MODULATION OF MAMMALIAN SPINAL MOTOR NETWORKS BY GROUP I METABOTROPIC GLUTAMATE RECEPTORS: IMPLICATIONS FOR LOCOMOTOR CONTROL AND THE MOTOR NEURON DISEASE AMYOTROPHIC LATERAL SCLEROSIS

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Modulation of mammalian spinal motor networks by group I metabotropic glutamate receptors: implications for locomotor control and the motor neuron disease Amyotrophic Lateral Sclerosis

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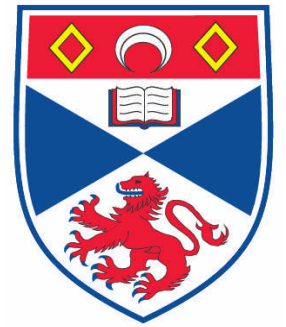

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Abstract

The present study examined the role of group I metabotropic glutamate receptors (mGluRs) in mammalian spinal motor networks and investigated the potential role of mGluRs in the fatal neurodegenerative disease amyotrophic lateral sclerosis (ALS). Group I mGluR activation was found to modulate locomotor-related activity recorded from ventral roots of *in vitro* mouse spinal cord preparations. Activation of group I mGluRs led to an increase in the frequency of locomotor-related bursts and a decrease in their amplitude. The cellular mechanisms underlying group I mGluR-mediated modulation were investigated using whole-cell patch-clamp recordings from spinal neurons. Recordings from motoneurons revealed a wide range of effects, some of which were expected to increase motoneuron excitability, such as membrane depolarisation and hyperpolarisation of action potential thresholds. However, the net modulatory effect of group I mGluR activation was a reduction in motoneuron excitability, likely reflecting a reduction in the density of fast inactivating Na^+ currents. The activation of group I mGluRs also reduced excitatory synaptic input to motoneurons, suggesting that modulation of motoneuron properties and synaptic transmission both contribute to group I mGluR-mediated reductions in locomotor motoneuron output. Recordings from spinal interneurons revealed a smaller range of modulatory effects for group I mGluRs. The clearest effect on interneurons, membrane depolarisation, may underlie group I mGluR-mediated increases in the frequency of locomotor activity. Finally, the potential role of group I mGluRs in the pathogenesis of ALS was investigated using a mouse model of the disease. Although no major perturbations in group I mGluR-mediated modulation were demonstrated in

ALS affected spinal cords, there appeared to be a difference in the intrinsic excitability of spinal interneurons between wild type and ALS affected animals. Together these data highlight group I mGluRs as important sources of neuromodulation within the spinal cord and potential targets for the treatment of ALS.

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Chapter 1

Introduction

Overview of the project

Neuromodulation is a key mechanism that allows the nervous system to produce behaviours with considerable flexibility. The present study investigates the role of G-protein coupled metabotropic glutamate receptors (mGluRs) in the modulation of spinal motor networks that control the fundamental behaviour, locomotion. In particular, the study focuses on whether the activation of one class of mGluRs, group I mGluRs, modulates locomotor-related neural networks of the mammalian spinal cord. This is assessed by using electrophysiological recordings of locomotor activity generated by motor networks present in intact *in vitro* spinal cord preparations and activity produced by single neurons within locomotor networks. In addition, it is investigated whether the modulatory roles of group I mGluRs differ in spinal cords affected by the neurodegenerative disease amyotrophic lateral sclerosis (ALS) in which motoneurons selectively degenerate due to unknown causes. The present study demonstrates a wide range of modulatory effects of group I mGluRs in mouse spinal locomotor networks, which helps to further our understanding of glutamatergic modulation in the neural control of mammalian locomotion.

Neural control of locomotion

Locomotor behaviours, such as swimming and walking, require the coordination of rhythmic body movements, typically involving muscular activity which alternates on the left and right sides of the body, to propel animals forwards. Although locomotion is a fundamental behaviour for all animals, there is still much to be learnt about how the nervous system controls rhythmic locomotor behaviour. Early studies of the neural control of locomotion demonstrated in cats that hind limb muscles still contracted

rhythmically and repetitively when animals were spinalised and dorsal roots severed to remove the influence of sensory input and descending input originating from supra-spinal systems (Brown, 1911). This work led to the idea that there are populations of local neurons intrinsic to the spinal cord that form motor networks capable of producing rhythmic locomotor-related activity. Populations of local interneurons that generate the locomotor rhythm are collectively referred to as central pattern generators (CPGs) for locomotion (Grillner, 2006; Goulding, 2009; Kiehn *et al.*, 2010). Rhythmic locomotor signals set by spinal CPG networks then converge onto motoneurons which act as the final common pathway for all motor command exiting the CNS (Sherrington, 1947).

It has been difficult to reveal rhythm generating interneuron populations in the mammalian spinal locomotor CPG. At present, interneurons that play important roles in the generation of rhythmic locomotor activity are best described in the spinal networks of evolutionarily simpler vertebrates such as lampreys and *Xenopus* tadpoles (Roberts, 2000; Roberts *et al.*, 2008b; Soffe *et al.*, 2009). This seems to be partly because the locomotor behaviour of these water-living animals, swimming, perhaps requires smaller sets of interneuron populations than those required for the neural control of walking. Nonetheless, a complete understanding of the architecture of swimming networks is important towards understanding of neural networks controlling walking in limbed animals, since the two types of locomotor networks are highly likely to share some commonality. Excitation in swimming networks is primarily provided by populations of ipsilaterally projecting descending interneurons in both the left and right halves of the spinal cord (Li *et al.*, 2009; Soffe *et al.*, 2009). Each half of the excitatory networks are mutually connected by populations of commisurally projecting

inhibitory interneurons, which ensures network excitation alternates between two halves of the spinal cord (Roberts *et al.*, 2008a). There are also populations of ipsilaterally projecting ascending interneurons which provide recurrent inhibition to excitatory networks of their own sides, regulating network excitation in one half of the spinal cord (Roberts *et al.*, 2008a).

In contrast, locomotor networks in the mammalian spinal cord appear to consist of much more complex and heterogeneous populations of local interneurons, as the locomotor behaviour of walking requires not only left-right alternation of limbs but also finer coordination of a number of agonist and antagonist muscles within one limb. Despite this complexity, some of the interneuron populations within mammalian spinal locomotor networks have been uncovered recently, thanks to combined advances in molecular genetics and developmental biology (Jessell, 2000). Neurons found in the ventral horn of the spinal cord are developmentally grouped based on expression of different transcription factors which can be used as markers for specific neural populations. Some of these interneuron populations, identified via developmental genetics, have been shown to play similar roles to some of the neural populations described in locomotor networks controlling swimming. Specified by the transcription factor Dbx1, commissurally projecting inhibitory V0 interneurons are necessary for left-right alternation during mammalian locomotion (Lanuza *et al.*, 2004). V1 interneuons, specified by the transcription factor En1, are ipsilaterally projecting inhibitory interneurons that regulate the speed of locomotion (Gosgnach *et al.*, 2006). V2a interneurons, specified by the transcription factor Chx10, are involved in the left-right coordination during locomotion via their ipsilateral glutamatergic projections onto commissural interneurons such as V0 interneurons (Crone *et al.*, 2008).

Ipsilaterally projecting excitatory V3 interneurons, specified by the transcription factor Sim1, help to balance robust network excitation required in rhythmically active spinal locomotor networks (Zhang *et al.*, 2008). Other populations of spinal interneurons will likely be revealed as additional transcription factors responsible for the differentiation of spinal neurons are identified. However, the possibility that identified neural populations include heterogeneous neuronal subsets must always be considered, in order to reach a full understanding of locomotor CPG networks in the mammalian spinal cord. Nevertheless, any neural signals generated by locomotor CPG networks finally converge onto spinal motoneurons where integration, modulation and determination of the final locomotor network output takes place.

Spinal motoneurons

Motoneurons that innervate limb muscles are referred to as the final common pathway of motor networks of the CNS (Sherrington, 1947). Anatomically, cell bodies of spinal motoneurons form a nucleus at the ventrolateral corner, mostly in lamina IX, of the ventral horn of the spinal cord. Cell bodies of motoneurons are somatotopically organised within each motor nucleus (Nicolopoulos-Stournaras & Iles, 1983), such that those found in the medial part of lamina IX innervate muscles of the trunk, whereas those found in the dorsolateral and ventrolateral parts innervate muscles of distal and proximal limbs respectively. The diameter of motoneuron cell bodies is typically 2 – 3 times larger than other neuron types found in the spinal cord, for example the soma diameter of motoneurons that control muscles of the hind limb in cats can be up to 70 μm (Zwaagstra & Kernell, 1981).

Spinal motoneurons should not be regarded as simple relay neurons that passively

transmit rhythmic locomotor signals generated from pre-motor CPG networks to the muscles they innervate. Motoneurons in fact play important roles in neural processes such as integration of rhythmic pre-motor locomotor signals and modulation of the final motor output generated by whole spinal locomotor networks. Spinal motoneurons receive cohorts of mixed excitatory and inhibitory locomotor command signals produced by pre-motor CPG networks via activation of fast acting ionotropic receptors, such that excitatory signals are primarily mediated via glutamatergic transmission while inhibitory signals are mediated via GABAergic and glycinergic transmission (Shefchyk & Jordan, 1985; Orsal *et al.*, 1986; Cazalets *et al.*, 1996; Hochman & Schmidt, 1998). Concurrently, spinal motoneurons also receive a wide variety of other chemical signals (including monoamines, peptides, and acetylchonine to name but a few) which mediate neuromodulatory signals via the activation of slower acting G-protein coupled metabotropic receptors (Rekling *et al.*, 2000). In addition, although glutamate is the major fast excitatory neurotransmitter within locomotor networks of the spinal cord (Beato *et al.*, 1997; Whelan *et al.*, 2000; Talpalar & Kiehn, 2010), glutamate can also act as an important neuromodulator for spinal locomotor networks, including motoneurons, by activating its metabotropic receptors (El Manira *et al.*, 2002; Nistri *et al.*, 2006; El Manira *et al.*, 2008).

Modulation of spinal locomotor networks

Due to their G-protein coupled nature and slow mode of action, metabotropic receptors, not only of glutamate but also of other neurotransmitters, play significant roles in the modulation of a wide range of neural networks throughout the nervous system. Locomotor networks within the spinal cord are, of course, no exception and

are modulated by a number of neuromodulatory signals acting via metabotropic receptors. This diverse neuromodulation allows the fundamental behaviour, locomotion, to be flexible and adaptable to meet varied physiological demands. These include the demands of different internal metabolic states, developmental stages and external stimuli from ever changing environments. Most neuromodulatory signals to spinal locomotor networks originate from 'extrinsic' supra-spinal systems, in particular descending monoaminergic inputs from distinct nuclei of the brainstem (Rekling *et al.*, 2000; Schmidt & Jordan, 2000; Grillner, 2006; Heckman *et al.*, 2009). These extrinsic modulatory inputs from the brainstem to spinal locomotor networks often reflect an animal's sleep-wake cycle, and attentional or motivational states (Rekling *et al.*, 2000; Grillner, 2006). These descending inputs from the brainstem also play important roles in the development of locomotor networks in the foetal and neonatal mammalian spinal cord. For example, serotonergic projections from the raphe nuclei are one of the first descending projections that reach the lumbar enlargement of the rat spinal cord during the late foetal period (Bregman, 1987; Rajaofetra *et al.*, 1989). This is the developmental period when rat spinal locomotor networks start to exhibit left-right alternating activity rather than synchronous activity (Iizuka *et al.*, 1998). Through the early neonatal period, the presence of 5-HT then helps refine left-right alternation of locomotor activity produced by spinal networks (Norreel *et al.*, 2003; Pearlstein *et al.*, 2005).

However, it has been demonstrated that there are also other neuromodulatory inputs to spinal motor networks that arise 'intrinsically' from local intra-spinal networks, which activate, for example, cholinergic, glutamatergic or purinergic receptors, to modulate networks of which they are intrinsic components (Dale & Gilday, 1996; Katz

& Frost, 1996; Rekling *et al.*, 2000; Alaburda & Hounsgaard, 2003; Miles *et al.*, 2007; El Manira *et al.*, 2008; Zagoraiou *et al.*, 2009). Cholinergic pre-motor terminals of local spinal interneurons called C-boutons are known to modulate input-output relationships of motoneurons via postsynaptic activation of G-protein coupled muscarinic receptors (Miles *et al.*, 2007; Zagoraiou *et al.*, 2009). Perhaps unsurprisingly, since glutamate is the major fast excitatory neurotransmitter within locomotor CPG networks in the spinal cord (Beato *et al.*, 1997; Whelan *et al.*, 2000; Talpalar & Kiehn, 2010), glutamate can also act as one of the most widely available intrinsic neuromodulators for neural components within spinal locomotor networks, including motoneurons, via activation of its metabotropic receptors (El Manira *et al.*, 2002; Nistri *et al.*, 2006; El Manira *et al.*, 2008).

Metabotropic glutamate receptors

Glutamate is one of 20 amino acids that make up the proteins of biological systems. However it also exists as one of the major excitatory neurotransmitters found in the CNS. Glutamate receptors are very broadly divided into two types based on their biochemical mode of actions, ion channel coupled ionotropic glutamate receptors (iGluRs) and G-protein coupled metabotropic glutamate receptors (mGluRs). iGluRs include NMDA, AMPA, and kainate receptors, named after their specific pharmacological agonists. Due to their classic role mediating fast excitatory neurotransmission, iGluRs have long been the focus of extensive research. On the other hand, mGluRs were first reported in the late 1980s as new types of glutamate receptors (Sugiyama *et al.*, 1987) but remain less understood compared to iGluRs. To date, there are 8 subtypes of mGluRs identified, mGluR1 to mGluR8 subtypes, which

are normally divided into 3 groups, group I, II, and III, according to their molecular homology, pharmacological profiles and coupled biochemical intracellular signalling (Pin & Duvoisin, 1995; Conn & Pin, 1997; Anwyl, 1999, 2009).

Because of the wide variety of intracellular cascades affected by the activation of mGluRs, these receptors are important players in neuromodulation of intrinsic neural properties and the efficacy of synaptic transmission throughout the CNS. Group I mGluRs, including mGluR1 and mGluR5 subtypes, are Gq ptotein coupled metabotropic receptors, which are positively coupled to phospholipase C (PLC), resulting in catalysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacyl glycerol (DAG) and inositol triphosphate (IP_3). Group II mGluRs, consisting of mGluR2 and mGluR3 subtypes, and group III mGluRs, namely mGluR4, mGluR6 to mGluR8 subtypes, are Gi protein coupled metabotropic receptors that inhibit production of cAMP via blockade of adenylate cyclase. In the CNS in general, group I mGluRs facilitate neuronal excitation via postsynaptic mechanisms (Anwyl, 1999, 2009). On the other hand, group II and III mGluRs are often found to be inhibitory at pre-synaptic terminals (Anwyl, 1999, 2009) for example acting as autoreceptors to inhibit pre-synaptic glutamate release (Chen *et al.*, 2002; Mateo & Porter, 2007). Group I mGluRs are also reported to have pre-synaptic actions. In the lamprey spinal cord, for example, activation of group I mGluRs leads to an increase in intracellular Ca^{2+} concentration at pre-synaptic terminals (Schwartz & Alford, 2000), which is followed by potentiation of pre-synaptic glutamate release (Takahashi & Alford, 2002).

Group I mGluRs and modulation of spinal locomotor networks

Neuromodulatory roles of mGluRs in spinal locomotor networks have previously

been studied in vertebrate animals such as lampreys (El Manira *et al.*, 2002; El Manira *et al.*, 2008), *Xenopus* tadpoles (Chapman & Sillar, 2007; Chapman *et al.*, 2008) and rats (Nistri *et al.*, 2006). The modulatory roles of group I mGluRs have been most extensively studied in isolated spinal cord preparations obtained from lampreys. In these preparations, endogenous activation of group I mGluRs is reported, which supports the importance of group I mGluRs in the modulation of locomotor networks in the lamprey spinal cord (Krieger *et al.*, 1998; Krieger *et al.*, 2000; Kettunen *et al.*, 2002). Exogenous activation of group I mGluRs causes both short- and long-term modulation of lamprey locomotor networks, increasing locomotor-related burst frequency recorded from ventral roots of *in vitro* spinal cord preparations (Krieger *et al.*, 1998; Krieger *et al.*, 2000; Kyriakatos & El Manira, 2007). Group I mGluR-mediated modulation of locomotor network activity likely involves modulation of synaptic transmission within spinal locomotor networks, such that inhibitory synaptic transmission is depressed (Kettunen *et al.*, 2005; Kyriakatos & El Manira, 2007) and excitatory transmission to spinal motoneurons is enhanced (Kyriakatos & El Manira, 2007). In addition, group I mGluR-mediated modulation of synaptic transmission involves retrograde modulatory mechanisms mediated via endocannabioid and nitric oxide signalling (Kettunen *et al.*, 2005; Kyriakatos & El Manira, 2007).

Beyond the modulation of locomotor network activity, cellular properties that are modulated by group I mGluR activation have also been well characterised in spinal neurons of lampreys. The activation of group I mGluRs results in depolarisation of the resting membrane potential (Krieger *et al.*, 1998; Kettunen *et al.*, 2003), which is mediated via blockade of leak currents in a PKC dependent manner (Kettunen *et al.*, 2003; Nanou *et al.*, 2009). The activation of group I mGluRs also causes oscillations

in intracellular Ca^{2+} levels, requiring Ca^{2+} entry through L-type but not N-type Ca^{2+} channels (Kettunen *et al.*, 2002). Furthermore, the activation of group I mGluRs is reported to modulate activities of iGluRs, for example enhancing currents mediated by NMDA receptors (Krieger *et al.*, 1998; Krieger *et al.*, 2000; Nanou *et al.*, 2009) as well as affecting AMPA-induced Na^{+} activated K^{+} currents (Nanou & El Manira, 2010).

Compared to studies performed in lamprey *in vitro* spinal cord preparations, less detailed analyses of the modulatory roles of group I mGluRs have been performed in the mammalian spinal cord, and these mammalian studies are so far limited to those conducted on *in vitro* spinal cord preparations obtained from neonatal rats. Endogenous activation of group I mGluRs has been demonstrated in rat spinal cord preparations by the application of group I mGluR antagonists, which reduce the frequency of rhythmic locomotor-related burst activity recorded from lumbar ventral roots of *in vitro* spinal cord preparations (Taccola *et al.*, 2004). However, application of a group I mGluR agonist also slows the frequency of locomotor bursts, although a high dose of this agonist rapidly disrupts the rhythm of locomotor network activity (Taccola *et al.*, 2004). The effects of group I mGluR activation upon synaptic transmission within the rat spinal cord are also diverse as evidenced by reports of both enhancement and depression of inhibitory synaptic transmission to spinal motoneurons (Marchetti *et al.*, 2003, 2005). Cellular properties are also modulated by the activation of group I mGluRs in rat spinal motoneurons to cause membrane depolarisation and oscillations in the resting membrane potential (Marchetti *et al.*, 2003). It therefore seems that the modulatory roles of group I mGluRs in mammalian spinal locomotor networks are complicated and not yet fully understood. Thus further investigation of the roles and mechanisms of group I mGluR-mediated modulation are warranted in the

mammalian spinal cord.

Amyotrophic Lateral Sclerosis

One clinical condition that affects locomotor networks in the spinal cord is the motoneuron disease, amyotrophic lateral sclerosis (ALS). ALS is also known as Lou Gehrig's disease in some countries after a baseball player who suffered from it. ALS is a fatal neurodegenerative disease in which motoneurons are selectively lost due to unknown causes and via undefined mechanisms. It is estimated that ALS affects 2 in 100,000 people per year. 90 % of all ALS cases are sporadic (no known causes), and the remaining 10 % are familial cases (hereditary genetic causes). 20 % of familial ALS cases involve genetic defects in the gene encoding an enzyme, superoxide dismutase-1 (SOD-1). The onset of ALS is sudden, typically at middle age (late 30s – early 50s). Disease progression is characterised by rapid and selective degeneration of motoneurons in the brainstem and spinal cord, which severely impairs patients' motor functions. Most patients die of breathing difficulties due to paralysis of their respiratory muscles within 2 - 3 years of diagnosis. No significant treatments are available for ALS. The only approved treatment is the administration of a drug, riluzole, which only provides modest prolongation of patients' life, approximately 3 months. A number of hypotheses for understanding the pathogenesis of ALS have been proposed. These include mitochondrial dysfunction, cellular oxidative stress, disorganisation of neurofilaments, toxicity from intracellular aggregates, and glutamatergic excitotoxicity (Bruijn *et al.*, 2004; Boillee *et al.*, 2006). However, these various proposed mechanisms are unlikely to be mutually exclusive, probably all converging to cause the final death of motoneurons.

Glutamatergic excitotoxicity

Although ALS is a multi-factorial neurodegenerative disease, the hypothesis that glutamatergic excitotoxicity is involved in the pathogenesis of ALS seems to have received the most support. Firstly, an elevated concentration of glutamate in cerebrospinal fluid is reported in 40 % of ALS patients (Spreux-Varoquaux *et al.*, 2002). Secondly, the astroglial glutamate transporter, excitatory amino acid transporter 2 (EAAT2), which is responsible for removal of glutamate from synaptic spaces, is reported as having reduced expression in ALS patients (Rothstein *et al.*, 1995; Fray *et al.*, 1998) and also in ALS model mice (Bruijn *et al.*, 1997). Finally, riluzole, the only drug clinically approved for the treatment of ALS, is thought to inhibit glutamate release (Doble, 1996). Thus, evidence points strongly towards glutamatergic excitotoxicity, initiated not only by an excessive release of glutamate into extracellular spaces but also by abnormal clearance of glutamate from extracellular spaces, as playing a key role in the pathology of ALS.

Glutamatergic excitotoxicity in ALS is mostly discussed in the context of iGluR-mediated Ca^{2+} influx and the subsequent disturbance of intracellular Ca^{2+} homeostasis in motoneurons (Arundine & Tymianski, 2003; Van Den Bosch *et al.*, 2006). Initiated by an excessive amount of glutamate present in the extracellular space, glutamatergic excitotoxicity is a vicious circle of intracellular biochemical consequences that ends in degeneration of motoneurons. Over-activation of iGluRs, especially NMDA receptors and Ca^{2+} permeable AMPA receptors, allows huge influx of Ca^{2+} ions from the extracellular space. As Ca^{2+} ions are important in a range of cellular processes, the homeostasis of intracellular Ca^{2+} needs to be tightly regulated under physiological conditions. However, disturbances in intracellular Ca^{2+}

homeostasis, particularly abnormally elevated intracellular Ca^{2+} levels, lead to excessive activation of many Ca^{2+} dependent catalytic processes, for example those activating proteases, lipases, phosphatases, and endonucleases. These enzymes mediate reactions which breakdown the physical structures of motoneurons, and mediate the formation of oxidative free radicals which also favour the death motoneurons.

Group I mGluRs and ALS

In contrast to the well-documented roles of iGluRs in glutamatergic excitotoxicity (Arundine & Tymianski, 2003; Van Den Bosch *et al.*, 2006) and despite the fact that group I mGluRs are suggested to increase intracellular Ca^{2+} levels in spinal motoneruons (Kettunen *et al.*, 2002), whether group I mGluRs are involved in the excitotoxic pathogenesis of ALS remains uncertain. Molecular expression of group I mGluRs is comparable in motoneurons of the spinal cord between control subjects and ALS patients (Aronica *et al.*, 2001; Valerio *et al.*, 2002) but elevated expression of group I mGluRs is reported in glial cells in the ALS affected spinal cord (Aronica *et al.*, 2001). Subtypes of group I mGluRs seem to be differentially expressed in different motoneuron nuclei showing varied susceptibility to neurodegeneration in ALS. In the brainstem and spinal cord, mGluR1 subtypes are expressed to a lesser degree in vulnerable motor nuclei, whereas mGluR5 subtypes are expressed at higher levels in resistant motor nuclei (Laslo *et al.*, 2001; Anneser *et al.*, 2004; Ma *et al.*, 2006). Such differential expression of group I mGluR subtypes may explain mixed evidence that group I mGluRs play both neuroprotective and neurotoxic roles in glutamate-induced neurotoxicity depending on different experimental conditions. Application of group I mGluR agonists prevents kainate-induced neuronal death in spinal cord slices obtained

from adult rats (Pizzi *et al.*, 2000; Valerio *et al.*, 2002). However, application of either an agonist or an antagonist of group I mGluRs prevents neuronal death in embryonic chick spinal cell cultures treated with CSF obtained from human ALS patients (Anneser *et al.*, 2006). Given the uncertainty regarding the potential neuroprotective or neurotoxic roles of group I mGluRs in glutamatergic excitotoxicity, it is important to assess the physiological effects of group I mGluR activation or inhibition in nervous tissues that are affected by ALS.

Chapter 2

The role of group I metabotropic glutamate receptors (mGluRs) in locomotor network activity in the mouse spinal cord

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Activation of group I metabotropic glutamate receptors modulates locomotor-related motoneuron output in mice

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Introduction

Modulation of spinal locomotor networks is important for animals to adapt their motor behaviours in response to variable environmental stimuli and physiological states such as metabolism or development. In many cases, modulatory inputs to locomotor networks in the spinal cord come from supra-spinal systems, for example descending serotonergic or noradrenergic inputs to spinal networks originate from distinct nuclei in the brainstem (Rekling *et al.*, 2000; Schmidt & Jordan, 2000; Heckman *et al.*, 2009). However, there is also evidence of intrinsic neuromodulatory inputs that come from neural networks within the spinal cord (Dale & Gilday, 1996; Katz & Frost, 1996; Alaburda & Hounsgaard, 2003; El Manira *et al.*, 2008; Zagoraiou *et al.*, 2009). In spinal locomotor networks, glutamate is released as a major neurotransmitter that mediates fast excitatory neurotransmisson (Beato *et al.*, 1997; Whelan *et al.*, 2000; Talpalar & Kiehn, 2010). However glutamate can also act as an important intrinsic neuromodulator that activates metabotropic receptors (El Manira *et al.*, 2002; Nistri *et al.*, 2006; El Manira *et al.*, 2008).

The modulatory roles of metabotropic glutamate receptors (mGluRs), particularly group I mGluRs, in vertebrate spinal locomotor networks are best described in lamprey *in vitro* spinal cord preparations in which rhythmic left-right alternating bursts of swimming-related activity are recorded from ventral roots. The activation of group I mGluRs leads to both short- and long-term increases in the frequency of locomotor-related ventral root bursts (Krieger *et al.*, 1998; Kyriakatos & El Manira, 2007), which reflects depression of inhibitory synaptic transmission (Kettunen *et al.*, 2005; Kyriakatos & El Manira, 2007) and also enhancement of excitatory transmission

to motoneurons (Kyriakatos & El Manira, 2007). Both the short- and long-term increases in locomotor burst frequency depend on endocannabioid signalling, which retrogradely modulates both excitatory and inhibitory synaptic transmission to spinal motoneurons (Kettunen *et al.*, 2005; Kyriakatos & El Manira, 2007). In addition, long-term, but not short-term, enhancement of excitatory neurotransmission requires signalling mediated by the release of nitric oxide (Kyriakatos & El Manira, 2007). Similar modulation of spinal locomotor networks by group I mGluRs has also been reported on bursts of fictive swimming recorded from ventral roots of immobilised *Xenopus* tadpole preparations (Chapman & Sillar, 2007; Chapman *et al.*, 2008). The activation of group I mGluRs causes an increase in the frequency of swimming-related ventral root bursts (Chapman & Sillar, 2007). This increase in swimming burst frequency is mediated via depression of inhibitory glycinergic transmission (Chapman *et al.*, 2008).

Investigations into the modulation of mammalian spinal locomotor networks are so far limited to studies using *in vitro* spinal cord preparations obtained from neonatal rats. However, the modulatory roles of group I mGluRs in rat spinal locomotor networks appear to be complicated, with the application of either group I mGluR agonists or antagonists both reducing the frequency of locomotor-related burst activity recorded from lumbar ventral roots (Taccola *et al.*, 2004). Moreover, the application of a group I mGluR agonist at higher doses completely disrupts rhythmic bursts of spinal locomotor activity (Taccola *et al.*, 2004). Effects of group I mGluR activation upon synaptic transmission within the rat spinal cord also seem diverse, since data support both inhibition (Marchetti *et al.*, 2003) and enhancement (Marchetti *et al.*, 2005) of inhibitory synaptic transmission.

Given the complexities and unsolved roles of group I mGluRs in mammalian spinal locomotor networks, the present study aimed to investigate effects of group I mGluR activation on rhythmic locomotor-related ventral root activity recorded from *in vitro* spinal cord preparations obtained from neonatal mice. Although the activation of group I mGluRs has been demonstrated to modulate locomotor burst frequency in rats, *Xenopus* tadpoles and lampreys, it remains to be determined whether the activation of group I mGluRs also modulates the intensity of rhythmic burst activity produced by spinal locomotor networks. Therefore a key focus of the present chapter is to determine the effects of group I mGluR activation on the intensity of locomotor-related motoneuron output. Furthermore, it is assessed which receptor subtypes of group I mGluRs (mGluR1 versus mGluR5) are specifically involved in the modulation of mammalian spinal locomotor networks. Finally, it is investigated whether the group I mGluR-mediated modulation of locomotor networks within the mouse spinal cord requires downstream effects that are dependent on endocannabinoid or nitric oxide signalling, as has been demonstrated in locomotor networks in the lamprey spinal cord (Kettunen et al. 2005; Kyriakatos and El Manira 2007).

Methods

In vitro mouse spinal cord preparations

All methods required to obtain tissue for *in vitro* experiments were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986. *In vitro* whole spinal cord preparations were obtained from post natal day (P) 2 – 6 C57BL/6 mice, using techniques similar to those described by Jiang *et al.* (1999). Animals were killed via cervical dislocation, decapitated and eviscerated before spinal cords were isolated from the mid-cervical to upper sacral segments in a chamber containing artificial cerebral spinal fluid (aCSF; equilibrated with 95 % O_2 – 5 % CO_2, ~4 ˚C). The dura matter was removed for the purpose of assisting diffusion of chemicals to the preparations.

Ventral root recordings

Glass suction electrodes were attached to lumbar (L) 1 or L2 ventral roots of *in vitro* spinal cord preparations (Fig. 1A). NMDA (5 μM), 5-HT (10 μM) and dopamine (50 μM) were added to the aCSF to induce rhythmic, left-right alternating bursts of locomotor-related ventral root activity (Jiang *et al.*, 1999; Miles *et al.*, 2007; Fig. 1B). Unless noted otherwise, locomotor-related bursts were left to stabilise (~1 hour) before subsequent drug applications. Raw signals were amplified and filtered (30 – 3,000 Hz; Qjin Design, ON, Canada), before being acquired at ≥ 1 kHz using a Digidata 1440A A/D board and AxoScope software (Molecular Devices, Sunnyvale, CA). These raw signals were also rectified and integrated online (Qjin Design) for subsequent analyses of ventral root bursts.

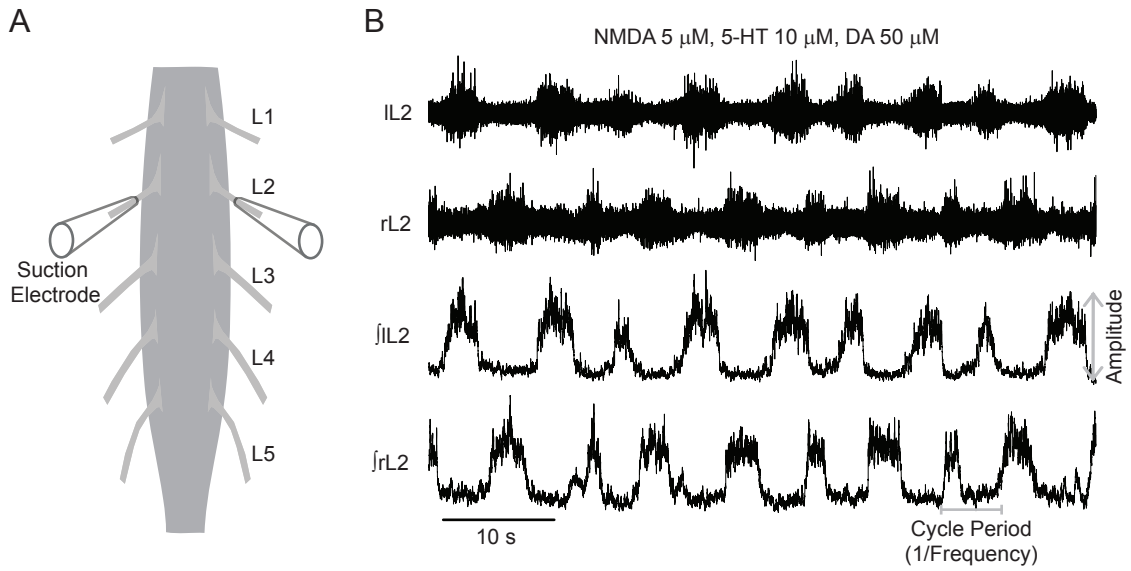


Figure 1: Rhythmic locomotor-related burst activity recorded from an *in vitro* mouse spinal cord preparation.

A) Schematic diagram of an *in vitro* mouse spinal cord preparation (courtesy of G. B. Miles, University of St Andrews). Extracellular recording suction electrodes were attached to the left and right first or second lumbar (L1 or L2) ventral roots. B) Raw (top) and integrated and rectified (bottom) traces showing rhythmic locomotor burst activity induced by pharmacological agents (5 μM NMDA, 10 μM 5-HT, and 50 μM dopamine). Amplitude and frequency of locomotor bursts were measured for analyses.

Data analysis

Data from ventral root recordings were analysed offline using Dataview software (courtesy of W. J. Heitler, University of St Andrews). To detect ventral root bursts, a threshold was set near to the bottom of rectified and integrated traces. The beginning and end of ventral root bursts were defined as points where the integrated traces positively and negatively crossed the threshold. The amplitude of ventral root bursts was measured from the baseline, threshold value to the peak value of bursts (Fig. 1B). The reciprocal of the interval between the start of two adjacent bursts was defined as the instantaneous frequency (Fig. 1B). Values of burst amplitude and frequency were normalised by dividing them by averaged values of amplitude and frequency recorded for 5 minutes before control (pre-control values). Data are reported as mean ± S.E. Differences in means between control and drug conditions were compared using Student's t-tests. Values of $p < 0.05$ were considered significant.

Solution and drugs

The standard aCSF solution used for dissection and recordings contained 127 mM NaCl, 3 mM KCl, 1.3 mM NaH_2PO_4, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM D-glucose (equilibrated with 95 % O_2 – 5 % CO_2). Pharmacological agents used included: NMDA (*N*-methyl-D-aspartic acid), 5-HT (5-hydroxytryptamine hydrochloride), dopamine (3,4-dihydroxyphenethylamine hydrochloride), and PTIO (2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide) purchased from Sigma-Aldrich (St Louis, MO); DHPG ((S)-3,5-dihydroxyphenylglycine), LY367385 ((S)-(+)-a-amino-4carboxy-2-methylbenzeneacetic acid), MPEP (2-methyl-6(phenyl-ethynyl)pyridine hydrochloride), and AM251 (N-(piperidin-1-yl)-5-(4-iodophenyl)-

1-(2,4-dichlorophenyl)4-methyl-1H-pyrazole-3-carboxamide) purchased from Tocris Bioscience (Bristrol, UK); and L-NAME (Nω-Nitro-L-arginine methyl ester hydrochloride) purchased from Ascent Scientific (Bristrol, UK).

Results

Group I mGluR-mediated modulation of spinal locomotor networks

Locomotor drugs (5 μM NMDA, 10 μM 5-HT, 50 μM dopamine) were added to the recording aCSF to induce rhythmic, left-right alternating bursts of locomotor-related ventral root activity in mouse *in vitro* spinal cord preparations. The group I mGluR agonist, DHPG (5 – 50 μM), was then bath applied to investigate whether the activation of group I mGluRs modulates spinal locomotor-related burst activity. The application of DHPG (5 μM; 15 min) led to a significant decrease in the amplitude of locomotor-related burst activity recorded from lumbar ventral roots (Fig. 2A & 2B). The amplitude of locomotor bursts decreased gradually, reaching the minimum level near the end of the 15 minute DHPG application (18.0 ± 4.0 % reduction, n = 11, $p < 0.001$; Fig. 2Bi & 2Bii), before returning to control levels within ~10 minutes of drug washout (Fig. 2Bi). Analyses of baseline ventral root activity between locomotor bursts (inter-burst activity) revealed no change in the baseline activity upon DHPG application (n = 11; Fig. 2C). The application of DHPG also caused a significant increase in the frequency of locomotor burst activity (Fig. 2A & 2D). Locomotor burst frequency increased gradually, peaking near the end of the 15 minute DHPG application (23.0 ± 8.8 % increase, n = 11, $p = 0.008$; Fig. 2Di & 2Dii), before returning to control levels within ~10 minutes of drug washout (Fig. 2Di). Together these data demonstrate that the activation of group I mGluRs modulates on-going locomotor output driven by spinal motor networks.

Given that group I mGluR activation has long-term effects on locomotor burst activity recorded from *in vitro* lamprey spinal cord preparations (Kyriakatos & El

Figure 2: Group I metabotropic glutamate receptors (mGluRs) modulate locomotor burst activity recorded from *in vitro* mouse spinal cord preparations.

A) Raw (top) and rectified and integrated (bottom) traces showing the effects of the group I mGluR agonist (S)-3,5-dihydroxyphenylglycine (DHPG; 5 μM) on spinal locomotor burst activity. Bi) DHPG application (15 min) reduced the amplitude of locomotor bursts. Each point in the time-course plots represents 1 min worth of recording, normalized to control (5 μM, n = 11; 10 μM, n = 9). Bii) Pooled data, averaged from 5 min worth of recording in each experimental condition, show a significant decrease in locomotor burst amplitude in response to DHPG application (5 μM). Ci) Time course plot and Cii) pooled data showing no significant effect of DHPG application (5 μM) on baseline inter-burst activity of ventral roots. Di) DHPG application (5 μM and 10 μM) increased the frequency of locomotor bursts. Dii) Pooled data showing a significant increase in locomotor burst frequency by DHPG application (5 μM). * = significantly different from control.

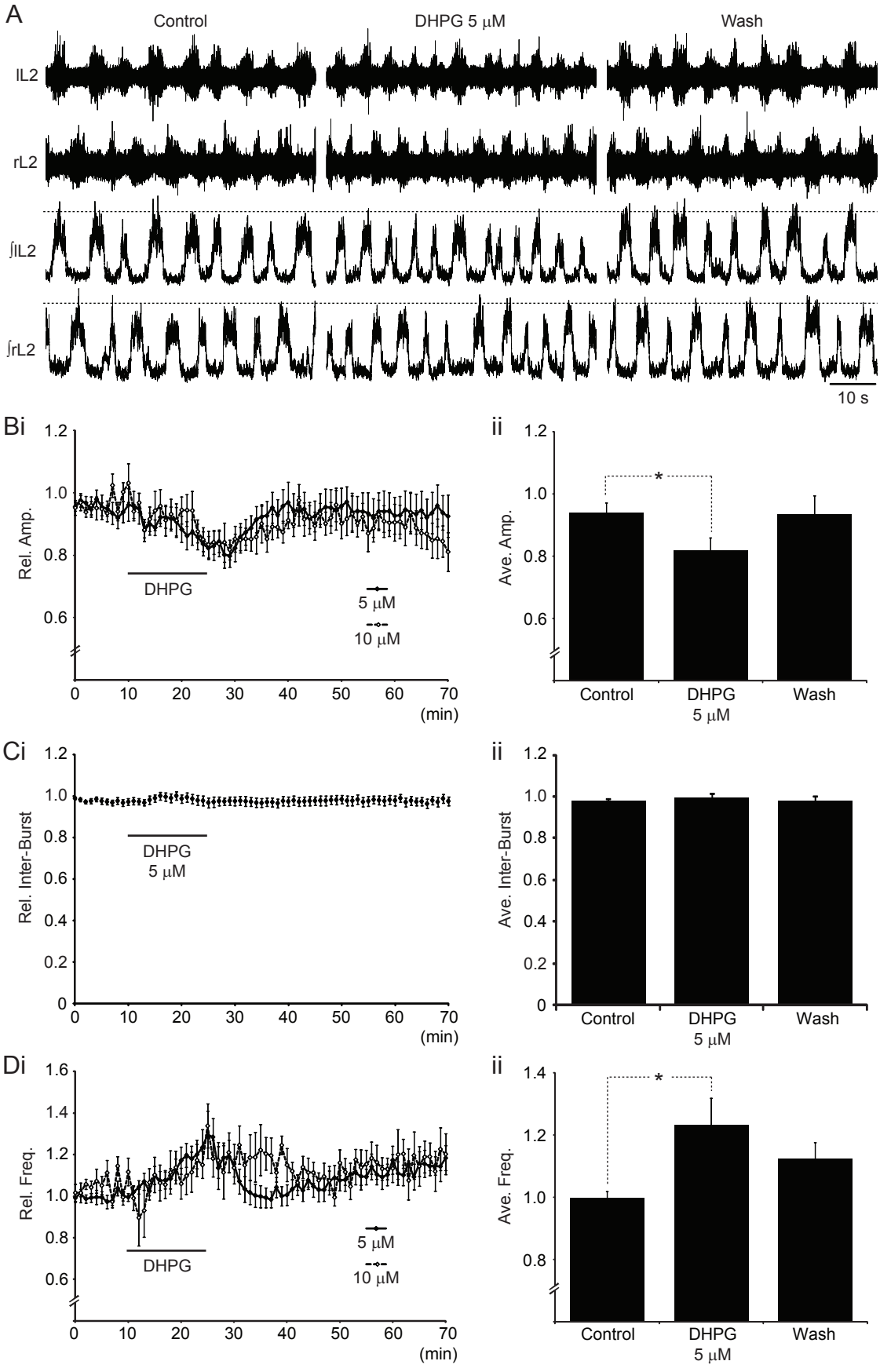
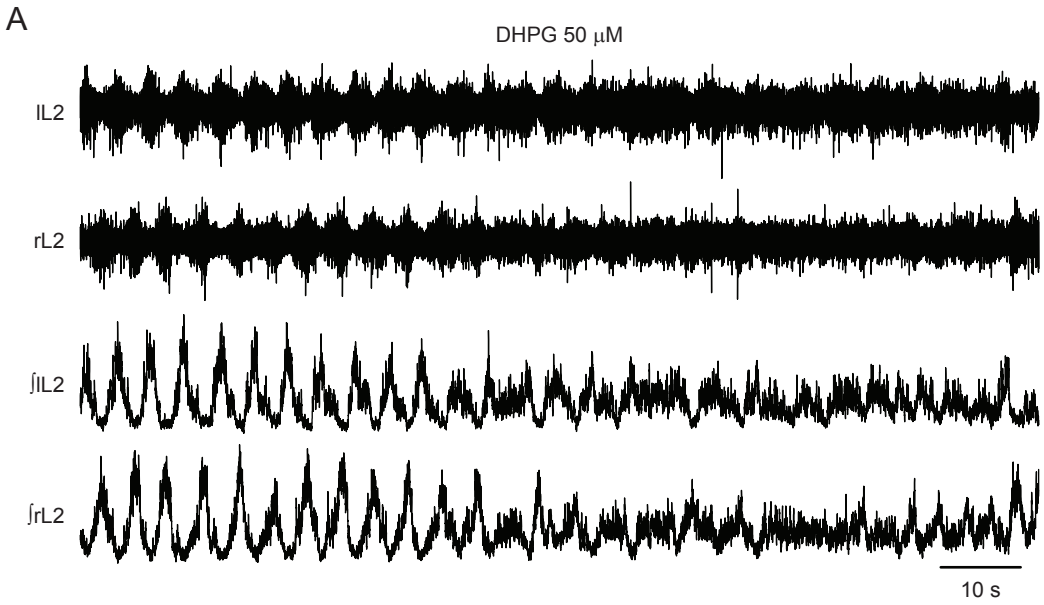
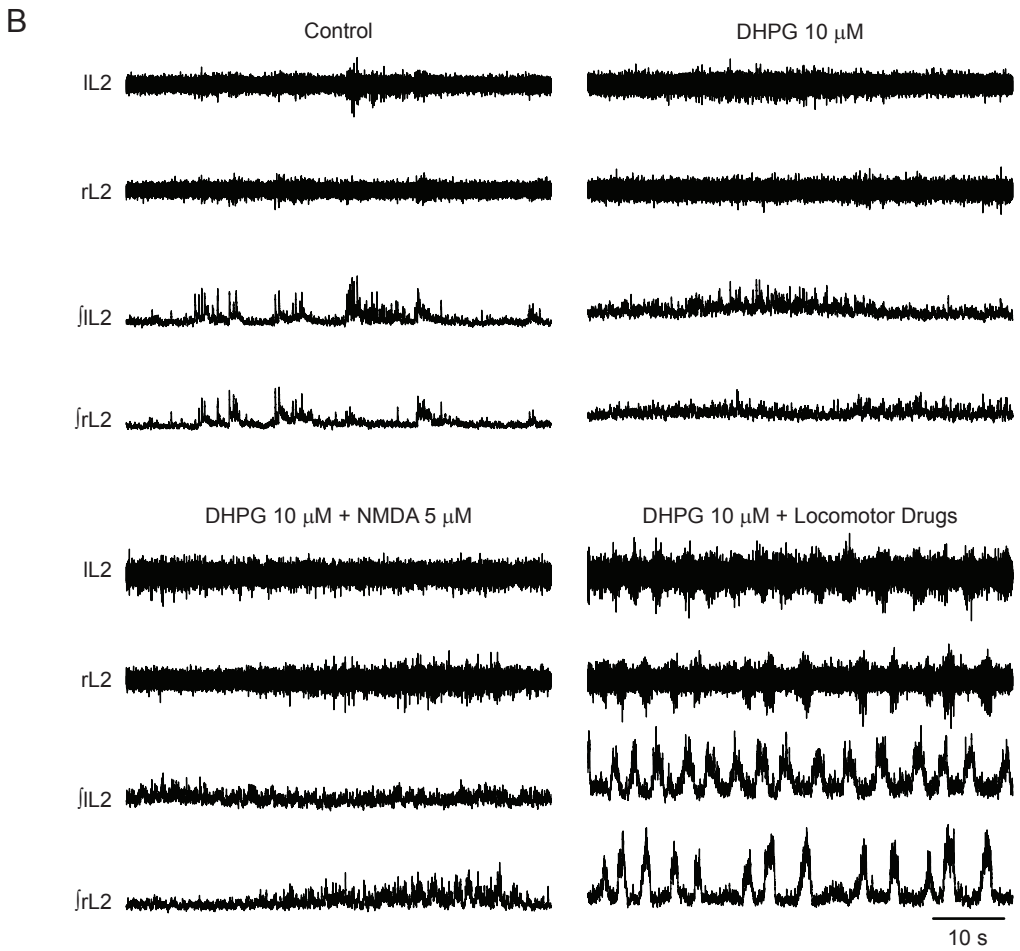

A
Control
DHPG 5 μM
Wash
lL2
rL2
∫lL2
∫rL2
10 s
Bi
Rel. Amp.
DHPG
5 μM
10 μM
(min)
ii
Ave. Amp.
Control
DHPG
5 μM
Wash
Ci
Rel. Inter-Burst
DHPG
5 μM
(min)
ii
Ave. Inter-Burst
Control
DHPG
5 μM
Wash
Di
Rel. Freq.
DHPG
5 μM
10 μM
(min)
ii
Ave. Freq.
Control
DHPG
5 μM
Wash

Figure 3: Different conditions of DHPG application.

A) A high dose of DHPG (50 μM) disrupted rhythmic locomotor activity recorded from lumbar ventral roots of *in vitro* mouse spinal cord preparations. B) Neither DHPG alone (10 μM) nor DHPG in combination with NMDA (5 μM) application was able to induce rhythmic locomotor burst activity in an *in vitro* mouse spinal cord preparation.


A
DHPG 50 μM
lL2
rL2
∫lL2
∫rL2
10 s


B
Control
DHPG 10 μM
lL2
rL2
∫lL2
∫rL2
DHPG 10 μM + NMDA 5 μM
DHPG 10 μM + Locomotor Drugs
lL2
rL2
∫lL2
∫rL2
10 s

Manira, 2007), it was assessed whether higher doses of DHPG might have long-term effects in mouse *in vitro* spinal cord preparations. Application of 10 µM DHPG (n = 9) again led to a significant decrease in locomotor burst amplitude and a significant increase in locomotor burst frequency (Fig. 2Bi & 2Di), with both effects of equivalent magnitude to those observed following 5 µM DHPG application. Although the effects of 10 µM DHPG application slightly outlasted those of 5 µM application, no long-term effects were observed (Fig. 2Bi & 2Di). DHPG at higher doses (20 – 50 µM) also decreased the amplitude of locomotor bursts. However, this was accompanied by a rapid disruption in locomotor output, where rhythmic burst activity ceased several minutes after the start of drug application (Fig. 3A).

Since apparent excitatory effects of group I mGluR activation were observed on the frequency of locomotor burst activity generated from spinal CPG networks, it was also investigated whether DHPG (10 µM) alone or DHPG in combination with NMDA (5 µM) application could elicit rhythmic locomotor burst activity. Although DHPG application increased baseline tonic activity recorded from lumbar ventral roots, no rhythmic locomotor bursts were observed under these conditions (n = 3; Fig. 3B).

Together these results demonstrate that, although group I mGluR activation cannot induce spinal locomotor activity, it does modulate on-going activity generated by locomotor networks in two ways. Firstly, the activation of group I mGluRs reduces the intensity of the final locomotor output produced by spinal motoneurons, and secondly, the activation of group I mGluRs speeds up the rhythm of locomotor activity mediated by interneurons that make up spinal locomotor CPGs.

Group I mGluR subtype-specific modulation of spinal locomotor networks

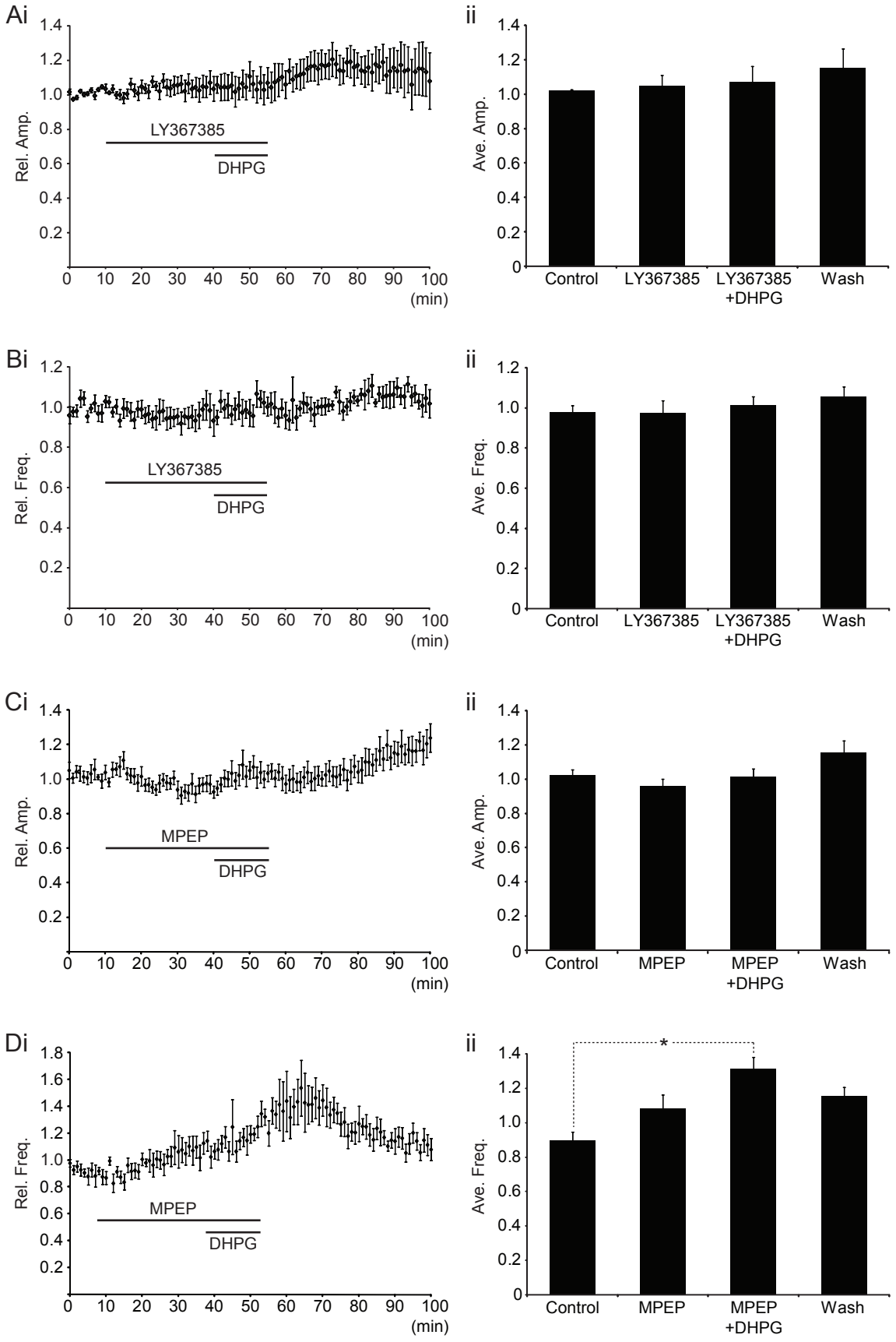
The group I mGluR specific agonist DHPG equally activates both mGluR1 and mGluR5 subtypes. Thus, the activation of either receptor subtype could be responsible for the effects of DHPG application on spinal locomotor networks. To determine which receptor subtypes are involved, and to assess whether there is endogenous activation of group I mGluRs in mouse *in vitro* spinal cord preparations, mGluR1 (LY367385) and mGluR5 (MPEP) subtype-specific antagonists were utilised. Each antagonist was applied for 30 minutes prior to, and then together with the application of DHPG (5 μM, 15 min; Fig. 4).

Application of the mGluR1 antagonist, LY367385 (50 μM), alone had no significant effects on the amplitude or frequency of locomotor-related ventral root bursts (n = 7; Fig. 4A & 4B). However, the application of LY367385 blocked the decrease in burst amplitude and increase in burst frequency normally induced by DHPG application (Fig. 4A & 4B). Application of the mGluR5 antagonist, MPEP (50 μM), also had no significant effects on locomotor burst activity when applied alone (n = 6; Fig. 4C & 4D). MPEP application did, however, block the DHPG-induced decrease in locomotor burst amplitude (Fig. 4C), but had no effect on the increase in locomotor burst frequency induced by DHPG application (31.5 ± 6.4 % increase, n = 6, $p = 0.002$; Fig. 4D).

Given that LY367385 is a competitive mGluR1 antagonist, it is possible that an endogenous role for mGluR1 subtypes was not uncovered due to weak competition of the antagonist with endogenous glutamate present within spinal networks. To address this issue, the *in vitro* spinal cord preparations were pre-incubated in LY367385 (50 μM) prior to the induction of locomotor burst activity, and then LY367385 was subsequently washed out while locomotor activity was still on-going. In these

Figure 4: Receptor subtype-specific effects of group I mGluR activation on locomotor burst activity.

Ai) Time course plot shows that the mGluR1 antagonist LY367385 (50 μM) blocked the DHPG (5 μM)-mediated decrease in locomotor burst amplitude. Aii) Pooled data, averaged over 5 min worth of recording in each experimental condition (control, subtype-specific antagonist, subtype-specific antagonist plus DHPG, and drug washout) show that DHPG application had no significant effect on locomotor burst amplitude in the presence of LY367385 (n = 7). Bi) Time course plot shows that LY367385 also blocked the DHPG-mediated increase in locomotor burst frequency. Bii) Pooled data show no significant effect of DHPG application on locomotor burst frequency in the presence of LY367385. Ci) Time course plot shows that the mGluR5 antagonist MPEP (50 μM) also blocked the DHPG-mediated decrease in locomotor burst amplitude. Cii) Pooled data show no significant effect of DHPG application on locomotor burst amplitude in the presence of MPEP (n = 6). Di) Time course plot shows that MPEP did not, however, block the DHPG-mediated increase in locomotor burst frequency. Cii) Pooled data show a significant increase in locomotor burst frequency in response to DHPG application even in the presence of MPEP. * = significantly different from control.


Ai
Rel. Amp.
LY367385
DHPG
(min)
ii
Ave. Amp.
Control
LY367385
LY367385 +DHPG
Wash
Bi
Rel. Freq.
LY367385
DHPG
(min)
ii
Ave. Freq.
Control
LY367385
LY367385 +DHPG
Wash
Ci
Rel. Amp.
MPEP
DHPG
(min)
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Control
MPEP
MPEP +DHPG
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MPEP +DHPG
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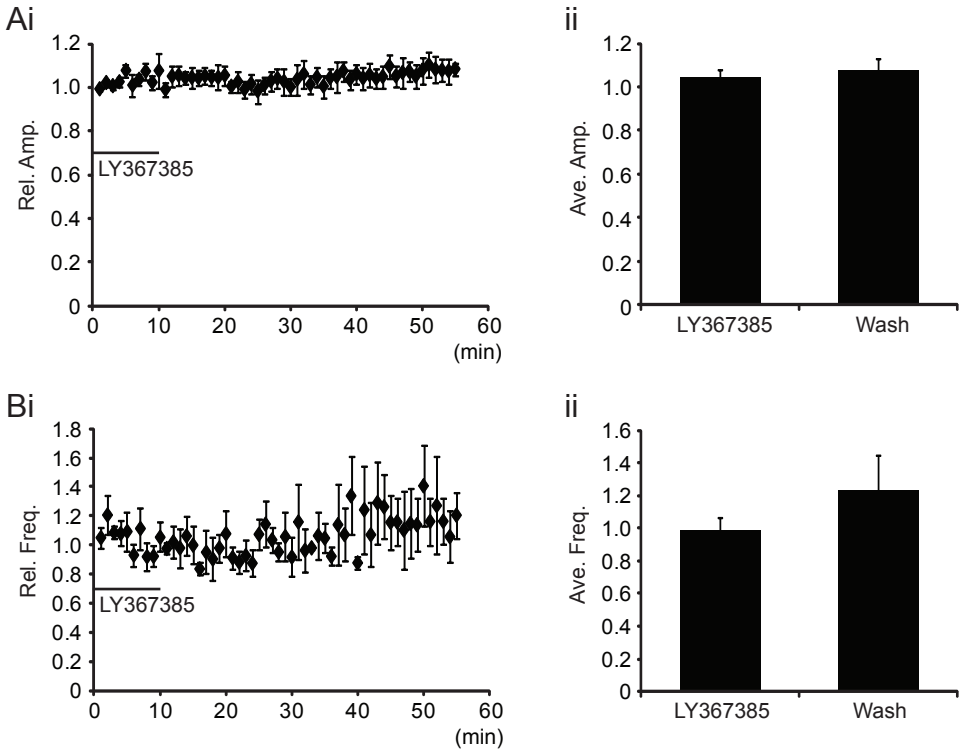


Figure 5: Assessment of the potential pharmacological competition between endogenous glutamate and the competitive mGluR1 antagonist LY367385.

Ai) Time course plot and Aii) pooled data show there was no significant change in locomotor burst amplitude upon the washout of LY367385 (50 μM), when the drug was included in the recording solution prior to and during the pharmacological induction of locomotor activity (n = 3). Bi) Time course plot and Bii) pooled data show there was also no significant difference in locomotor burst frequency upon the washout of LY367385.

experiments, LY367385 again had no effects on the amplitude or frequency of locomotor burst activity (n = 5; Fig. 5A & 5B).

Together these results suggest that group I mGluRs are not endogenously activated during locomotor activity recorded *in vitro* from isolated mouse spinal cord preparations. However once stimulated, group I mGluRs modulate the locomotor activity driven by spinal networks. mGluR1 subtypes are involved in the modulation of both the amplitude and frequency of rhythmic locomotor burst activity. However, mGluR5 subtypes are involved in the modulation of locomotor burst amplitude but not frequency.

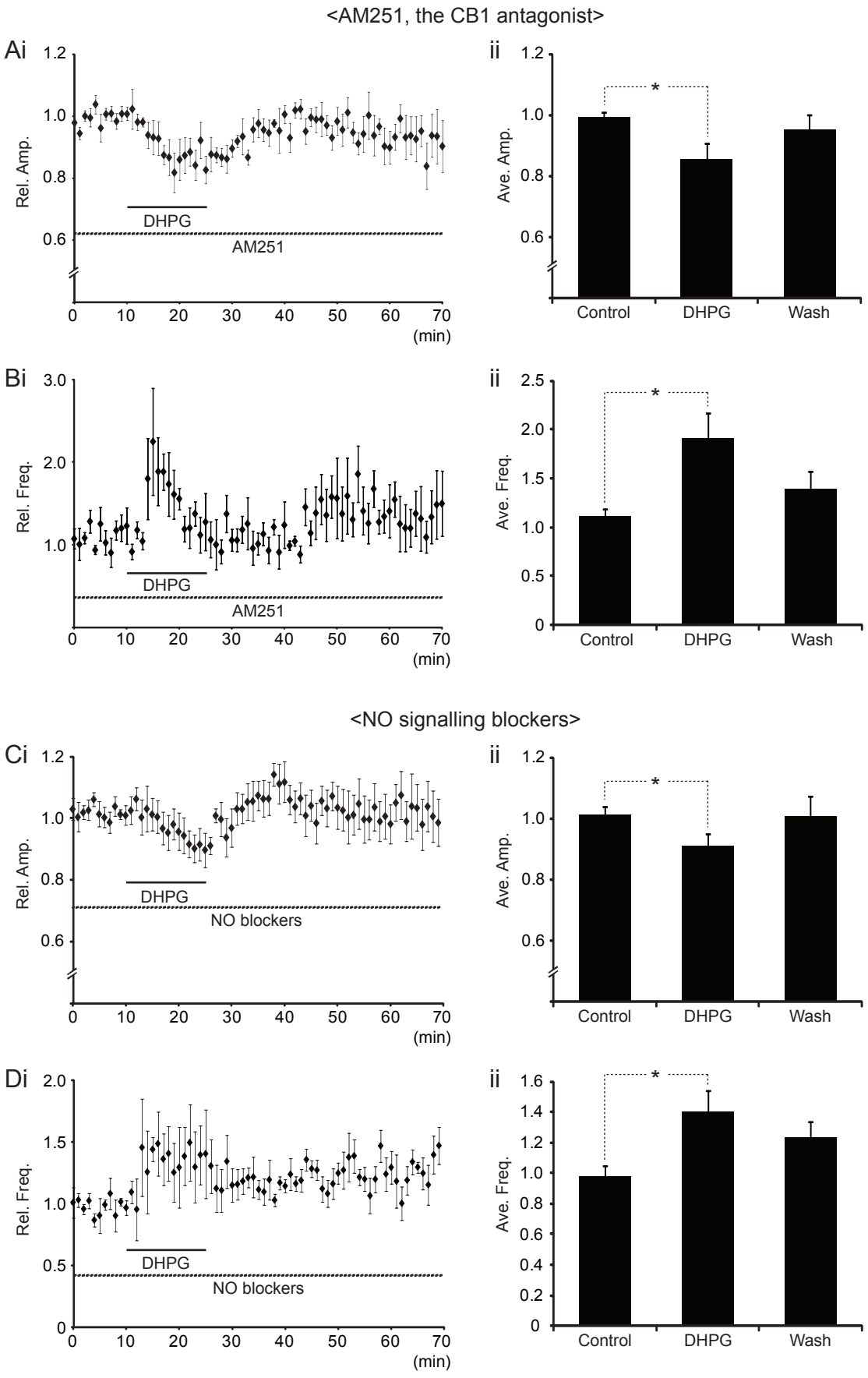
Analysis of signalling pathways downstream of group I mGluR activation

Modulation of spinal locomotor activity by the activation of group I mGluRs involves downstream effects that are mediated via endocannabinoid and nitric oxide signalling in lamprey *in vitro* spinal cord preparations (Kettunen *et al.*, 2005; Kyriakatos & El Manira, 2007). It was therefore assessed whether similar signalling mechanisms were required for the group I mGluR-mediated modulation of locomotor activity in mouse *in vitro* spinal cord preparations. An endocannabinoid receptor antagonist and pharmacological blockers of nitric oxide signalling were utilised to examine whether the group I mGluR-mediated modulation of spinal locomotor activity was occluded when these signalling pathways were blocked.

To assess involvement of endocannabinoid signalling, rhythmically active *in vitro* spinal cord preparations were bathed in recording aCSF that contained the endocannabinoid CB1 receptor antagonist, AM251 (5 μM). The application of DHPG (5 μM) in the presence of AM251 still led to a significant decrease in the amplitude of

Figure 6: Involvement of endocannabinoid or nitric oxide signalling in group I mGluR-mediated modulation of locomotor activity.

Ai) Time course plot shows that the CB1 receptor antagonist AM251 (5 μM) did not block the DHPG (5 μM)-mediated decrease in locomotor burst amplitude. Aii) Pooled data show that DHPG application still significantly reduced locomotor burst amplitude in the presence of AM251 (n = 6). Bi) Time course plot shows that AM251 also did not block the DHPG-mediated increase in locomotor burst frequency. Bii) Pooled data show a significant increase in locomotor burst frequency by DHPG application in the presence of AM251. Ci) Time course plot shows that nitric oxide signalling blockers (L-NAME 200 μM and PTIO 400 μM) also did not block the DHPG-mediated decrease in locomotor burst amplitude. Cii) Pooled data show a significant decrease in locomotor burst amplitude by DHPG application in the presence of nitric oxide signalling blockers (n = 6). Di) Time course plot shows that nitric oxide signalling blockers again did not block the DHPG-mediated increase in locomotor burst frequency. Dii) Pooled data show a significant increase in locomotor burst frequency in response to DHPG application in the presence of nitric oxide blockers. * = significantly different from control.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Ai
Rel. Amp.
DHPG
AM251
(min)
ii
Ave. Amp.
Control
DHPG
Wash
Bi
Rel. Freq.
DHPG
AM251
(min)
ii
Ave. Freq.
Control
DHPG
Wash
<NO signalling blockers>
Ci
Rel. Amp.
DHPG
NO blockers
(min)
ii
Ave. Amp.
Control
DHPG
Wash
Di
Rel. Freq.
DHPG
NO blockers
(min)
ii
Ave. Freq.
Control
DHPG
Wash

locomotor bursts (13.7 ± 5.7 % decrease, n = 6, p = 0.033; Fig. 6A) and also an increase in locomotor burst frequency (82.4 ± 39.7 % increase, n = 6, p = 0.024; Fig. 6B). The degree to which DHPG application reduced locomotor burst amplitude in the presence of AM251 was similar to that observed in the standard aCSF. However, the DHPG-induced increase in locomotor burst frequency was approximately 4 times greater, but far more variable, in the presence of AM251, compared to in the standard aCSF. This was likely to be due to greater variability in the control locomotor frequency recorded from *in vitro* spinal cord preparations incubated in AM251, as was also reported in lamprey *in vitro* spinal cord preparations (Kettunen *et al.*, 2005). Although these data suggest that endogenous activation of CB1 receptors may be required for stable generation of rhythmic locomotor activity, endocannabinoid signalling mediated via CB1 receptors is not required for group I mGluR-mediated modulation of locomotor networks in the mouse spinal cord.

Next, to examine involvement of nitric oxide signalling in group I mGluR-mediated modulation of spinal locomotor activity, the general nitric oxide synthase inhibitor, L-NAME (200 μM), and the nitric oxide scavenger, PTIO (400 μM), were included in the recording aCSF that perfused rhythmically active *in vitro* spinal cord preparations. Despite the presence of L-NAME and PTIO, the application of DHPG (5 μM) again led to a significant decrease in locomotor burst amplitude (10.1 ± 3.8 % decrease, n= 6, p = 0.026; Fig. 6C) and a significant increase in locomotor burst frequency (44.1 ± 12.9 % increase, n= 6, p = 0.010; Fig. 6D). The DHPG-induced decrease in locomotor burst amplitude in the presence of nitric oxide signalling blockers was slightly smaller than that recorded in the standard aCSF, whereas the increase in burst frequency was double that recorded in the standard aCSF. These differences may again reflect greater

variability in the output of preparations in which nitric oxide signalling is blocked. Nevertheless, these data suggest that nitric oxide signalling is also not required for group I mGluR-mediated modulation of rhythmic locomotor activity in mouse spinal networks.

Discussion

In this chapter it has been demonstrated that the activation of group I mGluRs modulates locomotor-related network activity in isolated spinal cord preparations obtained from neonatal mice. There were two main effects of group I mGluR activation on pharmacologically activated rhythmic locomotor burst activity recorded from lumbar ventral roots. One was a decrease in the amplitude of locomotor-related ventral root activity, and the other was an increase in the frequency of rhythmic locomotor burst activity.

The decrease in the amplitude of locomotor burst activity most likely reflects modulation of the intensity of motoneuron firing or altered recruitment of spinal motoneurons, which determine the final output from spinal locomotor networks. Although modulation of the input-output relationships of motoneurons has been demonstrated in studies concerning other intrinsic neuromodulators such as cholinergic inputs mediated by muscarinic C-boutons on mouse spinal motoneurons (Miles *et al.*, 2007; Zagoraiou *et al.*, 2009), the present study is the first to demonstrate group I mGluR-mediated modulation of the intensity of locomotor-related motoneuron output in mouse *in vitro* spinal cord preparations. It is therefore investigated, in the next chapter (Chapter 3), what cellular mechanisms underlie group I mGluR-mediated modulation of the input-output relationships of individual motoneurons in the mouse spinal cord.

Accompanying the decrease in locomotor burst amplitude, it has also been shown that the activation of group I mGluRs leads to an increase in the frequency of locomotor bursts recorded from lumbar ventral roots. A group I mGluR-mediated increase in locomotor burst frequency is also reported in spinal locomotor networks of other

vertebrates such as lampreys (Krieger *et al.*, 1998) and *Xenopus* tadpoles (Chapman & Sillar, 2007). In contrast, *in vitro* spinal cord preparations obtained from neonatal rats, the activation of group I mGluRs via low dose agonist application slows down locomotor burst frequency, whereas high dose agonist application completely disrupts rhythmic burst activity (Taccola *et al.*, 2004). Similar disruptions to rhythmic locomotor activity were observed in mouse *in vitro* spinal cord preparations when applying higher doses of the group I mGluR agonist, DHPG.

The group I mGluR-mediated modulation of locomotor burst frequency is likely to involve modulation of spinal interneurons and synaptic transmission within locomotor CPG networks. Depression of inhibitory synaptic transmission by the activation of group I mGluRs is suggested in spinal locomotor networks of lampreys (Kettunen *et al.*, 2005; Kyriakatos & El Manira, 2007) and *Xenopus* tadpoles (Chapman *et al.*, 2008). Enhancement of excitatory transmission is also mediated by group I mGluR activation in lamprey spinal locomotor networks (Kyriakatos & El Manira, 2007). Data from *in vitro* rat spinal cord preparations show both depression and enhancement of inhibitory synaptic transmission (Marchetti *et al.*, 2003, 2005). Together these data support that modulation of either excitatory and/or inhibitory synaptic transmission within locomotor CPG networks could underlie group I mGluR-mediated modulation of locomotor burst frequency. To fully understand the roles of group I mGluRs in the modulation of rhythmic locomotor frequency, it will be important to directly investigate the effects of group I mGluR activation on interneurons that make up locomotor CPGs. However, such direct analyses of interneurons in the mammalian spinal cord are challenging, particularly given that the neural populations which make up mammalian spinal CPGs remain poorly defined compared to CPG networks of other evolutionary

simpler vertebrates (Roberts, 2000; Grillner, 2006). The ongoing classification of distinct interneuron populations, assisted by recent advances in genetic tools based on developmental and molecular biology in the mammalian spinal cord (Jessell, 2000; Goulding, 2009; Kiehn *et al.*, 2010) will therefore be critical towards future studies aiming to understand group I mGluR-mediated modulation of mammalian spinal locomotor networks.

In addition, the present study demonstrates that the group I mGluR-mediated modulation of spinal locomotor activity is separable into the effects mediated by the activation of specific group I mGluR subtypes. The decrease in locomotor burst amplitude is mediated by both mGluR1 and mGluR5 subtype activation. On the other hand, the increase in locomotor burst frequency is mediated by mGluR1 but not mGluR5 activation, which is also shown in lamprey locomotor networks (Kettunen *et al.*, 2002). The application of group I mGluR subtype specific antagonists did not indicate endogenous activation of group I mGluRs in rhythmically active *in vitro* mouse spinal cord preparations utilised in the present study. The endogenous role of group I mGluRs is nonetheless suggested for the control of rhythmic locomotor frequency in spinal networks of lampreys (Krieger *et al.*, 1998; Krieger *et al.*, 2000; Kettunen *et al.*, 2002), *Xenopus* tadpoles (Chapman & Sillar, 2007) and rats (Taccola *et al.*, 2004). It is possible that the inability to detect an endogenous role for group I mGluRs in rhythmic *in vitro* mouse preparations may reflect compensatory activation of serotonergic and dopaminergic receptors upon the antagonism of group I mGluRs. In addition, the reduced nature and simple state of the *in vitro* preparation does not preclude involvement of group I mGluRs in the control of locomotor networks in the whole animal. Glutamatergic inputs to spinal locomotor CPGs that are lost or inactive

in the *in vitro* preparation, such as descending inputs implicated in the initial generation of spinal locomotor activity (Jordan *et al.*, 2008; Hagglund *et al.*, 2010), might activate both ionotropic and metabotropic glutamate receptors concurrently in networks of the vertebrate spinal cord (Delgado-Lezama *et al.*, 1997).

In lamprey *in vitro* spinal cord preparations, both endocannabinoid and nitric oxide signalling appears to be involved in the downstream effects of group I mGluR activation (Kettunen *et al.*, 2005; Kyriakatos & El Manira, 2007). However, the present study has not been able to provide evidence that either endocannabioid or nitric oxide signalling is directly involved in the group I mGluR-mediated modulation of rhythmic locomotor activity in the *in vitro* mouse spinal cord preparation. Although there may not be a direct link between group I mGluR activation and endocannabinoid or nitric oxide signalling, this does not exclude involvement of these signalling pathways in the modulation of mammalian spinal locomotor networks. In fact, the increased variability in locomotor burst frequency in the presence of pharmacological blockers for enndocannabinoid or nitric oxide signalling may suggest that these signalling pathways are important for intrinsic modulation of mammalian spinal networks. Endogenous modulation of spinal locomotor networks is indeed reported for enndocannabinoid signalling in lampreys (Kettunen *et al.*, 2005) and also for nitric oxide signalling in *Xenopus* tadpoles (McLean & Sillar, 2002, 2004) and in neonatal mice (Dunford *et al.*, 2009).

In summary, the present study demonstrates that the activation of group I mGluRs modulates pharmacologically induced rhythmic locomotor-related burst activity recorded from lumbar ventral roots of *in vitro* spinal cord preparations obtained from neonatal mice. The reduction in the amplitude of locomotor burst activity is likely to

be mediated via modulation of the input-output relationships of spinal motoneurons or modulation of synaptic transmission to motoneurons, both of which are investigated in the next chapter (Chapter 3). The increase in locomotor burst frequency observed in mouse *in vitro* spinal cord preparations is in accordance with results reported in other vertebrates such as lampreys (Krieger *et al.*, 1998) and *Xenopus* tadpoles (Chapman & Sillar, 2007). The group I mGluR-mediated modulation of spinal locomotor networks therefore seems evolutionally conserved from simpler vertebrate swimming networks to mammalian walking networks, further supporting the importance of intrinsic neuromodulation to provide animals with significant flexibility in the control of their motor behaviour.

Chapter 3

The role of group I mGluRs in the control of spinal motoneuron output

Parts of the following chapter have been published in:

Iwagaki, N. and Miles, GB. (2011)

Activation of group I metabotropic glutamate receptors modulates locomotor-related motoneuron output in mice

J Neurophysiol. 105 (5) : 2108 - 20.

Introduction

As discussed in the previous chapter (Chapter 2), activation of group I mGluRs modulates locomotor-related network activity in the mouse neonatal spinal cord. The aim of this chapter is therefore to investigate the cellular mechanisms that underlie group I mGluR-mediated modulation of spinal locomotor activity, with particular emphasis on modulation of the input-output relationships of spinal motoneurons that determine the final locomotor output from spinal motor networks.

In general, group I mGluRs play an excitatory role in postsynaptic neurons by coupling to the Gq protein which activates phospholipase C (PLC) catalysing phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacyl glycerol (DAG) and inositol triphosphate (IP_3) (Anwyl, 1999, 2009). In spinal neurons including motoneurons, the activation of group I mGluRs results in depolarisation of the resting membrane potential in lampreys, turtles, and rats (Krieger *et al.*, 1998; Svirskis & Hounsgaard, 1998; Kettunen *et al.*, 2003; Marchetti *et al.*, 2003, 2005). The group I mGluR-mediated depolarisation is associated with an increase in the membrane resistance of spinal neurons in lampreys (Kettunen *et al.*, 2003). However, data obtained from rat spinal motoneurons suggest that the group I mGluR-mediated depolarisation is associated with either an increase or no change in the membrane resistance (Marchetti *et al.*, 2003, 2005). In contrast, the activation of group I mGluRs does not affect either the resting membrane potential or the input resistance of motoneurons in the *Xenopus* tadpole spinal cord (Chapman *et al.*, 2008).

Further investigation into the cellular mechanisms of group I mGluR subtype specific modulation reveals that activation of the mGluR1 subtype is responsible for the

depolarisation of resting membrane potentials (Kettunen *et al.*, 2003; Marchetti *et al.*, 2003), whereas mGluR5 activation induces cellular oscillations in spinal neurons of lampreys and rats (Kettunen *et al.*, 2002; Marchetti *et al.*, 2003). The mGluR1-mediated membrane depolarisation is mediated via the blockade of leak K^+ currents, a process dependent on PLC, PKC and intracellular Ca^{2+} second messenger signals (Kettunen *et al.*, 2003; Nanou *et al.*, 2009). Whereas the mGluR5-mediated oscillation of intracellular Ca^{2+} requires stimulation of PLC and Ca^{2+} entry through L-type but not N-type Ca^{2+} channels (Kettunen *et al.*, 2002).

In addition, there have been reports of interactions between ionotropic glutamate receptors and group I mGluRs in spinal neurons of lampreys. The activation of group I mGluRs enhances NMDA receptor-mediated currents via PLC activation but independent of Ca^{2+} released from internal stores (Krieger *et al.*, 1998; Krieger *et al.*, 2000; Nanou *et al.*, 2009). The activation of mGluR1 subtypes is also reported to modulate AMPA-induced Na^+ activated K^+ currents such that a reduction of these currents is mediated via PKC activation but an enhancement of these currents occurs when intracellular Ca^{2+} is chelated (Nanou & El Manira, 2010).

Interestingly, the commonly reported excitatory effects of group I mGluR-mediated modulation of spinal neurons, in particular motoneurons, are in contrast to the group I mGluR-mediated reduction in the amplitude of locomotor-related burst activity recorded from lumbar ventral roots of rhythmically active *in vitro* mouse spinal cord preparations (Chapter 2). Given limited understanding of the possible mechanisms underlying group I mGluR-mediated modulation of the intensity of the final locomotor network output, the aim of this chapter is to elucidate roles of group I mGluRs in the modulation of motoneuron properties and synaptic inputs to motoneurons in the

mammalian spinal cord. This is investigated using whole-cell patch-clamp recordings from individual motoneurons recorded in whole *in vitro* spinal cord preparations obtained from neonatal mice.

Methods

In vitro whole spinal cord preparation

Animals were dissected and spinal cords were isolated as described in the previous chapter (Chapter 2). To perform whole-cell patch-clamp recordings from individual motoneurons in the intact *in vitro* spinal cord preparation, access for patch pipettes was created by scraping a thin line of pia matter from the ventral surface of the spinal cord preparation, just above the motor columns at the level of the 1st or 2nd lumbar (L1 - L2) roots (Miles *et al.*, 2002; Fig. 7A).

Whole-cell patch-clamp recordings

Spinal motoneurons were visualised under infrared differential interference contrast (IR-DIC) microscopy (Olympus, Tokyo, Japan). Most recorded cells were confirmed to be motoneurons by the presence of antidromic action potentials in response to stimulation of segmentally aligned ipsilateral ventral roots (<100 μA, 0.5 ms; ISO-Flex stimulator, A.M.P.I., Jerusalem, Israel; Fig. 7B). Patch electrodes (3 – 4 MΩ) were pulled on a horizontal puller (Sutter Instrument Company, Novato, CA) from non-filamented borosilicate glass capillaries (World Precision Instruments, Sarasota, FL). Patch-clamp signals were amplified and filtered (4 kHz low-pass Bassel filter) with a MultiClamp 700B amplifier (Molecular Devices) and acquired at ≥10 kHz using a Digidata 1440A A/D board and pClamp software (Molecular Devices). Details of voltage- and current-clamp protocols are explained in the results section. Series resistance compensation (60 %) was used during all voltage-clamp protocols.

Data analysis

Whole-cell patch-clamp recordings were analysed, using either Clampfit software (Molecular Devices), Dataview software (courtesy of W. J. Heitler, University of St Andrews) or, for mEPSC analyses, the Mini Analysis Program (Synaptosoft, Fort Lee, NJ). Na^+ current activation and inactivation curves were fitted with a Boltzmann function in the form: $1/(1 + \exp((V_{1/2} - V)/k)$, where $V_{1/2}$ is the half activation or half inactivation voltage, V is the test or conditioning voltage, and *k* is the slope of the fitted curve at $V_{1/2}$. Fitting of the Boltzmann function was performed using Microsoft Excel as described by Brown (2001). Data are reported as mean ± S.E. Differences in means between control and drug conditions were compared using Student's t-tests. The Kolmogorov-Smirnov test was used to test for differences in mEPSC amplitude or inter-event interval. Values of $p < 0.05$ were considered significant.

Solution and drugs

The standard aCSF solution used for dissection and recordings was identical to the solution used in the previous chapter (Chapter 2). The standard patch-clamp pipette solution contained 140 mM potassium methane sulfonate, 10 mM NaCl, 1 mM $CaCl_2$, 10 mM HEPES, 1 mM EGTA, 3 mM Mg-ATP, and 0.4 mM GTP-Na_2 (pH 7.2 – 7.3, adjusted with KOH).

For experiments investigating Na^+ currents in isolation, external and pipette solutions were designed to eliminate Ca^{2+} and K^+ currents and reduce Na^+ current amplitude to help minimise voltage-clamp errors. The modified aCSF contained 10 mM NaCl, 105 mM choline chloride, 3 mM KCl, 30 mM TEA-Cl, 10 mM HEPES, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM D-glucose, 4 mM 4-AP, 1.5 mM kynurenic acid, 10 µM bicuculline,

5 µM strychnine, 0.5 mM $CdCl_2$ (gassed with 100% O_2, pH 7.3 - 7.4 adjusted with NaOH). The modified pipette solution contained 100 mM caesium methane sulfonate, 30 mM TEA-Cl, 0.5 or 10 mM NaCl, 1 mM $CaCl_2$, 10 mM HEPES, 1 mM EGTA, 3 mM ATP-Mg, 0.4 mM GTP-Na_2, (pH 7.2 – 7.3 adjusted with KOH, osmolarity adjusted to ~290 mosmol l^{-1} with sucrose).

In addition to the pharmacological agents used in the previous Chapter 2, strychnine ((-)-strychnine) and bicuculline (1(S),9(R)-(−)-Bicuculline methiodide) were purchased from Sigma-Aldrich; TTX was purchased from Tocris Bioscience.

Results

In the previous chapter (Chapter 2), it was shown that the activation of group I mGluRs led to a decrease in the amplitude of locomotor-related motoneuron output recorded from *in vitro* mouse spinal cord preparations. In the present chapter, the cellular mechanisms that underlie this group I mGluR-mediated modulation of motoneuron output was investigated using whole-cell patch-clamp recordings from individual motoneurons in intact *in vitro* spinal cord preparations obtained from neonatal mice. The effects of group I mGluR activation were assessed with bath application of DHPG from a concentration of 10 µM to 50 µM, which maximised the likelihood of revealing the full range of group I mGluR-mediated modulatory effects on spinal motoneurons.

Group I mGluR-mediated effects on the sub-threshold properties of motoneurons

Firstly, the sub-threshold effects of group I mGluR activation on spinal motoneurons were investigated. Application of DHPG significantly depolarised the resting membrane potential of motoneurons (10 µM, 11.4 ± 1.5 mV, n = 10, $p < 0.001$; 50 µM, 16.0 ± 3.1 mV, n = 11, $p < 0.001$; Fig. 7C & 7D). The membrane potential began to repolarise in most recorded cells during the course of the drug application (15 – 20 min) as reported in spinal motoneurons of neonatal rats (Marchetti *et al.*, 2003), indicating possible desensitisation of these receptors or other components of group I mGluR signalling pathways. To investigate the currents responsible for the DHPG-induced depolarisation, the input resistance of motoneurons was measured using small voltage steps (-70 to -55 mV, 2.5 mV increments, 10 ms duration) delivered in voltage-clamp

mode (Fig. 7E). No significant change in input resistance was observed with application of 10 μM DHPG (n = 9). However, at the higher dose of DHPG (50 μM), a significant decrease in input resistance was revealed (52.8 ± 10.1 to 37.3 ± 3.6 MΩ, n = 11, p = 0.035; Fig. 7F). This reduction in input resistance was incompletely reversed with drug washout, suggesting that group I mGluR activation may be associated with long-term changes in conductances or that high doses of DHPG cannot be fully removed over the time course of recordings. Current-voltage (I-V) relationships were examined for DHPG-induced currents by subtracting I-V relationships in control from those in the presence of DHPG (50 μM). I-V relationships obtained from individual motoneurons were then pooled to reveal a linear I-V relationship for DHPG-induced currents, with an approximate reversal potential of -30 mV (n = 11, Fig. 7G). These data suggest that the activation of group I mGluRs leads to the activation or facilitation of a mixed-cationic current in mouse spinal motoneurons.

Group I mGluR-mediated modulation of motoneuron firing properties

The group I mGluR-mediated depolarisation of motoneurons is in apparent opposition to the decrease in locomotor-related motoneuron output recorded from rhythmically active *in vitro* mouse spinal cord preparations (Chapter 2). Thus, the effects of group I mGluR activation on the firing properties of individual motoneurons was investigated. Repetitive firing of action potentials was evoked in motoneurons by injection of square wave depolarising current pulses (1s duration). To study the effects of DHPG application upon repetitive firing of motoneurons in isolation from its depolarising effects, repolarising bias currents were applied to bring motoneuron resting potentials back to their control levels. The application of DHPG led to a reduction in

Figure 7: Sub-threshold effects of group I mGluR activation on individual motoneurons recorded in an intact whole spinal cord preparation.

A) Schematic showing the experimental setup with a patch pipette targeting a spinal motoneuron and glass suction electrodes attached to the second lumbar (L2) ventral roots (courtesy of G. B. Miles, University of St Andrews). B) Antidromic action potentials recorded from a motoneuron in response to stimulation of the ipsilateral ventral root. Arrow indicates stimulus artefact. C) and D) Resting membrane potential of motoneurons depolarised in response to DHPG application (50 μM, n = 11). E) Current recorded in response to brief sub-threshold voltage steps (10 ms, -70 to -55 mV) in a motoneuron held at -60 mV in control condition, in the presence of DHPG, and following drug washout. F) Membrane resistance was significantly decreased by DHPG application. G) DHPG-induced currents appeared to have a linear current-voltage relationship, with an extrapolated reversal potential of approximately -30 mV. * = significantly different from control.

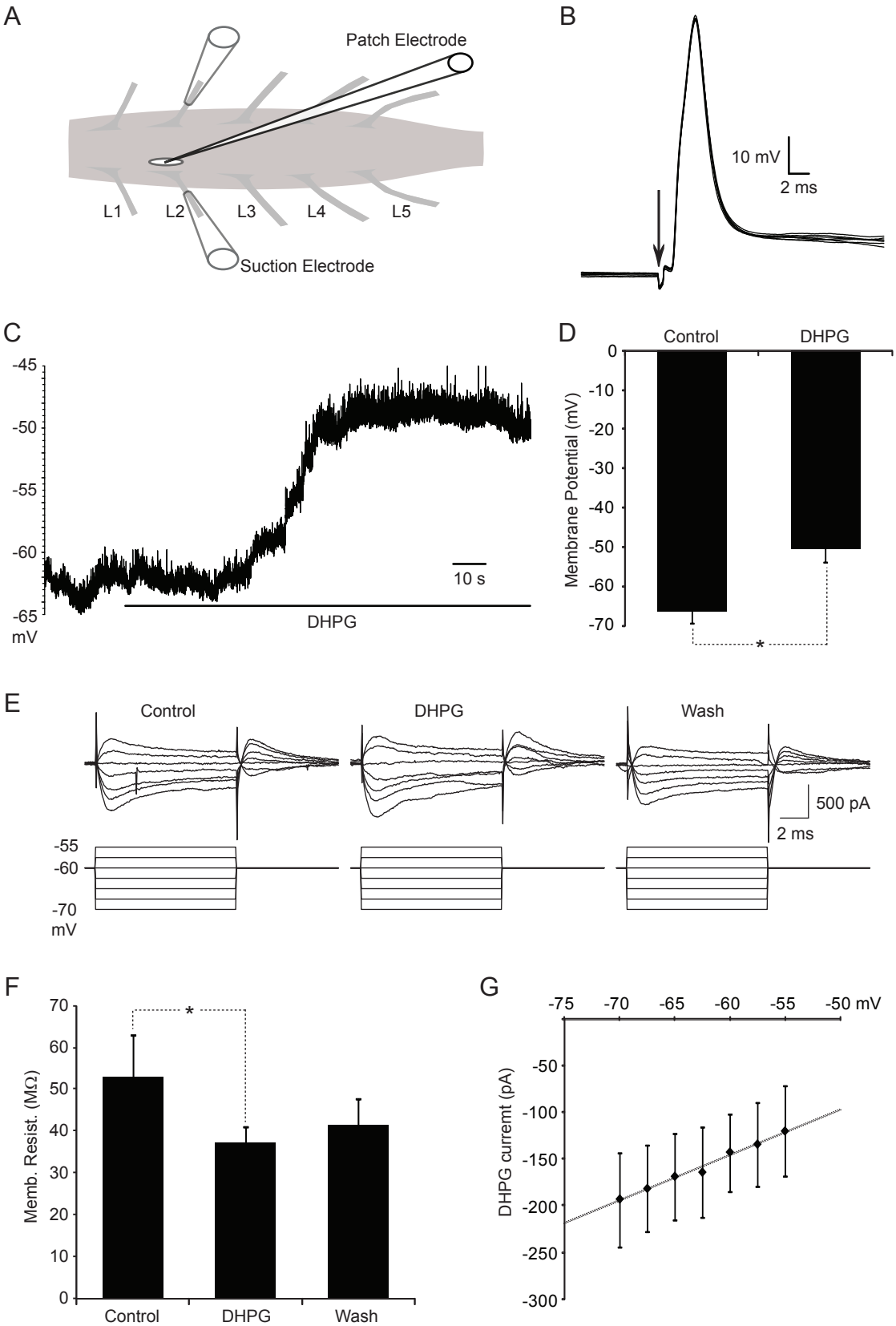
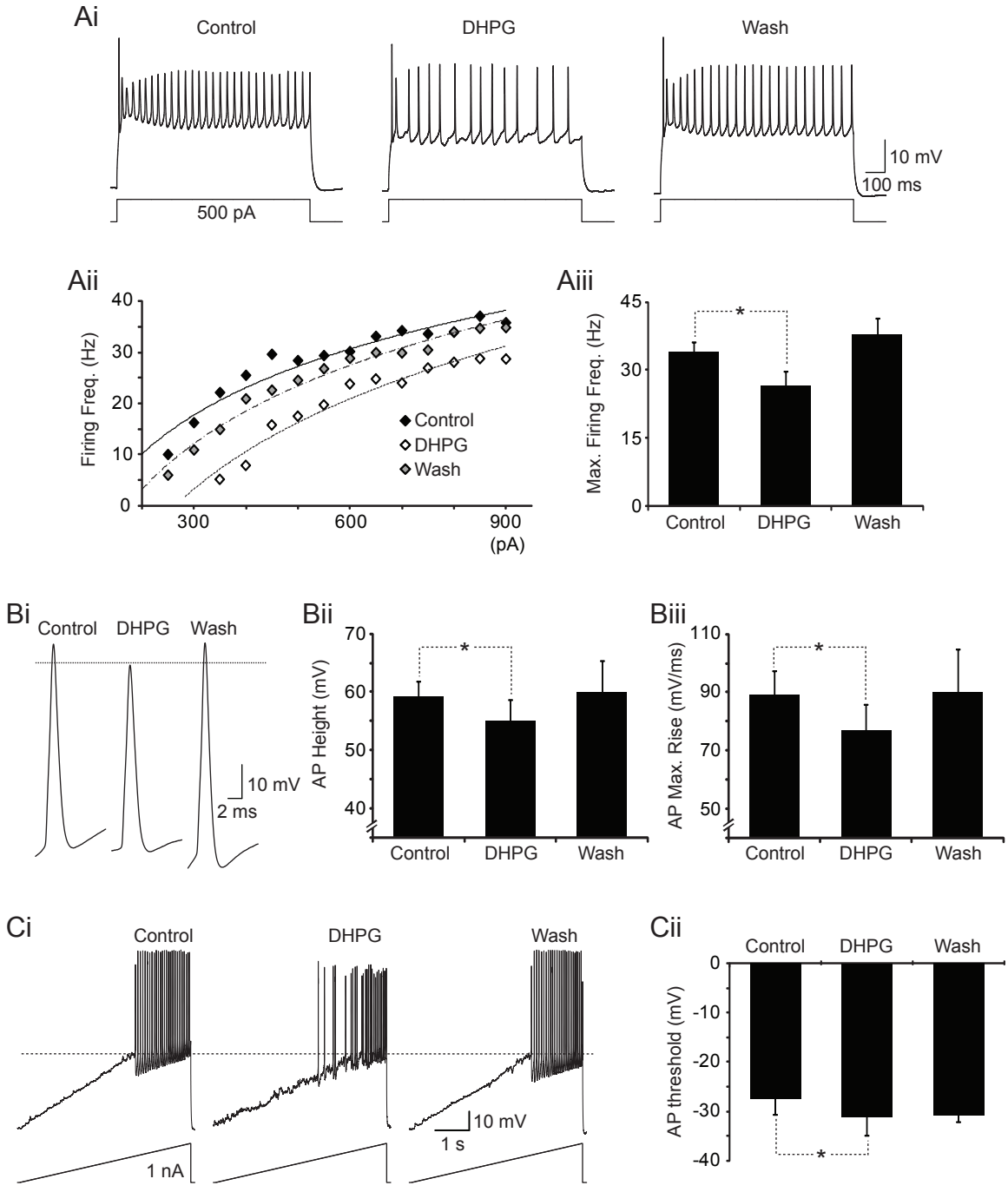

A
Patch Electrode
L1
L2
L3
L4
L5
Suction Electrode
B
10 mV
2 ms
C
-45
-50
-55
-60
-65
mV
10 s
DHPG
D
Control
DHPG
Membrane Potential (mV)
0
-10
-20
-30
-40
-50
-60
-70
*
E
Control
DHPG
Wash
500 pA
2 ms
-55
-60
-70
mV
F
Memb. Resist. (MΩ)
70
60
50
40
30
20
10
0
*
Control
DHPG
Wash
G
-75
-70
-65
-60
-55
-50 mV
DHPG current (pA)
-50
-100
-150
-200
-250
-300

Figure 8: Group I mGluR-mediated effects on motoneuron firing.

Ai) Repetitive firing in a motoneuron in response to brief (1 s) square current pulses in control condition, in the presence of DHPG (50 μM), and after drug washout. Aii) The firing frequency vs. injected current (f-I) relationship for this motoneuron was shifted to the right by DHPG application. Aiii) The maximum firing frequency of motoneurons was significantly reduced by DHPG application (n = 11). Bi) Traces showing the first action potentials evoked by square current pulses in control condition, in the presence of DHPG and after drug washout. Bii) The height and Biii) maximum rate of rise of action potentials were significantly reduced by DHPG application (n = 9). Ci) and Cii) The voltage threshold for action potential generation, measured by injecting a ramp of current, was significantly hyperpolarised by DHPG application. * = significantly different from control.


Ai
Control
DHPG
Wash
10 mV
100 ms
500 pA
Aii
Firing Freq. (Hz)
40
30
20
10
0
300
600
900
(pA)
Control
DHPG
Wash
Aiii
Max. Firing Freq. (Hz)
45
30
15
0
*
Control
DHPG
Wash
Bi
Control
DHPG
Wash
10 mV
2 ms
Bii
AP Height (mV)
70
60
50
40
*
Control
DHPG
Wash
Biii
AP Max. Rise (mV/ms)
110
90
70
50
*
Control
DHPG
Wash
Ci
Control
DHPG
Wash
10 mV
1 s
1 nA
Cii
Control
DHPG
Wash
AP threshold (mV)
0
-10
-20
-30
-40
*

the firing frequency of motoneurons in response to current injection (Fig. 8Ai). This reduction in motoneuron excitability was evident as a rightward shift in the steady-state firing frequency versus injected current (f-I) relationships of 6 out of 8 motoneurons in response to 10 μM DHPG application and 7 out of 11 motoneurons in response to 50 μM DHPG application (Fig. 8Aii). In the remaining cells, DHPG either had no clear effect on the f-I relationship or caused a leftward shift in the f-I relationship. Reduced motoneuron excitability upon application of DHPG was also demonstrated by a significant reduction in the maximum firing frequency of motoneurons (10 μM, 26.3 ± 7.6 % reduction, n = 8, p = 0.006; 50 μM, 16.1 ± 4.9 % reduction, n = 11, p = 0.031; Fig. 8Aiii). Furthermore, reduced motoneuron firing was observed in the presence of DHPG (50 μM) even when repolarising bias currents were not injected (n = 11).

To further understand the mechanisms that underlie the group I mGluR-mediated decrease in motoneuron excitability, parameters of action potentials were compared in control and in the presence of DHPG. Measurements of the height and the maximum rate of rise of the first action potential evoked by depolarising square current pulses (1s duration) revealed a significant reduction in action potential height (10 μM, 5.5 ± 2.4 mV reduction, n = 9, p = 0.028; 50 μM, 4.2 ± 1.8 mV reduction, n = 9, p = 0.026; Fig. 8Bi & 8Bii) and a significant slowing of the maximum rate of rise (10 μM, 16.2 ± 5.0 % reduction, n = 9, p = 0.030; 50 μM, 13.8 ± 5.9 % reduction, n = 9, p = 0.023; 8Bi & 8Biii) upon the application of DHPG. Changes in these two parameters were evident whether the measurements were taken from the same current pulses in control and in the presence of DHPG or from current pulses which elicited similar firing frequencies in the two conditions.

The effect of group I mGluR activation on the voltage threshold for the generation of

repetitive action potentials in motoneurons was also investigated. To remove the effects of DHPG-mediated depolarisation on action potential threshold, constant repolarising bias currents were applied. Action potentials were evoked by a ramp of depolarising current (100 – 500 pA/s; Fig. 8Ci). The voltage threshold was defined as the voltage at which the rate of rise of the first evoked action potential reached 10 mV/ms. Surprisingly, the voltage threshold for action potential generation was significantly hyperpolarised during the application of DHPG (10 μM, 2.1 ± 0.7 mV hyperpolarisation, n = 8, p = 0.008; 50 μM, 5.0 ± 1.6 mV hyperpolarisation, n = 9, p = 0.002; Fig. 8Cii). Despite hyperpolarisation of the action potential threshold, a reduction in motoneuron firing in response to the ramp current injection was still evident during DHPG application (Fig. 8Ci). Of note, there appears to be a reduction in the action potential after-hyperpolarisation in the recording depicted in Fig. 8Ci. However, this was not a consistent observation across the recordings. Finally, hyperpolarisation of the action potential threshold was also apparent in motoneuron recordings in response to square current pulses (Fig. 8Ai).

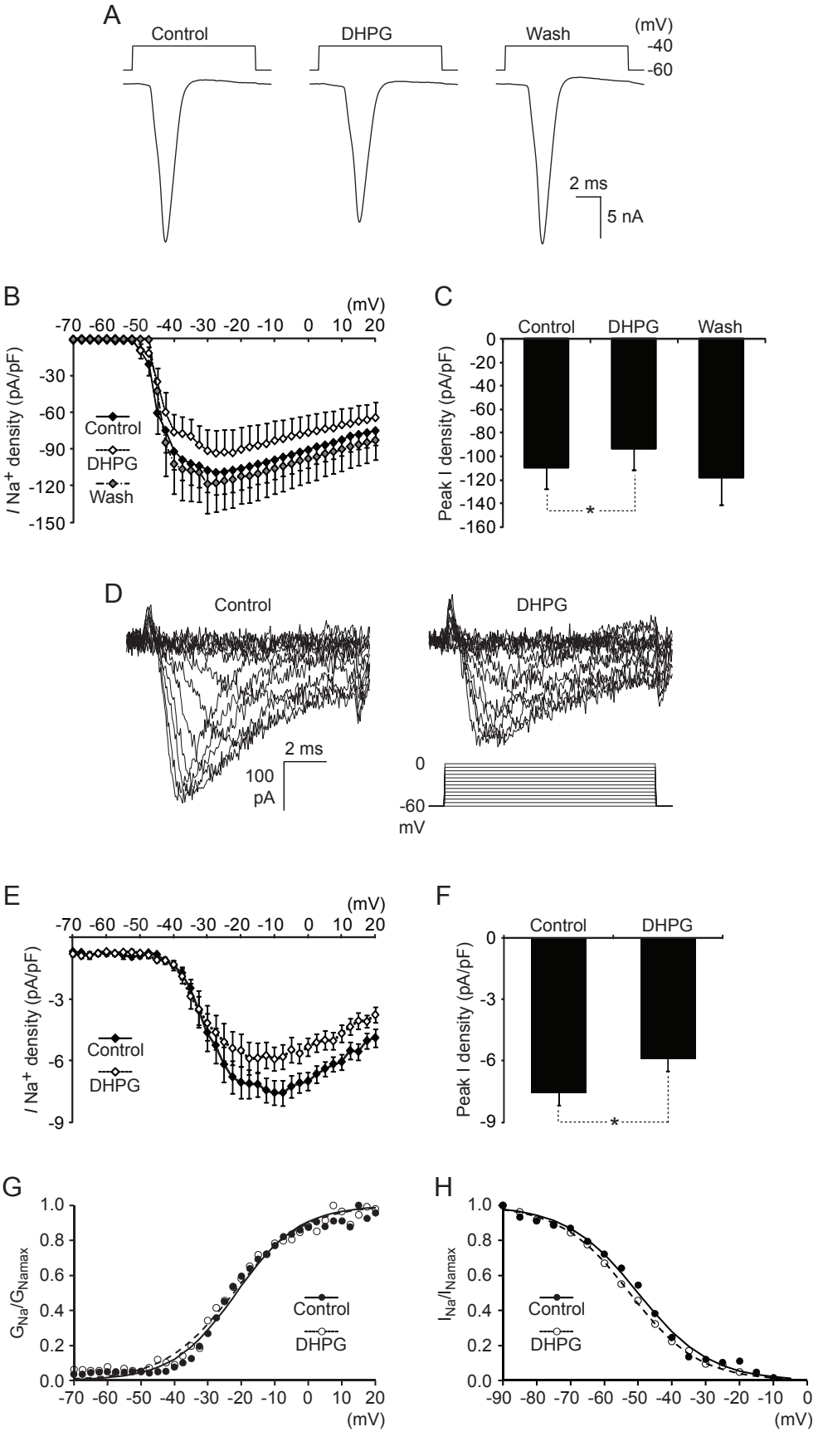
Taken together, the modulatory effects of group I mGluR activation upon the firing properties of spinal motoneurons appear to be mostly inhibitory. Group I mGluR-mediated inhibitory effects include the decreased excitability for generating repetitive firing and the reduced height and maximum rate of rise of action potentials. One group I mGluR-mediated excitatory effect was the hyperpolarisation of the voltage threshold for action potential generation.

Group I mGluR-mediated modulation of Na^+ currents in spinal motoneurons

Given that group I mGluR activation led to reductions in the height and maximum

Figure 9: Group I mGluR activation modulates the fast inactivating Na^+ current in motoneurons.

A) Fast inactivating Na^+ currents elicited in standard recording solutions by brief (10 ms) depolarising voltage steps in a motoneuron held at -60 mV in control condition, in the presence of DHPG (50 μM), and after drug washout. B) Plots of current-voltage (I-V) relationships and C) average peak current density for fast inactivating Na^+ currents demonstrate a DHPG-mediated reduction in the density of Na^+ currents (n = 11). D) Na^+ currents recorded in modified solutions (TEA, 4-AP, Cs^+, $CdCl_2$, bicuculline, strychnine, and kynurenic acid) designed to block Ca^{2+} channels, K^+ channels, and synaptic transmission and to reduce Na^+ current magnitude by lowering the concentration of extracellular Na^+ (10 mM). E) Plots of I-V relationships and F) average peak current density for Na^+ currents again demonstrate a DHPG (50 μM)-mediated reduction in Na^+ current density (n = 6). G) Normalised activation and H) steady-state inactivation curves plotted in control condition and in the presence of DHPG demonstrate no change in the voltage dependence of activation or steady-state inactivation of Na^+ currents. * = significantly different from control.


A
Control
DHPG
Wash
(mV)
-40
-60
2 ms
5 nA
B
(mV)
-70 -60 -50 -40 -30 -20 -10 0 10 20
I Na+ density (pA/pF)
-30
-60
-90
-120
-150
Control
DHPG
Wash
C
Control
DHPG
Wash
Peak I density (pA/pF)
0
-20
-40
-60
-80
-100
-120
-140
-160
*
D
Control
DHPG
2 ms
100
pA
0
-60
mV
E
(mV)
-70 -60 -50 -40 -30 -20 -10 0 10 20
I Na+ density (pA/pF)
-3
-6
-9
Control
DHPG
F
Control
DHPG
Peak I density (pA/pF)
0
-3
-6
-9
*
G
GNa/GNamax
1.0
0.8
0.6
0.4
0.2
0.0
Control
DHPG
-70 -60 -50 -40 -30 -20 -10 0 10 20
(mV)
H
INa/INamax
1.0
0.8
0.6
0.4
0.2
0.0
Control
DHPG
-90 -80 -70 -60 -50 -40 -30 -20 -10 0
(mV)

rate of rise of action potentials in motoneurons, it can be hypothesised that group I mGluR activation modulates Na^+ channels that mediate the fast, inactivating Na^+ current. This was tested by eliciting Na^+ currents in motoneurons in voltage-clamp mode, using a series of small voltage steps (-70 to +20 mV, 2.5 mV increments, 10 ms duration) from a holding potential of -60 mV in control conditions and in the presence of DHPG (Fig. 9A). Analyses of Na^+ currents were performed on leak subtracted traces. In standard recording aCSF and pipette solutions, the density of fast, inactivating Na^+ currents was significantly reduced during the application of DHPG (Fig. 9B & 9C). The peak current density, elicited by steps to -27.5 mV, was significantly reduced by 19.4 ± 8.4 % in response to 10 µM DHPG (n = 9, $p = 0.018$) and by 18.7 ± 5.3 % in response to 50 µM DHPG (n = 11, $p < 0.001$; Fig. 9B & 9C).

The occurrence of voltage- and space-clamp errors is increased when recording large currents from large neurons. Although these errors are likely to be equivalent in control and in the presence of DHPG, it is also possible that changes in cell properties induced by DHPG may differentially affect the ability to control voltage in the two conditions. It was therefore assessed whether DHPG application also modulated Na^+ currents when other channel types (Ca^{2+} and K^+) and synaptic transmission were pharmacologically blocked. In addition, modified recording solution with lowered extracellular Na^+ concentration (10 mM NaCl) was utilised to reduce the amplitude of Na^+ currents (Carlier *et al.*, 2006). In these modified pipette and recording solutions Na^+ currents, elicited by depolarising steps (-70 to +20 mV, 2.5 mV increments, 10 ms duration) from a holding potential of -60 mV, were more than 10-fold smaller than those recorded in standard solutions (Fig. 9D). With Na^+ currents considerably reduced and other channels and synaptic transmission blocked, the application of DHPG (50 µM)

still led to a significant decrease in the peak density of Na^+ currents (20.3 ± 7.8 % reduction, n = 6, p = 0.017; Fig. 9E & 9F).

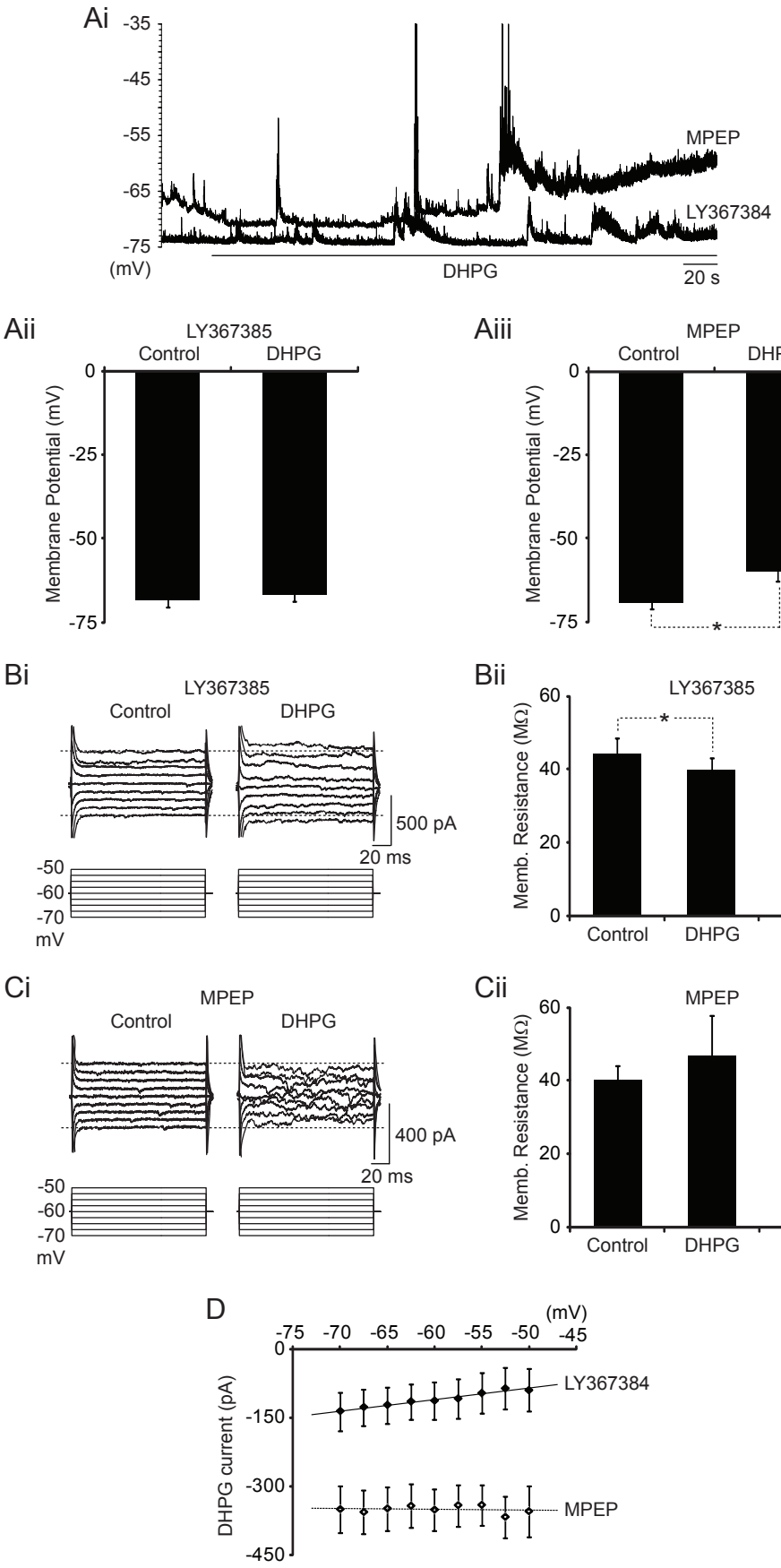
To investigate the mechanisms by which group I mGluR activation modulates Na^+ currents, the voltage dependence of Na^+ current activation and inactivation was analysed in control and in the presence of DHPG (50 μM) using the modified intracellular and recording solutions. Activation of Na^+ currents was investigated using depolarising steps from a holding potential of -60 mV (-70 to +20 mV, 2.5 mV increments, 10 ms duration; Fig. 9G). Steady-state inactivation of Na^+ currents was investigated using steps to 0 mV (10 ms duration) from pre-pulses (50 ms duration) ranging from -90 to -5 mV (5 mV increments; Fig. 9H). There were no significant changes in the half-maximal activation voltage of Na^+ currents in the presence of DHPG (-31.4 ± 1.1 mV in control, -33.2 ± 1.7 mV in DHPG, n = 6; Fig. 9G). The half maximal voltage of steady-state inactivation was also unchanged by DHPG application (-51.0 ± 0.8 mV in control, -51.1 ± 0.9 mV in DHPG, n = 6; Fig. 9H). These data indicate that the activation of group I mGluRs modulates Na^+ current density by means other than affecting the voltage dependence of activation or inactivation of Na^+ currents.

Group I mGluR subtype specific effects on intrinsic properties of motoneurons

The results thus far have demonstrated that the activation of group I mGluRs modulates a wide range of intrinsic properties of motoneurons in the spinal cord of neonatal mice. Although the effects of group I mGluR activation include depolarisation of the resting membrane potential and hyperpolarisation of the voltage threshold for repetitive action potential generation, the most pronounced effect is a reduction in motoneuron excitability, which is likely due to the modulation of Na^+

Figure 10: Receptor subtype-specific effects of group I mGluR activation on sub-threshold properties of motoneurons.

Ai, Aii and Aiii) A DHPG (10 μM)-meditated depolarisation of resting membrane potentials was blocked by the mGluR1 antagonist LY367385 (50 μM, n = 8) but not by the mGluR5 antagonist MPEP (10 μM, n = 9). Bi) and Bii) Membrane resistance, calculated from sub-threshold voltage steps (150 ms, -70 to -50 mV) in a motoneuron hold at -60 mV, was significantly reduced by DHPG application even in the presence of LY367385. Ci) and Cii) DHPG-mediated reduction in membrane resistance was blocked by MPEP. D) Small DHPG-induced currents, with an extrapolated reversal potential of approximately -15 mV were detected in the presence of LY367385. Larger DHPG-induced depolarising currents in the presence of MPEP show horizontal I-V relationships. * = significantly different from control.


Ai
-35
-45
-55
-65
-75
(mV)
MPEP
LY367384
DHPG
20 s
Aii
LY367385
Control
DHPG
0
-25
-50
-75
Membrane Potential (mV)
Aiii
MPEP
Control
DHPG
0
-25
-50
-75
Membrane Potential (mV)
*
Bi
LY367385
Control
DHPG
500 pA
20 ms
-50
-60
-70
mV
Bii
LY367385
*
60
40
20
0
Memb. Resistance (MΩ)
Control
DHPG
Wash
Ci
MPEP
Control
DHPG
400 pA
20 ms
-50
-60
-70
mV
Cii
MPEP
60
40
20
0
Memb. Resistance (MΩ)
Control
DHPG
Wash
D
(mV)
-75 -70 -65 -60 -55 -50 -45
0
-150
-300
-450
DHPG current (pA)
LY367384
MPEP

channels. As was shown in the previous chapter (Chapter 2), the group I mGluR-mediated modulation of the intensity of locomotor-related motoneuron output involves activation of both mGluR1 and mGluR5 subtypes. Therefore, the next aim was to identify which receptor subtypes are responsible for the various effects of group I mGluR-mediated modulation of mouse spinal motoneurons. Replicating the whole-cell patch-clamp recording protocols described above, the effects of DHPG (10 μM) application in the presence of either mGluR1 (LY367385, 50 μM) or mGluR5 (MPEP, 10 μM) specific antagonists were investigated.

The sub-threshold effects mediated by the activation of group I mGluR subtypes in spinal motoneurons were first analysed. The membrane depolarisation normally induced by DHPG application was blocked by LY367385 (n = 8; Fig. 10Ai & 10Aii). In comparison, the application of DHPG was still associated with a significant membrane depolarisation in the presence of MPEP (9.4 ± 2.0 mV depolarisation, n = 9, $p < 0.001$; Fig. 10Ai & 10Aiii). Input resistance was measured, using small voltage steps (-70 to -50 mV, 2.5 mV increments, 150 ms duration) from a holding potential of -60 mV (Fig. 10Bi & 10Ci). Although the depolarising effect of DHPG application was blocked by LY367385, the input resistance was still significantly decreased with DHPG application (44.4 ± 4.3 MΩ to 39.7 ± 3.4 MΩ, n = 8, $p = 0.025$; Fig. 10Bi & 10Bii). On the other hand, despite the membrane depolarisation recorded, the input resistance was unchanged by DHPG application in the presence of MPEP (n = 9; Fig. 10Ci & 10Cii). Analyses of the I-V relationships for DHPG-induced currents revealed small currents that would reverse at approximately -15 mV during DHPG application in the presence of LY367385 (n = 8; Fig. 10D). The absence of a significant membrane depolarisation in motoneurons from DHPG-induced small currents makes interpretation

difficult. However, one explanation is that the decreased input resistance shunted the mGluR1-mediated small currents therefore limiting changes in voltage across the cell membrane. In contrast, DHPG application in the presence of MPEP led to large depolarising inward currents that demonstrated horizontal I-V relationships (n = 9; Fig. 10D).

The receptor subtypes responsible for the group I mGluR-mediated reduction in the excitability of spinal motoneurons were examined next. Almost all of the modulatory effects upon motoneuron firing normally induced by DHPG application were no longer evident in the presence of the mGluR1 specific antagonist, LY367385. DHPG application had no consistent effect on the steady-state f-I relationships of motoneurons in the presence of LY367385 (n = 8; Fig. 11Ai), with maximum firing frequencies unchanged (n = 8; Fig. 11Aii). The height and maximum rate of rise of the first action potential evoked by square current pulse injection (1 s duration) also remained unchanged when DHPG was applied in the presence of LY367385 (n = 7; Fig. 11B). However, analysis of the voltage threshold for action potential generation in response to a ramp of current injection (3 s duration) revealed that the hyperpolarisation of action potential threshold was still induced by DHPG application even in the presence of LY367385 (Fig. 11Ci & 11Cii). The threshold for action potential generation significantly hyperpolarised by 3.5 ± 0.9 mV in response to DHPG application in the presence of LY367385 (n = 7, p = 0.004; Fig. 11Cii).

In keeping with the above experiments utilising the mGluR1 antagonist, LY367385, most of the DHPG-induced modulatory effects on motoneuron excitability were preserved in the presence of the mGluR5 antagonist, MPEP. Although obvious rightward shifts in steady-state f-I relationships in response to DHPG application (Fig.

Figure 11: Effects of group I mGluR activation on motoneuron firing in the presence of LY367385.

Ai) No consistent effect of DHPG application (10 μM) on steady state firing frequency vs. injected current (f-I) relationships was observed in motoneurons in the presence of LY367385 (50 μM). Aii) LY367385 blocked the DHPG-mediated decrease in maximum firing frequency of motoneurons (n = 8). Bi) Traces showing the first action potentials evoked by square current pulses (1 s) in control condition, during DHPG application and after drug washout in the presence of LY367385. Bii) The height and Biii) maximum rate of rise of these action potentials remained unchanged by DHPG application in the presence of LY367385 (n = 7). Ci) and Cii) The voltage threshold for action potential generation, measured by injecting a current ramp, was still significantly hyperpolarised by DHPG application in the presence of LY367385. * = significantly different from control.

<LY367385, mGluR1 antagonist>

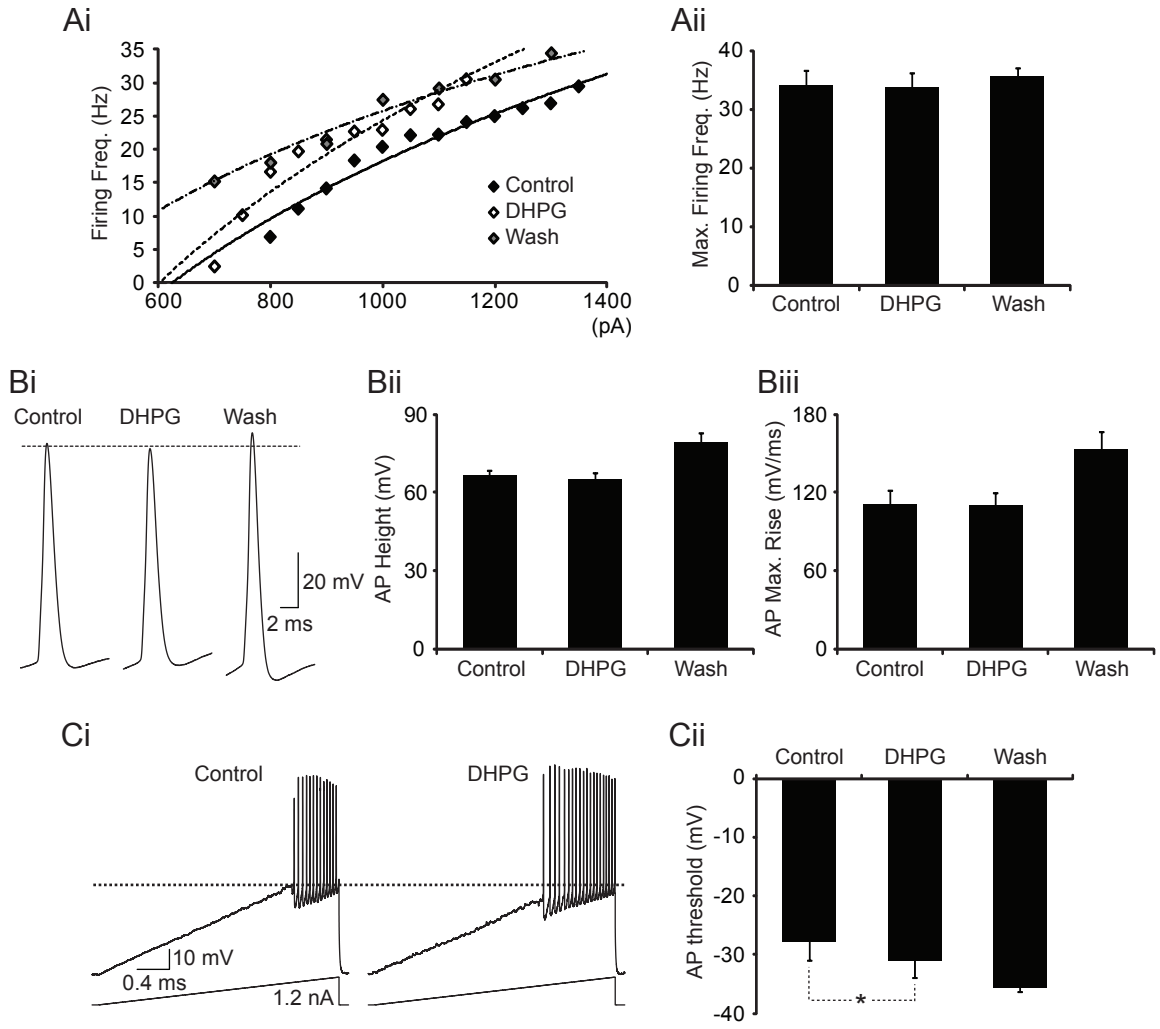
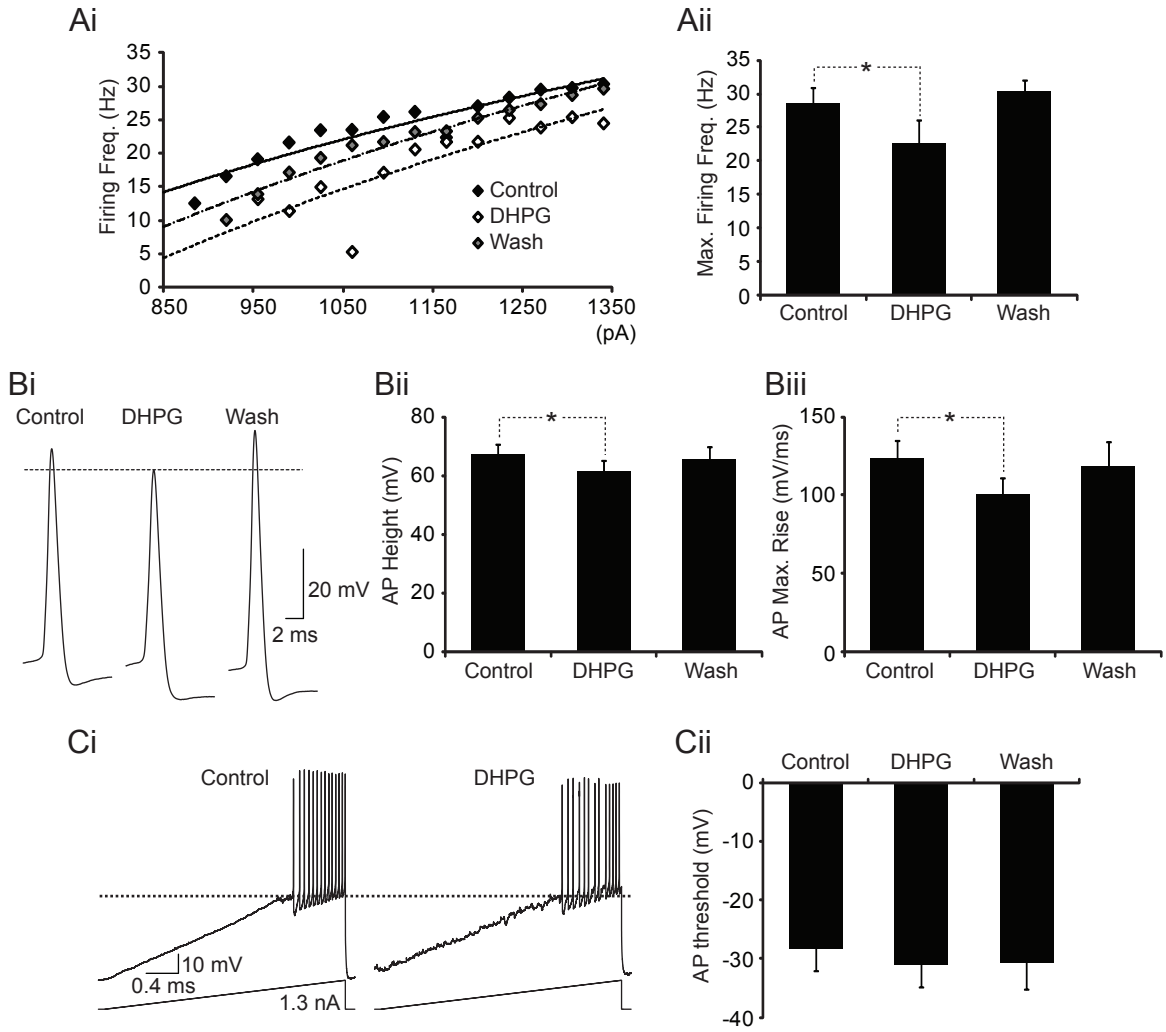


Figure 12: Effects of group I mGluR activation on motoneuron firing in the presence of MPEP.

Ai) Steady state f-I relationships recorded in a motoneuron in the presence of MPEP (10 μM). Aii) MPEP did not block the DHPG (10 μM)-induced decrease in maximum firing frequency of motoneurons (n = 9). Bi) Traces showing the first action potentials evoked by square current pulses (1 s) in control condition, during DHPG application and drug washout in the presence of MPEP. Bii) The height and Biii) maximum rate of rise of these action potentials were still significantly reduced by DHPG application in the presence of MPEP. Ci) and Cii) MPEP, however, blocked the DHPG-mediated hyperpolarisation of voltage thresholds for action potential generation. * = significantly different from control.

<MPEP, mGluR5 antagonist>



12Ai) were not often evident in motoneurons recorded in the presence of MPEP (2 out of 9 motoneurons), the maximum firing frequency of motoneurons was significantly reduced by DHPG application in the presence of MPEP (21.7 ± 9.5 % reduction, n = 9, p = 0.031; Fig. 12Aii). In addition, the height and maximum rate of rise of the first action potential evoked by square current pulse injection (1 s duration) were both significantly reduced when DHPG was applied in the presence of MPEP (5.4 ± 1.4 mV height reduction, n = 9, p = 0.002; 18.2 ± 3.4 % max. rise reduction, n = 9, p = 0.002; Fig. 12B). Again, as predicted from data obtained in the presence of LY367385, MPEP did block the hyperpolarising effect of DHPG application upon the voltage threshold for action potential generation, resulting in unchanged voltage thresholds for action potentials evoked by injections of ramp currents in control and during DHPG application (n = 9; Fig. 12Ci & Cii).

Consistent with a dominant role of the mGluR1 subtype in the group I mGluR-mediated reduction in motoneuron excitability, the modulation of fast inactivating Na^+ currents also appeared to be primarily mediated via the activation of the mGluR1 subtype. LY367385 blocked DHPG-induced reductions in the density of fast inactivating Na^+ currents (Fig. 13Ai), leaving the peak Na^+ current density unchanged with DHPG application (n = 8; Fig. 13Aii). In contrast, MPEP did not block the effect of DHPG application upon fast inactivating Na^+ currents (Fig. 13Bi). A significant reduction in the peak Na^+ current density was observed when DHPG was applied in the presence of MPEP (18.1 ± 2.5 % reduction, n = 9, $p < 0.001$; Fig. 13Bii).

Taken together these detailed analyses of the involvement of group I mGluR subtypes in the modulation of the intrinsic properties of spinal motoneurons indicate that the majority of the modulatory effects observed are mediated by the activation of

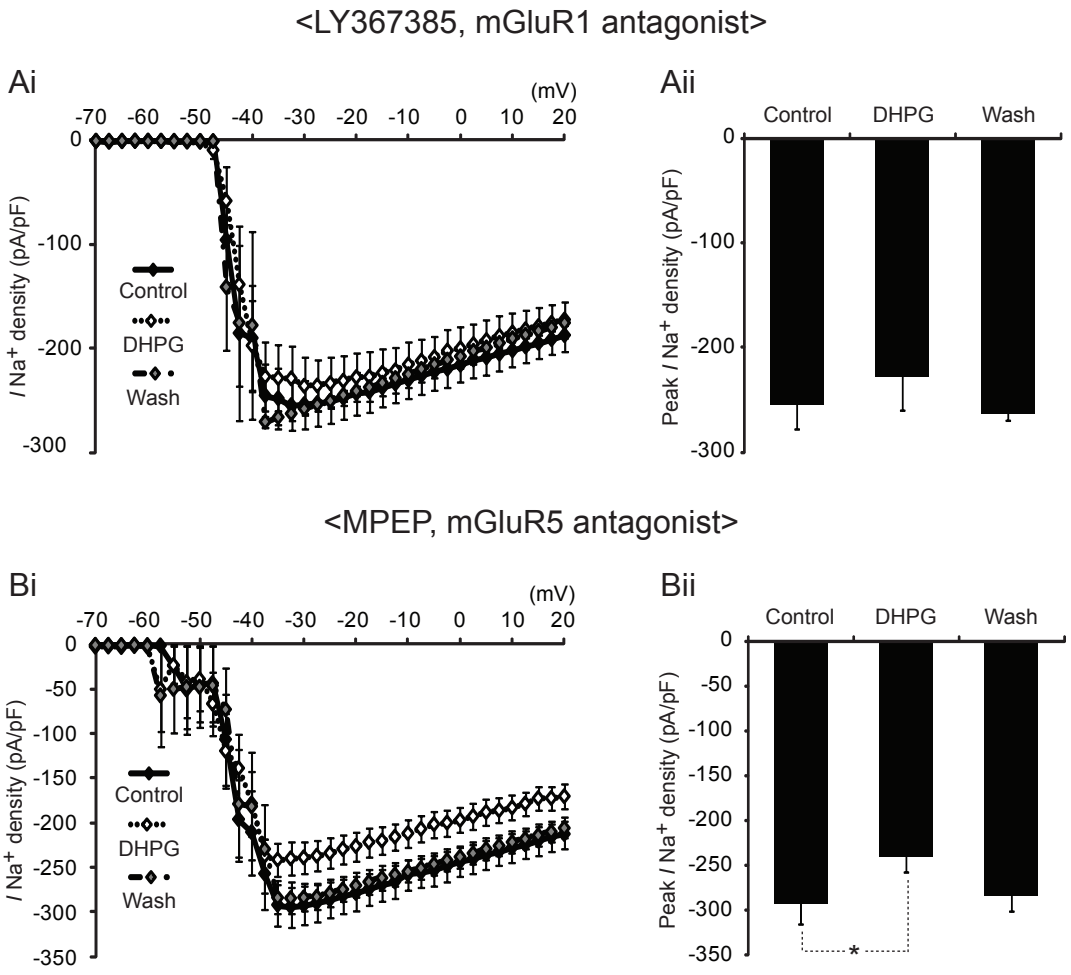


Figure 13: Receptor subtype-specific effects of group I mGluR activation upon modulation of fast inactivating Na^+ currents.

Ai) Plots of current-voltage (I-V) relationships and Aii) average peak current density for fast inactivating Na^+ currents demonstrate that LY367385 (50 μM) blocked the DHPG-mediated reduction in the density of Na+ currents (n = 8). Bi) Plots of I-V relationships and Bii) average peak current density for fast inactivating Na^+ currents demonstrate that DHPG application still significantly reduced the density of Na+ currents in the presence of MPEP (10 μM, n = 9).
* significantly different from control.

mGluR1 subtypes. However, the activation of mGluR5 subtypes appears to mediate one of the few potential excitatory effects of group I mGluR activation, the hyperpolarisation of the voltage threshold for action potential generation.

Group I mGluR-mediated modulation of synaptic transmission to motoneurons

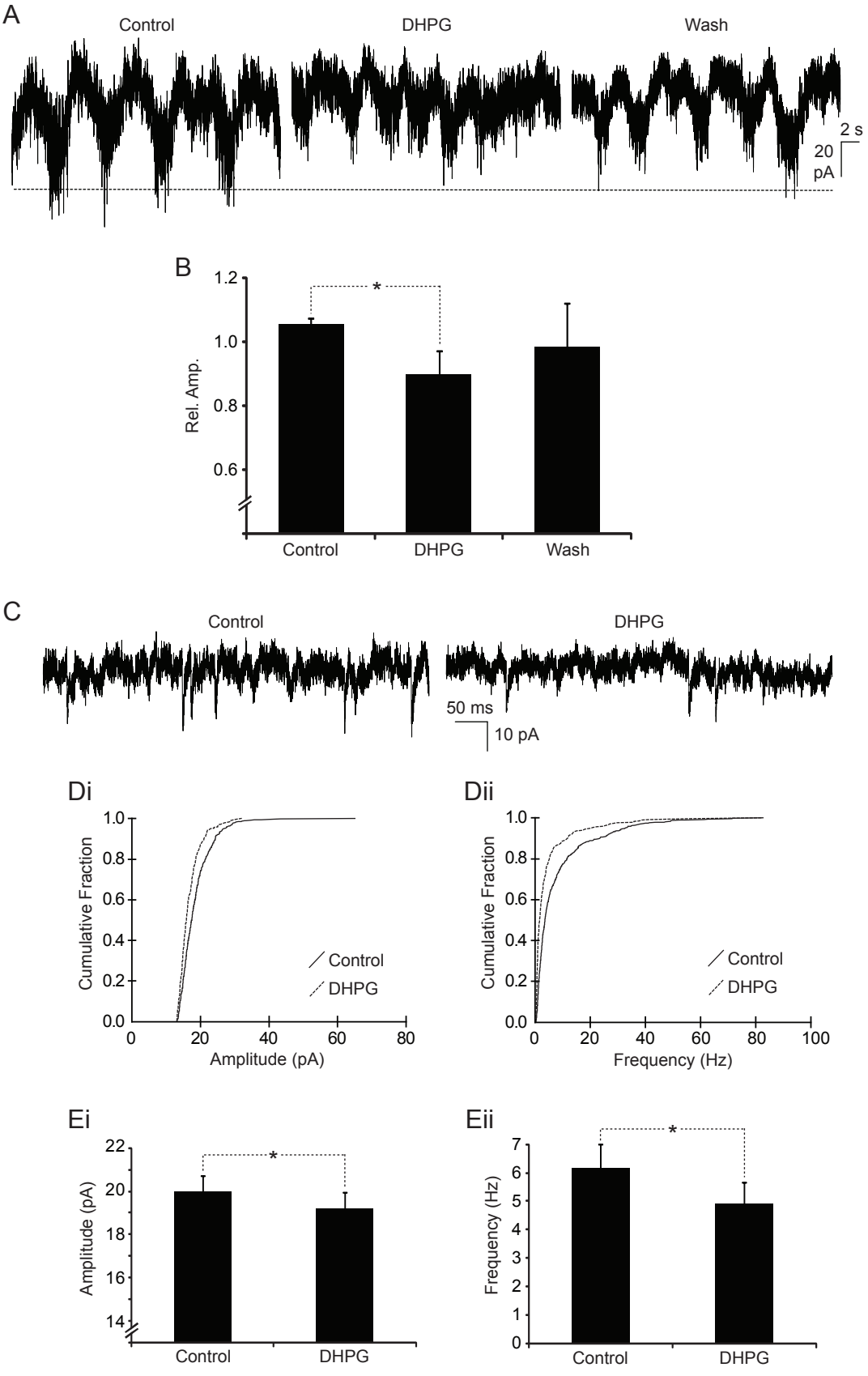
The present study has shown that several intrinsic properties of motoneurons are modulated by the activation of group I mGluRs. Further, to elucidate the synaptic mechanisms underlying the group I mGluR-mediated modulation of locomotor-related motoneuron output, it was also investigated whether the activation of group I mGluRs could alter excitatory synaptic inputs to spinal motoneurons. Rhythmic locomotor-related synaptic drive originating from pre-motor CPG interneurons was recorded from motoneurons held at -60 mV in pharmacologically activated (5 µM NMDA; 10 µM 5-HT; 50 µM dopamine) *in vitro* spinal cord preparations (Fig. 14A). The rhythmic synaptic drive received by motoneurons was in phase with bursts of locomotor activity recorded from segmentally aligned ventral roots. When DHPG (10 µM) was bath applied, the amplitude of rhythmic locomotor drive was significantly reduced by 10.4 ± 7.6 % (n = 10, $p = 0.035$; Fig. 14A & 14B). Thus, the activation of group I mGluRs indeed modulates locomotor-related excitatory synaptic transmission to motoneurons. The reduction in the intensity of locomotor-related motoneuron output observed upon the activation of group I mGluRs (Chapter 2) is therefore likely to reflect not only modulation of the intrinsic properties of motoneurons but also modulation of the excitatory locomotor-related synaptic drive that motoneurons receive from pre-motor CPG interneurons.

Next it was investigated whether the group I mGluR-mediated modulation of

excitatory synaptic transmission to motoneurons involves pre- or postsynaptic mechanisms. Miniature excitatory postsynaptic currents (mEPSCs) were recorded in voltage-clamp mode while motoneurons were held at -60 mV (Fig. 14C). To isolate mEPSCs, TTX (0.5 μM), strychnine (5 μM) and bicuculline (10 μM) were added to the recording aCSF. Upon the application of DHPG (50 μM), both the amplitude and frequency of mEPSCs were reduced (Fig. 14C, 14D & 14E). DHPG-mediated decreases in mEPSC amplitude and frequency were evident as leftward shifts in cumulative frequency plots generated from data collected from individual motoneurons (Fig. 14Di & 14Dii). As revealed using the Kolmogorov-Smirnov test, DHPG application led to a significant reduction in mEPSC amplitude in 7 out of 13 motoneurons and a significant decrease in mEPSC frequency in 8 out of 13 motoneurons. In addition, average mEPSC amplitude across all motoneurons decreased significantly from 20.0 ± 0.8 to 19.2 ± 0.8 pA (n = 13, Student's t-test p = 0.023; Fig. 14Ei), while average mEPSC frequency decreased significantly from 6.2 ± 0.8 to 4.9 ± 0.7 Hz (n = 13, Student's t-test p = 0.004; Fig. 14Eii). Together these results indicate that the activation of group I mGluRs reduces excitatory synaptic transmission to motoneurons via both pre- and postsynaptic mechanisms.

Figure 14: Effects of group I mGluR activation on synaptic input to motoneurons.

A) Rhythmic locomotor-related drive currents recorded from a motoneuron held at -60 mV in control condition, in the presence of DHPG (10 μM), and after drug washout. B) The amplitude of locomotor drive was significantly decreased by DHPG application (n = 10). C) Miniature excitatory postsynaptic currents (mEPSCs) recorded from a motoneuron in the presence of tetrodotoxin (TTX, 0.5 μM), bicuculline (10 μM), and strychnine (5 μM). Di) Cumulative probability plots showing a significant decrease in mEPSC amplitude and Dii) frequency, following DHPG application (50 μM). Ei) Pooled data demonstrating that DHPG caused a significant decrease in both mEPSC amplitude and Eii) frequency (n = 13). * significantly different from control.


A
Control
DHPG
Wash
2 s
20
pA
B
Rel. Amp.
1.2
1.0
0.8
0.6
*
Control
DHPG
Wash
C
Control
DHPG
50 ms
10 pA
Di
Cumulative Fraction
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
Amplitude (pA)
Control
DHPG
Dii
Cumulative Fraction
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
Frequency (Hz)
Control
DHPG
Ei
Amplitude (pA)
22
20
18
16
14
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Control
DHPG
Eii
Frequency (Hz)
7
6
5
4
3
2
1
0
*
Control
DHPG

Discussion

The present study has demonstrated that the activation of group I mGluRs modulates a wide range of cellular properties that determine the firing output generated by spinal motoneurons. Targets of group I mGluR-mediated modulation include both the intrinsic properties of motoneurons and the synaptic input that motoneurons receive from pre-motor CPG interneurons. Despite revealing both excitatory and inhibitory effects of group I mGluR activation on spinal motoneurons, the net modulatory effect of group I mGluR activation is to reduce both the excitability of motoneurons and excitatory synaptic transmission received by motoneurons. The net inhibitory role of group I mGluRs on spinal motoneurons is therefore likely to underlie the reduction in the amplitude of locomotor-related output recorded from lumbar ventral roots of rhythmically active *in vitro* mouse spinal cord preparations (Chapter 2).

One of the presumptive group I mGluR-mediated excitatory effects in motoneurons, which may appear in opposition to the group I mGluR-mediated reduction in motoneuron excitability, was depolarisation of the resting membrane potential of spinal motoneurons following the activation of group I mGluRs. Group I mGluR-mediated depolarisation of the resting membrane potential is commonly reported in spinal motoneurons of lampreys (Krieger *et al.*, 1998; Kettunen *et al.*, 2003), turtles (Svirskis & Hounsgaard, 1998), and rats (Marchetti *et al.*, 2003, 2005), as well as respiratory-related brainstem motoneurons of rats (Del Negro & Chandler, 1998; Dong & Feldman, 1999). In contrast, the activation of group I mGluRs did not lead to membrane depolarisation in spinal motoneurons of *Xenopus* tadpoles (Chapman *et al.*, 2008). Data from the present study have shown that the membrane depolarisation is

likely mediated via the activation of mGluR1, but not mGluR5 subtypes, which is in accordance with reports in lamprey and rat *in vitro* spinal cord preparations (Kettunen *et al.*, 2003; Marchetti *et al.*, 2003). However, the ionic mechansims that underlie the group I mGluR-mediated membrane depolarisation appear to be different in the present study of mouse spinal motoneurons compared to studies of motoneurons in other species. The group I mGluR-mediated membrane depolarisation of motoneurons is most often associated with an increase in membrane input resistance likely reflecting the blockade of leak K^+ currents (Del Negro & Chandler, 1998; Svirskis & Hounsgaard, 1998; Dong & Feldman, 1999; Kettunen *et al.*, 2003; Marchetti *et al.*, 2005). However, the present study found that group I mGluR-mediated depolarisation was associated with a decrease in input membrane resistance, with the underling current reversing at approximately at -30 mV. These data suggest that the activation of group I mGluRs in mouse spinal motoneurons involves the opening of non-selective cationic channels, as has been reported previously in other neuronal types within the mammalian brain (Congar *et al.*, 1997; Dong *et al.*, 2009; Kolaj & Renaud, 2010). Other currents that cannot be ruled out as underlying group I mGluR-mediated modulation include currents mediated by Na^+-Ca^{2+} exchangers (Keele *et al.*, 1997; Lee & Boden, 1997; Jian *et al.*, 2010) and Na^+-K^+-$2Cl^-$ co-transporters (Schomberg *et al.*, 2001).

The analyses of the involvement of group I mGluR specific subtypes in the membrane depolarisation and the decrease in membrane resistance in spinal motoneurons suggest a possible separation in signalling mechanisms responsible for these two modulatory effects. The depolarisation of the membrane potential depends on the activation of mGluR1 subtypes, while the decrease in membrane resistance appears to require the activation of mGluR5 subtypes. Interestingly, studies in rat

spinal motoneurons have reported that group I mGluR-mediated membrane depolarisations are associated with either an increase or no change in membrane resistance (Marchetti *et al.*, 2003, 2005). Additionally, in rat spinal motoneurons, the membrane depolarisation appears to be mediated by the activation of mGluR1 subtypes while oscillations in membrane potential are induced by the activation mGluR5 subtypes (Marchetti *et al.*, 2003). Also oscillation of intracellular Ca^{2+} concentrations is reported in spinal neurons of lampreys, mediated via the activation of mGluR5 subtypes and Ca^{2+} influx from L-type but not N-type Ca^{2+} channels (Kettunen *et al.*, 2002). It therefore seems that further work is needed to clarify which ion channels underlie the group I mGluR-mediated membrane depolarisation and to determine the sub-threshold effects of specific group I mGluR subtypes in mammalian spinal motoneurons.

Although the group I mGluR-mediated membrane depolarisation would suggest an increase in the excitability of spinal motoneurons, results from the previous chapter have demonstrated a reduction in the intensity of locomotor-related motoneuron output in response to the activation of group I mGluRs (Chapter 2). The group I mGluR-mediated membrane depolarisation might suggest a depolarising block of action potentials, nevertheless this was unlikely based on the magnitude of the depolarisation induced by group I mGluR activation. Furthermore, the time course of the group I mGluR-mediated depolarisation differed from that of the reduction in the amplitude of locomotor-related motoneuron output. Instead, the net inhibitory effect of group I mGluR activation on spinal motoneurons appears to involve the modulation of the ability of spinal motoneurons to produce repetitive firing of action potentials.

The present study has demonstrated that the frequency of repetitive action potential

firing decreases upon the activation of group I mGluRs in mouse motoneurons. This is in contrast to other detailed studies of the effects of group I mGluR activation on the firing of individual vertebrate motoneurons, performed in turtles, which demonstrated that the activation of group I mGluRs enhances sustained firing or plateau potentials via the facilitation of voltage-activated Ca^{2+} channels (Delgado-Lezama *et al.*, 1997; Svirskis & Hounsgaard, 1998). In addition to demonstrating a group I mGluR-mediated decrease in repetitive firing in mouse motoneurons, the present study demonstrated mGluR-mediated reductions in action potential height and maximum rate of rise that are consistent with reduced Na^+ channel availability (Miles *et al.*, 2005). In support of this, voltage-clamp analysis of fast, inactivating Na^+ currents suggests a reduction in Na^+ current density upon activation of group I mGluRs. Although the conclusions from data concerning the effects of group I mGluR activation upon Na^+ current density may be complicated by voltage- and space-clamp errors, the experimental design was modified to reduce the likelihood of these errors through the use of modified recording solutions. Using this methodology, other channels types (K^+ and Ca^{2+}) and synaptic transmission were blocked, while the magnitude of Na^+ currents was also reduced (Cantrell *et al.*, 1997; Carr *et al.*, 2003; Miles *et al.*, 2005; Carlier *et al.*, 2006). In addition, analyses of the involvement of specific group I mGluR subtypes on these inhibitory effects in mouse spinal motoneurons revealed that all of these modulatory effects are mediated via the activation of mGluR1 but not mGluR5 subtypes. Data presented in this chapter thus indicate that the mGluR1-mediated modulation of Na^+ channel properties and the subsequent inhibition of motoneuron firing are likely to contribute to group I mGluR-mediated reductions in the intensity of locomotor-related motoneuron output recorded from rhythmically active *in vitro* spinal

cord preparations of neonatal mice (Chapter 2).

Although this is the first report that the activation of group I mGluRs modulates Na^+ channels and the shape of action potentials in mouse spinal motoneurons, similar findings have been reported in cortical pyramidal neurons (Carlier *et al.*, 2006). In these neurons, group I mGluR activation hyperpolarises the inactivation of transient Na^+ currents, which results in a decrease in Na^+ current amplitude. The decreased Na^+ current amplitude underlies reductions in action potential height and maximum rate of rise and ultimately lowers the frequency of repetitive action potential firing (Carlier *et al.*, 2006). In contrast, the present study has shown in mouse spinal motoneurons that the activation of group I mGluRs reduces Na^+ current density without changing the voltage dependence of steady-state inactivation. Data from the present study are, however, similar to previous reports in hippocampal neurons where phosphorylation of Na^+ channels, following activation of either dopaminergic or muscarinic receptors, decreases peak Na^+ current without affecting the voltage dependence of activation or steady-state inactivation (Cantrell *et al.*, 1996; Cantrell *et al.*, 1997). In such cases, reduced Na^+ channel availability may instead reflect modulation of slow inactivation (Carr *et al.*, 2003), altered rates of inactivation, or a decrease in the unitary conductance of single channels.

The present study has also demonstrated that the activation of group I mGluRs hyperpolarises voltage thresholds for repetitive action potential generation in mouse spinal motoneurons Unlike the mGluR1-mediated inhibitory effects on the firing of spinal motoneurons, the hyperpolarisation of the action potential threshold was found to be mGluR5-mediated. Interestingly, it has recently been shown that an undefined intra-spinal system hyperpolarises the voltage threshold for action potentials during

fictive scratch in cats (Power *et al.*, 2010). In the rat spinal cord, protein kinase C, a downstream target of group I mGluRs (Pin & Duvoisin, 1995) is shown to modulate the voltage threshold for spike initiation (Dai *et al.*, 2009). Therefore it seems plausible that group I mGluRs form part of the intra-spinal system suggested in the cat (Power *et al.*, 2010) to control action potential threshold in a state-dependent manner. Although the mechanisms underlying group I mGluR-mediated modulation of action potential threshold remain unclear, the present data do not support a role for the modulation of the activation properties of fast inactivating Na^+ currents. Other potential mechanisms include the modulation of persistent Na^+ currents (Carlier *et al.*, 2006) and delayed rectifier K^+ channels (Dai *et al.*, 2009).

In addition to the modulation of intrinsic properties of motoneurons, it is demonstrated that the activation of group I mGluRs reduces excitatory synaptic transmission to spinal motoneurons. Group I mGluR-mediated modulation of excitatory and/or inhibitory synaptic transmission has been reported in spinal networks of lampreys, *Xenopus* tadpoles and rats (Marchetti *et al.*, 2003; Kettunen *et al.*, 2005; Marchetti *et al.*, 2005; Kyriakatos & El Manira, 2007; Chapman *et al.*, 2008). The present study concentrated on the effect of group I mGluR activation on excitatory synaptic transmission since the modulation of rhythmic excitatory locomotor drive to motoneurons seemed most likely to underlie the decrease in locomotor-related motoneuron output recorded in rhythmically active *in vitro* spinal cord preparations. It could be argued that modulation of inhibition, should it occur concurrent with rhythmic excitation (Berg *et al.*, 2007) or should it contribute to the circuitry of recurrent inhibition, could also be responsible for the group I mGluR-mediated decrease in the amplitude of locomotor-related synaptic drive to motoneurons. This possibility cannot

be ruled out, but it seems unlikely given that concurrent inhibition and excitation of motoneurons does not appear to be a feature of locomotion in mice (Endo & Kiehn, 2008). In addition, a previous study using rat *in vitro* spinal cord preparations reported that the activation of group I mGluRs depressed recurrent inhibition to spinal motoneurons (Marchetti *et al.*, 2005).

Findings from the present study indicate that group I mGluR-mediated modulation of excitatory synaptic drive to motoneurons involves both pre- and postsynaptic mechanisms. Although the exact mechanisms need to be investigated further, one possible pre-synaptic mechanism, based on the present findings, could be group I mGluR-mediated inhibition of transient Na^+ currents in pre-synaptic terminals. Other potential mechanisms include those revealed in lamprey *in vitro* spinal cord preparations where modulation of both excitatory and inhibitory transmission involves retrograde signalling of endocannabinoids and volume transmission of nitric oxide (Kettunen *et al.*, 2005; Kyriakatos & El Manira, 2007; Kyriakatos *et al.*, 2009). However, findings in the previous chapter (Chapter 2), suggesting that the modulatory effects of group I mGluR activation on spinal locomotor activity are independent of endocannabinoid and nitric oxide signalling, put this hypothesis unlikely. Modulatory interactions of group I mGluRs and iGluRs, such that group I mGluR activation modulates both NMDA and AMPA receptor-mediated currents (Krieger *et al.*, 1998; Krieger *et al.*, 2000; Nanou *et al.*, 2009; Nanou & El Manira, 2010), provide potential mechanisms for postsynaptic modulation.

Taken together, data from the present study and previous research demonstrate that the actions of group I mGluRs on motoneurons are complicated by the existence of multiple effects including the modulation of intrinsic neuronal properties and synaptic

transmission. This may allow considerable flexibility in the actions of group I mGluRs when the multiple effects they mediate are controlled by pathways that are separable, for example, based on the group I mGluR subtype activated (mGluR1 vs. mGluR5) or the second messenger signalling involved. In support of this, in the present study, it has been demonstrated that modulatory effects culminating in decreased motoneuron excitability are mediated via mGluR1 subtypes whereas modulatory effects which are likely to increase motoneuron excitability, specifically a hyperpolarisation of the voltage threshold for action potential generation, is mediated by the activation of mGluR5 subtypes. Further support for the functional division of group I mGluR-mediated signalling is provided in the lamprey spinal cord where separable intracellular signalling pathways downstream of mGluR1 activation are reported, such as PLC-mediated potentiation of NMDA currents and PKC-dependent blockade of leak channels (Nanou *et al.*, 2009). Interestingly, in opposition to the independence of separate aspects of group I mGluR-mediated signalling, studies of striatal cholinergic neurons have demonstrated an interaction between subtypes of group I mGluRs with mGluR5 modulating mGluR1 activity (Bonsi *et al.*, 2005).

In brainstem motoneurons, where group I mGluR activation also depresses excitatory inputs and mediates membrane depolarisation, group I mGluRs have been suggested to play a role in sculpting the final output of motoneurons by favouring strong synaptic inputs and amplifying their effects (Del Negro & Chandler, 1998). Findings from the present study suggest that group I mGluR activation might mediate similar enhancement of the signal-to-noise ratio of synaptic inputs to spinal motoneurons by reducing excitatory inputs but depolarising the resting membrane potential and hyperpolarising the action potential threshold. However, the inhibition of repetitive firing via the

modulation of Na^+ channels may be inconsistent with this. Alternatively, modulation of Na^+ currents and repetitive firing may represent a separate role for group I mGluRs in limiting the output of spinal motoneurons, perhaps in the presence of intense glutamatergic input, to provide homeostatic control of motoneuron output (Desai, 2003; Carlier *et al.*, 2006).

Evidence that a single neuromodulator provides neural networks with opposing neuromodulatory effects to limit the overall network output have been discussed in spinal locomotor CPG networks (Harris-Warrick, 2011). If glutamate provides spinal locomotor networks with such homeostatic control mediated via the activation of group I mGluRs, this might also serve to protect spinal networks from intensive glutamatergic stimulation such as glutamatergic excitotoxicity. Data support a neuroprotective role for group I mGluRs in pathological conditions affecting spinal motor networks such as amyotrophic lateral sclerosis (ALS; Anneser *et al.*, 1999; Anneser *et al.*, 2004; Ma *et al.*, 2006) where glutamate-mediated excitotoxicity is thought to contribute to motoneuron death (Boillee *et al.*, 2006). Further analyses of the role of mGluRs in motoneurons may therefore be of importance, not only for understanding the control of motor outflow from the CNS, but also toward the design of new treatments for degenerative diseases that afflict spinal motor systems. Therefore the focus of the next chapter will be to investigate whether group I mGluRs play any role in the pathology of the neurodegenerative disease, ALS.

Chapter 4

The role of group I mGluRs in the early pathogenesis of Amyotrophic Lateral Sclerosis

Introduction

The present study has so far discussed a wide variety of roles that group I mGluRs play in the modulation of locomotor networks of the mammalian spinal cord. This final chapter moves on to investigate whether these group I mGluR-mediated modulatory effects are perturbed in spinal locomotor networks under pathological conditions. One devastating disease that affects motor functions of the spinal cord is amyotrophic lateral sclerosis (ALS). In patients affected by ALS, motoneurons are selectively lost by unknown causes, leading to the loss of somatic motor control and subsequently the fatal paralysis of respiratory muscles 2 – 3 years after disease onset. 90 % of ALS cases are sporadic, but the remaining 10 % are attributable to familial (genetic) causes. 20 % of familial cases of ALS are known to be caused by specific mutations in the gene encoding the enzyme superoxide dismutase-1 (SOD-1). Genetically engineered transgenic mice that express the mutant SOD-1 gene have been widely used as experimental model animals of ALS, since the pathological phenotype exhibited by mutant SOD-1 transgenic mice resembles the disease progression of human ALS patients (Julien & Kriz, 2006). Several hypotheses are currently proposed to explain the pathogenesis of ALS, which include mitochondrial dysfunction, cellular oxidative stress, disorganisation of neurofilaments, toxic intracellular aggregates, and glutamatergic excitotoxicity (Bruijn *et al.*, 2004; Boillee *et al.*, 2006). However, it is highly likely that ALS is a multi-factorial disease with several pathological mechanisms converging to cause the final death of motoneurons.

Although ALS is a multi-factorial disease, the hypothesis that glutamatergic excitotoxicity is involved in the pathogenesis of ALS seems to have received the most

support. Firstly, a raised concentration of glutamate in cerebrospinal fluid is reported in 40 % of ALS patients (Spreux-Varoquaux *et al.*, 2002). Secondly, the astroglial glutamate transporter, excitatory amino acid transporter 2 (EAAT2), which is responsible for removal of glutamate from extracellular spaces, is reported as having reduced expression in ALS patients (Rothstein *et al.*, 1995; Fray *et al.*, 1998) and also in ALS model mice (Bruijn *et al.*, 1997). Finally, riluzole, the only drug clinically approved to date for the treatment of ALS, is thought to inhibit glutamate release (Doble, 1996). Thus, evidence points strongly towards glutamatergic excitotoxicity, initiated not only by an excessive release of glutamate into synaptic clefts but also by abnormal removal of glutamate from extracellular spaces, as playing a key role in the pathology of ALS.

The involvement of glutamatergic excitotoxicity in the pathogenesis of ALS is often discussed in the context of iGluR-mediated Ca^{2+} influx, in particular mediated via Ca^{2+} permeable AMPA receptors, which leads to a disturbance in intracellular Ca^{2+} homeostasis (Arundine & Tymianski, 2003; Van Den Bosch *et al.*, 2006). However, the role of group I mGluRs in glutamatergic excitotoxicity in ALS has received considerably less research attention, despite the fact that the activation of group I mGluRs is positively coupled to mechanisms that increase intracellular Ca^{2+} concentration (Anwyl, 1999, 2009). There are limited data available that indicate a possible role of group I mGluRs in ALS, although these data may support complex and dual neuroprotective and neurotoxic effects of group I mGluRs in the pathology of ALS. In human ALS patients, higher levels of group I mGluR expression are reported, particularly on glial cells in the spinal cord (Aronica *et al.*, 2001). Analyses of expression of group I mGluR subtypes in motoneuron populations of the brainstem and

the spinal cord demonstrate higher levels of mGluR1 expression, but lower levels of mGluR5 expression, in ALS-vulnerable motoneuron populations, such as the VII and XII brainstem motor nuclei and spinal motoneurons, compared to ALS-resistant motoneuron populations such as the III, IV and VI brainstem motor nuclei in both humans and rats (Laslo *et al.*, 2001; Ma *et al.*, 2006). Unlike most spinal motoneurons, lumbosacrally-located parasympathetic motoneurons of Onuf's nucleus, which are known to be resistant to neurodegeneration in ALS, show higher levels of mGluR5 expression, while levels of mGluR1 expression do not differ between Onuf's nucleus and the somatic spinal motoneuron population (Anneser *et al.*, 2004). Therefore it seems that high levels of mGluR1 expression may facilitate neuronal degeneration while mGluR5 expression may protect neurons from degeneration in ALS. Such a dual role of group I mGluRs may reflect mixed evidence that group I mGluRs promote or inhibit glutamate-induced death of motoneurons depending on experimental conditions. Application of group I mGluR agonists prevents kainate-induced neuronal death in spinal cord slices obtained from adult rats (Pizzi *et al.*, 2000; Valerio *et al.*, 2002). However, application of either an agonist or an antagonist of group I mGluRs prevents neuronal death in embryonic chick spinal cell cultures treated with CSF obtained from human ALS patients (Anneser *et al.*, 2006).

Although ALS is an adult onset disease, recent studies utilising transgenic mouse models of ALS (mutant SOD-1 transgenic mice) have revealed early pathological alterations in the ALS affected spinal cord that occur long before the onset of clinical symptoms (Durand *et al.*, 2006; ElBasiouny *et al.*, 2010). Data suggest pre-symptomatic alterations in the intrinsic excitability of spinal motoneurons as early as embryonic and neonatal stages. However, both hyperexcitability (Pieri *et al.*, 2003;

Kuo *et al.*, 2004; Kuo *et al.*, 2005; Zona *et al.*, 2006; Pambo-Pambo *et al.*, 2009; Quinlan *et al.*, 2011) and hypoexcitability (Bories *et al.*, 2007; Pambo-Pambo *et al.*, 2009) of spinal motoneurons are reported in mutant SOD-1 mice. Neuronal hyperexcitability is also reported in hypoglossal motoneurons in the brainstem of mutant SOD-1 mice (van Zundert *et al.*, 2008). In addition, there are likely to be hyperexcitable alterations in pre-motor interneurons that mediate input to motoneurons in the spinal cord and the brainstem of mutant SOD-1 mice (van Zundert *et al.*, 2008; Jiang *et al.*, 2009). It may therefore be hypothesised that such early pre-symptomatic hyperexcitability in pre-motor interneurons leads to a loss of excitatory synapses onto motoneurons in both symptomatic mutant SOD-1 mice (Schutz, 2005; Avossa *et al.*, 2006) and human ALS patients (Nagao *et al.*, 1998). Together, altered excitability of motoneurons and interneurons, and possibly changes in synaptic inputs to motoneurons, may therefore represent important pre-symptomatic features in the pathogenesis of ALS.

Given the limited understanding of the involvement of group I mGluRs in glutamatergic excitotoxicity, and the incomplete understanding of early pre-symptomatic alterations in neuronal excitability in the ALS affected spinal cord, the present study aims to investigate the modulatory effects of group I mGluR activation in spinal locomotor networks of neonatal mutant SOD-1 transgenic mice. In the previous chapters, it has been shown that the activation of group I mGluRs modulates spinal locomotor networks (Chapter 2) and possibly provides homeostatic control for excitatory glutamatergic transmission upon spinal motoneurons (Chapter 3). Therefore, this final chapter assesses whether the modulatory effects of group I mGluR activation are altered in favour of neuroprotection or excitotoxicity in early pre-symptomatic stages of spinal locomotor networks of neonatal mutant SOD-1 mice.

Methods

Transgenic ALS model mice

Animals used included genetically engineered ALS model mice, B6.Cg-Tg(SOD1*G93A)1Gur/J mice (mutant SOD-1 transgenic mice; The Jackson Laboratory, Maine, USA) and their wild type C57/BL6 littermates. Mutant SOD-1 mice express high copy numbers of the mutant human superoxide dismutase-1 gene that contains a substitution of Gly to Ala at amino acid 93 (G93A). The genotype of these animals was determined before any experimental procedures were performed. Small tissue samples were taken from tail tips. DNA was extracted and polymerase chain reaction (PCR) was performed using the REDExtract-N-Amp Tissue PCR Kit (Sigma-Aldrich) per the manufacturer's instructions. The primers used were:

SOD1 forward primer 5'-CAT CAG CCC TAA TCC ATC TGA-3'

SOD1 reverse primer 5'-CGC GAC TAA CAA TCA AAG TGA-3'

Internal control forward primer 5'-CTA GGC CAC AGA ATT GAA AGA TCT-3'

Internal control reverse primer 5'-GTA GGT GGA AAT TCT AGC ATC ATC C-3'

Thermal cycling was performed on a PCR machine (PCR sprint thermal cycler, Thermo Fisher Scientific, Massachusetts, USA) running the following programme:

1	95^{o}C	3 min	
2	95^{o}C	30 sec	
3	60^{o}C	30 sec	
4	72^{o}C	45 sec	(repeat steps 2-4 for 35 cycles)
5	72^{o}C	2 min	
6	10^{o}C	hold	

PCR products were run on a 1.5% agarose gel alongside a 1kb DNA ladder for analysis.

In vitro whole spinal cord preparations

In vitro whole spinal cord preparations were obtained from neonatal mice (P2 – P5) by performing the same dissection techniques as described in the previous chapters. The recording equipment was also arranged in the same way as described in the previous chapters. Locomotor-related burst activity was induced pharmacologically (NMDA 5 μM, 5-HT 10 μM and dopamine 50 μM), and recorded with glass suction electrodes attached to L1 or L2 ventral roots.

Spinal cord slice preparations

The spinal cord was dissected from neonatal mice (P4 – P8) as described in the previous chapters. However, dissection and slicing of the isolated spinal cord were performed in ice cold solution (dissecting solution, equilibrated with 95% O_2 - 5% CO_2, ~ 0˚C) that was designed to help minimise traumatic damage to neurons (Miles *et al.*, 2007). The isolated spinal cord was sliced 300 μm thick, using a Vibratome (VT1200, Leica Microsystems, Germany). Spinal cord slice preparations were then transferred to oxygenated standard aCSF (equilibrated with 95% O_2 - 5% CO_2, room temperature), to allow neurons to recover from traumatic damage.

Whole-cell patch-clamp recordings

The whole-call patch-clamp recording configuration used was as described in the previous chapter (Chapter 3). Recorded neurons were limited to interneurons found in the ventral half of the slice preparation. Large neurons (approximately 20 – 30 μM in

somal diameter) found in the most ventrolateral corners of the slice preparation were avoided since they are likely to be spinal motoneurons.

Data analysis

Data from ventral root recordings were analysed offline using Dataview software (courtesy of W. J. Heitler, University of St Andrews) as described in Chapter 2. Whole-cell patch-clamp recordings were analysed using either Clampfit software (Molecular Devices) or Dataview software. Data are reported as mean ± S.E. Differences in means between data obtained from wild type and mutant SOD-1 interneurons or control and drug conditions were compared using Student's t-tests. Values of $p < 0.05$ were considered significant.

Solution and drugs

The dissecting aCSF solution contained 25 mM NaCl, 1.9 mM KCl, 1.2 mM NaH_2PO_4, 10 mM $MgSO_4$, 1 mM $CaCl_2$, 26 mM $NaHCO_3$, 1.5 mM kynurenic acid, 25 mM D-glucose and 188 mM sucrose (equilibriated with 95% O_2 / 5% CO_2).

The standard aCSF solution used for recording contained 127 mM NaCl, 3 mM KCl, 1.3 mM NaH_2PO_4, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM D-glucose (equilibrated with 95% O_2/5% CO_2).

The standard patch-clamp pipette solution contained 140 mM potassium methane sulfonate, 10 mM NaCl, 1 mM $CaCl_2$, 10 mM HEPES, 1 mM EGTA, 3 mM Mg-ATP, and 0.4 mM GTP-Na_2 (pH 7.2 – 7.3, adjusted with KOH).

Pharmacological agents used included: NMDA (*N*-methyl-D-aspartic acid), 5-HT (5-hydroxytryptamine hydrochloride), dopamine (3,4-dihydroxyphenethylamine

hydrochloride), purchased from Sigma-Aldrich (St Louis, MO); DHPG ((S)-3,5-dihydroxyphenylglycine), purchased from Tocris Bioscience (Bristrol, UK).

Results

Effects of group I mGluR activation on *in vitro* spinal cord preparations of WT and mutant SOD-1 mice

To help assess whether group I mGluRs play a role in glutamatergic excitotoxicity and the early pathogenesis of ALS, it was investigated whether the modulatory effects of group I mGluR activation in spinal locomotor networks, demonstrated in Chapter 2, are perturbed in locomotor networks of *in vitro* spinal cord preparations obtained from neonatal mutant SOD-1 mice. The group I mGluR agonist DHPG (5 μM) was bath applied to pharmacologically activated rhythmic *in vitro* spinal cord preparations. In both wild type and mutant SOD-1 *in vitro* spinal cord preparations, the application of DHPG led to a significant decrease in the amplitude of locomotor-related ventral root bursts (WT 13.0 ± 3.2 % reduction, n = 12, p = 0.001; Fig. 15Ai & Aii: SOD-1 12.1 ± 5.0 % reduction, n = 11, p = 0.014; Fig. 15Ai & 15Aiii). The degree and time course of the DHPG-induced reduction in locomotor burst amplitude were very similar between wild type and mutant SOD-1 preparations (Fig. 15A). Also DHPG application caused a significant increase in the frequency of locomotor bursts both in wild type and mutant SOD-1 preparations (WT 40.6 ± 11.7 % increase, n = 12, p = 0.006; Fig. 15Bi & 15Bii: SOD-1 32.2 ± 10.0 % increase, n = 11, p = 0.007; Fig. 15Bi & 15Biii). Although the degree to which locomotor burst frequency increased was similar between wild type and mutant SOD-1 preparations (Fig. 15B), the time course of the increase in burst frequency differed, with the peak effect of DHPG application reached ~10 minutes earlier in mutant SOD-1 preparations (Fig. 15Bi). These results suggest that the modulatory effects of group I mGluR activation, at the level of spinal

motoneurons that determine the intensity of final locomotor output, are not altered in ALS affected *in vitro* spinal cord preparations. However, at the level of spinal interneurons that generate the rhythm of locomotor network output, group I mGluR activation may result in slightly different effects in wild type and ALS affected *in vitro* spinal cord preparations of neonatal mice.

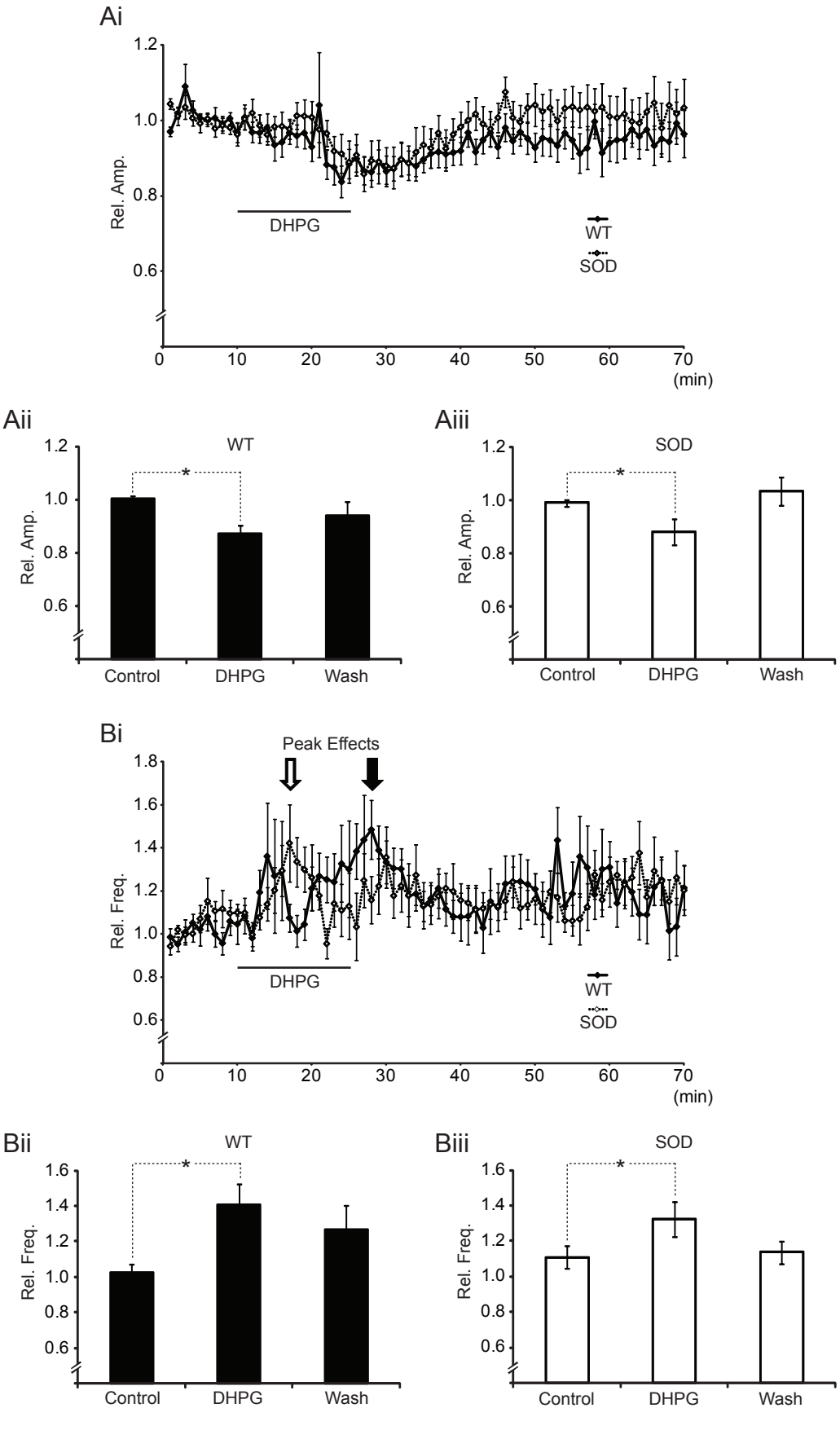
Intrinsic properties of WT and SOD-1 interneurons

Although the effects of group I mGluR activation on locomotor-related motoneuron output recorded from pharmacologically activated *in vitro* spinal cord preparations of wild type and mutant SOD-1 mice did not appear to differ, there did appear to be a slight difference in the effects of group I mGluR activation on spinal interneurons within locomotor CPG networks. There are two possible hypotheses that could explain the slight difference in interneuron activity between the two preparations: 1) intrinsic properties of interneurons that make up locomotor CPG networks are fundamentally different between wild type and mutant SOD-1 transgenic spinal cords, or 2) the responsiveness to group I mGluR activation is different between wild type and mutant SOD-1 interneuron populations.

To assess the first hypothesis, that there are early alterations in the basic intrinsic properties of interneurons in the spinal cord of mutant neonatal SOD-1 mice, whole-cell patch-clamp recordings were performed from single interneurons found in the ventral half of wild type and mutant SOD-1 spinal cord slice preparations (Fig. 16A). Starting with the examination of sub-threshold interneuron properties, resting membrane potentials of wild type and mutant SOD-1 interneurons were similar at values around -60 mV (WT -62.2 ± 1.1 mV, n = 17; SOD -61.8 ± 1.1 mV, n = 17; Fig. 16B).

Figure 15: Effects of group I mGluR activation in mutant SOD-1 *in vitro* spinal cord preparations.

Ai) Time course plots showing a significant decrease in locomotor burst amplitude in response to DHPG (5 μM) application in wild type and mutant SOD-1 *in vitro* spinal cord preparations. Aii) Pooled data, averaged for 5 min worth of recording in each experimental condition, show a DHPG-induced significant decrease in locomotor burst amplitude in wild type spinal cord preparations (n = 12). Aiii) Pooled data again showing a DHPG-induced significant decrease in locomotor burst amplitude in mutant SOD-1 spinal cord preparations (n = 11). Bi) Time course plots showing a significant increase in locomotor burst frequency in response to DHPG application in wild type and mutant SOD-1 *in vitro* spinal cord preparations. Arrows indicate peak effects of increased burst frequency (while = SOD; black = WT). Bii) Pooled data showing a DHPG-induced significant increase in locomotor burst frequency in wild type spinal cord preparations. Biii) Pooled data again showing a DHPG-induced significant increase in locomotor burst frequency in mutant SOD-1 spinal cord preparations. * significantly different from control.


Ai
Rel. Amp.
DHPG
WT
SOD
(min)
Aii
WT
Rel. Amp.
Control
DHPG
Wash
Aiii
SOD
Rel. Amp.
Control
DHPG
Wash
Bi
Peak Effects
Rel. Freq.
DHPG
WT
SOD
(min)
Bii
WT
Rel. Freq.
Control
DHPG
Wash
Biii
SOD
Rel. Freq.
Control
DHPG
Wash

Membrane resistance, calculated from I-V relationships generated from small voltage steps (-70 to -50 mV, 2.5 mV increments, 150 ms) applied in voltage-clamp mode, was also not significantly different between wild type (1.1 ± 0.2 GΩ, n = 22) and mutant SOD-1 (0.9 ± 0.2 GΩ, n = 24) interneurons (Fig. 16C). Membrane capacitance, taken as an indication of the cell size, again showed no significant differences between wild type (13.4 ± 1.0 pF, n = 22) and mutant SOD-1 (15.1 ± 1.8 pF, n = 24) interneurons (Fig. 16D).

To examine the repetitive firing properties of wild type and mutant SOD-1 interneuron populations, steady state f-I relationships were obtained from current-clamp protocols delivering steps of current pulses (1 sec; Fig. 17Ai). The f-I relationships obtained from individual interneurons were then averaged in each population (WT 20 cells, SOD-1 23 cells; Fig. 17Aii). No apparent differences in the average f-I relationships were observed (Fig. 17Aii), with the maximum firing frequencies unchanged in wild type (23.2 ± 2.5 Hz, n = 20) and mutant SOD-1 (23.3 ± 1.4 Hz, n = 23) interneurons (Fig. 17Aiii). Although there were no significant differences in the overall input-output relationships of interneurons in wild type and mutant SOD-1 spinal cord slice preparations, to ensure that smaller changes in interneuron firing were not missed, the properties of single action potentials were compared between wild type and mutant SOD-1 interneuron populations. Single action potentials were evoked by brief small square current pulses in current clamp mode (10 ms; Fig. 17Bi). Measurements of the height and maximum rate of rise of individual action potentials demonstrated no significant differences across wild type (height 89.7 ± 1.7 mV, max rise 94.1 ± 7.1 mV/ms, n = 23) and mutant SOD-1 (height 85.3 ± 2.2 mV, max rise 98.0 ± 7.9 mV/ms, n = 24) interneurons (Fig. 17Bi, 17Bii & 17Biii). For measurements of the amplitude

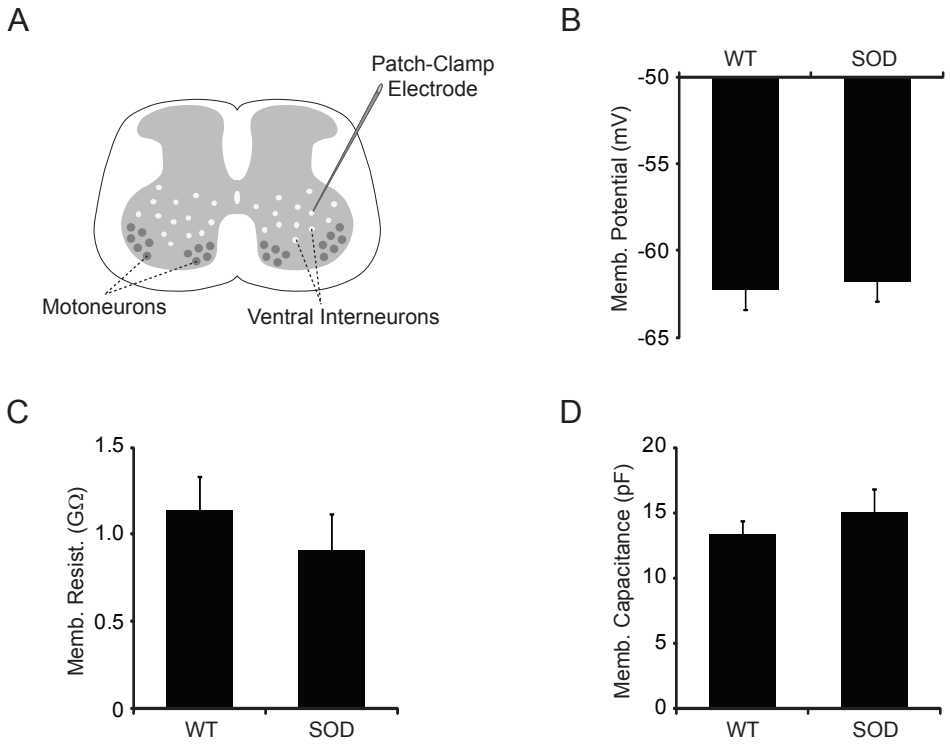
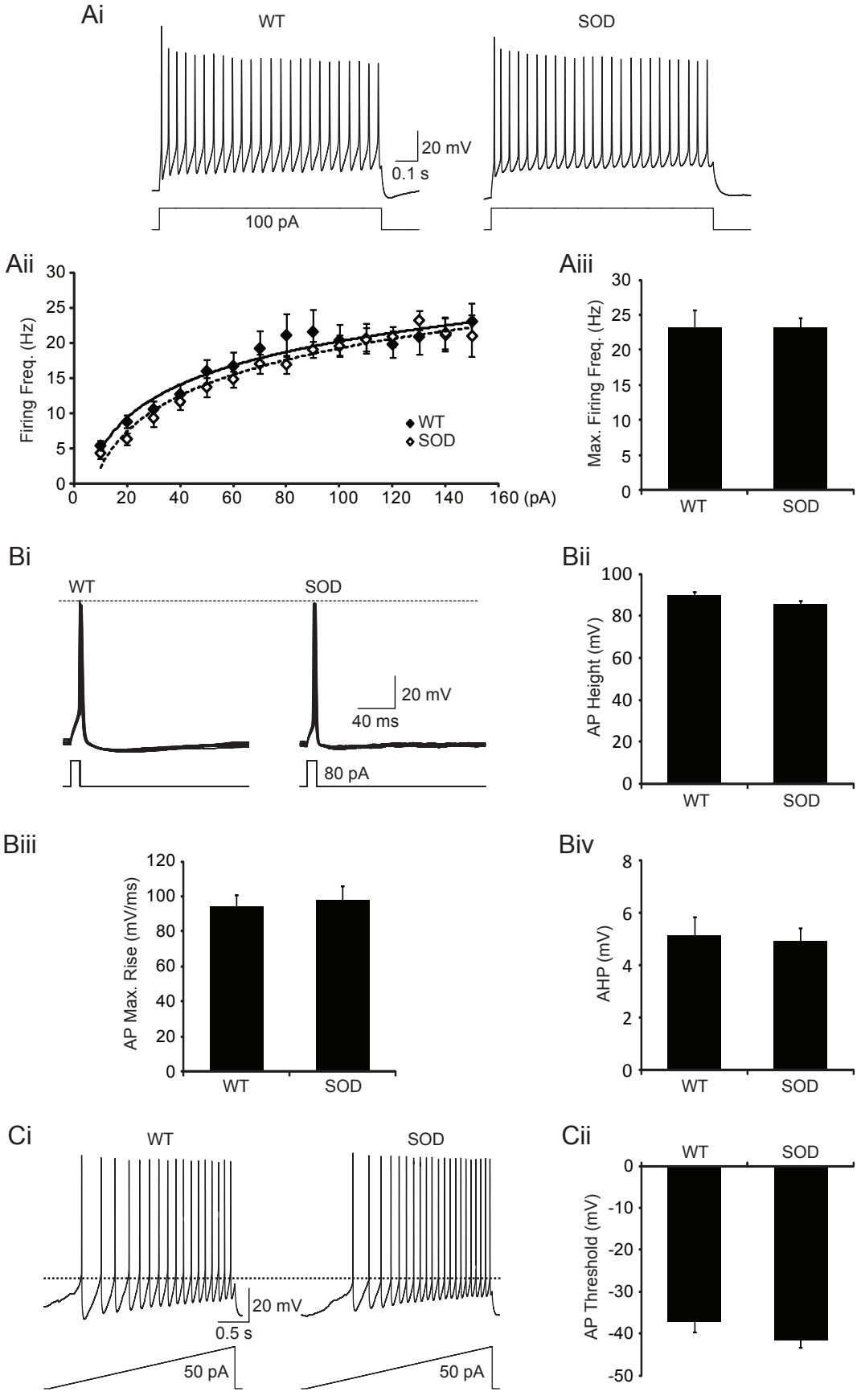


Figure 16: Sub-threshold properties of wild type and mutant SOD-1 interneurons.

A) Schematic showing a spinal cord slice preparation (courtesy of G. B. Miles, University of St Andrews). B) Pooled data showing no significant difference in resting membrane potentials of wild type (n = 17) and mutant SOD-1 (n = 17) interneurons. C) No significant difference in membrane resistance of wild type (n = 22) and mutant SOD-1 (n = 24) interneurons. D) No significant difference in membrane capacitance of wild type (n = 22) and mutant SOD-1 (n = 24) interneurons.

Figure 17: Intrinsic firing properties of wild type and mutant SOD-1 interneurons.

Ai) Repetitive firing evoked by brief (1 s) square current pulses in wild type and mutant SOD-1 interneurons. Aii) Firing frequency vs. injected current (f-I) relationships averaged across wild type (n = 20) and mutant SOD-1 (n = 23) interneuron populations. Aiii) Maximum firing frequency was not significantly different between wild type and mutant SOD-1 interneurons. Bi) Single action potentials elicited by brief square current pulses (10 ms) in wild type and mutant SOD-1 interneurons. Bii) The height and Biii) maximum rate of rise of action potentials and Biv) amplitude of the after-hyperpolarisation (AHP) were all not significantly different between wild type (n = 23) and mutant SOD-1 (n = 24) interneurons. Ci) and Cii) The voltage threshold for action potential generation, measured by injecting a ramp of current, was not significantly different between wild type (n = 17) and mutant SOD-1 (n = 21) interneurons.


Ai
WT
SOD
20 mV
0.1 s
100 pA
Aii
Firing Freq. (Hz)
WT
SOD
160 (pA)
Aiii
Max. Firing Freq. (Hz)
Bi
WT
SOD
20 mV
40 ms
80 pA
Bii
AP Height (mV)
Biii
AP Max. Rise (mV/ms)
Biv
AHP (mV)
Ci
WT
SOD
20 mV
0.5 s
50 pA
50 pA
Cii
AP Threshold (mV)

of the action potential after-hyperpolarisation (AHP), baseline membrane potentials were kept near -60 mV by delivering appropriate bias currents. AHP amplitude was again similar between wild type (5.2 ± 0.7 mV, n = 23) and mutant SOD-1 (4.9 ± 0.5 mV, n = 24) interneurons (Fig. 17Bi & 17Biv). The voltage threshold for action potential generation was also assessed from current-clamp protocols applying slow current ramps (3.3 to 83.3 pA/s; Fig. 17Ci). These measurements again demonstrated no significant differences between wild type (-37.2 ± 2.4 mV, n = 17) and mutant SOD-1 (-41.6 ± 1.7 mV, n = 21) interneuron populations (Fig. 17Cii).

A number of previous studies have suggested that an alteration in the properties of Na^+ currents is likely to be involved in early (embryonic and neonatal) pre-symptomatic changes in neuronal excitability in mutant SOD-1 mice (Kuo *et al.*, 2005; Zona *et al.*, 2006; van Zundert *et al.*, 2008; Pambo-Pambo *et al.*, 2009; Quinlan *et al.*, 2011). Given these findings, the present study analysed fast inactivating Na^+ currents, elicited by small voltage steps (-70 to +20 mV, 2.5 mV increments, 10 ms duration; Fig. 18Ai & 18Aii) from a holding potential at -60 mV. The analyses revealed no significant differences in the peak density of these Na^+ currents in wild type (-275.7 ± 21.6 pA/pF; n = 22) and mutant SOD-1 (-305.1 ± 24.5 pA/pF; n = 25) interneurons (Fig. 18Aii & 18iii). However, an intriguing finding was that the voltage at which the peak Na^+ current density was achieved was 7.5 mV apart in wild type (-20 mV) and mutant SOD-1 (-27.5 mV) interneuron populations (Fig. 18Aii). Thus, there seems to be a difference in the voltage dependence of fast inactivating Na^+ currents in these two interneuron populations. In comparison, both the density and voltage dependence of K^+ currents evoked by the delivery of long voltage pulses (-60 to 40 mV, 10 mV increments, 150 ms duration; Fig. 18Bi & 18Bii) from a holding potential of -60 mV

was not significantly different between the two interneuron populations (Fig. 18Bii). The maximum density of K^+ currents measured was 509.7 ± 40.7 pA/pF in wild type interneurons (n = 22) and -553.6 ± 64.8 pA/pF in mutant SOD-1 interneurons (n = 24; Fig. 18Bii & 18Biii).

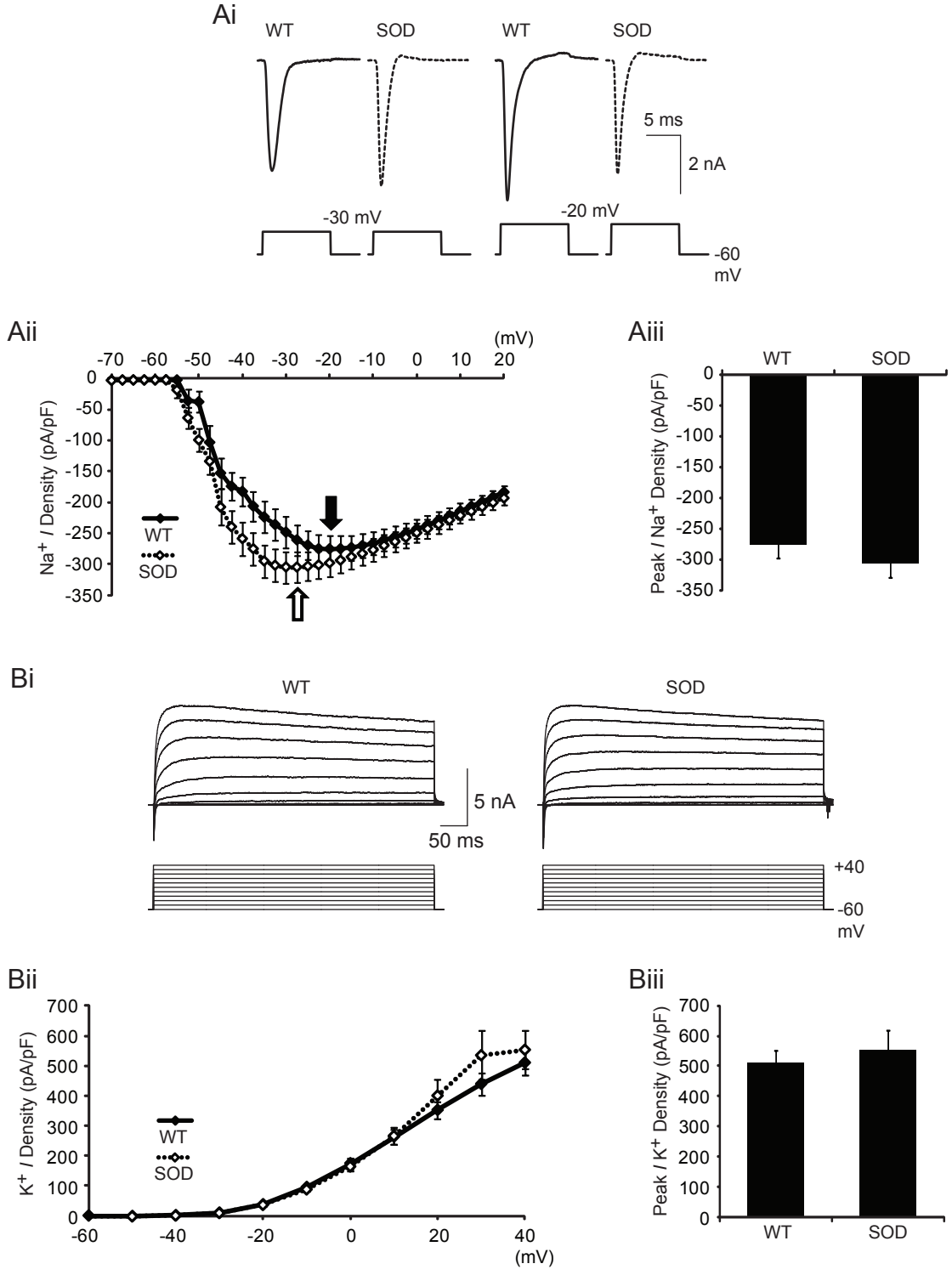
Taken together, the present data indicate no significant differences in the vast majority of intrinsic sub- and supra-threshold properties of interneurons recorded from spinal cord slice preparations obtained from wild type and mutant SOD-1 mice. However, in keeping with previous reports of alterations in the properties of Na^+ currents in motoneurons of mutant SOD-1 mice, the voltage-dependence of activation of fast inactivating Na^+ currents appeared to be altered in spinal interneurons of mutant SOD-1 mice.

Effects of group I mGluR activation in WT and mutant SOD-1 interneurons

Given the limited differences in intrinsic cellular properties of spinal interneurons recorded from wild type and mutant SOD-1 *in vitro* spinal cord slice preparations, the second hypothesis that these two interneuron populations differentially respond to the activation of group I mGluRs was assessed next. DHPG (10 μM) was bath applied to spinal cord slice preparations obtained from wild type and mutant SOD-1 mice, while whole-cell patch-clamp recordings were performed from ventral interneurons. Starting with the examination of sub-threshold effects, DHPG application caused a small but significant depolarisation of resting membrane potentials in both wild type (1.9 ± 0.8 mV depolarisation, n = 17, p = 0.036) and mutant SOD-1 (2.2 ± 0.7 mV depolarisation, n = 17, p = 0.005) interneuron populations (Fig. 19Ai, 19Aii & 19Aiii), although the degree of depolarisation was not different between the two populations (Fig. 19Ai,

Figure 18: Voltage-clamp analyses of Na^+ and K^+ currents in wild type and mutant SOD-1 interneurons.

Ai) Fast inactivating Na^+ currents elicited by brief (10 ms) depolarising voltage steps in wild type and mutant SOD-1 interneurons held at -60 mV. Aii) Plots of current-voltage (I-V) relationships show different peak activation voltages of wild type (black arrow, n = 22) and mutant SOD-1 (white arrow, n = 25) interneurons. Aiii) Pooled data showing the density of peak Na^+ currents was not significantly different between wild type and mutant SOD-1 interneurons. Bi) K^+ currents elicited by long depolarising voltage steps (150 ms, up to +40 mV) in wild type and mutant SOD-1 interneurons held at -60 mV. Bii) Plots of I-V relationships and Biii) pooled data show the voltage-dependence and density of peak K^+ currents was not significantly different between wild type and mutant SOD-1 interneurons.


Ai
WT
SOD
WT
SOD
5 ms
2 nA
-30 mV
-20 mV
-60
mV
Aii
(mV)
-70 -60 -50 -40 -30 -20 -10 0 10 20
0
-50
-100
-150
-200
-250
-300
-350
Na+ / Density (pA/pF)
WT
SOD
Aiii
WT
SOD
Peak / Na+ Density (pA/pF)
Bi
WT
SOD
5 nA
50 ms
+40
-60
mV
Bii
700
600
500
400
300
200
100
0
K+ / Density (pA/pF)
WT
SOD
-60 -40 -20 0 20 40
(mV)
Biii
Peak / K+ Density (pA/pF)
WT
SOD

19Aii & 19Aiii). The DHPG-induced membrane depolarisation was not accompanied by changes in input resistance (Fig. 19B) as calculated from I-V relationships obtained from voltage-clamp protocols delivering small voltage steps (-70 to -50 mV, 2.5 mV increments, 150 ms duration) from a holding potential at -60 mV.

Since it was demonstrated that the activation of group I mGluRs caused a net reduction in the excitability of spinal motoneurons (Chapter 3), the modulatory effects of group I mGluR activation upon firing properties of spinal interneurons were next analysed. Steady state f-I relationships were generated from current-clamp protocols applying steps of supra-threshold square current (1 s). The f-I relationships obtained from individual interneurons were then averaged across wild type (n = 12) and mutant SOD-1 (n = 12) interneuron populations. DHPG application did not shift the f-I relationships in these two populations (Fig. 20Ai & 20Aii). The unchanged excitability of spinal interneurons in response to DHPG application was statistically confirmed by the result that the maximum firing frequencies were unaffected by DHPG application in both wild type and mutant SOD-1 interneuron populations (Fig. 20Aiii). These data are in contrast with the effects observed in spinal motoneurons reported in the previous chapter (Chapter 3).

Although significant changes were not detected in the steady state f-I relationships of spinal interneurons in response to group I mGluR activation, it was still possible that small alterations in the parameters of individual action potentials might be uncovered. To assess this possibility, single action potentials were evoked by brief pulses of supra-threshold square current (10 ms; Fig. 20Bi) and the shape of single action potentials were analysed. Similar to the group I mGluR-mediated modulatory effects on motoneuron action potentials reported in the previous chapter (Chapter 3), the height

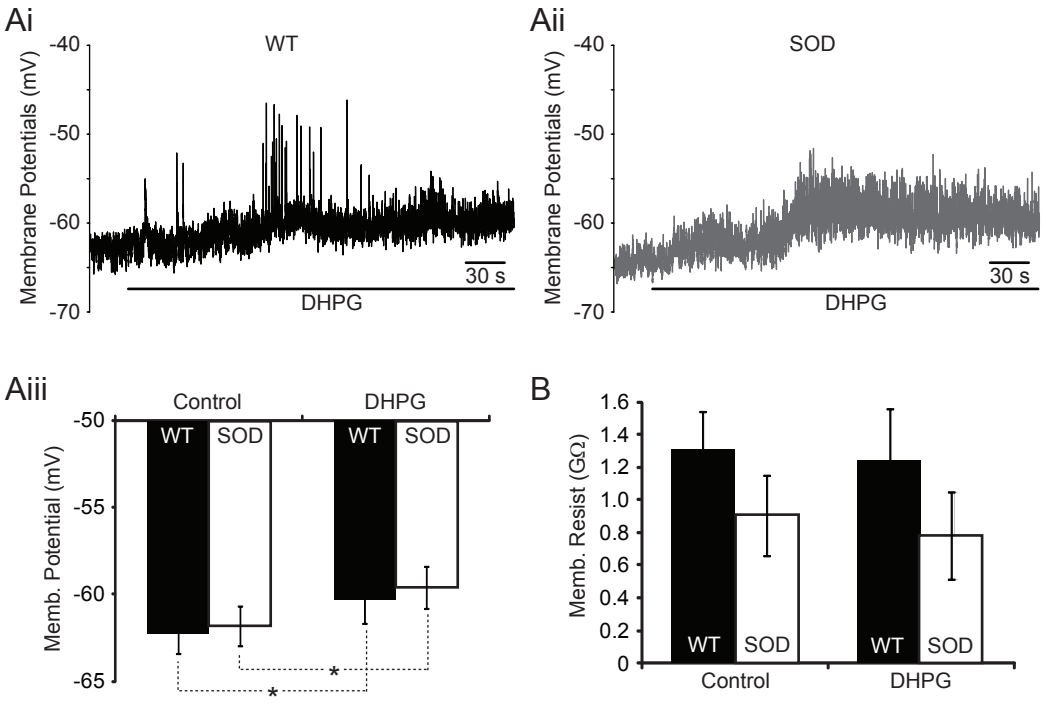
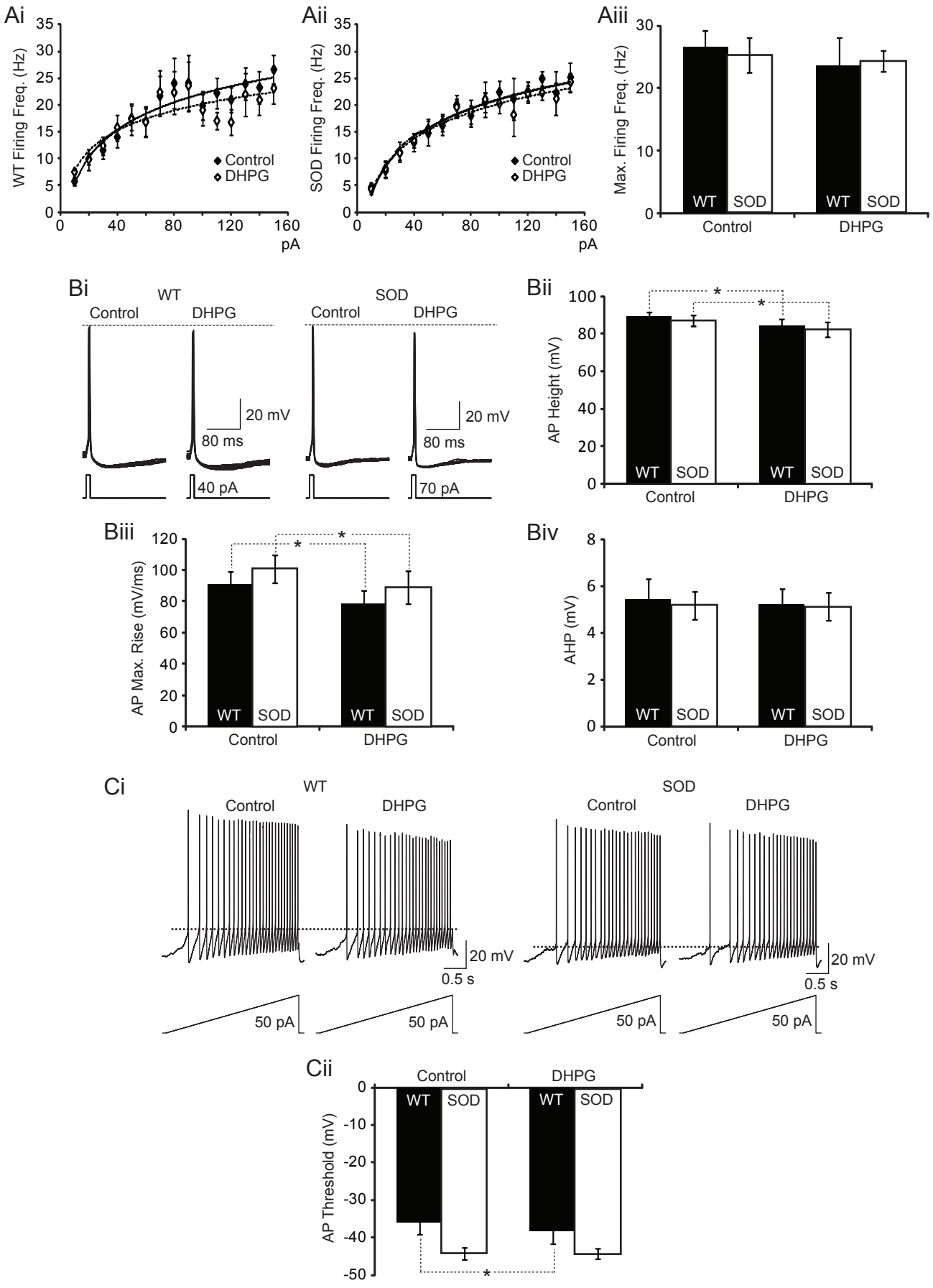


Figure 19: Sub-threshold effects of group I mGluR activation in wild type and mutant SOD-1 interneurons.

Ai) and Aii) Resting membrane potentials depolarised in response to DHPG application (10 μM) in wild type and mutant SOD-1 interneurons. Aiii) Pooled data show DHPG-induced significant membrane depolarisation to similar magnitude in wild type (n = 17) and mutant SOD-1 (n = 17) interneurons. B) Pooled data show no significant effect on membrane resistance in response to DHPG application in wild type (n = 15) and mutant SOD-1 (n = 16) interneurons. * = significantly different from control.

Figure 20: Effects of group I mGluR activation on the firing properties of wild type and mutant SOD-1 interneurons.

Ai) f-I relationships averaged across wild type interneurons (n = 12) in control condition and the presence of DHPG (10 μM). Aii) f-I relationships averaged across mutant SOD-1 interneurons (n = 12) in control condition and the presence of DHPG. Aiii) Pooled data show no significant effect on maximum firing frequency in response to DHPG application in wild type and mutant SOD-1 interneurons. Bi) Single action potentials elicited by brief square current pulses (10 ms) in control condition and the presence of DHPG in wild type and mutant SOD-1 interneurons. Bii) The height of action potentials was significantly reduced by DHPG application in both wild type (n = 15) and mutant SOD-1 (n = 16) interneuron populations. Biii) Maximum rate of rise of action potentials was also significantly reduced by DHPG application in both wild type and mutant SOD-1 interneuron populations. Biv) The amplitude of the AHP was not affected by DHPG application in either interneuron population. Ci) The voltage threshold for action potential generation was measured in wild type and mutant SOD-1 interneurons by injecting a ramp of current in control condition and the presence of DHPG. Cii) Pooled data show that action potential threshold was significantly hyperpolarised by DHPG application in wild type (n =11) but not in mutant SOD-1 (n = 12) interneurons. * = significantly different from control.


Ai
WT Firing Freq. (Hz)
Control
DHPG
pA
Aii
SOD Firing Freq. (Hz)
Control
DHPG
pA
Aiii
Max. Firing Freq. (Hz)
WT
SOD
WT
SOD
Control
DHPG
Bi
WT
SOD
Control
DHPG
Control
DHPG
20 mV
80 ms
40 pA
20 mV
80 ms
70 pA
Bii
AP Height (mV)
*
*
WT
SOD
WT
SOD
Control
DHPG
Biii
AP Max. Rise (mV/ms)
*
*
WT
SOD
WT
SOD
Control
DHPG
Biv
AHP (mV)
WT
SOD
WT
SOD
Control
DHPG
Ci
WT
SOD
Control
DHPG
Control
DHPG
20 mV
0.5 s
50 pA
50 pA
20 mV
0.5 s
50 pA
50 pA
Cii
Control
DHPG
AP Threshold (mV)
WT
SOD
WT
SOD
*

and maximum rate of rise of single action potentials were significantly reduced by DHPG application in both wild type (height 4.9 ± 1.6 mV reduction, n = 15, p = 0.007; max rate 15.1 ± 3.5 % reduction, n = 15, p = 0.001) and mutant SOD-1 (height 4.8 ± 1.8 mV reduction, n = 16, p = 0.017; max rate 14.5 ± 4.7 % reduction, n = 16, p = 0.010) interneurons (Fig. 20Bi, 20Bii & 20Biii). The degree to which DHPG application reduced the height and maximum rate of rise of single action potentials was not different between the two interneuron populations. For measurements of the amplitude of the AHP, the baseline membrane potentials were set around -60 mV by applying appropriate bias currents. The amplitude of the AHP was unchanged in response to DHPG application in both wild type and mutant SOD-1 interneurons (Fig. 20Bi & 20Biv). The voltage threshold for action potential generation was also analysed using current-clamp protocols involving slow ramps of current injection (3.3 to 83.3 pA/s; Fig. 20Ci). Interestingly, DHPG application led to differential effects on the voltage threshold for action potential generation in wild type and mutant SOD-1 interneurons. The action potential threshold was slightly but significantly hyperpolarised in response to DHPG application in wild type interneurons (2.2 ± 1.0 mV hyperpolarisation, n = 11, p = 0.044; Fig. 20Ci & 20Cii) whereas in mutant SOD-1 interneurons DHPG application had no effect on the threshold for action potential generation (n = 12; Fig. 20Ci & 20Cii).

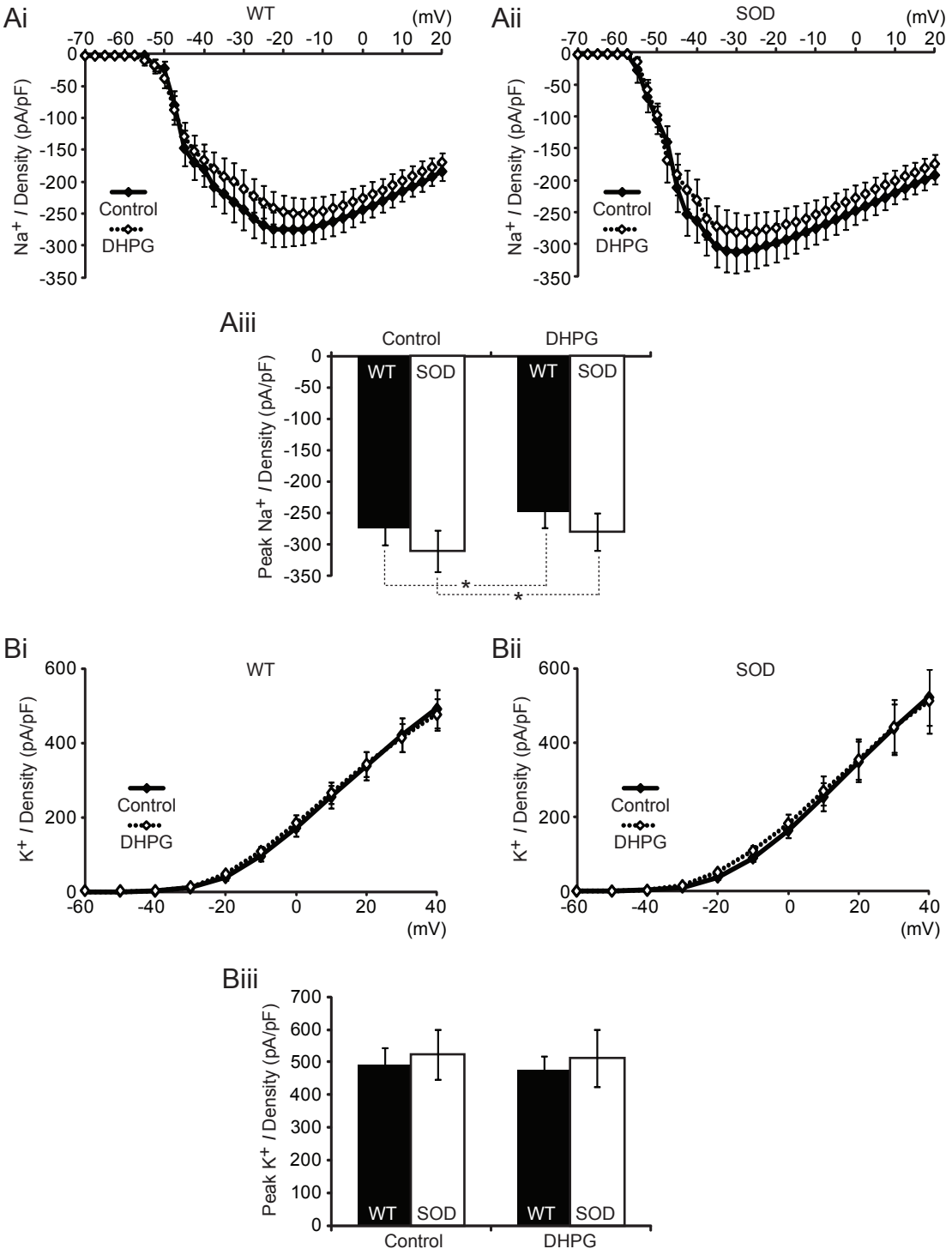
Next, it was assessed whether the group I mGluR-mediated effects on the shape of individual action potentials in spinal interneurons were mediated via the modulation of fast inactivating Na^+ currents as reported in spinal motoneurons (Chapter 3). Fast inactivating Na^+ currents were elicited by brief voltage steps (-70 to +20 mV, 2.5 mV increments, 10 ms duration; Fig. 21Ai & 21Aii) from a holding potential at -60 mV.

Consistent with the group I mGluR-mediated reductions in the height and maximum rate of rise of single action potentials in spinal interneurons, the density of fast inactivating Na^+ currents was reduced by DHPG application in both wild type and mutant SOD-1 interneurons (Fig. 21Ai, 21Aii & 21Aiii). The peak Na^+ current density was significantly reduced in wild type (10.3 ± 2.3 % reduction, n = 15, p = 0.001) and mutant SOD-1 (8.4 ± 3.4 % reduction, n = 16, p = 0.016) interneurons (Fig. 21Aiii), although the degree of the reduction in peak Na^+ current density was similar between the two interneuron populations. In addition, analyses of K^+ currents, elicited by long voltage steps (-60 to +40 mV, 10 mV increments, 150 ms duration; Fig. 21Bi & 21ii) from a holding potential at -60 mV, revealed no significant changes in the density and voltage dependence of K^+ currents in response to the application of DHPG in wild type (n = 15) or mutant SOD-1 (n = 16) interneuron populations (Fig. 21Bi, 21Bii & 21Biii).

In summary, the activation of group I mGluRs in spinal interneurons showed some of the same modulatory effects that were reported in spinal motoneurons (Chapter 3). However, unlike in spinal motoneurons, the overall input-output relationships of spinal interneurons, assessed by steady state f-I relationships, were unchanged by the activation of group I mGluRs. However, as reported in spinal motoneurons (Chapter 3), group I mGluR-mediated reductions in the height and maximum rate of rise of single action potentials were observed in spinal interneurons. Furthermore, the present data indicate that these reductions in action potential parameters are likely to be related to a reduction in the density of fast inactivating Na^+ currents in spinal interneurons. The modulatory effects of group I mGluR activation were, for the most part, similar between wild type and mutant SOD-1 interneuron populations. The only difference between the two populations was that a group I mGluR-mediated hyperpolarisation of the

Figure 21: Effects of group I mGluR activation on Na^+ and K^+ currents in wild type and mutant SOD-1 interneurons.

Ai) Plots of I-V relationships for fast inactivating Na^+ currents in response to DHPG application (10 μM) in wild type interneurons (n = 15). Aii) Plots of I-V relationships for fast inactivating Na^+ currents in response to DHPG application in mutant SOD-1 interneurons (n = 16). Aiii) Pooled data show DHPG-mediated significant reduction in the peak density of fast inactivating Na^+ currents in both wild type and mutant SOD-1 interneurons. Bi) Plots of I-V relationships for K^+ currents in response to DHPG application in wild type interneurons (n = 15). Aii) Plots of I-V relationships for K^+ currents in response to DHPG application in mutant SOD-1 interneurons (n = 16). Aiii) Pooled data show no significant change in the peak density of K^+ currents in both wild type and mutant SOD-1 interneuron populations. * = significantly different from control.


Ai
WT
(mV)
-70 -60 -50 -40 -30 -20 -10 0 10 20
0 -50 -100 -150 -200 -250 -300 -350
Na+ / Density (pA/pF)
Control
DHPG
Aii
SOD
(mV)
-70 -60 -50 -40 -30 -20 -10 0 10 20
0 -50 -100 -150 -200 -250 -300 -350
Na+ / Density (pA/pF)
Control
DHPG
Aiii
Control
DHPG
WT
SOD
WT
SOD
0 -50 -100 -150 -200 -250 -300 -350
Peak Na+ / Density (pA/pF)
*
*
Bi
WT
600 400 200 0
K+ / Density (pA/pF)
Control
DHPG
-60 -40 -20 0 20 40
(mV)
Bii
SOD
600 400 200 0
K+ / Density (pA/pF)
Control
DHPG
-60 -40 -20 0 20 40
(mV)
Biii
700 600 500 400 300 200 100 0
Peak K+ / Density (pA/pF)
WT
SOD
WT
SOD
Control
DHPG

voltage threshold for action potential generation was statistically confirmed in wild type but not in mutant SOD-1 interneurons.

Discussion

This final chapter has assessed the possible involvement of group I mGluRs in early pre-symptomatic alterations in the excitability of spinal interneurons that make up locomotor networks in the spinal cord of ALS model mice. Extracellular recordings from lumbar ventral roots of pharmacologically activated *in vitro* whole spinal cord preparations obtained from wild type and mutant SOD-1 neonatal mice demonstrated that the activation of group I mGluRs resulted in a reduction in the amplitude of locomotor-related burst activity and an increase in locomotor burst frequency in both types of *in vitro* spinal cord preparations. Interestingly, the present results represent the first report of rhythmic locomotor burst activity induced pharmacologically in isolated spinal cord preparations obtained from mutant SOD-1 mice. A previous study reported that rhythmic locomotor burst activity could not be induced from lumbar locomotor networks of *in vitro* mutant SOD-1 spinal cord preparations obtained from neonatal mice and concluded that the pathology of ALS involved disruption to neural networks in the developing lumbar spinal cord (Amendola *et al.*, 2004). The present data do not seem to support this conclusion. However, it is difficult to directly compare the results of the present study and those of Amendola *et al.* (2004) since the latter study utilised a different strain of mutant SOD-1 (G85R) mice and did not include dopamine for pharmacological activation of lumbar locomotor networks (Amendola *et al.*, 2004).

In the present study, the activation of group I mGluRs reduced the amplitude of rhythmic locomotor burst activity to a similar extent in wild type and mutant SOD-1 *in vitro* spinal cord preparations. The time course of this effect was also comparable

between the two preparations. Therefore, there is little evidence that group I mGluR activation exerts differential modulatory actions at the level of spinal motoneurons that determine the intensity of the final locomotor output produced from wild type and mutant SOD-1 spinal networks. In addition, the activation of group I mGluRs increased rhythmic locomotor burst frequency to a similar extent in wild type and mutant SOD-1 *in vitro* spinal cord preparations. However, an intriguing finding was that the time course of this effect on burst frequency differed between wild type and mutant SOD-1 preparations. The maximal effects of group I mGluR activation on locomotor burst frequency were reached approximately 10 minutes earlier in mutant SOD-1 spinal cord preparations. These data can be explained by two hypotheses: 1) spinal interneurons that make up locomotor CPG networks have different intrinsic neural properties in wild type and mutant SOD-1 mouse spinal cords, as suggested by Amendola *et al.* (2004); or 2) the responsiveness of these spinal interneurons to the activation of group I mGluRs is different between the two spinal cord preparations. To assess these two hypotheses, whole-cell patch-clamp recordings from individual spinal interneurons were performed in spinal cord slice preparations obtained from neonatal wild type and mutant SOD-1 transgenic mice.

Because interneurons were selected from the entire ventral half of spinal cord slice preparations, it is highly likely that whole-call patch-clamp recordings were made from mixed groups of spinal interneurons that make up locomotor networks in mice. It would be more ideal to limit the groups of spinal interneurons targeted for recordings through the use of combined transgenic and developmental tools that identify specific populations of CPG interneurons (Jessell, 2000; Goulding, 2009). However, the present data reported in this chapter still represent one of the few studies into the basic

intrinsic properties of interneurons in the ALS affected mammalian spinal cord or brainstem (van Zundert *et al.*, 2008). Although small alterations occurring in specific interneuronal populations may not have been detected due to the heterogeneity of the interneurons recorded, it was hoped in these recordings that global alterations in the basic properties of ventral interneurons or alternatively changes in the proportions of mixed neural groups might be uncovered in the spinal cord of mutant SOD-1 mice. Unfortunately in the present study, few major differences were discovered between the basic intrinsic properties of interneurons in wild type and mutant SOD spinal cord slice preparations. However, a subtle difference in the voltage-dependence of activation of fast inactivating Na^+ currents was uncovered between the two interneuron populations. The hyperpolarised peak activation voltage of fast inactivating Na^+ currents in mutant SOD-1 interneurons may represent a common, widespread perturbation in the excitability of interneurons at a very early pre-symptomatic stage of the ALS affected spinal cord in mice. However, further investigation is needed to prove this. For example, more detailed measurements of the voltage-dependence of Na^+ currents in wild type and mutant SOD-1 spinal interneurons should be performed using modified external and internal solutions designed to minimise voltage and space-clamp errors by to reducing the amplitude of Na^+ currents and isolating Na^+ currents from other currents such as K^+ and Ca^{2+} currents (Chapter 3).

In previous studies, analyses of altered neuronal properties at early pre-symptomatic stages of ALS affected spinal cord has concentrated on analyses of motoneurons in mutant SOD-1 mouse spinal cord preparations. There are some data supporting hypoexcitability of mutant SOD-1 motoneurons as indicated by, for example, a reduction in the gain of steady state f-I relationships (Bories *et al.*, 2007; Pambo-Pambo

et al., 2009). However, hyperexcitability of mutant SOD-1 motoneurons has been reported most frequently as an increase in the gain of steady state f-I relationships (Pieri *et al.*, 2003; Kuo *et al.*, 2004; Kuo *et al.*, 2005; Zona *et al.*, 2006; van Zundert *et al.*, 2008; Pambo-Pambo *et al.*, 2009; Quinlan *et al.*, 2011). The hyperexcitability of mutant SOD-1 motoneurons is likely mediated by modulation of Na^+ currents with effects including an increase in persistent Na^+ currents (Kuo *et al.*, 2005; van Zundert *et al.*, 2008; Quinlan *et al.*, 2011), an altered balance between persistent Na^+ and Ca^{2+} inward currents (Pambo-Pambo *et al.*, 2009) or the faster recovery of Na^+ channels from fast inactivation (Zona *et al.*, 2006). Given these previous studies of Na^+ current-mediated hyperexcitability of mutant SOD-1 motoneurons and the present study demonstrating the hyperpolarised peak activation voltage of fast inactivating Na^+ currents in mutant SOD-1 interneurons, it seems that functional alterations in Na^+ channels are highly likely to be involved in the neuronal hyperexcitability observed at early pre-symptomatic stages in ALS.

In the present chapter, it was also investigated whether interneurons of wild type and mutant SOD-1 spinal cord slice preparations respond differentially to the activation of group I mGluRs. Previous studies demonstrate that group I mGluRs play a neuroprotective role in kainate-induced excitotoxicity in adult rat spinal cord slice preparations (Pizzi *et al.*, 2000; Valerio *et al.*, 2002) and both neuroprotective and neurotoxic roles in neuronal death in embryonic chick spinal cord cultures treated with CSF obtained from human ALS patients (Anneser *et al.*, 2006). In the previous chapter (Chapter 3), it was demonstrated that the activation of group I mGluRs contributes to the homeostatic control of motoneuron excitability and glutamatergic excitatory inputs to spinal motoneurons. In the present chapter, the activation of group

I mGluRs resulted in similar, but fewer, modulatory effects on the intrinsic properties of interneurons in both wild type and mutant SOD-1 spinal cord slice preparations. A significant depolarisation of resting membrane potentials was observed in spinal interneurons following the activation of group I mGluRs, but the degree of membrane depolarisation was smaller in both wild type and mutant SOD-1 interneurons than that observed in spinal motoneurons (Chapter 3). The steady state f-I relationships were not altered by group I mGluR activation in both wild type and mutant SOD-1 interneuron populations, unlike the modulatory effect demonstrated in spinal motoneurons. These results indicate that the activation of group I mGluRs does not affect the generation of repetitive action potential firing in spinal interneurons. However, analyses of single action potentials revealed that in both wild type and mutant SOD-1 interneurons the height and maximum rate of rise of action potentials were significantly reduced by the activation of group I mGluRs. Voltage-clamp analyses of fast inactivating Na^+ currents suggested that the group I mGluR-mediated reductions in action potential parameters may relate to a reduction in the peak density of fast inactivating Na^+ currents in spinal interneurons, as was reported in motoneurons (Chapter 3).

There was only one differential effect of group I mGluR activation in wild type and mutant SOD-1 interneuron populations. The activation of group I mGluRs hyperpolarised the voltage threshold for action potential generation in wild type but not in mutant SOD-1 interneurons. In the previous chapter (Chapter 3) the group I mGluR-mediated hyperpolarisation of voltage thresholds for action potential generation was shown to be mediated via mGluR5 activation in spinal motoneurons. Hence it may be hypothesised that signalling pathways that involve mGluR5 activation are

altered in interneurons of the mutant SOD-1 mouse spinal cord. It is known that subtypes of group I mGluRs are differentially expressed in motor nuclei of the spinal cord and the brainstem, such that more mGluR1 expression is detected in motor nuclei vulnerable to ALS neurodegeneration, while more mGluR5 expression is detected in motor nuclei that are resistant to ALS neurodegeneration (Laslo *et al.*, 2001; Anneser *et al.*, 2004; Ma *et al.*, 2006). Comparisons of the effects of group I mGluRs in ALS vulnerable and resistant motor nuclei may therefore advance our understanding of the potential neuroprotective or neurotoxic subtype specific roles of group I mGluRs in ALS.

Although it is very hard to relate results obtained from mixed types of spinal interneurons that make up locomotor CPG networks with group I mGluR-mediated modulatory effects observed in rhythmically active *in vitro* whole spinal cord preparations, there are some findings worth discussing in this final chapter. A hyperpolarisation of voltage thresholds for action potential generation in response to group I mGluR activation in wild type interneurons might be a possible candidate that underlies the group I mGluR-mediated increase in locomotor burst frequency recorded from *in vitro* whole spinal cord preparations. However, this seems unlikely to be the case, because a hyperpolarisation of action potential thresholds was not observed in mutant SOD-1 interneurons, yet the group I mGluR-mediated increase in locomotor burst frequency had a quicker time course effect in mutant SOD-1 compared to wild type *in vitro* spinal cord preparations. The faster time course of the effect on burst frequency in mutant SOD-1 whole spinal cord preparations may reflect increased excitability of mutant SOD-1 interneurons as evidenced by a hyperpolarised activation voltage for fast inactivating Na^+ currents. However more detailed analyses will be

required to confirm this. In addition, the group I mGluR-mediated depolarisation of resting membrane potentials in wild type and mutant SOD-1 interneurons may be most likely to contribute to the increase in locomotor burst frequency recorded from both types of *in vitro* whole spinal cord preparations.

The present chapter represents the first study of the intrinsic neural properties of ventral interneurons and the modulatory effects of group I mGluR activation upon interneurons in the spinal cord of ALS affected neonatal mice. Given recent studies that have started to focus on the involvement of pre-motor interneurons and synaptic inputs to motoneurons in neurodegenerative diseases (Nagao *et al.*, 1998; Schutz, 2005; Avossa *et al.*, 2006; Stephens *et al.*, 2006), it will become of increasing importance to understand the physiological characteristics of different types of interneurons that make up motor networks as well as to uncover alterations that may occur to their neuronal properties due to pathological processes in the mammalian spinal cord.

Concluding remarks

The present study has demonstrated the role of group I mGluRs for intrinsic neuromodulation in locomotor networks of the mammalian spinal cord. Modulatory effects of group I mGluR activation have been assessed in locomotor networks, not only of the healthy mouse spinal cord, but also of the mouse spinal cord affected by the neurodegenerative disease, amyotrophic lateral sclerosis (ALS).

Activation of group I mGluRs in pharmacologically activated (NMDA, 5-HT, and dopamine) *in vitro* whole spinal cord preparations obtained from healthy wild type neonatal mice resulted in a decrease in the amplitude of locomotor-related rhythmic burst activity recorded from lumbar ventral roots. Concurrently, the activation of group I mGluRs in these *in vitro* spinal cord preparations increased the frequency of rhythmic locomotor burst activity. These results together suggest that the activation of group I mGluRs indeed modulates on-going locomotor network activity produced by both motoneurons and interneurons that make up central pattern generator (CPG) for locomotion in the mammalian spinal cord. The group I mGluR-mediated decrease in locomotor burst amplitude and the increase in locomotor burst frequency were also evident in whole *in vitro* spinal cord preparations obtained from ALS affected neonatal mice.

Whole-cell path-clamp recordings from motoneurons and interneurons within spinal locomotor networks have revealed a wide range of cellular mechanisms that may explain group I mGluR-mediated modulatory effects upon on-going locomotor network activity observed in the mouse spinal cord. In motoneurons, despite limited excitatory effects, such as membrane depolarisation, the net group I mGluR-mediated action was a

reduction in excitability, most likely reflecting a reduction in fast inactivating Na^+ currents. In addition to modulating the intrinsic properties of motoneurons, group I mGluRs were also found to modulate synaptic inputs received by motoneurons, specifically reducing locomotor-related excitatory drive to motoneurons via both pre- and post-synaptic modulatory mechanisms. These data together indicate that both reduced excitability of motoneurons and a decrease in synaptic drive to motoneurons are likely to underlie group I mGluR-mediated reductions in locomotor burst amplitude observed in the whole spinal networks.

Recordings from spinal interneurons of the ventral horn provided evidence that membrane depolarisation in response to group I mGluR activation is likely to contribute to the increase in locomotor burst frequency recorded from *in vitro* whole spinal cord preparations. Given the heterogeneous nature of the interneurons recorded from, it will be important for future studies to investigate the potential specificity of group I mGluR-mediated effects on different neural populations within CPG networks. Such analyses of group I mGluR-mediated effects on specific populations of neurons will become easier as more distinct interneuron populations are defined using genetic techniques based on increasing knowledge of spinal cord development.

In both motoneurons and interneurons, group I mGluR activation led to excitatory effects, such as membrane depolarisation and hyperpolarisation of action potential thresholds, as well as inhibitory effects, such as a decrease in Na^+ current density and a reduction in excitatory synaptic transmission from pre-motor interneurons to spinal motoneurons. The range of opposing actions of group I mGluRs reported in the present study suggests that group I mGluRs may act as important 'homeostatic neuromodulators' within locomotor networks of the mammalian spinal cord, perhaps

stabilising the state of the network to ensure output is produced within an appropriate range (Harris-Warrick and Johnson, 2010).

In the present study no major alterations were discovered in the actions of group I mGluRs on locomotor networks of ALS affected neonatal mice. However, this does not preclude involvement of these receptors in later stages of the disease. An intriguing finding was that interneurons recorded from spinal cord slice preparations obtained from ALS affected mice had hyperpolarised activation voltages for fast inactivating Na^+ currents compared to wild type interneurons. This finding is particularly interesting in the context of previous studies which have shown alterations in Na^+ channel function in motoneurons of ALS model mice at very early developmental stages (Kuo *et al.*, 2005; Zona *et al.*, 2006; van Zundert *et al.*, 2008; Quinlan *et al.*, 2011). Although it remains to be determined whether altered motoneuron, and perhaps interneuron, excitability due to perturbations in Na^+ channel function is critically involved in the pathology of ALS, this is an area in which further work should be conducted. It is intriguing that the only approved treatment for ALS, the drug riluzole, acts not only on glutamate release but also persistent Na^+ channels (Bellingham, 2011).

In summary, the present study has provided a number of novel results that demonstrate the important neuromodulatory role of group I mGluRs at the level of whole spinal locomotor networks as well as single neurons, motoneurons and interneurons, within the mammalian spinal cord in health and disease. As well as advancing our basic understanding of spinal motor control, the present work highlights the potential importance of studying neuromodulatory systems in neurodegenerative diseases. It is hoped that a greater knowledge of neuromodulation, including that

mediated by group I mGluRs, will ultimately lead to the development of useful clinical strategies for the treatment of neurodegenerative diseases such as ALS.

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