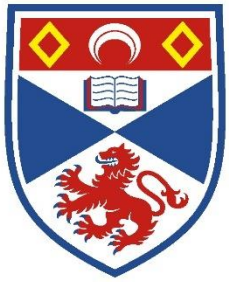
Regulation of Mammalian Spinal Locomotor Networks by Glial Cells

David Acton



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Abstract

Networks of interneurons within the spinal cord coordinate the rhythmic activation of muscles during locomotion. These networks are subject to extensive neuromodulation, ensuring appropriate behavioural output. Astrocytes are proposed to detect neuronal activity via $G_{\alpha q}$-linked G-protein coupled receptors and to secrete neuromodulators in response. However, there is currently a paucity of evidence that astrocytic information processing of this kind is important in behaviour. Here, it is shown that protease-activated receptor-1 (PAR1), a $G_{\alpha q}$-linked receptor, is preferentially expressed by glia in the spinal cords of postnatal mice. During ongoing locomotor-related network activity in isolated spinal cords, PAR1 activation stimulates release of adenosine triphosphate (ATP), which is hydrolysed to adenosine extracellularly. Adenosine then activates A_1 receptors to reduce the frequency of locomotor-related bursting recorded from ventral roots. This entails inhibition of D_1 dopamine receptors, activation of which enhances burst frequency. The effect of A_1 blockade scales with network activity, consistent with activity-dependent production of adenosine by glia. Astrocytes also regulate activity by controlling the availability of D-serine or glycine, both of which act as co-agonists of glutamate at *N*-methyl-D-aspartate receptors (NMDARs). The importance of NMDAR regulation for locomotor-related activity is demonstrated by blockade of NMDARs, which reduces burst frequency and amplitude. Bath-applied D-serine increases the frequency of locomotor-related bursting but not intense synchronous bursting produced by blockade of inhibitory transmission, implying activity-dependent regulation of co-agonist availability. Depletion of endogenous D-serine increases the frequency of locomotor-related but not synchronous bursting, indicating that D-serine is required at a subset of NMDARs expressed by inhibitory interneurons. Blockade of the astrocytic glycine transporter GlyT1 increases the frequency of locomotor-related activity, but application of glycine has no effect, indicating that GlyT1 regulates glycine at excitatory synapses. These results indicate that glia play an important role in regulating the output of spinal locomotor networks.

Contents

Chapter 1: General introduction

Spinal locomotor networks in mammals

Movement is a fundamental behaviour, allowing animals to interact with their environment and satisfy the requirements of survival. In vertebrates it arises from the biomechanical properties of the skeletomuscular system and activity within dedicated neural circuitry in the central nervous system (CNS) (Orlovsky, Deliagina and Grillner, 1999; Dickinson, 2000; Grillner, 2006; Kiehn, 2006; Kiehn, 2016). Planning and initiation of movements involves various brain regions, including the cortex, basal ganglia, midbrain and hindbrain (Orlovsky, Deliagina and Grillner, 1999; Takakusaki *et al.*, 2004), but executive control over the timing and coordination of muscle activity resides in networks of interneurons in the ventral horn of the spinal cord. These networks selectively excite and inhibit pools of motoneurons with precise timing to ensure appropriate contraction and relaxation within antagonistic pairs of muscles, resulting in smooth, controlled movement.

During rhythmic locomotor behaviours such as walking, flying and swimming, spinal networks activate muscles in a cyclically repeated sequence (Orlovsky, Deliagina and Grillner, 1999). The rhythmic output of spinal networks arises from the passive electrical properties of their constituent interneurons and the synaptic connections between those neurons (Grillner, 2003, 2006; Kiehn, 2016). Although spinal networks receive command signals and modulatory inputs from descending and peripheral pathways, they remain capable of generating a basic motor pattern when extrinsic influences are removed and are therefore an example of a central pattern generator (CPG) (Brown, 1911; Guertin, 2009). Other examples of CPGs include brainstem networks for chewing, swallowing and respiration (Jean, 2001; Marder and Bucher, 2001; Feldman and Del Negro, 2006; Lund and Kolta, 2006). Spinal cord preparations, primarily from cats, rats and mice, have been used extensively in conjunction with pharmacological and genetic manipulations to determine the organisation of the spinal motor circuitry (Orlovsky, Deliagina and Grillner, 1999; Kiehn and Butt, 2003; Whelan, 2010). Rodent

spinal cords may be isolated and sustained in artificial cerebrospinal fluid *in vitro*. Rhythmic locomotor-related activity can be evoked in hindlimb motor circuitry in these preparations by application of agonists of glutamate and monoamine receptors or by electrical or optogenetic stimulation (Kudo and Yamada, 1987; Smith and Feldman, 1987; Kiehn and Kjaerulff, 1996; Whelan, Bonnot and O'Donovan, 2000; Hägglund *et al.*, 2013). Isolated networks generate activity in hindlimb muscles, if present (Hayes, Chang and Hochman, 2009), or in transected ventral roots containing the motoneurons that innervate muscles in intact animals (Kudo and Yamada, 1987; Smith and Feldman, 1987). In preparations where the ventral roots are transected, field-potential recordings reveal a pattern of activity in the lumbar ventral roots corresponding to activity observed during treadmill locomotion in adult animals (Cowley and Schmidt, 1994; Kiehn and Kjaerulff, 1996; Whelan, Bonnot and O'Donovan, 2000). Bursts recorded from lumbar ventral roots L_1-L_4 are in phase with hip flexors and alternate with bursts in phase with hip extensors in roots L_5-L_6. Activity also alternates between left-right pairs of roots in all segments.

Many features of locomotor networks are conserved between non-limbed and limbed vertebrates (Orlovsky, Deliagina and Grillner, 1999; Marder and Bucher, 2001; Goulding, 2009; Kiehn, 2016); however, the architecture of locomotor circuits in the latter are considerably more complicated. In non-limbed vertebrates such as lampreys and frog tadpoles, muscle activation alternates between bilaterally paired axial muscles along the rostral-caudal axis, producing bends in the body in a metachronal wave that propels the animal during swimming. In limbed vertebrates including mammals, locomotion requires strict alternation of antagonistic groups of flexor and extensor muscles around the limb joints in addition to bilateral coordination of homologous muscles in the limbs and trunk.

Locomotor networks express rhythm, resulting in phasic activity within pools of motoneurons, and pattern, resulting in coordination of activity between pools of motoneurons (Kiehn, 2006; Kiehn, 2016). Changes in rhythm generation result in changes in cycle period. Changes in pattern generation result in differences in phase relationships between muscle groups. In

mammals, rhythm and pattern generation covary to produce different gaits at non-overlapping ranges of speed, allowing energetically efficient movement (Bellardita and Kiehn, 2015; Kiehn, 2016).

In non-limbed vertebrates, rhythm and pattern are proposed to be generated by common circuit elements with a single-layer organisation. This likely incorporates half-centre networks coupled across the midline by reciprocal inhibition in antiphase, such that when muscles are activated on one half of the body, those on the opposite side are rendered inactive (Roberts *et al.*, 1998; Grillner, 2003; Moult, Cottrell and Li, 2013). In limbed vertebrates, rhythm- and pattern-generating components are proposed to be segregated into two or more layers (Kriellaars *et al.*, 1994; Burke, Degtyarenko and Simon, 2001; Lafreniere-Roula and McCrea, 2005; Kiehn, 2006; McCrea and Rybak, 2008). Evidence supporting this model includes the observations that rhythm generation occurs in the absence of left-right alternation, for instance following hemisection of the spinal cord (Kato, 1987; Noga *et al.*, 1987; Kremer and Lev-Tov, 1997; Bonnot *et al.*, 2002), and that it is independent of flexor-extensor alternation (Machado *et al.*, 2015).

In mammals, the locomotor circuitry is distributed within the ventral horn of the spinal cord, with the upper limbs governed by networks located in cervical segments and the hindlimbs by networks in lumbar and sacral segments (Orlovsky, Deliagina and Grillner, 1999; Kiehn *et al.*, 2008; Goulding, 2009). Ventral horn interneurons have been described according to transcription-factor expression, axonal branching, neurotransmitter phenotype, passive membrane properties, sensory inputs and physiological function (Jankowska, 2001; Kiehn and Butt, 2003; Goulding, 2009). Four cardinal classes of postmitotic interneuron are generated within the ventral horn during embryonic development (these are V0, V1, V2 and V3 interneurons), as are motoneurons (Goulding, 2009). In addition, dorsally derived dI6 interneurons are integrated into the ventral circuitry. Each of these cell types is proposed to give rise to multiple subtypes in mature networks, generating considerable complexity (Bikoff *et al.*, 2016). The mechanisms by which this diverse population of interneurons generates

rhythm and pattern in mammals have been partially determined, and will be briefly described here.

A set of ipsilaterally projecting glutamatergic interneurons are proposed to form a rhythm generating core in both non-limbed and limbed vertebrates (Kiehn *et al.*, 2008; Kiehn, 2016). These neurons are proposed to be essential for both the initiation of rhythmic network activity and speed control. In mammals, rhythm generation is supressed during inhibition of glutamatergic transmission (see below) but can occur in hemisected cords in which commissural axons are severed (Kato, 1987; Noga *et al.*, 1987; Kremer and Lev-Tov, 1997; Bonnot *et al.*, 2002). Selective activation of glutamatergic neurons within the spinal cord is also sufficient to stimulate rhythmic network activity; whereas activity is suppressed when glutamatergic neurons are selectively silenced (Hägglund *et al.*, 2013). In mammals, rhythm-generating neurons are likely to be molecularly heterogeneous and may include neurons expressing the transcription factor short stature homeobox protein 2 (SHOX2) but not ceh-10 homeodomain containing homologue (CHX10); silencing of these cells results in perturbation of rhythmic bursting (Dougherty *et al.*, 2013), and interneurons containing the transcription factor basic helix–loop–helix domain containing, class B, 9 (Hb9), which display pacemaker properties (Wilson *et al.*, 2005; Brownstone and Wilson, 2008; Brocard, Tazerart and Vinay, 2010). Glutamatergic excitatory transmission is the subject of Chapter 4 and will be discussed at greater length under “Excitatory transmission in mammalian spinal locomotor networks”, below.

Commissural interneurons are proposed to coordinate activity in bilaterally homologous motor pools (Kiehn, 2016). At lower speeds of locomotion in quadrupeds, gaits are characterised by activity alternating between the left and right forelimbs and hindlimbs (Bellardita and Kiehn, 2015). This is mediated by inhibitory interneurons, since blockade of inhibitory transmission with strychnine, a glycine receptor (GlyR) antagonist, and picrotoxin or bicuculline, antagonists of γ-aminobutyric acid (GABA) type A ($GABA_A$) receptors results in synchronous activity on the left and right sides of the spinal cord (Cowley and Schmidt, 1995; Bracci, Ballerini and

Nistri, 1996). Different subsets of commissural V0 neurons are required for left-right alternation during walking, the lowest-frequency alternating gait, and trotting, which occurs at higher frequencies (Talpalar *et al.*, 2013; Bellardita and Kiehn, 2015). Walking requires dorsal V0 ($V0_D$) neurons, which are inhibitory, whereas trotting requires excitatory ventral V0 ($V0_V$) neurons, presumably in addition to $V0_D$ cells. Ablation of all V0 interneurons, including the inhibitory $V0_D$ subpopulation, prevents mice from producing gaits in which the left and right limbs alternate (Bellardita and Kiehn, 2015).

Gallop and bound are gaits used at the fastest speeds of quadrupedal locomotion, and are characterised by synchronous activation of left-right pairs of limbs (Bellardita and Kiehn, 2015). Synchronous activity is proposed to be mediated by excitatory commissural interneurons, the identity of which remains to be determined. V3 excitatory commissural interneurons contribute to the robustness of rhythmic bilaterally alternating locomotor-related activity, such that their removal from the spinal cord results in greater variance in amplitude and cycle period (Zhang *et al.*, 2008). However, their role in gaits in which the hindlimbs or forelimbs are synchronously active has not been assessed.

Flexor-extensor alternation is specified by circuit components distinct from those required for left-right alternation, and persists under conditions where left-right alternation is abolished, such as in hemicords (Cazalets, Borde and Clarac, 1995; Whelan, Bonnot and O'Donovan, 2000; Zagoraiou *et al.*, 2009). Flexor-extensor alternation is mediated by ipsilaterally derived inhibitory Ia interneurons belonging to the V1 and V2b populations (Zhang *et al.*, 2014; Britz *et al.*, 2015). Blocking output of Ia interneurons prevents flexor-extensor alternation but not left-right alternation, with V1 Ia interneurons dominantly inhibiting flexor activity and V2b Ia neurons dominantly inhibiting extensor activity. The premotor circuitry for flexion and extension is proposed to be organised into discrete modules since rhythmic activity can be generated discretely in localised pools of flexor and extensor motoneurons (Hägglund *et al.*, 2013).

Excitatory transmission in mammalian spinal locomotor networks

Excitatory glutamatergic transmission operates both within the spinal locomotor CPG (Kiehn *et al.*, 2008; Zhang *et al.*, 2008; Talpalar and Kiehn, 2010; Bellardita and Kiehn, 2015) and between descending pathways and the locomotor CPG (Jordan *et al.*, 2008; Hägglund *et al.*, 2010). Within spinal networks, excitatory transmission is required for rhythm generation, and determines the speed and amplitude of locomotor-related activity (Kiehn *et al.*, 2008; Talpalar and Kiehn, 2010). It also contributes to the robustness of activity (Zhang *et al.*, 2008; Dougherty *et al.*, 2013) and is implicated in gait selection (Talpalar *et al.*, 2013; Bellardita and Kiehn, 2015). Between descending pathways and spinal networks, excitatory transmission provides signals for both the initiation (Hägglund *et al.*, 2010) and, paradoxically, the cessation (Bouvier *et al.*, 2015) of locomotion.

Experiments in which selective agonists and antagonists of glutamate receptors were applied to isolated rodent spinal cord preparations have revealed roles for all three subtypes of ionotropic glutamate receptors, namely *N*-methyl-D-aspartate (NMDA) receptors (NMDARs), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors (AMPARs) and kainate receptors (Smith, Feldman and Schmidt, 1988; Cazalets, Sqalli-Houssaini and Clarac, 1992; Beato, Bracci and Nistri, 1997; Bracci, Beato and Nistri, 1998; Nishimaru, Takizawa and Kudo, 2000; Cowley *et al.*, 2005; Talpalar and Kiehn, 2010). In these studies, glutamatergic signalling is shown to determine the frequency and/or the amplitude of locomotor-related bursting. Glutamate receptors also mediate locomotor-related activity in intact rabbits and cats (Fenaux *et al.*, 1991; Douglas *et al.*, 1993; Giroux *et al.*, 2003)

It has been proposed that glutamatergic signalling differs between postnatal rats and mice (Nishimaru, Takizawa and Kudo, 2000). In rats, application of NMDAR agonists alone induces sustained locomotor-related activity (Kudo and Yamada, 1987; Smith, Feldman and Schmidt, 1988). In addition, NMDAR blockade consistently abolishes or reduces the frequency of rhythmic activity in rats, depending on the intensity of the stimulus used to excite the network (Smith, Feldman and Schmidt, 1988; Cazalets, Sqalli-Houssaini and Clarac, 1992; Beato,

Bracci and Nistri, 1997; Bracci, Beato and Nistri, 1998; Gabbay, 2004; Cowley *et al.*, 2005). Where activity is not abolished, left-right rhythmic alternation of bursts is maintained during NMDAR blockade (Beato, Bracci and Nistri, 1997; Bracci, Beato and Nistri, 1998; Cowley *et al.*, 2005).

In mice, sustained rhythmic activity is reported to require 5-HT in addition to NMDAR agonists (Jiang, Carlin and Brownstone, 1999), and NMDAR blockade is reported to enhance burst frequency during pharmacologically induced locomotor-related activity in mice (Whelan, Bonnot and Donovan, 2000). In addition, NMDARs and non-NMDARs may have distinct roles within murine locomotor networks. Non-NMDARs are proposed to be required for the generation of high (> 0.4 Hz) but not low (< 0.4 Hz) frequencies of locomotor-related activity, whereas NMDARs are required for consistent output at high frequencies, during which NMDAR blockade disrupts patterned output by increasing non-resetting deletions (breaks in rhythmic bursting lasting an integer of the control period), rather than altering frequency *per se* (Talpalar and Kiehn, 2010).

Glutamatergic signalling is necessary for the synchronous rhythmic bursting generated when inhibitory transmission is blocked by strychnine and picrotoxin (Talpalar *et al.*, 2011). Although NMDAR blockade abolishes disinhibited bursting in rat preparations, it is restored when AMPAR currents are enhanced, indicating this activity is mediated by both NMDARs and non-NMDARs (Bracci, Ballerini and Nistri, 1996), as is bilaterally alternating locomotor-related activity (Beato, Bracci and Nistri, 1997; Bracci, Beato and Nistri, 1998; Nishimaru, Takizawa and Kudo, 2000; Whelan, Bonnot and Donovan, 2000; Gabbay, 2004; Cowley *et al.*, 2005; Talpalar and Kiehn, 2010).

Neuromodulation of spinal locomotor networks

Alterations to motor network output allow animals to adjust their behaviour according to developmental stage, physiological state, and to meet the challenges of different environmental conditions (Grillner, 2006). For instance, changes in flexor-extensor

coordination within locomotor networks underlie transitions from one gait to another, and gait changes allow animals to efficiently vary the speed of their locomotion (Orlovsky, Deliagina and Grillner, 1999; Bellardita and Kiehn, 2015) – compare the movement of a predator evading detection with a predator in chase. Network output may vary in response to either descending commands mediated by synaptic transmission or the modification of cellular or synaptic properties by neuromodulators, which may originate from descending pathways or sources within the spinal cord (Jordan *et al.*, 2008; Hägglund *et al.*, 2010; Miles and Sillar, 2011; Bouvier *et al.*, 2015). Changes in the cellular composition of the network are a further source of flexibility (Talpalar *et al.*, 2013; Bellardita and Kiehn, 2015), but may be secondary to these mechanisms. This section will give an overview of neuromodulation and its complexities in the locomotor circuitry. The actions of the specific neuromodulators considered in later chapters, namely dopamine, adenosine and the *N*-methyl-D-aspartate (NMDA) receptor (NMDAR) co-agonists D-serine and glycine, will then be considered in turn. Many neuromodulatory systems operating within mammalian locomotor circuitry are also important in non-mammalian vertebrates (Dickinson, 2006; Miles and Sillar, 2011); although the focus of this thesis is the mammalian locomotor CPG, reference will be made to other models where appropriate.

The basic motor pattern of a CPG under a given set of conditions is determined by the passive electrical properties of its constituent neurons and by the synaptic connections between them. Neuromodulators adjust both of these features to regulate fast synaptic transmission and information processing within the network (Katz and Frost, 1996; Katz, 1999; Dickinson, 2006; Harris-Warrick, 2011; Miles and Sillar, 2011). In this way, neuromodulation enables a defined population of neurons to produce diverse outputs and modes of adaptive behaviour. Multifarious neuromodulatory influences acting in concert give rise to a vast repertoire of network and behavioural outputs and are thus a source of considerable behavioural flexibility (Harris-Warrick, 2011; Miles and Sillar, 2011).

The actions and interactions of neuromodulators within motor networks are numerous and complex. Individual neuromodulators have diverse effects within the locomotor CPG,

mediated by different cell types and signalling pathways (Harris-Warrick, 2011; Miles and Sillar, 2011). In general, neuromodulators that adjust the frequency of locomotor-related activity act on the premotor rhythm generating circuitry, whereas those that influence the amplitude of network output act directly on motoneurons or last order interneurons; neuromodulators that alter both properties typically act on both motoneurons and premotor interneurons (Miles and Sillar, 2011). Opposing neuromodulatory effects, mediated either by different neuromodulators or by a single substance acting via different signalling pathways or different cell types, may stabilise network output, ensuring coherent motor behaviour (Harris-Warrick, 2011). In addition, neuromodulators may themselves be subject to modulation. Broadly, metamodulation may entail a second-order neuromodulator exerting changes in the availability (release or uptake) of a first-order neuromodulator, or modulation of its effect on a target neuron (Katz, 1999). Metamodulation may be an efficient mechanism for fine-tuning the effects of first-order neuromodulators that have diverse actions within a network.

Neuromodulators acting within spinal motor networks are chemically diverse (Miles and Sillar, 2011). They include biogenic amines such as 5-hydroxytryptamine (5-HT), dopamine (DA), noradrenaline and the trace amines; amino acids such as GABA where it acts at metabotropic $GABA_B$ receptors and glutamate where it acts at metabotropic glutamate receptors (mGluRs); peptides such as somatostatin; the purines adenosine triphosphate (ATP) and adenosine; and the gas nitric oxide. The NMDAR co-agonists D-serine and glycine may be categorised as amino acid neuromodulators: unlike other neuromodulators, they act at an ionotropic receptor and are obligatory for receptor activation; however, they are regulated on a relatively slow timescale compared to glutamate released during synaptic transmission, and their availability may be regulated to tune the intensity of glutamatergic signalling (see below).

Receptor diversity is a further source of complexity within neuromodulatory systems: many neuromodulators bind to multiple receptors, which differ in their agonist affinity and signalling pathways (Dickinson, 2006; Miles and Sillar, 2011). Receptor expression varies between cell types, and activation of a given receptor can have different effects depending on cell type.

Typically, though not exclusively, neuromodulators are agonists of metabotropic, G-protein coupled receptors (GPCRs) (Miles and Sillar, 2011). GPCR activation triggers the dissociation of G-proteins into their G_{α} and $G_{\beta\gamma}$ components intracellularly (Neves, Ram and Iyengar, 2002). Dissociated subunits act as transducers within various isoform-specific signalling pathways and modulate neuronal activity by diverse mechanisms. G_{α} subunit isoforms fall into four classes, $G_{\alpha q}$, $G_{\alpha s}$, G_{i}, $G_{\alpha 12}$, which act via different signalling pathways (Liebmann and Böhmer, 2000). It should also be noted that GPCRs display basal activity independent of agonist binding, a consequence of their structural flexibility (Gether *et al.*, 1997; Kobilka and Deupi, 2007; Neve, 2010). In addition, GPCRs are capable of forming homo- and hetero- oligomers, permitting functional interactions (Bouvier, 2001).

Neuromodulators may derive from cells contained within the spinal cord (intrinsic) or from others that have projections to the spinal cord originating in brain or brainstem structures (extrinsic). Intrinsic sources of neuromodulators include motoneurons and various interneuron populations in the spinal cord that produce acetylcholine, which acts as a neuromodulator within the spinal cord via metabotropic muscarinic receptors (Witts, Zagoraiou and Miles, 2013), and glia, proposed as the principle source of modulatory adenosine in the mammalian spinal cord (Witts, Panetta and Miles, 2012). Extrinsic sources include the hypothalamic A11 region, the principle source of dopamine to the spinal cord, and neurons in the brainstem that synthesise 5-HT and noradrenaline (Jordan *et al.*, 2008; Miles and Sillar, 2011). Modulation by monoamines originating from descending projections has been studied extensively. Dopamine, 5-HT and noradrenaline are secreted within the spinal cord during locomotion and may, like glutamate released from descending pathways, be implicated in the initiation of locomotion in intact animals (Gerin and Privat, 1998; Jordan *et al.*, 2008; Miles and Sillar, 2011). In addition, all are potent modulators of ongoing locomotor-related activity, acting via multiple receptor subtypes and in a cell-type specific manner (Miles and Sillar, 2011).

Dopaminergic modulation

Dopamine exerts modulatory control over diverse processes, including working memory, learning, motivation, and addiction (Girault and Greengard, 2004). In the spinal cord, dopamine modulates locomotor networks to ensure correct expression of motor behaviour (Miles and Sillar, 2011). Microdialysis experiments have shown that dopamine is released within the ventral horn of the mammalian spinal cord during locomotion (Gerin and Privat, 1998). All segments of the mammalian spinal cord receive dopaminergic inputs from the brain (Hökfelt, Phillipson and Goldstein, 1979; Skagerberg and Lindvall, 1985); these originate principally in the hypothalamic A11 region, but other descending pathways are reported (Björklund and Skagerberg, 1979; Commissiong, Gentleman and Neff, 1979; Hökfelt, Phillipson and Goldstein, 1979; Skagerberg and Lindvall, 1985).

Dopamine receptors fall into two families: D_1-like receptors are the D_1 and D_5 subtypes and D_2-like receptors are the D_2, D_3, and D_4 subtypes (Neve, 2010; Pieper, Clerkin and MacFarlane, 2011). Receptors differ in affinity for dopamine, structure, cellular distribution, and G-protein coupling. D_1-like receptors bind dopamine with a lower affinity than D_2-like receptors (Pieper, Clerkin and MacFarlane, 2011). They are encoded by genes that lack introns (Zhou, Li and Civelli, 1992; Sunahara *et al.*, 1990), whereas D_2-like receptors are encoded by genes that contain introns, with alternative splicing of D_2 genes generating long and short isoforms (Giros *et al.*, 1989; Araki *et al.*, 1992). All five dopamine receptor subtypes are expressed in the rodent spinal cord, where they are broadly distributed in both the white and grey matters; however, D_1-like receptors are most strongly expressed in the ventral horn, whereas D_2-like receptors are most strongly expressed in the dorsal horn (Dubois *et al.*, 1986; Fleetwood-Walker, Hope and Mitchell, 1988; Zhu *et al.*, 2007, 2008).

Whereas D_1-like receptors signal through $G_{\alpha q}$ to stimulate phospholipase C and $G_{\alpha s}$ to stimulate adenylyl cyclase, D_2-like receptors signal through $G_{\alpha i}$ to inhibit adenylyl cyclase (Abdel-Majid *et al.*, 1998; Pieper, Clerkin and MacFarlane, 2011). Adenylyl cyclase is a 12-transmembrane-spanning protein that catalyses the synthesis of the second messenger cyclic

adenosine monophosphate (cAMP) and pyrophosphate from adenosine triphosphate (ATP). cAMP activates various proteins including exchange protein activated by cyclic AMP (EPAC) and protein kinase A (PKA). This latter regulates a number of proteins including sodium-dependent ion transporters, various ion channels, cAMP responsive element binding protein 1 (CREB1) and dopamine and cyclic AMP-regulated phosphoprotein of 32 kDa (DARPP-32) (Abdel-Majid *et al.*, 1998; Undieh, 2010).

Studies in which agonists and antagonists of dopamine receptors are applied to *in vitro* spinal cord preparations have demonstrated diverse actions of dopamine within mammalian spinal locomotor networks. Bath applied dopamine or a D_1-like receptor agonist is able to stimulate locomotor-related activity in neonatal rat preparations (Kiehn and Kjaerulff, 1996; Barrière, Mellen and Cazalets, 2004) but not in neonatal mouse preparations (Jiang, Carlin and Brownstone, 1999; Whelan, Bonnot and Donovan, 2000; Sharples *et al.*, 2015); however, dopamine can elicit low-frequency, uncoordinated bursting in ventral root pairs in neonatal mouse preparations (Sharples *et al.*, 2015), and the selective D_1-like receptor agonist SKF 81927 is able to stimulate locomotor activity in intact adult mice subject to spinal cord transection at lower thoracic levels (segments T_9/T_{10}) (Lapointe *et al.*, 2009). Dopamine may also be required in addition to 5-HT and NMDA to elicit locomotor-related activity in isolated spinal cords from older (> 1 wk) mice (Jiang, Carlin and Brownstone, 1999).

When bath-applied during locomotor-related activity induced by NMDA and 5-HT, dopamine stabilises rhythmic bursting, reducing the frequency and increasing the amplitude of bursts in both mouse and rat spinal cord preparations (Jiang, Carlin and Brownstone, 1999; Whelan, Bonnot and Donovan, 2000; Barrière, Mellen and Cazalets, 2004; Madriaga *et al.*, 2004; Humphreys and Whelan, 2012; Sharples *et al.*, 2015). Dopamine also increases the amplitude of flexor activity during ongoing locomotion in the cat (Barbeau and Rossignol, 1991). These effects are mediated by both D_1-like and D_2-like receptors, and selective agonists and antagonists have been used to characterise the contributions of both subfamilies (Madriaga *et al.*, 2004; Humphreys and Whelan, 2012; Sharples *et al.*, 2015). Although D_1-like receptors

typically have excitatory effects, they are reported not to increase the frequency of ongoing locomotor-related activity in mice (Humphreys and Whelan, 2012; Sharples *et al.*, 2015), but may instead enhance the stability of rhythmic bursting (Sharples *et al.*, 2015). By contrast, D_2-like receptors are reported to mediate the reduction in burst frequency produced by bath-applied dopamine (Humphreys and Whelan, 2012; Sharples *et al.*, 2015). The selective agonist quinpirole has been reported both to increase (Sharples *et al.*, 2015) and decrease (Humphreys and Whelan, 2012) the stability of bursting.

The effects of dopamine on network activity likely reflect multiple effects on diverse cell types mediated by both D_1-like and D_2-like receptors; accordingly, dopamine has been shown to modulate both synaptic strength and intrinsic properties of motoneurons and interneurons in mice. Dopamine depolarises motoneurons and interneurons projecting into the ventrolateral funiculus (Han *et al.*, 2007). When applied to spinal motoneurons, dopamine also decreases both first-spike latency by modulation of 4-AP-sensitive, low-threshold, fast inactivating K^+ currents (I_A) and the medium-duration afterhyperpolarisation (mAHP) by modulation of apamin-sensitive small-conductance Ca^{2+}-activated K^+ channel (SK_{Ca}) currents. Together these mechanisms increase the frequency-current slope, thereby enhancing motoneuronal excitability. Dopamine also enhances synaptic inputs onto motoneurons by modulation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor currents (Han *et al.*, 2007). Activation of D_1-like but not D_2-like receptors increases the open probability and open duration of AMPA receptor channels in a PKA-dependent manner, but does not alter insertion of receptors (Han and Whelan, 2009). By contrast, D_2-like but not D_1-like receptors are proposed to mediate inhibition of an excitatory recurrent collateral pathway onto the CPG (Humphreys and Whelan, 2012). In addition, dopamine modulates excitatory $Hb9^+$ interneurons to facilitate rhythmic oscillations in membrane potential, a characteristic that may be important in network rhythmogenesis (Han *et al.*, 2007). Information about the effects of dopamine on other populations of ventral horn interneurons is currently lacking. Dopamine modulates synaptic inputs to motoneurons from sensory afferents (Carp and Anderson, 1982;

Clemens, 2004; Barrière *et al.*, 2008) and may modulate synaptic transmission between motoneurons and Renshaw cells (Maitra *et al.*, 1993; Seth *et al.*, 1993).

Thus, dopamine has multiple targets within the spinal cord and acts via diverse mechanisms, in some cases inhibiting and in others enhancing neuronal activity. It is likely that the full range of modulatory mechanisms mediated by dopamine receptors remains to be elucidated. Furthermore, modulation by dopamine may itself be regulated by other modulators. For instance, D_1-like dopamine receptors are proposed to interact with A_1 adenosine receptors in mouse locomotor networks (see below) (Acevedo *et al.*, 2016). These considerations must be taken into account when the effects of dopamine or selective dopamine-receptor agonists and antagonists are evaluated at the network or behavioural level (Sharples *et al.*, 2014).

Adenosinergic modulation

The purines ATP and its derivative adenosine are involved in a myriad of biological processes, most notably energy transfer. In the nervous system they also function as neuromodulators, and are involved in diverse processes in health and disease, including sleep homeostasis, memory, and the regulation of mood (Cunha, 2001; Fredholm *et al.*, 2005; Burnstock, 2007).

There are four adenosine receptor subtypes, designated A_1, A_{2A}, A_{2B} and A_3, which differ in affinity for adenosine, structure, cellular distribution, and G-protein coupling. A_1 and A_{2A} receptors have the highest affinity for adenosine and are the best characterised (Cunha, 2001). Both A_1 and A_{2A} receptors are expressed throughout the spinal cord (Reppert *et al.*, 1991; Deuchars, Brooke and Deuchars, 2001; Paterniti *et al.*, 2011). A_1 receptors are tightly coupled to the $G_{\alpha i}$ pathway, which mediates inhibition of adenylyl cyclase and reduced production of cAMP. However, A_1 receptors are also coupled to other G proteins and act via adenylyl cyclase-independent pathways to inhibit spontaneous and evoked neurotransmitter release. Presynaptic inhibition may entail the adenylyl cyclase-independent inhibition of N-type Ca^{2+} channels (Ribeiro, 1995; Cunha, 2001). A_{2A} receptors are primarily coupled to $G_{\alpha s}$, but also to $G_{\alpha i}$ and $G_{\alpha 12}$, and have diverse effects mediated by PKA, PKC, N-type and P-type calcium

channels (Cunha, 2001). In many systems the inhibitory actions of A_1 receptors are countered by the facilitatory actions of A_{2A} receptors in a concentration-dependent manner (Cunha, 2001).

Extracellular adenosine is largely formed from ATP released from neurons or glia, although cells also release adenosine itself (Cunha, 2001; Klyuch, Dale and Wall, 2012; Wall and Dale, 2013). An array of ectonucleotidases catalyses ATP to adenosine in the extracellular space, and the dynamic regulation of the availability of ATP and adenosine by this mechanism is an important property of purinergic modulation in many systems (Wall and Dale, 2008).

Homeostatic, activity-dependent production of adenosine has been detected in several systems, including the mammalian brain and brainstem, and in the spinal cord of *Xenopus* tadpoles, and is proposed as a mechanism to avert fatigue and excitotoxicity (Wall and Dale, 2008). In some cases, this is coupled to the degradation of ATP. Adenosine derived from ATP consumed by neurons as a source of energy during activity is proposed to be released directly via neuronal equilibrative nucleoside transporters. Autocrine inhibition of activity via A_1 receptors is proposed as a homeostatic mechanism to prevent metabolic exhaustion (Cunha, 2001; Fredholm *et al.*, 2005). However, this mechanism does not appear to operate in some systems, including murine locomotor networks, in which the source of adenosine is ATP released into the extracellular space from either neurons or glia (Wall and Dale, 2008; Witts, Panetta and Miles, 2012; Carlsen and Perrier, 2014). For instance, ATP released during network activity in the spinal cord of *Xenopus* tadpoles is excitatory and sustains locomotor activity; the excitatory effect of ATP is counterbalanced by the inhibitory effect of adenosine, which accumulates following ATP release (Dale and Gilday, 1996). In several preparations, production of modulatory adenosine follows release of ATP from astrocytes (Panatier et al. 2011; Pascual et al. 2005; Serrano et al. 2006; Carlsen and Perrier 2014).

The role of adenosine signalling in the modulation of mammalian locomotor behaviour has been addressed by injection of intact animals with receptor antagonists, which typically have an excitatory effect. This effect has been proposed to be mediated by either A_1 (Snyder *et al.*, 1981; Goldberg, Prada and Katz, 1985), A_{2A} (Svenningsson, Nomikos and Fredholm, 1995;

Ledent *et al.*, 1997; Svenningsson *et al.*, 1997; El Yacoubi *et al.*, 2000; Lindskog *et al.*, 2002) or both A_1 and A_{2A} receptors (Karcz-Kubicha *et al.*, 2003; Antoniou *et al.*, 2005; Kuzmin *et al.*, 2006), indicating a role for both receptor subtypes. However, interpretation of studies in which antagonists are chronically applied is complicated by the reported acquisition of receptor tolerance, to which A_1 receptors are particularly susceptible (Karcz-Kubicha *et al.*, 2003). It is also difficult to draw conclusions about the locus or loci of adenosinergic modulation – it is likely that adenosine regulates locomotion in multiple regions of the nervous system involved in motor control, including the basal ganglia (Popoli *et al.*, 1996) and ventral spinal cord (Witts, Panetta and Miles, 2012; Acevedo *et al.*, 2016). It is conceivable that the effects of adenosine blockade in one network mask its effects in another.

Experiments in which spinal cord preparations were exposed to adenosine receptor agonists and antagonists have addressed the role of adenosine specifically within spinal locomotor networks. During locomotor-related activity induced by bath application of DA, NMDA and 5-HT in neonatal mouse preparations, blockade of A_1 receptors results in increased burst frequency (Witts, Panetta and Miles, 2012; Acevedo *et al.*, 2016), with no effect on amplitude, whereas blockade of A2A receptors has no effect (Witts, Panetta and Miles, 2012; Acevedo *et al.*, 2016). Conversely, bath application of adenosine results in reduced burst frequency in a dose-dependent manner, again with no effect on amplitude (Witts, Panetta and Miles, 2012; Acevedo *et al.*, 2016). The frequency effects are associated with changes in burst and cycle duration, implying no change in duty cycle (Acevedo *et al.*, 2016). Bath application of ATP has a similar effect to adenosine in reducing burst frequency (Witts, Panetta and Miles, 2012); however, both blockade of adenosine receptors and application of ATP have no effect in the presence of an ectonucleotidases inhibitor, indicating that endogenous adenosine is derived from ATP released into the extracellular space, that ATP itself does not modulate locomotor-related activity, and that both endogenous and exogenous ATP are efficiently degraded to adenosine within the spinal cord (Witts, Panetta and Miles, 2012). This ATP appears to derive from glia since adenosine receptor blockade has no effect on locomotor-related activity

following pharmacological ablation of glia by treatment with gliotoxins (see below) (Witts, Panetta and Miles, 2012).

Modulation of the frequency but not the amplitude of locomotor-related activity by adenosine suggests that it acts on the premotor circuitry rather than on motoneurons (Miles and Sillar, 2011). Consistent with this hypothesis, adenosine does not modulate disinhibited activity mediated by motoneurons and excitatory components of the locomotor circuitry alone (Witts, Panetta and Miles, 2012), implying that adenosine exerts its depressive effects via inhibitory interneurons, such as the V1 population (Gosgnach *et al.*, 2006).

It has been proposed that A_1-adenosine receptors interact with D_1-like DA receptors in the spinal locomotor circuitry (Acevedo *et al.*, 2016), as is observed in the basal ganglia (Popoli *et al.*, 1996). DA stabilises locomotor-related activity in isolated spinal cord preparations (Jiang, Carlin and Brownstone, 1999; Whelan, Bonnot and Donovan, 2000; Barrière, Mellen and Cazalets, 2004; Madriaga *et al.*, 2004; Humphreys and Whelan, 2012; Sharples *et al.*, 2015), but is presumed to be absent unless exogenously applied, as descending inputs that release DA onto the spinal cord are severed in *in vitro* preparations (Gerin, Becquet and Privat, 1995; Gerin and Privat, 1998). When dopamine is absent or when D_1-like receptors are selectively blocked, A_1 blockade no longer alters the frequency of locomotor-related bursting (Acevedo *et al.*, 2016). A_1 blockade is similarly ineffective when the $G_{\alpha s}$ signalling pathway through which D_1-like receptor signal is inhibited at the level of PKA. However, when forskolin is applied to activate adenylyl cyclase in a receptor-independent manner, the effect of A_1 blockade on the frequency of locomotor-related bursting is restored. Together, these data indicate that adenosine acting through A_1 receptors may function as a metamodulator to regulate the activity of locomotor networks by inhibition of D_1-like receptor signalling. This may occur through direct inhibition of adenylyl cyclase mediated by the $G_{\alpha i}$ pathway to which A_1 receptors are coupled.

The effects of bath-applied adenosine on neurons in acute slices from postnatal mice have also been assessed. In the absence of dopamine, adenosine induces a hyperpolarising

current in ventral horn interneurons, likely by facilitating leak K^+ currents, and acts at A_1 receptors to reduce both inhibitory and excitatory spontaneous postsynaptic currents (IPSCs; EPSCs) by both pre- and post-synaptic mechanisms (Witts, Nascimento and Miles, 2015). In addition, adenosine reduces the amplitude of evoked EPSCs onto interneurons by a presynaptic mechanism (Carlsen and Perrier, 2014). ATP has a similar effect, but only after a delay, consistent with its hydrolysis to adenosine by ectonucleotidases (Carlsen and Perrier, 2014). In motoneurons, by contrast, adenosine induces a depolarising current and reduces spontaneous postsynaptic currents by a presynaptic mechanism (Witts, Nascimento and Miles, 2015). Thus, adenosine has disparate effects on different populations of neurons in the ventral horn. A_1-receptor blockade alone has no effect on spontaneous postsynaptic currents, either because there is insufficient adenosine present in acute slices for the tonic activation of these receptors, or because adenosine at the concentrations at which it exists in the spinal cord requires concurrent activation of D_1-like receptors to exert modulatory effects(Acevedo *et al.*, 2016).

Stimulation of Ca^{2+} signalling in glia by selective activation of protease-activated receptor-1 (PAR1), an endogenous GPCR preferentially expressed by astrocytes, also reduces the amplitude of evoked EPSCs by a presynaptic mechanism in a manner depending on extracellular ectonucleotidase activity and A_1 receptor activation (Carlsen and Perrier, 2014). Conversely, chelation of Ca^{2+} in astrocytes enhances evoked EPSCs; however, it has not been shown directly that this effect is mediated by A_1 receptors. These data indicate that ventral horn astrocytes in slice preparations release ATP in a Ca^{2+}-dependent manner, and that glial cell-derived ATP is degraded to adenosine, which acts at A_1 receptors to inhibit excitatory transmission by a DA-independent presynaptic mechanism. These results may be consistent with inhibition of network activity by adenosine derived from glia in the presence of DA, but it will be important to determine whether adenosine modulates evoked EPSCs by the same mechanism in the presence and absence of DA.

In spinal cord preparations from postnatal rats, adenosine does not modulate the frequency of locomotor-related bursting evoked by 5-HT and NMDA in the absence of dopamine, and A_1 blockade has no effect on drug-induced or electrically stimulated locomotor-related activity (Taccola *et al.*, 2012). These data may indicate that adenosinergic modulation of the frequency of locomotor-related activity requires DA in rats, as in mice; however, the effects of adenosine on locomotor-related activity in the presence of DA in rats are not known, preventing direct comparison with results from mouse preparations. Furthermore, adenosine is reported to have effects in isolated rat spinal cords in the absence of DA that have not been reported in mouse preparations. In rats, adenosine enhances burst amplitude during locomotor-related activity and depresses the frequency of disinhibited bursting in an A_1-dependent manner (Taccola *et al.*, 2012), effects not observed in mouse preparations (Witts, Panetta and Miles, 2012; Acevedo *et al.*, 2016). In addition, the duration of bouts of locomotor-related activity induced by dorsal-root stimulation is reduced by bath-applied adenosine (Taccola *et al.*, 2012), and reflex potentials produced by dorsal root stimulation are depressed by adenosine released under hypoxic conditions (Otsuguro, Wada and Ito, 2011). Both of these mechanisms are mediated by A_1 receptors. Although A_1 blockade prevents the depression of disinhibited and electrically evoked bursting by bath-applied adenosine, it has no effect alone. It is possible that high concentrations of adenosine, such as those used in bath applications or produced during hypoxia, are able to modulate neural activity independently of DA, as in acute slices from mice, whereas low levels of endogenous adenosine require DA to be present.

Xenopus tadpoles provide an example of purinergic signalling during motor behaviour in a non-mammalian vertebrate. ATP released within the spinal cord during episodes of swimming serves to excite the locomotor CPG and extend the duration of swimming bouts (Dale and Gilday, 1996; Dale, 1998). As swimming progresses, ATP is hydrolysed to adenosine, which activates A_1 receptors to drive down network activity (Brown and Dale, 2000). This mechanism may exist to prevent metabolic exhaustion and excitotoxicity (Wall and Dale, 2008).

It may be instructive to compare the effects of adenosine on spinal locomotor networks with its effects on rhythmically active respiratory networks in the mammalian brainstem. As in the spinal cord of *Xenopus* tadpoles, adenosinergic inhibition of respiratory networks is reported to follow ATP-mediated excitation in *in vitro* preparations from neonatal mice (Funk *et al.*, 1997) and rats (Miles *et al.*, 2002; Lorier *et al.*, 2007), and in anaesthetised adult rats (Funk *et al.*, 1997). By contrast, ATP does not modulate ongoing locomotor network activity in mice (Witts, Panetta and Miles, 2012). It may, however, be relevant that the rat respiratory CPG is reported to be less sensitive to adenosinergic modulation than mouse networks because of differences in ectonucleotidase expression (Zwicker *et al.*, 2011). Adenosine also exerts a tonic depression of network activity in cats (Schmidt, Bellingham and Richter, 1995), rats (Kawai *et al.*, 1995; Huxtable *et al.*, 2009) and mice (Mironov, Langohr and Richter, 1999); this declines during development in rats (Huxtable *et al.*, 2009).

Modulation of NMDARs via the co-agonist binding site

Unlike non-NMDARs, canonical GluN1/GluN2 subunit-containing NMDARs require the binding of a co-agonist in addition to the binding of glutamate for their activation (Johnson and Ascher, 1987; Kleckner and Dingledine, 1988; Clements and Westbrook, 1991; Paoletti, Bellone and Zhou, 2013). In the brain, the endogenous co-agonist may be either glycine or D-serine, with one or the other dominating at a given synapse, such that its selective depletion results in a reduction in NMDAR current (Mothet *et al.*, 2000; Kalbaugh, Zhang and Diamond, 2009; Li *et al.*, 2009, 2013, Henneberger *et al.*, 2010, 2013; Papouin *et al.*, 2012; Le Bail *et al.*, 2015; Meunier *et al.*, 2016). The identity of the dominant co-agonist is proposed to vary during development (Le Bail *et al.*, 2015), with receptor subunit composition (Kalbaugh, Zhang and Diamond, 2009; Papouin *et al.*, 2012; Le Bail *et al.*, 2015), between synaptic and extrasynaptic receptor populations (Papouin *et al.*, 2012), and in an activity-dependent manner (Kalbaugh, Zhang and Diamond, 2009; Li *et al.*, 2009, 2013). In the spinal cord, co-agonist identity has hitherto received little attention.

Occupancy of the co-agonist binding site is proposed to be regulated in an activity-dependent manner, in this way modulating NMDAR activity (Kalbaugh, Zhang and Diamond, 2009; Li *et al.*, 2009, 2013). An unsaturated co-agonist binding site could permit the regulation of glutamatergic signalling by adjustments to the availability of the co-agonist (Berger, Dieudonné and Ascher, 1998; Li *et al.*, 2009, 2013). The co-agonist binding site is reported to be unsaturated in some preparations (Berger, Dieudonné and Ascher, 1998; Kalbaugh, Zhang and Diamond, 2009; Li *et al.*, 2009, 2013; Le Bail *et al.*, 2015), but saturated in others (Kemp *et al.*, 1988; Li *et al.*, 2009; Shigetomi *et al.*, 2013), and activity-dependent changes in occupancy of the co-agonist binding site have been reported in the brain (Kalbaugh, Zhang and Diamond, 2009; Li *et al.*, 2009, 2013).

The co-agonist binding site is unsaturated in *in vitro* forelimb locomotor circuit preparations of pre- and postnatal rats (Shimomura *et al.*, 2015), in motoneurons in a hemisected spinal cord preparation from postnatal rats (Brugger *et al.*, 1990), and in the locomotor network of *Xenopus* tadpoles during swimming; in this latter preparation episodes of swimming are prolonged by exogenous application of either of the two NMDAR co-agonists D-serine or glycine (Issberner and Sillar, 2007). Thus, either increases or decreases in co-agonist availability could modulate excitatory transmission within the spinal cord locomotor circuitry to modulate behaviour.

The availability of both D-serine and glycine at excitatory synapses is determined by metabolic processes in both neurons and astrocytes, and entails functional coupling between these two cell types (Le Bail *et al.*, 2015). Evidence that co-agonist availability may be regulated in an activity-dependent manner (Kalbaugh, Zhang and Diamond, 2009; Li *et al.*, 2009, 2013, Henneberger *et al.*, 2010, 2013) implies information processing by astrocytes.

Glycine

Glycine is released at inhibitory synapses by vesicular exocytosis and may spill over into excitatory synapses to bind to NMDARs (Ahmadi *et al.*, 2003; Bowery and Smart, 2006). Alternatively, it may be released at excitatory synapses from neurons by Asc-1 (Rosenberg *et*

al., 2013), or from astrocytes by reverse operation of glycine transporter-1 (GlyT1) triggered by increase in intracellular Na^+, for instance following activation of astrocytic AMPA receptors (Attwell, Barbour and Szatkowski, 1993; Roux and Supplisson, 2000; Marcaggi and Attwell, 2004; Li *et al.*, 2009; Shibasaki *et al.*, 2016).

Levels of extracellular glycine are regulated by forward operation of GlyT1 and glycine transporter-2 (GlyT2), both of which are highly expressed in the spinal cord and brainstem, where glycinergic transmission is prevalent (Eulenburg *et al.*, 2005). GlyT2 is exclusively expressed on the terminals of glycinergic interneurons (Zafra, Aragón, *et al.*, 1995; Jursky and Nelson, 1996), where it removes glycine from inhibitory and possibly also excitatory synapses (Danglot *et al.*, 2004). By contrast, GlyT1 regulates glycine concentrations at glutamatergic synapses and is strongly expressed by astrocytes in the grey matter of the spinal cord and brainstem (Zafra, Gomeza, *et al.*, 1995; Cubelos, Giménez and Zafra, 2005), although neuronal expression at lower levels has also been reported (Cubelos, Giménez and Zafra, 2005).

GlyT1 is a potent regulator of extracellular glycine capable of maintaining local concentration considerably below its concentration in the cerebrospinal fluid or a perfusate (Supplisson and Bergman, 1997; Berger, Dieudonné and Ascher, 1998; Bergeron *et al.*, 1998). Previously, glycine was found to be ineffective at potentiating NMDAR currents in brainstem preparations when bath applied at concentrations under 100 µM, orders of magnitude above its EC_{50}, consistent with very tight regulation of glycine in the extracellular space by GlyT1 (Berger, Dieudonné and Ascher, 1998).

In the spinal cord, spillover of synaptically released glycine is reported to facilitate NMDAR currents in the dorsal horn of postnatal rats (Ahmadi *et al.*, 2003). In pre- and postnatal rat forelimb locomotor circuits, activity is facilitated by blockade of GlyT1 (Shimomura *et al.*, 2015), suggesting a role for glycine transport in the regulation of excitatory transmission. Similarly, blockade of GlyT1 enhances NMDAR-dependent swimming in *Xenopus* tadpoles (Issberner and Sillar, 2007). However, it is unknown whether GlyT1 regulates glycine concentrations at

excitatory synapses in the spinal cord to determine rhythmic locomotor-related activity in mammals.

D-serine

The D-serine synthesis pathway is distributed between both neurons and astrocytes (Wolosker, 2011; Ehmsen *et al.*, 2013). Synthesis of D-serine from L-serine by serine racemase occurs in both neurons and astrocytes (Wolosker, 2011). However, the first reaction in the biosynthesis of L-serine is catalysed by 3-phosphoglycerate dehydrogenase, which is exclusive to astrocytes (Ehmsen *et al.*, 2013). D-serine is degraded by D-amino acid oxidase, which may be expressed by both neurons and glia, but is enriched in spinal cord astrocytes (Verrall *et al.*, 2007; Sasabe *et al.*, 2014). The availability of D-serine is therefore largely determined by the opposing actions of serine racemase, the enzyme that synthesises D-serine from L-serine, and D-amino acid oxidase (DAAO), the enzyme that degrades D-serine *in vivo* (Wolosker, Blackshaw and Snyder, 1999; Sasabe *et al.*, 2014); however, see (Crow, Marecki and Thompson, 2012) for a review of the complexities of D-serine metabolism.

D-serine may be released from astrocytes via ASCT2 (Verrall *et al.*, 2010) or by Ca^{2+}-dependent exocytosis (Yang *et al.*, 2003; Mothet *et al.*, 2005; Henneberger *et al.*, 2010). It is secreted from neurons via alanine–serine–cysteine transporter-1 (Asc-1) (Rosenberg *et al.*, 2013) or ASCT2 (Verrall *et al.*, 2010). Release from neurons may occur upon depolarisation by a Ca^{2+}-independent mechanism (Rosenberg *et al.*, 2010).

Concentrations of D-serine in the mammalian spinal cord are considerably lower than those of glycine, and considerably lower than those detected in the brain (Schell *et al.*, 1997; Sasabe *et al.*, 2007; Miyoshi *et al.*, 2012; Thompson *et al.*, 2012). Accordingly, glycine was proposed as the exclusive co-agonist of NMDARs in the spinal cord (Schell *et al.*, 1997). However, selective degradation of D-serine reduces pain symptoms in rats subjected to nerve ligation (Lefèvre *et al.*, 2015; Moon *et al.*, 2015), and levels of both D-serine and serine racemase are higher in the spinal cord following surgery (Lefèvre *et al.*, 2015; Moon *et al.*, 2015).

Aberrant D-serine metabolism in the spinal cord is implicated in the pathogenesis of amyotrophic lateral sclerosis (ALS) (Sasabe *et al.*, 2007; Mitchell *et al.*, 2010; Paul and de Belleroche, 2014). Amyotrophic lateral sclerosis (ALS) is the most common adult neuromuscular disease, with a lifetime risk of 1 in 500. It is characterised by loss of motoneurons in the spinal cord, brainstem and cerebral cortex, with substantial dysfunction in associated glia (Bruijn, Miller and Cleveland, 2004; Robberecht and Philips, 2013). These cellular effects are associated with progressive muscle weakness, paralysis and muscle atrophy. Disease progression is rapid and death, usually from respiratory failure, typically occurs within 30 months of symptom onset (Gordon, 2013).

Levels of D-serine were found to be elevated in the spinal cords of both a small sample of ALS patients and in the G93A SOD1 mouse model of ALS (Sasabe *et al.*, 2007, 2012). Consistent with these reports, a mutation (R199W) in DAAO that abolishes enzyme function has been identified in a small subset of familial ALS (FALS) cases (Mitchell *et al.*, 2010) and DAO is downregulated in the mouse model (Sasabe *et al.*, 2012). In addition, the number of cells, predominantly glia, expressing serine racemase is increased in the SOD1 mouse model, possibly as a result of cell stress (Sasabe *et al.*, 2007; Paul and de Belleroche, 2012). By contrast, serine racemase knockout in the G93A SOD1 mouse model accelerates disease onset, but slows disease progression (Thompson *et al.*, 2012).

Elevated D-serine in mouse models of ALS is proposed to mediate excitotoxicity in motoneurons in an NMDAR-dependent manner, implying the co-agonist binding site is unsaturated in healthy animals, although the requirement of NMDARs for D-serine toxicity has been tested only in primary cultures (Sasabe *et al.*, 2007, 2012; Paul *et al.*, 2014). Thus, further study of the regulation of NMDARs via the co-agonist binding site will provide important information required to understand the pathogenesis of ALS.

Information processing by astrocytes

Glia are a class of cell within the central and peripheral nervous systems represented by macroglia, which include astrocytes, oligodendrocytes and Schwann cells, and microglia. Astrocytes, like the other cell-types, are traditionally considered to have a merely passive, supportive function within neural networks, with well-established roles in, *inter alia,* ion homeostasis and the synthesis and clearance of neurotransmitters (Verkhratsky and Butt, 2013). However, substantial evidence now indicates that astrocytes dynamically regulate neuronal excitability and synaptic strength by the release of so-called gliotransmitters, in this way acting as the third partner in a tripartite synapse with pre- and postsynaptic neuronal elements (Araque *et al.*, 1999, 2014; Haydon and Nedergaard, 2015; Bazargani and Attwell, 2016). In its original formulation, the tripartite-synapse model entails the binding of a neurotransmitter released during synaptic activity to an astrocytic $G_{\alpha q}$-linked GPCR; the production of inositol trisphosphate (IP_3) and the release of Ca^{2+} from stores within the astrocyte mediated by IP_3 receptors; Ca^{2+}-dependent release of a gliotransmitter such as glutamate (Parpura *et al.*, 1994; Pasti *et al.*, 1997; Bezzi *et al.*, 1998), ATP (Newman, 2001; Pryazhnikov and Khiroug, 2008) or D-serine (Mothet et al., 2005) by vesicular exocytosis or via ion channels; and finally the activation of metabotropic or ionotropic receptors on either the pre- or postsynaptic element of the same or a different synapse (Araque *et al.*, 1999; Hamilton and Attwell, 2010). Although signalling in this manner has been denoted “gliotransmission”, this is arguably a misnomer: modulation of neuronal activity by substances derived from glia occurs on a variable timescale, but owing to its dependency on metabotropic receptors, is necessarily slower than acute transmission mediated by ionotropic receptors (Agulhon *et al.*, 2012; Araque *et al.*, 2014). “Gliomodulation” has therefore been proposed as a more appropriate term to distinguish bidirectional signalling between neurons and glia (Agulhon *et al.*, 2012), but is not widely used.

The steps in the pathway described above have been deduced largely from Ca^{2+} imaging of astrocytes and electrophysiological recordings from neurons, and are supported by numerous

studies (Haydon and Nedergaard, 2015). However, the extent to which gliotransmission is important for the operation of neural networks and in the production of behaviour in healthy animals remains controversial (Hamilton and Attwell, 2010; Agulhon *et al.*, 2012; Nedergaard and Verkhratsky, 2012; Sloan *et al.*, 2014; Bazargani and Attwell, 2016). The coding of information by astrocytes in the form of Ca^{2+} fluctuations, which vary in kinetics and subcellular localisation, is poorly understood, and the physiological relevance of experimental manipulations used to investigate Ca^{2+} signalling is disputed (Nedergaard and Verkhratsky, 2012; Rusakov, 2015). Furthermore, several experiments indicate mechanisms of signalling between astrocytes and neurons that depart from the model as originally described. Importantly, clear evidence is lacking that gliotransmission is important in the generation of behaviours is lacking.

Ca^{2+} signalling in astrocytes has been extensively studied by imaging cells expressing a genetically encoded Ca^{2+} indicator or loaded with Ca^{2+}-sensitive dyes by means of a patch pipette. Astrocytes display intracellular Ca^{2+} elevations in response to neuronal activity in both neonatal and adult rodents *in vitro* and *in vivo* (Araque *et al.*, 2014; Bazargani and Attwell, 2016; Shigetomi, Patel and Khakh, 2016). Ca^{2+} signalling is observed in response to sensory stimulation in the brain and dorsal horn of the spinal cord (Cirillo *et al.*, 2012; Sekiguchi *et al.*, 2016), and during locomotion in the cortex (Dombeck *et al.*, 2007). Although desirable, Ca^{2+} imaging has not yet been performed in the ventral horn during locomotion *in vivo*, and is likely to be challenging because of the difficulty of accessing ventral horn tissue. However, preliminary studies have reported rhythmic Ca^{2+} fluctuations in putative astrocytes during pharmacologically induced locomotor-related activity in *in vitro* hemicord preparations (Chub, Liu and O'Donovan, 2012). Furthermore, Ca^{2+} fluctuations were selectively blocked during inhibition of metabotropic glutamate receptor 1 (mGluR1) during ventral root stimulation in disinhibited preparations, implying a role for that receptor in neuron-glia signalling in the spinal cord, as reported in the brain (Chub and O'Donovan, 2011).

Information about the dynamics of Ca^{2+} signalling in astrocytes has been provided for the most part by studies in the cortex, hippocampus and cerebellum. Astrocytes are acutely sensitive to neuronal activity, and respond to basal synaptic activity stimulated by a single action potential (Panatier *et al.*, 2011). Ca^{2+} elevations in astrocytes vary in rise and decay times, amplitude, frequency and spatial diffusion (Shigetomi *et al.*, 2008; Rusakov, 2015; Shigetomi, Patel and Khakh, 2016). These properties are related to patterns of synaptic activity, consistent with astrocytic information processing; however, spontaneous Ca^{2+} fluctuations also occur (Parri, Gould and Crunelli, 2001; Di Castro *et al.*, 2011; Panatier *et al.*, 2011; Srinivasan *et al.*, 2015). Although they appear to have a stellate morphology under some imaging conditions, such as when stained with antibodies against the intermediate filament protein GFAP, astrocytes are in fact spongiform, with intricate folds of membrane, sometimes referred to as fine processes, surrounding a soma and larger processes (Rusakov, 2015). The conditions for Ca^{2+} fluxes within and between these astrocytic compartments are yet to be elucidated (Rusakov, 2015). In the soma, Ca^{2+} elevations are in most cases IP_3-receptor dependent, although Ca^{2+} release mediated by ryanodine receptors has also been reported (Hua *et al.*, 2004; Pankratov and Lalo, 2015; Bazargani and Attwell, 2016). By contrast, Ca^{2+} elevations observable under two-photon excitation microscopy in the fine processes persist in mice lacking the type 2 IP_3 receptor (IP_3R2), which is preferentially expressed by astrocytes (Srinivasan *et al.*, 2015; Rungta *et al.*, 2016), and are instead dependent on Ca^{2+} influx from the extracellular medium, mediated by channels that are yet to be identified. These may include NMDA and AMPA receptors (Lalo *et al.*, 2006) and transient receptor potential cation channel, subfamily A, member 1 (TRPA1), which has been implicated in setting basal Ca^{2+} levels in astrocytes (Shigetomi *et al.*, 2008, 2012), although it does not mediate spontaneous Ca^{2+} elevations in the adult hippocampus (Rungta *et al.*, 2016). Like those in the soma, Ca^{2+} elevations in the fine processes are also enhanced by sensory stimulation and by activation of $G_{\alpha q}$-linked GPCRs, although these act by an IP_3R2-independent pathway (Srinivasan *et al.*, 2015). Future investigations of Ca^{2+} signalling in the spinal cord would ideally consider the

relationship between astrocytic Ca^{2+} dynamics and activity in adjacent neurons, as well as differences in Ca^{2+} signalling between astrocytic compartments.

The role of Ca^{2+} signalling in astrocytic information processing and release of gliotransmitters is supported by experiments in which astrocytic Ca^{2+} elevations are either induced or blocked, or astrocytic function is otherwise impaired. Release of gliotransmitters in these experiments is typically inferred from changes in neuronal activity (Haydon and Nedergaard, 2015), although in some studies changes in the composition of the extracellular medium are directly measured, or behaviour is assessed.

A range of techniques exists for the experimental induction of astrocytic Ca^{2+} elevations, including ultraviolet (UV) photolysis of caged Ca^{2+} or IP_3 introduced via a patch pipette (Hua *et al.*, 2004; Wang *et al.*, 2013; Martin *et al.*, 2015), depolarisation of the astrocyte under whole-cell patch-clamp conditions (Kang *et al.*, 1998; Jourdain *et al.*, 2007), mechanical stimulation (Hua *et al.*, 2004; Lee *et al.*, 2015), activation of endogenous or transgenically expressed GPCRs (Rae and Irving, 2004; Shigetomi *et al.*, 2008; Agulhon, Fiacco and McCarthy, 2010; Agulhon *et al.*, 2013), and activation of transgenically expressed channelrhodopsins (Gourine *et al.*, 2010; Li *et al.*, 2012; Beppu *et al.*, 2014). The extent to which these techniques replicate endogenous Ca^{2+} signalling is disputed (Nedergaard and Verkhratsky, 2012). For instance, although UV photolysis of caged Ca^{2+} can raise cytosolic Ca^{2+} within the physiological range (Parpura and Haydon, 2000), it may not replicate the kinetics or subcellular localisation of Ca^{2+} release elicited by neural activity. Interestingly, some techniques fail to elicit gliotransmitter release in preparations where other techniques are effective (Shigetomi *et al.*, 2008; Wang *et al.*, 2013). Activation of either PAR1 or the purinergic receptor $P2Y_1$, both endogenous astrocytic $G_{\alpha q}$-linked GPCRs, elicits Ca^{2+} elevations of similar amplitude in the somas of astrocytes in the CA1 region of the hippocampus, but only PAR1 activation stimulates gliotransmitter release. The cause of this discrepancy is unknown, but could, for instance, be related to differences in the subcellular localisation of the receptors in relation to Ca^{2+} stores and sites of gliotransmitter release (Bazargani and Attwell, 2016).

Caution is therefore necessary when interpreting experiments in which astrocytic Ca^{2+} signalling is experimentally induced, and ideally astrocytes would be stimulated by more than one technique. Although stimulation of neuronal activity has been shown to evoke astrocytic Ca^{2+} signalling and concomitant release of gliotransmitters (Navarrete et al., 2012; Martín *et al.*, 2015), this is not possible under all experimental conditions. In general, the usefulness of the techniques currently available to stimulate astrocytic Ca^{2+} signalling varies between preparations: for instance, dialysis of astrocytes with caged Ca^{2+} or IP_3 via a patch pipette may be possible in slices, but not in preparations that do not allow access to astrocytes via a patch pipette, such as isolated spinal cord preparations used to study locomotor-related activity *in vitro*.

In acute spinal cord slices from postnatal mice, experimental activation of Ca^{2+} signalling in ventral horn astrocytes reduces the amplitude of evoked EPSCs in neighbouring interneurons by a presynaptic mechanism (Carlsen and Perrier, 2014). This effect is sensitive to inhibitors of A_1 receptors and ectonucleotidases, suggesting astrocytic release of ATP, hydrolysis of ATP to adenosine and activation of presynaptic A_1 receptors. The effects of stimulating spinal cord astrocytes on IPSCs in neighbouring neurons and on network output during locomotor-like activity *in vitro* remain to be determined. The latter may replicate the previously reported inhibitory effects of bath applied adenosine; however, it may be expected that astrocytes match the multiplicity of genetically and physiologically distinct neuronal cell types in the spinal cord (Kiehn and Butt, 2003), and secrete multiple gliotransmitters.

Astrocytic Ca^{2+} signalling may be inhibited by various strategies, including loading astrocytes with a Ca^{2+} chelator such as 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA) via a patch pipette (Kang *et al.*, 1998; Ge and Duan, 2007; Di Castro *et al.*, 2011; Panatier *et al.*, 2011; Morquette *et al.*, 2015), IP_3R2 knockout (Petravicz, Fiacco and McCarthy, 2008; Navarrete *et al.*, 2012; Petravicz, Boyt and McCarthy, 2014; Martin *et al.*, 2015) or pharmacological inhibition (Hua *et al.*, 2004; Di Castro *et al.*, 2011; Tang *et al.*, 2015), and depletion of intracellular (Carmignoto, Pasti and Pozzan, 1998; Haustein *et al.*, 2014) or

extracellular (Lee *et al.*, 2015; Srinivasan *et al.*, 2015) Ca^{2+}. None of the techniques currently available is cell-specific, acute and reversible, and it should be noted that disruption of astrocytic Ca^{2+} signalling may influence processes other than gliotransmitter release, such as K^+ homeostasis via astrocytic Ca^{2+}-dependent K^+ channels (Hamilton and Attwell, 2010; Nedergaard and Verkhratsky, 2012). Furthermore, those techniques that target IP_3 receptors may neglect the role of Ca^{2+} released in IP_3 receptor-independent manner (Srinivasan *et al.*, 2015; Bazargani and Attwell, 2016; Shigetomi, Patel and Khakh, 2016). As with techniques for stimulating astrocytes, the usefulness of the techniques available for supressing astrocytic Ca^{2+} signalling varies between preparations.

In acute spinal cord slices, loading astrocytes with a Ca^{2+} chelator enhances the amplitude of EPSCs in neighbouring neurons (Carlsen and Perrier, 2014). It was proposed that this is because Ca^{2+}-dependent release of ATP-adenosine from glia is prevented, but this has not been tested directly. The effects of inhibiting Ca^{2+} signalling in astrocytes during locomotor-related network activity are also yet to be tested.

Glial information processing and gliotransmitter release may be disrupted by techniques other than those that affect Ca^{2+} signalling directly. These include inhibition of G-protein signal transduction (Di Castro *et al.*, 2011), and disruption of vesicle release mediated by *N*-ethylmaleimide–sensitive factor attachment protein receptor (SNARE) proteins, either by loading astrocytes with pharmacological agents via a patch pipette (Araque *et al.*, 2000; Jourdain *et al.*, 2007; Gourine *et al.*, 2010; Lalo *et al.*, 2014; Sultan *et al.*, 2015) or by conditional expression of toxins (Angelova *et al.*, 2015) or a dominant-negative cytosolic domain of vesicle-associated membrane protein 2 (VAMP2; aka synaptobrevin 2) (Pascual *et al.*, 2005; Fellin *et al.*, 2009; Fujita *et al.*, 2014; Lalo *et al.*, 2014; Sultan *et al.*, 2015). In dominant-negative SNARE (dn-SNARE) mice, this protein is expressed under the GFAP promotor fragment and interacts with the SNARE complex to prevent exocytosis in astrocytes (Pascual *et al.*, 2005) (but see below); channel-mediated release of gliotransmitters is presumably unaffected by this manipulation (Hamilton and Attwell, 2010; Oh *et al.*, 2012; Han

et al., 2013). Whereas the above manipulations target only one element in putative pathways of gliotransmitter release, pharmacological ablation of glia provides a comprehensive disruption of astrocytic function (Clarke, 1991; Fonnum, Johnsen and Hassel, 1997; Zhang *et al.*, 2003; Huxtable *et al.*, 2010; Witts, Panetta and Miles, 2012; Li *et al.*, 2013; Wall and Dale, 2013). Substances such as fluoroacetate, fluorocitrate, methionine sulfoximine, L-α-aminoadipic acid (L-α-AA) selectively disrupt glial metabolism to cause cell death. In respiratory and locomotor networks, rhythmic activity is lost following glial ablation (Huxtable *et al.*, 2010; Witts, Panetta and Miles, 2012), but can be restored when glutamine is added to the preparation, presumably to permit continued synthesis of glutamate and GABA in the absence of astrocytes. In spinal locomotor networks, modulation of locomotor-related activity by endogenous adenosine is lost following glial ablation by fluoroacetate and methionine sulfoximine (Witts, Panetta and Miles, 2012). This is unlikely to be because of receptor desensitisation caused by release of purines upon glial cell death (Wall and Dale, 2013), because preparations retain sensitivity to adenosine.

In some studies, gliotransmitter release has been directly demonstrated by the use of high performance liquid chromatography (HPLC) (Huxtable *et al.*, 2010); “sniffer cells” expressing ligand-gated ATP channels (e.g. P2X2 receptors) (Lalo *et al.*, 2014), which are applied to the preparation and recorded from by patch-clamp techniques; the bioluminescent enzyme luciferase, which detects ATP (Wang, Haydon and Yeung, 2000); and microelectrode biosensors (Wall and Dale, 2013; Lalo *et al.*, 2014), of which several designs exist. These techniques vary in their analyte specificity, temporal resolution and the degree to which they damage the tissue in which they are applied (Dale, 2013). Direct measurements of gliotransmitter release have not been made in spinal cord preparations. Given that ATP-adenosine is proposed to be released by glia during locomotor-related activity, direct measurements of both purines could be used to characterise their release independently of neuronal activity.

Although there is extensive evidence for bidirectional communication between neurons and glia in *in vitro* preparations, there are few clear examples from behavioural studies. Until further behavioural evidence for gliotransmission in healthy animals is provided, it will remain controversial (Agulhon *et al.*, 2012; Nedergaard and Verkhratsky, 2012; Sloan *et al.*, 2014). Importantly, IP_3R2 germline knock out (IP_3R2KO) mice have been subjected to tests for a range of behaviours including locomotion, learning, memory and anxiety, but do not differ from wild type littermates on most measures (Agulhon *et al.*, 2013; Petravicz, Boyt and McCarthy, 2014). Although IP_3R2KO are reported to differ from wild type mice in their response to the forced swim test, they do not respond to other tests for depressive-like behaviour (Cao *et al.*, 2013; Petravicz, Boyt and McCarthy, 2014). These findings contradict evidence from many studies using slice preparations that support a role for astrocytic IP_3 receptors in gliotransmission; however, some studies have also not found evidence of gliotransmission in slices from IP_3R2KO mice (Fiacco *et al.*, 2007; Petravicz, Fiacco and McCarthy, 2008; Agulhon, Fiacco and McCarthy, 2010). It may therefore be that IP_3 receptor-dependent Ca^{2+} signalling in astrocytic somas, although observable *in vitro* and *in vivo*, does not stimulate gliotransmitter release in healthy behaving animals. However, it cannot be ruled out that compensatory mechanisms during development in IP_3R2KO mice mask the effects of deficient astrocytic Ca^{2+} signalling. In addition, it is possible that activity is altered in multiple regions of the CNS in IP_3R2KO mice, confounding the detection of effects within any given network, though total masking of multiple effects may be unlikely. Alternatively, it may be that activity-dependent IP_3 receptor-independent Ca^{2+} elevations in astrocytic fine processes have a more important in role in gliotransmission than currently appreciated. Spontaneous Ca^{2+} fluctuations in fine processes mediated by TRPA1 are proposed to regulate long-term potentiation via constitutive D-serine release and inhibitory synapse efficacy via GABA transporter type 3 (GAT-3) (Shigetomi *et al.*, 2012, 2013).

Evidence that behaviours including sleep (Halassa *et al.*, 2009), pain aversion (Foley, McIver and Haydon, 2011) and recognition memory (Halassa *et al.*, 2009; Florian *et al.*, 2011) are

modulated by substances released from astrocytes in a SNARE- and therefore Ca^{2+}-dependent manner has been provided by experiments using the dn-SNARE mouse model. However, it was recently shown that dn-SNARE is also expressed in cortical neurons (Fujita *et al.*, 2014), and it is unclear how this finding affects interpretation of the earlier studies.

Given the limitations of the techniques currently available for studying gliotransmission in behaving animals, *in vitro* and *ex vitro* rhythmically active brainstem and spinal cord preparations may provide insight into the role of bidirectional neuron-glia signalling in behaviour. Iterative, behaviourally relevant patterns of activity can be generated by isolated networks in the absence of descending and peripheral inputs, and this has permitted the study of diverse neuromodulatory systems and their roles in networks controlling defined behaviours (Marder and Bucher, 2001; Dickinson, 2006; Whelan, 2010; Harris-Warrick, 2011; Miles and Sillar, 2011). In addition to the examples from spinal locomotor networks given above, studies of brainstem networks for respiration and mastication have provided evidence for behaviourally-relevant gliotransmission. In the brainstem, astrocytic Ca^{2+} elevations are evoked by reductions in both pH (Gourine *et al.*, 2010; Kasymov *et al.*, 2013) and the partial pressure of oxygen (Angelova *et al.*, 2015), in both cases causing release of ATP, which stimulates breathing. In intact rats, optogenetic stimulation of astrocytes triggers release of ATP, which stimulates breathing (Gourine *et al.*, 2010) whereas expression of tetanus toxin in astrocytes to prevent vesicular release prevents increases in respiration normally observed in response to reduced oxygen availability (Angelova *et al.*, 2015). In the brainstem circuity for mastication, glia release the Ca^{2+}-binding protein S100β in a Ca^{2+}-dependent manner to regulate extracellular Ca^{2+} concentration, conferring rhythmic bursting properties on neighbouring neurons (Morquette *et al.*, 2015).

Several studies have provided evidence for Ca^{2+}-dependent signalling between astrocytes and neurons that departs from the established tripartite-synapse model. Signals other than neuronal activity are reported to elicit Ca^{2+} elevations in astrocytes and release of modulators. In addition to the examples given above of chemosensitivity in brainstem astrocytes,

astrocytes in the nucleus of the solitary tract respond to low glucose availability (McDougal *et al.*, 2013), and hippocampal astrocytes display elevations in intracellular Ca^{2+} in response to a reduction in extracellular Ca^{2+}, such as occurs during intense synaptic activity, resulting in the release of ATP from astrocytes (Torres *et al.*, 2012). Interestingly, a reduction in extracellular Ca^{2+} is reported in the spinal cord during locomotor-related activity (Brocard *et al.*, 2013), but the responsiveness of spinal cord astrocytes to changes in the ionic composition of the extracellular medium has not yet been assessed.

In addition, activity dependent astrocytic Ca^{2+} elevations are reported to stimulate activities that regulate neuronal activity by mechanisms other than the release of receptor agonists. These include the release of substances to constrict or dilate the microvasculature according to local demand for oxygen and glucose (Stobart and Anderson, 2013), regulation of the Na^+- and K^+-dependent adenosine triphosphatase (Na^+/K^+-ATPase) to increase K^+ uptake and hyperpolarise neurons (Wang *et al.*, 2012), and release of the Ca^{2+}-binding protein S100β to regulate extracellular Ca^{2+} concentration, as described above (Morquette *et al.*, 2015).

Finally, astrocytes may also regulate neuronal activity in an activity-dependent manner without Ca^{2+} fluctuations. Neurotransmitter uptake is as important as release in shaping patterns of synaptic transmission, and may be regulated in an activity-dependent manner independently of Ca^{2+} signalling. Clearance of synaptic glutamate by astrocytes has been shown to be regulated by neuronal activity (Perego *et al.*, 2000; Al Awabdh *et al.*, 2016), and the availability of glycine, a co-agonist of glutamate at NMDA receptors, is proposed to be regulated by GlyT1 in an activity dependent manner (see above). In addition, activation of astrocytic GPCRs may result in the modulation of neuronal activity by Ca^{2+}-independent pathways. Selective activation of a $G_{\alpha q}$-coupled DREADD (designer receptor exclusively activated by designer drugs) expressed by glia lacking IP_3R2 receptors results in diverse behavioural modifications on a timescale longer than that predicted for Ca^{2+}-dependent responses; these may involve signalling via $G_{\beta\gamma}$ or protein kinase C (PKC), for instance (Agulhon *et al.*, 2013). Interestingly, it has been observed that astrocytes express receptors for the neurotransmitters released

specifically by the neurons they make contact with, implying that glial receptors function to detect synaptic transmission (Verkhratsky and Butt, 2013). Thus, spinal cord astrocytes are unique in expressing receptors for glycine, the dominant inhibitory neurotransmitter in the spinal cord (Pastor *et al.*, 1995; Bowery and Smart, 2006). Both GlyRs and $GABA_A$ receptors expressed by spinal cord astrocytes carry a Cl^- current, as in neurons (Pastor *et al.*, 1995) but the physiological importance of these currents remains unknown. These examples suggest that, regardless of whether Ca^{2+}-dependent gliotransmission influences neural activity in healthy animals, information processing by astrocytes may be crucial in the generation of behaviours by spinal networks, exceeding their established role as cellular housekeepers.

Astrocyte identity

Astrocytes are distinguished by the expression of several markers, most notably the intermediate filament protein glial fibrillary acidic protein (GFAP), but also S100B, a calcium-binding protein, aldehyde dehydrogenase 1 family member L1 (Aldh1L1), and the glutamate transporters excitatory amino acid transporter 1 (EAAT1; aka glutamate and aspartate transporter, GLAST) and excitatory amino acid transporter 2 (EAAT2; aka glutamate transporter 1, GLT1) (Wang and Bordey, 2008; Tien *et al.,* 2012). However, expression of these markers is not consistent, and in some cases, is not limited to astrocytes (Wang and Bordey, 2008). Although GFAP is widely used to identify astrocytes, it is a non-specific marker, being detectable in some neural progenitor cells and oligodendrocytes, and levels of expression differ between populations of astrocytes: in general, it is highly expressed in white-matter fibrous astrocytes, but sporadically expressed in grey-matter protoplasmic astrocytes (Wang and Bordey, 2008; Tien *et al.,* 2012). Within the spinal cord, astrocytes are characterised by GFAP expression, although this is more pronounced in the white than in the grey matter in the early postnatal period (Tien *et al.,* 2012); they are further distinguished electrophysiologically by expression of non-decaying K^+ currents (Chvátal *et al.,* 1995). Oligodendrocytes, by contrast, do not express GFAP, but do exhibit positive immunostaining for O1 and O4 antigens. Furthermore, they display symmetrical, passive, non-decaying K^+

currents. Importantly, a further population of cells characterised by weak GFAP immunoreactivity and a complex pattern of Na^+, and K^+ currents is proposed to represent neural progenitors of the astrocyte lineage (Chvátal *et al.,* 1995). In addition, the adult spinal cord contains GFAP+ multipotent neural stem cells that are quiescent within the intact spinal cord (Fiorelli *et al.,* 2013). Such populations of non-astrocytic GFAP+ cells must therefore be taken into account when interpreting studies using GFAP as a marker of astrocytes in the spinal cord.

It may be apparent from this brief description that studies of gliotransmission do not typically consider diversity among astrocytes, despite evidence for considerable molecular and physiological diversity (Bachoo *et al.,* 2004; Khakh and Sofroniew, 2015). Studies of gliotransmission within spinal circuits would ideally consider the possibility of physiological specialisation among astrocytes, but this may require the identification of molecular markers to identify subpopulations (Bachoo *et al.,* 2004). The availability of such markers may facilitate understanding of astrocyte function in the spinal cord, just as identification of a range of molecular markers has advanced the study of neuronal subpopulations within spinal circuits (Goulding, 2009; Kiehn, 2016).

Outline of study

Mammalian spinal locomotor networks are subject to modulation by neuromodulators originating from both supraspinal and spinal sources, ensuring network output appropriate to the needs of the animal (Miles and Sillar, 2011). Astrocytes are proposed to release gliotransmitters in response to intracellular Ca^{2+} elevations resulting from activation of $G_{\alpha q}$-linked GPCRs by neurotransmitters released during synaptic transmission. However, the importance of such astrocytic signalling for the operation of neural networks and in shaping behaviour remains controversial, in part because of methodological limitations to the study of behaving animals (Bazargani and Attwell, 2016). In rhythmically active spinal cords *in vitro*, activity recorded from ventral roots corresponds to signals received from the locomotor CPG

by the muscles in intact animals (Kiehn and Kjaerulff, 1996). Isolated spinal cords may therefore provide a tractable model for the study of gliotransmission in relation to behaviour. In Chapter 2, the effects of activating the $G_{\alpha q}$ pathway in astrocytes during ongoing rhythmic network activity in spinal cord preparations from postnatal mice are determined. It is shown that activation of the glial receptor PAR1 triggers release of ATP, which is hydrolysed to adenosine by extracellular ectonucleotidases, consistent with previous reports that spinal cord glia produce modulatory adenosine (Witts, Panetta and Miles, 2012; Carlsen and Perrier, 2014). Adenosine derived from glial cells is shown to modulate both network output and synaptic inputs to ventral horn interneurons.

Previously, it was proposed that during locomotor-related activity adenosine acts via $G_{\alpha i}$-coupled A_1 receptors to inhibit signalling through $G_{\alpha s}$-linked D_1-like receptors to reduce the frequency of locomotor related activity (Acevedo *et al.*, 2016). In Chapter 3, this interaction is characterised, and activation of D_1-like receptors is shown to be a precondition for the modulation of spinal locomotor networks by glial-cell derived and bath-applied adenosine.

Activation of NMDARs requires binding of a co-agonist, either D-serine or glycine, in addition to glutamate. Astrocytes are proposed to regulate the availability of both D-serine and glycine at excitatory synapses (Le Bail *et al.*, 2015). In Chapter 4, the regulation of NMDARs by D-serine and glycine during rhythmic activity of the murine locomotor CPG is characterised. Evidence is provided that D-serine and glycine separately gate NMDARs in an activity-dependent and synapse-specific manner, and the glycine transporter GlyT1 is shown to be crucial for the regulation of network output. A direct investigation of the role of astrocytes in these processes is beyond the scope of this study, but is implied by the preferential expression of GlyT1 by astrocytes and the requirement of astrocytes for synthesis of D-serine.

Together, the experiments presented in this study provide evidence that glia actively modulate the output of mammalian locomotor networks and therefore provide further evidence for that glial information processing is important in shaping a fundamental behaviour.

Chapter 2: Stimulation of glia reveals modulation of mammalian spinal locomotor networks by adenosine

This chapter is adapted from previously published work (Acton and Miles, 2015)

Introduction

There is now considerable evidence from electrophysiological and Ca^{2+}-imaging studies that glia can both respond to activity at the synapses they enwrap with elevations in cytosolic Ca^{2+} and modulate the excitability of neighbouring neurons via the Ca^{2+}-dependent release of so-called gliotransmitters (Araque *et al.*, 2014; Volterra, Liaudet and Savtchouk, 2014; Bazargani and Attwell, 2016). However, the importance of these activities for the operation of neural networks and in shaping behaviours remains controversial (Hamilton and Attwell, 2010; Nedergaard and Verkhratsky, 2012; Fujita *et al.*, 2014; Petravicz, Boyt and McCarthy, 2014; Bazargani and Attwell, 2016). This chapter will examine the role of gliotransmission and glial cell-derived adenosine in spinal motor networks. These networks coordinate the rhythmic activation of flexor and extensor muscles within and between limbs during locomotion, and for this reason their output is immediately relatable to a defined behaviour (Orlovsky, Deliagina and Grillner, 1999).

Like other central pattern generators (CPGs) controlling stereotyped motor behaviours, spinal motor networks are subject to extensive neuromodulation, allowing network output to be varied according to the requirements of different environmental conditions, physiological states and developmental stages (Grillner, 2006; Miles and Sillar, 2011). Although many modulators are neuronal in origin, previous studies have reported modulation of spinal cord and brainstem CPGs following release of glutamate and the purines ATP and adenosine from glia (Gourine *et al.*, 2010; Huxtable *et al.*, 2010; Witts, Panetta and Miles, 2012).

Adenosine is among the most widespread neuromodulators in the nervous system and participates in diverse processes in health and disease (Cunha, 2001; Fredholm *et al.*, 2005).

Modulatory adenosine may be released from cells either directly or result from the hydrolysis of ATP by extracellular ectonucleotidases (Cunha, 2001; Klyuch, Dale and Wall, 2012; Wall and Dale, 2013). Several studies have detected Ca^{2+}-dependent release of ATP from glia, with subsequent degradation of ATP to adenosine and activation of neuronal A_1 or A_{2A} adenosine receptors (Pascual *et al.*, 2005; Serrano *et al.*, 2006; Panatier *et al.*, 2011; Carlsen and Perrier, 2014).

Adenosinergic modulation has previously been detected in motor networks of the spinal cord and brainstem. In the spinal cord of *Xenopus* tadpoles, ATP released during episodes of swimming excites the locomotor CPG and extends the duration of bouts of swimming (Dale and Gilday, 1996; Dale, 1998). As swimming progresses, ATP is hydrolysed to adenosine, which activates A_1 receptors to drive down network activity (Brown and Dale, 2000). Similarly, it was recently shown that adenosine derived from the hydrolysis of ATP acts on A_1 receptors to reduce the frequency of locomotor-related bursting in the spinal locomotor CPG of postnatal mice (Witts, Panetta and Miles, 2012; Acevedo *et al.*, 2016). At the cellular level, adenosine reduces the frequency and amplitude of synaptic inputs to interneurons and induces a hyperpolarising current in interneurons in acute slices from mice (Witts, Nascimento and Miles, 2015). Spinal locomotor networks of postnatal rats are also modulated by adenosine but may be less sensitive compared to equivalent networks in mice (Taccola *et al.*, 2012).

In mice, modulation of locomotor-related activity by endogenous adenosine is abolished following pharmacological ablation of glia, indicating that glia rather than neurons are the principal source of modulatory adenosine in murine spinal motor networks (Witts, Panetta and Miles, 2012). In support of these observations, excitatory transmission at synapses onto ventral horn interneurons is augmented following chelation of Ca^{2+} in neighbouring astrocytes; conversely, it is depressed by a mechanism involving both ATP hydrolysis and activation of A_1 receptors when neighbouring glia are stimulated (Carlsen and Perrier, 2014).

Mammalian brainstem networks controlling respiration, another rhythmic motor behaviour, are also depressed under the influence of adenosine. As in the spinal cord of *Xenopus* tadpoles,

inhibition of network activity by adenosine is reported to follow ATP-mediated excitation (Lorier *et al.*, 2007). In addition, adenosine exerts a tonic depression of network activity that is most evident in foetal stages (Kawai *et al.*, 1995; Schmidt, Bellingham and Richter, 1995; Mironov, Langohr and Richter, 1999; Huxtable *et al.*, 2009).

This chapter considers whether direct stimulation of glial cells leads to modulation of ongoing locomotor-related network activity in spinal cord preparations isolated from postnatal mice, and whether any such modulation involves adenosinergic signalling. Experimental stimulation of Ca^{2+} signalling in glia is proposed to replicate endogenous responses to neuronal activity and evokes release of neuromodulators in different systems (Araque *et al.*, 2014; Bazargani and Attwell, 2016); however, the effects of glial stimulation during locomotor-related activity are unknown. It is shown here that enhancement of glial Ca^{2+} signalling via activation of the $G_{\alpha q}$ pathway results in the modulation of locomotor network activity, and that this entails the secretion by glia of ATP and its subsequent hydrolysis to adenosine. Interestingly, no evidence of other glial cell-derived modulators affecting spinal locomotor networks is apparent. Evidence is also provided to show that adenosinergic modulation of locomotor networks scales with network activity, likely reflecting proportional release of adenosine. This implies that glia possess a mechanism for detecting activity in adjacent neurons, a key element of the tripartite synapse model (Araque *et al.*, 1999), and that they provide negative feedback to regulate the output of spinal motor circuitry. The use of three-enzyme microelectrode biosensors for the detection of adenosine (Sarissa Biomedical Ltd, Coventry, UK) (Llaudet *et al.*, 2003) in murine spinal cord preparations is also considered. These are shown to lose sensitivity when applied to hemicords from neonatal mice, and they are therefore unsuitable for the detection of adenosine from glia during locomotor-related network activity. Finally, it is shown that adenosinergic modulation of network activity recorded from ventral roots is associated with changes in the frequency but not the amplitude of synaptic inputs to ventral horn interneurons.

Together, these findings suggest that adenosine is the primary glial cell-derived modulator of spinal motor networks and implicate glia as active participants in the modulation of these networks and thus of locomotor behaviour.

Methods

Ethics Statement

All procedures performed on animals were conducted under project licence 60/13802 and personal licence 60/13802 in accordance with the UK Animals (Scientific Procedures) Act 1986 and were approved by the University of St Andrews Animal Welfare and Ethics Committee.

Tissue preparation

For physiological experiments, spinal cords were isolated from postnatal day (P)1-P4 C57BL/6 mice as previously described (Jiang, Carlin and Brownstone, 1999). In summary, animals were killed by cervical dislocation, decapitated and eviscerated before being transferred to a dissection chamber containing recording artificial cerebrospinal fluid (aCSF) for whole spinal cords, or dissection aCSF for hemicords. aCSF was maintained at ~4°C and equilibrated with 95% oxygen and 5% carbon dioxide. Spinal cords were then isolated between midthoracic and upper sacral segments, and ventral and dorsal roots were trimmed. To produce hemicords, spinal cords were pinned ventral-side-up in the dissecting chamber, and an insect pin (base diameter: 0.1 mm) was passed through the midline from caudal to rostral segments, separating the left and right sides of the spinal cord.

Ventral root recordings from whole spinal cords

Isolated spinal cords were pinned ventral-side up in a recording chamber perfused with recording aCSF (equilibrated with 95% oxygen, 5% carbon dioxide; RT) at 10 ml/min. Glass suction electrodes were attached to the first or second lumbar ventral roots (L_1, L_2) on each side of the spinal cord to record flexor-related activity. In some experiments a further suction

electrode was attached to the fifth lumbar ventral root (L_5) to record the corresponding extensor-related activity. Locomotor-related activity was evoked by bath application of *N*-methyl-D-aspartic acid (NMDA; 5 µM), 5-hydroxytryptamine (5-HT; 10 µM) and dopamine (DA; 50 µM), unless otherwise stated, and was characterised by rhythmic bursting alternating contralaterally between upper ventral roots and ipsilaterally between upper ventral roots and L_5. For disinhibited preparations (Bracci, Ballerini and Nistri, 1996; Witts, Panetta and Miles, 2012), strychnine (1 µM) and picrotoxin (60 µM) were applied to evoke rhythmic bursting that was synchronous in all roots. In some experiments theophylline (20 µM); SCH58261 (25 µM); 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; 50 µM); ARL67156 (50 µM); methionine sulfoximine (MSO; 100 µM) and glutamine (1.5 mM); or fluoroacetate (FA; 5 mM) and glutamine (1.5 mM) were bath-applied upon the onset of locomotor-related bursting at concentrations previously employed in this preparation (Witts, Panetta and Miles, 2012). In all experiments, stable rhythmic bursting was established over a period of ~1 h prior to bath-application of the PAR1-specific agonist TFLLR (Lee *et al.*, 2007; Lalo *et al.*, 2014). In all experiments, any drugs present in the control stage were also present during TFLLR application and washout. Rhythmic bursting was considered stable when the frequency, amplitude and duration of bursts were unchanged over several minutes. TFLLR application was limited to 5 min, consistent with methods previously employed (Lee *et al.*, 2007; Lalo *et al.*, 2014). Data were amplified and filtered (band-pass filter 30-3,000 Hz, Qjin Design) and acquired at a sampling frequency of 6 kHz with a Digidata 1440A analogue-digital converter and Axoscope software (Molecular Devices, Sunnyvale, CA). Custom-built amplifiers (Qjin Design) enabled simultaneous online rectification and integration (50-ms time constant) of raw signals.

Ventral root recordings from hemicords

Isolated hemicords were pinned, with the exposed midline surface facing up, to transparent Sylgard in a recording chamber perfused with recording aCSF (equilibrated with 95% oxygen, 5% carbon dioxide; RT) at 3 ml min^{-1}. A glass suction electrode was attached to the L_1 or L_2

ventral root. Locomotor-related activity was evoked by bath application 5-HT (10 µM), NMDA (5 µM) and DA (50 µM), and was characterised by rhythmic bursting. Any drugs present during the control period were also present during application of additional pharmacological agents and during washout. In all experiments, stable rhythmic bursting was established over a period of ~1 h prior to the control period. Rhythmic bursting was considered stable when the frequency, amplitude and duration of bursts were unchanged over several minutes. Data were acquired as for whole spinal cord experiments.

Whole-cell patch-clamp recordings

All recordings were made in a chamber perfused with recording aCSF (equilibrated with 95% oxygen, 5% carbon dioxide; RT) at 3 ml min^{-1}. Recordings were taken from ventral horn interneurons on the exposed midline surface of hemicords visualized under infrared-differential interference contrast microscopy. Recordings were made from a heterogeneous population of interneurons (average whole cell capacitance 13.5 ± 2.1 pF; average input resistance 758.5 ± 54.2 MΩ; *n* = 33). Interneurons were not readily classifiable into distinct subpopulations based on location, passive properties, or their responses to adenosine, TFLLR or DPCPX. Patch-clamp electrodes (3–5 MΩ) were pulled on a horizontal puller (Sutter Instrument, Novato, CA) from borosilicate glass (World Precision Instruments, Sarasota, FL). Signals were amplified and filtered (4 kHz low-pass Bessel filter) with a MultiClamp 700B amplifier (Molecular Devices) and acquired at 10 kHz with a Digidata 1440A A/D board and pCLAMP software (Molecular Devices). Correction for the liquid junction potential, which was calculated as 14.2 mV for solutions used here, was not made (Clampex JPCalcW).

Biosensor recordings

Isolated hemicords were pinned, with the exposed midline surface facing up, to transparent Sylgard in a 15 ml recording chamber perfused with recording aCSF (equilibrated with 95% oxygen, 5% carbon dioxide; RT) at 3 ml min^{-1}. A glass suction electrode was attached to the L_1 or L_2 ventral root to stabilise the preparation.

Adenosine and inosine biosensors were obtained from Sarissa Biomedical Ltd (Coventry, UK). The fabrication and use of these biosensors were previously described (Frenguelli, Llaudet and Dale, 2003; Llaudet *et al.*, 2003). Briefly, adenosine sensors consisted of a Pt/Ir wire with a diameter of 50 µm mounted within a glass pipette. A 2 mm length of wire protruding from the tip of the pipette bore an enzyme cascade consisting of adenosine deaminase, purine nucleoside phosphorylase and xanthine oxidase within a lactobionamide and amphiphilic polypyrrole matrix. Inosine (null) biosensors lacked adenosine deaminase but were otherwise identical. The exposed wire tips of biosensors were angled so that they would lie flat against the exposed midline surface of hemicords during experiments (Fig. 8, A). Biosensors were prepared for use in accordance with the manufacturer's guidelines. Biosensor tips were rehydrated for > 10 min and thereafter stored in a solution of 2 mM NaPi buffer, pH 7.4, 100 mM NaCl, 1 mM $MgCl_2$, 2 mM glycerol. Following rehydration, exposure of biosensor tips to the air was minimised to prevent drying. Biosensor tips were either submerged in the recording chamber when no tissue was present or placed in contact with the exposed midline surface of the ventral horn within segment L_1 or L_2 (Fig. 8, A and B). A MultiClamp 700B amplifier (Molecular Devices, Sunnyvale, CA) was used to polarise biosensors. Sensors were positioned in recording aCSF and cycled between -500 mV and +500 mV at 100 mV/s for 10 cycles before use to maximise sensitivity. Sensors were polarised to +500 mV throughout experiments. Signals were allowed to stabilise over 10-20 min prior to recording. Data was acquired at 10 kHz with a Digidata 1440A A/D board and pCLAMP software (Molecular Devices).

Data analysis

Recordings from ventral roots were analysed off-line with DataView software (courtesy of Dr W.J. Heitler, University of St Andrews). Ventral-root bursts were identified from rectified/integrated traces and their instantaneous frequencies, peak-to-peak amplitudes, and durations were then measured from the corresponding raw traces. Amplitude was measured as a non-calibrated unit and is presented here as an arbitrary unit (a.u.). For time-course plots,

data were averaged across 1-min bins and normalised to a 10-min pre-control period to permit comparison between preparations. Duty cycle was calculated as burst duration divided by cycle period. For whole spinal cord preparations, statistical comparisons were performed on raw data averaged over 3-min periods for locomotor-related activity or 6-min periods for disinhibited activity. For hemicords, statistical comparisons were performed on raw data averaged over six consecutive bursts and intra-burst intervals. Whole cell patch-clamp recordings were analysed with Clampfit software (Molecular Devices) or, for analyses of synaptic events, the Mini Analysis Program (Synaptosoft, Fort Lee, NJ). Biosensor currents were analysed offline with pCLAMP software (Molecular Devices). Averaged currents during the final 30 s of control, drug and wash were analysed. Data are reported as means ± SE. Data were analysed with repeated-measures ANOVA, one-way ANOVA, or Student's *t*-tests. Where appropriate, Bonferroni post-hoc tests were used for pairwise comparisons. Sphericity was assessed with Mauchly's test, and Greenhouse-Giesser corrections were applied where necessary. p values < 0.05 were considered significant. Cohen's d effect size was also determined where appropriate (Cohen, 1988). Tests were performed in SPSS Statistics for Windows, Version 21.0 (IBM Corp. Armonk, NY) or Excel 2013 (Microsoft Corp. Redmond, WA).

Immunohistochemistry

P4-P11 C57BL/6 mice were dissected in recording aCSF as above, and spinal cords were incubated in fixative containing 4% (w/v) paraformaldehyde and 0.1 M phosphate buffer (pH 7.4) for 16 h at 4°C, before being washed with 0.1 M phosphate-buffered saline (PBS; pH 7.4) and stored in PBS at 4°C. Slices from segments L_1-L_3 were cut at a thickness of 50 µm on a vibratome (Leica, Nussloch, Germany). Free-floating slices were incubated for 48 h at 4°C in solution containing primary antibodies, PBS, 1% (w/v) bovine serum albumin (BSA) and 0.1% (v/v) Triton X-100. Slices were then washed with PBS and incubated for 24 h at 4°C in solution containing secondary antibodies, PBS and 1% BSA. Slices were washed again with PBS and mounted with Vectashield (Vector Labs, Peterborough, UK). The stained tissue was examined

with an epifluorescence microscope and structured illumination (Imager.M2 fitted with ApoTome.2, Carl Zeiss Microscopy, Göttingen, Germany). Primary antibodies were used at the following concentrations: chicken polyclonal anti-microtubule-associated protein 2 (MAP2; Millipore AB5543) (McDowell *et al.*, 2010; Karumbaiah *et al.*, 2012; Xu *et al.*, 2013), 1:200; rabbit polyclonal anti-protease activated receptor-1 (PAR1; Bioss bs-0828R) (Liu *et al.*, 2015), 1:100; chicken polyclonal anti-glial fibrillary acidic protein (GFAP; Aves GFAP) (Benoit *et al.*, 2009; White, McTigue and Jakeman, 2010; Daadi *et al.*, 2012), 1:100. Secondary antibodies were used at the following concentrations: polyclonal anti-chicken FITC (Aves F-1005), 1:200; polyclonal anti-rabbit Cy3 (Jackson Immunoresearch 111-165-003), 1:500.

Drug and Solution Preparation

Recording aCSF contained (in mM) 127 NaCl, 26 $NaHCO_3$, 10 glucose, 3 KCl, 2 CaCl, 1.25 NaH_2PO_4, and 1 $MgCl_2$. Dissection aCSF contained (in mM) 25 NaCl, 188 sucrose, 1.9 KCl, 1.2 NaH_2PO_4, 10 $MgSO_4$, 1 $CaCl_2$, 26 $NaHCO_3$, 25 D-glucose, and 1.5 kynurenic acid. The patch-clamp pipette solution contained (in mM) 140 potassium methanesulfonate, 10 NaCl, 1 $CaCl_2$, 10 HEPES, 1 EGTA, 3 Mg-ATP, and 0.4 GTP-Na_2 (pH 7.2-7.3, adjusted with KOH). Adenosine, DA, NMDA, TFLLR, theophylline, MSO, FA and glutamine were supplied by Sigma-Aldrich (Poole, UK); 5-HT, DPCPX and SCH58261 were supplied by Abcam (Cambridge, UK); ARL67156 was supplied by Tocris Bioscience (Bristol, UK). Drugs were dissolved in reverse-osmosis water, except adenosine, picrotoxin, DPCPX and SCH58261, which were dissolved in DMSO. The concentration of DMSO in working solutions did not exceed 0.1% (v/v).

Results

Stimulation of glia modulates locomotor network output

Protease activated receptor-1 (PAR1), an endogenous G-protein coupled receptor, is preferentially expressed by astrocytes in the brain, and application of the peptide agonist TFLLR has been shown to elicit Ca^{2+} signalling selectively in cortical astrocytes

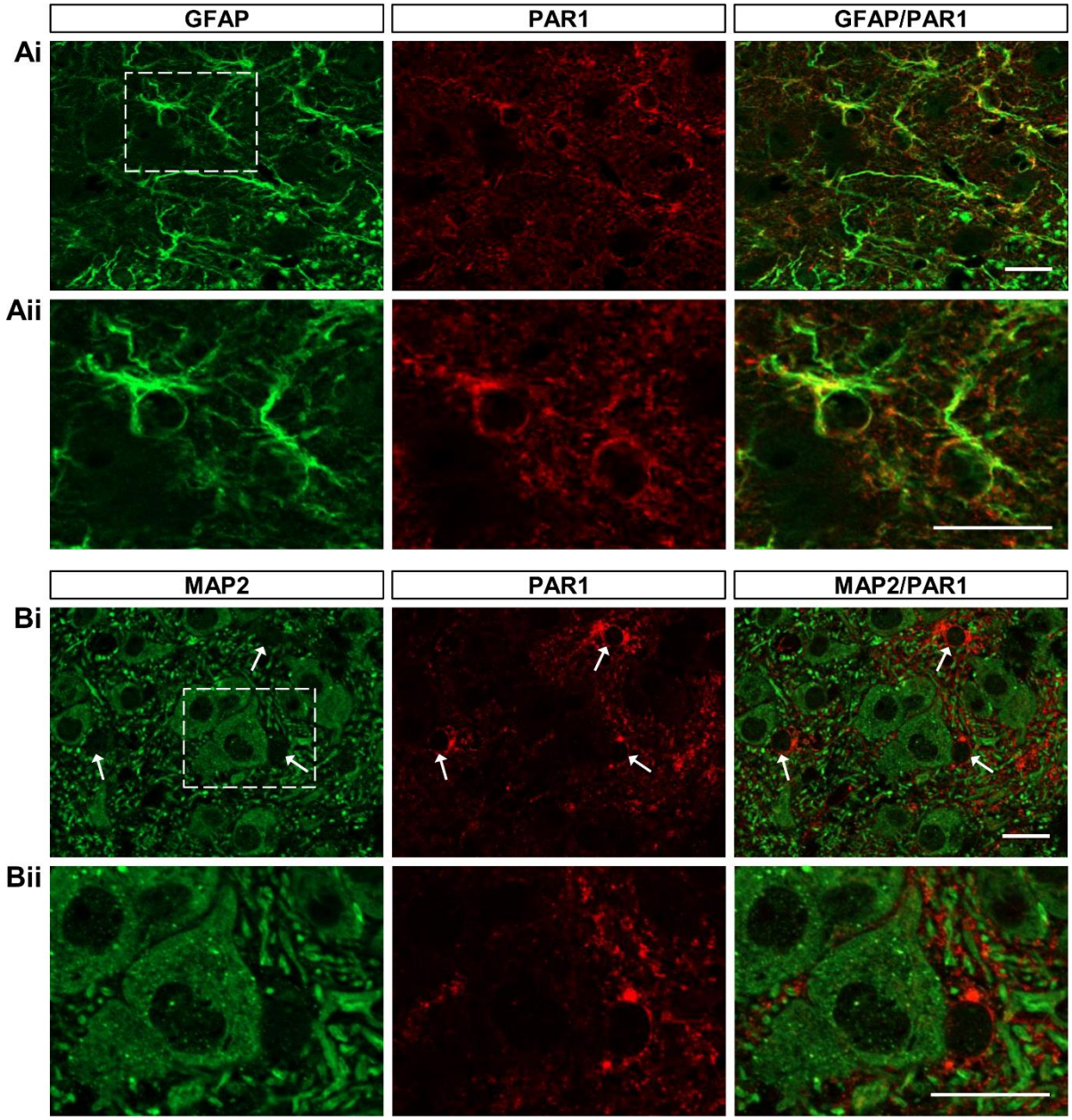

Fig. 1. PAR1 immunoreactivity co-localises with GFAP but not with MAP2 in the lumbar ventral spinal cord. A: representative images showing 50 µm transverse sections taken from the upper lumbar spinal cord of a P6 C57BL/6 mouse. Sections were stained with antibodies raised against GFAP (green) and PAR1 (orange). B: representative images showing 50 µm transverse sections taken from the upper lumbar spinal cord of a mouse. Sections were stained with antibodies raised against MAP2 (green) and PAR1 (orange). Arrows in Bi indicate areas of PAR1 staining between MAP2+ cells. Scale bars: 20 µm.

(Lee *et al.*, 2007; Shigetomi *et al.*, 2008; Lalo *et al.*, 2014). To assess the suitability of PAR1 activation as a strategy for selectively stimulating glia during ongoing rhythmic activity of spinal motor networks *in vitro*, expression of PAR1 in the spinal cord was examined. In spinal cord slices taken from segments L_1-L_3 of P4-11 mice, PAR1 immunoreactivity co-localised with the astrocyte marker GFAP throughout the ventral horn (Rexed's laminae VII-IX; Fig. 1A; $n = 4$). By contrast, PAR1 immunoreactivity was not exhibited by cells positive for the pan-neuronal marker MAP2 (Fig. 1B; $n = 4$), but was instead restricted to cells located between those labelled with MAP2 (Fig. 1Bii, arrows). This pattern of expression, which was consistent across P4-11 tissue, resembles that previously reported in the brain and supports the use of endogenous PAR1 for the specific stimulation of Ca^{2+}-dependent processes in spinal cord glia (Weinstein *et al.*, 1995; Junge *et al.*, 2004).

To determine the contribution of glial-neuronal signalling to the modulation of the mammalian spinal circuitry controlling locomotion, the PAR1-specific agonist TFLLR (10 µM) (Lee *et al.*, 2007; Lalo *et al.*, 2014) was briefly bath applied to isolated mouse spinal cord preparations while ongoing pharmacologically-induced (10 µM 5-HT, 50 µM DA, 5 µM NMDA) fictive locomotor-related activity was recorded from lumbar ventral roots (Fig. 2A). A 5-min application of TFLLR caused a transient reduction in the frequency of locomotor-related bursting, beginning within the first minute, and with a maximum effect of 11.5 ± 2.9% after 5 min (Fig. 2, A and B; $F[2,18] = 9.1$, $p < 0.01$, $d = 0.43$, $n = 10$). Burst duration was found to increase during TFLLR application (21.0 ± 6.7%; $F[2,18] = 9.6$, $p < 0.01$, $n = 10$) whereas duty cycle did not change significantly ($F[2,18] = 0.8$, $p > 0.05$, $n = 10$). In addition, TFLLR application had no effect on the peak-to-peak amplitude of bursts (Fig. 2C; $F[2,18] = 3.9$, $p > 0.05$, $n = 10$). Alternation of bursts, both between contralateral roots and between ipsilateral roots L_2 and L_5, was maintained throughout the drug application and wash periods (Fig. 2A).

To confirm that the reduced frequency of locomotor-related bursting detected upon TFLLR application was mediated by glia, and that TFLLR had no direct effects on neurons, TFLLR was applied to preparations in which glia had been ablated by exposure to gliotoxins

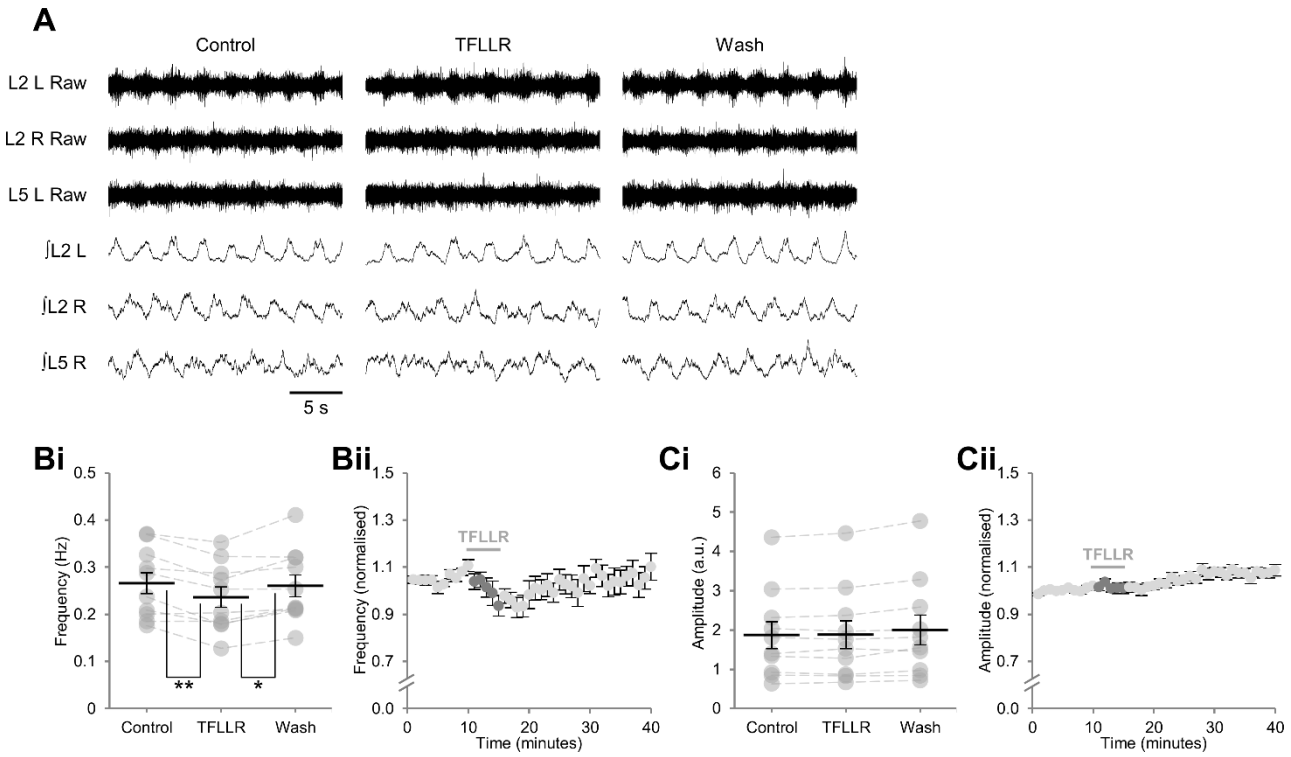

Fig. 2. Stimulation of glia during ongoing locomotor-related activity results in a transient reduction in burst frequency. A: raw (top) and rectified/integrated (bottom) traces recorded from left (L) and right (R) L_2 ventral roots and the right L_5 ventral root showing the effect of the PAR1 agonist TFLLR (10 µM). Bi: locomotor-burst frequency over 3 min during a control period, immediately following TFLLR application, and following a 20 min washout period. Individual data points are shown in grey, and means are represented by black lines. Bii: time course plot of normalised data aggregated into 1-min bins showing a reduction in burst frequency upon application of TFLLR. Ci: locomotor-burst amplitude over 3 min during a control period, immediately following TFLLR application, and following a 20 min washout period. Cii: time course plot of normalised data aggregated into 1-min bins showing no change in burst amplitude upon application of TFLLR. n = 10 preparations. Error bars: ± SEM. Statistically significant differences in pairwise comparisons: *$p < 0.05$, **$p < 0.01$.

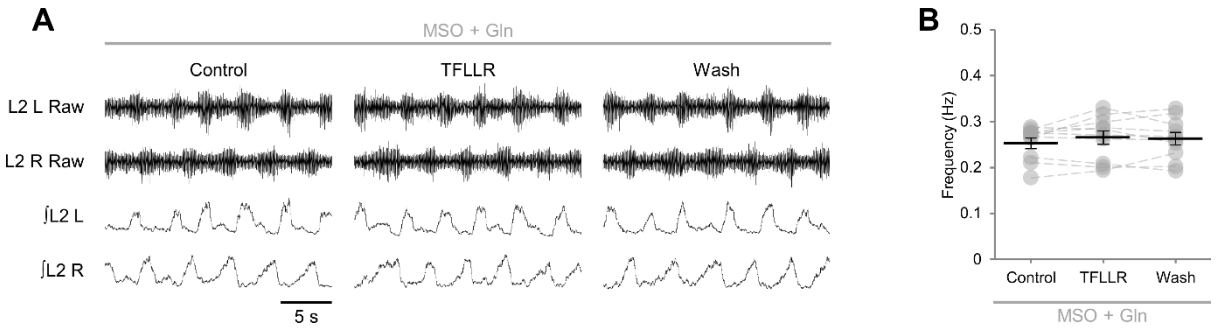

Fig. 3. TFLLR has no effect on locomotor-related bursting following pharmacological ablation of glia. A: raw (top) and rectified/integrated (bottom) traces recorded from left (L) and right (R) L_2 ventral roots showing the effect of the PAR1 agonist TFLLR (10 µM) following glial ablation with methionine sulfoximine (MSO; 100 µM), which was co-applied with glutamine (Gln; 1.5 mM). B: locomotor-burst frequency in the presence of MSO and Gln over 3 min during a control period, immediately following TFLLR application, and following a 20 min washout period. Individual data points are shown in grey, and means are represented by black lines. *n* = 10. Error bars: ± SEM.

(Hülsmann *et al.*, 2000; Huxtable *et al.*, 2010; Witts, Panetta and Miles, 2012). Stable locomotor-like bursting resembling that observed in the absence of toxins persisted following application of MSO (100 µM) or FA (5 mM), both of which selectively disrupt glial metabolism, when the aCSF was supplemented with glutamine (1.5 mM) to sustain synthesis of glutamate and GABA (Hülsmann *et al.*, 2000; Huxtable *et al.*, 2010; Witts, Panetta and Miles, 2012). Burst frequency was found to be unaffected by TFLLR applied 1 hr after either MSO (Fig. 3, A and B; $F[2,18] = 1.0$, $p > 0.05$, $n = 10$) or FA (5 mM; $F[2,18] = 0.2$, $p > 0.05$, $n = 10$), demonstrating that the effect of TFLLR on locomotor network activity is mediated by glia and that TFLLR does not directly affect neurons. Together these findings indicate that, when stimulated via an endogenous GPCR, spinal cord glia are capable of releasing a factor or factors that reduce the frequency of rhythmic activity generated by locomotor circuits.

Network modulation following PAR1 activation is mediated by adenosine derived from ATP

The identity of the neuromodulatory factor or factors released by glia upon PAR1 activation was next considered. Previously, it was shown that spinal motor networks are modulated by endogenous adenosine that appears to derive from glia (Witts, Panetta and Miles, 2012). It was therefore investigated whether adenosine mediated a component of the network response to PAR1 activation by applying TFLLR in the presence of the non-selective adenosine receptor antagonist theophylline (20 µM). In these preparations TFLLR had no effect on the frequency of locomotor-related bursting (Fig. 4, A and B; $F[2,18] = 2.8$, $p > 0.05$, $n = 10$), implying that adenosine is the dominant modulator released by glia following PAR1 activation. To investigate the adenosine receptor subtypes activated by glial cell-derived adenosine, TFLLR was applied in the presence of antagonists selective for A_1 or A_{2A} receptors, both of which are broadly expressed high-affinity adenosine receptor subtypes (Cunha, 2001). Like theophylline, the A_1-subtype specific antagonist DPCPX (50 µM) efficiently abolished the modulation of burst frequency by PAR1 activation (Fig. 4, C and D; $F[2,18] = 1.9$, $p > 0.05$, $n = 10$). By contrast, in the presence of the A_{2A}-subtype specific antagonist SCH58261 (25 µM),

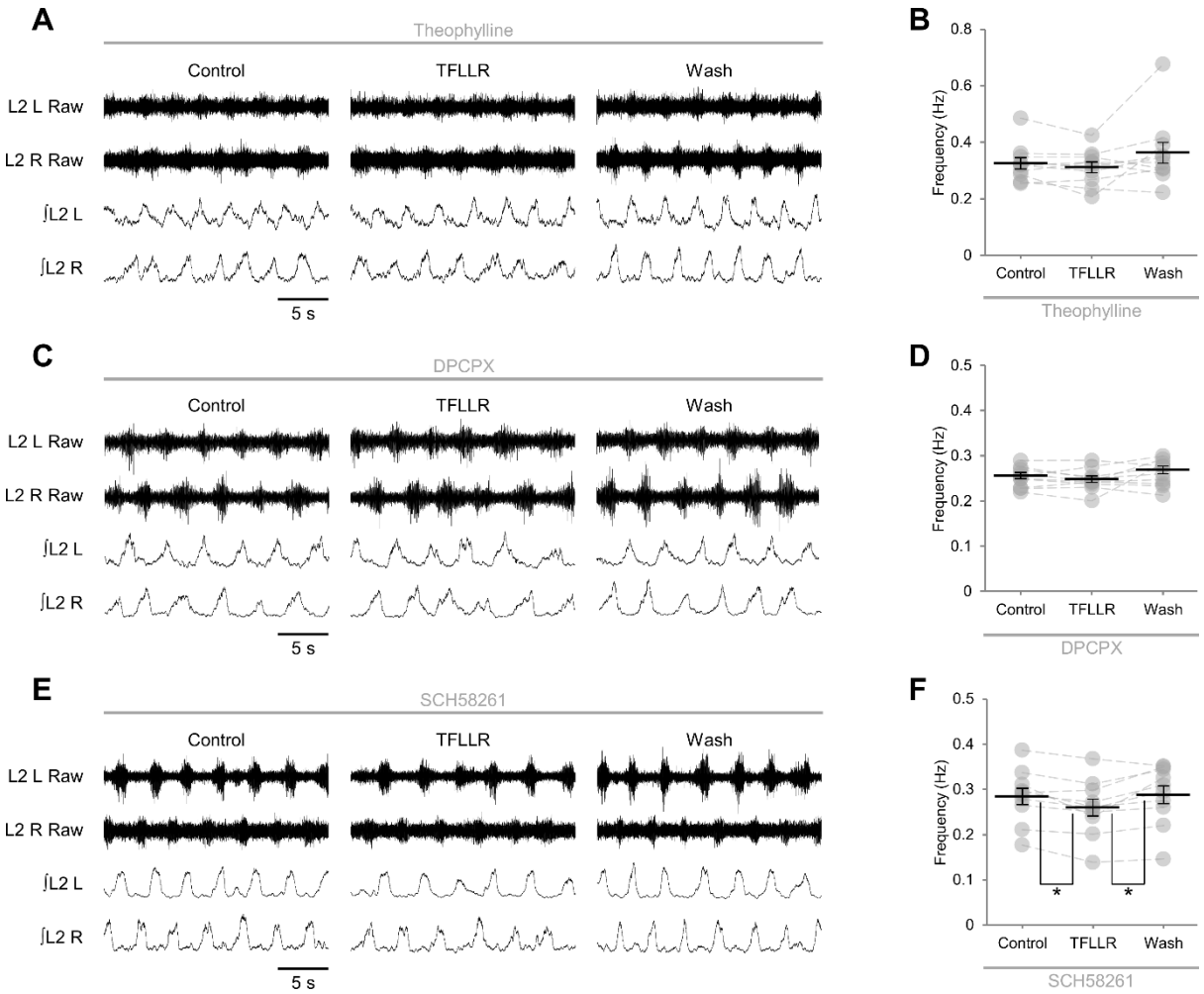
A
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Fig. 4. Glial stimulation results in release of adenosine and activation of neuronal A_1 receptors. A: raw (top) and rectified/integrated (bottom) traces recorded from left (L) and right (R) L_2 ventral roots showing the effect of the PAR1 agonist TFLLR (10 µM) applied in the presence of theophylline (20 µM). B: locomotor-burst frequency in the presence of the non-selective adenosine receptor antagonist theophylline over 3 min during a control period, immediately following TFLLR application, and following a 20 min washout period (n = 10). C: raw (top) and rectified/integrated (bottom) traces recorded from left (L) and right (R) L_2 ventral roots showing the effect of the PAR1 agonist TFLLR (10 µM) applied in the presence of the A_1-receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; 50 µM). D: locomotor-burst frequency in the presence of DPCPX over 3 min during a control period, immediately following TFLLR application, and following a 20 min washout period (n = 10). E: raw (top) and rectified/integrated (bottom) traces recorded from left (L) and right (R) L_2 ventral roots showing the effect of the PAR1 agonist TFLLR (10 µM) applied in the presence of SCH58261 (25 µM). F: locomotor-burst frequency in the presence of the A_{2A}-receptor antagonist SCH58261 over 3 min during a control period, immediately following TFLLR application, and following a 20 min washout period (n = 10). Individual data points are shown in grey, and means are represented by black lines. Error bars: ± SEM. Statistically significant differences in pairwise comparisons: *$p < 0.05$.

PAR1 activation caused a transient reduction in the frequency of locomotor-related bursting of a similar magnitude to that measured in the absence of receptor antagonists (9.1 ± 2.3%; Fig. 4, E and F; F[2,18] = 7.0, $p < 0.01$, $d = 0.43$ $n = 10$). In addition, PAR1 activation in the presence of SCH58261 did not affect burst amplitude (F[2,18] = 0.267, $p > 0.05$, $n = 10$).

Adenosine may be released from cells directly or result from the ectonucleotidase-mediated hydrolysis of ATP following its release into the extracellular space. To further investigate the release of adenosine by glia during network activity, TFLLR was applied in the presence of the ectonucleotidase inhibitor ARL67156 (50 µM). In preparations to which ARL657156 was pre-applied, PAR1 activation did not modulate the frequency of locomotor-related bursting (Fig. 5, A and B; F[2,20] = 1.7, $p > 0.05$, $n = 11$), implying that glia do not release adenosine directly, but instead release ATP, which is subsequently degraded to adenosine. Together these data indicate that glia associated with spinal motor control networks release ATP following PAR1 activation, and that the adenosine produced by hydrolysis of ATP in the extracellular space acts on neuronal A_1 but not A_{2A} receptors to inhibit locomotor-related activity.

Glial cell-derived adenosine does not modulate excitatory components of the locomotor circuitry

Following the discovery that PAR1 activation results in the release of ATP-adenosine from glia, the cellular targets of glial cell-derived adenosine within spinal motor circuitry were considered. To investigate the relative sensitivity of excitatory versus inhibitory components of spinal motor networks to adenosine derived from glia, experiments were performed in preparations in which inhibitory transmission was blocked by the glycine receptor antagonist strychnine (1 µM) and the $GABA_A$ channel antagonist picrotoxin (60 µM) (Bracci, Ballerini and Nistri, 1996; Witts, Panetta and Miles, 2012). Blockade of inhibitory transmission results in slow (0.032 ± 0.003 Hz, $n = 13$), rhythmic, large-amplitude bursts that are synchronous across all ventral roots (Fig. 6A). To assess whether adenosine released from glia during network activity modulated disinhibited bursting, DPCPX (50 µM) was applied to block A_1 receptors. A_1-receptor blockade altered neither burst frequency (Fig. 6A and B; F[2,10] = 0.8, $p > 0.05$,

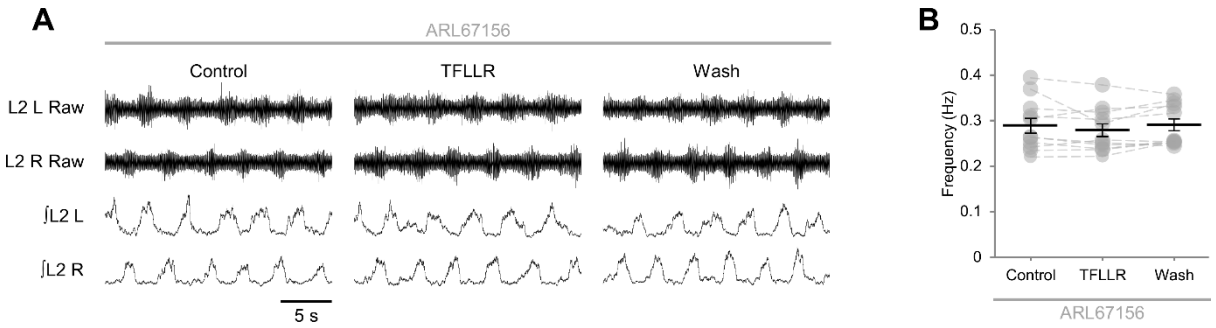


Fig. 5. Modulation of locomotor network output upon glial stimulation requires extracellular degradation of ATP to adenosine. A: raw (top) and rectified/integrated (bottom) traces recorded from left (L) and right (R) L_2 ventral roots showing the effect of the PAR1 agonist TFLLR (10 µM) applied in the presence of the ectonucleotidase inhibitor ARL67156 (50 µM). B: locomotor-burst frequency in the presence of ARL67156 (50 µM) over 3 min during a control period, immediately following TFLLR application, and following a 20 min washout period (*n* = 11). Error bars: ± SEM.

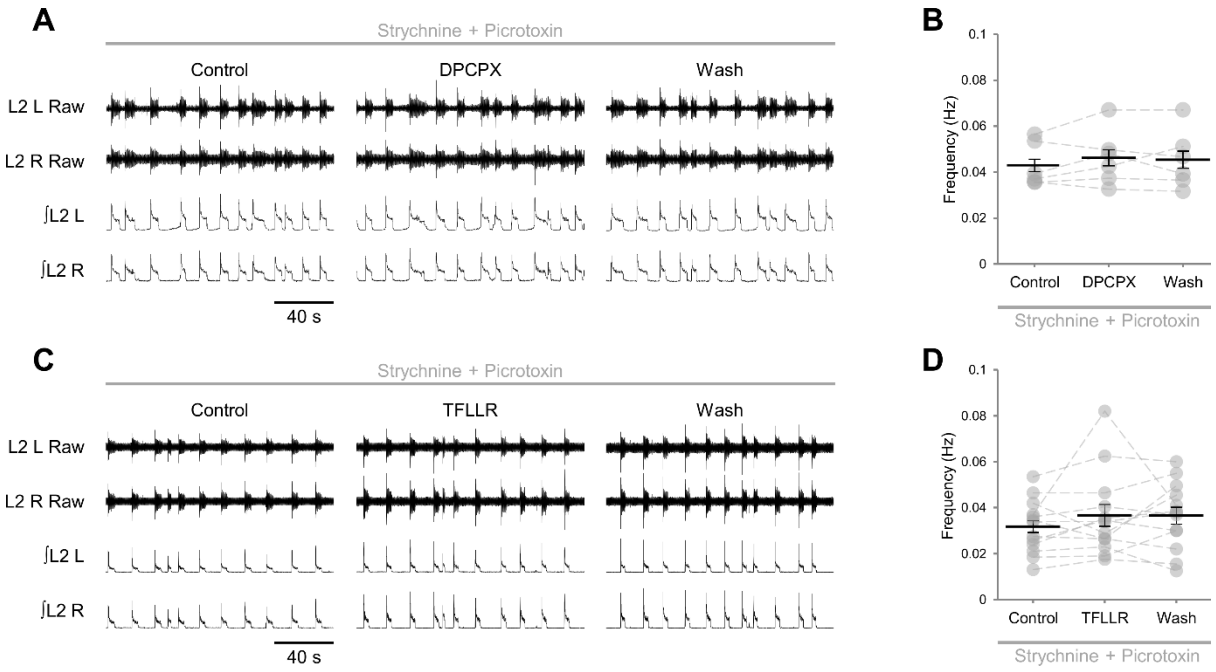


Fig. 6. ATP-adenosine released endogenously or following stimulation of glia modulates inhibitory components of locomotor networks. A: raw (top) and rectified/integrated (bottom) traces recorded from left (L) and right (R) L_2 ventral roots showing the effect of the A_1-receptor antagonist DPCPX (50 µM) in preparations in which inhibitory transmission was blocked by the $GABA_A$-receptor antagonist pictrotoxin (10 µM) and the glycine-receptor antagonist strychnine (1 µM). The locomotor drugs 5-HT (10 µM), NMDA (5 µM) and DA (50 µM) were also present throughout. B: locomotor-burst frequency over 10 min during a control period, at the end of a 40 min application of DPCPX, and at the end of a 40 min washout. C: raw (top) and rectified/integrated (bottom) traces recorded from left (L) and right (R) L_2 ventral roots showing the effect of the PAR1 agonist TFLLR (10 µM) applied to preparations in which inhibitory transmission was blocked by pictrotoxin and strychnine. D: locomotor-burst frequency in disinhibited preparations over 6 min during a control period, immediately following TFLLR application, and following a 20 min washout period (n = 13). Individual data points are shown in grey, and means are represented by black lines. Error bars: ± SEM.

n = 6) nor amplitude (*F*[2,10] = 2.1, *p* > 0.05, *n* = 6). Similarly, burst frequency (Fig. 6, C and D; *F*[2,24] = 1.2, *p* > 0.05, *n* = 13) and amplitude (*F*[2,24] = 2.294, *p* > 0.05, *n* = 13) were unchanged following a 10-min bath application of TFLLR to stimulate glia. These results suggest that adenosine produced during locomotor-related activity or upon PAR1 activation primarily modulates the activity of inhibitory interneurons or microcircuits not active within disinhibited preparations.

Glial cell-derived adenosine mediates feedback inhibition of locomotor network activity

It was then considered whether modulation of the murine locomotor CPG by endogenous glial cell-derived adenosine scales with network activity, as predicted by the tripartite synapse model of bidirectional signalling between neurons and glia (Araque *et al.*, 1999, 2014). Stable locomotor-related bursting was generated by bath application of 5-HT (10 µM), DA (50 µM) and three different concentrations of NMDA to generate a range of control frequencies (0 µM NMDA: 0.097 ± 0.017 Hz, *n* = 11; 3 µM NMDA: 0.131 ± 0.008 Hz, *n* = 16; 5 µM NMDA: 0.175 ± 0.001 Hz, *n* = 14) (Talpalar and Kiehn, 2010), and the effect of endogenous adenosine at each level of network activity was revealed by application of DPCPX (50 µM) to block A_1 receptors, as previously described (Witts, Panetta and Miles, 2012). The change in frequency of locomotor-related bursting following DPCPX application increased with NMDA concentration (Fig. 7, A-D; *F*[2,41] = 14.2, *p* < 0.001, *n* = 11-17). The frequency of locomotor-related bursting following DPCPX application was not significantly altered in 0 µM NMDA (3.1 ± 4.4%; Fig. 7, A and D; *F*[2,20] = 1.0, *p* > 0.05, *n* = 11) but increased by 13.1 ± 3.1% in 3 µM NMDA (Fig. 7, B and D; *F*[2,30] = 6.1, *p* < 0.05, *n* = 16) and by 24.4 ± 2.5% in 5 µM NMDA (Fig. 7, C and D; *F*[2,32] = 11.9, *p* < 0.01, *n* = 17). To confirm that the effect of endogenous adenosine was related to the baseline frequency of locomotor-related activity and was not instead mediated by a direct action of NMDA on glia, the effect of DPCPX on low-frequency activity generated by 10 µM 5-HT and 50 µM DA in standard aCSF containing 3 mM K^+ was compared with the effect of DPCPX on a higher baseline frequency (0.24 ± 0.017

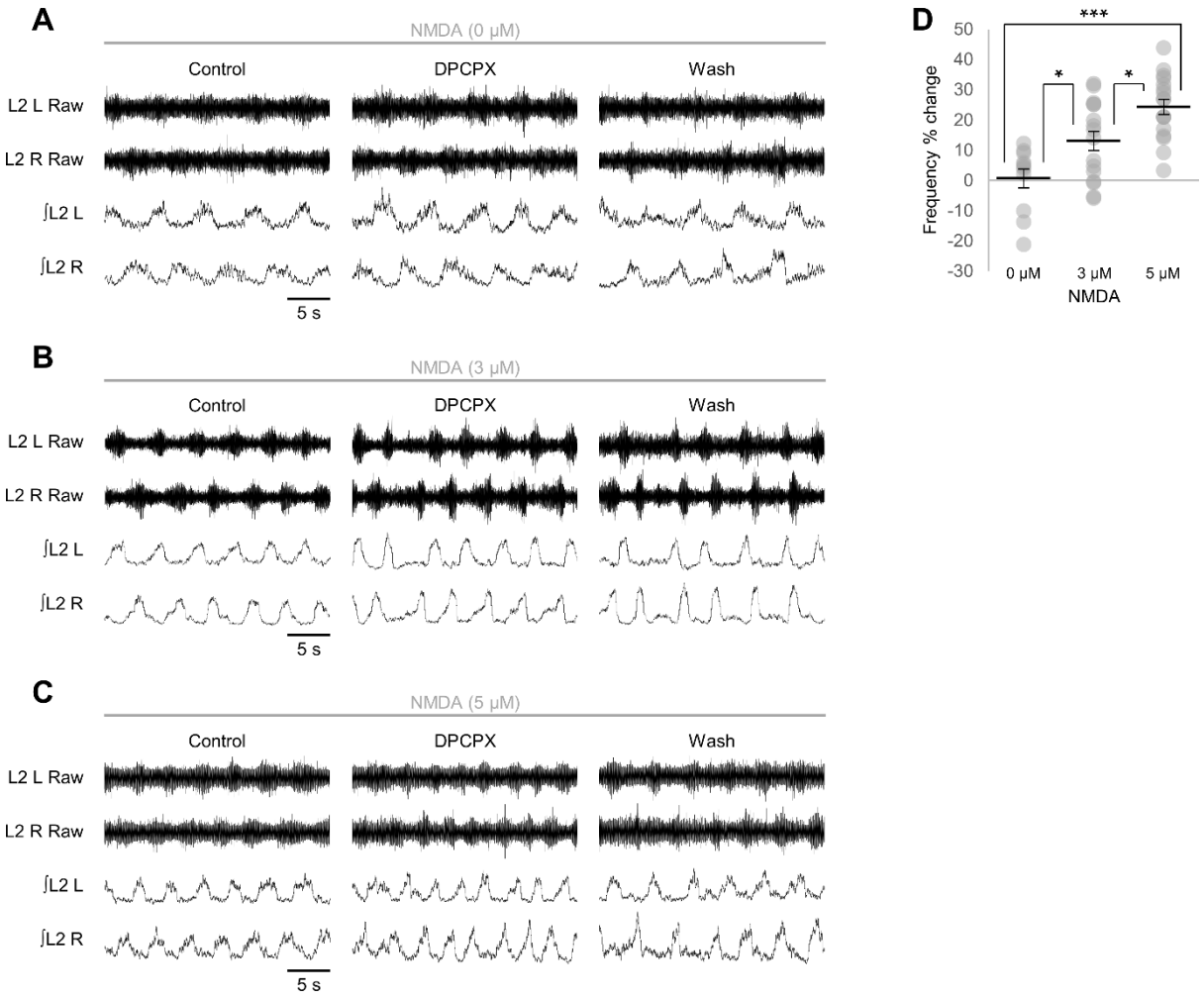


Fig. 7. Adenosinergic modulation of locomotor networks scales with network activity. A-C: raw (top) and rectified/integrated (bottom) traces recorded from left (L) and right (R) L_2 ventral roots showing the effect of the A1-receptor antagonist DPCPX (50 µM) in preparations in which locomotor-related activity was evoked by 5-HT (10 µM) and DA (50 µM) alone (A) or with 3 µM NMDA (B) or 5 µM NMDA (C). D: percentage change in locomotor-burst frequency in response to DPCPX application in preparations in which locomotor-related activity was evoked at different frequencies using 0 µM NMDA (n = 11), 3 µM NMDA (n = 16) and 5 µM NMDA (n = 17), calculated by comparing a 10 min control period with the last 10 min of a 30 min application of DPCPX. Individual data points are shown in grey, and means are represented by black lines. Error bars: ± SEM. Statistically significant differences in pairwise comparisons: *$p < 0.05$, ***$p < 0.001$.

Hz, n = 6) induced by 5-HT and DA at the same concentrations in aCSF containing 5 mM K^+. In high-K^+ solution, DPCPX raised burst frequency by 23.0 ± 6.6 % (F[2,10] = 8.7, p < 0.01, n = 6), differing significantly from its effect in 3 mM K^+ solution (n = 11; Student's t-test, p < 0.05). Together these findings indicate that the influence of adenosine on locomotor-related bursting scales with the level of network activity, suggesting detection of neural activity by glia and proportional release of ATP-adenosine.

Three-enzyme microelectrode biosensors for the detection of adenosine are not suitable for use in a mouse spinal cord preparation

It was then considered whether activity-dependent production of adenosine could be confirmed in hemicords by means of three-enzyme microelectrode biosensors. Hemicords, unlike intact spinal cords, provide a surface along which the ventral-horn grey matter containing cells of the locomotor circuitry is exposed (Fig. 8, A and B).

In brief, Sarissa adenosine biosensors consist of three enzymes held in a composite matrix surrounding a Pt/Ir microelectrode: adenosine deaminase converts adenosine to inosine and NH_3; purine nucleoside phosphorylase converts inosine and Pi to hypoxanthine and ribose-phosphate; and xanthine oxidase converts hypoxanthine to xanthine and H_2O_2, and xanthine to urate and H_2O_2; finally, H_2O_2 is converted to electrogenic H^+ and H_2O on the Pt/Ir microelectrode when the latter is polarised to +500 mV. Thus, adenosine generates a current in the biosensor proportionate to its concentration. Because other electrogenic substances (e.g. DA), as well as substrates of purine nucleoside phosphorylase and xanthine oxidase, may also be present in neural tissue, a sensor lacking adenosine deaminase but otherwise identical to the adenosine sensor was used in these experiments as a control.

To test the sensitivity and specificity of adenosine and inosine (null) biosensors, they were positioned in a 15 ml bath containing aCSF and washed with control solutions. Bath application of adenosine (10 µM; Fig. 8C) induced robust currents in adenosine sensors (mean = 2290.0 ± 224.8 pA, n = 14) and modest currents in null sensors (mean = 172.2 ± 43.3 pA, n = 14).

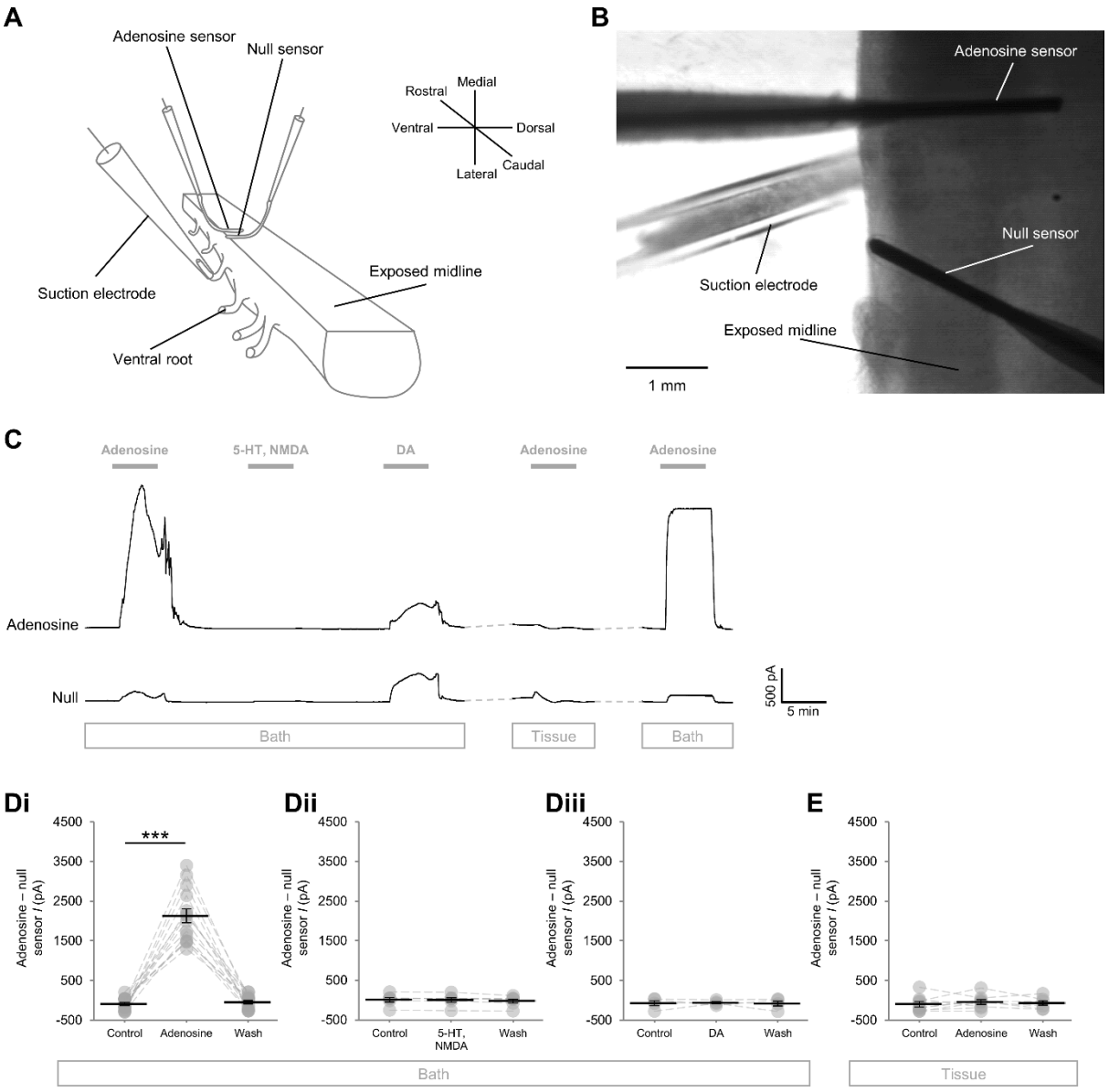

A
Adenosine sensor
Null sensor
Rostral
Medial
Ventral
Dorsal
Caudal
Lateral
Suction electrode
Exposed midline
Ventral root
B
Adenosine sensor
Null sensor
Suction electrode
Exposed midline
1 mm
C
Adenosine
5-HT, NMDA
DA
Adenosine
Adenosine
Adenosine
Null
500 pA
5 min
Bath
Tissue
Bath
Di
Dii
Diii
E
Adenosine – null sensor I (pA)
4500
3500
2500
1500
500
-500
***
Control
Adenosine
Wash
5-HT, NMDA
DA
Bath
Tissue

Fig. 8. Microelectrode biosensors for adenosine lose sensitivity when in contact with mouse spinal cord tissue. A: schematic illustrating the positioning of adenosine and null (inosine) biosensors within the same lumbar segment on the cut surface of a hemicord. B: light-microscope image showing the same preparation from above. C: example traces showing the responses of adenosine (top) and null (bottom) biosensors to bath applied adenosine, 5-HT and NMDA, and DA when sensors were placed in the bath without contact with tissue, and to adenosine when sensors were positioned as shown in A and B, and when sensors were placed again in the bath away from tissue. D: corrected (adenosine - null) peak currents recorded from biosensors positioned in the bath, without contact with tissue, upon application of (i) 10 µM adenosine (n = 14), (ii) 10 µM 5-HT and 5 µM NMDA (n = 6), and (iii) 50 µM DA (n = 4). Individual data points representing currents are shown in grey, and means are represented by black lines. E: corrected (adenosine - null) currents recorded from biosensors positioned in contact with spinal cord tissue as in A and B upon bath application of 10 µM adenosine (n = 9). Error bars: ± SEM. Statistically significant difference from control: ***$p < 0.001$.

Mean corrected current (adenosine - null sensor) was 2220.0 ± 197.7 pA (Fig. 8Di; $p < 0.001$, $n = 14$), similar to previous reports (Llaudet *et al.*, 2003; Wall and Dale, 2007), and indicating a selective response to adenosine. The response of biosensors to drugs used to elicit locomotor-related activity in spinal cord preparations was also tested. Although biosensors displayed modest responses to co-applied 5-HT (10 µM) and NMDA (5 µM; Fig. C; adenosine senor: 272.2 ± 83.1 pA, $n = 6$; null senor: 268.4 ± 71.6 pA, $n = 6$), the corrected response was not significant (Fig. 8Dii; $p > 0.05$, $n = 6$). Similarly, biosensors displayed modest responses to DA (50 µM; Fig. C; adenosine senor: 482.9 ± 44.6 pA, $n = 4$; null senor: 472.1 ± 91.4 pA; $n = 6$), but the corrected response was not significant (Fig. 8Diii; $p > 0.05$, $n = 4$). Together, these results indicate that biosensors could be used for the selective detection of adenosine in preparations to which 5-HT, NMDA and DA have been applied.

The sensitivity of biosensors to adenosine when they were placed in contact with spinal cord preparations was then assessed. Biosensors were positioned so that the lower surface of the wire probe made contact with the exposed midline surface of hemicords, leaving the upper surface of the probe exposed. Surprisingly, sensitivity to adenosine was lost under these conditions (Fig. 8, C and E; $p > 0.05$, $n = 9$), but in 2/2 experiments adenosine-sensitivity was restored when sensors were moved from the tissue to the bath (Fig. 8C). These findings indicate that adenosine biosensors of this design are unsuitable for use in the mouse spinal cord.

Adenosine modulates the frequency of synaptic inputs onto interneurons during locomotor-related activity in hemicords

To determine the effects of glial cell-derived adenosine on synaptic transmission and the passive membrane properties of interneurons during network activity, whole-cell patch-clamp recordings in voltage-clamp mode were made from rhythmically active cells at the exposed midline surface of hemicord preparations (Zagoraiou *et al.*, 2009). Rhythmic activity was recorded in the L_1 or L_2 ventral root following bath application of 5-HT (10 µM), NMDA (5 µM)

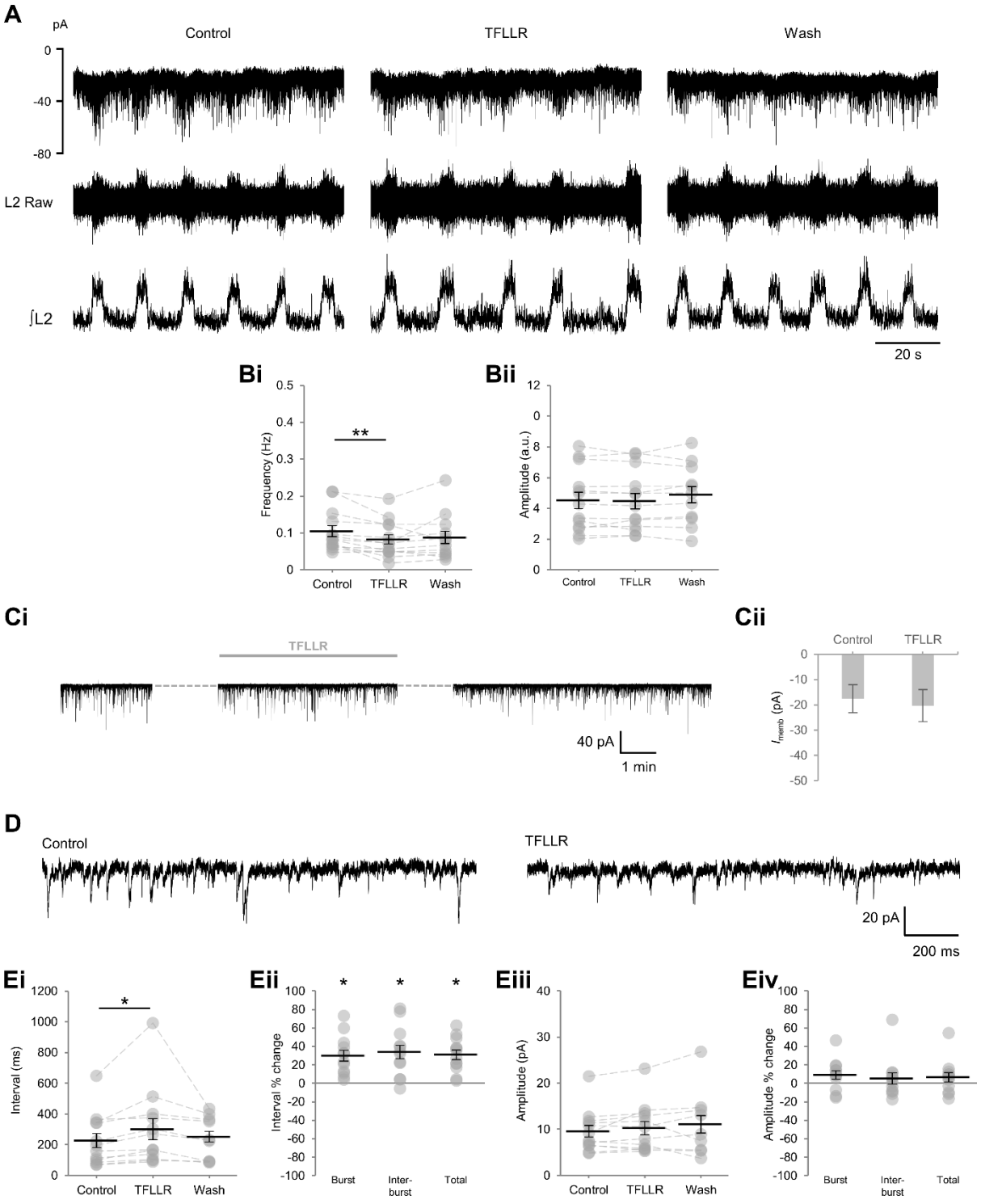

A
pA
Control
TFLLR
Wash
0
-40
-80
L2 Raw
∫L2
20 s
Bi
Frequency (Hz)
**
Control
TFLLR
Wash
Bii
Amplitude (a.u.)
Control
TFLLR
Wash
Ci
TFLLR
40 pA
1 min
Cii
Control
TFLLR
I_{memb} (pA)
D
Control
TFLLR
20 pA
200 ms
Ei
Interval (ms)
*
Control
TFLLR
Wash
Eii
Interval % change
*
*
*
Burst
Inter-burst
Total
Eiii
Amplitude (pA)
Control
TFLLR
Wash
Eiv
Amplitude % change
Burst
Inter-burst
Total

Fig. 9. PAR1 stimulation reduces ventral-root bust frequency and synaptic inputs onto interneurons in hemicords. A: voltage-clamp recording from a ventral horn interneuron held at -60mV (top trace) and raw (top) and rectified/integrated (bottom) traces recorded from the L_2 ventral root in a hemicord showing the effects of the TFLLR (10 µM). Bi: locomotor-burst frequency over six bursts at the end of a control period, a 5-min application of TFLLR, and a 20 min washout period. *n* = 13 preparations. Individual data points are shown in grey, and means are represented by black lines. Bii: locomotor-burst amplitude over six bursts at the end of a control period, a 5-min application of TFLLR, and a 20 min washout period. *n* = 13. Ci: voltage-clamp recording from a ventral horn interneuron held at -60mV in a rhythmically active hemicord showing no change in holding current upon application of TFLLR. *n* = 12 cells. Cii: Holding current (I_{memb}) over 30 s at the end of a control period and a 5-min application of TFLLR. *n* = 12. D: voltage-clamp recording from a ventral horn interneuron held at -60mV in a rhythmically active hemicord showing synaptic inputs during a ventral-root burst in a control period and at the end of a 5-min application of TFLLR. *n* = 12. Ei: the interval between synaptic events recorded from a ventral horn interneuron held at -60mV over six bursts and interburst intervals at the end of a control period, a 5-min application of TFLLR and a 20-min washout. *n* = 12. Eii: percentage change in the interval between synaptic events elicited by a 5-min application of TFLLR during ventral-root bursts, between ventral-root bursts and in total. *n* = 12. Eiii: the amplitude of synaptic events recorded from a ventral horn interneuron held at -60mV over six bursts and interburst intervals at the end of a control period, a 5-min application of TFLLR and a 20-min washout. *n* = 12. Eiv: percentage change in the amplitude of synaptic events elicited by a 5-min application of TFLLR during ventral-root bursts, between ventral-root bursts and in total. *n* = 12. Error bars: ± SEM. Significantly different from control: **p* < 0.05, ***p* < 0.01.

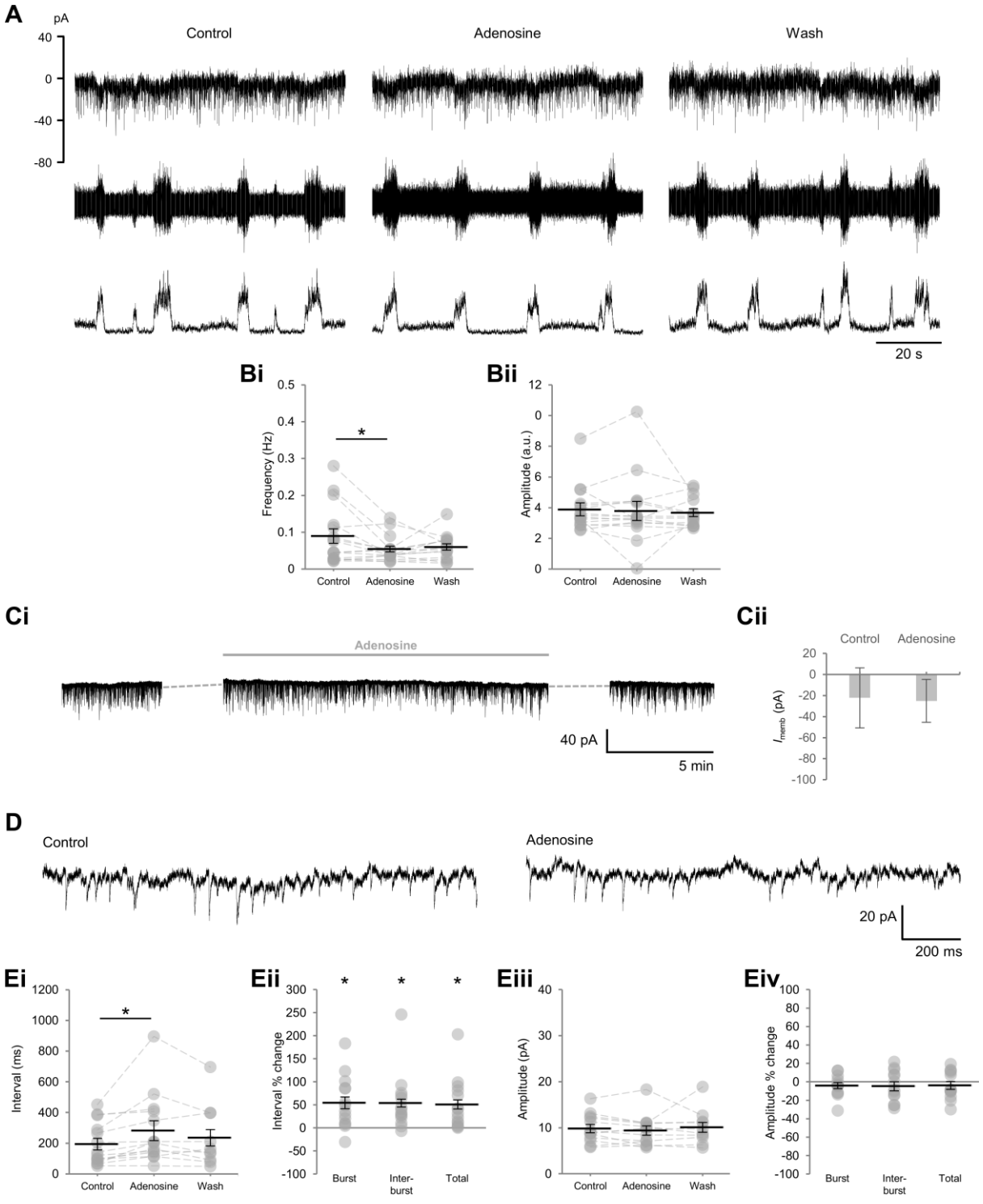

A
pA
40
0
-40
-80
Control
Adenosine
Wash
20 s
Bi
Frequency (Hz)
0.5
0.4
0.3
0.2
0.1
0
*
Control
Adenosine
Wash
Bii
Amplitude (a.u.)
12
0
8
6
4
2
0
Control
Adenosine
Wash
Ci
Adenosine
40 pA
5 min
Cii
Control
Adenosine
20
0
-20
-40
-60
-80
-100
I_{memb} (pA)
D
Control
Adenosine
20 pA
200 ms
Ei
Interval (ms)
1200
1000
800
600
400
200
0
*
Control
Adenosine
Wash
Eii
Interval % change
300
250
200
150
100
50
0
-50
-100
*
*
*
Burst
Inter-burst
Total
Eiii
Amplitude (pA)
40
30
20
10
0
Control
Adenosine
Wash
Eiv
Amplitude % change
100
80
60
40
20
0
-20
-40
-60
-80
-100
Burst
Inter-burst
Total

Fig. 10. Adenosine reduces ventral-root bust frequency and synaptic inputs onto interneurons in hemicords. A: voltage-clamp recording from a ventral horn interneuron held at -60mV (top trace) and raw (top) and rectified/integrated (bottom) traces recorded from the L_2 ventral root in a hemicord showing the effects of the adenosine (75 µM). Bi: locomotor-burst frequency over six bursts at the end of a control period, a 15-min application of adenosine, and a 20 min washout period. *n* = 16 preparations. Individual data points are shown in grey, and means are represented by black lines. Bii: locomotor-burst amplitude over six bursts at the end of a control period, a 15-min application of adenosine, and a 20 min washout period. *n* = 16. Ci: voltage-clamp recording from a ventral horn interneuron held at -60mV in a rhythmically active hemicord showing no change in holding current following application of adenosine. *n* = 14 cells. Cii: holding current (I_{memb}) over 30 s at the end of a control period and a 15-min application of adenosine. *n* = 14. D: voltage-clamp recording from a ventral horn interneuron held at -60mV in a rhythmically active hemicord showing synaptic inputs during a ventral-root burst in a control period and at the end of a 15-min application of adenosine. *n* = 14. Ei: the interval between synaptic events recorded from a ventral horn interneuron held at -60mV over six bursts and interburst intervals at the end of a control period, a 15-min application of adenosine and a 20-min washout. *n* = 14. Eii: percentage change in the interval between synaptic events elicited by a 15-min application of adenosine during ventral-root bursts, between ventral-root bursts and in total. *n* = 14. Eiii: the amplitude of synaptic events recorded from a ventral horn interneuron held at -60mV over six bursts and interburst intervals at the end of a control period, a 15-min application of adenosine and a 20-min washout. *n* = 14. Eiv: percentage change in the amplitude of synaptic events elicited by a 15-min application of adenosine during ventral-root bursts, between ventral-root bursts and in total. *n* = 14. Error bars: ± SEM. Significantly different from control: $*p < 0.05$.

and DA (50 µM). Interneurons within the same segment as the ventral root received synaptic inputs both during and between ventral-root bursts (holding potential: -60 mV; Fig. 9A, 10A and 11A). Cells were determined to receive inputs in a rhythmic manner related to ventral-root bursting if the interval between synaptic inputs was smaller within than between bursts. For rhythmically active cells, the average intraburst interval was 198.9 ± 24.0 ms and the average interburst interval was 305.1 ± 36.8 ms ($p < 0.001$; $n = 36$).

To determine whether adenosinergic modulation is similar between hemicords and whole spinal cords, TFLLR, adenosine and DPCPX were applied to rhythmically active hemicords. Stimulation of glia by TFLLR (10 µM) resulted in a reduction in the frequency (18.6 ± 4.4%; Fig. 9, A and Bi; $p < 0.01$; $n = 13$) of ventral-root activity in hemicords, with no change in its amplitude (Fig. 9, A and Bii; $p > 0.05$; $n = 13$). Similarly, bath application of adenosine (75 µM) (Witts, Panetta and Miles, 2012) resulted in a reduction in the frequency of activity (28.6 ± 8.2%; Fig. 10, A and Bi; $p < 0.05$; $n = 16$) with no change in amplitude (Fig. 10, A and Bii; $p > 0.05$; $n = 16$). Conversely, blockade of A_1 receptors by DPCPX (1 µM) (Acevedo *et al.*, 2016) resulted in a substantial increase in the frequency of activity (105.2 ± 34.9%; Fig. 11, A and Bi; $p < 0.05$; $n = 10$) that typically did not reverse during washout, with no change in amplitude (Fig. 11, A and Bii; $p > 0.05$; $n = 10$). Thus, adenosinergic modulation of network activity is similar in hemicords and whole spinal-cord preparations.

Given previous reports that bath applied adenosine induces a hyperpolarising current in ventral horn interneurons held at -60 mV in acute slices (Witts, Nascimento and Miles, 2015), it was next assessed whether TFLLR, adenosine or DPCPX has a similar effect on rhythmically active cells. However, no deflection in the membrane holding current (I_{memb}) was detected in interneurons in hemicords when TFLLR (Fig. 9C; $p > 0.05$; $n = 12$), adenosine (Fig. 10C; $p > 0.05$; $n = 14$) or DPCPX (Fig. 11C; $p > 0.05$; $n = 10$) were applied.

Because, in slices, adenosine acting at A_1 receptors modulates both the frequency and amplitude of postsynaptic currents recorded from interneurons, whereas A_1 blockade alone has no effect (Witts, Nascimento and Miles, 2015), the effects of TFLLR, adenosine and

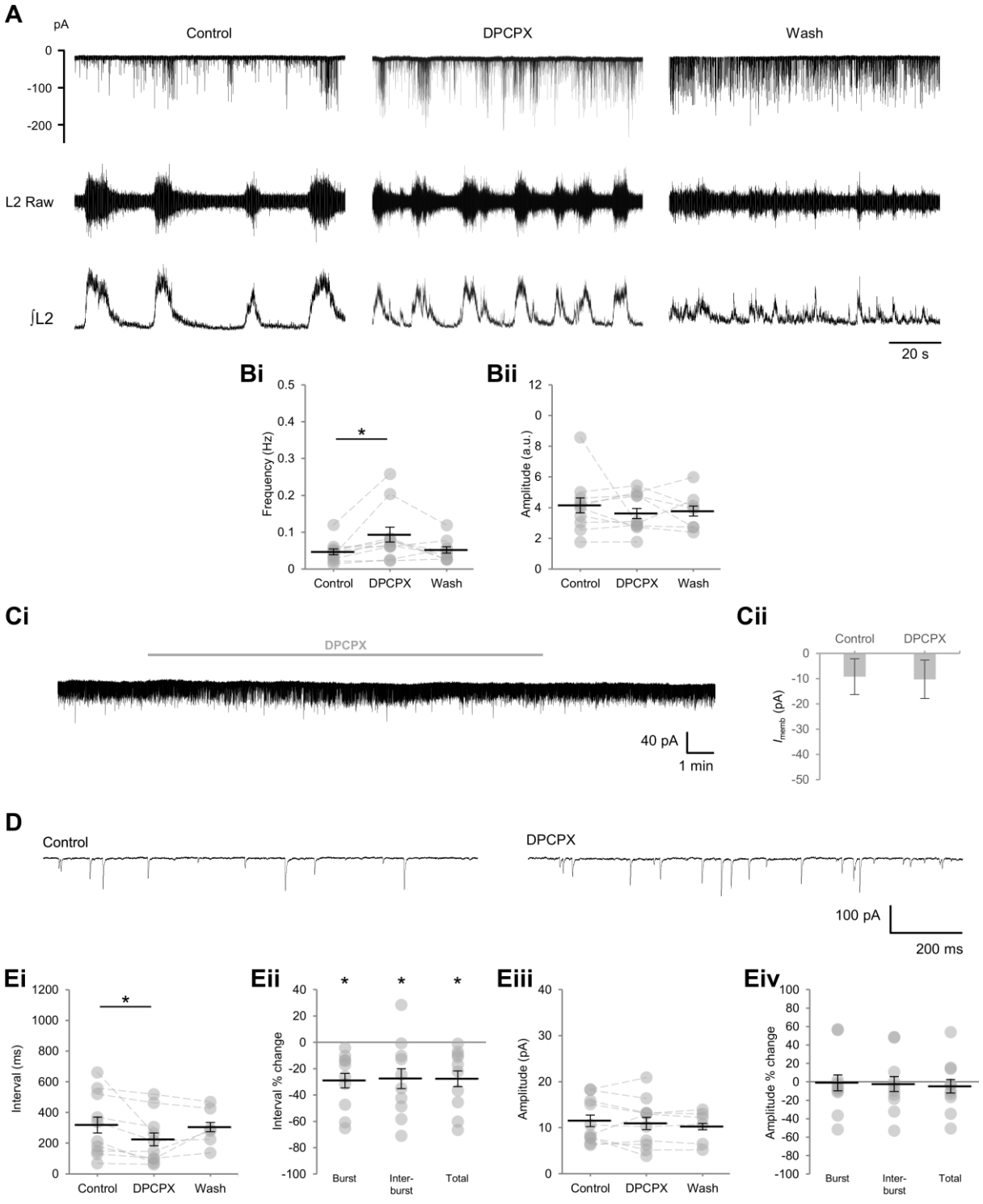

A
pA
Control
DPCPX
Wash
0
-100
-200
L2 Raw
∫L2
20 s
Bi
Frequency (Hz)
0.5
0.4
0.3
0.2
0.1
0
*
Control
DPCPX
Wash
Bii
Amplitude (a.u.)
12
0
8
6
4
2
0
Control
DPCPX
Wash
Ci
DPCPX
40 pA
1 min
Cii
Control
DPCPX
I_{memb} (pA)
0
-10
-20
-30
-40
-50
D
Control
DPCPX
100 pA
200 ms
Ei
Interval (ms)
1200
1000
800
600
400
200
0
*
Control
DPCPX
Wash
Eii
Interval % change
40
20
0
-20
-40
-60
-80
-100
*
*
*
Burst
Inter-burst
Total
Eiii
Amplitude (pA)
40
30
20
10
0
Control
DPCPX
Wash
Eiv
Amplitude % change
100
80
60
40
20
0
-20
-40
-60
-80
-100
Burst
Inter-burst
Total

Fig. 11. A_1 receptor blockade reduces ventral-root bust frequency and synaptic inputs onto interneurons in hemicords. A: voltage-clamp recording from a ventral horn interneuron held at -60mV (top trace) and raw (top) and rectified/integrated (bottom) traces recorded from the L_2 ventral root in a hemicord showing the effects of the DPCPX (1 µM). Bi: locomotor-burst frequency over six bursts at the end of a control period, a 20-min application of DPCPX, and a 20 min washout period. *n* = 10 preparations. Individual data points are shown in grey, and means are represented by black lines. Bii: locomotor-burst amplitude over six bursts at the end of a control period, a 20-min application of DPCPX, and a 20 min washout period. *n* = 10. Ci: voltage-clamp recording from a ventral horn interneuron held at -60mV in a rhythmically active hemicord showing no change in holding current following application of DPCPX. *n* = 10 cells. Cii: holding current (I_{memb}) over 30 s at the end of a control period and a 20-min application of DPCPX. *n* = 10. D: voltage-clamp recording from a ventral horn interneuron held at -60mV in a rhythmically active hemicord showing synaptic inputs during a ventral-root burst in a control period and at the end of a 20-min application of DPCPX. *n* = 10. Ei: the interval between synaptic events recorded from a ventral horn interneuron held at -60mV over six bursts and interburst intervals at the end of a control period, a 20-min application of DPCPX and a 20-min washout. *n* = 10. Eii: percentage change in the interval between synaptic events elicited by a 20-min application of DPCPX during ventral-root bursts, between ventral-root bursts and in total. *n* = 10. Eiii: the amplitude of synaptic events recorded from a ventral horn interneuron held at -60mV over six bursts and interburst intervals at the end of a control period, a 20-min application of DPCPX and a 20-min washout. *n* = 10. Eiv: percentage change in the amplitude of synaptic events elicited by a 20-min application of DPCPX during ventral-root bursts, between ventral-root bursts and in total. *n* = 10. Error bars: ± SEM. Significantly different from control: $*p < 0.05$.

DPCPX on synaptic inputs to rhythmically active interneurons were assessed next. Depolarising synaptic events represent combined excitatory and inhibitory postsynaptic currents (EPSCs; IPSCs), as a function of a -60 mV holding potential and the reversal potential of Cl^- in the solutions used here. When TFLLR was applied to hemicords to induce release of ATP-adenosine from glia, the interval between synaptic events in rhythmically active interneurons increased (34.2 ± 5.1%; Fig. 9, A, D, Ei and Eii; $p < 0.05$; $n = 12$), but the amplitude was unchanged (Fig. 9, A, D, Eiii and Eiv; $p > 0.05$; $n = 12$). Similarly, bath-applied adenosine increased the interval between synaptic events (46.9 ± 9.7%; Fig. 10, A, D, Ei and Eii; $p < 0.05$; $n = 14$) but did not modulate amplitude (Fig. 10, A, D, Eiii and Eiv; $p > 0.05$; $n = 14$). Conversely, blockade of A1 receptors with DPCPX reduced the interval between synaptic events (30.7 ± 6.7%; Fig. 11, A, D, Ei and Eii; $p < 0.05$; $n = 10$), but also did not alter their amplitude (Fig. 11, A, D, Eiii and Eiv; $p > 0.05$; $n = 10$). Although cells received more synaptic inputs during than between bursts, event frequency was modulated to a similar degree during intra- and interburst periods by TFLLR (Fig. 9Eii), adenosine (Fig. 10Eii) and DPCPX (Fig. 11Eii). These results indicate that endogenous and exogenous adenosine modulate the frequency but not the amplitude of synaptic inputs in hemicords, consistent with changes in the frequency of ventral-root bursting.

Discussion

This chapter provides evidence that glia contribute to the operation of spinal motor networks by the secretion of neuromodulatory ATP-adenosine in an activity-dependent manner. This study extends a previous characterisation of the modulation of network output by purines and corroborates the proposal that glia are the source of neuromodulatory adenosine in the spinal motor circuitry (Witts, Panetta and Miles, 2012). Furthermore, adenosine is proposed as the primary, if not sole, gliotransmitter responsible for the modulation of mouse spinal locomotor networks.

Glia have been shown to release a number of substances including glutamate, D-serine, ATP and GABA upon experimental stimulation in both the brain and spinal cord, with diverse effects on synaptic transmission (Araque *et al.*, 1999, 2014). Stimuli are proposed to reproduce the cytosolic Ca^{2+} elevations evoked in glia following the activation of endogenous receptors by transmitters released during neuronal activity, a phenomenon that has been observed throughout the CNS, both *in vitro* and *in vivo* (Nedergaard and Verkhratsky, 2012; Volterra, Liaudet and Savtchouk, 2014). Here, it is shown that experimental stimulation of glia by activation of PAR1 during ongoing locomotor-like activity within spinal motor networks results in a reversible reduction in the frequency of locomotor-related bursting associated with an increase in burst duration, with no effect on duty cycle or burst amplitude.

PAR1 is an endogenous G-protein coupled receptor associated with $G_{\alpha q}$ proteins, and its activation results in the release of Ca^{2+} from internal stores in cortical slice preparations, followed by Ca^{2+}-dependent release of glutamate or ATP (Lee *et al.*, 2007; Shigetomi *et al.*, 2008; Lalo *et al.*, 2014). Similar glial cell-specific effects of PAR1 activation are proposed in the ventral horn of the spinal cord (Carlsen and Perrier, 2014). It is shown in the present study that GFAP+ cells in the spinal cord preferentially express PAR1. Although these cells are likely to be astrocytes, consistent with reports of preferential expression by astrocytes in the brain and brainstem (Weinstein *et al.*, 1995; Junge *et al.*, 2004), the possibility that neural precursor cells of the astrocyte lineage also express PAR1 cannot be excluded; previously it was suggested that a subset of these cells expresses GFAP in the spinal cords of postnatal rats (Chvátal *et al.*, 1995). Although clear PAR1 immunofluorescence in MAP2+ neurons is not detected, this finding cannot exclude the possibility of low-level PAR1 expression by neurons. To confirm that the effects of PAR1 activation on network output are mediated by glia and that the PAR1-selective agonist TFLLR has no off-target effects on neurons, TFLLR was applied to preparations following pharmacological ablation of glia by FA and MSO. Toxins that disrupt normal glial metabolism have been used in several studies to elucidate the role of glia in neuromodulation and homeostasis (Clarke, 1991; Fonnum, Johnsen and Hassel, 1997; Zhang

et al., 2003; Huxtable *et al.*, 2010; Witts, Panetta and Miles, 2012; Li *et al.*, 2013; Wall and Dale, 2013). Although both MSO, an inhibitor of glutamine synthetase (Ronzio, Rowe and Meister, 1969) and FA, a precursor of the aconitase inhibitor fluorocitrate (Fonnum, Johnsen and Hassel, 1997), are able to cross neuronal membranes (Hassel *et al.*, 1992) and have actions that may not result directly from the disruption of glial metabolism (Clarke, 1991; Largo, Ibarz and Herreras, 1997; Young *et al.*, 2005), neither prevents the rhythmic activity of motor networks when co-applied with glutamine to ensure continued synthesis of glutamate and GABA (Hülsmann *et al.*, 2000; Huxtable *et al.*, 2010; Witts, Panetta and Miles, 2012). Furthermore, FA and MSO do not prevent the modulation of motor networks by exogenously applied neuromodulators (Huxtable *et al.*, 2010; Witts, Panetta and Miles, 2012). It is therefore proposed that the modulation of locomotor network output by TFLLR reflects selective activation of PAR1 expressed by spinal glia rather than any off-target effects on neurons, consistent with previous reports that TFLLR does not act on neurons in the brain (Lee *et al.*, 2007; Lalo *et al.*, 2014).

The effects of PAR1 activation on network output closely resemble those of exogenously applied adenosine, which likewise depresses the frequency of locomotor-related bursting without modulating burst amplitude or left-right alternation (Witts, Panetta and Miles, 2012). In support of A_1 adenosine receptor-mediated modulation of the locomotor CPG following PAR1 activation, the modulation of network output is efficiently prevented by either the general adenosine-receptor antagonist theophylline or the A_1 antagonist DPCPX, but not by the A_{2A} antagonist SCH58261. Similarly, inhibition of A_1 receptors, but not A_{2A} receptors is sufficient to abolish the modulation of locomotor-related activity by endogenous adenosine (Witts, Panetta and Miles, 2012), and DPCPX abolishes the PAR1-evoked modulation of excitatory currents recorded from ventral horn interneurons (Carlsen and Perrier, 2014). Of the four known adenosine receptor subtypes, A_1 and A_{2A} receptors have the highest affinity for adenosine, and in many regions A_1-mediated inhibition balances A_{2A}-mediated facilitation (Cunha, 2001). Although both receptors are expressed throughout the spinal cord (Reppert *et*

al., 1991; Deuchars, Brooke and Deuchars, 2001; Paterniti *et al.*, 2011), A_{2A} receptors do not appear to modulate the activity of the locomotor CPG (Fig. 4, E and F; Witts, Panetta and Miles, 2012).

Given that glia are shown in numerous systems to be competent to release a range of modulators (Araque *et al.*, 1999, 2014), and that diverse neuromodulators regulate spinal motor networks (Miles and Sillar, 2011), it may be surprising that PAR1 stimulation of glia results in the modulation of locomotor network activity by adenosine alone. Although glia are proposed to exercise fine control over synaptic transmission (Araque *et al.*, 1999; Perea, Navarrete and Araque, 2009; Navarrete and Araque, 2011), the findings presented in this chapter suggest that the diversity of neuronal signalling mechanisms in the spinal cord is not reflected by a similar diversity of gliotransmitters. Instead, adenosine derived from glia appears to provide broad negative feedback control of locomotor networks. It should, however, be noted that the choice of technique employed to stimulate glia may influence gliotransmitter release (Shigetomi *et al.*, 2008; Wang *et al.*, 2013) and for this reason the possibility that spinal glia release other gliotransmitters in response to different stimuli cannot be excluded.

Under non-pathological conditions, extracellular adenosine is largely derived from the hydrolysis of ATP by ectonucleotidases (Dunwiddie, Diao and Proctor, 1997); however, it may also be released directly via exocytosis or equilibrative nucleoside transporters (Klyuch, Dale and Wall, 2012; Wall and Dale, 2013). Several studies show Ca^{2+}-dependent release of ATP by glia, with subsequent conversion of ATP to adenosine and activation of either A_1 or A_{2A} receptors (Pascual *et al.*, 2005; Serrano *et al.*, 2006; Panatier *et al.*, 2011; Carlsen and Perrier, 2014). The reduction in frequency of locomotor-like bursting detected following PAR1 activation is abolished in the presence of the ectonucleotidase inhibitor ARL67156, implying that ATP and not adenosine is released by glia following stimulation. In other networks, including the mammalian respiratory CPG (Lorier *et al.*, 2007) and tadpole locomotor CPG (Dale and Gilday, 1996), modulation of neuronal activity by adenosine opposes modulation by ATP. By contrast, the present study indicates that spinal motor networks in the mouse are

modulated by adenosine derived from the extracellular hydrolysis of ATP, but not by ATP directly, a finding supported by a previous investigation of purinergic modulation of the murine locomotor CPG (Witts, Panetta and Miles, 2012).

Neither blockade of A_1 receptors nor stimulation of glia influences the frequency of synchronous, disinhibited bursting generated and coordinated exclusively by excitatory components of the motor circuitry, consistent with a previous finding that exogenously applied adenosine has no effect on disinhibited bursting (Witts, Panetta and Miles, 2012). Although it remains uncertain whether common circuit elements underlie both disinhibited and left-right alternating rhythms, these data suggest that adenosine modulates the frequency of locomotor-related bursting by acting on inhibitory interneurons. Because adenosine does not affect the contralateral alternation of locomotor related bursts, Witts *et al.* (2012) proposed a model whereby adenosine modulated the activity of ipsilaterally projecting inhibitory interneurons, of which V1 local circuit inhibitory neurons are an example (Alvarez *et al.*, 2005; Witts, Panetta and Miles, 2012). The ablation or inactivation of V1 interneurons results in a reduction in the frequency of locomotor-like activity (Gosgnach *et al.*, 2006), making them a candidate for future studies aiming to decipher the cellular targets of modulatory adenosine. However, recent studies (Carlsen and Perrier, 2014; Witts, Nascimento and Miles, 2015) and the data presented here (here) indicate that adenosine modulates a broader population of interneurons within the ventral horn.

Evidence is provided to show that the influence of endogenous adenosine on network output increases with the frequency of network activity, likely reflecting enhanced release of ATP-adenosine from glia and implying a mechanism for the detection of neuronal activity by glia. In other preparations, glial Ca^{2+} signalling is triggered by the spillover of neurotransmitters from synapses (Araque *et al.*, 1999). A similar mechanism operating in the spinal cord may therefore involve the spillover of glutamate, glycine or GABA, all of which are endogenous neurotransmitters to which spinal glia have been shown to respond (Pastor *et al.*, 1995; Ziak, Chvátal and Syková, 1998).

Activity-dependent increases in extracellular adenosine are reported in several preparations (Wall and Dale, 2008). Of particular relevance to this study is the *Xenopus* tadpole spinal cord, in which ATP is released during swimming episodes and enhances the excitability of motor networks (Dale and Gilday, 1996). The hydrolysis of ATP by ectonucleotidases as swimming proceeds raises the concentration of extracellular adenosine, which opposes ATP to reduce network excitability and terminate swimming. By contrast, in murine spinal locomotor networks, ATP does not appear to act as a neuromodulator (Witts, Panetta and Miles, 2012; Carlsen and Perrier, 2014). Since ATP is consumed by neurons during activity, raising cytoplasmic adenosine levels, an efficient coupling mechanism would entail direct release of adenosine via neuronal equilibrative nucleoside transporters, with autocrine inhibition of activity via A_1 receptors (Cunha, 2001; Fredholm *et al.*, 2005). This mechanism does not appear to operate in mouse spinal motor networks or other systems in which the source of adenosine is ATP released into the extracellular space from either neurons or glia (Wall and Dale, 2008; Witts, Panetta and Miles, 2012). Whether adenosine production is direct or indirect, the functional consequences of the negative feedback it provides may include the stabilisation of network activity and the prevention of excitotoxicity and metabolic exhaustion.

Direct measurement of adenosine within the spinal cord could be used to confirm that glia release adenosine in an activity-dependent manner, perhaps avoiding confounding factors associated with measurements of neuronal activity. To this end, the suitability of Sarissa three-enzyme biosensors for use in murine spinal cord tissue was investigated. Microelectrode biosensors detect release of adenosine in real time, cause minimal damage to tissue and are highly selective (Dale, 2013). In addition, they permit relative and, under some experimental conditions, absolute quantitation of adenosine. These properties have been useful in demonstrating activity dependent adenosine production in a variety of preparations (Wall and Dale, 2008). Evidence is provided that Sarissa three-enzyme biosensors are unsuitable for use in the mouse spinal cord. Detection of control applications of adenosine by biosensors is abolished when biosensors make contact with spinal cord tissue. It is unlikely that this

inhibition of sensor function is due to restricted diffusion of adenosine within spinal cord tissue, as the upper surface of the probe was exposed in these experiments. Instead, it is likely that the mouse spinal cord, unlike the brain, contains at least one diffusible factor that reversibly inhibits the function of one or more enzymes of the biosensor. Consistent with these data, it is reported that sensors had reduced sensitivity to adenosine in the spinal cord of *Xenopus* tadpoles compared to the brain (Llaudet *et al.*, 2003). An alternative method such as microdialysis in conjunction with HPLC may be more useful in confirming the activity-dependent production of adenosine from glia implied by electrophysiological recordings from neurons.

Adenosinergic modulation of synaptic activity and the passive membrane properties of ventral horn interneurons was previously reported in slice preparations (Carlsen and Perrier, 2014; Witts, Nascimento and Miles, 2015); however, it was not determined whether effects previously reported are relevant to cells in rhythmically active preparations. In slices, adenosine reduces the frequency of synaptic events by a presynaptic mechanism (Witts, Nascimento and Miles, 2015). Similarly, in rhythmically active hemicords, the frequency of synaptic events recorded from interneurons decreases when adenosine is bath applied or released from glia following PAR1 activation. Conversely, blockade of A_1 receptors increases the frequency of postsynaptic currents. Despite the considerable genetic and physiological heterogeneity of spinal cord interneurons (Kiehn, 2006; Bikoff *et al.*, 2016), and the expectation that adenosine modulates only a subset of inhibitory interneurons (see above), these effects are consistent across the interneurons sampled. Similarly, interneurons in acute slices display consistent responses to adenosine (Carlsen and Perrier, 2014; Witts, Nascimento and Miles, 2015). In slices, adenosine reduces the frequency of synaptic events in the presence of TTX, implying that this effect is not network dependent (Witts, Nascimento and Miles, 2015). Furthermore, changes in synaptic activity in hemicords are evenly distributed across intra- and interburst periods, suggesting that A_1 receptor activation results in a generalised reduction in gain during network activity. However, the possibility cannot be

excluded that the changes in the frequency of synaptic inputs to interneurons observed in hemicords reflect changes in network activity rather than direct effects of adenosine. In support of this, changes in the frequency of synaptic inputs are consistent with changes in the frequency of bursting in ventral roots. In addition, synaptic effects observed in hemicords in the presence of DA may not be comparable with those observed in slices in its absence, as adenosine does not modulate network activity in the absence of DA (see Chapter 3).

Interneurons in rhythmically active networks differ from interneurons in slices in some of their responses to adenosine and DPCPX. In slices, adenosine induces a hyperpolarising current in interneurons that likely results from the opening of leak K^+ channels (Witts, Nascimento and Miles, 2015). This current is abolished in the presence of TTX, suggesting that it entails network activity. In rhythmically active hemicords, however, a hyperpolarising current is not observed. In addition, adenosine reduces the amplitude of postsynaptic currents in interneurons in slices (Witts, Nascimento and Miles, 2015), but in rhythmically active hemicords the amplitude of synaptic events is unaltered by adenosine applied at the same concentration or following PAR1 activation. The reason for these differences is unclear; it is possible that adenosinergic modulation of cells within murine spinal networks displays state dependency (see below).

Blockade of A_1 receptors abolishes the effects of bath-applied adenosine in interneurons in slices but does not otherwise alter synaptic activity (Witts, Nascimento and Miles, 2015). By contrast, blockade of A_1 receptors results in a pronounced increase in the frequency of postsynaptic currents recorded from interneurons in hemicords. This may reflect greater endogenous activation of A_1 receptors in hemicords, perhaps because of activity-dependent release of adenosine. Alternatively, it may reflect differential effects of A_1-receptor signalling in a state-dependent manner, perhaps owing to the presence of other neuromodulators. For instance, A_1 and D_1-like receptors are proposed to interact in the spinal cord to modulate locomotor-related activity (Acevedo *et al.*, 2016). Whereas DA is bath applied to activate D_1-like receptors in rhythmically active whole spinal cords and hemicords, it is likely that there is

little endogenous activation of D_1-like receptors in slices. These differences in modulatory effects in hemicords and slices require further investigation, and illustrate the importance of recording from neurons during network activity where possible.

Despite considerable evidence from *in vitro* brain preparations that glia sense activity in neighbouring neurons and, in turn, modulate that activity in a Ca^{2+}-dependent manner, there is a paucity of evidence that gliotransmission is important for the operation of brain networks and the behaviours they direct (Nedergaard and Verkhratsky, 2012; Fujita *et al.*, 2014; Petravicz, Boyt and McCarthy, 2014). Rhythmically active CPGs in the spinal cord and brainstem provide a tractable model for studying the contribution of gliotransmission to network output and thus behaviour. Previously it was shown that glia mediate purinergic modulation of rhythmically-active inspiratory networks in the brainstem, most likely owing to the Ca^{2+}-dependent release of glutamate (Huxtable *et al.*, 2010). It was also shown that brainstem glia release ATP in a Ca^{2+}-dependent manner upon acidification of the extracellular medium, stimulating activity in the phrenic nerve (Gourine *et al.*, 2010). In addition, it was recently demonstrated that glia release the Ca^{2+}-binding protein S100β in a Ca^{2+}-dependent manner to confer rhythmic bursting properties on associated neurons in the brainstem circuity for mastication (Morquette *et al.*, 2015). This study provides evidence that activity-dependent release of ATP-adenosine from glia in the mammalian spinal cord provides negative feedback onto the circuitry that controls locomotion. This feedback may stabilise activity, delay metabolic rundown and/or have a neuroprotective role. The present study informs understanding of the regulation of locomotor networks and provides an insight into the role of glia in shaping activity at the network level.

Chapter 3: Adenosine derived from glia activates A_1 receptors to inhibit signalling by excitatory D_1-like dopamine receptors

Introduction

In the previous chapter, it was shown that glia release ATP-adenosine when stimulated, and that this results in activation of A_1 receptors and inhibition of locomotor-related network activity. In this chapter, the cellular pathway by which glial adenosine acts to inhibit neuronal activity is investigated.

Within isolated murine spinal cords, endogenous adenosine acts at A_1 receptors to reduce the frequency but not the amplitude of ongoing locomotor-related activity (Witts, Panetta and Miles, 2012; Acevedo *et al.*, 2016; Chapter 2). By contrast, A_{2A} receptors, although expressed in the ventral horn (Paterniti *et al.*, 2011), do not modulate locomotor networks (Witts, Panetta and Miles, 2012; Acevedo *et al.*, 2016; Chapter 2). Glia are proposed as the principle source of adenosine in spinal motor networks, since adenosinergic modulation does not occur when glia are pharmacologically ablated (Witts, Panetta and Miles, 2012; Chapter 2); conversely, selective activation of protease-activated receptor-1 (PAR1), a $G_{\alpha q}$-linked G-protein coupled receptor (GPCR) preferentially expressed by glia, stimulates production of adenosine and activation of A_1 receptors (Chapter 2). Adenosine may be released from cells either directly or in the form of ATP, which is hydrolysed to adenosine in the extracellular space by ectonucleotidases (Cunha, 2001; Klyuch, Dale and Wall, 2012; Wall and Dale, 2013). Application of an ectonucleotidase inhibitor prevents activation of A_1 receptors in the spinal cord, either by adenosine derived from glia or when ATP is bath-applied, indicating that glia release ATP and not adenosine into the extracellular space (Witts, Panetta and Miles, 2012; Chapter 2).

Recently, it was proposed that A_1 receptors interact with D_1-like dopamine (DA) receptors in spinal locomotor circuitry (Acevedo *et al.*, 2016), as is reported in the basal ganglia (Popoli *et al.*, 1996). A_1 receptors are tightly coupled to the $G_{\alpha i}$ pathway, which mediates inhibition of adenylyl cyclase and reduces production of cyclic adenosine monophosphate (cAMP). By contrast, D_1-like receptors signal through $G_{\alpha s}$ to stimulate adenylyl cyclase and cAMP production, resulting in activation of protein kinase A (PKA). PKA regulates diverse proteins, including ion channels and neurotransmitter receptors (Abdel-Majid *et al.*, 1998; Pieper, Clerkin and MacFarlane, 2011).

DA is released within the ventral horn during locomotion (Gerin, Becquet and Privat, 1995; Gerin and Privat, 1998). DA is released principally from inputs originating in the A11 region of the hypothalamus, although other descending inputs are described (Björklund and Skagerberg, 1979; Commissiong, Gentleman and Neff, 1979; Hökfelt, Phillipson and Goldstein, 1979; Skagerberg and Lindvall, 1985). The five DA receptor subtypes are classified as either D_1- or D_2-like, and these subfamilies signal through distinct pathways (Abdel-Majid *et al.*, 1998; Pieper, Clerkin and MacFarlane, 2011). All receptor subtypes are expressed in the rodent spinal cord (Dubois *et al.*, 1986; Fleetwood-Walker, Hope and Mitchell, 1988; Zhu *et al.*, 2007, 2008).

DA has diverse modulatory actions within mammalian spinal locomotor networks mediated by both D_1- or D_2-like receptors (Madriaga *et al.*, 2009; Humphreys and Whelan, 2012; Sharples *et al.*, 2014, 2015). Although D_1-like receptors typically have excitatory effects, they are reported not to increase burst frequency in the spinal cord of postnatal mice (Humphreys and Whelan, 2012; Sharples *et al.*, 2015), but instead enhance the stability of rhythmic bursting (Sharples *et al.*, 2015). However, selective activation of D_1-like receptors initiates locomotor activity in spinalised adult mice (Lapointe *et al.*, 2009) and in isolated spinal cords from postnatal rats (Barrière, Mellen and Cazalets, 2004).Signalling by D_1-like receptors in spinal cord interneurons that determine the frequency has not been reported; however activation of D_1-like receptors expressed by motoneurons increases the open probability and open duration

of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor channels in a PKA-dependent manner, but does not alter insertion of receptors (Han and Whelan, 2009).

In the spinal cord, A_1 inhibition fails to alter the frequency of locomotor-related network activity when DA is absent or when D_1-like receptors are blocked (Acevedo *et al.*, 2016). A_1 blockade is similarly ineffective when PKA activity is inhibited (Acevedo *et al.*, 2016). However, modulation of locomotor-related bursting by A_1 receptors is restored when forskolin is applied to activate adenylyl cyclase independently of D_1-like receptor activation (Acevedo *et al.*, 2016). Thus, adenosine is proposed to inhibit adenylyl cyclase in the spinal cord, blocking signalling through the D_1-like receptor pathway. Suppression of PKA activity in this way results in a reduction in the frequency of locomotor-related activity. However, D_1-like receptors have not been shown to have the excitatory effect on network activity that is implied by this mechanism (Humphreys and Whelan, 2012; Sharples *et al.*, 2015). Furthermore, adenosine is reported to modulate neuronal passive electrical properties and synaptic activity in the ventral horn in the absence of DA (Witts, Nascimento and Miles, 2015; Acevedo *et al.*, 2016), and it has not been shown directly that adenosine released from glia requires D_1-like receptor activation in order to modulate neuronal activity.

This study assesses the role of D_1-like receptor signalling and the mechanism by which glial adenosine acts during ongoing locomotor-related activity in isolated spinal cord preparations from postnatal mice. Contrary to previous reports (Humphreys and Whelan, 2012; Sharples *et al.*, 2015), it is shown that selective activation of D_1-like receptors enhances the frequency of locomotor-related activity. Activation of D_1-like receptors is shown to be necessary for the previously-reported modulatory actions of bath-applied adenosine, endogenous adenosine acting at A_1 receptors and adenosine released from glia following PAR1 stimulation. Finally, glial cell-derived adenosine is shown to reduce burst frequency through a PKA-dependent mechanism.

Methods

Ethics Statement

All procedures performed on animals were conducted under project licence 60/13802 and personal licence 60/13802 in accordance with the UK Animals (Scientific Procedures) Act 1986 and were approved by the University of St Andrews Animal Welfare and Ethics Committee.

Tissue preparation

Spinal cords were isolated from postnatal day (P)1-P4 C57BL/6 mice as previously described (Jiang, Carlin and Brownstone, 1999). In summary, animals were killed by cervical dislocation, decapitated and eviscerated, before being transferred to a dissection chamber containing artificial cerebrospinal fluid (aCSF; equilibrated with 95% oxygen, 5% carbon dioxide, ~4°C). Spinal cords were then isolated between midthoracic and upper sacral segments, and ventral and dorsal roots were trimmed.

Ventral root recordings

Isolated spinal cords were pinned ventral-side up in a recording chamber perfused with aCSF (equilibrated with 95% oxygen, 5% carbon dioxide; RT) at 10 ml min^{-1}. Glass suction electrodes were attached to the first or second lumbar ventral roots (L_1, L_2) on each side of the spinal cord to record flexor-related activity. Locomotor-related activity was evoked by bath application of 5-hydroxytryptamine (5-HT; 15 µM) and *N*-methyl-D-aspartic acid (NMDA; 5 µM), and was characterised by rhythmic bursting alternating between contralateral ventral roots. In some experiments, protein kinase inhibitor-(14-22)-amide (14-22 amide) or (±)-1-Phenyl-2,3,4,5-tetrahydro-(1*H*)-3-benzazepine-7,8-diol hydrobromide (SKF 38393; 100 nM) was bath-applied at the onset of locomotor-related bursting. All drugs present during the control period were also present during application of further drugs and during washout. In all experiments, stable rhythmic bursting was established over a period of ~1 h prior to the control period. Rhythmic bursting was considered stable when the frequency, amplitude and duration

of bursts were unchanged over several minutes. Data were amplified and filtered (band-pass filter 30-3,000 Hz, Qjin Design), and acquired at a sampling frequency of 6 kHz with a Digidata 1440A analogue-digital converter and Axoscope software (Molecular Devices, Sunnyvale, CA). Custom-built amplifiers (Qjin Design) enabled simultaneous online rectification and integration (50-ms time constant) of raw signals.

Data analysis

Data were analysed offline with DataView software (courtesy of Dr W.J. Heitler, University of St Andrews). Ventral-root bursts were identified from rectified/integrated traces and their instantaneous frequencies, peak-to-peak amplitudes, and durations were then measured from the corresponding raw traces. Amplitude was measured as a non-calibrated unit and is presented here in arbitrary units (a.u.). For time-course plots, data were averaged across 1-min bins and normalised to a 10-min pre-control period to permit comparison between preparations. Statistical comparisons were performed on raw data averaged over 3-min periods for experiments testing the effects of PAR1 stimulation with TFLLR or 5-min periods in experiments to test other drugs. Data were analysed with repeated-measures ANOVA or Student's t-tests. Bonferroni post-hoc tests were applied to pairwise comparisons. Where appropriate, sphericity was assessed with Mauchly's test and Greenhouse-Giesser corrections were applied. p values < 0.05 were considered significant. Tests were performed in SPSS Statistics for Windows, Version 21.0 (IBM Corp. Armonk, NY) or Excel 2013 (Microsoft Corp. Redmond, WA).

Solution, drug and enzyme preparation

The aCSF used for dissections and recordings contained (in mM) 127 NaCl, 26 $NaHCO_3$, 10 glucose, 3 KCl, 2 CaCl, 1.25 NaH_2PO_4, and 1 $MgCl_2$. Adenosine and TFLLR-NH_2 were supplied by Sigma-Aldrich (Poole, UK); DPCPX was supplied by Abcam (Cambridge, UK); PKI 14-22 amide, myristoylated and SKF 38393 hydrobromide were supplied by Tocris Bioscience (Bristol, UK). All drugs were dissolved in reverse-osmosis water, except adenosine

and DPCPX, which were dissolved in DMSO. The concentration of DMSO in working solutions did not exceed 0.1% (v/v).

Results

Selective activation of D_1-like receptors increases the frequency of locomotor-related network activity

Adenosine is proposed to reduce the frequency but not the amplitude of locomotor-related activity by inhibiting signalling through the D_1-like receptor pathway (Acevedo *et al.*, 2016), implying a role for D_1-like receptors in the modulation of burst frequency but not amplitude. To determine the contribution of the D_1-like receptor pathway to the output of locomotor networks, the selective D_1-like receptor agonist SKF 38393 (100 nM) (Neumeyer *et al.*, 2003; Clemens *et al.*, 2012) was applied to isolated spinal cord preparations from postnatal mice during stable locomotor-related activity induced by NMDA (5 µM) and 5-HT (10 µM). The frequency of bursts recorded from L_2 ventral roots gradually increased over the 30 min period in which D_1-like receptors were activated, with a maximum effect of 17.5 ± 2.6%. The frequency returned to the baseline value when SKF 38393 was washed out (Fig. 1, A and B; $F[2,16] = 18.9$, $p < 0.001$, $n = 9$). Although burst amplitude was unchanged during application of SKF 38393, it increased following washout (15.1 ± 3.2%; Fig. 1, A and C; $F[2,16] = 6.6$, $p < 0.01$, $n = 9$). These data indicate that D_1-like receptors modulate the frequency of locomotor-related bursting, but not its amplitude, in conflict with previous reports that D_1-like receptors have no effect on burst frequency (Humphreys and Whelan, 2012; Sharples *et al.*, 2015).

Activation of D_1-like receptors is required for the modulation of locomotor frequency by glial cell-derived adenosine

Previous studies assessing the modulation of murine locomotor networks by adenosine were conducted in preparations in which DA was present (Witts, Panetta and Miles, 2012; Acevedo *et al.*, 2016; Chapter 2). However, blockade of D_1-like receptors in the presence of DA was reported to prevent the modulation of burst frequency by adenosine (Acevedo *et al.*, 2016).

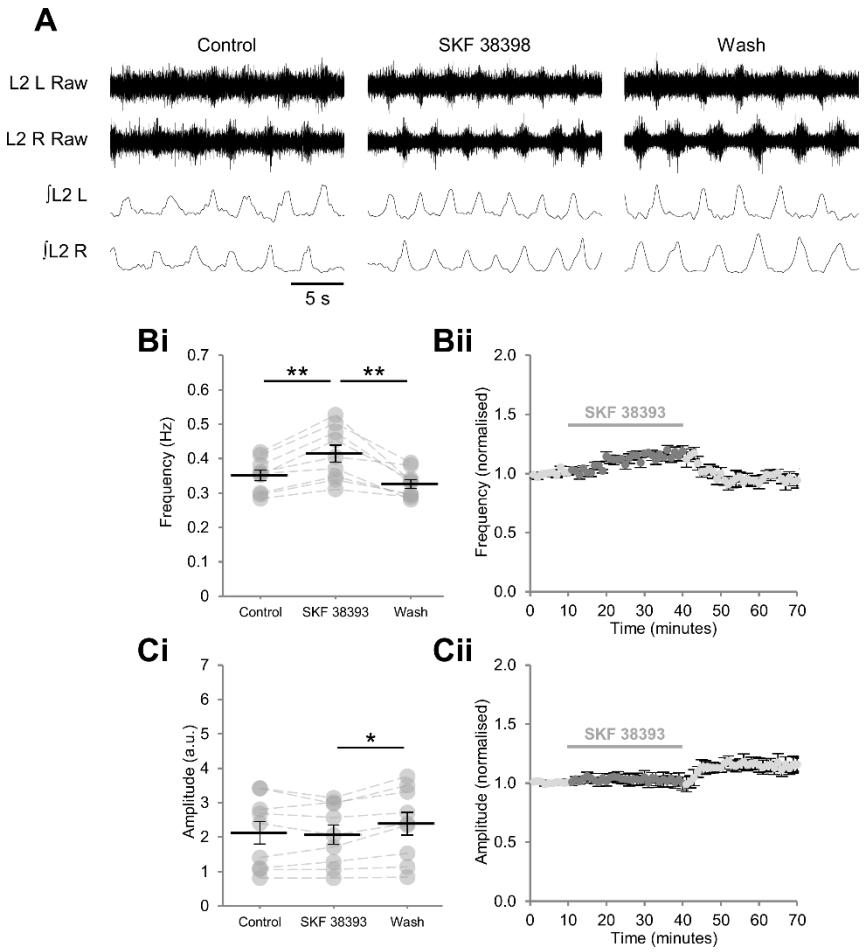
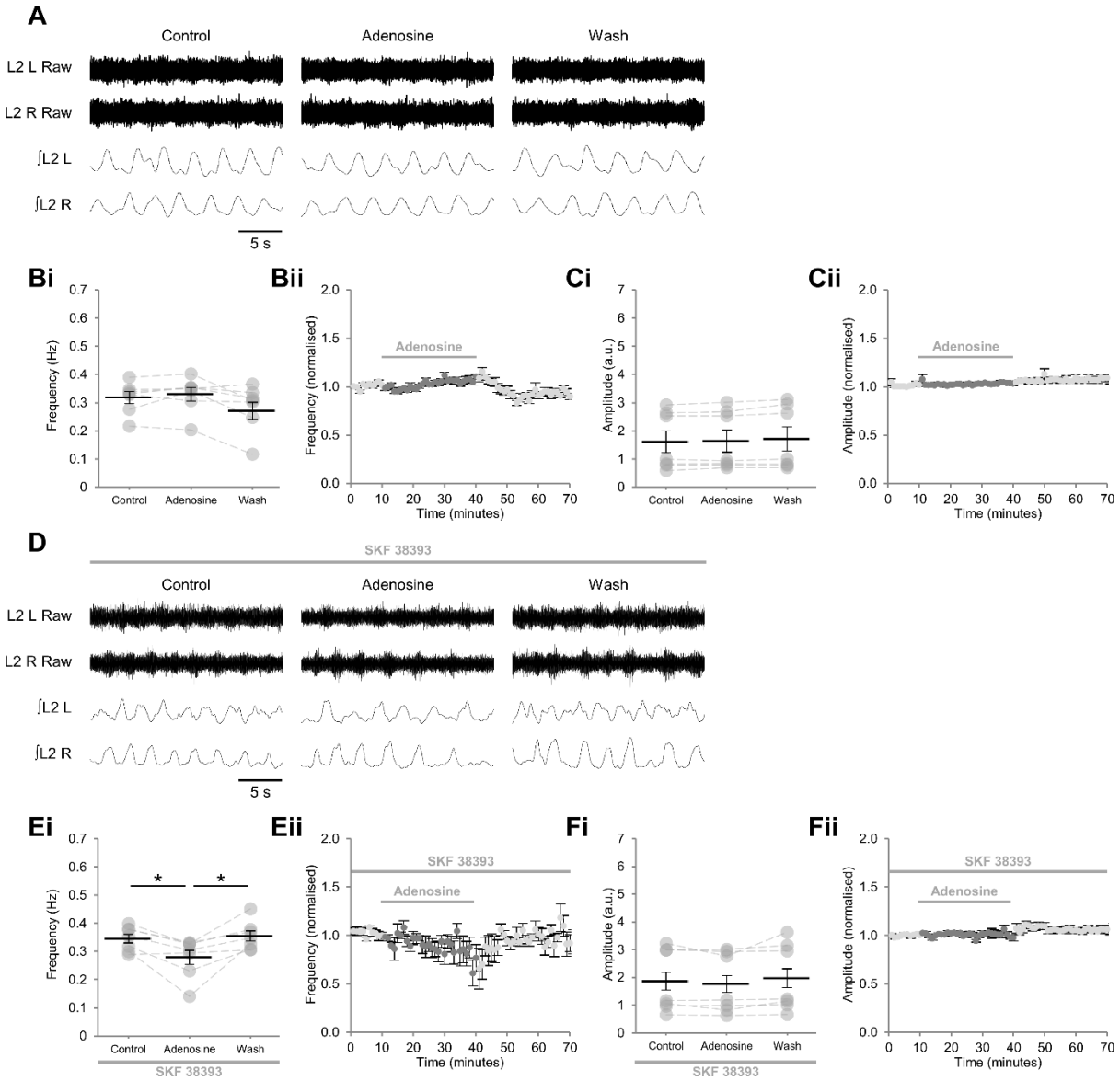


Fig. 1. Activation of D_1-like receptors increases the frequency but not the amplitude of locomotor-related activity. A: raw (top) and rectified/integrated (bottom) traces recorded from left (L) and right (R) L_2 ventral roots showing the effect of the selective agonist of D_1-like receptors SKF 38393 (100 nM) on locomotor-related activity induced by 5-HT (10 µM) and NMDA (5 µM). Bi: locomotor-burst frequency over 5 min during a control period, during a 30-min application of SKF 38393, and during a 30-min washout. Individual data points are shown in grey, and means are represented by black lines. *n* = 9 preparations. Bii: time course plot of normalised data aggregated into 1-min bins showing an increase in burst frequency during SKF 38393 application. *n* = 9. Ci: locomotor-burst amplitude over 5 min during a control period, during a 30-min application of SKF 38393, and during a 30-min washout. *n* = 9. Cii: time course plot of normalised data aggregated into 1-min bins showing no change in burst amplitude during SKF 38393 application. *n* = 9. Error bars: ± SEM. Statistically significant difference: **p* < 0.05, ***p* < 0.01.

To confirm that activation of D_1-like receptors is required for adenosinergic modulation in locomotor networks, adenosine (75 µM) was bath-applied to isolated spinal cords in which locomotor-related activity was induced by bath-applied NMDA and 5-HT. Importantly, descending dopaminergic neurons are severed in this preparation (Björklund and Skagerberg, 1979; Commissiong, Gentleman and Neff, 1979; Hökfelt, Phillipson and Goldstein, 1979; Skagerberg and Lindvall, 1985). Adenosine modulated neither the frequency (Fig. 2, A and B; $p > 0.05$, $n = 7$) nor the amplitude (Fig. 2, A and C; $p > 0.05$, $n = 7$) of locomotor-related activity in these experiments. When adenosine was applied in the presence of SKF 38393, burst frequency was reduced (19.5 ± 6.0%; Fig. 2, D and E; $F[2,12] = 10.7$, $p < 0.01$, $n = 7$) but amplitude was unchanged (Fig. 2, D and F; $p > 0.05$, $n = 7$), as reported in preparations in which DA is present (Witts, Panetta and Miles, 2012; Acevedo *et al.*, 2016). Similarly, the A_1-receptor antagonist DPCPX (1-50 µM) (Witts, Panetta and Miles, 2012; Acevedo *et al.*, 2016) failed to modulate either the frequency (Fig. 3, A and B; $p > 0.05$, $n = 7$) or amplitude (Fig. 3, A and C; $p > 0.05$, $n = 7$) of bursting in the absence of DA of SKF 38393. By contrast, as when it is applied in the presence of DA (Witts, Panetta and Miles, 2012; Acevedo *et al.*, 2016), DPCPX (1 µM) (Acevedo *et al.*, 2016) applied in the presence of SKF 38398 elicited an increase in burst frequency (23.5 ± 4.8%; Fig. 3, D and E; $F[2,12] = 13.7$, $p < 0.001$, $n = 7$) without modulating burst amplitude (Witts, Panetta and Miles, 2012; Acevedo *et al.*, 2016); however, an increase in burst amplitude was recorded following washout of DPCPX (23.1 ± 2.9%; Fig. 3, D and F; $F[2,12] = 17.9$, $p < 0.001$, $n = 7$).

Stimulation of glia by selective activation of the endogenous GPCR PAR1 triggers the release of ATP-adenosine and activation of A_1-receptors (Carlsen and Perrier, 2014; Chapter 2). Stimulation of glia in this way is proposed to replicate the endogenous activation of glial receptors by synaptically released neurotransmitters (Araque *et al.*, 1999, 2014). Like bath-applied adenosine (Witts, Panetta and Miles, 2012; Acevedo *et al.*, 2016), adenosine produced following experimental stimulation of glia reduces the frequency but not the amplitude of ongoing locomotor-related activity when DA is present (Chapter 2). Like


A
Control
Adenosine
Wash
L2 L Raw
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Amplitude (normalised)
SKF 38393
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Time (minutes)

Fig. 2. Adenosine requires activation of D_1-like receptors to modulate locomotor-related activity. A: raw (top) and rectified/integrated (bottom) traces recorded from left (L) and right (R) L_2 ventral roots showing the effect of adenosine (75 µM) on locomotor-related activity induced by 5-HT (10 µM) and NMDA (5 µM). Bi: locomotor-burst frequency over 5 min during a control period, during a 30-min application of adenosine, and during a 30-min washout. Individual data points are shown in grey, and means are represented by black lines. *n* = 7 preparations. Bii: time course plot of normalised data aggregated into 1-min bins showing no change in burst frequency during adenosine application. *n* = 7. Ci: locomotor-burst amplitude over 5 min during a control period, during a 30-min application of adenosine, and during a 30-min washout. *n* = 7. Cii: time course plot of normalised data aggregated into 1-min bins showing no change in burst amplitude during adenosine application. *n* = 7. D: raw (top) and rectified/integrated (bottom) traces recorded from left (L) and right (R) L_2 ventral roots showing the effect of adenosine on locomotor-related activity induced by 5-HT and NMDA in the presence of the selective agonist of D_1-like receptors SKF 38393 (100 nM). Ei: locomotor-burst frequency over 5 min during a control period, during a 30-min application of adenosine, and during a 30-min washout. SKF 38393 was present throughout. *n* = 7. Eii: time course plot of normalised data aggregated into 1-min bins showing a reduction in burst frequency during adenosine application in the presence of SKF 38393. *n* = 7. Fi: locomotor-burst amplitude over 5 min during a control period, during a 30-min application of adenosine, and during a 30-min washout. SKF 38393 was present throughout. *n* = 7. Fii: time course plot of normalised data aggregated into 1-min bins showing no change in burst amplitude during adenosine application in the presence of SKF 38393. *n* = 7. Error bars: ± SEM. Statistically significant difference: $*p < 0.05$.

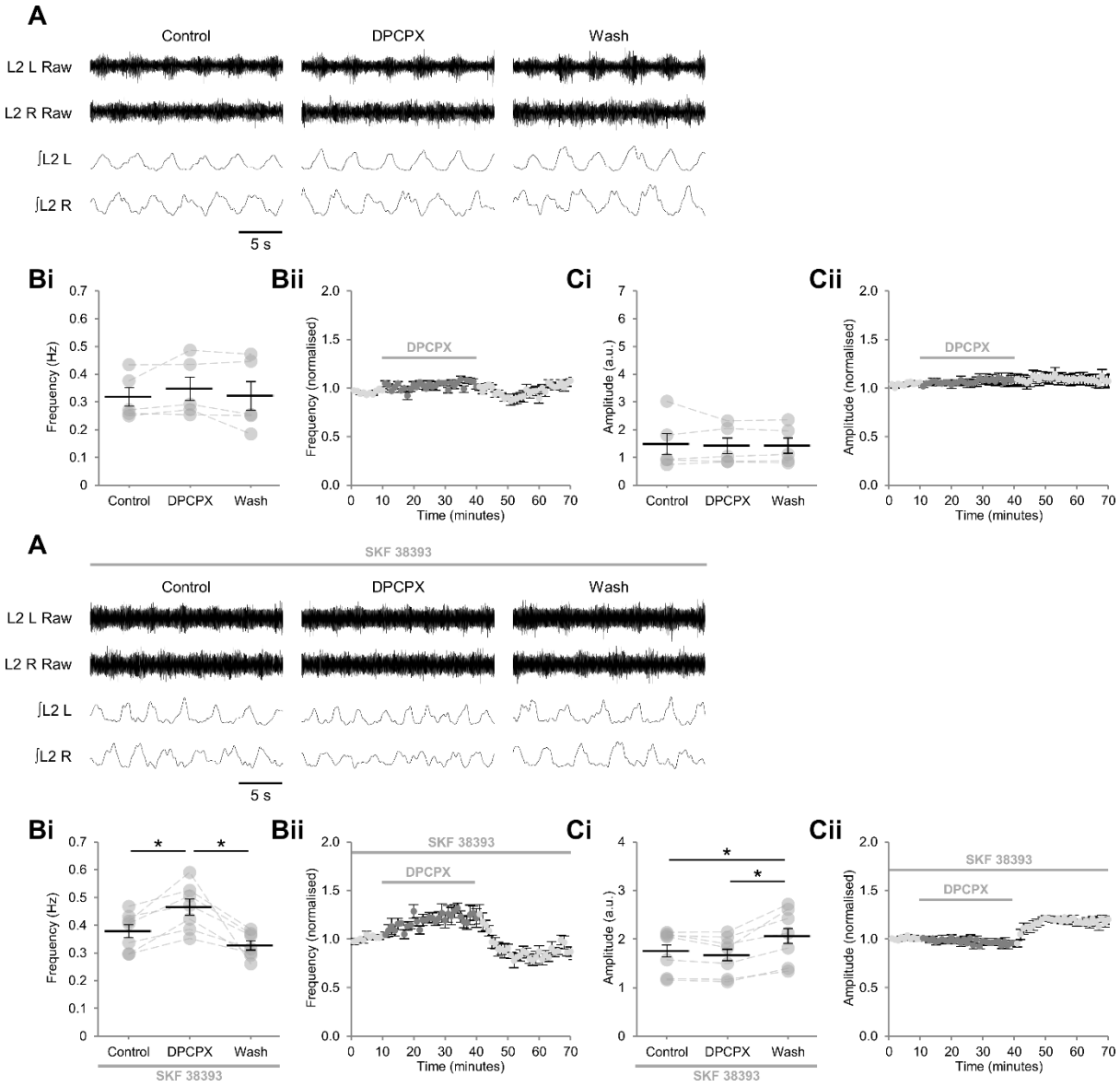
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Fig. 3. A_1 adenosine receptors require activation of D_1-like receptors to modulate locomotor-related activity. A: raw (top) and rectified/integrated (bottom) traces recorded from left (L) and right (R) L_2 ventral roots showing the effect of the selective A_1 antagonist DPCPX (1 µM) on locomotor-related activity induced by 5-HT (10 µM) and NMDA (5 µM). Bi: locomotor-burst frequency over 5 min during a control period, during a 30-min application of DPCPX (1-50 µM), and during a 30-min washout. Individual data points are shown in grey, and means are represented by black lines. *n* = 5 preparations. Bii: time course plot of normalised data aggregated into 1-min bins showing no change in burst frequency during DPCPX application. *n* = 5. Ci: locomotor-burst amplitude over 5 min during a control period, during a 30-min application of DPCPX, and during a 30-min washout. *n* = 5. Cii: time course plot of normalised data aggregated into 1-min bins showing no change in burst amplitude during DPCPX application. *n* = 5. D: raw (top) and rectified/integrated (bottom) traces recorded from left (L) and right (R) L_2 ventral roots showing the effect of DPCPX (1 µM) on locomotor-related activity induced by 5-HT and NMDA in the presence of the selective agonist of D_1-like receptors SKF 38393 (100 nM). Ei: locomotor-burst frequency over 5 min during a control period, during a 30-min application of DPCPX (1 µM), and during a 30-min washout. SKF 38393 was present throughout. *n* = 7. Eii: time course plot of normalised data aggregated into 1-min bins showing an increase in burst frequency during DPCPX application in the presence of SKF 38393. *n* = 7.Fi: locomotor-burst amplitude over 5 min during a control period, during a 30-min application of DPCPX, and during a 30-min washout. SKF 38393 was present throughout. *n* = 7. Fii: time course plot of normalised data aggregated into 1-min bins showing no change in burst amplitude during DPCPX application in the presence of SKF 38393. *n* = 7. Error bars: ± SEM. Statistically significant difference: $*p < 0.05$.

bath-applied adenosine (Witts, Panetta and Miles, 2012; Acevedo *et al.*, 2016), adenosine produced following experimental stimulation of glia reduces the frequency but not the amplitude of ongoing locomotor-related activity when DA is present (Chapter 2). Like adenosine and DPCPX, TFLLR (10 µM) (Carlsen and Perrier, 2014; Chapter 2), a selective agonist of PAR1, failed to modulate the frequency (Fig. 4, A and B; $p > 0.05$, $n = 7$) or the amplitude (Fig. 4, A and C; $p > 0.05$, $n = 7$) of locomotor-related activity in the absence of a DA or a SKF 38393. In the presence of SKF 38393, however, a transient reduction in burst frequency was detected (12.3 ± 2.3%; Fig. 4, D and E; $F[2,20] = 14.9$, $p < 0.001$, $n = 11$), with no change in amplitude (Fig. 4, D and F; $p > 0.05$, $n = 11$), as previously reported in the presence of DA (Chapter 2). Together, these results confirm that bath-applied adenosine, endogenous adenosine present in the spinal cord during locomotor-related activity and adenosine released following stimulation of glia modulate locomotor-related activity by a mechanism which requires the simultaneous activation of D_1-like receptors.

Glial-cell derived adenosine modulates locomotor-related activity in a PKA-dependent manner

D_1-like receptors signal through $G_{\alpha s}$ to stimulate adenylyl cyclase and cAMP production, resulting in activation of PKA and the modulation of neuronal activity (Abdel-Majid *et al.*, 1998; Pieper, Clerkin and MacFarlane, 2011), whereas A_1 receptors signalling through $G_{\alpha i}$ are proposed to inhibit adenylyl cyclase and its downstream effectors, acting in opposition to D_1-like receptors (Acevedo *et al.*, 2016). This suggests that adenosine released following glial stimulation modulates network activity by reducing the activity of PKA. To confirm this, TFLLR was applied to spinal cord preparations in which locomotor-related activity had been induced by NMDA (5 µM), 5-HT (10 µM) and DA (50 µM) in the presence of 14-22 amide (1 µM), a PKA inhibitor (Acevedo *et al.*, 2016). Under these conditions, the modulation of burst frequency upon PAR1 activation was abolished (Fig. 5, A and B; $p > 0.05$, $n = 8$); burst amplitude was also unchanged (Fig. 5, A and C; $p > 0.05$, $n = 8$). These data are further evidence that glial cell-derived adenosine inhibits signalling through the D_1-like

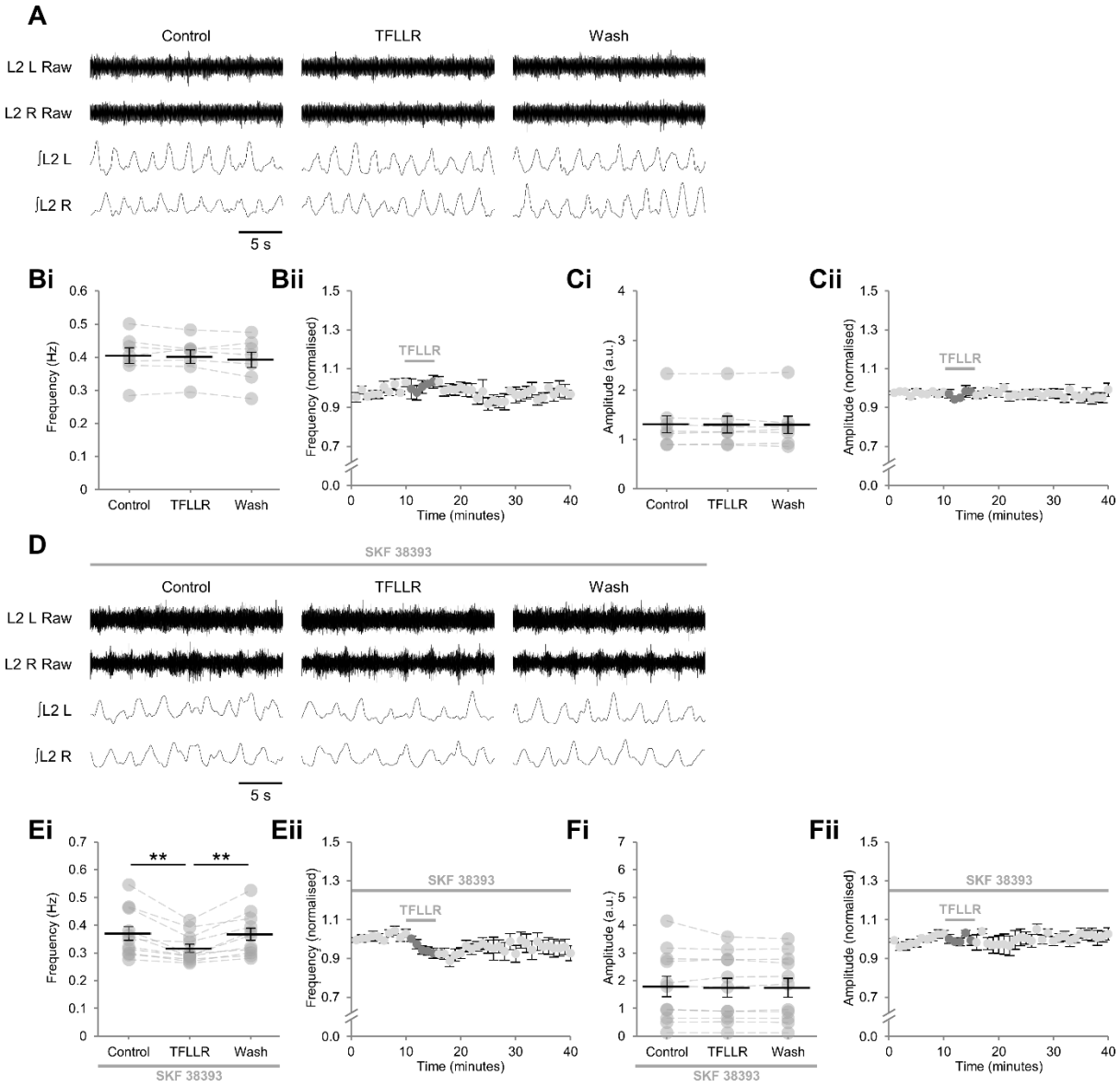
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Fig. 4. Adenosine released from glia upon PAR1 activation requires activation of D_1-like receptors to modulate locomotor-related activity. A: raw (top) and rectified/integrated (bottom) traces recorded from left (L) and right (R) L_2 ventral roots showing the effect of the PAR1 agonist TFLLR (10 µM) on locomotor-related activity induced by 5-HT (10 µM) and NMDA (5 µM). Bi: locomotor-burst frequency over 5 min during a control period, upon a 5-min application of TFLLR, and during a 25-min washout. Individual data points are shown in grey, and means are represented by black lines. *n* = 7 preparations. Bii: time course plot of normalised data aggregated into 1-min bins showing no change in burst frequency upon TFLLR application. *n* = 7. Ci: locomotor-burst amplitude over 5 min during a control period, upon a 5-min application of TFLLR, and during a 25-min washout. *n* = 7. Cii: time course plot of normalised data aggregated into 1-min bins showing no change in burst amplitude upon TFLLR application. *n* = 7. D: raw (top) and rectified/integrated (bottom) traces recorded from left (L) and right (R) L_2 ventral roots showing the effect of TFLLR on locomotor-related activity induced by 5-HT and NMDA in the presence of the selective agonist of D_1-like receptors SKF 38393 (100 nM). Ei: locomotor-burst frequency over 5 min during a control period, upon a 5-min application of TFLLR, and during a 25-min washout. SKF 38393 was present throughout. *n* = 11. Eii: time course plot of normalised data aggregated into 1-min bins showing a transient reduction in burst frequency upon TFLLR application in the presence of SKF 38393. *n* = 11. Fi: locomotor-burst amplitude over 5 min during a control period, upon a 5-min application of TFLLR, and during a 25-min washout. SKF 38393 was present throughout. *n* = 11. Fii: time course plot of normalised data aggregated into 1-min bins showing no change in burst amplitude upon TFLLR application in the presence of SKF 38393. *n* = 11. Error bars: ± SEM. Statistically significant difference: $^{**}p < 0.01$.

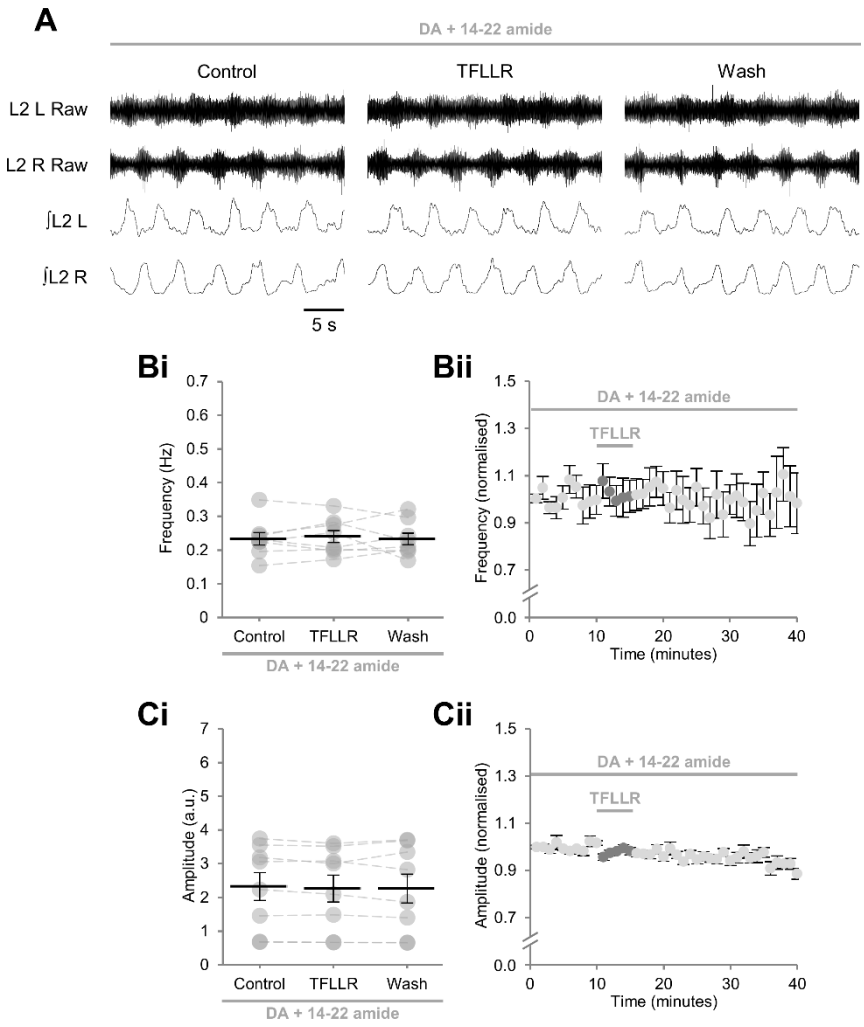


Fig. 5. Adenosine released from glia upon PAR1 activation requires PKA activity to modulate locomotor-related activity. A: raw (top) and rectified/integrated (bottom) traces recorded from left (L) and right (R) L_2 ventral roots showing the effect of the PAR1 agonist TFLLR (10 µM) on locomotor-related activity induced by 5-HT (10 µM), NMDA (5 µM) and DA (50 µM) in the presence of the PKA inhibitor 14-22 amide (1 µM). Bi: locomotor-burst frequency over 5 min during a control period, upon a 5-min application of TFLLR, and during a 25-min washout. Individual data points are shown in grey, and means are represented by black lines. *n* = 8 preparations. Bii: time course plot of normalised data aggregated into 1-min bins showing no change in burst frequency upon TFLLR application. *n* = 8. Ci: locomotor-burst amplitude over 5 min during a control period, upon a 5-min application of TFLLR, and during a 25-min washout. *n* = 8. Cii: time course plot of normalised data aggregated into 1-min bins showing no change in burst amplitude upon TFLLR application. *n* = 8. Error bars: ± SEM.

receptor/adenylyl cyclase/PKA pathway.

Discussion

The experiments described in this chapter demonstrate that endogenous glial cell-derived adenosine functions during locomotor-related activity as a second-order modulator, constraining excitatory DA signalling which is mediated through D_1-like receptors and PKA. Metamodulation of this kind may serve to provide fine control over a modulator that has very broad effects within the spinal cord (Katz, 1999; Sharples *et al.*, 2014).

Adenosine acting at A_1 receptors is proposed to act in opposition to DA acting at D_1-like receptors to reduce the speed of locomotion. This implied a previously undemonstrated excitatory role for D_1-like receptors in modulating the frequency of locomotor-related activity in mice. Accordingly, selective activation of D_1-like receptors with SKF 38393 is shown here to increase the frequency of locomotor-related activity. This finding contradicts previous studies reporting no change in the frequency of ongoing locomotor-related activity when D_1-like receptors are activated with another phenylbenzazepine, SKF 81297 (Humphreys and Whelan, 2012; Sharples *et al.*, 2015). SKF 81297 applied at the concentration used in these studies is likely also activate inhibitory D_2-like receptors (Neumeyer *et al.*, 2003), which have been shown to reduce the frequency of locomotor-related bursting (Humphreys and Whelan, 2012; Sharples *et al.*, 2015). The excitatory effect of D_1-like receptors on burst frequency may therefore have been masked in these studies. The evidence presented here that D_1-like receptors are indeed excitatory in postnatal mice is consistent with studies showing that D_1-like receptors stimulate locomotor activity in intact adult mice (Lapointe *et al.*, 2009) and neonatal rats (Barrière, Mellen and Cazalets, 2004). Conversely, it is shown that bath-applied adenosine, which acts via A_1 but not A_{2A} receptors in the murine locomotor circuitry (Witts, Panetta and Miles, 2012; Carlsen and Perrier, 2014; Acevedo *et al.*, 2016; Chapter 2), reduces the frequency of locomotor-related activity, whereas A_1 blockade increases it. Importantly, the

effects of A_1 receptor activation or inhibition are only observed when D_1-like receptors are also activated.

Despite evidence that D_1-like receptors enhance AMPA currents in motoneurons (Han and Whelan, 2009), no change in the amplitude of network activity is detected upon activation of D_1-like receptors; similarly, adenosine is reported not to modulate burst amplitude (Witts, Panetta and Miles, 2012; Acevedo *et al.*, 2016; Chapter 2). However, although burst amplitude is unaltered during the activation of D_1-like receptors and during inhibition of A_1 receptors by DPCPX, in both cases burst amplitude increases upon drug washout. The mechanism by which this occurs is unclear, but it may be relevant that in both experiments the change in amplitude follows a period of enhanced signalling through the D_1-like receptor pathway; in the case of DPCPX, this occurs because A_1 blockade relieves the pathway of inhibition by endogenous adenosine. Taken together, the data presented here indicate that A_1 and D_1-like receptors have opposite effects on the frequency of network activity, and that neither receptor modulates the amplitude or intensity of locomotor-related output. This is consistent with the proposal that they act via a common pathway (Acevedo *et al.*, 2016).

Previous studies demonstrated that A_1 receptors modulate the frequency of ongoing network activity in murine preparations in the presence of DA (Witts, Panetta and Miles, 2012; Acevedo *et al.*, 2016; Chapter 2), but not in the presence of a D_1-like receptor inhibitor (Acevedo *et al.*, 2016). Consistent with these reports, blockade of A_1 receptors fails to modulate activity in the rat spinal cord in the absence of DA. (Taccola *et al.*, 2012). Here, it is shown that the previously reported effects of adenosine, DPCPX and PAR1 activation by TFLLR on network activity are absent when DA is excluded from the aCSF, but that they are restored in the presence of a D_1-like receptor agonist. Thus, activation of D_1-like receptors is a precondition of adenosinergic modulation of ongoing locomotor-related activity. However, it is difficult to resolve these findings with previous observations that adenosine modulates postsynaptic currents in interneurons in acute slices from postnatal mice, both when bath-applied at the same concentration as used here (Witts, Nascimento and Miles, 2015) and when it is released

endogenously following stimulation of astrocytes (Carlsen and Perrier, 2014). Furthermore, adenosine applied to isolated rat spinal cords in the absence of DA modulates burst amplitude during locomotor-related activity, the frequency of disinhibited bursting, and the duration of bouts of locomotor-related activity induced by dorsal-root stimulation (Taccola *et al.*, 2012). Adenosine also modulates reflex potentials induced by dorsal root stimulation in rats (Otsuguro, Wada and Ito, 2011; Taccola *et al.*, 2012). All of these DA-independent effects are abolished by A_1 blockade; however, in these studies A_1 blockade alone has no effect in the absence of DA, indicating that adenosine present at basal levels within spinal cord preparations does not have modulatory effects. It is therefore possible that a high concentration of exogenous adenosine can under some circumstances act independently of DA; however, the ability of adenosine to act independently of DA in slices from postnatal mice (Witts, Nascimento and Miles, 2015) but not, as shown here, in rhythmically active preparations from the same species implies that a state-dependent mechanism is involved.

Microdialysis experiments have shown that DA is released in the spinal cord during locomotion (Gerin, Becquet and Privat, 1995; Gerin and Privat, 1998). There is also evidence for activity-dependent release of adenosine, since adenosinergic modulation scales with neuronal activity (Chapter 2). One possibility is that the excitatory actions of DA and the inhibitory actions of adenosine do not perfectly overlap, such that DA promotes locomotion at low frequencies of network activity, but its effect is constrained by adenosine at higher frequencies. Adenosine may function in this way to stabilize network output, ensuring controlled locomotion at higher speeds, or to prevent metabolic exhaustion. Alternatively, adenosine may inhibit D_1-like receptor signalling across all speeds of locomotion, with adenosine shaping dopaminergic modulation by limiting only some of its actions. Second-order modulation of this kind may represent an efficient and selective mechanism of control over a first-order modulator that has diverse actions within a network (Katz, 1999). In the case of DA, those actions are mediated by multiple cell types via both D_1-like and D_2-like receptors. It is also possible that adenosine modulates only a subset of D_1-like receptors. Consistent with this, it was previously observed

that motoneurons and excitatory interneurons are insensitive to adenosine during locomotor-related activity implying that adenosine acts via inhibitory interneurons to modulate burst frequency (Witts, Panetta and Miles, 2012; Chapter 2). Therefore, although D_1-like receptors modulate AMPA currents recorded from motoneurons in slices (Han and Whelan, 2009), it is likely that adenosine modulates only signalling through D_1-like receptors expressed by inhibitory interneurons.

Inhibition of PKA prevents the modulation of locomotor-related bursting otherwise detected following stimulation of glia. Similarly, PKA inhibition abolishes the effect of A_1 blockade (Acevedo *et al.*, 2016). These findings support a role for PKA in the modulation of network activity by adenosine acting through the D_1-like receptor pathway. PKA has diverse molecular targets, including ion channels and neurotransmitter receptors (Abdel-Majid *et al.*, 1998; Pieper, Clerkin and MacFarlane, 2011). Although AMPA receptors may be modulated by PKA (Banke *et al.*, 2000; Esteban *et al.*, 2003), and motoneuronal AMPA receptors are modulated by D_1-like receptors in the lumbar spinal cord (Han and Whelan, 2009), motoneuronal AMPA receptors are unlikely to mediate the effects of adenosine during locomotor-related activity (see above). The targets of PKA activity, which may include AMPA receptors expressed by inhibitory interneurons, therefore remain to be elucidated.

The data presented in this chapter provide evidence that adenosine produced upon stimulation of glia acts via A_1 receptors to inhibit signalling through D_1-like receptors (Fig. 6). Significantly, glia are proposed as the principal source of modulatory adenosine in spinal motor networks (Witts, Panetta and Miles, 2012; Chapter 2). These findings support a previously described interaction between A_1 and D_1-like receptors in spinal motor networks (Acevedo *et al.*, 2016). Other examples of metamodulation in spinal locomotor networks are provided by *Xenopus* tadpoles, in which nitric oxide (NO) modulates the release of noradrenaline (McLean and Sillar, 2004) and lampreys, in which NO modulates the activity of endocannabinoids (Song, Kyriakatos and El Manira, 2012). The present study reveals a mechanism by which a second-order neuromodulator refines the effects of a first-order neuromodulator with diverse

and potent actions, providing behaviourally relevant network output with flexibility and specificity.

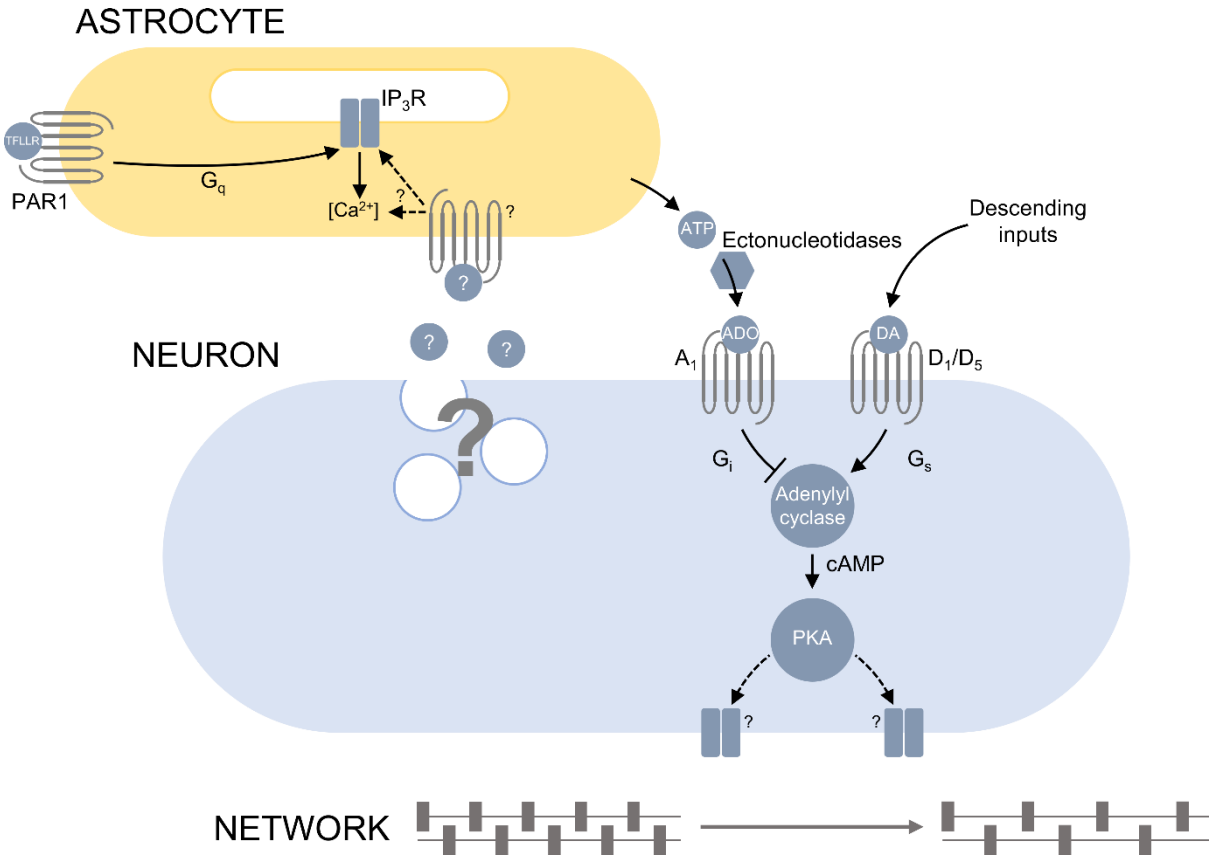


Fig. 6. Schematic illustrating proposed release of ATP-adenosine from astrocytes and inhibition of D_1-like receptor signalling in locomotor networks. ATP is released from putative spinal cord astrocytes upon experimental activation of the $G_{\alpha q}$-linked receptor PAR1 by TFLLR, proposed to mimic the endogenous action of neurotransmitters on astrocytic GPCRs (Carlsen and Perrier, 2014; Chapter 2). Extracellular ectonucleotidases mediate the hydrolysis of ATP to adenosine, which activates neuronal G_i-linked A_1 receptors to inhibit signalling through $G_{\alpha s}$-linked D_1-like receptors at the level of adenylyl cyclase. Reduced cAMP production by adenylyl cyclase results in reduced activation of PKA. PKA acts on unidentified targets, perhaps including ion channels and ionotropic receptors, to reduce neuronal excitability, resulting in a reduced frequency of locomotor-related activity.

Chapter 4: Differential regulation of NMDA receptors by D-serine and glycine in mammalian spinal locomotor networks

Introduction

Chapters 2 and 3 considered the importance of gliotransmission in the modulation of spinal locomotor networks. However, glial information processing is proposed to be important in processes other than the Ca^{2+}-dependent release of modulators. These may include the regulation of the *N*-methyl-D-aspartate (NMDA) receptor (NMDAR) co-agonists D-serine and glycine. This chapter will consider the importance of NMDAR co-agonist regulation within spinal locomotor networks. Although the availability of D-serine and glycine at excitatory synapses in the spinal cord is likely to be determined, at least in part, by astrocytes (Le Bail *et al.*, 2015), a direct demonstration of their involvement in NMDAR regulation is beyond the scope of this chapter.

Mammalian locomotion requires the activation of limb and trunk muscles with precise timing. During locomotor tasks such as walking, running and swimming, the coordination and pacing of muscle activation are determined by central pattern generator (CPG) networks within the spinal cord (Kiehn, 2016). Whereas much of the patterning of network output is determined by glycine/GABAergic inhibitory transmission, excitatory drive is largely glutamatergic (Kiehn *et al.*, 2008, 2010). Changes in the intensity of glutamatergic transmission affect the frequency and amplitude of locomotor-related network output (Kiehn *et al.*, 2008; Talpalar and Kiehn, 2010) and contribute to gait selection (Talpalar *et al.*, 2013; Bellardita and Kiehn, 2015). Thus excitatory signalling underlies behavioural modifications that allow mammals to respond to the varying demands of their habitat (Orlovsky, Deliagina and Grillner, 1999; Grillner, 2006; Bellardita and Kiehn, 2015).

In mammalian spinal locomotor networks, endogenous glutamate acts via both NMDARs and non-NMDARs, namely α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors, with blockade of either resulting in alterations to locomotor-related output (Cazalets, Sqalli-Houssaini and Clarac, 1992; Douglas *et al.*, 1993; Beato, Bracci and Nistri, 1997; Talpalar and Kiehn, 2010). In most studies of isolated rodent spinal cord preparations, blockade of NMDARs results in either pronounced reductions in the frequency and amplitude of locomotor-related rhythmic activity or its cessation, likely depending on the strength of the stimulus used to excite network activity (Beato, Bracci and Nistri, 1997; Bracci, Beato and Nistri, 1998; Cowley *et al.*, 2005; Talpalar and Kiehn, 2010). However, blockade of NMDARs is also reported to increase the frequency of locomotor network activity in mice (Whelan, Bonnot and Donovan, 2000), while at high frequencies of pharmacologically induced network activity, NMDAR blockade results in severe disruption to the coordination of rhythmic bursting without altering frequency (Talpalar and Kiehn, 2010). It remains to be determined whether these disparate findings reflect a fundamental difference in glutamatergic signalling between mice and rats or methodological differences between these studies.

Unlike non-NMDARs, canonical GluN1/GluN2 subunit-containing NMDARs require the binding of a co-agonist in addition to the binding of glutamate for their activation (Johnson and Ascher, 1987; Kleckner and Dingledine, 1988; Clements and Westbrook, 1991; Paoletti, Bellone and Zhou, 2013). In the brain, the endogenous co-agonist may be either glycine or D-serine, with one or the other dominating at a given synapse(Mothet *et al.*, 2000; Kalbaugh, Zhang and Diamond, 2009; Li *et al.*, 2009, 2013, Henneberger *et al.*, 2010, 2013; Papouin *et al.*, 2012; Le Bail *et al.*, 2015; Meunier *et al.*, 2016). The identity of the dominant co-agonist is proposed to vary during development (Le Bail *et al.*, 2015), with receptor subunit composition (Kalbaugh, Zhang and Diamond, 2009; Papouin *et al.*, 2012; Le Bail *et al.*, 2015), between synaptic and extrasynaptic receptor populations (Papouin *et al.*, 2012), and in an activity-dependent manner (Kalbaugh, Zhang and Diamond, 2009; Li *et al.*, 2009, 2013). In the spinal cord, co-agonist identity has hitherto received little attention.

Dynamic regulation of co-agonist binding site occupancy is proposed as a mechanism for the modulation of NMDAR activity (Berger, Dieudonné and Ascher, 1998; Li *et al.*, 2009, 2013). An unsaturated co-agonist binding site could permit the regulation of glutamatergic signalling by adjustments to the availability of the co-agonist (Berger, Dieudonné and Ascher, 1998; Li *et al.*, 2009, 2013). The co-agonist binding site is reported to be unsaturated in some preparations (Berger, Dieudonné and Ascher, 1998; Kalbaugh, Zhang and Diamond, 2009; Li *et al.*, 2009, 2013; Le Bail *et al.*, 2015) but saturated in others (Kemp *et al.*, 1988; Li *et al.*, 2009; Shigetomi *et al.*, 2013), and activity-dependent changes in co-agonist binding have been reported in the brain (Kalbaugh, Zhang and Diamond, 2009; Li *et al.*, 2009, 2013). The co-agonist binding site is unsaturated in *in vitro* forelimb locomotor circuit preparations of pre- and postnatal rats (Shimomura *et al.*, 2015), in motoneurons in a hemisected spinal cord preparation from postnatal rats (Brugger *et al.*, 1990), and in the locomotor network of *Xenopus* tadpoles during swimming; in this latter preparation episodes of swimming are prolonged by exogenous D-serine or glycine (Issberner and Sillar, 2007). Thus, either increases or decreases in co-agonist availability could modulate excitatory transmission within mammalian spinal locomotor networks to modulate behaviour.

The availability of D-serine is largely determined by the opposing actions of serine racemase, the enzyme that synthesises D-serine from L-serine, and D-amino acid oxidase (DAAO), the enzyme responsible for degrading D-serine *in vivo* (Wolosker, Blackshaw and Snyder, 1999; Sasabe *et al.*, 2014); however, see Crow et al. (2012) for a review of the complexities of D-serine metabolism (Crow, Marecki and Thompson, 2012). Concentrations of D-serine in the mammalian spinal cord are considerably lower than those of glycine, and considerably lower than those detected in the brain (Schell *et al.*, 1997; Sasabe *et al.*, 2007; Miyoshi *et al.*, 2012; Thompson *et al.*, 2012). Accordingly, glycine was proposed as the exclusive co-agonist of NMDARs in the spinal cord (Schell *et al.*, 1997). However, selective degradation of D-serine reduces pain symptoms in rats subjected to nerve ligation (Lefèvre *et al.*, 2015; Moon *et al.*, 2015), and levels of both D-serine and serine racemase are higher following surgery (Lefèvre

et al., 2015; Moon *et al.*, 2015). Furthermore, aberrant D-serine metabolism in the spinal cord is implicated in the pathogenesis of amyotrophic lateral sclerosis (ALS) (Sasabe *et al.*, 2007; Mitchell *et al.*, 2010; Paul and de Belleroche, 2014). Thus the role of D-serine in spinal cord physiology in both health and disease merits further investigation.

Glycine, the other NMDAR co-agonist, is released by exocytosis at glycinergic synapses (Bowery and Smart, 2006), by Asc-1 (Rosenberg *et al.*, 2013) and possibly by reverse operation of glycine transporter-1 and -2 (GlyT1 and GlyT2) (Attwell, Barbour and Szatkowski, 1993). Forward operation of GlyT1 and GlyT2 serves to clear glycine from the extracellular space and determines concentrations of free glycine (Eulenburg *et al.*, 2005). Both transporters are highly expressed in the spinal cord and brainstem, where glycinergic transmission is prevalent (Eulenburg *et al.*, 2005). Whereas GlyT2 is preferentially expressed at inhibitory synapses (Danglot *et al.*, 2004), GlyT1 is expressed at excitatory synapses (Zafra, Aragón, *et al.*, 1995; Cubelos, Giménez and Zafra, 2005) and is capable of holding the concentration of glycine within the restricted space of the synaptic cleft below its concentration in the surrounding area (Supplisson and Bergman, 1997; Berger, Dieudonné and Ascher, 1998; Bergeron *et al.*, 1998). Thus, glycine is ineffective at potentiating NMDAR currents in brainstem preparations when bath applied at concentrations under 100 µM, orders of magnitude above its EC_{50} (Berger, Dieudonné and Ascher, 1998). By contrast, NMDAR currents are potentiated by GlyT1 blockade (Lim, Hoang and Berger, 2004). These findings illustrate the GlyT1 exercises very tight regulation of glycine at excitatory terminals.

In the spinal cord, spillover of synaptically released glycine is reported to facilitate NMDAR currents in the dorsal horn of postnatal rats (Ahmadi *et al.*, 2003). In pre- and postnatal rat forelimb locomotor circuits, activity is facilitated by blockade of GlyT1 (Shimomura *et al.*, 2015), suggesting a role for glycine transport in the regulation of excitatory transmission. Similarly, blockade of GlyT1 enhances NMDAR-dependent swimming in *Xenopus* tadpoles (Issberner and Sillar, 2007). However, it is unknown whether GlyT1 regulates glycine concentrations at

excitatory synapses in the spinal cord to determine rhythmic locomotor-related activity in mammals.

The gating of NMDARs via the co-agonist binding site may provide a potent mechanism for the dynamic regulation of glutamatergic transmission in spinal locomotor networks, and thus of behaviour (Issberner and Sillar, 2007). However, the importance of NMDARs during locomotor-related activity in mice is currently unclear, and the roles and regulation of glycine and D-serine within the mammalian spinal cord are yet to be characterised. This chapter assesses the regulation of NMDARs via the co-agonist binding site during network activity in spinal cord preparations isolated from postnatal mice. Evidence is provided to show that in mice, as in rats, NMDARs contribute to speed control during pharmacologically induced locomotor-related activity, and that increasing co-agonist availability enhances NMDAR activation and network activity. Furthermore, it is shown that endogenous glycine and D-serine regulate NMDARs in a synapse-specific manner, and that occupancy of the co-agonist binding differs between locomotor-related and disinhibited activity, implying activity-dependent regulation. Finally, GlyT1 is shown to be a potent regulator of activity at excitatory synapses. Together, these findings demonstrate the importance of co-agonist binding site regulation during activity of a mammalian network with a behaviourally relevant output.

Methods

Ethics Statement

All procedures performed on animals were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986 and were approved by the University of St Andrews Animal Welfare and Ethics Committee.

Tissue preparation

Spinal cords were isolated from postnatal day (P)1-P4 C57BL/6 mice as previously described (Jiang, Carlin and Brownstone, 1999). In summary, animals were killed by cervical dislocation,

decapitated and eviscerated, before being transferred to a dissection chamber containing artificial cerebrospinal fluid (aCSF; equilibrated with 95% oxygen, 5% carbon dioxide, ~4°C). Spinal cords were then isolated between midthoracic and upper sacral segments, and ventral and dorsal roots were trimmed.

Ventral root recordings

Isolated spinal cords were pinned ventral-side up in a recording chamber perfused with aCSF (equilibrated with 95% oxygen, 5% carbon dioxide; RT) at 10 ml min^{-1}. Glass suction electrodes were attached to the first or second lumbar ventral roots (L_1, L_2) on each side of the spinal cord to record flexor-related activity. In some experiments a further suction electrode was attached to the fifth lumbar ventral root (L_5) to record the corresponding extensor-related activity. Locomotor-related activity was evoked by bath application of 5-hydroxytryptamine (5-HT; 15 µM) and dopamine (DA; 50 µM), and was characterised by rhythmic bursting alternating contralaterally between upper ventral roots and ipsilaterally between upper ventral roots and L_5. For disinhibited preparations (Bracci, Ballerini and Nistri, 1996; Witts, Panetta and Miles, 2012), strychnine (1 µM) and picrotoxin (60 µM) were applied to evoke rhythmic bursting that was synchronous in all roots. In some experiments, D(−)-2-amino-5-phosphonopentanoic acid (D-APV; 50 µM) or erythro-beta-hydroxy-L-aspartic acid (HOAsp; 400 µM) were bath-applied at the onset of locomotor-related bursting. Any drugs present during the control period were also present during application of further drugs and during washout. In all experiments, stable rhythmic bursting was established over a period of ~1 h prior to the control period. Rhythmic bursting was considered stable when the frequency, amplitude and duration of bursts were unchanged over several minutes. Data were amplified and filtered (band-pass filter 30-3,000 Hz, Qjin Design), and acquired at a sampling frequency of 6 kHz with a Digidata 1440A analogue-digital converter and Axoscope software (Molecular Devices, Sunnyvale, CA). Custom-built amplifiers (Qjin Design) enabled simultaneous online rectification and integration (50-ms time constant) of raw signals.

Data analysis

Data were analysed offline with DataView software (courtesy of Dr W.J. Heitler, University of St Andrews). Ventral-root bursts were identified from rectified/integrated traces and their instantaneous frequencies, peak-to-peak amplitudes, and durations were then measured from the corresponding raw traces. Amplitude was measured as a non-calibrated unit and is presented here in arbitrary units (a.u.). For time-course plots, data were averaged across 1-min bins and normalised to a 10-min pre-control period to permit comparison between preparations. Statistical comparisons were performed on raw data averaged over 5-min periods or 10-min periods for disinhibited preparations. Data were analysed with Student's *t*-tests. p values < 0.05 were considered significant. Tests were performed in SPSS Statistics for Windows, Version 21.0 (IBM Corp. Armonk, NY) or Excel 2013 (Microsoft Corp. Redmond, WA).

Solution, drug and enzyme preparation

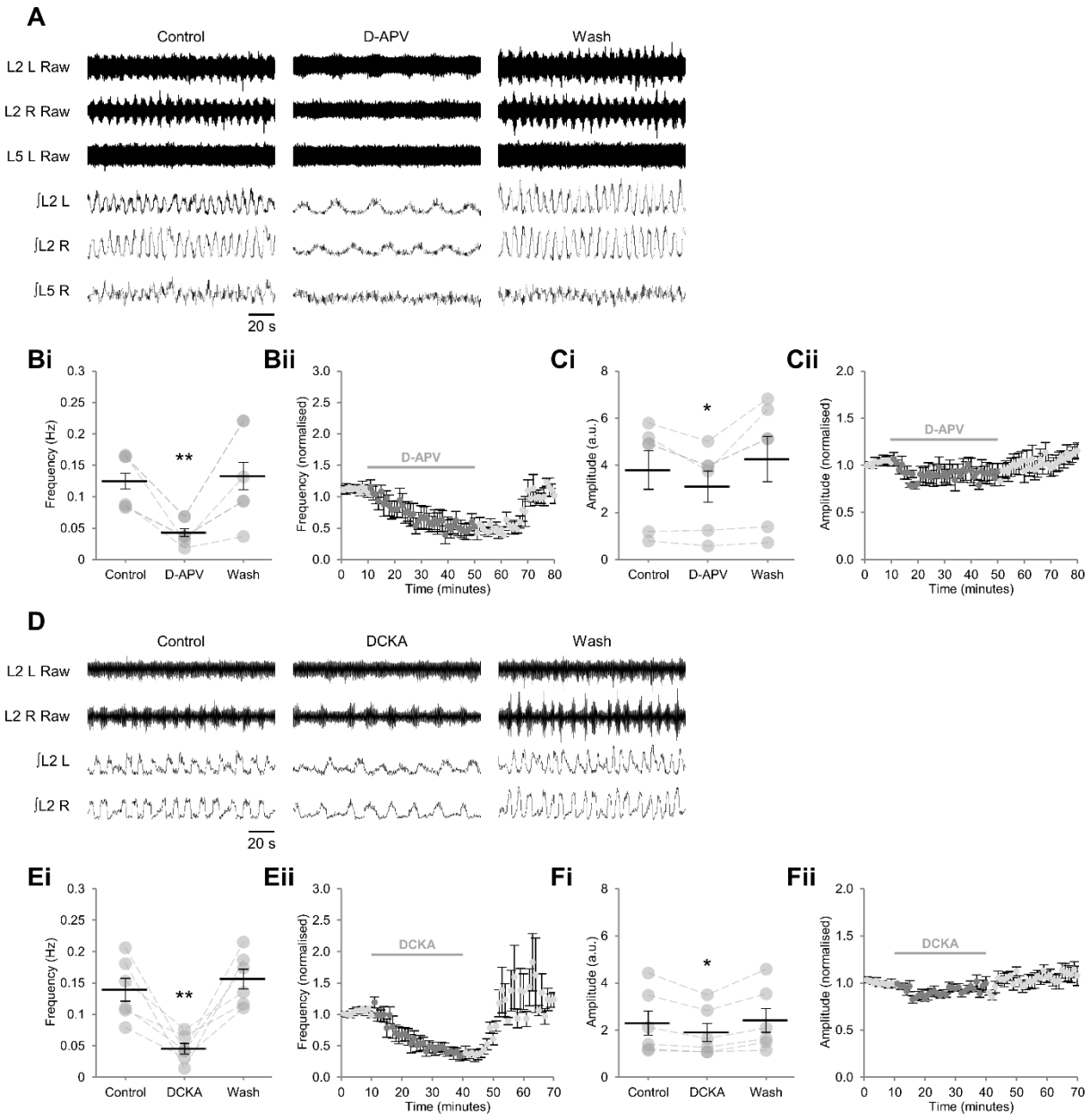
The aCSF used for dissections and recordings contained (in mM) 127 NaCl, 26 $NaHCO_3$, 10 glucose, 3 KCl, 2 CaCl, 1.25 NaH_2PO_4, and 1 $MgCl_2$. 5,7-dichlorokynurenic acid (DCKA) was supplied by Abcam (Cambridge, UK); 5-HT, D-amino acid oxidase from porcine kidney (DAAO), D-APV, D-serine, glycine, L-serine, NMDA, picrotoxin and strychnine were supplied by Sigma-Aldrich (Poole, UK); *N*-[(3R)-3-([1,1'-Biphenyl]-4-yloxy)-3-(4-fluorophenyl)propyl]-N-methylglycine hydrochloride (ALX 5407) was supplied by Tocris Bioscience (Bristol, UK); HOAsp was supplied by Wako Chemicals USA (Richmond, VA). ALX 5407 and picrotoxin were dissolved in DMSO, the concentration of which did not exceed 0.1% (v/v) in working solutions; DCKA and HOAsp were dissolved in 1 eq. NaOH; all other drugs and DAAO were dissolved in reverse-osmosis water.

Results

NMDAR activation enhances the frequency and amplitude of pharmacologically induced locomotor-related activity

The contribution of NMDARs to the production of rhythmic bilaterally alternating locomotor-related network activity in isolated mouse spinal cord preparations has been subject to conflicting reports (Nishimaru, Takizawa and Kudo, 2000; Whelan, Bonnot and Donovan, 2000; Talpalar and Kiehn, 2010). To assess the role of NMDARs in this preparation, D-APV (50 µM), a selective, competitive NMDAR antagonist that interacts with the glutamate-binding site on GluN2 subunits, was bath applied during recordings of pharmacologically induced (5-HT, 15 µM; DA, 50 µM) locomotor-related output from lumbar ventral roots. D-APV produced a marked reduction in the frequency of flexor-related bursting in L_2 roots (64.9 ± 4.6%; $p < 0.01$, $n = 6$), from 0.125 ± 0.016 Hz to 0.043 ± 0.011 Hz (Fig. 1, A and B). The amplitude of locomotor-related L_2 bursts was also reduced, but to a lesser extent (16.7 ± 4.4%; Fig 1, A and C; $p < 0.05$, $n = 6$). Alternation of bursts between contralateral L_2 roots was maintained throughout the drug application and wash periods; however, extensor-related activity recorded from L_5 roots became indistinct upon application of D-APV (Fig. 1A) and was not assessed further. No differences were found between the effects of D-APV at 50 µM and 100 µM ($n = 6$) on either the frequency ($p > 0.05$) or amplitude ($p > 0.05$) of bursting in L_2 roots, indicating that receptors were saturated at the lower concentration, consistent with previous reports (Talpalar and Kiehn, 2010).

To further investigate the contribution of NMDARs to locomotor-related activity, and to confirm the requirement for occupation of the co-agonist binding site, DCKA (5 µM), a competitive inhibitor acting at the co-agonist binding site on GluN1 subunits (Henderson, Johnson and Ascher, 1990), was applied to rhythmically active preparations. Like D-APV, DCKA potently reduced the frequency of locomotor-related bursting in L_2 roots (63.7 ± 7.2%; Fig. 1, D and E; $p < 0.01$, $n = 6$) and modestly reduced burst amplitude (14.6 ± 2.6%; Fig. 1, D and F; $p < 0.05$, $n = 6$) without disrupting left-right alternation (Fig. 1D). Together, these results indicate a role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L2 L Raw
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Frequency (normalised)
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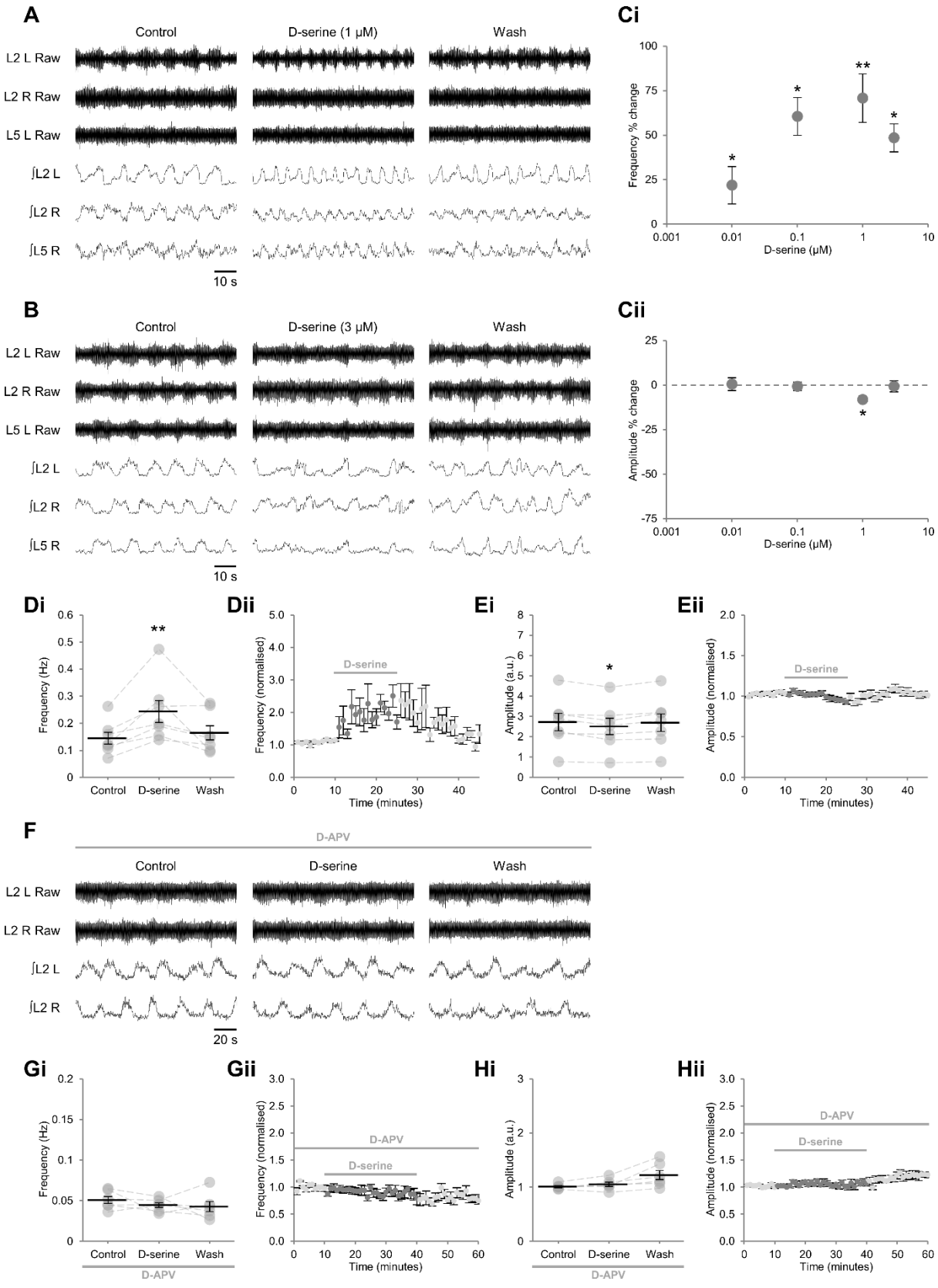
Wash
Fii
Amplitude (normalised)
DCKA
Time (minutes)

Fig. 1. NMDARs determine the speed and amplitude of locomotor-related activity in spinal cord preparations from postnatal mice. A: raw (top) and rectified/integrated (bottom) traces recorded from left (L) and right (R) L_2 ventral roots and RL_5 showing the effect of the competitive glutamate-binding site antagonist D-APV (50 µM) on locomotor-related activity induced by 5-HT (15 µM) and DA (50 µM). Bi: locomotor-burst frequency over 5 min during a control period, during a 40-min application of D-APV, and during a 30-min washout. Individual data points are shown in grey, and means are represented by black lines. *n* = 6 preparations. Bii: time course plot of normalised data aggregated into 1-min bins showing a reduction in burst frequency during D-APV application. *n* = 6. Ci: locomotor-burst amplitude over 5 min during a control period, during a 40-min application of D-APV, and during a 30-min washout. *n* = 6. Cii: time course plot of normalised data aggregated into 1-min bins showing a reduction in burst amplitude during D-APV application. *n* = 6. D: raw (top) and rectified/integrated (bottom) traces recorded from left (L) and right (R) L_2 ventral roots showing the effect of the competitive co-agonist binding site antagonist DCKA (5 µM) on locomotor-related activity. Ei: locomotor-burst frequency over 5 min during a control period, during a 30-min application of DCKA, and during a 30-min washout. *n* = 6. Eii: time course plot of normalised data aggregated into 1-min bins showing a reduction in burst frequency during DCKA application. *n* = 6. Fi: locomotor-burst amplitude over 5 min during a control period, during a 30-min application of DCKA, and during a 30-min washout. *n* = 6. Fii: time course plot of normalised data aggregated into 1-min bins showing a reduction in burst amplitude during DCKA application. *n* = 6. Error bars: ± SEM. Statistically significant difference from control: $*p < 0.05$, $**p < 0.01$.

for NMDARs in controlling the frequency and amplitude of locomotor-related activity in mouse spinal cord preparations, consistent with findings from rat preparations (Beato, Bracci and Nistri, 1997; Bracci, Beato and Nistri, 1998; Cowley *et al.*, 2005), and contradicting findings from an earlier study of the mouse (Whelan, Bonnot and Donovan, 2000). Furthermore, these effects are mediated by canonical GluN1/GluN2 subunit-containing NMDARs.

The NMDAR co-agonist binding site is unsaturated during fictive locomotion

Occupancy of the co-agonist binding site by D-serine or glycine is a precondition for the activation of NMDARs by glutamate (Johnson and Ascher, 1987; Kleckner and Dingledine, 1988; Clements and Westbrook, 1991); however, the co-agonist binding site may not be fully saturated for a given receptor population, and co-agonist availability may be adjusted to modulate glutamatergic transmission during network activity (Berger, Dieudonné and Ascher, 1998; Kalbaugh, Zhang and Diamond, 2009; Li *et al.*, 2009, 2013). Therefore, it was next investigated whether co-agonist binding sites are saturated during fictive locomotion by bath applying D-serine at a range of concentrations (0.01 µM n = 7 preparations; 0.1 µM, n = 5; 1 µM, n = 7; 3 µM, n = 6; duration 15 min); D-serine, unlike glycine, is not subject to rapid clearance from the synapse (Supplisson and Bergman, 1997). D-serine rapidly increased burst frequency at concentrations of 0.01-3 µM, with the greatest effect at 1 µM (70.9 ± 13.6%; Fig. 2, A, Ci and D; $p < 0.01$, n = 7). At higher concentrations (Fig. 2, B and Ci), and when applied for longer periods (not shown), rhythmic activity became disordered, and a smaller effect on burst frequency was recorded. D-serine altered burst amplitude only at 1 µM, although the reduction detected at that concentration was modest (8.1 ± 2.1%; Fig. 2, A, Cii and E; $p < 0.05$, n = 7). Alternation of bursts, both between contralateral L_2 roots and between ipsilateral roots L_2 and L_5, was maintained throughout the drug application and wash periods (Fig 2, A and B). To confirm that the effects of D-serine were mediated exclusively by NMDARs (Kakegawa *et al.*, 2011), it was applied during NMDAR blockade. In the presence of saturating D-APV (50 µM), D-serine (1-10 µM) failed to modulate either the frequency (Fig. 2, F and G; $p > 0.05$, n = 6) or amplitude (Fig. 2, F and H; $p > 0.05$, n = 6) of rhythmic bursting. These data


A
Control
D-serine (1 µM)
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D-APV
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Gii
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Fig. 2. Exogenous D-serine acts at unsaturated NMDAR co-agonist binding sites to modulate locomotor-related activity. A and B: Raw (top) and rectified/integrated (bottom) traces recorded from left (L) and right (R) L_2 ventral roots and RL_5 showing the effect of D-serine at 1 µM (A) and 3 µM (B) on locomotor-related activity induced by 5-HT (15 µM) and DA (50 µM). C: Percentage change in frequency (Ci) and amplitude (Cii) in response to varying concentrations of D-serine, calculated by comparing a 5-min window during a control period with a 5-min window during a 15-min application of D-serine. *n* = 5-7 preparations. Di: locomotor-burst frequency over 5 min during a control period, during a 15-min application of D-serine (1 µM), and during a 20-min washout. Individual data points are shown in grey, and means are represented by black lines. *n* = 7. Dii: time course plot of normalised data aggregated into 1-min bins showing an increase in burst frequency during D-serine (1 µM) application. *n* = 7. Ei: locomotor-burst amplitude over 5 min during a control period, during a 15-min application of D-serine (1 µM), and during a 20-min washout. *n* = 7. Eii: time course plot of normalised data aggregated into 1-min bins showing a reduction in burst amplitude during D-serine (1 µM) application. *n* = 7. F: raw (top) and rectified/integrated (bottom) traces recorded from left (L) and right (R) L_2 ventral roots showing the effect of D-serine (10 µM) on locomotor-related activity in the presence of D-APV (50 µM). Gi: locomotor-burst frequency over 5 min during a control period, during a 30-min application of D-serine (1-10 µM), and during a 20-min washout. D-APV was present throughout. *n* = 6. Gii: time course plot of normalised data aggregated into 1-min bins showing no change in burst frequency when D-serine is applied in the presence of D-APV *n* = 6. Hi: locomotor-burst amplitude over 5 min during a control period, during a 30-min application of D-serine (1-10 µM), and during a 20-min washout. D-APV was present throughout. *n* = 6. Hii: time course plot of normalised data aggregated into 1-min bins showing no change in burst amplitude when D-serine is applied in the presence of the competitive glutamate-binding site antagonist D-APV *n* = 6. Error bars: ± SEM. Statistically significant difference from control: $*p < 0.05$, $**p < 0.01$.

indicate that NMDARs within the spinal motor circuitry are unsaturated during fictive locomotion, permitting enhancement of NMDAR activation by increased co-agonist availability.

Endogenous D-serine acts via NMDARs to reduce the frequency of locomotor-related activity

In various brain preparations, the endogenous NMDAR co-agonist is either D-serine or glycine, with one or the other gating receptors in a non-redundant manner (Mothet *et al.*, 2000; Kalbaugh, Zhang and Diamond, 2009; Li *et al.*, 2009, 2013, Henneberger *et al.*, 2010, 2013; Papouin *et al.*, 2012; Le Bail *et al.*, 2015; Meunier *et al.*, 2016). Co-agonist identity within spinal networks was assessed by selective depletion of endogenous D-serine. Unexpectedly, selective degradation of endogenous D-serine by bath application of the enzyme DAAO (0.29 U ml^{-1}) (Papouin *et al.*, 2012; Sasabe *et al.*, 2014) resulted in a pronounced increase in the frequency of locomotor-related bursting (71.6 ± 20.7%; Fig. 3, A and B; $p < 0.01$, $n = 8$), but did not alter burst amplitude (Fig. 3, A and C; $p > 0.05$, $n = 8$). Likewise, application of HOAsp (400 µM) (Strísovský *et al.*, 2005; Henneberger *et al.*, 2010) to inhibit endogenous serine racemase increased the frequency of rhythmic bursting (77.9 ± 21.3%; Fig. 3, D and E; $p < 0.01$, $n = 6$) without affecting amplitude (Fig. 3, D and F; $p > 0.05$, $n = 6$). To confirm that the increase in locomotor frequency upon depletion of endogenous D-serine was mediated by NMDARs, HOAsp was bath applied in the presence of D-APV (50 µM). In these experiments, neither burst frequency (Fig. 3, G and H; $p > 0.05$, $n = 10$) nor amplitude ($p > 0.05$, $n = 10$) were altered, indicating that endogenous D-serine acts at NMDARs. However, it is unlikely that endogenous D-serine acts universally at NMDARs since, if this were the case, its depletion would result in reduced frequency and amplitude of locomotor-related bursting, reproducing the effects of NMDA blockade. The finding that depletion of endogenous D-serine instead increases the frequency of network output implies that its action is restricted to a subset of NMDARs. Previously, it was shown that selective inhibition of a subset of glutamatergic neurons within the spinal cord proposed to synapse onto inhibitory interneurons enhances locomotor activity (Bouvier *et al.*, 2015).

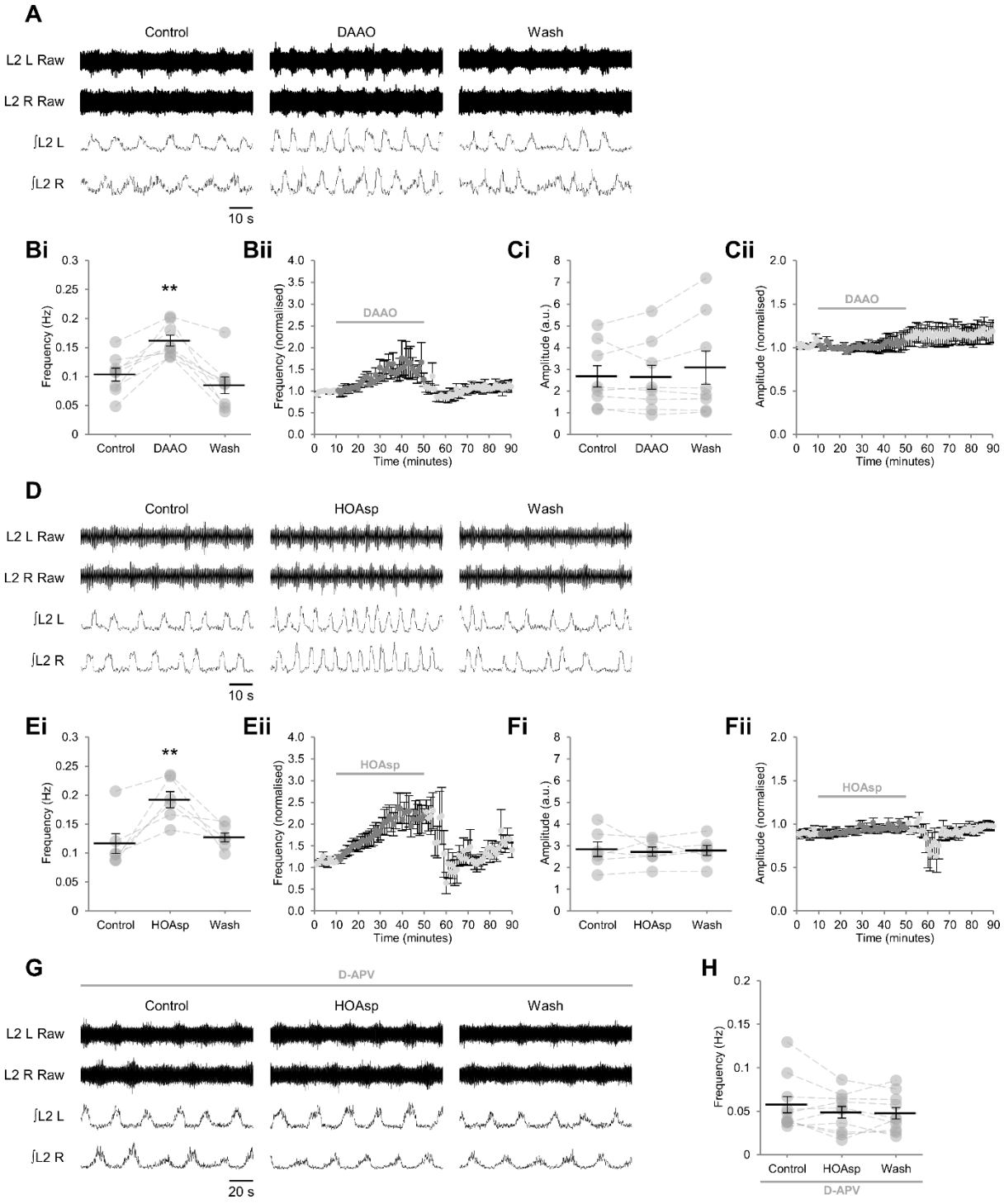
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Fig. 3. Endogenous D-serine acts via NMDARs to modulate the frequency but not the amplitude of locomotor-related activity. A: raw (top) and rectified/integrated (bottom) traces recorded from left (L) and right (R) L_2 ventral roots showing the effect of the D-serine scavenger DAAO (0.29 U ml^{-1}) on locomotor-related activity induced by 5-HT (15 µM) and DA (50 µM). Bi: locomotor-burst frequency over 5 min during a control period, during a 40-min application of DAAO, and during a 40-min washout. Individual data points are shown in grey, and means are represented by black lines. *n* = 8. Bii: time course plot of normalised data aggregated into 1-min bins showing an increase in burst frequency during DAAO application. *n* = 8. Ci: locomotor-burst amplitude over 5 min during a control period, during a 40-min application of DAAO, and during a 40-min washout. *n* = 8. Cii: time course plot of normalised data aggregated into 1-min bins showing no change in burst amplitude during DAAO application. *n* = 8. D: raw (top) and rectified/integrated (bottom) traces recorded from left (L) and right (R) L_2 ventral roots showing the effect of the serine racemase inhibitor HOAsp (400 µM) on locomotor-related activity. Ei: locomotor-burst frequency over 5 min during a control period, during a 40-min application of HOAsp, and during a 40-min washout. *n* = 6. Eii: time course plot of normalised data aggregated into 1-min bins showing an increase in burst frequency during HOAsp application. *n* = 6. Fi: locomotor-burst amplitude over 5 min during a control period, during a 40-min application of HOAsp, and during a 40-min washout. *n* = 6. Fii: time course plot of normalised data aggregated into 1-min bins showing no change in burst amplitude during HOAsp application. *n* = 6. G: raw (top) and rectified/integrated (bottom) traces recorded from left (L) and right (R) L_2 ventral roots showing the effect of HOAsp on locomotor-related activity in the presence of the competitive glutamate-binding site antagonist D-APV (50 µM). H: locomotor-burst frequency over 5 min during a control period, during a 30-min application of HOAsp, and during a 20-min washout. D-APV was present throughout. *n* = 10. Error bars: ± SEM. Statistically significant difference from control: $^{**}p < 0.01$.

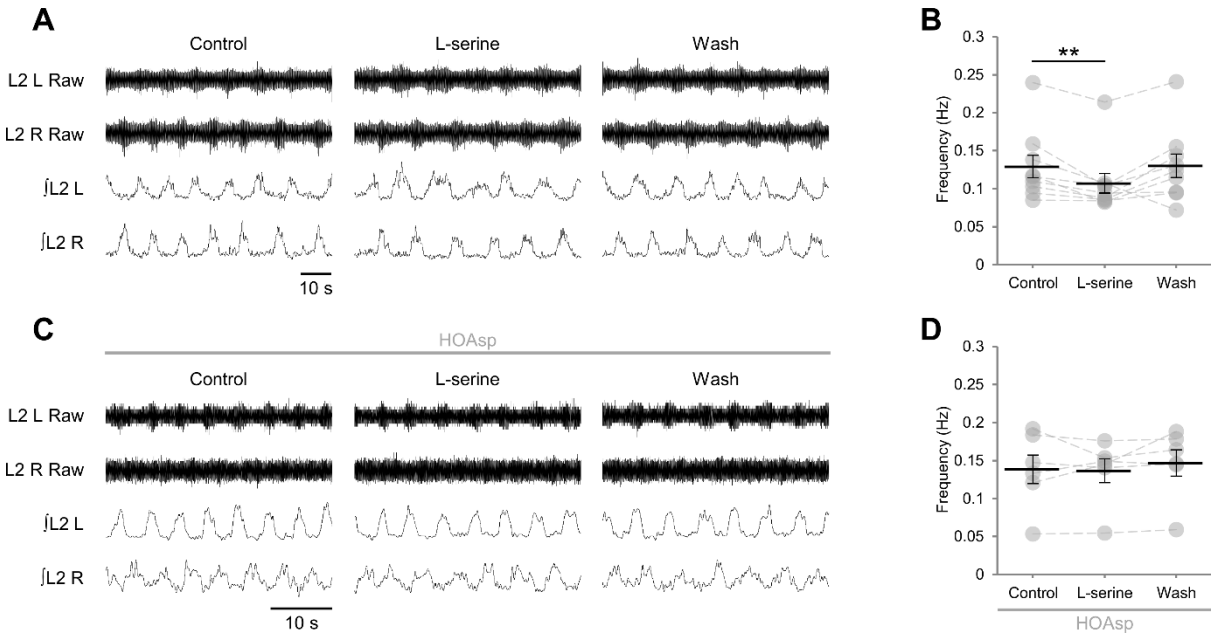
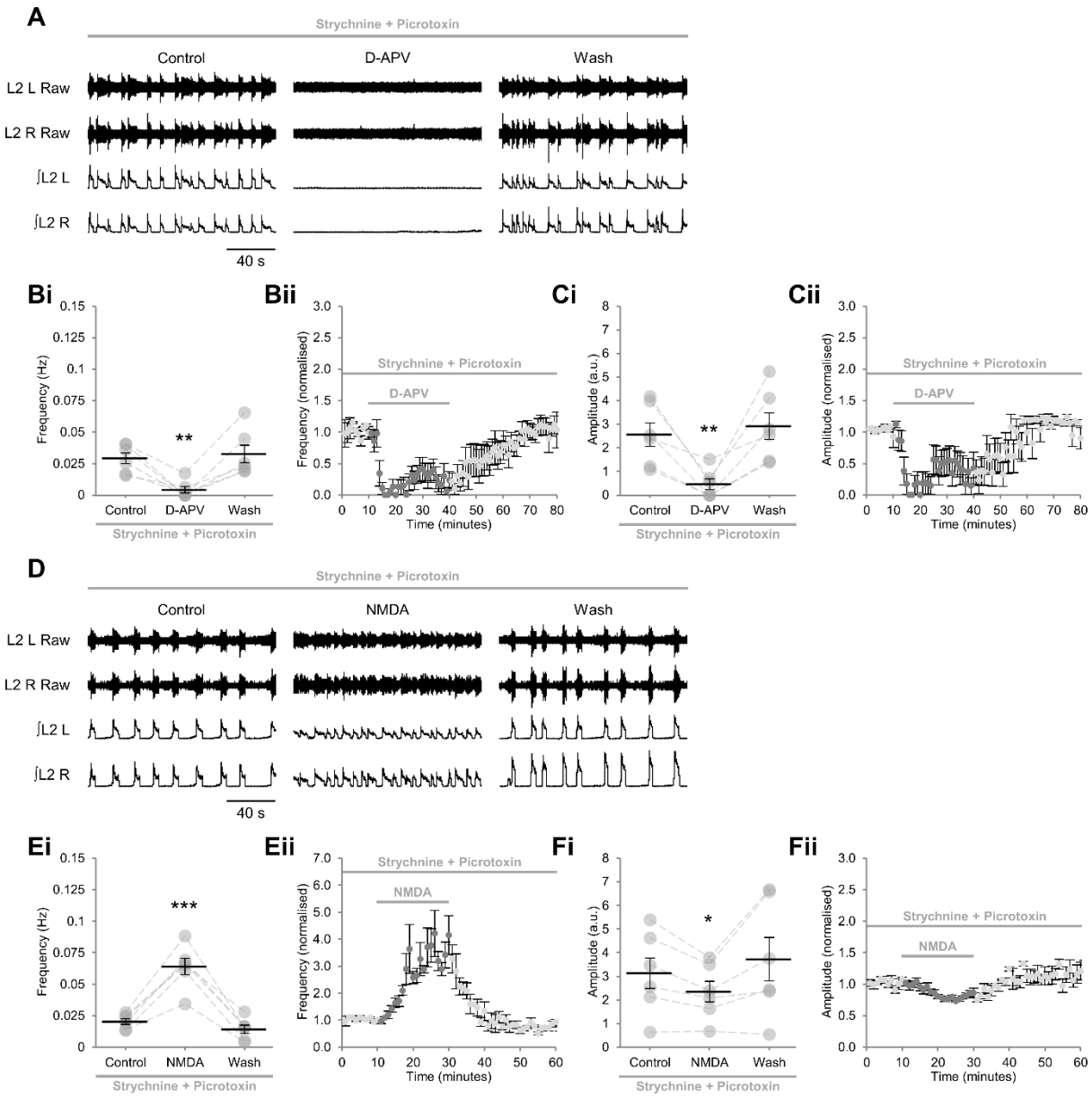


Fig 4: Racemisation of L-serine within the spinal cord results in a decrease in the frequency of locomotor-related activity. A: raw (top) and rectified/integrated (bottom) traces recorded from left (L) and right (R) L_2 ventral roots showing the effect of the D-serine precursor L-serine (50 µM) on locomotor-related activity induced by 5-HT (15 µM) and DA (50 µM). B: locomotor-burst frequency over 5 min during a control period, during a 15-min application of L-serine (40-100 µM), and during a 30-min washout. Individual data points are shown in grey, and means are represented by black lines. *n* = 9 preparations. C: raw (top) and rectified/integrated (bottom) traces recorded from left (L) and right (R) L_2 ventral roots showing the effect of L-serine (50 µM) on locomotor-related activity in the presence of the serine racemase inhibitor HOAsp (400 µM). D: locomotor-burst frequency over 5 min during a control period, during a 15-min application of L-serine (50 µM), and during a 30-min washout. HOAsp was present throughout. *n* = 6. Error bars: ± SEM. Statistically significant difference from control: $^{**}p < 0.01$.

To further investigate the activity of endogenous D-serine, experiments were performed in which the substrate of serine racemase, L-serine, was applied to preparations. Whereas bath-applied D-serine is expected to potentiate all NMDARs at which the co-agonist binding site is unsaturated, application of L-serine is likely to enhance the availability of D-serine preferentially at synapses proximate to its synthesis (Rosenberg *et al.*, 2010). Supplementation of L-serine (40-100 µM) consistently reduced the frequency of locomotor-related bursting (16.2 ± 3.6%; Fig. 4, A and B; $p < 0.01$, $n = 9$), without affecting amplitude ($p > 0.05$, $n = 9$). Burst frequency was unchanged when L-serine (50 µM) was applied in the presence of HOAsp (400 µM; Fig. 4, C and D; $p > 0.05$, $n = 6$), indicating that serine racemase is required for its conversion to D-serine, which in turn facilitates NMDAR activation. Collectively, these data support a role for endogenous D-serine in the regulation of a restricted population of NMDARs in the spinal cord, with the remaining fraction of NMDARs likely being gated by glycine.

D-serine does not modulate excitatory components of locomotor networks during disinhibited bursting

If endogenous D-serine acts as a dominant co-agonist at a subset of NMDARs, it is likely that these are expressed by inhibitory interneurons that act to constrain the frequency of locomotor-related activity. To test the relative sensitivity of excitatory versus inhibitory components of the spinal motor circuitry to D-serine, experiments were performed in which inhibitory transmission was blocked by the glycine receptor (GlyR) antagonist strychnine (1 µM) and the $GABA_A$ channel antagonist picrotoxin (60 µM) (Bracci, Ballerini and Nistri, 1996; Witts, Panetta and Miles, 2012; Foster *et al.*, 2014). Blockade of inhibitory transmission resulted in slow (0.032 ± 0.002 Hz, $n = 25$), long-duration, high-amplitude bursts that were synchronous across ventral roots (Fig. 5 and 6). When D-APV (50 µM) was applied to determine the importance of NMDARs for the generation of this pattern of network activity, bursting rapidly ceased in all experiments (Fig. 5, A, Bii and Cii; $n = 6$), as previously reported (Bracci, Ballerini and Nistri, 1996). In 2/6 experiments, however, bursting recovered during the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Time (minutes)
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Amplitude (a.u.)
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Control
NMDA
Wash
Strychnine + Picrotoxin
Fii
Amplitude (normalised)
Strychnine + Picrotoxin
NMDA
Time (minutes)

Fig. 5: NMDA receptors are active during disinhibited bursting. A: raw (top) and rectified/integrated (bottom) traces recorded from left (L) and right (R) L_2 ventral roots showing the effect of the competitive glutamate-binding site antagonist D-APV (50 μM) applied to preparations in which inhibitory transmission was blocked by the $GABA_A$-receptor antagonist pictrotoxin (60 μM) and the glycine-receptor antagonist strychnine (1 μM). Bi: Ventral-root burst frequency over 10 min during a control period, during a 30-min application of D-APV, and during a 40-min washout. Individual data points are shown in grey, and means are represented by black lines. *n* = 6 preparations. Bii: time course plot of normalised data aggregated into 1-min bins showing a reduction in burst frequency during D-APV application. *n* = 6. Ci: Ventral-root amplitude over 10 min during a control period, during a 30-min application of D-APV, and during a 40-min washout. *n* = 6. Cii: time course plot of normalised data aggregated into 1-min bins showing a reduction in burst amplitude during D-APV application. *n* = 6. D: raw (top) and rectified/integrated (bottom) traces recorded from left (L) and right (R) L_2 ventral roots showing the effect of NMDA (10 μM) applied to preparations in which inhibitory transmission was blocked. Ei: Ventral-root burst frequency over 10 min during a control period, during a 20-min application of NMDA, and during a 30-min washout. *n* = 6. Eii: time course plot of normalised data aggregated into 1-min bins showing an increase in burst frequency during NMDA application. *n* = 6. Fi: Ventral-root burst amplitude over 10 min during a control period, during a 20-min application of NMDA, and during a 30-min washout. *n* = 6. Fii: time course plot of normalised data aggregated into 1-min bins showing a reduction in burst amplitude during NMDA application. *n* = 6. Error bars: ± SEM. Statistically significant difference from control: $*p < 0.05$, $***p < 0.001$.

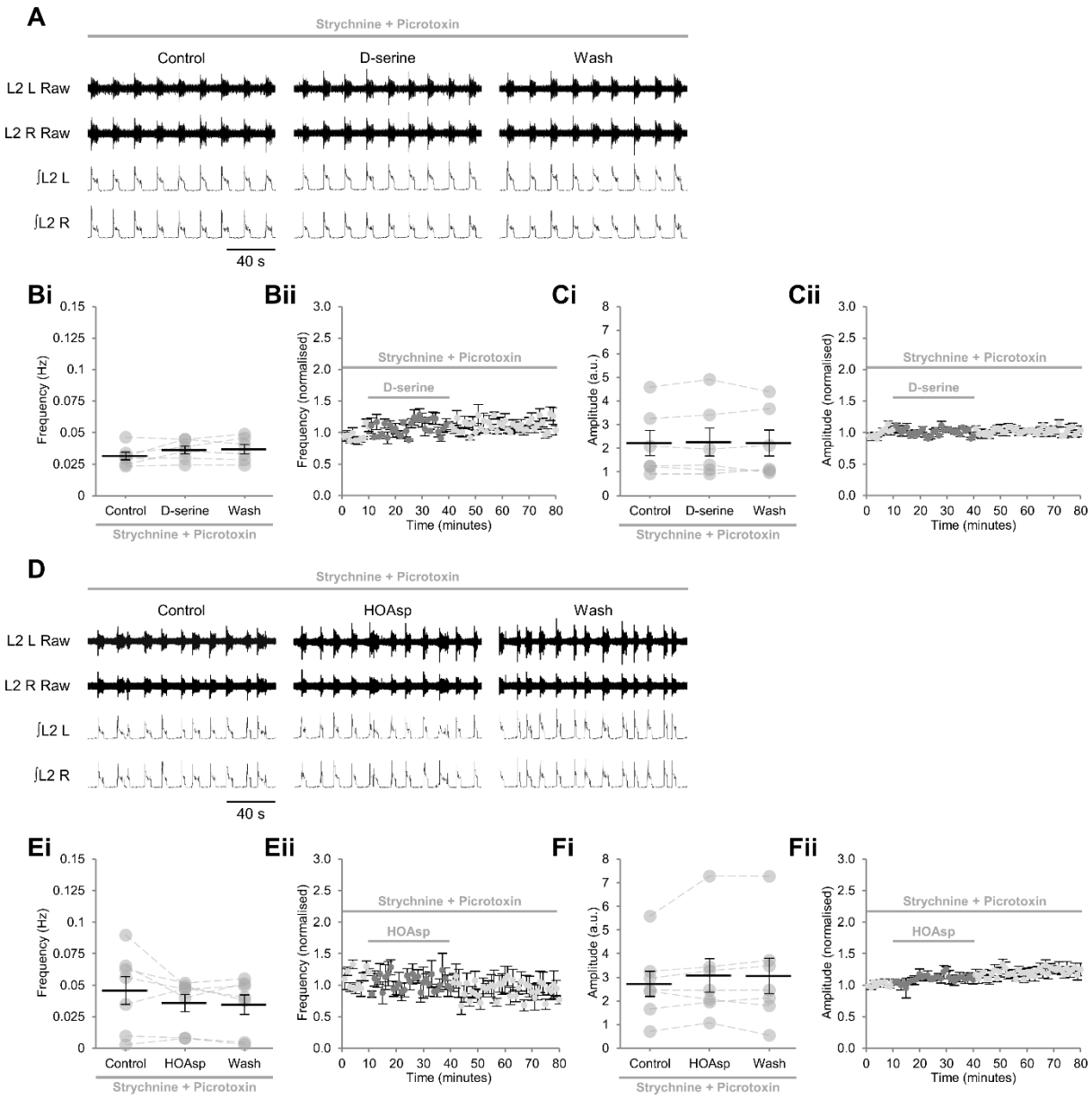

A
Strychnine + Picrotoxin
Control
D-serine
Wash
L2 L Raw
L2 R Raw
∫L2 L
∫L2 R
40 s
Bi
Frequency (Hz)
Control
D-serine
Wash
Strychnine + Picrotoxin
Bii
Frequency (normalised)
Strychnine + Picrotoxin
D-serine
Time (minutes)
Ci
Amplitude (a.u.)
Control
D-serine
Wash
Strychnine + Picrotoxin
Cii
Amplitude (normalised)
Strychnine + Picrotoxin
D-serine
Time (minutes)
D
Strychnine + Picrotoxin
Control
HOAsp
Wash
L2 L Raw
L2 R Raw
∫L2 L
∫L2 R
40 s
Ei
Frequency (Hz)
Control
HOAsp
Wash
Strychnine + Picrotoxin
Eii
Frequency (normalised)
Strychnine + Picrotoxin
HOAsp
Time (minutes)
Fi
Amplitude (a.u.)
Control
HOAsp
Wash
Strychnine + Picrotoxin
Fii
Amplitude (normalised)
Strychnine + Picrotoxin
HOAsp
Time (minutes)

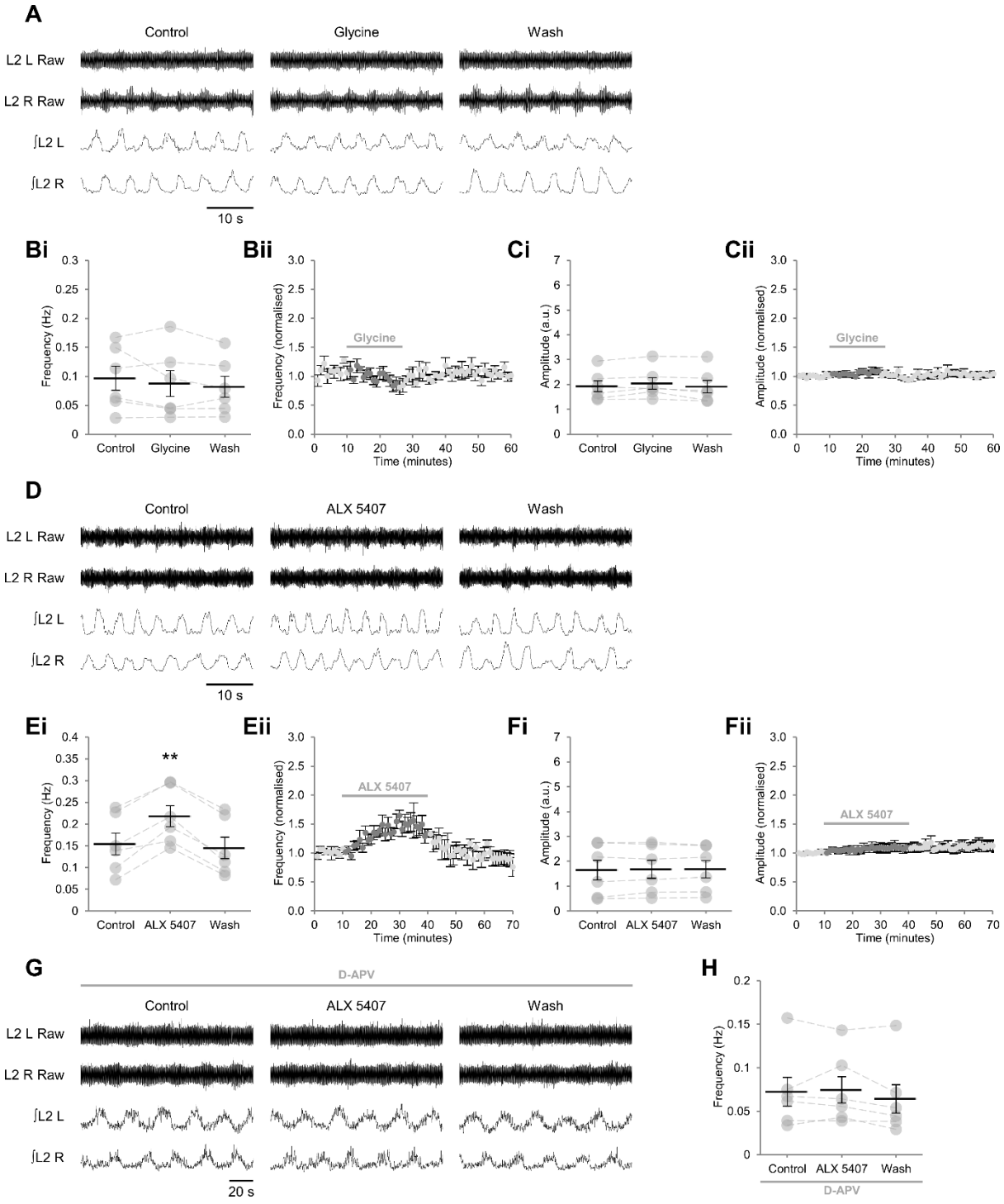
Fig. 6: Neither exogenous nor endogenous D-serine modulates disinhibited activity mediated by excitatory components of locomotor networks. A: raw (top) and rectified/integrated (bottom) traces recorded from left (L) and right (R) L_2 ventral roots showing the effect of D-serine (10 µM) applied to preparations in which inhibitory transmission was blocked by the $GABA_A$-receptor antagonist pictrotoxin (60 µM) and the glycine-receptor antagonist strychnine (1 µM). Bi: Ventral-root burst frequency over 10 min during a control period, during a 30-min application of D-serine (1-10 µM), and during a 40-min washout. Individual data points are shown in grey, and means are represented by black lines. *n* = 6 preparations. Bii: time course plot of normalised data aggregated into 1-min bins showing no change in burst frequency during D-serine application. *n* = 6. Ci: Ventral-root burst amplitude over 10 min during a control period, during a 30-min application of D-serine (1-10 µM), and during a 40-min washout. *n* = 6. Cii: time course plot of normalised data aggregated into 1-min bins showing no change in burst amplitude during D-serine application. *n* = 6. D: raw (top) and rectified/integrated (bottom) traces recorded from left (L) and right (R) L_2 ventral roots showing the effect of the serine racemase inhibitor HOAsp (400 µM) applied to preparations in which inhibitory transmission was blocked. Ei: Ventral-root burst frequency over 10 min during a control period, during a 30-min application of HOAsp, and during a 40-min washout. *n* = 7. Eii: time course plot of normalised data aggregated into 1-min bins showing no change in burst frequency during HOAsp application. *n* = 7. Fi: Ventral-root burst amplitude over 10 min during a control period, during a 30-min application of HOAsp, and during a 40-min washout. *n* = 7. Fii: time course plot of normalised data aggregated into 1-min bins showing no change in burst amplitude during HOAsp application. *n* = 7. Error bars: ± SEM.

period of D-APV application at a reduced frequency (Fig. 5B) and amplitude (Fig. 5C), indicating that NMDARs contribute to network excitation within disinhibited preparations, but may not be indispensable. NMDA (10 µM) was also applied during disinhibited bursting to determine whether NMDAR activation could be further increased. NMDA consistently increased burst frequency (221.2 ± 28.1%; Fig. 5, D and E; $p < 0.001$, $n = 6$) while reducing burst amplitude (20.1 ± 5.0%; Fig. 5, D and F; $p < 0.05$, $n = 6$).

Next, D-serine (1-10 µM) was applied to assess whether NMDAR currents could be modulated via the co-agonist binding site during disinhibited activity. Neither burst frequency (Fig. 6, A and B; $p > 0.05$, $n = 6$) nor amplitude (Fig. 6, A and C; $p > 0.05$, $n = 6$) were altered, indicating that NMDARs expressed by excitatory components of the locomotor circuitry have saturated co-agonist binding sites under disinhibited conditions. To assess whether endogenous D-serine acted as an NMDAR co-agonist within excitatory components of the locomotor circuitry, HOAsp (400 µM) was applied during disinhibited activity. Bursting was unchanged in both frequency (Fig. 6, D and E; $p > 0.05$, $n = 7$) and amplitude (Fig. 6, D and F; $p > 0.05$, $n = 7$) during HOAsp application, indicating that D-serine is not required as a co-agonist by NMDAR populations involved in the production of disinhibited bursting. These results support differential regulation of spinal cord NMDARs via the co-agonist binding site, whereby endogenous D-serine is required by NMDARs expressed by inhibitory components of the circuitry only and receptors are saturated under some conditions but not others.

GlyT1 regulates extracellular glycine concentration and NMDAR activation

Because the action of endogenous D-serine appears limited to a subpopulation of NMDARs within spinal networks, the role of glycine in the gating of NMDARs during bilaterally alternating locomotor-related activity was then assessed. Although glycine is the principal inhibitory transmitter within the spinal cord (Bowery and Smart, 2006) and has a similar binding affinity and potency to D-serine at NMDAR co-agonist binding sites (Brugger *et al.*, 1990; Priestley *et al.*, 1995; Chen *et al.*, 2007), application of glycine did not modulate the frequency or amplitude of rhythmic activity at 1 µM (frequency: $p > 0.05$, $n = 6$; amplitude: $p > 0.05$, $n = 6$), 10 µM


A
Control
Glycine
Wash
L2 L Raw
L2 R Raw
∫L2 L
∫L2 R
10 s
Bi
Frequency (Hz)
Control
Glycine
Wash
Bii
Frequency (normalised)
Glycine
Time (minutes)
Ci
Amplitude (a.u.)
Control
Glycine
Wash
Cii
Amplitude (normalised)
Glycine
Time (minutes)
D
Control
ALX 5407
Wash
L2 L Raw
L2 R Raw
∫L2 L
∫L2 R
10 s
Ei
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Frequency (Hz)
Control
ALX 5407
Wash
Eii
Frequency (normalised)
ALX 5407
Time (minutes)
Fi
Amplitude (a.u.)
Control
ALX 5407
Wash
Fii
Amplitude (normalised)
ALX 5407
Time (minutes)
G
D-APV
Control
ALX 5407
Wash
L2 L Raw
L2 R Raw
∫L2 L
∫L2 R
20 s
H
Frequency (Hz)
Control
ALX 5407
Wash
D-APV

Fig. 7. GlyT1 glycine transporters control the availability of glycine at excitatory synapses. A: raw (top) and rectified/integrated (bottom) traces recorded from left (L) and right (R) L_2 ventral roots showing the effect of glycine (100 µM) on locomotor-related activity induced by 5-HT (15 µM) and DA (50 µM). Bi: locomotor-burst frequency over 5 min during a control period, during a 15-min application of glycine, and during a 40-min washout. Individual data points are shown in grey, and means are represented by black lines. *n* = 6. Bii: time course plot of normalised data aggregated into 1-min bins showing no change in burst frequency during glycine application. *n* = 6. Ci: locomotor-burst amplitude over 5 min during a control period, during a 15-min application of glycine, and during a 40-min washout. *n* = 6. Cii: time course plot of normalised data aggregated into 1-min bins showing no change in burst amplitude during glycine application. *n* = 6. D: raw (top) and rectified/integrated (bottom) traces recorded from left (L) and right (R) L_2 ventral roots showing the effect of the GlyT1 inhibitor ALX 5407 (30 µM) on locomotor-related activity. Ei: locomotor-burst frequency over 5 min during a control period, during a 30-min application of ALX 5407, and during a 30-min washout. *n* = 6. Eii: time course plot of normalised data aggregated into 1-min bins showing an increase in burst frequency during ALX 5407 application. *n* = 6. Fi: locomotor-burst amplitude over 5 min during a control period, during a 30-min application of ALX 5407, and during a 30-min washout. *n* = 6. Fii: time course plot of normalised data aggregated into 1-min bins showing no change in burst amplitude during ALX 5407 application. *n* = 6. G: raw (top) and rectified/integrated (bottom) traces recorded from left (L) and right (R) L_2 ventral roots showing the effect of ALX 5407 on locomotor-related activity in the presence of the competitive glutamate-binding site antagonist D-APV (50 µM). H: locomotor-burst frequency over 5 min during a control period, during a 30-min application of HOAsp, and during a 30-min washout. D-APV was present throughout. *n* = 6. Error bars: ± SEM. Statistically significant difference from control: $^{**}p < 0.01$.

(frequency: $p > 0.05$, $n = 6$; amplitude: $p > 0.05$, $n = 6$) or 100 µM (frequency: $p > 0.05$, $n = 6$; amplitude: $p > 0.05$, $n = 6$; Fig. 7, A-C), nor was left-right alternation of bursting altered (Fig 7. A).

The glycine transporter GlyT1 is highly expressed at excitatory synapses in both the spinal cord and brainstem (Zafra, Gomeza, *et al.*, 1995; Cubelos, Giménez and Zafra, 2005) and has been proposed to maintain sub-saturating concentrations of glycine in the latter (Berger, Dieudonné and Ascher, 1998; Lim, Hoang and Berger, 2004). The selective GlyT1 antagonist ALX 5407 (30 µM) (Atkinson *et al.*, 2001) was applied to rhythmically active preparations to determine the influence of GlyT1 on extracellular glycine and locomotor-related network activity. GlyT1 antagonism resulted in a gradual increase in burst frequency (52.0 ± 13.9%; Fig. 7, D and E; $p < 0.01$, $n = 6$) but had no effect on amplitude (Fig. 7, D and F; $p > 0.05$, $n = 6$). By contrast, no changes in locomotor frequency (Fig. 7, G and H; $p > 0.05$, $n = 6$) or amplitude ($p > 0.05$, $n = 6$) were observed when ALX 5407 (30 µM) was applied in the presence of D-APV (50 µM). Together, these results indicate that GlyT1 governs occupation of NMDAR co-agonist binding sites by exercising tight control over the concentration of glycine at excitatory synapses.

Discussion

This chapter provides evidence that both endogenous D-serine and glycine gate NMDARs within mammalian spinal networks to regulate the frequency of locomotor-related activity, that the NMDAR co-agonist binding site at which they act is unsaturated during locomotor-related but saturated during disinhibited activity, and that the glycine transporter GlyT1 is a potent regulator of glycine concentration at excitatory synapses. These findings suggest that dynamic regulation of NMDARs via the co-agonist binding site contributes to flexibility in the output repertoire of mammalian spinal motor circuits.

Glutamatergic transmission operates both within spinal locomotor networks, where it determines the frequency, amplitude and robustness of rhythmic activity (Talpalar and Kiehn,

2010), and between spinal networks and descending pathways, which transmit commands for both the initiation (Jordan *et al.*, 2008; Hägglund *et al.*, 2010) and, paradoxically, the cessation (Bouvier *et al.*, 2015) of locomotion. Glutamatergic signalling within spinal networks is mediated by both NMDARs and non-NMDARs (Cazalets, Sqalli-Houssaini and Clarac, 1992; Beato, Bracci and Nistri, 1997; Nishimaru, Takizawa and Kudo, 2000; Whelan, Bonnot and Donovan, 2000; Talpalar and Kiehn, 2010). Previously, it was proposed that the contribution of NMDARs to network output differed between isolated spinal cord preparations from postnatal rats and mice (Nishimaru, Takizawa and Kudo, 2000). In rats, application of NMDAR agonists alone is reported to induce sustained fictive locomotion (Kudo and Yamada, 1987; Smith, Feldman and Schmidt, 1988), whereas in mice, sustained activity is reported to require 5-HT in addition to NMDAR agonists (Jiang, Carlin and Brownstone, 1999). In addition, NMDAR blockade consistently inhibits rhythmic activity in rats (Smith, Feldman and Schmidt, 1988; Cazalets, Sqalli-Houssaini and Clarac, 1992; Beato, Bracci and Nistri, 1997; Bracci, Beato and Nistri, 1998; Gabbay, 2004; Cowley *et al.*, 2005), whereas in mice, it has been reported to enhance burst frequency during pharmacologically induced (5-HT, 15 µM; DA, 75 µM) fictive locomotion (Whelan, Bonnot and Donovan, 2000). In the present study, it is shown that NMDAR blockade reduces the frequency of pharmacologically induced locomotor-related activity in mouse spinal cord preparations, as previously reported in rat preparations (Beato, Bracci and Nistri, 1997; Gabbay, 2004; Cowley *et al.*, 2005). It is also shown that NMDAR blockade reduces burst amplitude, an observation previously reported in the mouse (Whelan, Bonnot and Donovan, 2000) and rat (Beato, Bracci and Nistri, 1997). As in rat preparations (Beato, Bracci and Nistri, 1997; Bracci, Beato and Nistri, 1998; Cowley *et al.*, 2005), left-right rhythmic alternation of bursts is maintained during NMDAR blockade. This study does not consider the effects of NMDAR blockade during high frequency (>0.4 Hz) fictive locomotion, during which it is reported to affect the robustness of rhythmic activity by disrupting patterned output by increasing non-resetting deletions – interruptions to patterned bursting lasting an integer of the cycle period – rather than altering frequency *per se* (Talpalar and Kiehn, 2010). The data presented here are consistent with previous reports that NMDARs are not essential

for the operation of the rodent locomotor CPG, but instead function to modulate pacing, at least at lower frequencies (< 0.4 Hz) of network activity (Beato, Bracci and Nistri, 1997; Bracci, Beato and Nistri, 1998; Cowley *et al.*, 2005; Talpalar and Kiehn, 2010). D-APV, a competitive inhibitor of the glutamate-binding site, and DCKA, a competitive inhibitor of the co-agonist binding site, have similar effects on burst frequency and amplitude, revealing a requirement for binding of both glutamate and a co-agonist, and implying that canonical GluN1/GluN2 subunit-containing NMDARs dominate in the spinal motor circuitry.

The finding that extensor-related bursting recorded from root L_5 becomes indistinct upon NMDAR blockade likely reflects relatively weak locomotor drive to the lower lumbar segments according to the rostrocaudal gradient of excitability within the locomotor circuitry (Bonnot *et al.*, 2002), rather than a greater contribution of NMDARs to the generation of activity in that root, since bursting in L_5 is reported to continue during NMDAR blockade when a strong pharmacological stimulus is applied (Talpalar and Kiehn, 2010).

Blockade of NMDARs is shown to result in sustained abolition of disinhibited bursting in most but not all preparations. Similarly, it is reported that NMDAR blockade abolishes disinhibited bursting in rat preparations, except when AMPAR currents are enhanced (Bracci, Ballerini and Nistri, 1996). Thus, NMDARs make a substantial contribution to disinhibited activity in both rats and mice, but are not essential for it. As in bilaterally alternating locomotor-related activity (Beato, Bracci and Nistri, 1997; Bracci, Beato and Nistri, 1998; Nishimaru, Takizawa and Kudo, 2000; Whelan, Bonnot and Donovan, 2000; Gabbay, 2004; Cowley *et al.*, 2005; Talpalar and Kiehn, 2010), disinhibited activity can be sustained during NMDAR blockade when an alternative stimulus of sufficient intensity is provided.

Together these results confirm the importance of NMDARs during both locomotor-related and disinhibited activity in the mouse, suggesting that NMDARs function in a similar manner in both mice and rats, by contrast with previous reports (Nishimaru, Takizawa and Kudo, 2000; Whelan, Bonnot and Donovan, 2000). Furthermore, these data demonstrate the necessity for a co-agonist to be bound to receptors in addition to glutamate.

It is shown that the NMDAR co-agonist binding site is unsaturated during locomotor-related activity and may be subject to cell type-specific and activity-dependent regulation. An unsaturated co-agonist binding site could permit the regulation of glutamatergic signalling and locomotor frequency by adjustments to co-agonist availability (Berger, Dieudonné and Ascher, 1998; Li *et al.*, 2009, 2013). Exogenous D-serine acts at NMDARs to increase the frequency of locomotor-related activity in a dose-dependent manner. The effective concentrations of exogenous D-serine are consistent with the binding affinities and potencies reported for D-serine and glycine, with EC_{50} values in the range of ~0.1-1.3 µM (Brugger *et al.*, 1990; Priestley *et al.*, 1995; Chen *et al.*, 2007). However, bursting becomes disrupted at higher concentrations of D-serine, as previously reported for NMDA itself (Talpalar and Kiehn, 2010), illustrating a requirement for regulation of NMDAR activation to ensure correct network activity. Excessive NMDAR activity resulting from aberrant regulation at the co-agonist binding site is a cause of excitotoxicity (Shleper, Kartvelishvily and Wolosker, 2005) and is proposed as a mechanism in the pathogenesis of amyotrophic lateral sclerosis (ALS) (Sasabe *et al.*, 2007; Paul *et al.*, 2014).

The effect of exogenous D-serine varies with the mode of network activity. Exogenous D-serine does not modulate disinhibited bursting generated and coordinated exclusively by excitatory components of the motor circuitry, despite strong activation of NMDARs during this mode of activity. By contrast, exogenous D-serine increases the burst frequency of bilaterally alternating locomotor-related activity. Given that excitatory interneurons provide rhythmic drive and speed control within locomotor networks (Kiehn *et al.*, 2008; Kiehn, 2016), modulation of their activity is likely to underlie increases in the frequency of rhythmic bursting during both modes of activity The finding that burst frequency increases upon NMDA but not D-serine application during disinhibited bursting indicates that the co-agonist binding sites of NMDARs expressed by excitatory interneurons are saturated. This may relate to presumably stronger activation of glutamate receptors during disinhibited bursting, which is characterised by seizure-like, high-amplitude, long-duration bursts. Thus, although it is uncertain whether

populations of rhythmogenic interneurons are identical between disinhibited and locomotor-related preparations, these data indicate differential availability of the co-agonist depending on network activity. Similarly, it is reported that co-agonist availability scales with glutamate release in brain preparations (Li *et al.*, 2009, 2013).

Whereas NMDA and glutamate enhance burst amplitude during locomotor-related activity at lower concentrations and reduce it at higher concentrations (Talpalar and Kiehn, 2010), exogenous D-serine only modestly reduces burst amplitude. Since this effect occurs only at the concentration of D-serine that most potently increases burst frequency, it may be an indirect effect of rapid network activity. The failure of D-serine to enhance burst amplitude at lower doses implies that motoneuronal NMDARs are saturated during locomotor-related activity. Consistent with this possibility, burst amplitude is also unchanged when glycine availability is enhanced by blockade of GlyT1. Amplitude is also unaffected by D-serine application during disinhibited bursting, although this may be a function of more intense glutamate release (see above). By contrast, the co-agonist binding site of motoneuronal NMDARs is unsaturated when network activity is silenced by TTX (Brugger *et al.*, 1990). Thus, the co-agonist site may be subject to cell-type specific as well as activity-dependent regulation, implying flexibility in the regulation of co-agonist availability to extend the range of possible network outputs.

This study provides evidence that endogenous D-serine, as well as glycine, regulates the frequency of locomotor-related activity. If D-serine were the exclusive co-agonist in spinal locomotor networks, or if it acted in conjunction with glycine at all NMDARs, its depletion would mimic the effect of NMDAR inhibition by D-APV of DCKA; that is, it would reduce the frequency and amplitude of locomotor-related. Instead, depletion of endogenous D-serine, either with an enzymatic scavenger or by inhibition of its synthesising enzyme, results in a pronounced increase in burst frequency with no change in burst amplitude. Conversely, supplementation of the d-serine precursor L-serine results in a decrease in burst frequency that is dependent on D-serine synthesis. Exogenous L-serine presumably enhances D-serine production at

synapses where serine racemase is concentrated and D-serine is utilised, whereas exogenous D-serine potentiates NMDARs at all synapses where the co-agonist binding site is unsaturated. The finding that endogenous D-serine inhibits locomotor-related activity suggests that it is primarily required at excitatory synapses onto inhibitory interneurons. Consistent with this possibility, serine racemase inhibition has no effect when excitatory components of the network are examined in isolation. As a similar example of excitatory signalling acting to reduce network activity, excitation of spinal cord networks by descending glutamatergic V2a interneurons mediates inhibition of locomotor-related activity, likely through excitation of inhibitory interneurons (Bouvier *et al.*, 2015)..Together, the findings of this study suggest a model in which D-serine acts via a subpopulation of NMDARs expressed by inhibitory interneurons to constrain locomotor speed, with the remaining moiety of co-agonist binding sites presumably regulated by glycine.

Despite having two possible targets in the spinal cord (strychnine-sensitive inhibitory GlyR and the strychnine-insensitive NMDAR co-agonist binding site), and binding NMDARs with a similar affinity to D-serine (Brugger *et al.*, 1990; Priestley *et al.*, 1995; Chen *et al.*, 2007) which potently modulates locomotor-related activity via NMDARs at 1 µM, exogenous glycine does not perturb locomotor-related activity at concentrations up to 100 µM. Although it cannot be ruled out that the actions of glycine at inhibitory receptors mask opposing activity at NMDARs, it is unlikely that GlyRs were activated in these experiments, as they have a considerably lower affinity for glycine than NMDARs, with EC_{50} values 2-3 orders of magnitude higher (Young and Snyder, 1974; Engblom and Akerman, 1991; Blednov, Bleck and Harris, 1996; Lewis *et al.*, 1998; Tapia and Aguayo, 1998; Graham *et al.*, 2011). Furthermore, increasing extracellular glycine concentration by GlyT1 blockade in the presence of D-APV does not reveal inhibitory effects of glycine. The lack of effect of exogenous glycine in spinal cord preparations parallels observations in the brainstem, where D-serine facilitates NMDAR currents in the low-micromolar range, but glycine is ineffective at concentrations below 100 µM until GlyT1 is inhibited (Berger, Dieudonné and Ascher, 1998). The lack of effect of exogenous glycine is

thus attributable to powerful glycine clearance by GlyT1, which is highly expressed in both regions (Cubelos, Giménez and Zafra, 2005). GlyT1 is a potent regulator of glycine availability at excitatory synapses, and is capable of holding glycine at subsaturating concentrations within the restricted space of the synaptic cleft below those of both the cerebrospinal fluid and a perfusate with a glycine concentration an order of magnitude greater (Supplisson and Bergman, 1997; Bergeron *et al.*, 1998). Consistent with tight regulation of glycine concentration by GlyT1 at excitatory synapses within locomotor circuitry, it is shown that GlyT1 antagonism results in an NMDAR-dependent increase in locomotor-burst frequency but not amplitude, similar to the effect of global facilitation of NMDARs with unsaturated co-agonist binding sites by exogenous D-serine. Similarly, inhibition of GlyT1 potentiates NMDAR currents in the brainstem (Lim, Hoang and Berger, 2004) and enhances swimming in *Xenopus* tadpoles in a manner resembling glycine or D-serine application (Issberner and Sillar, 2007). The regulation of glycine concentration at inhibitory synapses by GlyT2 is not considered here (Danglot *et al.*, 2004; Eulenburg *et al.*, 2005); however, these results reveal an important role for GlyT1 in the gating of NMDARs and the regulation of rhythmic activity in the mouse spinal cord, a role that is conserved between amphibians and mammals (Issberner and Sillar, 2007).

Modulation of GlyT1 activity to adjust the availability of glycine at excitatory synapses could provide a mechanism for the regulation of motor behaviour (Issberner and Sillar, 2007). GlyT1 is modulated, for instance, by protein kinase C (PKC) (Sato *et al.*, 1995), which is activated by endogenous modulators of locomotor networks released during network activity, including 5-HT (Anji *et al.*, 2001) and acetylcholine (Sculptoreanu *et al.*, 2001). GlyT1 is also modulated by brain derived neurotrophic factor (BDNF) (Aroeira, Sebastião and Valente, 2015), which is released in an activity-dependent manner (Goodman *et al.*, 1996; Marini *et al.*, 1998). Thus, GlyT1 is equipped to mediate the dynamic regulation of glycine availability at excitatory synapses and may extend the repertoire of adaptive behaviours produced by spinal networks.

The regulation of NMDARs is essential to the proper functioning of spinal locomotor networks in health and disease. Tuning of NMDAR activation by adjustments to the availability of the

co-agonist would enhance the flexibility of glutamatergic signalling in healthy animals, providing a mechanism for speed control and perhaps contributing to the determination of gait (Talpalar and Kiehn, 2010; Talpalar *et al.*, 2013; Bellardita and Kiehn, 2015). By contrast, dysregulation of NMDARs results in excitotoxicity (Shleper, Kartvelishvily and Wolosker, 2005), and may be implicated in the pathogenesis of ALS (Sasabe *et al.*, 2007; Paul *et al.*, 2014). Dichotomous regulation of NMDARs by glycine and D-serine is described in the brain (Kalbaugh, Zhang and Diamond, 2009; Papouin *et al.*, 2012; Li *et al.*, 2013; Le Bail *et al.*, 2015), although the rationale for regulation of NMDARs by two different co-agonists remains to be elucidated. This study provides the first evidence that NMDARs in the spinal cord are also regulated by both D-serine and glycine acting at distinct populations of NMDARs, and that co-agonist availability varies with neuronal activity. Furthermore, GlyT1 is shown to control the availability of glycine at excitatory synapses. Together, these findings reveal previously unexplored complexity in glutamatergic signalling during activity of mammalian locomotor networks.

Chapter 5: General discussion

Introduction

Many studies indicate that glia release of modulators in response to neuronal activity, yet the importance of gliotransmission in regulating network activity and in shaping behaviour remains controversial (Araque *et al.*, 2014; Bazargani and Attwell, 2016). This study provides evidence that glia release ATP-adenosine in an activity-dependent manner to modulate hindlimb locomotor networks in spinal cord preparations from postnatal mice. The activity of adenosine produced in this way is shown to be mediated by an antagonistic interaction between A_1 adenosine receptors and D_1-like dopamine receptors. This study also demonstrates that locomotor-related activity in murine spinal networks entails activation of *N*-methyl-D-aspartate receptors (NMDARs). NMDARs are shown to be gated in a synapse-specific manner by glycine and D-serine, co-agonists of glutamate that are regulated by astrocytes. Since spinal locomotor networks direct the activation of muscles in intact animals, it is proposed that the modulation of network activity by glia described in this study is important in locomotion and adds to evidence that glial information processing is relevant to behaviour.

Adenosine derived from glia reduces the frequency of ongoing locomotor related activity

According to the tripartite synapse model, $G_{\alpha q}$-coupled G-protein coupled receptors (GPCRs) expressed by astrocytes detect local release of neurotransmitters, stimulating inositol trisphosphate (IP_3) receptor-dependent release of intracellular Ca^{2+}; in turn, astrocytic Ca^{2+} elevations stimulate vesicular release of gliotransmitters such as glutamate, D-serine and adenosine triphosphate (ATP), which activate neuronal receptors to modify neuronal excitability or synaptic transmission (Araque *et al.*, 1999, 2014; Bazargani and Attwell, 2016). This model is supported by evidence that astrocytic Ca^{2+} elevations are closely related to activity in neighbouring neurons, that Ca^{2+} elevations are related to activation of $G_{\alpha q}$-coupled

GPCRs, and that experimental stimulation of astrocytic Ca^{2+} elevations results in modulation of neuronal activity (Araque *et al.*, 1999, 2014; Bazargani and Attwell, 2016). In the present study, the endogenous $G_{\alpha q}$-coupled G-protein protease-activated receptor-1 (PAR1) is exploited to stimulate $G_{\alpha q}$-dependent release of gliotransmitters during ongoing locomotor-related activity in spinal cord preparations. PAR1 has previously been shown to be preferentially expressed by astrocytes in the brain (Weinstein *et al.*, 1995; Junge *et al.*, 2004), and has been used to stimulate Ca^{2+} elevations and gliotransmitter release (Lee *et al.*, 2007; Shigetomi *et al.*, 2008; Carlsen and Perrier, 2014; Lalo *et al.*, 2014). In Chapter 2, it is shown that the $G_{\alpha q}$-coupled GPCR PAR1 is also preferentially expressed by glia within the spinal cord. Brief activation of PAR1 during ongoing locomotor-related activity is shown to result in a reversible reduction in the frequency but not the amplitude of locomotor-related bursting. Because this effect can be prevented by the gliotoxins methionine sulfoximine and fluoroacetate, inhibition of neuronal A_1 but not A_{2A} adenosine receptors, or inhibition of extracellular ectonucleotidases, it is proposed that PAR1 activation triggers the release of ATP from glia, rapid hydrolysis of ATP to adenosine catalysed by extracellular ectonucleotidases, and activation of neuronal A_1 but not A_{2A} receptors.

The finding that PAR1 activation results in the release of ATP from glia in isolated spinal cords, followed by the rapid conversion of ATP to adenosine, is consistent with a previous study showing that PAR1 activation in slice preparations reduces amplitude of evoked excitatory postsynaptic potentials (EPSCs) by a presynaptic mechanism sensitive to inhibition of A_1 receptors and ectonucleotidases (Carlsen and Perrier, 2014). In addition, adenosinergic modulation of locomotor-related activity is prevented following pharmacological ablation of glia, indicating that glia are the primary source of modulatory adenosine in spinal locomotor networks (Witts, Panetta and Miles, 2012). Consistent with these observations, production of adenosine from ATP released following stimulation of glial Ca^{2+} signalling has previously been reported in the brain (Pascual *et al.*, 2005; Serrano *et al.*, 2006; Panatier *et al.*, 2011). Importantly, GPCRs that stimulate astrocytic Ca^{2+} signalling differ in their capacity to elicit

gliotransmitter release (Shigetomi *et al.*, 2008). Future studies should seek to corroborate the effects of PAR1 stimulation by using other techniques for stimulating astrocytes during network activity, such as activation of genetically encoded GPCRs (Agulhon *et al.*, 2013; Bonder and McCarthy, 2014; Scofield *et al.*, 2015; Yang, Qi and Yang, 2015) or channelrhodopsins (Gourine *et al.*, 2010; Li *et al.*, 2012; Beppu *et al.*, 2014). Alternative approaches may reveal that spinal cord glia are competent to release modulators other than adenosine.

The present study does not consider the effects of suppressing glial Ca^{2+} signalling during ongoing activity in locomotor networks. In acute spinal cord slices, introduction of a Ca^{2+} chelator into astrocytes via a patch pipette increases the amplitude of evoked EPSCs in neighbouring neurons, perhaps because by reducing astrocytic release of ATP-adenosine (Carlsen and Perrier, 2014); however, the mechanism by which this occurs was not tested directly. Techniques that would permit the acute and selective inhibition of Ca^{2+} elevations in all astrocytes within spinal locomotor networks are lacking. However, comparison of locomotor-related activity in isolated spinal cords from wild-type mice and from mice lacking IP_3 receptor type 2 (IP_3R2), which is preferentially expressed by astrocytes, may be informative (Petravicz, Fiacco and McCarthy, 2008; Takata *et al.*, 2011; Navarrete *et al.*, 2012; Haustein *et al.*, 2014; Petravicz, Boyt and McCarthy, 2014; Martin *et al.*, 2015). Although locomotor behaviour was reported to be unaltered in IP_3R2-knockout mice (Agulhon *et al.*, 2013; Petravicz, Boyt and McCarthy, 2014), in these studies IP_3R2 knockout was not acute and affected all regions of the nervous system, raising the possibility that neuronal compensatory mechanisms masked astrocytic deficits. Network-specific effects may therefore be revealed in isolated spinal cord preparations.

IP_3R2-knockout mice could also be used to confirm that PAR1 activation results in adenosine production via $G_{\alpha q}$-mediated release of intracellular Ca^{2+} in spinal cord astrocytes. Interestingly, activation of a $G_{\alpha q}$-coupled DREADD (designer receptor exclusively activated by designer drugs) expressed by glia in IP_3R2-knockout results in inhibition of locomotion by an unknown mechanism (Agulhon *et al.*, 2013). The rapid production of adenosine reported here is

consistent with Ca^{2+}-dependent release of gliotransmitters (Lalo *et al.*, 2014), but direct demonstration that PAR1 activation triggered Ca^{2+} elevations and that these directed release of ATP-adenosine was beyond the scope of this study. Use of Ca^{2+} imaging to show that PAR1 activation stimulates Ca^{2+} transients in spinal cord astrocytes was also not possible here, but would further support a $G_{\alpha q}$- and Ca^{2+}-dependent mechanism of ATP-adenosine release.

The effects of adenosine secreted from glia following PAR1 activation are consistent with studies showing that bath-applied adenosine reduces the frequency but not the amplitude of locomotor-related bursting in postnatal mice in an A_1 but not A_{2A}-dependent manner (Witts, Panetta and Miles, 2012; Acevedo *et al.*, 2016). In addition, modulation of network activity by bath-applied ATP or endogenous adenosine requires ectonucleotidase activity, indicating that adenosine within mammalian spinal cords derives from ATP and that ATP alone does not modulate network activity (Witts, Panetta and Miles, 2012). Although adenosine acting at A_1 receptors has a similar depressive effect on locomotion in *Xenopus* tadpoles, ATP is also an effective modulator of spinal networks in tadpoles (Dale and Gilday, 1996). Thus, some but not all features of purinergic signalling are common to tadpoles and mice.

Adenosine modulates the frequency of synaptic inputs to heterogeneous ventral horn interneurons

The cell population that mediates the effect of adenosine on the frequency of network activity remains to be determined. Synchronous bursting produced by blockade of inhibitory transmission is shown here to be unaltered by adenosine produced upon PAR1 activation or by adenosine released during network activity, as revealed by inhibition of A_1 receptors; similarly, it is unaltered by bath-applied adenosine (Witts, Panetta and Miles, 2012). These findings imply that both endogenous and exogenous adenosine act via inhibitory interneurons, perhaps by disynaptic inhibition (Witts, Panetta and Miles, 2012). However, bath-applied adenosine and adenosine produced upon PAR1 activation are shown in Chapter 2 to increase the interval between postsynaptic currents (PSCs) recorded from a mixed population of

rhythmically active ventral-horn interneurons during network activity in hemicords, whereas PSC interval is increased by blockade of A_1 receptors. These changes in PSC interval may simply reflect changes in network activity; however, bath-applied adenosine also increases the interval between PSCs in heterogeneous interneurons in slice preparations, and this effect persists in the presence of tetrodotoxin (TTX), suggesting adenosine acts broadly within the ventral horn via a presynaptic mechanism (Witts, Nascimento and Miles, 2015). Adenosine also acts by a presynaptic mechanism to reduce the amplitude of evoked EPSCs in mixed ventral horn interneurons in slice preparations (Carlsen and Perrier, 2014). It is possible that adenosine acts via multiple mechanisms, not all of which may be relevant to network output. The observation that adenosine induces a hyperpolarising current in interneurons in slices (Witts, Nascimento and Miles, 2015) but not in rhythmically active hemicords indicates that the effects of adenosine are state-dependent. Furthermore, the studies showing modulation of PSCs by adenosine in slices were conducted in the absence of dopamine, yet it is shown in Chapter 3 that adenosine does not modulate network activity in the absence of either dopamine or a D_1-like receptor agonist (see below). Since activation of D_1-like dopamine receptors is prerequisite for the modulation of network activity by adenosine, future studies to determine the actions of adenosine at the cellular level, as well as the cell population responsible for its network effects, will also need to consider the activities and distribution of D_1-like receptors in addition to the effects of network activity.

Glia detect neuronal activity within spinal locomotor networks

The observation in Chapter 2 that modulation of locomotor-related activity by endogenous adenosine scales with the frequency of network activity is consistent with activity-dependent release of ATP-adenosine by glia. Glia are therefore proposed to mediate a negative feedback loop, whereby activity-dependent release of adenosine depresses and perhaps stabilises network output, resulting in even, controlled movement. This model implies that ventral horn glia detect neuronal activity, for instance by detection of neurotransmitter release, as predicted

by the tripartite synapse model. Ca^{2+} imaging of astrocytes could be used in future studies to confirm astrocytic responsiveness to neuronal activity, as has been demonstrated in the brain (Araque *et al.*, 2014; Bazargani and Attwell, 2016). Preliminary reports have indicated that ventral horn astrocytes display rhythmic Ca^{2+} elevations during network activity, and that these are blocked by inhibition of metabotropic glutamate receptor 1 (mGluR1) (Chub, Liu and O'Donovan, 2012; Chub and O'Donovan, 2011). The relationship between mGluR1 signalling in astrocytes and release of ATP-adenosine has not been assessed.

Release of adenosine in proportion to neuronal activity could be demonstrated directly by measurement of adenosine within the spinal cord at different frequencies of network activity. It was not possible to achieve this with three-enzyme molecular biosensors for adenosine (Llaudet *et al.*, 2003). In Chapter 2 adenosine biosensors are shown to lose sensitivity when placed in contact with spinal cord tissue, perhaps because of the presence in spinal cord tissue of a reversible inhibitor of one of their constituent enzymes. An alternative method for detecting substances released during locomotor-related activity, such as an analysis of the extracellular medium by high performance liquid chromatography (HPLC) (Huxtable *et al.*, 2010), could be used instead in future studies.

Adenosine is a second-order modulator of dopamine within locomotor networks

Endogenous adenosine fails to modulate locomotor-related activity in the absence of dopamine or following inhibition of D_1-like receptors or their downstream effector protein kinase A (PKA), suggesting that endogenous adenosine modulates network activity via an interaction between A_1 and D_1-like receptors (Acevedo *et al.*, 2016). In Chapter 3, these observations are corroborated by data showing that bath-applied adenosine, adenosine produced upon PAR1 activation, and A_1-receptor blockade fail to modulate locomotor-related activity when dopamine or an agonist of D_1-like receptors is absent, but that the effects of adenosine reported in the presence of dopamine are restored when a D_1-receptor agonist is

present. Adenosine therefore appears to function as a metamodulator of dopamine during locomotor-related activity. Previously, it was shown that nitric oxide (NO) modulates the release of noradrenaline in the spinal locomotor networks of *Xenopus* tadpoles (McLean and Sillar, 2004) and the activity of endocannabinoids in the spinal locomotor networks of lampreys (Song, Kyriakatos and El Manira, 2012). Metamodulation of this kind could serve to diversify the effects of first-order modulators, extending the repertoire of behavioural outputs of spinal networks (Katz, 1999).

Previously, D_1-like receptors were proposed not to modulate the frequency of rhythmic bursting in postnatal mice (Humphreys and Whelan, 2012; Sharples *et al.*, 2015). In the present study, D_1-like receptors are shown to have an excitatory effect, increasing the frequency of locomotor-related bursting. The reason for this discrepancy is uncertain and could be investigated in future studies; however, the agonist used in the earlier studies was applied at a concentration that may have activated inhibitory D_2-like receptors in addition to D_1-like receptors, masking their frequency effect (Neumeyer *et al.*, 2003). The data presented here supporting an excitatory effect of D_1-like receptors are consistent with findings that selective activation of D_1-like receptors initiates locomotor-related activity in intact adult mice (Lapointe *et al.*, 2009) and in isolated spinal cords from postnatal rats (Barrière, Mellen and Cazalets, 2004). Furthermore, an excitatory role is implied by the proposed interaction between A_1 and D_1-like receptors, whereby the former inhibit the activity of the latter to depress the frequency of network activity.

The mechanisms by which D_1-like receptors modulate ventral horn interneurons, including the molecular targets of PKA, remain to be determined. D_1-like receptors are broadly expressed in the ventral horn (Dubois *et al.*, 1986; Zhu *et al.*, 2007, 2008), likely by multiple populations of physiologically distinct interneurons in which signalling by D_1-like receptors could have different effects (Sharples *et al.*, 2014). It remains to be determined whether adenosine modulates all or a fraction of those cell types. The failure of adenosine to modulate disinhibited activity suggests that its activity is restricted to inhibitory interneurons expressing D_1-like

receptors. It was not possible by the pharmacological approach taken in the present study to distinguish between D_1, D_5, both members of the D_1-like receptor subfamily; in future studies, this could be assessed using mice in which either D_1 or D_5 receptors are knocked out .

D-serine and glycine regulate excitatory transmission in spinal locomotor networks

Activation of NMDARs by glutamate requires the binding of a co-agonist, either D-serine of glycine, and the availability of both substances at excitatory synapses is regulated by glia (Le Bail *et al.*, 2015). This study provides evidence that D-serine and glycine gate NMDARs during network activity in the spinal cord in a synapse-specific and activity dependent manner, as occurs in the brain (Mothet *et al.*, 2000; Kalbaugh, Zhang and Diamond, 2009; Li *et al.*, 2009, 2013; Henneberger *et al.*, 2013; Le Bail *et al.*, 2015; Meunier *et al.*, 2016).

Selective blockade of NMDARs within murine spinal locomotor networks is shown in Chapter 4 to consistently reduce the frequency and amplitude of pharmacologically induced network activity. Although this contradicts previous studies using spinal cords from postnatal mice (Nishimaru, Takizawa and Kudo, 2000; Whelan, Bonnot and Donovan, 2000), it is consistent with findings from postnatal rats (Beato, Bracci and Nistri, 1997; Bracci, Beato and Nistri, 1998; Cowley *et al.*, 2005). The reason for this conflict is unclear, since this study used similar methods to the previous mouse studies, and it seems unlikely that the different mouse strains used in these studies would differ very greatly in properties of a fundamental mechanism of excitatory transmission.

Both D-serine and glycine regulate NMDARs in the spinal cord. Selective depletion of D-serine increases the frequency of locomotor-activity, but does not alter disinhibited bursting, suggesting that it regulates NMDARs expressed by a population of inhibitory interneurons that mediates disynaptic inhibition, such as V1 interneurons (Gosgnach *et al.*, 2006). By deduction, glycine is proposed to gate the remaining fraction of NMDARs. It was beyond the scope of this study to assess NMDAR currents in individual neurons; however, whole-cell patch-clamp

recordings could be used in future to confirm that co-agonist identity is synapse-specific, as suggested by the data presented here. Although enzymatic depletion of glycine would presumably abolish locomotor-related bursting during network activity, as blockade of glycine and $GABA_A$ receptors produces synchronous bursting (Bracci, Ballerini and Nistri, 1996), scavenging both glycine and D-serine could be performed in slice preparations to characterise co-agonist identity in a cell-type specific manner (Papouin *et al.*, 2012; Li *et al.*, 2013; Le Bail *et al.*, 2015).

The extent to which the co-agonist binding site is occupied differs between locomotor-related activity and disinhibited activity, in which glutamate release is likely to be greater. It is therefore proposed that co-agonist availability is regulated in an activity-dependent manner, as reported in the brain (Kalbaugh, Zhang and Diamond, 2009; Li *et al.*, 2009, 2013). Activity-dependent release has been detected in brain slices by assessing the degree to which the co-agonist binding site is occupied while varying the intensity of an electrical or pharmacological stimulus (Li *et al.*, 2009, 2013). A similar approach could be used in spinal cord slices in future studies to corroborate the effects at the level of network output presented here.

Finally, glycine applied at concentrations sufficient to facilitate NMDARs is shown not to alter network activity, whereas blockade of the glycine transporter-1 (GlyT1) enhances the frequency of locomotor-related bursting, implying that GlyT1 has a key role in regulating glycine availability in hindlimb locomotor networks, as previously reported in rat forelimb networks (Shimomura *et al.*, 2015), in the spinal cord of *Xenopus* tadpoles (Issberner and Sillar, 2007) and in the brain (Berger, Dieudonné and Ascher, 1998; Bergeron *et al.*, 1998). Dynamic regulation of GlyT1 activity is implied by activity-dependent regulation of co-agonist availability, but it was not possible to demonstrate this directly here. Future studies could assess the roles of regulators of GlyT1 in spinal locomotor networks; these may include protein kinase C (PKC) (Sato *et al.*, 1995) and brain derived neurotrophic factor (BDNF) (Aroeira, Sebastião and Valente, 2015).

Although it was not possible in this study to directly characterise the role of astrocytes in regulating glycine and D-serine, the results presented here provide a basis for future investigations into the role of astrocytes in excitatory transmission in the spinal cord. GlyT1 is preferentially expressed by astrocytes and is highly expressed in the spinal cord and brainstem (Zafra, Gomeza, *et al.*, 1995; Cubelos, Giménez and Zafra, 2005), although relatively modest expression of GlyT1 is also reported in neurons (Cubelos, Giménez and Zafra, 2005). D-serine biosynthesis requires 3-phosphoglycerate dehydrogenase, which is exclusively expressed by astrocytes (Ehmsen *et al.*, 2013), and D-serine may be released by astrocytes in addition to neurons (Yang *et al.*, 2003; Mothet *et al.*, 2005; Henneberger *et al.*, 2010; Verrall *et al.*, 2010; Rosenberg *et al.*, 2013). Regulation of co-agonist availability has been shown to entail functional interactions between neurons and glia in the brain (Yang *et al.*, 2003; Mothet *et al.*, 2005; Henneberger *et al.*, 2010; Li *et al.*, 2013; Shigetomi *et al.*, 2013; Le Bail *et al.*, 2015). It may be possible in future to demonstrate a similar interaction between neurons and glia in the spinal cord by comparing experiments to assess occupancy of the co-agonist binding site and co-agonist identity in intact preparations, as here, with similar experiments performed in preparations in which glia have been pharmacologically ablated, an approach that has been taken in the brain (Li *et al.*, 2013; Le Bail *et al.*, 2015). However, further characterisation of functional interactions between neurons and glia will require recordings from individual neurons in conjunction with acute pharmacological or genetic manipulations. The finding presented in Chapter 2 that the effects of PAR1 activation can be abolished by inhibition of A_1 receptors suggests that spinal cord astrocytes do not release D-serine in response to $G_{\alpha q}$ signalling, but this should be confirmed using alternative techniques to stimulate glia (Shigetomi *et al.*, 2008). Nevertheless, astrocytic participation in activity-dependent regulation of NMDARs would implie a degree of information processing and may occur by mechanisms distinct from those described by the tripartite synapse model.

Summary

Stimulation of glia is shown in this study to result in release of ATP-adenosine and the modulation of hindlimb locomotor networks in spinal cord preparations from postnatal mice, the output of which is directly relevant to a defined behaviour. This study adds to evidence that neuromodulators released by glia contribute to the production of behaviour (Gourine *et al.*, 2010; Halassa and Haydon, 2010; Angelova *et al.*, 2015; Morquette *et al.*, 2015), supporting studies at the cellular level demonstrating that astrocytic information processing results in release of neuromodulators (Araque *et al.*, 2014; Bazargani and Attwell, 2016). The use of isolated networks may in future help to determine why these studies are contradicted by others reporting no effect of glial Ca^{2+} signalling in behaving animals (Agulhon *et al.*, 2013; Petravicz, Boyt and McCarthy, 2014).

Spinal locomotor networks are also shown to be modulated by D-serine and glycine, co-agonists of glutamate at NMDARs. The proposed role of astrocytes in regulating these substances is not directly demonstrated here, but this finding indicates a further important role for glial information processing in the generation of behaviour.

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