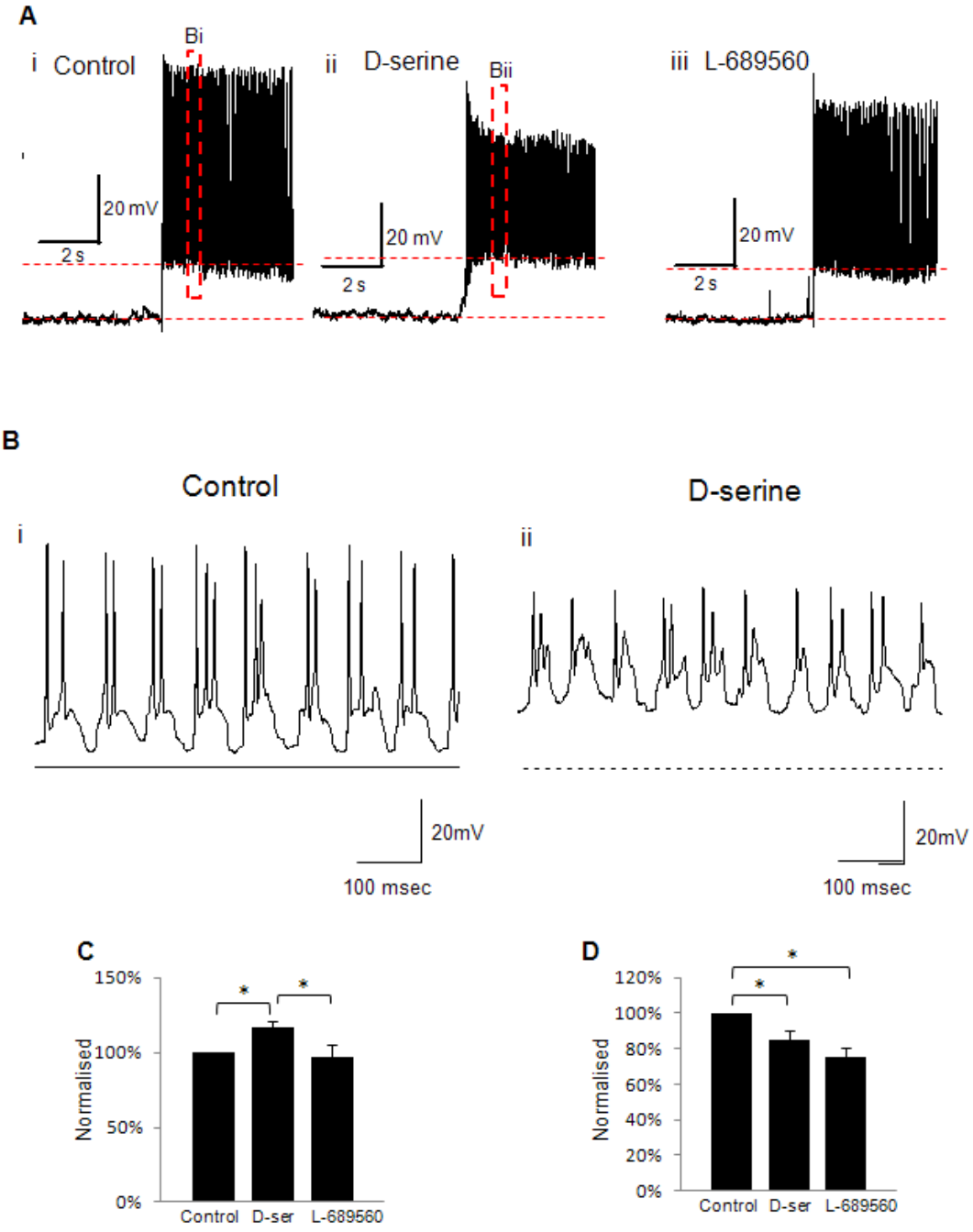

Figure. 12. **D-serine effects the tonic depolarisation and spike height**. A, D-serine (25-500 µM) (Aii) increased the tonic depolarisation to 117% (±4.3%) of control (Ai). This effect was reversed by L-689560 (10 µM) or D-AP5 (50 µM), to 98% (±8.3%) of control (Aiii, $P<0.05$, n=11). Effects on tonic depolarisation can also be seen in B, which in addition shows that spike height was affected by D-serine (Bii) decreasing it to 85% (±4.7%) of control (Bi). This effect was only reversed in 2/11 experiments (Biii). C, the tonic depolarisation was significantly increased by D-serine and could be reversed by L-689560 or D-AP5 ($P<0.05$, n=11). D, the effect of D-serine on spike height was significant ($P<0.05$, n=11), however could not be reversed by the antagonists. Spinal neurons recorded from where identified as 4 ascending interneurons, 1 descending interneuron, 1 motoneuron, 4 unidentified interneurons, and one unidentified neuron.

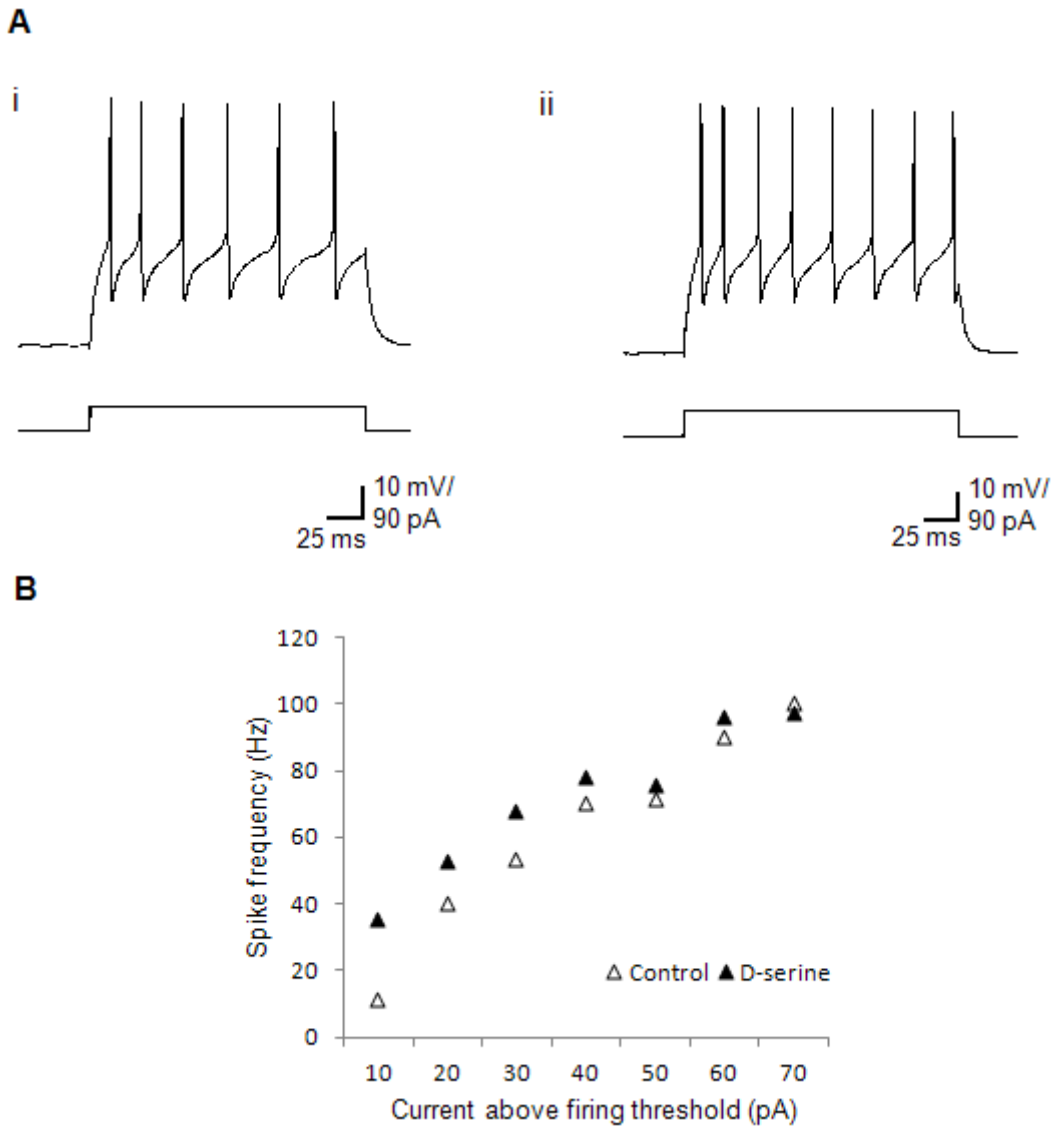

Figure 13.**D-serine affects spike frequency**. A, Spike frequency is dependent on the amount of injected current. D-serine causes a small increase in the neurons' spike frequency in response to 200 ms current pulses of increasing magnitude. B, this increase in frequency is large in response to lower current, then gets smaller with increasing injected current and is not seen when the injected current is >60 pA above firing threshold. (n=5, values are means). Spinal neurons recorded from where identified as 3 ascending interneurons, 1 unspecified interneuron, and one unidentified spinal neuron.

3.3 TTX-resistant effects of glycine site activation on spinal neurons

In addition to the effects on the firing properties of neurons, D-serine appeared to affect the membrane potential, membrane conductance, and the occurrence of spontaneous postsynaptic potentials in all neurons, irrespective of cell type. In order to investigate this further, spike-mediated synaptic transmission was blocked with (1μM) tetrodotoxin (TTX) and spontaneous inhibitory potentials were blocked with strychnine (5 μM) and bicuculline (20 μM). This allowed me to study the effects of D-serine on passive membrane properties and on the occurrence of miniature (m)EPSPs, which were presumed to be predominantly glutamatergic (Li et al., 2004).

3.3.1 Effects of D-serine on membrane potential

NMDA (50-100 μM) depolarises the membrane potential of spinal neurons by about 20 mV (Dale and Roberts, 1984; Scrymgeour-Wedderburn et al., 1997). The preceding results on the effects of D-serine on the synaptic drive underlying swimming suggested that it increased tonic depolarisation, presumably by occupying vacant glycine sites and increasing NMDA receptor-mediated excitation. However, the possibility that endogenous D-serine causes a tonic activation of glutamate receptors has not been addressed previously. D-serine appeared to cause a small depolarisation of the membrane potential which I decided to investigate further by blocking synaptic transmission with TTX. In the presence of TTX (1 μM), D-serine (50-100 μM) caused a depolarisation of the membrane potential within three to seven minutes after drug application. This initial depolarisation was of 3.1 (±1.1) mV in magnitude (fig.14 Ai, Bi, $P<0.001$, $n=14$) and it occurred at approximately the same time as the increase in the occurrence of spontaneous swim episodes in experiments where synaptic transmission had not been blocked. Prolonged application of D-serine led to further depolarisation of the membrane, however the recordings often became instable. A wash off of the effect of D-serine often

proved difficult to obtain and reversal seemed to work best when D-serine was applied only for a brief time with a maximum of ten minutes. Full or partial reversal was obtained in 8 out of 14 experiments by adding either the NMDA receptor glycine site antagonist L-689560 (10 µM) or the NMDA receptor antagonist D-AP5 (25-50 µM). The antagonists hyperpolarised the membrane potential by 0.86 (±0.26) mV (fig.14 Aii, Bi, $P<0.001$, $n=14$). In addition, when D-AP5 was added prior to D-serine in 5 experiments, D-serine did not depolarise the membrane potential as it did when applied on its own (fig.14 Bii, $P=0.4975$, $n=5$). These results strongly suggest that there is a tonic activation of glutamate receptors that is masked until D-serine is applied, which presumably results in an increase in current flow through the activated NMDA receptors. This idea is further strengthened by the observation that D-serine increases the membrane noise level, an effect which indicates the opening of ion channels, resulting in an increase in motor network excitability.

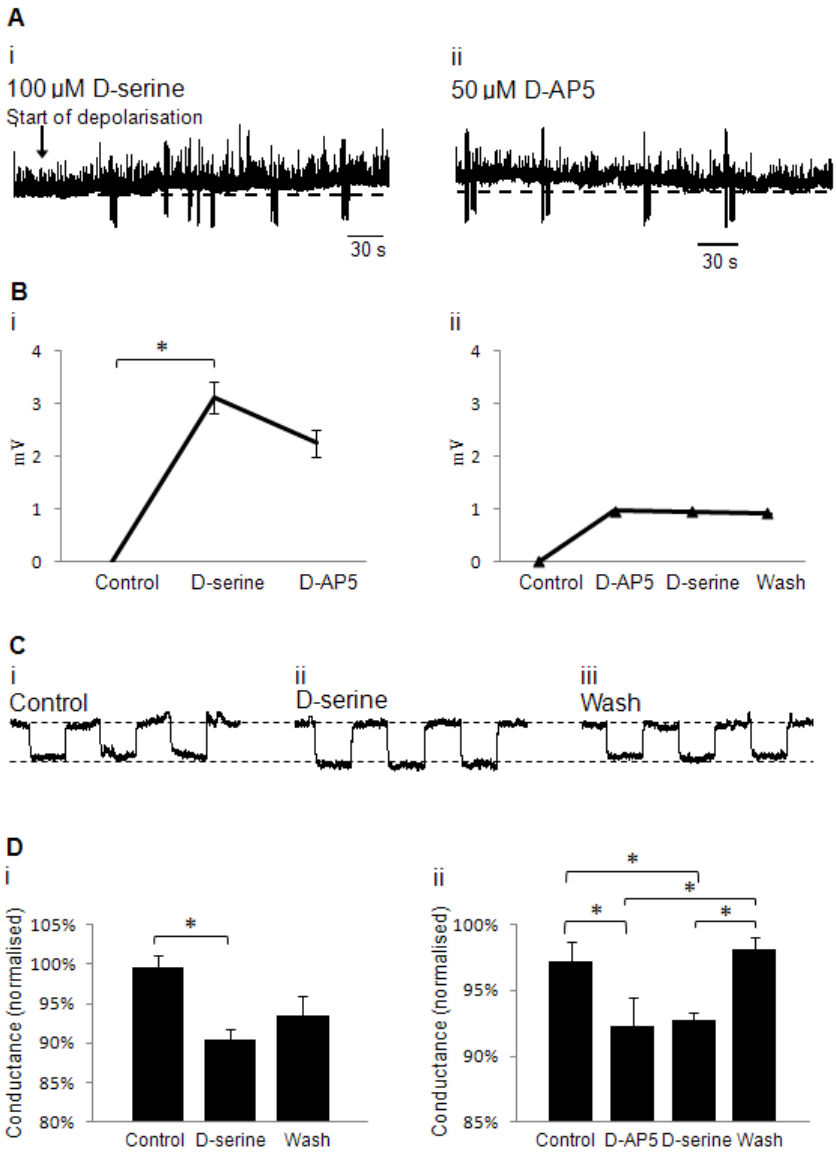

Figure 14. **D-serine affects the membrane potential and conductance of spinal neurons**. D-serine causes a small, depolarisation of the membrane potential (Ai), which can be reversed by adding the NMDA antagonist D-AP5 (Aii). Bi shows the membrane depolarisation increase and the partial reverse by the antagonist ($P<0.001$, $n=14$). Pre-treatment with D-AP5 prior to D-serine blocks these depolarising effects on the membrane potential (Bii). D-serine also leads to a paradoxical and reversible decrease in membrane conductance as displayed by negative current pulses in control (Ci), D-serine (Cii), and wash (Ciii). (note, the level of the membrane potential has been adjusted in for illustration purposes) This presumable conductance increase is caused by conductance pulses artificially hyperpolarising the membrane potential to a level at which magnesium ions can more fully block the NMDA ionophore -and hence give the appearance of a conductance decrease. Consequently, addition of the NMDA antagonist D-AP5 has the same effect on conductance as D-serine (Di vs. Dii) decreasing membrane conductance by 5.4 % (±1.8 %) however D-serine has no further effect when applied after the antagonist (Cii) and wash returned conductance to control levels (Dii, $P<0.05$, $n=5$). Values are means ± SEM, all experiments are conducted after synaptic potentials have been blocked with TTX..

3.3.2 Effects of D-serine on membrane conductance

The increase in membrane potential upon addition of D-serine was accompanied by a decrease in the membrane conductance of 10.2% (±1.4%), ($P<0.05$, n=17, fig.14 C and Di). Although this effect is seemingly paradoxical and the opposite of what might be expected following enhanced NMDA receptor activation by D-serine, it matches the effects seen after NMDA applications in previous studies (Dale and Roberts, 1984; Soffe and Roberts, 1989; Scrymgeour-Wedderburn et al., 1997). This conductance decrease could be explained by the conductance pulses artificially hyperpolarising the membrane potential to a level at which magnesium ions can more fully block the NMDA ionophore and hence give the appearance of a conductance decrease. The conductance decrease could be partially but not significantly reversed by adding the NMDA antagonist D-AP5. D-AP5 reduced the conductance by 3.3% (±2.1%) (fig.14 Dii). Moreover, pre-application of D-AP5 prior to adding D-serine caused a real decrease in conductance by 5.4% (±1.8%) (fig.14 Dii, $P<0.05$, n=5) as expected of an NMDA receptor antagonist. Subsequent addition of D-serine in these experiments failed to elicit any further effect on the membrane conductance and washing with normal saline brought the conductance back to pre-D-AP5 levels (fig.14 Dii, $P<0.05$, n=5).

These effects of D-serine on the membrane potential and conductance are small compared with NMDA alone, an unsurprising but not necessarily unimportant observation (for effects of NMDA on the *Xenopus laevis* tadpole neurons see: Soffe and Roberts 1989; Scrymgeour-Wedderburn et al. 1997).

3.3.3 Effects of D-serine on quantal release

In addition to the effects on membrane potential and conductance, an increase in the rate of spontaneous depolarising synaptic potentials was evident, suggesting that D-serine affected the quantal release of transmitter to increase the probability of miniature (m)EPSPs. This

could be explained by a depolarisation of synaptic terminals by D-serine. In order to investigate this possibility closer, the effects of D-serine on mEPSPs were examined in the presence of TTX (1 µM), bicuculline (20 µM) and strychnine (5 µM). Bicuculline and strychnine block GABAergic and glycinergic events respectively and, in addition to blocking mIPSPs, they should have similar effect to PTIO by removing the influence of NO on inhibitory transmission (McLean and Sillar, 2002). Hence, pre-treatment with PTIO was considered unnecessary and excluded in these experiments.

In the presence of TTX, strychnine, and bicuculline, D-serine (50-100 µM) increased the rate of mEPSPs measured as the number of events per minute in 3/5 experiments. When pooled this increase was calculated to 52.6% (±35.2%) (P=0.0979, n=5). Addition of the NMDA antagonist D-AP5 caused a decrease in the frequency of mEPSPs in 5/5 experiments. The decrease was measured to be 56.3% (±12.7%). These effects on spontaneous release were not quite significant when the data were pooled due to the large amount of variance between the experiments (fig.15 A and B, P=0.0979, n=5). However, when D-AP5 (50 µM) is added prior to D-serine the effects on spontaneous release the effect is completely absent compared to when D-serine is applied on its own (compare 15 Bi and Bii). In D-AP5 the occurrence of mEPSPs increased by 4.6% (±7.5%) and subsequent addition of D-serine lead to a further increase by 3.2% (±1.2%). None of these effects were significant (fig.15, P=0.2816, n=4). It should be noted that a cumulative probability plot in combination with a Kolmogorov-Smirnov test might have lead to a more accurate statistical judgement of the effects on spontaneous release, however due to acquisition- and analysis program compatibility problems these were not possible to carry out.

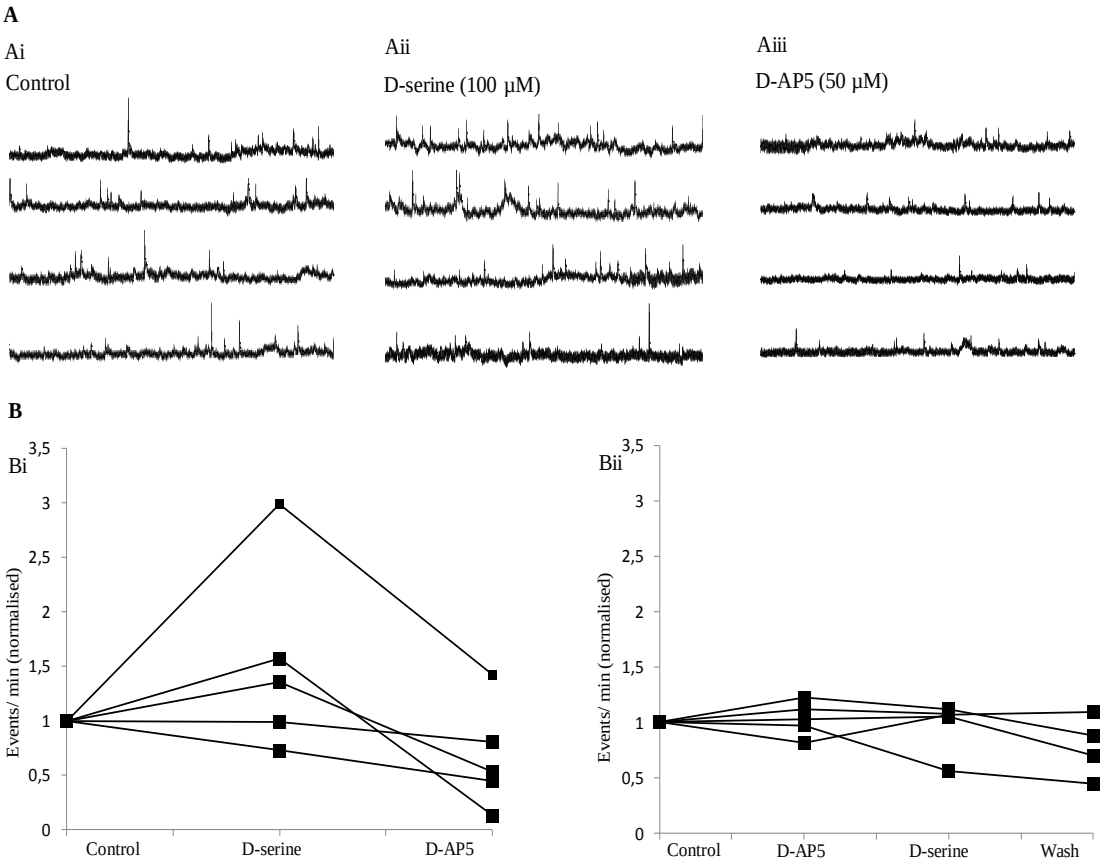

Figure 15. D-serine effects on vesicular release. D-serine leads to a statistically insignificant increase in the probability of vesicular release, or miniature excitatory post synaptic potentials (mEPSPs), in the presence of TTX, strychnine and bicuculline. Ai-Aiii, raw data traces four consecutive ones for each condition illustrating the effects of D-serine on the amount of mEPSPs. Bi, diagram showing the effect of D-serine and the antagonist D-AP5 on the occurrence of mEPSPs measured in events/minute for five individual neurons. D-serine increases the amount of quantal release insignificantly by 52.6% (±35.2%) which is decreased again by the NMDA antagonist D-AP5 by 56.3% (±12.7%) (P=0.0979, n=5). Strength of effect did not correlate with cell type. Bii, when the antagonist D-AP5 is added prior to D-serine any effect of D-serine on the amount of mEPSPs is blocked. Values are means ± SEM.

3.3.4 Effects of exogenous glycine on neuronal properties in the presence of TTX

To further investigate whether the effects of D-serine were mediated via the NMDA receptor glycine binding site, the effects of bath applied glycine itself were explored in the presence of TTX. Since glycine also is the major fast inhibitory neurotransmitter in the CPG playing an important role in mediating reciprocal mid-cycle inhibition during swimming (Dale, 1984; Soffe, 1989), the glycine receptor antagonist strychnine (5 µM) was applied prior to glycine to ensure it would not exert any undesired inhibitory effects. In addition, bicuculline (20 µM) was applied, as above, to block GABAergic synaptic transmission that could be affected by an up-regulation of the activity of NOS-positive neurons.

Similar to D-serine, glycine (100 µM) caused a depolarisation of the membrane potential of 3.6 (±0.11) mV in magnitude (fig.16 A and B, $P<0.001$, n=4). This depolarisation was slower in onset than that of D-serine and lacked the initial component that D-serine seemed to have. In contrast to D-serine, however, prolonged exposure (>10 min) to glycine did not lead to an irreversible depolarisation. This could explain why the depolarising effects of glycine were easier to reverse using L-689560 or D-AP5. D-AP5 (25-50 µM) caused a hyperpolarisation of 2.6 (±0.21) mV in magnitude in 4/4 experiments (fig.16 A and B, $P<0.01$, n=4). In contrast to D-serine, glycine had no significant effects on the conductance of the neurons recorded from ($P=0.5354$, n=4, fig.16 C). Whether this is due to a lower n-number, the fact that glycine seems to be a less potent agonist, or some other factor is unknown.

The effects of glycine on mEPSPs were also investigated. Glycine (100 µM) led to a small increase in mEPSPs by 15.3% (±15.3%) and D-AP5 led to a decrease in mEPSPs of 29.6% (±18.8%). However, none of these effects were statistically significant ($P=0.3335$, n=4, fig.16 D).

3.3.5 Effects of endogenous glycine on neuronal properties in the presence of TTX

It has been reported that increasing the endogenous levels of glycine by blocking the glycine transporter GlyT1b, which regulates glycine concentrations in the vicinity of the NMDA receptors, exerts similar effects on swimming activity to D-serine or glycine (Issberner and Sillar, 2007). Therefore, to investigate whether increasing endogenous levels of glycine would give rise to the same cellular effects as exogenous applications, the effects of the potent GlyT1b transporter inhibitor ALX 5407 on the membrane properties were assessed. In the presence of TTX, strychnine and bicuculline ALX caused a small but significant membrane depolarisation of 2.08 (±0.27) mV ($P<0.001$, n=7, fig.17 A). This depolarisation was partially reversed in 5/7 experiments by addition of D-AP5. D-AP5 caused a hyperpolarisation of the membrane potential by 1.37 (±0.44) mV however this decrease was statistically insignificant ($P>0.05$, n=7, fig.17 A). Furthermore, ALX caused a small but insignificant increase in input resistance of 3.4 (±2.4) %, which could be reversed to control levels by D-AP5 ($P=0.4009$, n=7, fig.17 B). Similar to D-serine and glycine, ALX had effects on the occurrence of spontaneous release. ALX increased the mEPSPs by 73% (±37.6%), and effect that was partially reversed by D-AP5 by 21.9% (±18.8%). Similar to D-serine and glycine these effects were not significant ($P=0.2253$, n=6, fig.17 C).

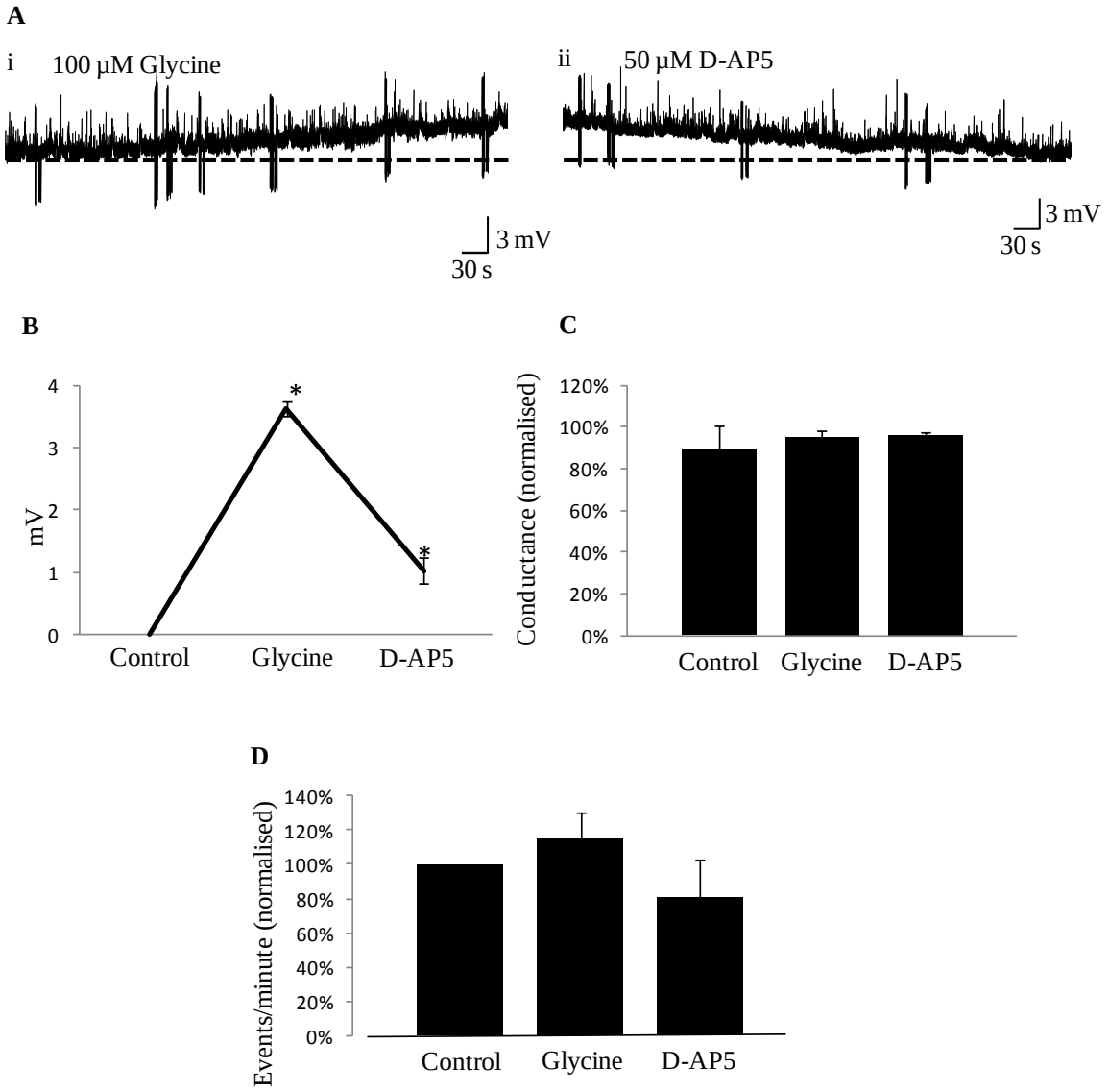

Figure 16. **Glycine mimics the effects of D-serine**. Effects of glycine in the presence of TTX (1µM), strychnine (5 µM), and bicuculline (20 µM). A and B, Similar to D-serine, glycine (100 µM) has a depolarising effect on the membrane potential of cells causing a depolarisation of 3.6 (±0.11) mV ($P<0.001$, n=4). This effect can be partially reversed by the antagonist D-AP5 (50 µM) which hyperpolarises the membrane by 2.6 (±0.21) mV ($P<0.01$, n=4). C and D, Glycine has no significant effect on membrane conductance (C, n=4) or the amount of mEPSPs (D, n=4). Values are means ± SEM.

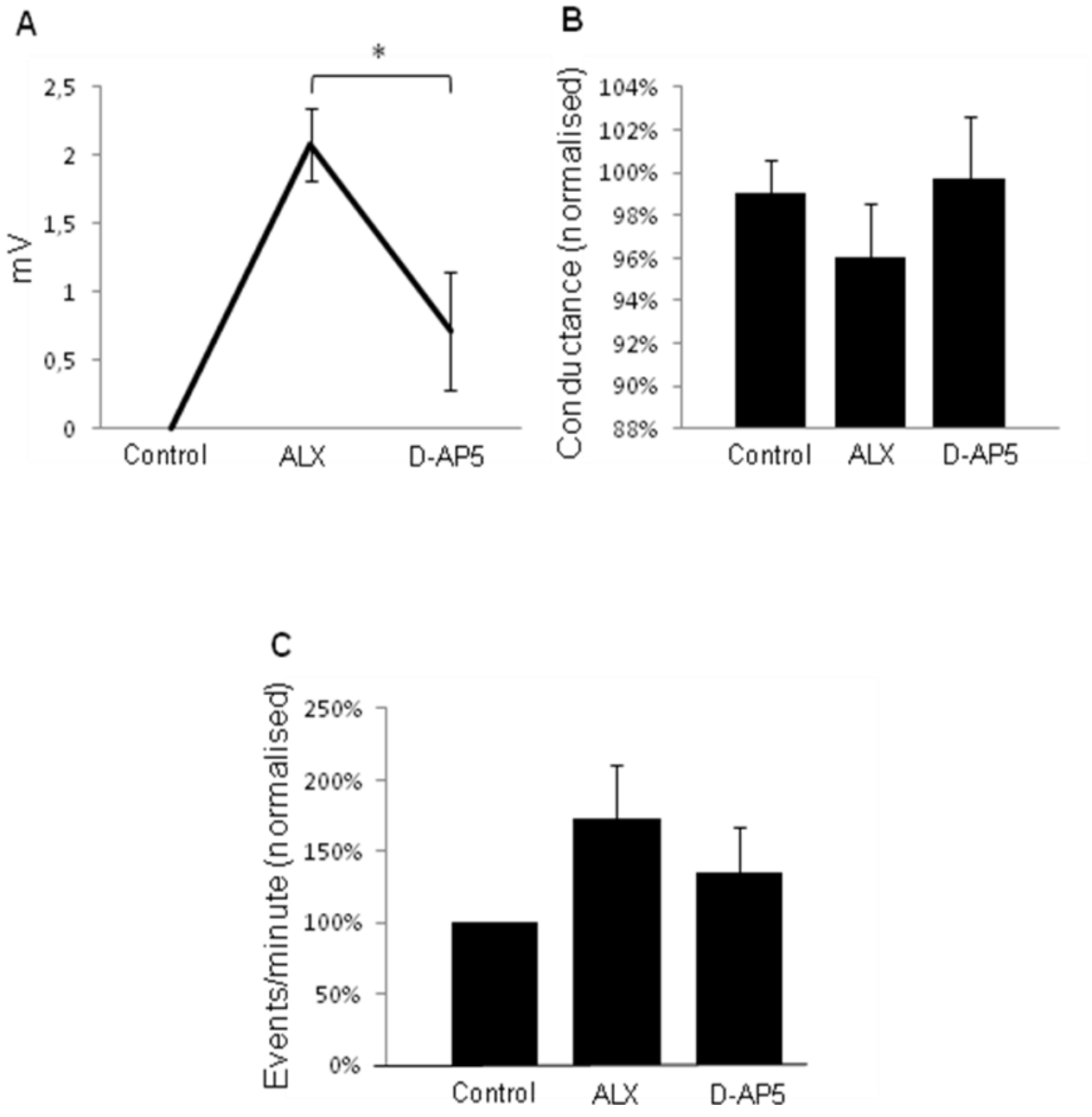

Figure 17. **Endogenous glycine mimics the effects of D-serine**. Effects of ALX in the presence of TTX (1 µM), strychnine (5 µM), and bicuculline (20 µM). A, ALX depolarises the membrane reversibly by 2.08 (±0.27) mV ($P<0.001$, $n=7$) reminiscent of effects caused by D-serine and glycine. Furthermore, ALX increases input resistance (B) and the amount of epsps (C) reversibly, however in neither case significantly. Values are means ± SEM.

3.4 D-serine enhances intrinsic membrane potential oscillations

3.4.1 NMDA-dependent intrinsic oscillations

In many species the activation of NMDA receptors triggers oscillations in the membrane potential that are TTX-resistant and therefore intrinsic properties of the neurons involved. In spinal motor systems, these oscillations were first described in the lamprey where they are dependent on the level of the membrane potential and the presence of extracellular Mg^{2+} (Sigvardt and Grillner, 1981; Grillner and Wallén, 1985). Similar NMDA-dependent, TTX resistant oscillations occur in *Xenopus laevis* hatchling stage 37/38 descending interneurons (dINs) after application of exogenous NMDA (20-100 µM). Reminiscent of what has been observed in the lamprey, these oscillations are also dependent on the level of the membrane potential and the presence of Mg^{2+} in the surrounding medium. Furthermore, the frequency of the oscillations in *Xenopus* lies at the lower end of the frequency spectrum for normal swimming (~10 Hz). Other types of interneurons and motoneurons at stage 37/38 also show signs of oscillatory behaviour in response to NMDA, however these oscillations are rarer and of a lower frequency (~5 Hz) (Li et al. 2010). In *Xenopus laevis* stage 42 larvae, NMDA (100 µM) produces a membrane depolarisation of about 20 mV (Sillar et al., 1997; personal observations). This depolarisation occurs also in the presence of TTX and is followed by an increase in membrane noise due to the opening of NMDA channels (Scrymgeour-Wedderburn et al., 1997). However, in some experiments an oscillatory behaviour of the membrane potential reminiscent of that previously reported for stage 37/38 could be seen (fig.18 A and C, n=5). The NMDA-dependent oscillations recorded in stage 42 larval neurons had a mean frequency of 11.1 (±1.2) Hz, similar to, or slightly higher than was reported for stage 37/38 dINs (Li et al., 2010). The oscillations were typically more prominent immediately after the NMDA-induced depolarisation and then gradually reduced in amplitude (fig.18 A).

Furthermore, the oscillations remained when the membrane potential was artificially hyperpolarised with tonic negative current towards the original, pre-NMDA, resting potential of the neuron (fig.18 C, n=5, also see fig.22 Bii). In lamprey, the amplitude of the intrinsic NMDA-dependent oscillations is dependent on the voltage level of the membrane. Artificially hyperpolarising the membrane potential with tonic negative current leads to an increase in oscillation amplitude (Wallén and Grillner, 1987). This effect on amplitude was not consistently observed on the NMDA-dependent oscillations in stage 42 tadpoles.

The occurrence and frequency of the oscillations seen in *Xenopus* stage 42 appeared to be dependent on neuron type as reported by Li et al. in 2010, however the n-number in the study presented here is too low to statistically confirm this (fig.18 B, C, and D). Similar to stage 37/38, these preliminary data from stage 42 suggest that the dIN demonstrate more robust oscillations of a slightly lower frequency upon NMDA application than other interneurons. (fig.18 B, C, and D, n=1). Unfortunately, no motoneurons were obtained to be analysed for this part of the study.

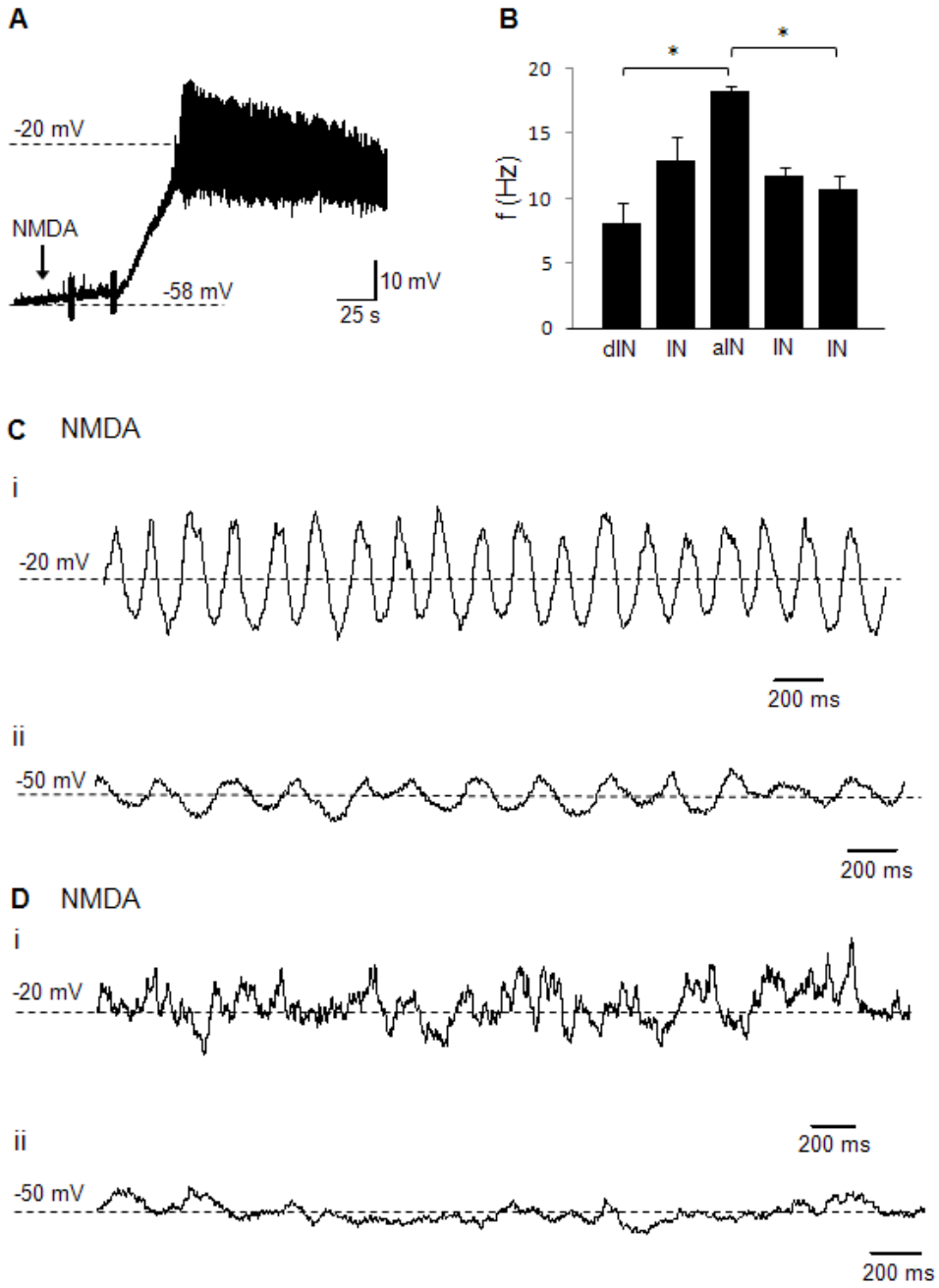

Figure 18. **The effects of 100 µM NMDA in the presence of 1 µM TTX.** A, in stage 42 *Xenopus* tadpoles NMDA produces a ~20 mV membrane depolarisation which is followed by an increase in membrane noise and the appearance of oscillations in the membrane potential. The frequency of these oscillations varies slightly between neurons and could be cell type dependent, as is illustrated in B displaying the oscillation frequency of five neurons, (B, $P<0.01$ and $P<0.05$, n=1 for each neuron) however, the n-number is far too low for these findings to be more than indications. These oscillations are more clearly visible in some cells than others, compare C and D. Furthermore, in neurons where they are clearly visible, the amplitude is higher just after the depolarisation and then deteriorates (A). The amplitude of these oscillations could be dependent on membrane potential (Ci and Cii) and (Di and Dii), however, in this study the data were not unanimous enough to draw such a conclusion.

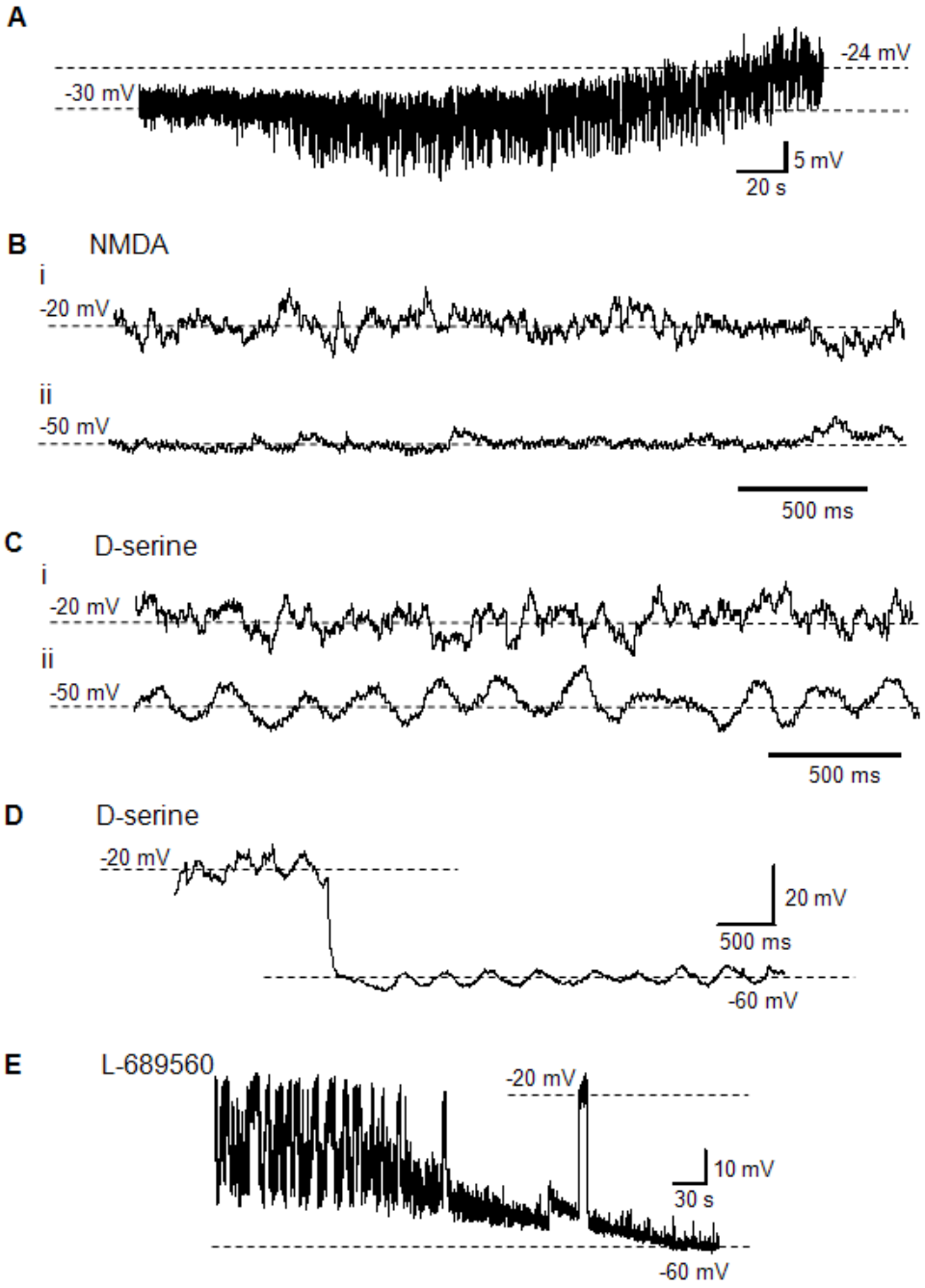

Figure 19. **The effects of 100 µM D-serine on the NMDA-dependent membrane voltage oscillations in the presence of TTX**. A, D-serine causes the membrane oscillations to become more prominent, followed by a small depolarisation in addition to that caused by NMDA. B, in some cells NMDA fails to bring out oscillations clearly. Holding the cell at a more hyperpolarised level by injecting negative current to bring it closer to its original resting potential also fails to bring out these oscillations (Bii). When D-serine is added, oscillations become discernable (Ci) and even more so when the cell is held at a more hyperpolarised membrane potential (Cii). This is further illustrated in D where the membrane potential is artificially hyperpolarised by 40 mV. These oscillations can be abolished by adding 10 µM of the NMDA glycine site antagonist L-689560 which also hyperpolarises the membrane (E).

3.4.2 D-serine has additional effects on intrinsic NMDA-dependent membrane oscillations

Bath application of D-serine (100 µM) in the presence of an NMDA-induced depolarisation, in preparations where there were no or only weak NMDA-dependent oscillations, caused not only a small additional depolarisation (fig.19 A, n=5), but also made the NMDA oscillations more pronounced (fig.19 B and C). These NMDA- and D-serine-dependent oscillations were slightly lower in frequency than those dependent only on NMDA, 3.5 (±0.39) Hz. Additionally, these D-serine oscillations appeared to have a range of membrane potential where they work optimally, approximately between -20 mV and -60 mV. Indeed, these oscillations seem to become slower and more robust at lower membrane potentials (fig.19 C and D, also see fig.22 Aii). However, the amplitude of the D-serine oscillations was generally unaffected by membrane potential, although there was a large variation in this parameter between experiments (fig.22 Bii). The NMDA- and D-serine-dependent oscillations could be disrupted by switching to Mg^{2+} -free saline (fig.21 Aii) or abolished by the addition of the antagonist L-689560 (10 µM) (fig.21 B, n=4).

The finding that D-serine can induce or modulate oscillatory membrane properties argues in favour of the suggestion that the NMDA receptor glycine site is not merely a binding site which is fully occupied to assure a full NMDA receptor response at all times. Instead, it suggests that an agonist of this site, be it D-serine or glycine, is kept at sub-saturating levels so that when these sites are fully occupied, as in this case by D-serine, intrinsic membrane potential oscillations are triggered.

3.4.3 D-serine can induce slow frequency membrane oscillations

Interestingly, in two preparations another type of slower oscillations was evident following the addition of D-serine (fig.20, Aii). These oscillations had a frequency of 0.8 (±0.008) Hz

and were reminiscent of those produced by 5-HT in the presence of NMDA (fig.20 Aiii). These 5-HT- and NMDA- dependent oscillations have been described previously and the proposed mechanism involves 5-HT altering the region of negative slope conductance in the I-V relationship of neurons by modulating the Mg^{2+} block of the NMDA ionophore, thereby facilitating membrane bi-stability (Reith and Sillar, 1998; see also Wallén and Grillner, 1987). These NMDA- and 5-HT-dependent oscillations can be blocked by the NMDA antagonist APV or by removing Mg^{2+} from the bath medium, they operate optimally between two voltage levels, and they have, due to their slow frequency, been suggested to modulate swimming over several consecutive cycles by boosting the excitation and lead to a slow oscillatory pattern of swimming (Scrymgeour-Wedderburn et al., 1997). In this study, application of 5-HT (2 µM) produced oscillations with a mean frequency of 0.76 (±0.02) Hz, lower than that of D-serine (fig.22 Ai, $P<0.05$, $n=3$). These oscillations were blocked by the NMDA glycine site antagonist L-689569 (10 µM) indicating that the occupation of the NMDA glycine site, possibly by D-serine, is of vital importance for the maintenance of these oscillations as well (fig.20 B).

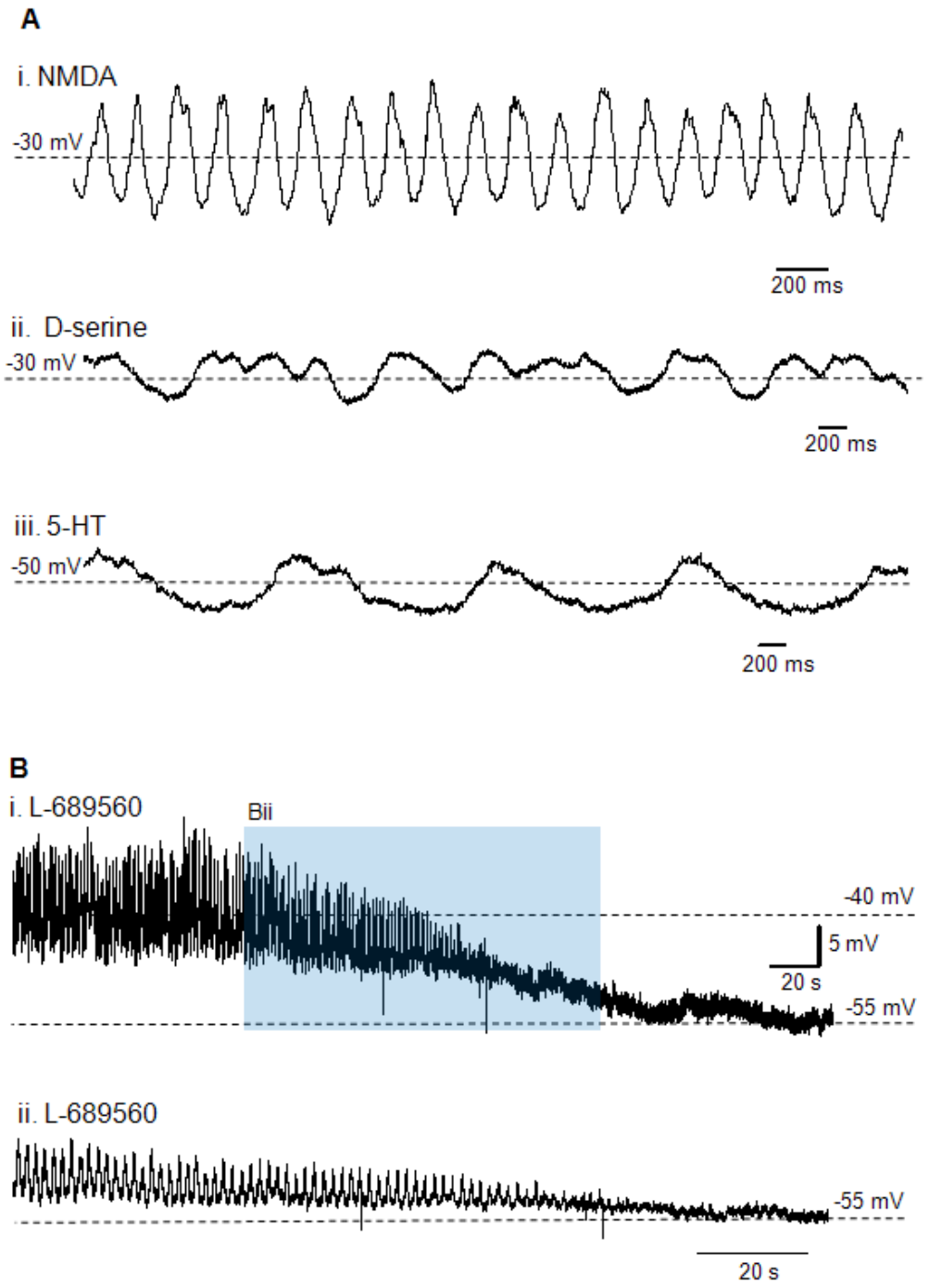

Figure 20. Effects on oscillation frequency by NMDA, D-serine, and 5-HT. NMDA produces fast oscillations of a frequency of 5.3 (±0.6) Hz (Ai). Adding D-serine leads to oscillations of a slower frequency 3.5 (±0.39) Hz (Aii) and 5-HT produces oscillations that are even slower at a frequency of 0.76 (±0.02) Hz (Aiii). All these oscillation frequencies are significantly different from each other ($P<0.05$, $P<0.01$, and $P<0.001$, n=5 for NMDA and D-serine and n=3 for 5-HT). All oscillations could be blocked by adding the NMDA glycine site antagonist L-689560 (Bi) for expanded trace see (Bii).

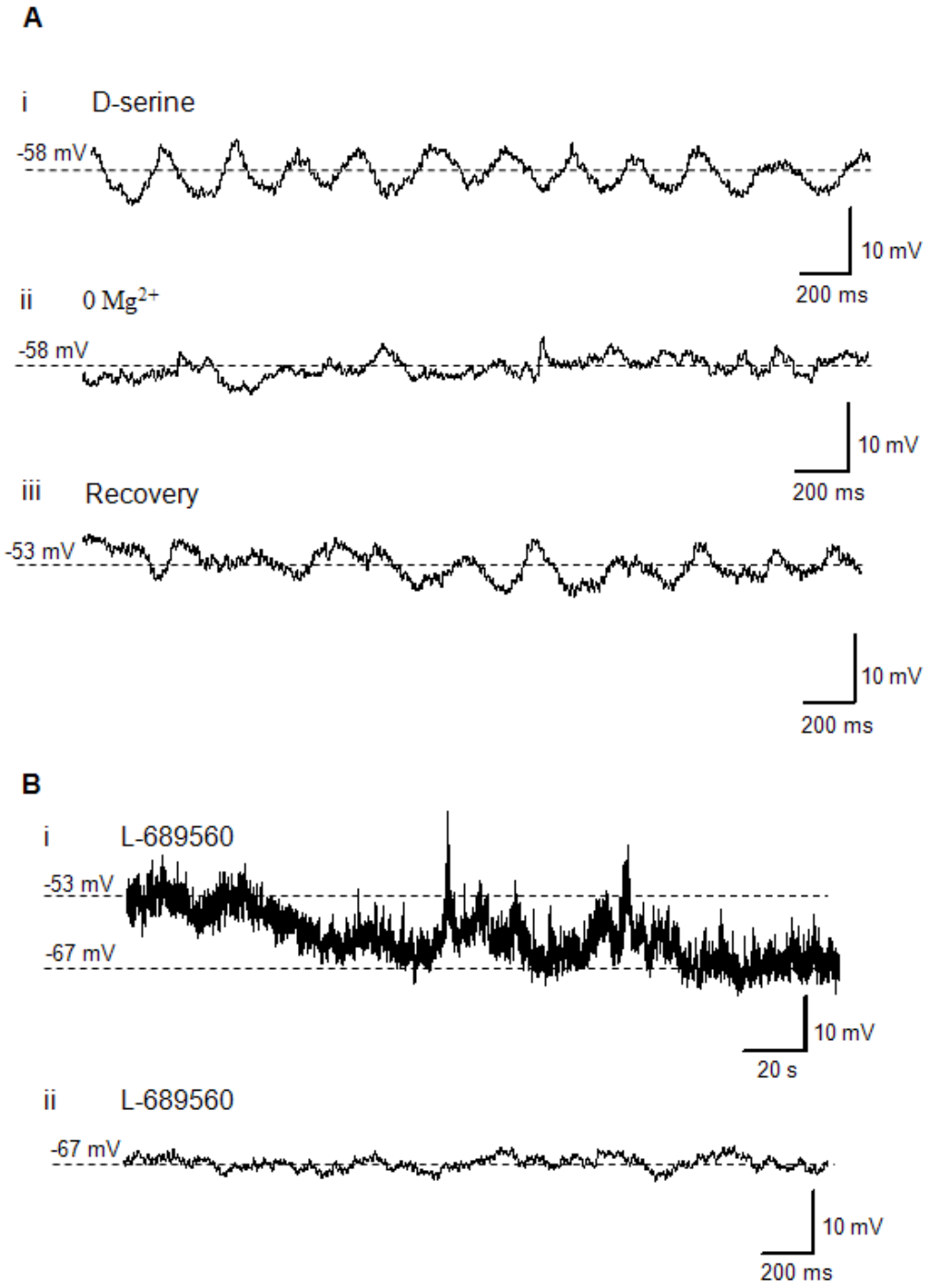

Figure 21. Effects of Mg^{2+} and L-689560 on TTX-resistant membrane oscillations. A, membrane potential oscillations became apparent in D-serine when the membrane potential was artificially hyperpolarised to -58 mV (Ai). These oscillatory properties were disrupted in Mg^{2+} free saline (Aii). Oscillations recovered when Mg^{2+} was once again added to the bath solution (Aiii). B, again, these membrane potential oscillations could be blocked by the glycine site antagonist L-689560 (Bii). L-689560 hyperpolarised the membrane potential (Bi).

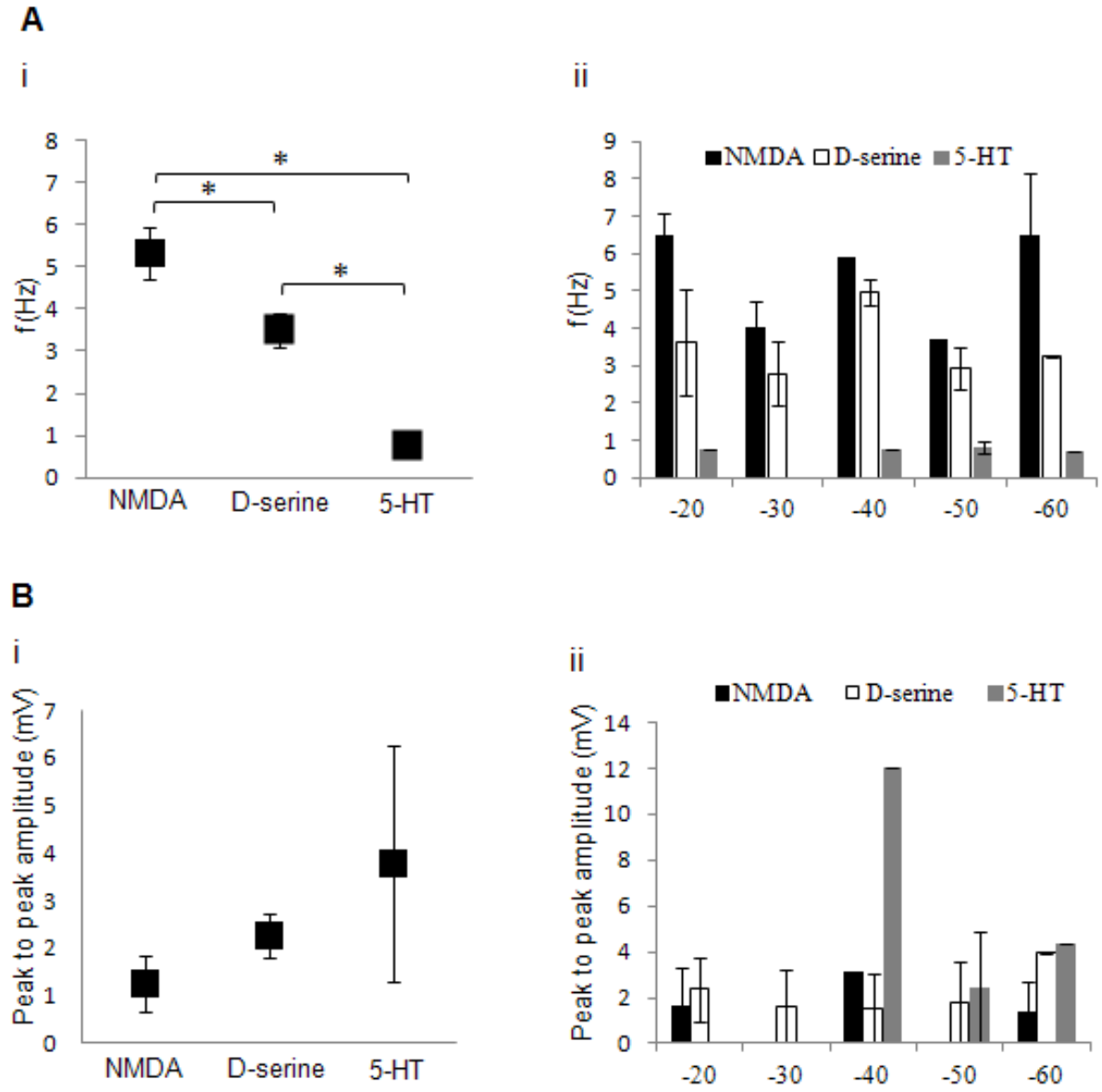

Figure 22. Effects of NMDA, D-serine, and 5-HT on oscillation amplitude and frequency. A, mean frequency with variance of membrane oscillations in NMDA, D-serine and 5-HT. Ai, NMDA produces oscillations with a mean frequency of 5.3 (±0.6) Hz, D-serine slightly slower oscillations of 3.5 (±0.4) Hz, and 5-HT even slower oscillations of 0.76 (±0.02) Hz. Frequencies are significantly different from each other in all conditions ($P<0.01$; $P<0.001$; $P<0.05$, n=5). Frequency also varies slightly but insignificantly between different membrane voltage levels for all drug conditions (Aii, n=5). B, oscillation amplitude varies slightly but insignificantly between drug conditions (Bi). Greater variations in amplitude are found within drug conditions (Bii) however the n-number for each condition is insufficient to draw any conclusions from this. Values are means ± SEM.

4. Discussion

4.1 The effects of D-serine on fictive swimming and possible mechanisms underlying its modulatory effects

In this study I have examined the modulatory effects of D-serine on fictive swimming in *Xenopus laevis* tadpoles at the larval stage 42. I have successfully repeated the work of Issberner and Sillar (2007) showing that D-serine, by acting at the NMDA glycine site boosts fictive swimming by increasing the duration of fictive swim episodes and leading to a swim pattern that varies more in amplitude and frequency within each episode in comparison to control. In addition, there is an obvious increase in the number of spontaneous swim episodes, a clear indication that D-serine is an excitatory modulator of the system that enhances locomotor output, and that there are vacant NMDA glycine sites at rest which, when activated, cause additional NMDA receptor activation.

Furthermore, I have extended previous work by means of whole cell patch clamp recordings of spinal neurons and explored how the effects of D-serine modulation are expressed at the cellular and synaptic levels. I have found that D-serine leads to an increase in tonic depolarisation along with a decrease in spike height during fictive swimming episodes. These effects resemble those caused by exogenously applied NMDA where the underlying cause is a summation of EPSPs (Dale and Roberts, 1984). It is reasonable to propose that positive modulation of the NMDA glycine site would increase current flow through NMDA receptors to mediate its effects on the swimming system. Indeed, as previously mentioned and suggested in fig. 15 Aii, D-serine also increases the membrane potential noise level in recordings which is indicative of the opening of ion channels, in this case most likely NMDA channels. Furthermore, the small increase that can be seen in spike frequency in response to increasing current injection in D-serine in comparison to control is in agreement with D-serine

causing a membrane potential depolarisation leading to a small shunting of spikes and an increase in spike frequency.

In keeping with its effects on fictive swimming D-serine also, as would be expected, depolarised the membrane potential when synaptic transmission had been blocked with TTX, an effect that is similar to, though much smaller than, the effect observed when exogenous NMDA is applied to the system (Scrymgeour-Wedderburn et al., 1997). In experiments where synaptic transmission had not been blocked, this effect paralleled the increase in the number of spontaneous swimming episodes, suggesting that the depolarisation brings the system closer to the activity initiation threshold. These depolarising effects of D-serine again suggest that there is an underlying tonic activation of glutamate receptors which is masked and only becomes apparent when the NMDA glycine site is fully occupied.

A noteworthy issue in a lot of these experiments was the absence of a wash-off of the depolarising effect of D-serine on the membrane potential. Although partial wash-off was obtained in several experiments, the membrane potential effect rarely reversed completely. A possible explanation for this result could be that there was also a small continuous depolarisation which tends to occur over time in long recordings. Alternatively, D-serine enhances NMDA channel openings and hence increases the influx of Ca^{2+} into neurons. Over time, intracellular Ca^{2+} concentrations could build up to damaging levels and create a maintained depolarisation of the membrane potential. Since a wash off effect was more likely to occur when D-serine was left on for a shorter period of time (<10 min), this seems a likely explanation for the results reported here. However, if this was the case, application of NMDA should reasonably have a similar, if not even more damaging outcome; however this has not been reported previously in this system. In addition, in experiments where glycine was applied instead of D-serine, there was no uncontrolled depolarisation or instability and wash-

off effect was more readily obtained, suggesting that D-serine has effects on the system that are perhaps not caused by its binding to NMDA receptors.

Interestingly, D-serine has been shown to be a likely pro-death signal in cells. It has been demonstrated that the levels of serine racemase (SR) are increased in the hippocampus of patients with Alzheimer's disease (AD) and *in vitro* studies have shown that amyloid β (Aβ), the neurotoxic misfolded protein characteristic of AD pathology, can stimulate the synthesis of D-serine by microglia (reviewed in Martineau et al., 2006). DAAO has been shown to have a protective action against these Aβ-mediated neurotoxic effects (Wu et al., 2004; reviewed in Martineau et al., 2006). Moreover, DAAO has been shown to prevent neuronal death in ischemic brain slices (Katsuki et al., 2004 reviewed in Martineau et al., 2006). These findings would suggest that D-serine can be particularly damaging to cells and could provide an explanation for why the effects of D-serine and not NMDA, for example are difficult to reverse even though their effects on the system are, at least initially mediated through the same receptor.

The proposal that D-serine mediates its effects via an enhancement of excitatory mechanisms is further supported by the findings that D-serine seemed to increase the probability of quantal release in a majority of the preparations where synaptic transmission has been blocked. Although this increase overall is not statistically significant, probably due to a large variation between preparations, it certainly suggests an increase in excitability of the neurons in the system and implies that D-serine directly activates NMDA receptors by binding to vacant glycine sites. Furthermore, the variability in the magnitude of the D-serine-mediated effects on spontaneous release could suggest that certain types of neurons respond more to D-serine modulation at the NMDA glycine site, perhaps depending on receptor density. Li et al (2010) found that dINs specifically were responsible for providing the underlying NMDA-mediated

oscillatory rhythm that drives swimming, suggesting that this neuronal type is more responsive to effects mediated by the NMDA receptors (Li et al., 2010). Although this remains speculative, perhaps D-serine also has greater impact on the dINs than it has on motoneurons or other types of interneurons.

In addition, there is also a possibility that an NMDA mediated increase in mEPSPs could be masked by higher amplitude AMPA receptor-mediated components. However, an attempt at selectively measuring the effects of only NMDA mediated components (disregarding any events <100 ms in duration) did not produce a result that differed much from the original analysis, probably due to a large variation in the increase of mEPSPs between experiments. Another possibility is that low amplitude NMDA components are masked by the increase in the tonic depolarisation.

The effects on vesicular release would suggest that D-serine exerts its effects on the pre-synaptic neuron by binding to pre-synaptic NMDA receptors increasing Ca^{2+} influx and thus increasing the probability of vesicular release. The absence of an effect on mEPSP amplitude indicates that it does not affect the loading of the vesicles with neurotransmitter, and additionally, that D-serine does not exert any post-synaptic effects.

Another observed effect of D-serine that has previously been reported to result from NMDA receptor activation (Dale and Roberts, 1984; Scrymgeour-Wedderburn et al., 1997) was a change in conductance. Although the observed change was a decrease in conductance, indicative of a closing rather than an opening of ion channels, this is an anticipated effect that has been seen in other systems as well as the tadpole and a plausible explanation is that the negative current pulses used in the experiments take the membrane potential down to a level where Mg^{2+} is better able to block more NMDA ionophores, hence creating a paradoxical decrease in conductance. Most importantly, this effect of D-serine is blocked by the NMDA

receptor antagonist D-AP5 which has the same effects on conductance as D-serine when applied on its own, further suggesting a tonic activation of receptors being the underlying cause of the excitatory effects of D-serine. In this case, however, it is reasonable to assume the decrease in conductance is real and accounted for by the blocking of NMDA channels by the antagonist. This effect on conductance is a strong indication that D-serine affects neurons by changing their membrane properties. Another possible explanation for the paradoxical conductance decrease could be that D-serine blocks a leak K^+ conductance however no current evidence exists to support this idea.

Considering that these results might suggest a masked tonic activation of NMDA receptors, it is interesting to speculate upon where the glutamate would be released from to maintain this tonic activation. One possibility is that there is an alternative, and perhaps more proximal source of glutamate responsible for the tonic activation, however, another option would be that glutamate is released from the dINs and the tonic activation is then unmasked by D-serine released from a more local area to the receptors. This idea would fit with the findings in other species that D-serine is mainly released by astroglia and considered a 'gliotransmitter' (Schell et al., 1995). Another question is where these tonically activated NMDA receptors might be expressed. Tonic activation of extrasynaptic GABA receptors has been reported (e.g. Otis et al., 1991; Valeyev et al., 1993; Hausser et al., 1997; for review: Farrant and Nusser, 2005) and it is therefore not entirely unrealistic that there could be a tonic activation of extrasynaptic NMDA receptors as well. However, evidence has accumulated for extrasynaptic NMDA receptors to activate pro-death pathways in cells (Hardingham et al., 2002; for review: Hardingham and Bading, 2010). Hence, it would appear that evidence is against a tonically masked extrasynaptic NMDA receptor activation mediating excitation and a boost to the swim circuitry. One possible way to discern how proximal the postsynaptic receptors are to

the presynaptic neuron would be to analyse the kinetics of the rising phase of the mEPSPs. A rapid rising phase would suggest a close connection whereas slower kinetics would indicate a more distal position of the receptors.

4.2 D-serine: a more potent ligand than glycine?

In other systems, D-serine has been described as a more potent agonist at the NMDA glycine site than glycine itself. For example Mothet et al. (2000) report that in some parts of the brain D-serine is likely to be the major agonist at this site since experimental degradation of D-serine leads to a reduction in NMDA transmission while reduced glycine levels leave NMDA transmission unchanged (Mothet et al., 2000, reviewed in Mustafa et al., 2006). It is conceivable that this occurs also in the tadpole locomotor system since in this study D-serine was found to exert more prominent effects on the system than glycine (in the presence of strychnine), irrespective of whether glycine was added exogenously or increased endogenously by blocking its uptake by selective inhibition of the glycine transporter GlyT1b with ALX. For example, D-serine led to an effectively irreversible depolarisation of the membrane potential whereas the same effects of glycine were more often reversed, also when mediated via the selective Glyt1b transporter blocker ALX. Furthermore, D-serine leads to a significant change in conductance, a parameter that neither glycine nor ALX significantly changed. These findings suggest not only that the NMDA glycine site is available for modulation but that this modulation might be carried out more efficiently by D-serine.

However, whether D-serine is an endogenous modulator at the NMDA glycine site in the tadpole spinal cord or not remains to be proven since comparing its effects to those mediated by glycine only implies that this is the case. There have been attempts in the past to increase endogenous levels of D-serine by means of adding DAAO, however these attempts have been problematic and unsatisfactory (Issberner, unpublished observations, personal

communication). Perhaps the reason for this is that DAAO increases the levels of D-serine uncontrollably and leads to neurotoxicity quicker than D-serine does when bath applied.

Another way to address the question would be to, inhibit the uptake of D-serine into the glial cells, presumably the astroglia. In other systems, uptake of D-serine has been suggested to be regulated by the sodium-dependent alanine-serine-cysteine-1 receptor (ASC-1) since, for example, knock-out of this receptor in mouse greatly impedes the uptake of D-serine (Rutter et al., 2007) and can lead to NMDA receptor-mediated hyperexcitability (Xie et al., 2005, reviewed in Martineau et al., 2006). However, alternative but unspecified sodium-independent mechanisms have been suggested to exist (Ribeiro et al., 2002; Javitt et al., 2002). Uptake of D-serine could be inhibited by administration of small L-amino acids (Ribeiro et al., 2002) however, this might be an unsuitable method in the *Xenopus* model system as it would be difficult to control for any other effects that these small compounds could have in the CNS.

4.3 Possible effects/interaction between D-serine and NO in the tadpole CNS

Another possible D-serine mediated effect that was addressed in this work is the possible link between D-serine levels and the levels of nitric oxide (NO). Due to the reported findings that D-serine can activate NOS-positive neurons leading to an up-regulation of NO (Mothet et al., 2000), and bearing in mind that NO has been found to be an important neuromodulator in the tadpole CPG decreasing the duration of swim episodes and increasing the cycle period of bursts (MacLean and Sillar, 2000), this might be a novel and interesting interaction to investigate. The results in this study pointed towards D-serine having greater effects of fictive swimming when the NO system was depressed. Although the effects were not consistent enough to statistically show and firmly conclude that this interaction occurs and, if so, that it is of importance in the tadpole CPG, the fact that NO and D-serine have both been shown to have modulatory effects on the system that are of importance to the resulting locomotor

output suggest that such an interaction could have strong resulting effects on the tadpole swimming rhythm. Furthermore, if these two neuromodulators interact with one another, it is hard to imagine that D-serine would not have an effect on the production of NO when, through the dissection, given better access to the NOS-positive neurons in the brainstem.

4.4 D-serine effects on membrane oscillatory bahaviour

NMDA receptor activation has previously been found to trigger an oscillatory behaviour of the membrane potential in many different species, which persists when synaptic transmission is blocked. These intrinsic oscillations have been suggested to contribute to the underlying on-cycle depolarising drive to the swimming rhythm in lamprey by a slow depolarisation leading to alleviation of the Mg^{2+} block of the NMDA ionophores which triggers a more rapid rising phase limited by the influx of K^+ through K^+ channels. The subsequent opening of calcium-dependent potassium channels K_{Ca2+} leads to a repolarisation phase and to Mg^{2+} blocking the NMDA ionophores again (Wallén and Grillner, 1987). In this study, I found that D-serine can promote this TTX-resistant oscillatory behaviour or even generate it in neurons which do not readily display these oscillations upon the addition of exogenous NMDA. This finding supports the idea that D-serine is an important excitatory neuromodulator of the tadpole CPG.

The boosting effect that D-serine had on the NMDA-dependent oscillations is unlikely to be simply explained by NMDA not being applied in high enough concentrations since NMDA dependent oscillations were reported by Li et al. (2010) to occur even after application of lower concentrations of NMDA (Li et al., 2010). Furthermore, 100 μM is at the top of the NMDA dose-response curve and should hence produce the maximal drug response (Soffe, 1989). The degree of membrane depolarisation that occurs in response to the addition of NMDA or D-serine has been suggested to be dependent upon the density of NMDA receptors on the neuron. Hence the ability for oscillations to occur might also be linked to the receptor

density (Grillner and Wallén, 1987). This idea would support the conclusions drawn by Li et al. (2010) and suggest that the descending interneurons that have been found to more prominently display an oscillatory behaviour upon the addition of NMDA would have more NMDA receptors and hence act as pacemakers for the swimming rhythm. However, this does not explain the role for D-serine and why it can unmask these oscillations in other types of neurons that are not necessarily a part of this suggested pacemaker unit.

Interestingly, D-serine not only enhanced the amplitude of NMDA-dependent oscillations, it was also able to produce lower frequency oscillations in the membrane potential of 0.8 Hz as opposed to 3.5 Hz. These lower frequency oscillations are more reminiscent of the oscillations that have been shown to be dependent on the co-activation of NMDA and 5-HT receptors. The frequency of these slower oscillations is too low to be directly underlying the drive for the cycle by cycle swimming. However, their frequency is similar to the slow waxing and waning pattern of fictive swimming that can be observed in the extracellular recordings and that D-serine seems to promote. Therefore, it has been proposed that 5-HT could inhibit the GlyT1b transporter which regulates glycine concentrations in the vicinity of the NMDA receptors in order to boost the NMDA mediated response (Issberner and Sillar, 2007). This proposal is certainly strengthened by my finding that D-serine, even in the absence of 5-HT, can produce these slow frequency oscillations on its own. Since NMDA- and 5-HT-dependent oscillations are not present at the larval stage 37/38, most likely due to a not fully developed serotonergic system, it might be of interest to investigate whether D-serine has any modulatory effects on the swimming rhythm at the embryonic stage. The answer is most likely that the response is weaker than at the larval stage.

4.5 Speculations regarding the true role of D-serine in the spinal cord

In the brain, D-serine has been suggested to play an important role in LTP and synaptic plasticity (Yang et al., 2003, reviewed in Martineau et al., 2006). Furthermore, in DAAO deficient mice the highest elevations in D-serine have been reported to occur in the brainstem and spinal cord (Wake et al., 2001, reviewed in Martineau et al., 2006). It is conceivable that this strong presence of D-serine is an indication that it plays an important role also in the generation of locomotion. Moreover, D-serine levels have been reported to be lower in cerebellar slices from adult rats than in young (Mothet et al., 2000), an observation that, if also applicable to the tadpole spinal cord, might suggest that D-serine's role is restricted to early stages of development. In recent years, there have been debates both for and against D-serine exerting any modulatory effects via the NMDA glycine site. Should D-serine be involved in development, it is reasonable to suppose that there could be a certain time-window during which it exerts its effects on the system. Building on the idea that there are connections between D-serine and 5-HT, these modulatory effects would perhaps not been seen before sufficient development of the serotonergic system has taken place. Likewise, if the levels of D-serine decline when the animal approaches adulthood, the effects of D-serine might become weaker.

Although these speculations are far beyond the scope of this work, they provide possible new avenues to take in terms of investigating the role and importance of D-serine as a neuromodulator in the tadpole CPG.

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