# Gliotransmission and adenosinergic modulation: insights from mammalian spinal motor networks

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**Running head:** Gliotransmission and adenosinergic modulation in the locomotor CPG

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## Abstract

Astrocytes are proposed to converse with neurons at tripartite synapses, detecting neurotransmitter release and responding with release of gliotransmitters, which in turn modulate synaptic strength and neuronal excitability. However, a paucity of evidence from behavioral studies calls into question the importance of gliotransmission for the operation of the nervous system in healthy animals. Central pattern generator (CPG) networks in the spinal cord and brainstem coordinate the activation of muscles during stereotyped activities such as locomotion, inspiration and mastication, and may therefore provide tractable models in which to assess the contribution of gliotransmission to behaviorally relevant neural activity. Here, we review evidence for gliotransmission within spinal locomotor networks, including studies indicating that adenosine derived from astrocytes regulates the speed of locomotor activity via metamodulation of dopamine signaling.


## Introduction

Locomotor behaviors such as walking, flying and swimming permit mammals to interact with their environment, thereby satisfying basic requirements of survival. In vertebrates, locomotion arises from the biomechanical properties of the skeletomuscular system and activity within dedicated neural circuitry in the central nervous system (CNS) (Orlovsky et al. 1999; Dickinson 2000; Grillner 2006; Kiehn 2006, 2016). Whereas the planning, initiation and maintenance of movements involve various brain regions, including the cortex, basal ganglia, midbrain and hindbrain (Orlovsky et al. 1999; Takakusaki et al. 2004), executive control over the timing and coordination of muscle activity is invested in networks of interneurons in the ventral horn of the spinal cord. These networks, like others in the spinal cord and brainstem that produce a stereotyped motor output, are referred to as central pattern generators (CPGs).

Spinal motor networks, including the motoneurons that provide their final common output, are subject to extensive neuromodulation by substances such as the biogenic amines 5-

hydroxytryptamine (5-HT/serotonin), dopamine (DA) and noradrenaline, and amino acids including γ-aminobutyric acid (GABA) and glutamate (Miles and Sillar 2011). A given neuromodulator may act at multiple receptor subtypes and in a cell-type specific fashion. In addition, the effects of an individual neuromodulator may be modified by a second-order neuromodulator, a process termed metamodulation (Katz 1999; Miles and Sillar 2011). The resulting multiplicity of signaling mechanisms extends the repertoire of motor patterns that can be generated by a finite population of cells, contributing to the behavioral plasticity necessary for an animal to successfully negotiate its environment.

Although in many cases neuromodulators derive from neurons, they may also be secreted by astrocytes (Araque et al. 1999, 2014), as well as from other glial cells (Jackson et al. 2017). Evidence gathered over the past two decades indicates that astrocytes release neuromodulators in response to activity in neighboring neurons, acting as the third partner, along with pre- and postsynaptic neurons, at tripartite synapses throughout the nervous system. So-called gliotransmission represents a form of information processing that extends the canonical role of astrocytes as cellular housekeepers, responsible for tasks including ion homeostasis, metabolic support and neurotransmitter clearance (Verkhratsky and Butt 2013). However, the importance of gliotransmission to the operation of the nervous system in healthy animals remains controversial, in part because of a paucity of evidence that it contributes to the production of behavior (Hamilton and Attwell 2010; Agulhon et al. 2012; Nedergaard and Verkhratsky 2012; Sloan and Barres 2014; Bazargani and Attwell 2016).

Because the activity of neurons within the spinal cord determines quantifiable behaviors, spinal cord preparations may provide a tractable model for the study of gliotransmission and its role in the operation of neural networks. The lumbar spinal locomotor CPG controls the rhythmic activation of hindlimb muscles during walking and running, and rhythmic, behaviorally relevant patterns of activity can be generated by isolated CPG networks in the absence of descending and peripheral

inputs. This has permitted the study of diverse neuromodulatory systems and their roles in networks controlling defined behaviors (Marder and Bucher 2001; Dickinson 2006; Whelan 2010; Harris-Warrick 2011; Miles and Sillar 2011). Here, we review recent studies indicating that astrocytes within the spinal cord are a source of neuromodulatory adenosine. We then discuss the modulation of motor networks by adenosine, including recent evidence that it functions principally as a metamodulator of dopamine during motor behaviors. For the most part, we discuss evidence from mice and rats, the models used in nearly all studies of gliotransmission to date. However, a recent report implicates bidirectional signaling between neurons and glia in the regulation of behavior in *Drosophila,* suggesting the possibility that a greater range of organisms may be investigated in future (Ma et al. 2016).

## Spinal locomotor networks and neuromodulation

Networks of premotor interneurons 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 (Orlovsky, Deliagina and Grillner, 1999). During rhythmic locomotor behaviors, such as walking, flying and swimming, spinal networks activate muscles in a cyclically repeated sequence. Like other CPGs within the brainstem and spinal cord, including the networks that coordinate muscle contractions during chewing, swallowing and respiration (Jean 2001; Marder and Bucher 2001; Feldman and Del Negro 2006; Lund and Kolta 2006), locomotor networks remain capable of generating a basic motor pattern when inputs from descending and peripheral pathways are removed (Brown 1911; Guertin 2009).

The effects of neuromodulators have been studied extensively within spinal cord preparations, most notably from lampreys, *Xenopus* tadpoles, cats, rats and mice (Katz and Frost 1996; Katz 1999; Dickinson 2006; Harris-Warrick 2011; Miles and Sillar 2011). Postnatal rodent spinal cords may be isolated and sustained in artificial cerebrospinal fluid *in vitro,* and rhythmic locomotor-related activity can be evoked in the hindlimb motor circuitry 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 et al. 2000; Hägglund et al. 2013). Isolated networks generate rhythmic activity in hindlimb muscles, if present, or in transected ventral roots containing the motoneurons that innervate them (Kudo and Yamada 1987; Smith and Feldman 1987; Hayes et al. 2009). In preparations in which the ventral roots are transected, field-potential (electroneurogram) recordings reveal a pattern of rhythmic bursting in the lumbar ventral roots corresponding to activity observed during treadmill locomotion in adult animals (Cowley and Schmidt 1994; Kiehn and Kjaerulff 1996; Whelan et al. 2000). Bursting in lumbar ventral roots $L_{1-4}$ is in phase with hindlimb flexors and is out of phase with bursting in roots $L_5$ and $L_6$, which corresponds to bursting in extensors.

The basic output of the locomotor CPG is determined by the intrinsic electrical properties of the neurons that constitute the network and the synaptic connections between them (Grillner 2006). Neuromodulators adjust both, enabling a circumscribed population of neurons to produce diverse outputs and modes of adaptive behavior (Katz and Frost 1996; Katz 1999; Dickinson 2006; Harris-Warrick 2011; Miles and Sillar 2011). Alterations to motor-network output allow animals to adjust their behavior 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 et al. 1999; Bellardita and Kiehn 2015). Multifarious neuromodulatory influences acting in concert give rise to a vast repertoire of network and behavioral outputs and are thus a source of considerable behavioral flexibility (Harris-Warrick, 2011; Miles and Sillar, 2011). The iterative nature of fictive locomotor activity facilitates comparison of control and treatment conditions, and isolated spinal networks have been used to study the effects of diverse neuromodulators at the network level, by application of pharmacological and genetic tools. Although neuromodulation in locomotor networks has

121 received considerable attention, evidence that neuron-glia interactions play a role has only recently
122 emerged (Witts et al. 2012; Acton and Miles 2015).

## 123 Gliotransmission

124 Glia are a class of cell within the central and peripheral nervous systems consisting of macroglia –
125 which include astrocytes, oligodendrocytes and Schwann cells – and microglia (Verkhratsky and Butt
126 2013). Astrocytes, like the other glial cell-types, are traditionally considered to have a merely
127 passive, supportive function within neural networks, with well-established roles in ion homeostasis
128 and the synthesis and clearance of neurotransmitters (Verkhratsky and Butt 2013). However,
129 substantial evidence now indicates that astrocytes are active participants at tripartite synapses,
130 dynamically regulating neuronal excitability and synaptic strength by the release of gliotransmitters
131 (Araque et al. 1999, 2014; Haydon and Nedergaard 2015; Bazargani and Attwell 2016). In its original
132 formulation, the tripartite-synapse model entails (1) the binding of a neurotransmitter released
133 during synaptic activity to an astrocytic $G_{\alpha q}$-linked G-protein coupled receptor (GPCR); (2) the
134 production of inositol trisphosphate ($IP_3$) and activation of $IP_3$ receptors, triggering release of $Ca^{2+}$
135 from stores within the astrocyte; (3) $Ca^{2+}$-dependent release of a gliotransmitter such as glutamate
136 (Parpura et al. 1994; Pasti et al. 1997; Bezzi et al. 1998; Oh et al. 2012; Han et al. 2013), adenosine
137 triphosphate (ATP) (Newman 2001; Pryazhnikov and Khiroug 2008) or D-serine (Mothet et al. 2005;
138 Henneberger et al. 2010) by vesicular exocytosis or via ion channels; and (4) the activation of
139 metabotropic or ionotropic receptors on either the pre- (Jourdain et al. 2007; Carlsen and Perrier
140 2014) or postsynaptic neuron (Parri, 2001; Angulo 2004; Fellin et al. 2004) (Fig. 1) of the same
141 (Navarrete and Araque 2010; Martin et al. 2015) or a different (Zhang et al. 2003; Pascual et al.
142 2005; Serrano et al. 2006) synapse. Although signaling in this manner has been denoted
143 “gliotransmission”, this is arguably a misnomer: modulation of neuronal activity by substances
144 derived from astrocytes occurs on a variable timescale, but owing to its dependency on
145 metabotropic receptors, is necessarily slower than acute transmission mediated by ionotropic

146 receptors (Agulhon et al. 2012; Araque et al. 2014). “Gliomodulation” has therefore been proposed
147 as a more appropriate term to distinguish bidirectional signaling between neurons and glia (Agulhon
148 et al. 2012), but is not widely used (“gliotransmission” will be used in this review).

149 The steps in the pathway described above have been deduced largely from $Ca^{2+}$ imaging of
150 astrocytes and electrophysiological recordings from neurons, and are supported by numerous
151 studies (Haydon and Nedergaard 2015). However, the extent to which gliotransmission is important
152 for the operation of neural networks and in the production of behavior in healthy animals remains
153 controversial (Hamilton and Attwell 2010; Agulhon et al. 2012; Nedergaard and Verkhratsky 2012;
154 Sloan and Barres 2014; Bazargani and Attwell 2016). The coding of information by astrocytes in the
155 form of $Ca^{2+}$ fluctuations, which vary in kinetics and subcellular localization, is poorly understood,
156 and the physiological relevance of experimental manipulations used to investigate $Ca^{2+}$ signaling is
157 disputed (Nedergaard and Verkhratsky 2012; Rusakov 2015). Crucially, evidence is lacking that
158 gliotransmission dependent on astrocytic $G_{\alpha q}$-linked GPCRs and $Ca^{2+}$ signaling is important in the
159 generation of behavior.

## 160 Gliotransmission and behavior

161 Reports have indicated astrocytic involvement in diverse behaviors (reviewed in Oliveira et al. 2015);
162 these include sleep (Halassa et al. 2009; Foley et al. 2017; however see Fujita et al. 2014), pain
163 aversion (Foley et al. 2011), recognition memory (Halassa et al. 2009; Florian et al. 2011) motor
164 coordination (Watase et al. 1998), whisker-dependent perception (Han et al. 2014), olfactory
165 responses (Martin et al. 2012), and anxiety-like and depressive behavior (Banasr and Duman 2008;
166 John et al. 2012; Cao et al. 2013). However, despite extensive evidence for bidirectional
167 communication between neurons and astrocytes in *in vitro* preparations, evidence that
168 gliotransmission is important for the production or modulation of behavior is sparse (for further
169 discussion, see Hamilton and Attwell 2010; Agulhon et al. 2012; Nedergaard and Verkhratsky 2012;
170 Sloan and Barres 2014; Bazargani and Attwell 2016). Uncertainty about the contribution of

gliotransmission to the generation of behaviors stems from a lack of acute and selective techniques for the manipulation of astrocytes *in vivo* and uncertainty about the physiological relevance of techniques that have been applied to the study of gliotransmission to date (reviewed in Nedergaard and Verkhratsky 2012 and Bazargani and Attwell 2016). Importantly, mice lacking $IP_3R2$, the $IP_3$ receptor isoform that is preferentially expressed in astrocytes, ($IP_3R2KO$ mice) have been tested for a range of behaviors, including locomotion, learning, memory and anxiety, but do not differ from wild type littermates on most measures (Agulhon et al. 2013; Petravicz et al. 2014); however, the possibility that the importance of $IP_3R$ signaling is masked in these studies by compensatory mechanisms during development cannot be excluded. Until further behavioral evidence for gliotransmission in healthy animals is provided, it will remain controversial.

Given the limitations of the techniques currently available for studying gliotransmission in behaving animals, *in vitro* and *in vivo* rhythmically active brainstem and *in vitro* spinal cord preparations may provide insight into the role of bidirectional neuron-glia signaling in behavior. Studies of brainstem networks for respiration and mastication have provided cogent evidence for behaviorally 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 also triggers release of ATP to stimulate breathing (Gourine et al. 2010), whereas expression of tetanus toxin in astrocytes to inhibit vesicular release prevents increases in respiration normally observed in response to reduced oxygen availability (Angelova et al. 2015). The release of modulators from astrocytes described by these studies departs from the tripartite synapse model, however, as it is stimulated by astrocytic chemoception, rather than detection of a neurotransmitter. Glia are also proposed to modulate neuronal responses to ATP within the preBötzinger complex (preBötC), an area of the medulla critical for production of the respiratory rhythm, by releasing glutamate in a $Ca^{2+}$ dependent manner (Huxtable et al. 2010). A further example of neuron-glia crosstalk in a rhythmic network is provided by the brainstem circuity for

197 mastication, where sensory input is proposed to activate astrocytic *N*-methyl-D-aspartate (NMDA)
198 receptors, eliciting release of the $Ca^{2+}$-binding protein S100β, with the resulting decrease in
199 extracellular $Ca^{2+}$ concentration conferring rhythmic bursting properties on neighboring neurons
200 (Morquette et al., 2015). Evidence from networks that generate rhythmic, stereotyped motor
201 behaviors - including evidence from the locomotor CPG, described below – may therefore help to
202 illuminate the extent to which astrocytes participate in information processing within the nervous
203 system of intact animals.

# 204 Evidence for gliotransmission in the spinal cord

## 205 Glial $Ca^{2+}$ signaling

206 Although a substantial literature is dedicated to the role of astrocytic information processing in *in*
207 *vitro* brain preparations, research into gliotransmission in the spinal cord is in its infancy.
208 Information about the dynamics of $Ca^{2+}$ signaling in astrocytes has been provided for the most part
209 by studies in the cortex, hippocampus and cerebellum. $Ca^{2+}$ signaling in astrocytes has been
210 extensively studied by imaging cells expressing a genetically encoded $Ca^{2+}$ indicator or loaded with
211 $Ca^{2+}$-sensitive dyes by means of a patch pipette. Astrocytes are acutely sensitive to neuronal activity,
212 to the extent that they can respond to basal synaptic activity stimulated by a single action potential
213 (Panatier et al. 2011). They display intracellular $Ca^{2+}$ elevations in response to neuronal activity in
214 both neonatal and adult rodents *in vitro* and *in vivo* (Araque et al. 2014; Bazargani and Attwell 2016;
215 Rusakov 2015; Shigetomi et al. 2016). $Ca^{2+}$ signaling is observed in awake mice in response to
216 sensory stimulation in the brain and dorsal horn of the spinal cord (Srinivasan et al. 2015; Sekiguchi
217 et al. 2016), and during locomotion in the cortex (Dombeck et al. 2007).Given these observations,
218 astrocytes within the ventral horn of the spinal cord may be expected to display rhythmic oscillations
219 corresponding to those of neuronal components of the locomotor CPG during network activity.
220 Although imaging of ventral horn astrocytes in behaving animals may be challenging because of the
221 difficulty of accessing tissue, it is possible to detect $Ca^{2+}$ signaling in astrocytes in *in vitro*

preparations during network activity. Preliminary studies have reported rhythmic $Ca^{2+}$ fluctuations in putative astrocytes during pharmacologically induced locomotor-related activity in *in vitro* hemicord preparations (Chub et al. 2012). Furthermore, $Ca^{2+}$ fluctuations are 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 signaling in the spinal cord, as reported in the brain (Chub and O'Donovan 2011). Consistent with these results, a subset of astrocytes in the preBötC displays rhythmic $Ca^{2+}$ activity immediately preceding rhythmic respiratory-related neuronal bursts (Okada et al. 2012; Oku et al. 2015). Interestingly, preBötC astrocytes also display low-frequency synchronized $Ca^{2+}$ transients during network activity, indicating a complex relationship between activity in neurons and astrocytes in this network. This finding suggests that astrocytes in locomotor networks may also display patterns of $Ca^{2+}$ signaling that do not directly correspond to rhythmic locomotor-related activity in neurons. Apart from preliminary evidence from the $Ca^{2+}$-imaging studies cited above, indirect evidence also supports neuron-to-astrocyte signaling in the spinal cord; this is discussed below.

## Stimulation of spinal cord astrocytes

A range of techniques is available for the experimental induction of astrocytic Ca2+ elevations (for further information see: Araque et al. 1999; Nedergaard and Verkhratsky 2012 and Bazargani and Attwell 2016), including ultraviolet (UV) photolysis of caged Ca2+ or IP3 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, including DREADDs (designer receptors exclusively activated by designer drugs) (Rae and Irving 2004; Shigetomi et al. 2008; Agulhon et al. 2010, 2013; Chen et al. 2016), and activation of transgenically expressed channelrhodopsins (Gourine et al. 2010; Li et al. 2012; Beppu et al. 2014).

247 Recent studies of gliotransmission in the spinal cord have exploited protease-activated receptor-1
248 (PAR1) to trigger or enhance astrocytic $Ca^{2+}$ signaling (Carlsen and Perrier 2014; Acton and Miles
249 2015). PAR1 is an endogenous GPCR associated with $G_{\alpha q}$ proteins, and selective activation by a
250 synthetic ligand results in $Ca^{2+}$ elevations in astrocytes (Lee et al. 2007; Shigetomi et al. 2008; Lalo et
251 al. 2014). In the spinal cord (Acton and Miles 2015), as in the brain (Weinstein et al. 1995; Junge et
252 al. 2004), PAR-1 is expressed by cells that also express glial fibrillary acidic protein (GFAP), an
253 intermediate filament protein used widely as an astrocyte marker (Wang and Bordey 2008); by
254 contrast, neurons do not appear to express the receptor in the ventral horn (Acton and Miles 2015).
255 Cells expressing PAR1 in the spinal cord are therefore proposed to be astrocytes. However, the
256 possibility that a subset of neural precursor cells of the astrocyte lineage also expresses PAR1 has
257 not been excluded; gliogenesis continues during postnatal stages at which network-level and single-
258 cell recordings are typically made (Tien et al. 2012), and it has been proposed that some neural
259 precursor cells express GFAP in the spinal cord during postnatal development (Chvátal et al. 1995).
260 In addition, the adult spinal cord contains GFAP+ multipotent neural stem cells that are quiescent
261 within the intact spinal cord (Fiorelli et al. 2013). Such populations of non-astrocytic GFAP+ cells
262 must therefore be taken into account when interpreting studies using GFAP as a marker of
263 astrocytes in the spinal cord. Whether PAR1 activation results in $Ca^{2+}$ signaling exclusively in
264 terminally differentiated astrocytes remains to be confirmed, as does the proportion of astrocytes
265 that expresses PAR1. Nevertheless, PAR1 activation offers several advantages as a means of
266 stimulating astrocytes in the spinal cord: activation of PAR1 does not affect activity in locomotor
267 networks following pharmacological ablation of astrocytes, indicating that it does not directly
268 modulate the activity of other cell types (Acton and Miles 2015) - similarly, it does not alter the
269 excitability of neurons in the brain (Lee et al. 2007; Shigetomi et al. 2008; Lalo et al. 2014); it is
270 expressed from early postnatal stages in cells across the ventral horn, allowing those cells to be
271 stimulated concurrently during activity of the locomotor CPG by bath-application of a selective PAR1
272 agonist; finally, because it is an endogenous receptor, experimental PAR1 activation may result in

$Ca^{2+}$ elevations with spatiotemporal properties close to those generated by GPCR activation during neural activity *in vivo*, which may not be the case for transgenically expressed receptors.

During pharmacologically induced network activity in *in vitro* spinal cord preparations from postnatal mice, stimulation of glia by PAR1 activation results in a transient reduction in the frequency but not the amplitude of rhythmic bursting (Fig 2., A-D) (Acton and Miles 2015). This effect is blocked by theophylline, a non-selective adenosine-receptor antagonist, and cyclopentyl dipropylxanthine (DPCPX), a selective antagonist of $A_1$-adenosine receptors, indicating that it is mediated by adenosine. Extracellular adenosine is largely formed when ATP is released from cells and then hydrolyzed by ectonucleotidases in the extracellular space, although adenosine may also be secreted from cells directly (Cunha 2001; Klyuch et al. 2012; Wall and Dale 2013). Given that the activity of spinal neurons is unaltered when glia are stimulated in the presence of an ectonucleotidase inhibitor, spinal glia appear to release ATP rather than adenosine itself (Carlsen and Perrier 2014; Acton and Miles 2015).

It is interesting that inhibition of $A_1$ receptors during fictive locomotion is sufficient to block the effects of PAR1 stimulation, suggesting that ATP-adenosine is the only gliotransmitter produced in these experiments (Acton and Miles 2015). By contrast, stimulation of astrocytes in the dorsal horn has been shown to elicit release of glutamate (Bardoni et al. 2010; Nie et al. 2010), and astrocytes in the brain release substances including glutamate, D-serine and GABA (Araque et al. 1999, 2014), all of which modulate spinal motor networks (Wang and Dun 1990; Bertrand and Cazalets 1999; Iwagaki and Miles 2011; Miles and Sillar 2011; Acton and Miles 2017). Astrocytes in the ventral horn may not, therefore, match neurons in the diversity of neuromodulatory substances they secrete. However, some techniques fail to elicit gliotransmitter release in preparations where others are effective (Shigetomi et al. 2008; Wang et al. 2013). Activation of either PAR1 or the purinergic receptor P2Y1, 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

298 stimulates gliotransmitter release. The cause of this discrepancy is unknown, but could, for instance,
299 be related to differences in the subcellular localization of the receptors in relation to $Ca^{2+}$ stores and
300 sites of gliotransmitter release (Bazargani and Attwell 2016). For this reason, it is desirable that
301 several techniques for stimulating astrocytes be compared in future studies. In addition, the
302 experiments described above do not fully consider state-dependent differences in gliotransmission.
303 It is conceivable that the influence of astrocyte-derived modulators varies according to the
304 fluctuating neuromodulatory milieu of the spinal cord (Gerin et al. 1995; Gerin and Privat 1998;
305 Acevedo et al., 2016; Bazargani and Attwell 2016). State-dependent modulation of networks by
306 astrocytes may involve changes in the competence of astroyctes to detect neuronal activity or
307 release modulators, or changes in neuronal sensisitivity. The possibility that adenosinergic
308 modulation is dependent on the actions of dopamine is discussed below.

309 In agreement with adenosine release during network activity, PAR1 activation elicits the release of
310 ATP-adenosine from ventral horn glia in slice preparations from postnatal mice, reducing the
311 amplitude of evoked excitatory post-synaptic currents (EPSCs) recorded from interneurons via a
312 presynaptic mechanism mediated by $A_1$ receptors (Carlsen and Perrier 2014). Also, consistent with
313 findings from rhythmically active preparations, this depends on extracellular ectonucleotidase
314 activity, indicating that adenosine but not ATP modulates ventral horn interneurons. However,
315 despite apparent agreement, it remains unclear whether adenosine acts via a common mechanism
316 in spinal cord slices and rhythmically active isolated spinal cord preparations (see below).

## 317 Suppression of gliotransmission in the spinal cord

318 Endogenous release of substances from astrocytes can be suppressed by inhibition of astrocytic $Ca^{2+}$
319 signaling by various strategies, including dialysis of astrocytic syncytia by $Ca^{2+}$ buffers and inhibition
320 of $IP_3R$ signaling (for further information, see: Hamilton and Attwell 2010; Nedergaard and
321 Verkhratsky 2012; Bazargani and Attwell 2016). In addtion, secretion of gliotransmitters can be
322 supressed by inhibition of G-protein signal transduction (Di Castro et al. 2011), disruption of vesicle

323 release mediated by *N*-ethylmaleimide–sensitive factor attachment protein receptor (SNARE)
324 proteins, (Araque et al. 2000; Jourdain et al. 2007; Gourine et al. 2010; Lalo et al. 2014; Sultan et al.
325 2015) or by conditional expression of toxins (Angelova et al. 2015) or a dn-SNARE, a dominant-
326 negative cytosolic domain of vesicle-associated membrane protein 2 (VAMP2; aka synaptobrevin 2)
327 (Pascual et al. 2005; Fellin et al. 2009; Fujita et al. 2014; Lalo et al. 2014; Sultan et al. 2015).

328 Suppression of astrocytic $Ca^{2+}$ signaling has not been achieved during locomotor-related network
329 activity in spinal cord preparations, and techniques for selective and acute inhibition of astrocytic
330 $Ca^{2+}$ signaling are currently unavailable. In acute spinal cord slices from mice, loading astrocytes with
331 a $Ca^{2+}$ chelator enhances the amplitude of EPSCs in neighboring neurons (Carlsen and Perrier, 2014).
332 Although this is proposed to reflect a reduction in $Ca^{2+}$-dependent release of ATP-adenosine from
333 glia, this has not been tested directly. It has, however, been shown that adenosinergic modulation of
334 network activity is prevented in murine preparations in which astrocytes have been treated with
335 gliotoxins, which comprehensively disrupt astrocytic function (Clarke 1991; Fonnum et al. 1997;
336 Zhang et al. 2003; Huxtable et al. 2010; Witts et al. 2012; Li et al. 2013; Wall and Dale 2013).
337 Network activity persists following application of the gliotoxins methionine sulfoximine or
338 fluoroacetate if glutamine, the astrocyte-derived precursor of glutamate and GABA, is supplemented
339 to support rhythmic activity (Witts et al. 2012). Methionine sulfoximine irreversibly inhibits the
340 astrocytic enzyme glutamine synthetase and causes glycogen accumulation and cell damage in
341 astrocytes but not other glial cells (Ronzio et al. 1969; Phelps 1975; Aschner and Kimelberg 1996; De
342 Vellis 2002). Fluoroacetate, a precursor of the glial aconitase inhibitor fluorocitrate (Fonnum et al.
343 1997), is proposed to disrupt astrocyte metabolism by reversible inhibition of the Krebs cylcle,
344 resulting in decreased production of ATP (Paulsen et al. 1987; Keyser and Pellmar 1994; Aschner and
345 Kimelberg 1996). Following astrocytic ablation by either substance, blockade of adenosine receptors
346 by DPCPX or theophylline has no effect on network output, revealing that endogenous adenosine no
347 longer modulates network activity, although preparations remain sensitive to exogenous adenosine
348 (Fig 2., E-F) (Witts et al. 2012). It should be noted that extensive disruption of astrocytic metabolism

is an imprecise technique for suppressing gliotransmission, and off-target effects of gliotransmitters have been reported, including changes in extracellular potassium levels (Largo et al. 1997) and glycogen deposition in cranial motoneurons (Young et al. 2005). However, spinal cord preparations remain sensitive to adenosine following gliotoxin treatment, consistent with disruption of endogenous release of adenosine from astrocytes (Witts et al. 2012). Thus, astrocytes are proposed as the source of modulatory adenosine in the mammalian spinal cord during locomotor-related activity, despite reported release of adenosine from neurons and microglia in other systems (Wall and Dale 2013; Jackson et al. 2017).

Adenosine is also released in the spinal cord during hypoxia and hypocapnia, and depresses reflex potentials recorded from the ventral roots (Lloyd et al. 1988; Otsuguro et al. 2006, 2009, 2011). Release of adenosine during hypoxia, although not hypocapnia, is inhibited by fluoroacetate, implying an astrocytic source (Takahashi et al. 2010). However, in this context it is not clear whether adenosine acts on spinal cord neurons or on peripheral afferents. In addition, it should be noted that in primary cultures of brain cells, oxidative stress was found to elicit release of adenosine from microglia and not from astrocytes or neurons (Jackson et al. 2017).

Together, these experiments demonstrate that activation of $G_{\alpha q}$-coupled GPCRs expressed by putative astrocytes in the spinal cord results in the production of adenosine, which in turn modulates neuronal activity via $A_1$ adenosine receptors. Production of modulatory adenosine following release of ATP from astrocytes appears to be widespread throughout the CNS, having also been reported in brain preparations (Pascual et al. 2005; Serrano et al. 2006; Panatier et al. 2011).

## Indirect evidence for neuron-to-glia signaling in motor networks

The tripartite synapse model implies detection of neuronal activity by astrocytes, which respond with elevations in cytosolic $Ca^{2+}$. Indirect support for neuron-to-astrocyte signaling during locomotor network activity is provided by the observation that modulation of the frequency of locomotor-

related activity by endogenous adenosine scales with network activity (Acton and Miles 2015). This presumably represents activity-dependent release of ATP-adenosine by astrocytes, and implies that ventral horn astrocytes detect neuronal activity; for instance, by detection of neurotransmitter release, as predicted by the tripartite synapse model. Astrocytes are therefore proposed to mediate a negative feedback loop, whereby activity-dependent release of adenosine depresses and perhaps stabilizes network output, resulting in smooth, controlled movement (Acton and Miles 2015). However, further $Ca^{2+}$ imaging is needed to confirm spinal astrocytic responsiveness to neuronal activity, as has been demonstrated in the brain (Araque et al. 2014; Bazargani and Attwell 2016; see above).

Interestingly, it has been observed that astrocytes express receptors for the neurotransmitters released specifically by the neurons they make contact with, implying that astrocytic receptors function to detect synaptic transmission (Verkhratsky and Butt 2013). Thus, spinal cord astrocytes are unusual 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 they do in neurons (Pastor et al. 1995). Spinal cord astrocytes also express NMDA receptors and are sensitive to glutamate (Ziak et al. 1998); however, the physiological importance of these currents remains unknown.

## Adenosinergic modulation of spinal motor circuitry

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). Experiments indicating release of modulatory adenosine from astrocytes in the ventral horn are complemented by studies into the functions and mechanisms of purinergic signaling during motor activity. However, some data point to unresolved complexity.

398 There are four adenosine receptor subtypes, designated $A_1$, $A_{2A}$, $A_{2B}$ and $A_3$, which differ in affinity
399 for adenosine, structure, cellular distribution, and G-protein coupling (Cunha 2001; Dunwiddie and
400 Masino 2001). $A_1$ and $A_{2A}$ receptors have the highest affinity for adenosine and are the best
401 characterized (Cunha 2001). Both receptors are expressed throughout the spinal cord (Reppert et al.
402 1991; Deuchars et al. 2001; Paterniti et al. 2011). $A_1$ receptors are tightly coupled to the $G_{\alpha i}$ pathway,
403 which mediates inhibition of adenylyl cyclase and reduced production of cAMP (Cunha 2001).
404 However, $A_1$ receptors are also coupled to other G proteins and act via adenylyl cyclase-independent
405 pathways to inhibit spontaneous and evoked neurotransmitter release (Cunha 2001). $A_1$ receptors
406 typically mediate presynaptic inhibition via the adenylyl cyclase-independent inhibition of N-type
407 $Ca^{2+}$ channels (Ribeiro 1995; Cunha 2001). $A_{2A}$ receptors are primarily coupled to $G_{\alpha s}$, but also to $G_{\alpha i}$
408 and $G_{\alpha 12}$, and have diverse effects mediated by PKA, PKC, N-type and P-type calcium channels
409 (Cunha 2001). In many systems, the inhibitory actions of $A_1$ receptors are countered by the
410 facilitatory actions of $A_{2A}$ receptors in a concentration-dependent manner (Cunha 2001).

411 The role of adenosine signaling in the modulation of mammalian locomotor behavior has been
412 addressed by injection of intact animals with receptor antagonists, such as caffeine, which typically
413 have an excitatory effect. This effect has been proposed to be mediated by either $A_1$ (Snyder et al.
414 1981; Goldberg et al. 1985), $A_{2A}$ (Svenningsson et al. 1995, 1997; Ledent et al. 1997; El Yacoubi et al.
415 2000; Lindskog et al. 2002) or both $A_1$ and $A_{2A}$ receptors (Karcz-Kubicha et al. 2003; Antoniou et al.
416 2005; Kuzmin et al. 2006), perhaps indicating a role for both receptor subtypes. However,
417 interpretation of studies in which antagonists are chronically applied is complicated by the reported
418 acquisition of receptor tolerance, to which $A_1$ receptors are particularly susceptible (Karcz-Kubicha et
419 al. 2003). It is also difficult to draw conclusions about the locus or loci of adenosinergic modulation:
420 it is likely that adenosine regulates locomotion in multiple regions of the nervous system involved in
421 motor control, including the basal ganglia (Popoli et al. 1996) and ventral spinal cord (Witts et al.
422 2012; Acevedo et al. 2016), and the effects of adenosine blockade in one network may mask its
423 effects in another.

Experiments in which *in vitro* rodent 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 5-HT, NMDA and DA in neonatal mouse preparations, blockade of $A_1$ receptors results in increased burst frequency (Witts et al. 2012; Acevedo et al. 2016), with no effect on amplitude, whereas blockade of $A_{2A}$ receptors has no effect (Witts, Panetta and Miles, 2012; Acevedo et al., 2016). Conversely, bath application of adenosine or an $A_1$-selective agonist, but not an $A_{2A}$ agonist, 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 ectonucleotidase inhibitor. This indicates 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 murine spinal cord (Witts, Panetta and Miles, 2012). The effects of bath-applied and endogenous adenosine therefore closely resemble those of ATP-adenosine released upon glial stimulation during network activity (Acton and Miles 2015).

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, neither exogenous adenosine nor adenosine released following PAR1 activation modulates 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, which may include the V1 population, for example (Fig. 3a). Ablation or inhibition of V1 interneurons reduces the frequency of locomotor-related activity, similar to the effects of adenosine acting at inhibitory $A_1$ receptors

450 (Gosgnach et al. 2006). However, disinhibited activity, which is generated by spinal cord
451 preparations following application of $GABA_A$- and glycine-receptor antagonists, is produced by a
452 modified network, and caution must therefore be applied when comparing the effects of adenosine
453 under disinhibited conditions and during fictive locomotion.

454 The effects of bath-applied adenosine on neurons in acute slices from postnatal mice have also been
455 assessed. Adenosine modulates a broad population of ventral horn interneurons, representing both
456 excitatory and inhibitory populations. In these cells, adenosine acts via presynaptic $A_1$ receptors to
457 reduce the frequency of miniature postsynaptic currents (mPSCs) (Witts et al. 2015) and the
458 amplitude of evoked excitatory post-synaptic currents (eEPSCs) in a paired simulation protocol
459 (Carlsen and Perrier 2014). Thus, adenosine acting at $A_1$ receptors inhibits synaptic transmission
460 onto interneurons. In addition, $A_1$ receptor activation induces a hyperpolarizing current in ventral
461 horn interneurons (Witts et al. 2015). In contrast, adenosine induces a depolarizing current in
462 motoneurons by an unknown mechanism that is sensitive to TTX, although adenosine does not
463 appear to directly modulate synaptic inputs onto motoneurons (Witts et al. 2015). Interestingly, $A_1$-
464 receptor blockade alone has no effect in recordings from neurons within spinal cord slices. It is
465 possible that there is insufficient adenosine present in slices for the tonic activation of these
466 receptors, or that adenosine at the concentrations at which it exists in the spinal cord requires
467 concurrent activation of $D_1$-like receptors to exert modulatory effects (Acevedo et al. 2016; see
468 below).

469 Data suggest differences in purinergic modulation between rats and mice. In isolated rat spinal cord
470 preparations, bath-applied adenosine enhances burst amplitude during pharmacologically induced
471 locomotor-related activity and depresses the frequency of disinhibited bursting in an $A_1$-dependent
472 manner (Taccola et al. 2012), effects not observed in mouse preparations (Witts et al. 2012; Acevedo
473 et al. 2016). In addition, the duration of bouts of locomotor-related activity induced by dorsal-root
474 stimulation in rat spinal cord preparations is reduced by exogenous adenosine (Taccola et al., 2012).

475 $A_1$ blockade alone has no effect on drug-induced or electrically stimulated locomotor-related activity
476 in rats (Taccola et al. 2012), whereas in mice $A_1$ blockade increases the frequency of fictive
477 locomotion (Witts et al. 2012; Acevedo et al. 2016). Differences between mice and rats in purinergic
478 modulation of respiratory networks have been attributed to species-specific ectonucleotidase
479 expression (Huxtable et al. 2009; Zwicker et al. 2011), which result in the mouse network being more
480 strongly modulated by adenosine, and the rat network by ATP. Further experiments are required to
481 assess whether similar species differences account for the reported differences in the sensitivity of
482 rat and mouse spinal locomotor networks to adenosine. Differential responses to adenosine may
483 also relate to differing roles of co-activation of $D_1$-like receptors (Acevedo et al. 2016), but the
484 potential role $D_1/A_1$ co-activation remains to be investigated in the rat spinal cord.

## 485 Metamodulation of $D_1$-like dopamine receptors by $A_1$ receptors

486 Recent data suggests that endogenous adenosine exerts its effects on locomotor-related activity via
487 second-order modulation of dopamine signaling within the mouse spinal cord (Acevedo et al. 2016).
488 Dopamine is a potent modulator of spinal motor circuitry, acting via diverse mechanisms to regulate
489 motor behavior (Miles and Sillar 2011; Sharples et al. 2014). All segments of the mammalian spinal
490 cord receive dopaminergic or L-DOPAergic inputs from descending fibers originating in the brain,
491 most notably from the A11 region of the hypothalamus (Björklund and Skagerberg 1979;
492 Commissiong et al. 1979; Hökfelt et al. 1979; Skagerberg and Lindvall 1985; Koblinger et al. 2014),
493 and dopamine levels increase within the ventral horn during locomotion (Gerin et al. 1995; Gerin
494 and Privat 1998).

495 The five dopamine-receptor subtypes fall into two families: the $D_1$-like receptors are the $D_1$ and $D_5$
496 subtypes and the $D_2$-like receptors are the $D_2$, $D_3$, and $D_4$ subtypes (Neve 2010; Pieper et al. 2011).
497 Whereas $D_1$-like receptors signal through $G_{\alpha q}$ to stimulate phospholipase C and $G_{\alpha s}$ to stimulate
498 adenylyl cyclase, $D_2$-like receptors signal through $G_{\alpha i}$ to inhibit adenylyl cyclase (Abdel-Majid et al.
499 1998; Pieper et al. 2011). Adenylyl cyclase catalyzes the synthesis of the second messenger cyclic

adenosine monophosphate (cAMP), which activates various proteins including protein kinase A (PKA). PKA 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). Dopaminergic modulation of locomotor-related activity in spinal networks is mediated by both $D_1$-like and $D_2$-like receptors (Madriaga et al. 2004; Humphreys and Whelan 2012; Sharples et al. 2015).

$A_1$-adenosine receptors are reported to interact with $D_1$-like dopamine receptors in the spinal locomotor circuitry (Acevedo et al., 2016), as is observed in the basal ganglia (Popoli et al. 1996), where they co-localize (Ferré et al. 1992). Dopamine stabilizes locomotor-related activity (Jiang et al. 1999; Whelan et al. 2000; Barrière et al. 2004; Madriaga et al. 2004; Humphreys and Whelan 2012; Sharples et al. 2015) and is co-applied with 5-HT and NMDA to induce locomotor-related activity in many studies. It is presumed to be absent in isolated lumbar spinal cord preparations unless exogenously applied, as descending dopaminergic inputs are severed in *in vitro* preparations, and dopamine receptor antagonists do not alter locomotor-related activity induced in the absence of a dopamine receptor agonist (Barrière et al. 2004). 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 $G_{\alpha s}$ signaling pathway through which $D_1$-like receptors 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.

Collectively, data from isolated spinal cord preparations suggest a model in which astrocytes secrete ATP during locomotor-related activity in an activity-dependent manner. Adenosine produced by hydrolysis of this ATP acts through $A_1$ receptors to regulate the activity of locomotor networks by inhibiting ongoing signaling mediated by $D_1$-like receptors and PKA (Fig. 3b). This may occur through direct inhibition of adenylyl cyclase mediated by the $G_{\alpha i}$ pathway to which $A_1$ receptors are coupled,

525 and it is possible that $A_1$ and $D_1$-like receptors form heterodimers (Franco et al. 2000); however,
526 colocalization of $A_1$ and $D_1$-like receptors has not yet been demonstrated in the spinal cord.

527 It is unclear how these results relate to experiments in which adenosine was found to modulate
528 neuronal activity in the absence of dopamine (Lloyd et al. 1988; Otsuguro et al. 2006, 2009, 2011;
529 Taccola et al. 2012; Carlsen and Perrier 2014; Witts et al. 2015). It is possible that high
530 concentrations of adenosine, such as those used for bath applications or produced during hypoxia
531 and hypocapnia, modulate neural activity independently of dopamine, whereas low levels of
532 endogenous adenosine require co-activation of $D_1$-like receptors by dopamine. Consistent with this,
533 $A_1$ blockade has not been shown to modulate neuronal activity in the absence of dopamine (Taccola
534 et al. 2012; Witts et al. 2012; Acevedo et al. 2016), although it does inhibit effects mediated by
535 exogenous adenosine or adenosine released in response to a stimulus (Lloyd et al. 1988; Otsuguro et
536 al. 2006, 2009, 2011; Taccola et al. 2012; Witts et al. 2015); presumably, this indicates low basal
537 levels of adenosine within the spinal cord. Interestingly, all dopamine-dependent and dopamine-
538 independent effects so far described in *in vitro* spinal cord preparations from healthy animals are
539 mediated by $A_1$ receptors, despite expression of $A_{2A}$ receptors in the spinal cord. It is conceivable
540 that $A_{2A}$ receptors may have a functional role only in responses to injury: following spinal cord injury,
541 $A_{2A}$ receptors are upregulated in the spinal cord, and administration of a selective $A_{2A}$ antagonist
542 exerts neuroprotective effects, perhaps by reducing excitotoxicity (Paterniti et al. 2011). Thus, $A_1$
543 receptors may participate in multiple signaling pathways, depending on agonist concentration.

544 The proposed antagonistic interaction between $A_1$ and $D_1$-like receptors implies that $D_1$-like
545 receptors, which are typically excitatory, enhance the frequency of locomotor-related bursting in
546 neonatal mice. However, this has not been clearly demonstrated. Although $D_1$-like receptors
547 typically have excitatory effects, they have not been reported to increase the frequency of ongoing
548 locomotor-related activity in mice (Humphreys and Whelan 2012; Sharples et al. 2015), but may
549 instead enhance the stability of rhythmic bursting (Sharples et al. 2015). Bath applied dopamine or a

550 $D_1$-like receptor agonist stimulates locomotor-related activity in neonatal rat preparations (Kiehn
551 and Kjaerulff 1996; Barrière et al. 2004) but not in neonatal mouse preparations (Jiang et al. 1999;
552 Whelan et al. 2000; Sharples et al. 2015); however, the selective $D_1$-like receptor agonist SKF 81927
553 is able to stimulate locomotor activity in spinalized adult mice (Lapointe et al. 2009). Thus, although
554 $D_1$-like receptors have excitatory effects within the spinal cord, the enhancement of burst frequency
555 that $A_1$ receptors are proposed to counteract in spinal cords from neonatal mice has not been
556 detected.

557 The effects of dopamine on network activity likely reflect multiple effects mediated by both $D_1$-like
558 and $D_2$-like signaling pathways within diverse cell populations: dopamine has been shown to
559 modulate synaptic strength and passive electrical properties of motoneurons and interneurons (Carp
560 and Anderson 1982; Maitra et al. 1993; Seth et al. 1993; Clemens 2004; Han et al. 2007; Barrière et
561 al. 2008; Han and Whelan 2009; Humphreys and Whelan 2012). The extent of second-order
562 modulation of $D_1$-like receptors by adenosine is currently unclear: further experiments are required
563 to reveal whether adenosine acts on all cells expressing $D_1$-like receptors or only on a subset
564 involved in the regulation of speed. Nevertheless, the model by which adenosine is proposed to
565 modulate the effects of $D_1$-like receptor activation during fictive locomotion is consistent with other
566 examples of metamodulation, which is proposed as an efficient mechanism for refining the effects of
567 a first-order neuromodulator; this may be important when the first-order modulator is widespread
568 and/or is capable of acting promiscuously on various cell typesto exert diverse effects within a
569 network (Katz 1999). Metamodulation may entail a second-order neuromodulator regulating the
570 availability (release or uptake) of a first-order neuromodulator or, as in the present example,
571 modulation of its effect on a target neuron. Other examples of metamodulation in spinal locomotor
572 networks are provided by *Xenopus* tadpoles, in which nitric oxide (NO) modulates the release of
573 noradrenaline (McLean and Sillar 2004) and lampreys, in which NO modulates the activity of
574 endocannabinoids (Song et al. 2012).

# The physiological importance of glial adenosine in spinal locomotor networks

Adenosinergic neuromodulation is proposed to have a homeostatic role in diverse systems, preventing metabolic exhaustion and excitotoxicity (Cunha 2001; Fredholm et al. 2005; Wall and Dale 2008). Activity-dependent production of adenosine has been detected in the mammalian brain and brainstem, and in the spinal cord of *Xenopus* tadpoles (Wall and Dale 2008). In some cases, this may be coupled to the degradation of ATP. ATP is consumed by neurons as a source of energy during activity, and adenosine produced by its hydrolysis may be released directly via neuronal equilibrative nucleoside transporters. Subsequent autocrine inhibition of activity via $A_1$ receptors is proposed as a mechanism to prevent metabolic exhaustion (Cunha 2001; Fredholm et al. 2005). However, this mechanism does not appear to operate in some systems, including spinal motor networks in tadpoles and mice, in which the source of adenosine is ATP released into the extracellular space from either neurons or astrocytes (Pascual et al. 2005; Serrano et al. 2006; Wall and Dale 2008; Panatier et al. 2011; Witts et al. 2012; Carlsen and Perrier 2014). Nevertheless, it remains possible that adenosine functions to avoid metabolic exhaustion, even if its source is not ATP expended as energy.

*Xenopus* tadpoles provide an example of purinergic signaling during motor behavior in a non-mammalian vertebrate. ATP released within the spinal cord during episodes of swimming excites the locomotor CPG, extending the duration of swimming bouts (Dale and Gilday 1996; Dale 1998). As swimming progresses, ATP is hydrolyzed 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). The cellular origin of ATP in the tadpole spinal cord is unclear, and given recent evidence from *Drosophila* larvae that neuron-glia crosstalk regulates behavior of non-mammalian species (Ma et al. 2016), glial release of purines should not be ruled out. The temporal dynamics of adenosine release in the mammalian spinal cord have not been

investigated; it is possible that gradual increase in adenosinergic tone during motor behaviors could also have a protective function by preventing metabolic exhaustion.

## Summary

Recent studies have provided evidence for the release of ATP-adenosine from astrocytes in the spinal cord, as previously demonstrated elsewhere in the CNS. Adenosine operates by multiple pathways within spinal motor circuitry. During fictive locomotion, it is proposed to function as a second-order modulator of $D_1$-like receptors (Acevedo et al. 2016). This mechanism is likely to be important in refining the influence of dopamine, which has manifold cellular activities during locomotor behaviors. The consequent reduction in locomotor speed may be important in ensuring fluency of movement or have a role in averting cellular fatigue (Witts et al. 2012; Acton and Miles 2015; Acevedo et al. 2016). Adenosine also acts by a dopamine-independent pathway or pathways (Lloyd et al. 1988; Otsuguro et al. 2006, 2009, 2011; Taccola et al. 2012; Witts et al. 2012; Carlsen and Perrier 2014), which may be activated only by high concentrations of adenosine. It will be interesting to compare tissue concentrations of adenosine during inactivity, locomotion and injurious conditions in which adenosine is released, such as hypoxia and hypercapnia. The agreement between dopamine-dependent effects mediated by $A_1$-adenosine receptors during fictive locomotion and the effects of adenosine released upon glial stimulation suggests that the latter acts via $D_1$-like receptors to modulate rhythmic activity, but this remains to be tested directly.

Although evidence exists for glial release of adenosine (Witts et al. 2012; Carlsen and Perrier 2014; Acton and Miles 2015), detailed $Ca^{2+}$ imaging of astrocytes is required to confirm a role for $Ca^{2+}$-dependent neuron-glia signaling in the ventral horn. However, several studies have provided evidence for crosstalk between neurons and astrocytes in a manner that departs from the established tripartite-synapse model (Gourine et al. 2010; Gourine and Kasparov 2011; Torres et al. 2012; McDougal et al. 2013; Angelova et al. 2015). Neurotransmitter uptake is as important as release in shaping patterns of synaptic transmission, and may be regulated in an activity-dependent

manner independently of $Ca^{2+}$ signaling (Attwell et al. 1993; Perego et al. 2000; Roux and Supplisson 2000; Marcaggi and Attwell 2004; Li et al. 2009; Al Awabdh et al. 2016; Shibasaki et al. 2016). Although PAR1 activation does not appear to elicit release of glutamate or its co-agonists at NMDA receptors, glycine and D-serine (Acton and Miles 2015), it has been proposed that co-agonist concentrations are regulated by astrocytes in an activity-dependent manner during motor behaviors (Acton and Miles 2017). 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 expressed by astrocytes lacking $IP_3R2$ receptors results in diverse behavioral modifications on a timescale longer than that predicted for $Ca^{2+}$-dependent responses; these may involve signaling via $G_{\beta\gamma}$ or protein kinase C (PKC), for instance (Agulhon et al., 2013). These examples suggest that information processing by astrocytes is underexplored, and may be crucial in the generation of behaviors by spinal networks, exceeding their established role as cellular housekeepers.

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## Author contributions

DA drafted and edited manuscript and prepared figures. GBM edited manuscript. DA and GBM approved final manuscript.

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# Figures

**Figure 1. Established model of bidirectional signaling between neurons and glia at the tripartite synapse.** Neurotransmitters activate astrocytic $G_{\alpha q}$-linked G-protein coupled receptors (GPCRS),

stimulating the production of inositol-3-phosphate ($IP_3$), activation of astrocytic $IP_3$ receptors ($IP_3R2$) and release of $Ca^{2+}$ from internal stores. An increase in intracellular $Ca^{2+}$ triggers release of gliotransmitters including glutamate, ATP and D-serine by a vesicular or channel-mediated mechanism, which in turn bind to pre- or postsynaptic GPCRs - or in the case of D-serine, *N*-methyl-D-aspartate (NMDA) receptors – to modulate synaptic strength or neuronal excitability.

**Figure 2. Adenosine derived from glial cells acts via $A_1$ receptors to modulate locomotor-related activity in a murine spinal cord preparation.** A: an *in vitro* spinal cord preparation in which suction electrodes are used to recording compound action potentials from motoneurons in transected ventral roots. B: sample raw (top) and rectified/integrated (bottom) ventral root traces showing rhythmic locomotor-related bursting evoked by bath-application of 5-hydroxytryptamine (10 μM), NMDA *N*-methyl-D-aspartate (5 μM) and dopamine (50 μM) in control conditions, following application of TFLLR (10 μM) to selectively activated glial protease-activated receptor-1 (PAR1), and during washout. Ci: 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. Cii: time course plot of normalized data aggregated into 1-min bins showing a reduction in burst frequency upon application of TFLLR. *n* = 10 preparations. Di: locomotor-burst amplitude is unaffected by TFLLR application. Dii: time course plot showing no change in burst amplitude upon application of TFLLR.. Ei: sample traces showing the effects of the non-selective adenosine receptor antagonist theophylline (20 μM) on locomotor-related activity. Eii: theophylline increases the frequency of locomotor-related bursting, revealing the modulatory role of endogenous adenosine derived from sources within the ventral horn. *n* = 6. Eiii: the selective $A_1$ adenosine receptor antagonist cyclopentyl dipropylxanthine (DPCPX; 50 μM) also reveals modulation of locomotor-related activity by endogenous adenosine, and prevents further frequency increases in the presence of theophylline, demonstrating that adenosine acts via $A_1$ receptors. *n* = 6. Fi: traces showing the effects of the glial toxin fluoroacetate (FA; 5 mM) when co-applied with glutamine (1.5 mM) and a lack of modulation by endogenous adenosine following glial

ablation, as revealed by application of theophylline (20 μM). Fii: theophylline has no effect on the frequency of locomotor activity when applied in the presence of the glial toxins FA and MSO. $n = 6$. $^{*}p < 0.05$, $^{**}p < 0.01$. B-D adapted from Acton & Miles (2015). E-F adapted from Witts, Panetta, & Miles (2012).

**Figure 3. Schematic illustrating proposed release of ATP-adenosine from astrocytes and inhibition of $D_1$-like receptor signaling within locomotor networks.** A: Outline of the spinal locomotor CPG as a bilateral network comprising flexor and extensor and rhythm- and pattern-generating modules for the coordination of muscle activation during locomotion. Molecularly defined populations of postmitotic ventral horn neurons (V1, V2a, V2b, V0v, V0d, V3) are represented as blue circles and some of their proposed interactions are indicated by arrows, signifying excitation, or bars, signifying inhibition. Astrocytes are proposed to modulate the rhythm-generating circuity by exerting an inhibitory effect, via secretion of ATP-adenosine, on an inhibitory population that regulates locomotor frequency, possibly the V1 population of interneurons (see text). For details of the roles of the neuronal populations indicated, see Goulding (2009) and Kiehn (2016). B: ATP is released from putative spinal cord astrocytes during network activity in response to an unidentified neuronal signal, and upon experimental activation of the $G_{\alpha q}$-linked receptor PAR1 by TFLLR, which is proposed to mimic the endogenous action of neurotransmitters on astrocytic GPCRs. Extracellular ectonucleotidases mediate the hydrolysis of ATP to adenosine, which activates neuronal $G_{\alpha i}$-linked $A_1$ receptors to inhibit signaling 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. Abbreviations: IN, interneuron MN, motoneuron.

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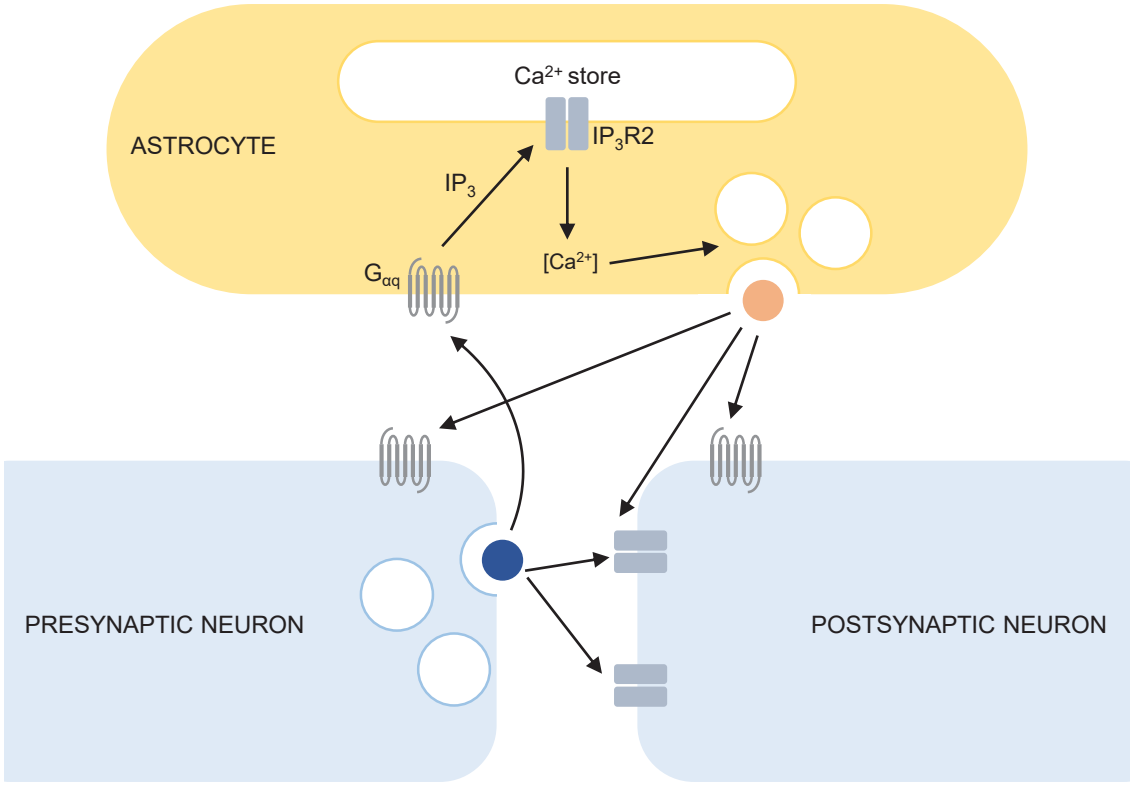
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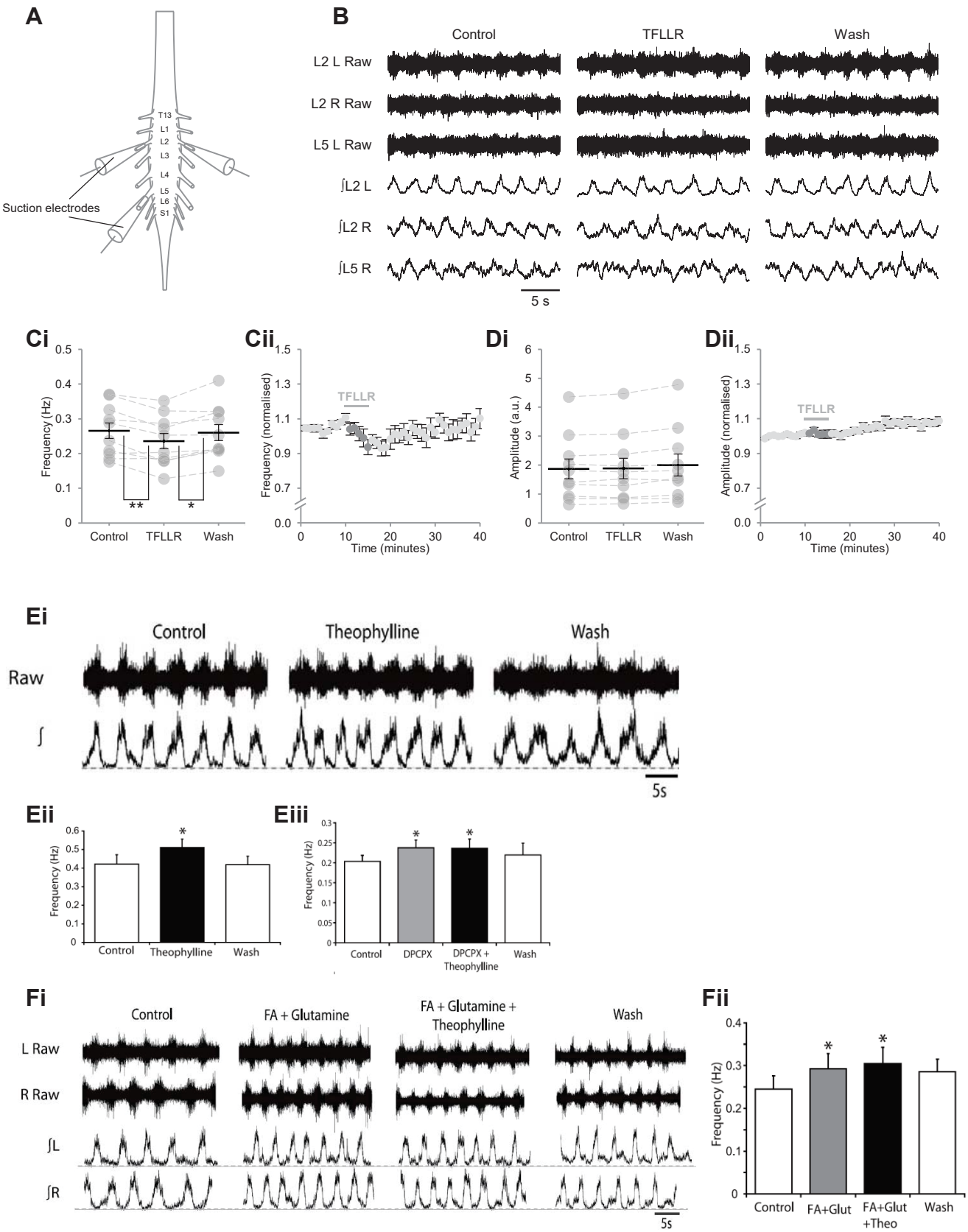
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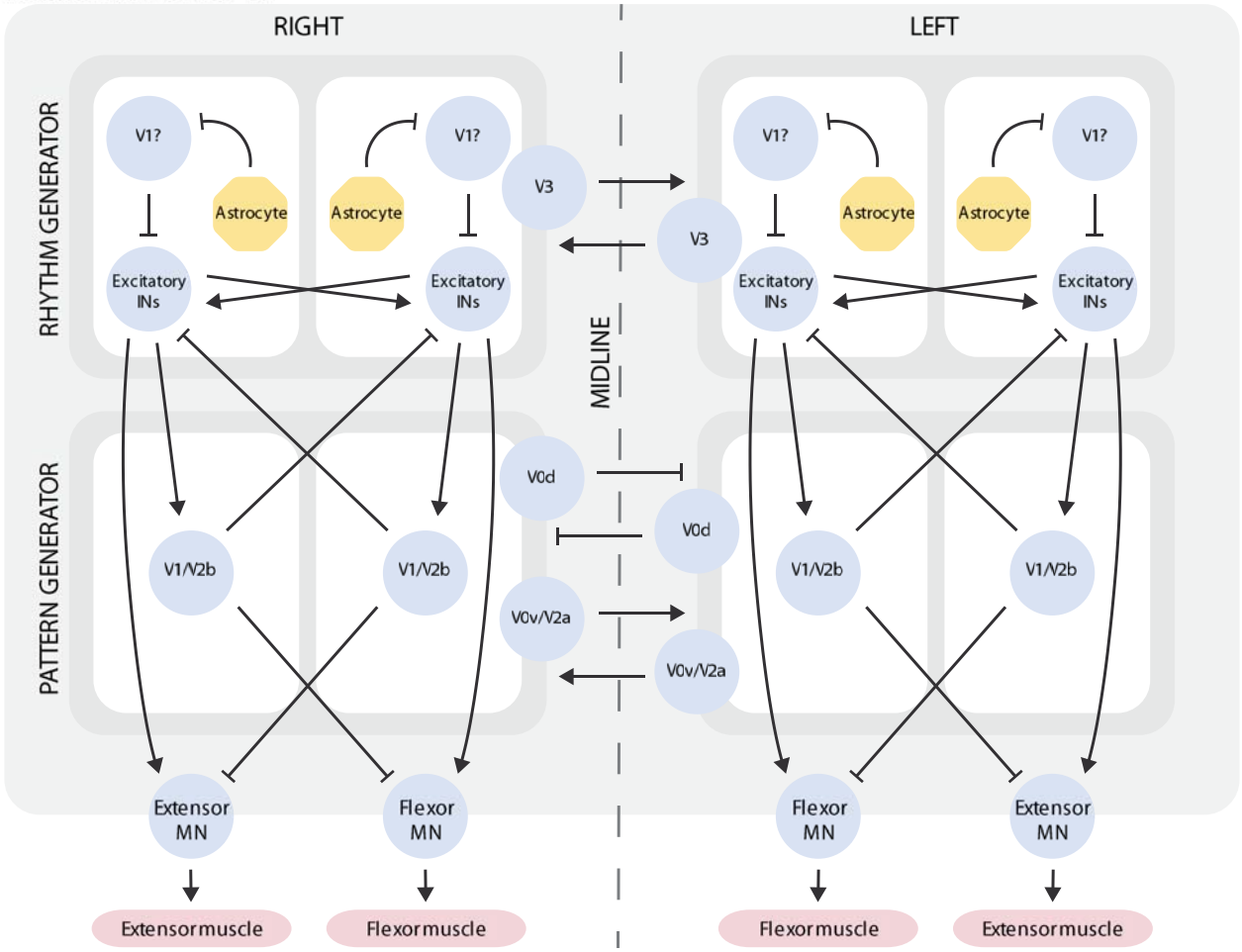
LOCOMOTOR CPG
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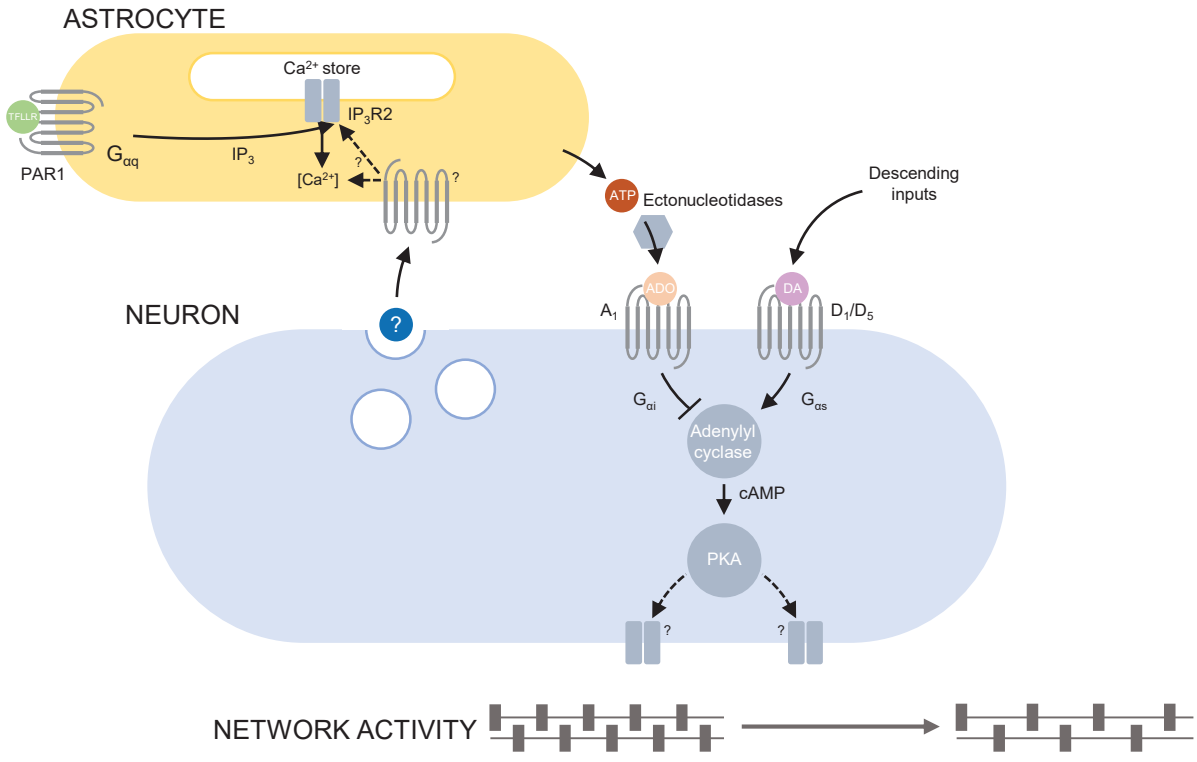
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NETWORK ACTIVITY