Title:

A Common Role for Astrocytes in Rhythmic Behaviours?

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Abstract

Astrocytes are a functionally diverse form of glial cell involved in various aspects of nervous system infrastructure, from the metabolic and structural support of neurons to direct neuromodulation of synaptic activity. Investigating how astrocytes behave in functionally related circuits may help us understand whether there is any conserved logic to the role of astrocytes within neuronal networks. Astrocytes are implicated as key neuromodulatory cells within neural circuits that control a number of rhythmic behaviours such as breathing, locomotion and circadian sleep-wake cycles. In this review, we examine the evidence that astrocytes are directly involved in the regulation of the neural circuits underlying six different rhythmic behaviours: locomotion, breathing, chewing, gastrointestinal motility, circadian sleep-wake cycles and oscillatory feeding behaviour. We discuss how astrocytes are integrated into the neuronal networks that regulate these behaviours, and identify the potential gliotransmission signalling mechanisms involved. From reviewing the evidence of astrocytic involvement in a range of rhythmic behaviours, we reveal a heterogenous array of gliotransmission mechanisms, which help to regulate neuronal networks. However, we also observe an intriguing thread of commonality, in the form of purinergic gliotransmission, which is frequently utilised to facilitate feedback inhibition within rhythmic networks to constrain a given behaviour within its operational range.

Introduction

Glial cells comprise a highly diverse population of non-neuronal cells that provide the infrastructure to the central nervous system (CNS) and peripheral nervous systems (PNS). Astrocytes, the star-like cells which constitute the most populous form of glia (von Bartheld et al., 2016), were long considered silent structural elements of the nervous system. Since it was first demonstrated that they were capable of complex intracellular Ca^{2+} fluctuations (Porter & McCarthy, 1995), their role in neural circuits and behaviour has been hotly investigated. Now, a vast field of research has generated an ever growing list of tasks that astrocytes perform within various parts of the nervous system, including synapse maintenance and regulation (Allen, 2014; Araque et al., 2014; Chung et al., 2015), interacting with the blood brain barrier (Alvarez et al., 2013), regulating metabolism (Allaman et al., 2011), maintaining the extracellular matrix (ECM) (Wiese et al., 2012) and crucially, activity-dependent modulation of neural network activity (Halassa & Haydon, 2010).

Attempting to establish a sense of logic to the role of astrocytes within a given neural network is of critical importance to our basic understanding of neurophysiology. Given their diverse roles and potentially lethal activity profiles in neurological and neurodegenerative disorders (Molofsky et al., 2012; Phatnani & Maniatis, 2015), there is also considerable therapeutic and pharmaceutical interest in the mechanisms by which astrocytes contribute to neural circuit function.

If the neural networks underlying distinct types of behaviour are different, astrocytic function may also be heterogenous to suit the needs of a given system (Chai et al., 2017; Khakh & Sofroniew, 2015; Pestana et al., 2020). By identifying networks with similar traits and characteristics, it may be possible to address whether there is a conserved logic to the roles of astrocytes across comparable systems.

Many neural networks display the distinct characteristic of rhythmicity. Basic physiological behaviours, such as locomotion (Grillner, 2003, 2006; Kiehn, 2016; Selverston, 2005), respiration (Harris-Warrick, 2010; Smith et al., 1991, 2009), mastication (Dellow & Lund, 1971; Lund & Kolta, 2006) and feeding (Carneiro & Araujo, 2012; Stephan, 2001; Yang et al., 2015), require rhythmic and patterned orchestration of neural programs, muscles and even whole organisms. Rhythm can be generated, in principle, by single neurons with intrinsic rhythmic,

pacemaker-like properties (Getting, 1989). Typically, however, complex coordinated patterns of behaviour, such as walking or breathing, utilise an anatomically localised group of neurons with cellular and network properties that produce rhythmic output, called a central pattern generator (CPG) (Brown, 1914; Marder & Bucher, 2001; Wilson, 1961). Some forms of rhythmic behaviours, such as sleep-wake cycles and food anticipatory behaviour (FAA) are driven at a more systemic level by circadian rhythms, which are partly entrained by environmental timing cues (known as Zeitgebers), such as light or food availability (Roenneberg & Merrow, 2016; Schulz & Steimer, 2009). Although CPG-mediated behaviours and circadian behaviours are distinctly different in their underlying mechanisms, time scales of operation and network organisation, they both ultimately result in rhythmic phasing of cellular activity and behaviour.

Rhythmic behaviours require mechanisms to control their initiation and cessation as well the pattern and timing of the neuronally encoded rhythm. For example, tasks such as walking require the organism to modulate the power, duration and frequency of muscle activation and the phase relationships between different muscles or systems in accordance with the demands of the organism under a given circumstance (Miles & Sillar, 2011). Neuromodulation endows circuits with the ability to finely tune rhythmic neural circuits to ensure that behaviours are malleable and adaptable to changing demands. There has been considerable research into how various neuronal networks are modulated by neurochemical transmitters that are released in a state-dependent manner from intrinsic and extrinsic neuronal sources. Such systems fine tune the intrinsic firing properties of individual neurons, as well as the synaptic transmission between them. More recent advances, notably over the last decade, have demonstrated that astrocytes also provide a key source of modulation of neural networks and rhythmic behaviours.

Astrocytes express a host of different neurotransmitter receptors, including glutamatergic, GABAergic, muscarinic, serotonergic, adrenergic, and purinergic receptors (P1 subtypes for adenosine, P2 subtypes for ATP and ADP) (Porter & McCarthy, 1997). Combined with the expression of different ion channels to detect ionic changes in the extracellular environment (Kadala et al., 2015; Kuffler, 1967; Olsen et al., 2015), and their expansive, arboreal structural domains that contact and envelope synapses (Calì et al., 2019; Heller & Rusakov, 2017; Ventura & Harris, 1999), astrocytes are equipped to be key sensors and integrators of different signalling mechanisms within neural networks. Activation of G-protein coupled

receptors (GPCRs) or some ligand-gated ion channels expressed by astrocytes can lead to elevations in whole cell intracellular Ca^{2+}, herein termed Ca^{2+} transients, which may be dependent on IP3 receptor type 2 (IP3R2) (Straub et al., 2006). Astrocytic Ca^{2+} transients are thought to correlate with the release of transmitters such as Glutamate, D-Serine and ATP (so-called gliotransmission), though the precise mechanisms of gliotransmission remain a topic of contention (Fiacco & McCarthy, 2018; Savtchouk & Volterra, 2018).

Given the wide range of signals that astrocytes respond to, and their ability to signal to neurons through gliotransmission, astrocytes could act as global integrators within neural circuits and thus position themselves as essential neuromodulators of rhythmic networks. Indeed, computational evidence suggests that astrocytes can both increase and decrease synaptic activity and bursting activity within neural networks using different gliotransmission mechanisms over different time scales (Lenk et al., 2020).

In this review, we examine the role of astrocytes in a number of neural circuits that control rhythmic behaviours. Taking each behaviour in turn, we examine to what extent astrocytes impact the neuronal circuitry and behavioural output, and shed light on the neuron-to-astrocyte and astrocyte-to-neuron signalling mechanisms that are implicated in regulating rhythmic behaviour. We would also like to highlight a number of other previous reviews that cover related themes. Kadala et al., (2015) review the role of astrocytes in homeostatic regulation of ionic concentrations that contribute to rhythmogenesis; Christensen et al., (2013) review evidence of gliotransmission and chemosensory roles of astrocytes in locomotor, masticatory and respiratory behaviours; Grubisic et al (2017) review the role of enteric glia in gut motility; and Lindberg et al., (2018) review the role of astrocytes in sleep-wake cycles.

Investigating Astrocyte Signalling in Neural Circuits and Behaviours.

There are a number of methodologies used for detecting (sensors) and manipulating (actuators) astrocytic activity. This section aims to provide a basic overview of some of the primary sensor and actuator tools used to investigate astrocytic function in neural circuits and behaviours.

Astrocytes respond to a range of stimuli through changes in intracellular Ca^{2+} which occurs through channel-mediated entry (Dunn et al., 2013), through IP3R2-dependent intracellular stores (Straub et al., 2006) or mitochondria (Agarwal et al., 2017), and can be visualised using

a number of approaches. Organic dyes, such as Fluo-4 and Fura-2, can be injected or perfused into cells to enable the detection of Ca^{2+} events (Porter & McCarthy, 1995; Reeves et al., 2011). These Ca^{2+} indicator dyes can be used in conjunction with astrocyte-labelling dyes such as sulforhodamine 101 (Kafitz et al., 2008; Parri et al., 2010; Schnell et al., 2012) or post-hoc immunohistochemical labelling of astrocytes (Broadhead et al., 2012; Ujita et al., 2017). Organic dyes, however, can be difficult to load into cells and tissue (Paredes et al., 2008) and can result in erroneous identification of astrocytes (Hülsmann et al., 2017). Genetically encoded Ca^{2+} indicators (GECI's) can therefore provide optimal alternatives to organic dyes by enabling specific and consistent targeting of expression in genetically defined subsets of cells and improved stability for long term image acquisition. GECI's, such as GCaMP variants, can be specifically expressed in astrocytes using recombination technology (e.g. Cre-lox recombination), to enable direct cytosolic measurements of intracellular astrocyte Ca^{2+} activity (Li et al., 2013).

Critically, there is some disparity between the approaches used for imaging and analysing Ca^{2+} transients in astrocytes. Many studies focus on large-amplitude, slowly summating events in the somas of astrocytes as a readout of astrocytic function. However, there is considerable evidence that smaller, millisecond-timescale Ca^{2+} events exist in the smaller branches and microdomains of astrocytes and that these events may correlate more directly with local neuronal activity (Bindocci et al., 2017; Volterra et al., 2014). Capturing both the fast and slow types of Ca^{2+} events in astrocytes with sufficient resolution and signal-to-noise ratio is technically challenging due to limitations and trade-offs in camera sensitivity, spatial resolution and field of view and the intensity and longevity of the fluorescent probes used. Equally, the considerable variability of Ca^{2+} transient duration, amplitude, rise and decay times recorded from a single astrocyte, as well as between different astrocytes, can render computational analysis difficult to automate and standardise between studies (Y. Wang et al., 2018).

Although recording astrocytic Ca^{2+} transients is a useful tool for investigators to measure cell activity, there may be exceptions whereby Ca^{2+} levels do not correlate with gliotransmission (Agulhon et al., 2008, 2010; Fiacco & McCarthy, 2018; Li et al., 2013; Savtchouk & Volterra, 2018). For example, Ca^{2+}-independent forms of gliotransmission have been demonstrated through ion channels and hemichannels permeable to gliotransmitters (Brancaccio et al., 2019; Dahl, 2015; De Vuyst et al., 2009; Woo et al., 2012). Nevertheless,

Ca^{2+} imaging of astrocytes remains a valuable metric of astrocytic function within neural circuits. Ca^{2+} changes often relate to changes in the function and impact of astrocytes on neural networks and behaviours, irrespective of a demonstrable direct relationship between Ca^{2+} and mechanisms of gliotransmission.

An alternative approach to recording astrocyte activity is the use of genetically encoded fluorescent biosensors (namely Luciferase) to visualise changes in gene expression over long periods of time (Pazzagli et al., 1992). This approach is utilised frequently in the study of circadian rhythms (Abe et al., 2002; Brancaccio et al., 2017; Yoo et al., 2004). Astrocytes are also structurally dynamic in response to activity changes. Live fluorescence imaging, as well as post-hoc imaging of fixed cells and tissue using electron microscopy, confocal and super-resolution microscopy are also commonly used to infer whether astrocytes are active in response to neuronal network activity (Heller & Rusakov, 2017; Yu et al., 2020). Structural changes in the finer arbors of astrocytes, namely the perisynaptic astrocytic processes (PAPs) that contact synapses, provide insight into the degree to which astrocytes may be able to detect and modulate neighbouring synaptic activity and buffer neurotransmitter spillover (Haseleu et al., 2013).

Precisely how astrocytes signal to neurons through gliotransmission, and whether gliotransmission occurs during physiological circumstances, remains a topic of debate (Fiacco & McCarthy, 2018; Savtchouk & Volterra, 2018). Nevertheless, there is evidence that astrocytes are capable of both vesicular release and hemichannel pore-mediated release of transmitters. Astrocytes have been shown to contain vesicles that are filled with neurotransmitters such as glutamate (Bezzi et al., 2004) and ATP (Coco et al., 2003), and the use of chemical dyes and other tools have demonstrated vesicular gliotransmission from astrocytes in response to stimulation (Hayoz et al., 2012; Shigetomi et al., 2008). Genetic approaches to interfere with vesicular exocytosis, such as the expression of dominant negative SNARE, the expression of tetanus toxin light chain, or other mutations in genes encoding vesicular release machinery, have also been shown to hamper astrocytic release of transmitters (Angelova et al., 2015; Coco et al., 2003; Pascual et al., 2005; Schwarz et al., 2017; Sheikhbahaei et al., 2018; Zhang et al., 2004). However, the reported non-specificity of some of these genetic models has led to questions regarding the validity of these approaches, and whether or not astrocytes truly perform vesicular-dependent gliotransmission under physiological circumstances (Fiacco & McCarthy, 2018; Fujita et al., 2014). Astrocytes also

express hemichannels, pores in the plasma membrane. Hemichannels can be formed by voltage-dependent pannexins (Dahl, 2015; Iglesias et al., 2009) or through connexins which are activated through Ca^{2+}-triggered intermediate signalling mechanisms (De Vuyst et al., 2009; Rash et al., 2001). Pharmacological and genetic disruption of hemichannels also appears to block gliotransmitter release (Abudara et al., 2014; Slavi et al., 2018; Wang et al., 2013; Xing et al., 2019). Other mechanisms of gliotransmission, such as glutamate release through K^+ or Ca^{2+} channels have also been reported (Woo et al., 2012), suggesting that astrocytes may contain a broad toolkit for versatile functionality, or that there may be functional diversity between astrocytes forming specialised subsets in certain regions of the nervous system (Pestana et al., 2020).

Manipulating astrocytes is crucial for understanding their functional significance within a given circuit or behaviour. The challenge with stimulating astrocytes is to provide a 'physiological' stimulus that is specific to astrocytes and thus does not target neurons or other glial cell subtypes whilst preferably preserving a physiological readout of behaviour. In many parts of the nervous system, astrocytes express an abundance of group I metabotropic glutamate receptors (mGluRs) (Sun et al., 2013). Therefore, agonists such as dihydroxyphenylglycine (DHPG) or (RS)-2-chloro-5-hydroxy-phenylglycine (CHPG), can be used to stimulate astrocytes through the Gq-coupled mGluR receptors that lead to IP3R activation and the intracellular release of Ca^{2+} (Broadhead & Miles, 2020; Parri et al., 2010; Xie et al., 2012). However, mGluRs are not specific to astrocytes, and such agonists may lead to neuronal activation (Mannaioni et al., 2001).

Protease activated receptor 1 (PAR1) is a GPCR that is highly expressed in astrocytes throughout the CNS (Junge et al., 2004) and is coupled to multiple G-proteins (Gq, Gi, and $G_{12/13}$) (Traynelis & Trejo, 2007). The specific PAR1 agonist, TFLLR, has been utilised in several studies to activate astrocytes (Acton et al., 2018; Acton & Miles, 2015; Beamer et al., 2017; Hermann et al., 2009; Sweeney et al., 2017; Vance et al., 2015). Current evidence suggests that PAR1 receptor activation may lead to a range of different gliotransmission mechanisms, from the slow release of glutamate through bestrophin-1 channels (Oh et al., 2012) to fast vesicular release of ATP (Lalo et al., 2014). However, the PAR1 receptor is also expressed by groups of neurons in the hippocampus, cortex and striatum, and thus may not always be the ideal tool with which to activate astrocytes (Junge et al., 2004).

Pharmacogenetic and optogenetic methods enable more targeted manipulation of astrocytes through specific Cre-dependent or tetracycline-controllable transgenic mouse lines or viral vectors (Yu et al., 2020). Pharmacogenetics employs a genetically modified receptor that can be activated by a chemically inert ligand to evoke cell specific effects. The Gq coupled MrgA1 and hM3Dq receptors have been used to stimulate astrocytes through what is likely to be Gq evoked IP3R-dependent Ca^{2+} signalling (Bonder & McCarthy, 2014; Broadhead & Miles, 2020; Chai et al., 2017; Fiacco et al., 2007; Yang et al., 2015). Optogenetic stimulation methods, using either light gated ion channels (channelrhodopsins) or light-gated GPCR (melanopsin) have also been utilised to stimulate astrocytes (Gourine et al., 2010; Mederos et al., 2019; Pelluru et al., 2016; Perea et al., 2014). However, the physiological relevance of channelrhodopsin-based stimulation mechanisms are unclear for astrocytes that, unlike neurons, do not typically demonstrate rapid cation exchanges across the membrane (Yu et al., 2020). For example, the use of channelrhodopsin2 to activate astrocytes has been shown to induce transient release of K^+ exerting significant effects on neuronal function, despite the fact that astrocytes seldom express voltage gated ion channels of this nature (Octeau et al., 2019). Alternatively, the light sensitive proton pump, archaerhodopsin, while used as an inhibitory optogenetic tool in neurons by hyperpolarising the cell membrane, has been used to stimulate astrocytes (Poskanzer & Yuste, 2016).

Inhibition of astrocytes has historically been achieved using toxins such as fluorocitrate which disrupt metabolism in glial cells, though the specificity for astrocytes is highly dependent on the dosage used (Fonnum et al., 1997). The glutamate analogue L-alpha-aminoadipate offers an alternative, astrocyte-specific chemical ablation method (Khurgel et al., 1996). Astrocytic Ca^{2+} activity can also be inhibited via infusion of the Ca^{2+} chelator BAPTA during intracellular recordings (Serrano et al., 2006). By injecting just one astrocyte with BAPTA, a number of neighbouring astrocytes, connected to one another via gap junctions in a syncytium, can be inhibited (Serrano et al., 2006). Pharmacogenetics has also been utilised to inhibit astrocytic activity. The Gi/o-coupled hM4Di receptor has been used to inhibit astrocytes in what is likely an adenylyl cyclase-dependent manner (Broadhead & Miles, 2020; Yang et al., 2015). Conversely, however, hM4Di targeting has also been shown to stimulate astrocytic Ca^{2+} activity (Chai et al., 2017; Durkee et al., 2019), suggesting that regional diversity of astrocytes may impact the downstream intracellular signalling processes of

pharmacogenetic manipulation (Batiuk et al., 2020; Chai et al., 2017; Khakh & Sofroniew, 2015; Yu et al., 2020).

For further, more in-depth reviews of the tools for investigating astrocyte activity see Li et al., (2013), Losi et al., (2017) and Yu et al., (2020). In addition, the dual reviews of Savtchouk and Volterra (2018) and Fiacco and McCarthy (2018) provide a balanced coverage of the debate surrounding the existence of gliotransmission and its mechanisms under physiological conditions, which requires some consideration when interpreting the results of some studies into astrocytic function in neuronal networks.

Spinal Astrocytes in Locomotion

The term locomotion is used to describe a number of motor patterns, such as walking, flying or swimming, that are used by organisms throughout the animal kingdom to enable guided and controlled movement towards a goal. Locomotion is a highly conserved, rhythmic, behaviour studied in various vertebrate species (Goulding, 2009; Katz, 2016; Miles & Sillar, 2011) and invertebrate systems such as leeches , insects and crustaceans (Ayali et al., 2015; Friesen et al., 2007; Hughes & Wiersma, 1960; Pulver et al., 2015). The mammalian lumbar spinal cord contains a CPG comprising a vast network of interneurons (INs) which produce a rhythmic output that is relayed to, and in some cases also influenced by, motor neurons (MNs). This rhythmic motor output encodes the timing, pattern and strength of muscle contractions in order to perform locomotor behaviours.

In the postnatal rodent, locomotor-related rhythms, consistent with walking patterns, can be studied *in vitro* from the isolated spinal cord (Smith & Feldman, 1987). Output from the MNs can be recorded electrophysiologically using suction electrodes attached to the lumbar ventral roots and locomotor-related rhythms can be induced *in vitro* by electrically stimulating descending or sensory pathways, or by applying a combination of excitatory receptor agonists (typically NMDA, serotonin and dopamine). The resultant motor output is both asymmetrical (i.e. showing left and right ventral root alternation) and phase shifted between upper and lower lumbar roots, which would control the alternation of flexor and extensor muscle activation.

Spinal locomotor networks are subject to considerable modulation from various neural sources, both within the spinal cord and from descending inputs. Numerous different

neurotransmitters including monoamines (Han et al., 2007; Kiehn et al., 1999; Schmidt & Jordan, 2000; Sharples et al., 2015), acetylcholine (Jordan et al., 2014; Nascimento et al., 2019, 2020), nitric oxide (Foster et al., 2014; Yoshida et al., 2018) and adenosine (Acton & Miles, 2017; Brown & Dale, 2000) have been shown to modulate locomotor output in terms of the frequency of motor bursts, their duration and their amplitude. Moreover, many of these neuromodulators may work together in order to elicit different effects – termed metamodulation (Acton & Miles, 2017; McLean & Sillar, 2004; Sharples et al., 2015). The range of modulators and their potential effects endows spinal motor systems with a broad array of outputs, enabling locomotion to be tailored to the needs of the animal at any given moment (Miles & Sillar, 2011).

Using Ca^{2+} imaging, spinal cord astrocytes have been shown to exhibit more frequent Ca^{2+} events during locomotion measured *in vivo* (Sekiguchi et al., 2016) and fictive locomotion *in vitro* (Broadhead & Miles, 2020) (Figure 1A). Furthermore, spinal astrocytes are activated in direct response to trains of action potentials evoked from ventral INs in spinal cord slices, suggesting that spinal cord astrocytes may be directly responsive to the activity of the locomotor CPG network (Broadhead & Miles, 2020).

Targeted pharmacological activation of astrocyte activity using the PAR1 agonist reduces the frequency of locomotion recorded from the ventral roots (Acton et al., 2018; Acton & Miles, 2015). More recently, pharmacogenetic activation and inhibition of spinal astrocytes using DREADDs has also been shown to modulate fictive locomotion (Broadhead & Miles, 2020). This astrocytic modulation of fictive locomotion is dependent on glial-derived ATP which is subsequently converted to adenosine and targets neuronal A1 receptors (Acevedo et al., 2016; Acton & Miles, 2015; Carlsen & Perrier, 2014). The degree by which astrocytes inhibit the frequency of fictive locomotion is enhanced when network activity is raised by increasing concentrations of NMDA – suggesting a state-dependent modulation of the locomotor CPG (Acton & Miles, 2015). Though the mechanism of gliotransmission has not yet been illuminated, it has been shown that connexin-43 (Cx43) hemichannels are responsible for the majority of astrocytic ATP release in response to spinal cord injury (Huang et al., 2012). Interestingly, the effect of adenosine on spinal neuromodulation may be co-dependent on dopaminergic signalling via the D1 receptors (Acton et al., 2018), with molecular evidence suggesting this involves a heteromeric interaction between A1 receptors and D1 receptors (Rivera-Oliver et al., 2018).

Purinergic modulation of locomotion through adenosine A1 receptors has long been recognised since the work by Nick Dale and colleagues on swimming behaviour in the frog embryo (Brown & Dale, 2000; Dale, 1998; Dale & Gilday, 1996). A1 receptor activation is associated with a hyperpolarization of the resting membrane potential and a reduction in presynaptic vesicular release probability of spinal INs (Witts et al., 2015), including premotor INs (Carlsen & Perrier, 2014). In addition, A1 receptor signalling may be associated with changes in excitatory synapse number and postsynaptic molecular organisation (Broadhead et al., 2020). Astrocytic adenosine signalling may only target a subset of INs that control the frequency of locomotion, as manipulating astrocyte activity appears to have no effect on the pattern of left-right alternation, or the amplitude of locomotor-related bursts (Acevedo et al., 2016; Acton & Miles, 2015, 2017; Broadhead & Miles, 2020; Witts et al., 2012, 2015). Spinal astrocytes appear to contact synapses and enrich the structure and molecular content of excitatory postsynaptic densities in a manner that is independent of anatomical laminae or the presynaptic source (Broadhead et al., 2020), suggesting that astrocytes may modulate a broader range of circuits and behaviours in the spinal cord. Moreover, astrocyte-derived adenosine has been shown to tonically inhibit MNs through A1/D1 heteromeric receptors (Rivera-Oliver et al., 2018), suggesting that astrocytes may target both INs and MNs and provide metamodulation of a range of motor behaviours.

The identity of the neuron-to-astrocyte signalling mechanism that drives astrocyte-derived purinergic release remains unclear. Studies in tissue and cell cultures suggest that spinal cord glial cells express a range of receptors including acetylcholine receptors (Hösli et al., 1988), group I metabotropic glutamate receptors (mGluR1,5) (Silva et al., 1999) and glycine receptors (Kirchhoff et al., 1996). Recent findings suggest that neuronal glutamate release, acting via mGluR5 receptors to drive purinergic modulation, is the most likely candidate of neuronal-astrocyte signalling (Broadhead & Miles, 2020). To this end, it has been shown that mGluR5 activation evokes large astrocytic Ca^{2+} transients and that blocking mGluR5 receptors abolishes the adenosine-mediated modulation of the locomotor CPG (Broadhead & Miles, 2020).

The current model suggests that bi-directional communication between astrocytes and neurons provides inhibitory feedback that modulates the locomotor CPG (Figure 2). In this model, increased neuronal activity leads to increased glutamate release, which subsequently activates nearby astrocytes via mGluR5 receptors. Astrocytes then release ATP, which is

extracellularly converted to adenosine and reduces synaptic activity and neuronal excitability via A1 receptor activation. This model of neuron-to-astrocyte and astrocyte-to-neuron signalling would enable the locomotor network to constrain its own output within an optimal operational range in a state-dependent manner.

The purinergic gliotransmission mechanisms involved in astrocytic control of locomotion are displayed in Figure 3. However, this may not be the sole mechanism by which astrocytes integrate with spinal motor circuits. Astrocytes have been shown to respond to rises in the levels of extracellular K^+ that occur due to increased IN and MN bursting during periods of elevated motor network activity (Brocard et al., 2013). This may relate to high expression levels of inwardly rectifying K^+ channels (Kir4.1) amongst ventral horn astrocytes (Olsen et al., 2007). Astrocytic Kir4.1 expression is associated with fast α-MNs, and acts to regulate the peak strength of motor output by lowering extracellular K^+ levels surrounding the MNs. (Kelley et al., 2018). Given that spinal astrocytes also respond modestly to other ligands such as GABA, glycine, dopamine, and acetylcholine (Broadhead & Miles, 2020; Hösli et al., 1987, 1988; Kirchhoff et al., 1996) it is conceivable that numerous endogenous mechanisms drive multiple, parallel modulatory processes in spinal astrocytes to help control the locomotor CPG and other motor programs.

Brainstem Astrocytes in Respiration

Breathing in mammals is controlled by a distributed network of neocortical, brainstem and spinal cord regions. The brainstem, however, harbours a series of highly interconnected modules responsible for respiratory rhythm generation . Populations of excitatory INs in the pre-Botzinger complex (preBötC) of the medulla generate inspiratory-related motor rhythms, while neurons in the lateral parafacial respiratory group (pFL) generate rhythmic activity required for active expiration (Del Negro et al., 2018) The preBötC alone is considered sufficient for inspiratory rhythm generation in mammals (Smith et al., 1991). The ventral parafacial (pFV; historically referred to as the retrotrapezoid nucleus), detects pH changes in the blood due to fluctuations in circulating CO_2 levels. The chemosensitive properties of the pFV controls activity of downstream respiratory CPG INs and thus helps regulate rhythmic breathing behaviour (Guyenet et al., 2009). As respiratory demand increases, circulating CO_2 levels rise and pH levels are reduced. The acidity of the blood is detected within the pFV,

which then initiates an increased pace of respiration, via its influence on the preBötC, to ensure the respiratory demands of the organism are met.

Early investigations using gliotoxins to ablate glial cells within respiratory networks suggested that astrocytes provided metabolic support to neurons (Hülsmann et al., 2000) through means of the glutamate/GABA-glutamine cycle (Hertz, Schousboe, et al., 1978; Hertz, Wu, et al., 1978). Astrocytes in the preBötC have since been shown to contribute to purinergic modulation of the inspiratory CPG (Huxtable et al., 2010) and may be involved in initiating respiration (Okada et al., 2012). PreBötC astrocytes exhibit intrinsic rhythmic Ca^{2+} activity in the absence of neuronal activity (Okada et al., 2012), and induce firing in inspiratory neurons through the vesicular release of ATP (Okada et al., 2012; Sheikhbahaei et al., 2018), suggesting that these astrocytes may be involved in driving rhythmicity in respiratory networks. Excitation of the PreBötC by astrocytic ATP may be mediated through neuronal P2X2 receptors (Gourine et al., 2003) or through neuronal P2Y1 receptors (Huxtable et al., 2010; Huxtable et al., 2009; Lorier et al., 2007; Rajani et al., 2018; Zwicker et al., 2011). Furthermore, astrocytes express P2Y1 receptors themselves, enabling propagation of astrocyte signalling across larger networks (Rajani et al., 2018). Indeed, a subpopulation of astrocytes (approximately 20% of the total astrocyte population) may form a glial-subnetwork of respiratory active cells distinct from the neural network (Forsberg et al., 2017).

In the chemosensitive region of the pFV, astrocytes have been shown to respond to changes in blood pH levels (Ritucci et al., 2005), ultimately driving respiratory activity based on O_2/CO_2 levels in the blood (Gourine et al., 2010). Gourine et al., (2010) demonstrated that astrocytes in the rat pFV were capable of releasing ATP in response to acidic conditions, which would result in long lasting depolarisation of PFV neurons. By performing phrenic nerve recordings in the anaesthetised animal, it was shown that optogenetic activation of pFV astrocytes alone was sufficient to induce high frequency respiration (Gourine et al., 2010). It was therefore reasoned that pFV astrocytes act as sensory cells that detect changes in blood O_2/CO_2 levels and initiate a respiratory response in accordance (Figure 1B). Astrocytes have subsequently been shown to express the electrogenic Na^+/HCO_3^- cotransporter NBCe1, which raises intracellular Na^+ in response to acidification and in turn leads to Ca^{2+} influx through the Na^+/Ca^{2+} exchanger (NCX) (Turovsky et al., 2016). This NBCe1-NCX coupling is part of the proposed mechanism by which astrocytes act as chemosensors to initiate respiratory activity (Turovsky et al., 2016), independent of neuronal chemosensory mechanisms (Guyenet et al.,

2016). Furthermore, a new study has indicated that in a separate region of the mouse medulla, the caudal parapyramidal area, CO_2 binding directly to glial Cx26 hemichannels leads to pore opening and chemosensory stimulator of respiration (van de Wiel et al., 2020).

The release of ATP from astrocytes appears to be dependent on vesicular exocytosis, as pharmacological blockade of hemichannels shows little effect on ATP-dependent initiation of respiration (Gourine et al., 2010) and vesicular fusion events have been visualised and both pharmacologically and genetically nullified in PreBötC astrocytes (Sheikhbahaei et al., 2018).

Intriguingly, there is some divergence in the roles of ATP and Adenosine within respiratory control circuits, which has been highlighted by differences observed between the rat and mouse respiratory CPG (Zwicker et al., 2011). Astrocyte-derived purinergic signalling appears to have a more excitatory effect on the respiratory system in the rat than it does in the mouse. Astrocytes are known to release ATP (Franke & Illes, 2014; Queiroz et al., 1999), which is subsequently converted extracellularly, first to ADP and then to adenosine, by ectonucleotidases (Deaglio & Robson, 2011). ATP typically displays excitatory actions on respiratory neurons acting via P2Y receptors (Abbracchio et al., 2003; Von Kugelgen & Wetter, 2000), namely $P2Y_1$ receptors (Huxtable et al., 2009; Lorier et al., 2007), while adenosine typically inhibits neuronal activity through A1 receptors and inhibits respiration (Burr & Sinclair, 1988; Lagercrantz et al., 1984). The expression of the tissue-nonspecific alkaline phosphatase (TNAP), an ectonucleotidase which degrades ATP to adenosine, is considerably higher in the mouse, thus favouring higher extracellular levels of inhibitory adenosine (Zwicker et al., 2011). Therefore, the predominant effect of purines on the murine respiratory CPG seems to be an adenosine-mediated suppression, whilst excitatory modulation mediated by ATP predominates in the rat (Figure 3).

While considerable research from various groups has generally consolidated the theory that the respiratory CPG is highly regulated by astrocyte-derived purinergic signalling (Cinelli et al., 2017; Gourine et al., 2010; Huxtable et al., 2010; Okada et al., 2012; Sheikhbahaei et al., 2018; Zwicker et al., 2011), other gliotransmitters have since been identified to regulate breathing behaviours. Astrocytic release of D-Serine in the brainstem increases respiratory rate through its actions on the glycine binding site of NMDA receptors (Beltrán-Castillo et al., 2017). PFV astrocytes are also thought to release prostaglandin E2 (PGE2) which not only acts to stimulate respiration in a manner similar to ATP but also blunts the respiratory response

to hypercapnia (whereby CO_2 levels are increased beyond normal levels) (Forsberg et al., 2017).

Brainstem Astrocytes in Mastication

The repetitive activation of jaw muscles to chew food in the mouth, known as mastication, is another example of a rhythmic behaviour patterned by neuronal activity in the brainstem. The caudal pons in the brainstem harbours the trigeminal main sensory nucleus (NVsnpr) circuit involved in mastication (Lund & Kolta, 2006). The rhythmogenic nature of the NVsnpr neurons is thought to result from inward Na^+ currents which mediate bursting properties (Brocard et al., 2006). The intrinsic bursting properties of NVsnpr neurons, however, are amplified when extracellular Ca^{2+} concentrations are depleted. This Ca^{2+} gating of inward Na^+ currents occurs due to Ca^{2+} ions occupying the pores of Na^+ channels (Armstrong, 1999; Brocard et al., 2006; Tazerart et al., 2008).

Morquette and colleagues (2015), found that sensory activation of the NVsnpr leads to astrocyte activation in a partly NMDA-receptor dependent manner. In turn, NVsnpr astrocytes are capable of releasing the Ca^{2+} binding protein, S100β, which sequesters extracellular Ca^{2+} and thus elicits rhythmic bursting in neurons (Figure 1C). The release mechanism of S100β is unclear. Another study by the same group demonstrated astrocytic Cx43 expression in astrocytes was essential for eliciting bursting activity and establishing confined rhythmic neuronal motifs within the NVsnpr (Condamine et al., 2018). However, in other studies of astrocyte function, S100β release from astrocytes was independent of hemichannels but sensitive to bafilomycin A1, which has been shown to inhibit vesicular release (Sakatani et al., 2008).

The role of astrocytes in mastication offers an interesting example where traditional gliotransmitters, such as ATP or glutamate, are not thought to be implicated as the predominant mechanisms of astrocyte-to-neuron communication. Instead, astrocytes in the caudal Pons release S100β, which buffers ion concentration in the ECM, leading to the ionic balance required to establish network rhythmicity (Figure 3).

Enteric Glial Cells in Rhythmic Gastrointestinal Motility

Beyond the CNS, neural network rhythmicity is also an essential feature of the enteric nervous system (ENS). The so-called "second brain" contains more neurons than the spinal cord or PNS and provides the gastrointestinal (GI) tract with a significant degree of autonomy from other branches of the PNS and CNS (Furness, 2012). Despite developmental and molecular distinctions between the central and enteric systems, the ENS can serve as an ideal model to study neuronal-glial interactions and synaptic integration alongside muscles and other non-neuronal cell interactions, as these cellular behaviours can be readily investigated in isolated segments of the GI tract (Bayguinov et al., 2012; Hennig et al., 2015; Spencer & Hu, 2020).

The ENS comprises a vast network of neurons that form two sheets, known as "plexi", throughout the wall of virtually the entire GI tract (Furness, 2012). The submucosal plexus is situated between the mucosa and smooth circular muscle layers, while the myenteric plexus resides between the circular muscle layer and outer-most longitudinal muscle layer. Broadly speaking, gastric secretion and nutrient uptake processes are regulated by the submucosal plexus while intestinal motility is controlled by of the myenteric plexus (Costa et al., 2000). Both the myenteric and submucosal plexi consist of a range of sensory neurons, MNs and INs that are grouped into ganglia alongside enteric glial cells (EGCs), with approximately 2-3 EGCs enveloping the somas of single neurons. EGCs bare similarities to CNS astrocytes in both their structure and function (Furness, 2012; Neunlist et al., 2014; Ochoa-Cortes et al., 2016). There is no clear topographical organisation to the functionally different subclasses of neurons within ganglia throughout both the myenteric and submucosal plexi, with different cell types organised by developmental lineage (Lasrado et al., 2017).

The isolated large colon of the rodent is capable of generating neuronally-mediated rhythmic, propagating contractions of the smooth muscle layers, termed the colonic migrating motor complex (CMMC) (Smith et al., 2014). These large-scale propagating waves of neural activity can be initiated by circumferential stretch of the colon or serotonin released from enterochromaffin cells along the mucosal lining in response to mechanical stimulation from faecal matter (Heredia et al., 2009, 2013). Serotonin activates 5-HT3 receptors of projecting myenteric Dogiel type II neurons to initiate the CMMC (Bayguinov et al., 2010; Bywater et al., 1989; Heredia et al., 2009, 2013). It should also be noted that enteric neurons

may interact either directly with smooth muscle or via intermediary cells called the interstitial cells of Cajal (ICCs) (Smith & Koh, 2017).

It has been shown that approximately 30% of EGCs in the myenteric plexus display rises in intracellular Ca^{2+} following the stimulation of CMMCs in the isolated mouse colon (Broadhead et al., 2012) (Figure 1D). These long lasting Ca^{2+} transients typically follow bursts of myenteric neuron and synaptic activity and outlast the duration of the CMMC (Broadhead et al., 2012; Hennig et al., 2015). EGCs can be activated by a number of intrinsic ENS neurotransmitters, including acetylcholine, serotonin, ATP and substance P (Boesmans et al., 2013; Boesmans et al., 2019; Broadhead et al., 2012; Gulbransen & Sharkey, 2009). Enteric neurons appear to form localised neuron-to-glia signalling units by releasing purines via neuronally expressed pannexins on the soma (Werend Boesmans et al., 2019). Additionally, Ca^{2+} imaging also reveals that EGCs are contacted by a number of active varicosities through which vesicular release of transmitters would occur (Bayguinov et al., 2012; Broadhead et al., 2012), suggesting that a range of local neuron-to-glia and long distance neuron-to-glia signalling mechanisms may occur.

Although it has been well documented that EGCs respond to neuronally mediated activity in the ENS, it is still unclear as to what function EGCs have in the regulation of the CMMC, or the gliotransmission mechanisms involved. It has been shown that EGCs release the gaseous inhibitory neurotransmitter nitric oxide (NO), which would in turn inhibit excitatory cholinergic myenteric neurons (Bayguinov et al., 2010; Shuttleworth et al., 1993) and thus inhibit the CMMC as well as regulate epithelial ionic transport (MacEachern et al., 2011, 2015). An inhibitory role for EGCs in the regulation of the CMMC would correlate with the latency of EGC activation toward the terminal phase of the CMMC (Broadhead et al., 2012). However, pharmacogenetic stimulation of EGCs enhances the amplitude, frequency and velocity of propagating CMMCs (McClain et al., 2015). A study by Grubisic and Parpura (2017) examined the effects of altering gliotransmission on colonic motility and GI function. Genetic up-regulation of Cx43-dependent hemichannels resulted in increased gut motility *in vivo*, while knockdown of Cx43 hemichannels appeared to reduce gut motility as well as reduce the velocity of CMMCs recorded *in vitro* (Grubišić & Parpura, 2017). Genetic disruption of Ca^{2+}-dependent exocytosis in EGCs also appeared to slow CMMCs recorded in vitro, though no effect was observed on gut motility. Instead, EGC exocytosis appeared to show a role in regulating the fluid content of fecal pellets produced by mice. The study concluded that EGCs

may use differential signalling mechanisms to modulate distinct aspects of gut physiology and motility coordinated by the ENS (Grubišić & Parpura, 2017). While it is unclear what specific gliotransmitter is involved and the specific mechanism by which it is released, ATP is thought to be a likely candidate (Grubišić & Parpura, 2017; McClain et al., 2014) (Figure 3).

Astrocytes in Circadian Sleep-Wake Cycles.

Circadian rhythms are characterised by a 24-hour periodicity, regulating metabolism and behaviours which are entrained by external cues (known as Zeitgebers). Circadian rhythms act on a much slower timescale than the CPGs underlying motor behaviours such as locomotion, breathing, chewing or the enteric-generated CMMC. As such, the regulation of the internal body clock depends less on a CPG-like neuronal network and more on the periodicity of transcription and translation of core genes that encode the activity of populations of cells from a molecular level (Kunz & Achermann, 2003; Ralph et al., 1990; Webb et al., 2009). Many so called 'clock genes' implicated in mammalian circadian rhythm generation have been identified in fruit flies (drosophila), indicating conserved evolutionary mechanisms for circadian body clocks (Hastings, 1998; Robinson & Reddy, 2014; Roenneberg & Merrow, 2016). These genes include the PER1-3, BMAL, CRY1, CRY2 and the aptly named CLOCK (Hastings, 1998).

While cells in many regions of the brain show rhythmic circadian patterns of activity and gene expression, the suprachiasmatic nucleus (SCN) of the hypothalamus is considered the master circadian pacemaker of the brain (Abe et al., 2002). The SCN receives inputs from the retina and as such uses light as a Zeitgeber to drive a neuronal network that reinforces circadian rhythmicity in various other CNS regions (Hastings, 1998). The cellular circadian activity of the SCN can be isolated for investigation *in vitro* using either tissue slices from the rodent SCN or dissociated cell cultures (Savelyev et al., 2010; Welsh et al., 1995).

Astrocytes in the SCN display rhythmic oscillations of intracellular Ca^{2+} (Van den Pol et al., 1992) and undergo structural and molecular changes in astrocytic connections with synapses (Lavialle et al., 2011). The Ca^{2+} activity of neurons and astrocytes in the SCN appears to be largely alternating, such that neuronal activity peaks during the circadian light phase, while astrocyte activity peaks during the circadian dark phase (Brancaccio et al., 2017) (Figure 1E). Using bioluminescence reporter lines, SCN astrocytes in organotypic cultured slices and

dissociated cells exhibit circadian oscillations in the expression of clock genes (Clock, BMAL1, PER1 and Per2 and Cry1) in a manner that is independent of neuronal input (Brancaccio et al., 2019; Welsh et al., 2010). Astrocytic Per2 expression leads to circadian fluctuations in glutamate synthesis and reuptake, regulating excitability of the whole network by lowering glutamate levels during the dark phase (Chi-Castañeda & Ortega, 2018; Leone et al., 2015). Some lines of evidence suggest that astrocytes may also be capable of directly entraining the rhythmicity in SCN neurons (Brancaccio et al., 2017, 2019; Clasadonte et al., 2017; Prosser et al., 1994). Most recently, it has been observed that Ca^{2+}-independent release of astrocytic glutamate via Cx43 hemichannels leads to regulation of clock gene expression in neurons via activation of NMDA receptors containing the NR2C/D subunit (Brancaccio et al., 2019).

Alternative studies suggest that the transcriptional rhythms of clock genes in astrocytes regulate the synthesis, release and uptake of ATP, with higher levels of extracellular ATP associated with the latter period of the dark phase (Marpegan et al., 2011; Prolo et al., 2005; Womac et al., 2009). Meanwhile, adenosine displays rising extracellular levels throughout the course of the day, peaking early in the dark phase (Marpegan et al., 2011; Womac et al., 2009). The actions of adenosine on neurons is thought to be via A1 and A2 receptors expressed in the SCN, modulating the presynaptic release of GABA (Barca-Mayo et al., 2017; Hablitz et al., 2020). With extracellular levels rising throughout the day, it is thought that adenosine exerts sleep pressure, increasing the urge to sleep (Sims et al., 2013). A more recent study identified a cannabinoid signalling pathway that recruits astrocytes leading to adenosine-dependent inhibition of presynaptic GABA release, which the authors hypothesise may help to fine tune neuronal activity in the SCN during the light phase (Hablitz et al., 2020). However, given the range of P2 receptors (P2X and P2Y for ATP) and P1 receptors (A1, A2 and A3 receptors for adenosine), which together elicit a range of excitatory and inhibitory effects, purinergic modulation could elicit a broad spectrum of effects on the circadian network throughout the cycle (Lindberg et al., 2018).

The effects of purinergic signalling may also act through mechanisms that target transcriptional processes in both glia and neurons. For example, ATP acting via P2X7 receptors has been shown to induce Per1 expression and contribute, at least partially, to the rise of Per1 expression throughout the circadian day (Krueger et al., 2010; Nakazato et al., 2011). Similarly, Per1 gene expression is perturbed by genetic deletion of the adenosine transporter Equilibrative Nucleoside Transporter 1 (ENT1) and antagonism of the adenosine A2A

receptor, leading to circadian locomotor hyperactivity and seemingly more severe sleep-wake cycles (Ruby et al., 2014). In this capacity, astrocytic ATP release helps to limit the 'gain' of the circadian clock, thus stabilising sleep-wake behaviour by preventing extreme changes in clock gene expression (Lindberg et al., 2018).

The precise mechanisms of astrocytic ATP release in sleep-cycle regulation are not yet known. ATP release was found to be dependent on IP3 signalling but independent of SNARE proteins for vesicular exocytosis (Marpegan et al., 2011). Similarly, genetic deletion of pannexin-1 leads to perturbed ATP release and accumulation of intracellular adenosine leading to sleep disturbances (Kovalzon et al., 2017). However, a study on the effects of chronic sleep deprivation on astrocytes demonstrated that astrocytes show changes in vesicle-associated membrane protein-2 (VAMP2) which would implicate vesicular based gliotransmission (Kim et al., 2014).

Both glutamatergic and purinergic gliotransmission mechanisms are therefore known to play a role in the rhythmic circadian oscillations that drive sleep-wake cycles (Figure 3). It is not yet known whether one signalling mechanism is more significant than the other in regulating the circadian rhythm. Perhaps glutamatergic gliotransmission directly drives the neuronal network of the SCN while purinergic gliotransmission acts to self-restrain the circadian rhythm within operational ranges. To further complicate the issue, A1 and A2 adenosine receptors may also form heterodimers with D1/2 dopaminergic receptors and mGluR5 receptors (Brown et al., 2012; Ferré et al., 2002; Popoli et al., 2001). Heterodimeric interactions between adenosine and other neurotransmitter receptors would enable a flexible metamodulatory control system to regulate neurotransmission and clock gene expression in order to finely orchestrate circadian rhythmicity in astrocytes and neurons (Lindberg et al., 2018; Morioka et al., 2015).

Astrocytes in Entrained Oscillatory Feeding Behaviour

Feeding behaviour represents a neuronally entrained behaviour that cycles over the course of minutes to hours. The food entrainable oscillator (FEO) is promoted by scheduled food intake and generates cyclical food anticipatory activity that is partly independent of the circadian clock system and can persist during periods of fasting (Horst et al., 1999; Zheng et al., 2001). For many mammals, the FEO promotes regular feeding patterns to safeguard

against possible periods of food scarcity and offers a strong evolutionary advantage over merely eating whenever nutrition is required (Klok et al., 2007; Kobelt et al., 2008; Sánchez et al., 2004).

The FEO is thought to be organised by a distributed network in the brain, between the limbic forebrain regions of the amygdala and hippocampus, and the dorsomedial hypothalamic nucleus (DMH) and arcuate nucleus of the mediobasal hypothalamus (ARC) (Mistlberger, 1994; Storch & Weitz, 2009). Neurons in the hypothalamic nuclei appear to be sensitive to circulating 'hunger hormones' such as leptin and ghrelin, which act to decrease and increase hunger respectively (Verwey & Amir, 2009).

It was shown by Kim et al, (2014) that astrocytes in the hypothalamus express leptin receptors and are responsible for regulating both inhibitory and excitatory synapse number and synaptic transmission amongst a specific population of ARC neurons expressing agouti related peptide (AGRP). Conditional deletion of astrocyte leptin receptors did not disturb overall feeding behaviour of the mice, but instead blunted leptin-induced anorexia and enhanced fasting-induced or ghrelin-induced over-eating (hyperphagia) (Kim et al., 2014). It was next demonstrated by Yang et al., (2015) that pharmacogenetic manipulation of astrocytes (excitation via hM3Dq and inhibition via hM4Di) could modulate the FEO of the hypothalamus in an adenosine-dependent manner (Figure 1F). Activation of astrocytes in the mouse *in vivo* suppresses ghrelin-evoked feeding and facilitates leptin induced anorexia. These effects were shown to be dependent on adenosine signalling that acted via A1 receptors on AGRP neurons (Stephan, 2001; Sweeney et al., 2016; Yang et al., 2015). Additionally, optogenetic stimulation of astrocytes appears to modulate feeding and fasting behaviour and correlates with adenosine release (Sweeney et al., 2016) (Figure 3). Likewise, it has been shown that astrocytes in the nucleus of the solitary tract (NST) within the brainstem dorsal vagal complex undergo structural changes in response to excess feeding induced by an overnight fast, and that pharmacogenetic activation of NST astrocytes constrains excess feeding behaviour (MacDonald et al., 2020). Evidence also suggests that astrocytes in the ARC express insulin receptors, and show proteomic changes in SNARE proteins that promote vesicular ATP release in response to insulin receptor deletion (Cai et al., 2018)

These findings suggest that astrocytic purinergic signalling has a key role in the modulation of the FEO, enabling the network to be constrained within operational ranges (Figure 4) (Yang

et al., 2015). However, these studies have only demonstrated the ability to modulate 'evoked' feeding, for example, using experimental doses of ghrelin or leptin to alter feeding behaviour. Thus, it is yet to be demonstrated whether astrocytes have a significant role in regulating feeding behaviour when the behaviour itself is within normal ranges. It may be that astrocytes only evoke subtle effects on the FEO under normal conditions, while predominantly responding to extremes of behaviour to prevent hyperphagia or fasting.

Clock genes associated with circadian rhythms have also been shown to impact feeding behaviour. Indeed a number of genes, such as CLOCK, Per1, Per2 and Per3, and BMAL show circadian oscillations of transcription not only in the circadian centres but also the DMH (Yang et al., 2015). Perturbation in these clock genes can lead to arrhythmic patterns of feeding (Mieda et al., 2006; Piggins, 2002). Just as clock genes may regulate astrocytic purinergic signalling during sleep-wake cycles, it is possible that clock genes could also factor in purinergic regulation of feeding behaviour.

Is there a Conserved Astrocytic Logic in Rhythmic Circuits?

In this review we have discussed the roles of astrocytes within neural networks that underly a range of rhythmic behaviours. Although our understanding of the basic roles of astrocytes within neural circuits remains incomplete, we hypothesise that an examination of the mechanisms and roles of astrocytes within related circuits may reveal some sense of conserved logic. While there is considerable heterogeneity in the function of astrocytes within circuits controlling rhythmic behaviours (Figure 3), which may relate to emerging data demonstrating significant functional and molecular heterogeneity amongst astrocytes (Batiuk et al., 2020; Chai et al., 2017; Pestana et al., 2020), there is also an intriguing thread of commonality in the form of purinergic gliotransmission (Figure 4).

In the spinal control of locomotion, astrocyte activity is dependent on the state of the locomotor CPG (Broadhead & Miles, 2020). Astrocytic ATP release leads to adenosine-dependent neuromodulation, which helps to limit the frequency of locomotor-related output, possibly acting as an inhibitory feedback mechanism to prevent over-activation of the network (Acton & Miles, 2017; Broadhead & Miles, 2020). Within brainstem circuits that control respiration, astrocytic release of ATP can directly activate the respiratory network (Gourine et al., 2010). However, subsequent adenosine signalling may provide activity

dependent inhibition to the respiratory network (Sheikhbahaei et al., 2018; Zwicker et al., 2011). Despite the much longer duration of oscillations associated with circadian sleep-wake cycles, astrocytic ATP and adenosine also regulate neuronal networks underlying these rhythms, via effects on transcriptional patterns of clock genes and by directly modulating neuronal activity (Lindberg et al., 2018). Adenosine appears to provide inhibitory modulation to the SCN and surrounding networks, helping to evoke sleep and maintain balanced sleep-wake cycles. Furthermore, rhythmic feeding behaviours appear to be controlled by astrocyte-derived adenosine, acting as an inhibitory mechanism to prevent over-feeding (Yang et al., 2015). Taking these results into consideration, it could be concluded that astrocyte-derived purines help to modulate rhythmic networks by providing an activity-dependent, inhibitory feedback system (Figure 4). Therefore, activity-dependent astrocytic adenosine signalling in rhythmic networks helps prevent over-activity of networks and inherently promote stable rhythmicity by increasing inhibition during high levels of activity and reducing inhibition during low levels of activity. This hypothesis is supported by current computational models of astrocyte-to-neuron interactions in a study by Lenk et al., (2020). The *in silico* study modelled astrocytic interactions within neuronal networks based on *in vitro* and *in vivo* conditions wherein astrocytes were presumed to be capable of releasing both glutamate and purines with excitatory and inhibitory effects respectively. In these models, the combination of astrocytic glutamate to excite neuronal networks and astrocytic adenosine to inhibit neuronal networks could together promote and stabilise bursting behaviour within the neuronal network (Lenk et al., 2020). If we consider that many of the rhythmic networks and behaviours discussed in this review already contain neuronal sources of glutamatergic excitation to drive network activity, this leaves inhibitory purinergic signalling as the complementary astrocytic mechanism to help promote and constrain rhythmicity with optimal ranges.

However, the hypothesis that astrocyte-derived adenosine is a conserved gliotransmitter signalling mechanism that modulates all rhythmic networks is far from fool-proof. With regards to rhythmic CMMCs, it has not yet been directly shown whether EGC purinergic release is responsible for the potential role in modulating CMMCs (Boesmans et al., 2013; Grubišić & Parpura, 2017; McClain et al., 2014; McClain et al., 2015). EGCs and CNS astrocytes may not be molecularly and functionally comparable to one another and they may harness gliotransmission mechanisms. Other gliotransmitters besides purines have also been

identified in other rhythmic behaviours such as respiration (Beltrán-Castillo et al., 2017; Forsberg et al., 2017) and sleep-wake cycles (Brancaccio et al., 2019). Within brainstem circuits that control mastication, astrocytes release S100β to facilitate extracellular Ca^{2+} buffering, which modulates rhythmic neuronal bursting (Morquette et al., 2015). Although it remains to be determined how widely such mechanisms are employed, recent work has also implicated astrocytic release of S100β in the control of cortical neuron firing (Ryczko et al., 2020). Therefore, astrocytes may regulate neuronal activity and rhythmicity through the control of the extracellular environment as opposed to more directly purposed gliotransmission mechanism acting via neurotransmitters, for example.

There is, however, consistent evidence that adenosine acts as an activity-dependent modulator of neurons, and that astrocytes are one of the primary sources of adenosine (Wall & Dale, 2009). Adenosine receptors form heteromeric signalling complexes with other neurotransmitter receptors, enabling metamodulation of different circuits (Acton & Miles, 2017; Brown et al., 2012; Lindberg et al., 2018; Rivera-Oliver et al., 2018). Combined with the fact that astrocytes are capable of responding to a range of neurotransmitters themselves, astrocytes are endowed with the potential to act as global, activity-dependent feedback modulators of neuronal networks.

Rhythmic networks require steady modulation in order for the repetitive or oscillatory behaviour to be tuneable to the demands of the organism at any given moment. Astrocytes may therefore utilise adenosine signalling as their primary method of ensuring that the steady states of rhythmic networks are maintained within operational ranges, preventing overexertion of the network (Figure 4). Other gliotransmitters such as glutamate, D-Serine, or even the initial release of ATP, may be harnessed to provide more excitatory drive to certain networks under particular conditions. It is possible that wide scale mapping of the gliotransmitter signalling mechanisms utilised by astrocytes across a broader range of circuits and conditions could help us establish additional basic principles regarding how astrocytes integrate within neural networks.

There is still considerable debate surrounding the issue of whether gliotransmission occurs under physiological conditions throughout the entirety of the nervous system (Fiacco & McCarthy, 2018; Savtchouk & Volterra, 2018). Many CNS mediated behaviours are inaccessible to single-cell-level investigation *in vivo*, or can be difficult to study *in vitro*, as it may not be possible to correlate cellular function with physiological behaviours when the

whole system is no longer intact. There is an innate advantage to studying many rhythmic behaviours, such as respiration and locomotion, because the CPG's that underly such behaviours can be preserved for *in vitro* studies. As a result, the activity of single cells is highly accessible to experimental investigation, while their relationships with physiological behaviours and outputs are preserved and measurable. Even the circadian rhythms of the SCN are persistent for several days when isolated for *in vitro* studies. Therefore, rhythmic networks and behaviours may be useful model systems for advancing our understanding of astrocyte function and gliotransmission throughout the nervous system. Changes in astrocyte structure and function are known to occur in a host of different neurological and neurodegenerative diseases. Though in some cases astrocytes may help to ameliorate neuronal dysfunction, in other cases they may exacerbate or hasten disease progression. Only with a clear understanding of the basic principles of astrocyte function within a range of neuronal circuits can we better understand how these cells contribute to disease and how astrocytes could be targeted therapeutically for treating these conditions.

Figures

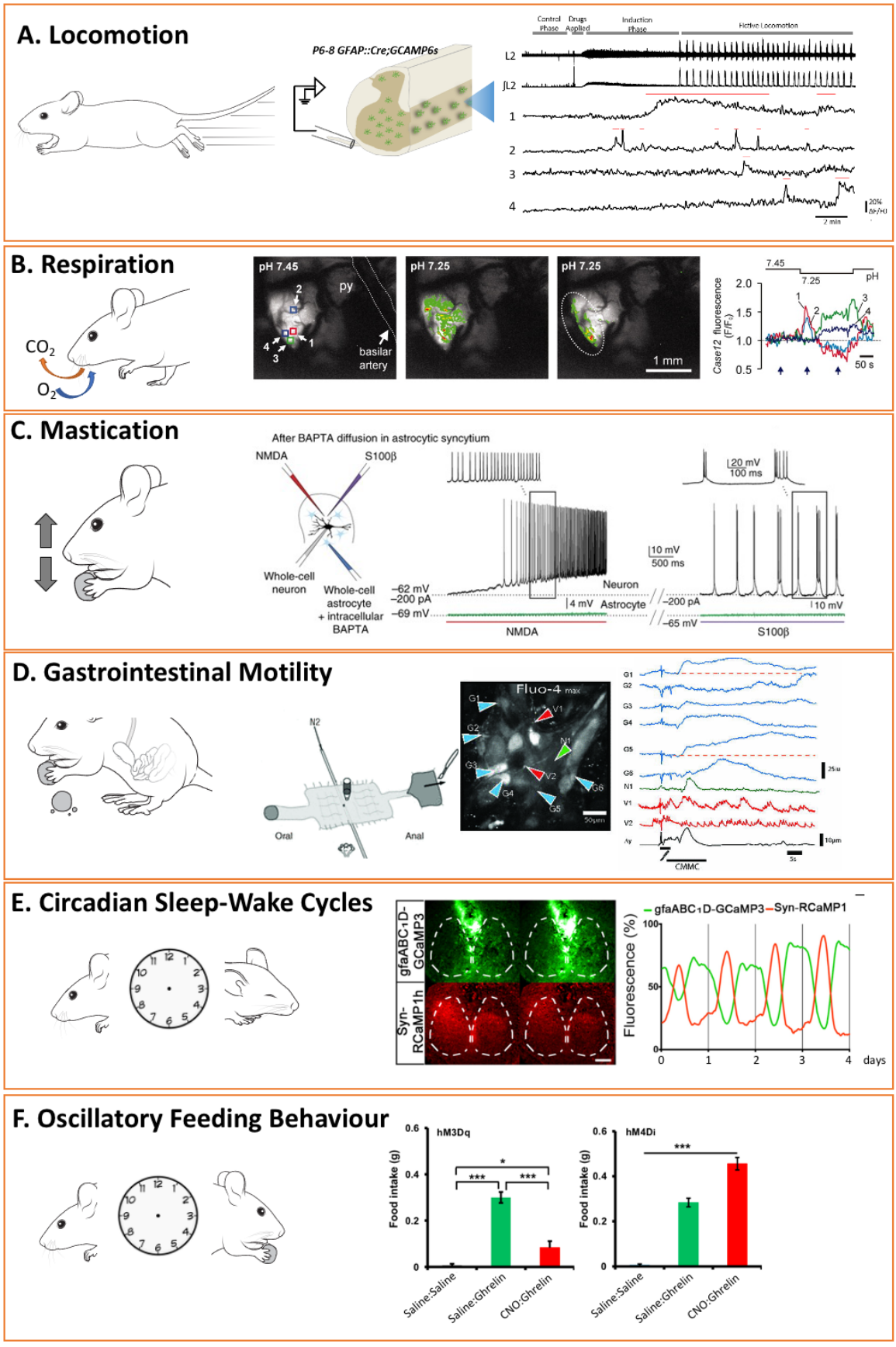
A. Locomotion
P6-8 GFAP::Cre;GCAMP6s
Control Phase
Drugs Applied
Induction Phase
Fictive Locomotion
L2
∫L2
1
2
3
4
B. Respiration
CO2
O2
pH 7.45
pH 7.25
pH 7.25
py
basilar artery
1 mm
Case12 fluorescence (F/F0)
7.45
7.25
pH
50 s
C. Mastication
After BAPTA diffusion in astrocytic syncytium
NMDA
S100β
Whole-cell neuron
Whole-cell astrocyte + intracellular BAPTA
–62 mV
–200 pA
–69 mV
NMDA
Neuron
4 mV
Astrocyte
20 mV
100 ms
10 mV
500 ms
–200 pA
–65 mV
10 mV
S100β
D. Gastrointestinal Motility
Fluo-4 max
N2
Oral
Anal
CMMC
E. Circadian Sleep-Wake Cycles
gfaABC1D-GCaMP3
Syn-RCaMP1h
gfaABC1D-GCaMP3
Syn-RCaMP1
Fluorescence (%)
days
F. Oscillatory Feeding Behaviour
hM3Dq
hM4Di
Food intake (g)
Saline:Saline
Saline:Ghrelin
CNO:Ghrelin

Figure 1. Examples of Astrocytic Involvement in Six Rhythmic Behaviours. A. Locomotion. Astrocytes have been shown to be active during chemically induced rhythmic locomotor bursting activity induced in the isolated neonatal mouse spinal cord. Astrocytic Ca^{2+} activity in a hemisected spinal cord of a GFAP::Cre;GCAMP6s mouse is imaged while electrophysiological recordings of locomotor bursting activity are obtained. Images adapted from Broadhead and Miles (2020). The top two black traces on the right panel shown the raw and processed locomotor bursting while the bottom 4 traces depict the Ca^{2+} activity of individual astrocytes. B. Respiration. Astrocytes in the chemosensitive RTN of the brainstem respond to experimentally induced pH changes, with lower pH levels producing Ca^{2+} transients from astrocytes. This chemosensitive astrocyte response helps to regulate respiration in accordance with the O_2/CO_2 demands of the animal. Images adapted from Gourine et al., (2010). C. Mastication. Brainstem circuitry that underlies rhythmic chewing actions of the jaw requires astrocytic release of S100β to induce neuronal bursting. Inhibition of astrocytes through the Ca^{2+} chelator BAPTA leads to tonic activity from trigeminal neurons upon addition of NMDA (left trace of this panel). Addition of S100β induces rhythmic bursting in neurons (right trace of this panel). Images adapted from Morquette et al., (2015). D. Gastrointestinal motility. Enteric glial cells of the myenteric plexus can be visualised using Ca^{2+} imaging in the isolated mouse colon. Following the mechanical induction of a CMMC, numerous identified glial cells show prolonged Ca^{2+} transients. Images adapted from Broadhead et al., (2012). E. Circadian sleep-wake cycles. Neurons and astrocytes in the isolated SCN are visualised using Ca^{2+} imaging in vitro over several days. Neurons and astrocytes display alternating phases of Ca^{2+} levels, with neurons peaking during the circadian day while astrocytes peak at circadian night. Images adapted from Brancaccio et al., (2017). F. Oscillatory feeding behaviour. Chemogenetic stimulation or inhibition of hypothalamic astrocytes bi-directionally regulates feeding behaviour. Mice injected with Ghrelin increases appetite and food intake compared to mice injected with saline. Mice expressing the excitatory DREADD receptor hM3Dq in astrocytes and injected with the ligand CNO displayed reduced ghrelin-induced feeding. Opposingly, mice expressing the inhibitory DREADD receptor, hM4Di, in astrocytes displayed elevated ghrelin-induced feeding behaviour. Image adapted from Yang et al., (2015). Images of mice were used acquired or adapted from Scidraw (www.scidraw.io).

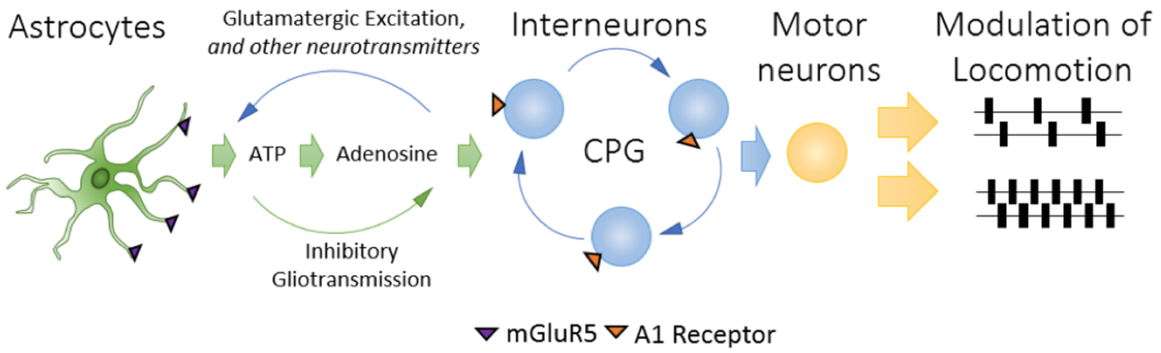

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Figure 2. Schematic of the Role of Astrocytes in Modulating the Frequency of Locomotion. The spinal cord locomotor CPG activity leads to increased glutamatergic signalling. This activates astrocytes through mGluR5 receptors, leading to the release of ATP which is converted extracellularly to adenosine. Adenosine then acts at A1 receptors which inhibit CPG interneurons and thus reduce the frequency of locomotion.

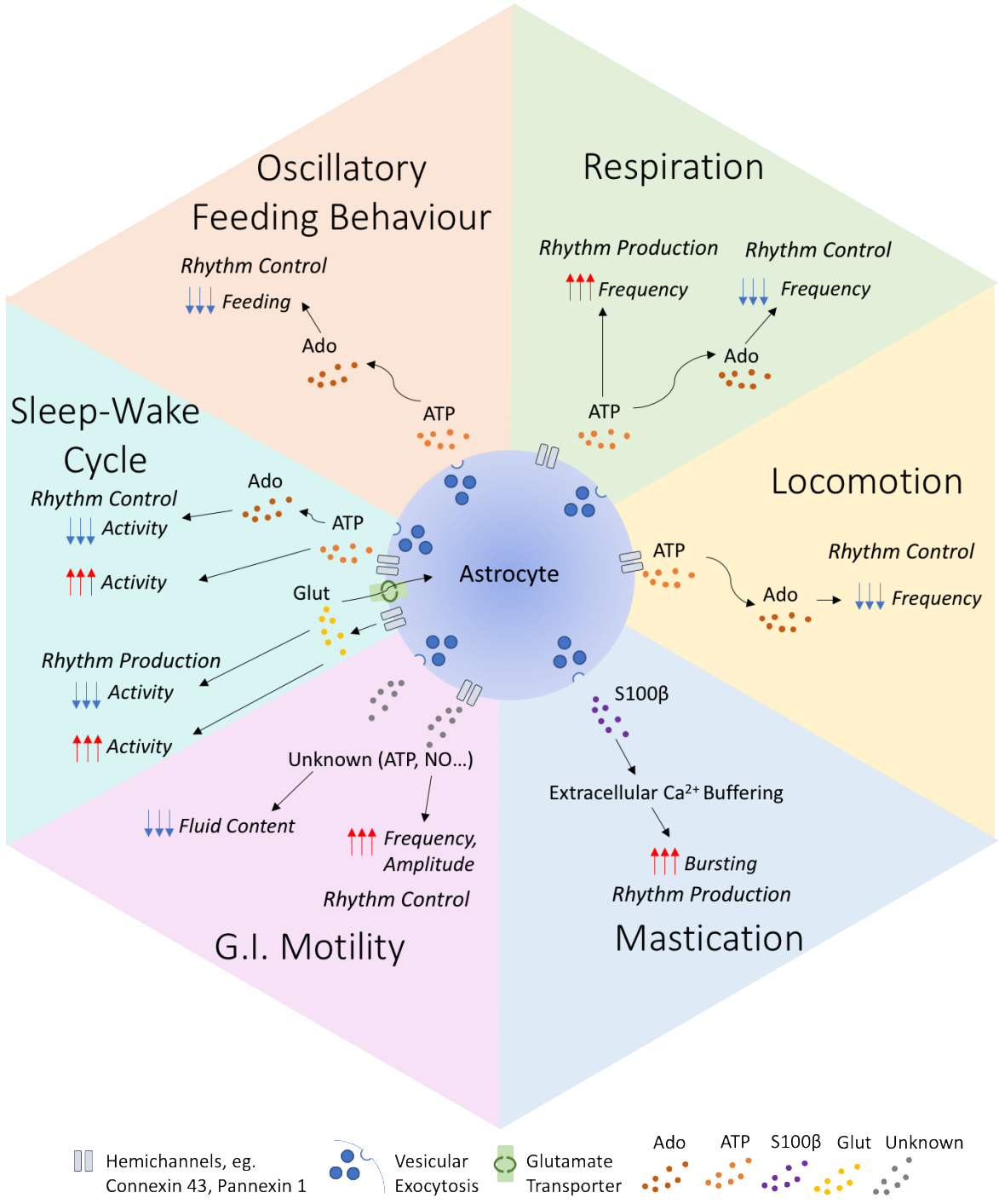

Figure 3. Gliotransmission Mechanisms in Rhythmic Behaviours. This diagram combines data from multiple studies, referenced throughout the article, to summarise the roles that astrocytes play in six different rhythmic behaviours. In each case, the gliotransmitter release mechanism, gliotransmitter identity and functional significance is denoted.

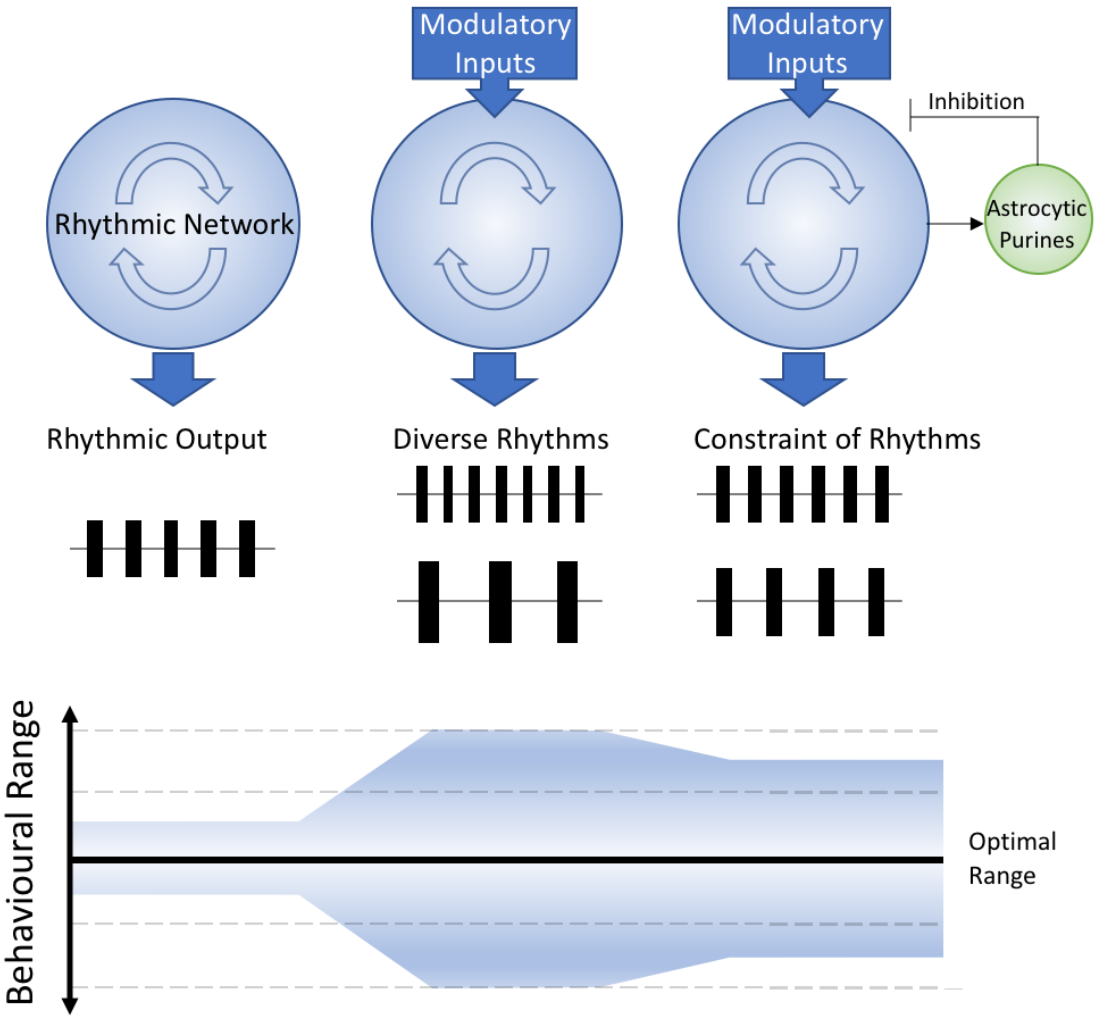

Figure 4. Purinergic Gliotransmission Enables Rhythmic Network Activity to be Constrained Within Optimal Operational Ranges. This schematic depicts a simplified model by which modulatory inputs ensure that neuronal networks produce appropriate rhythmic output. Network output can be modulated by different inputs to elicit a broader range of behaviours. Astrocytic purinergic signalling, predominantly using adenosine, inhibits neuronal activity in a manner which constrains the diversity of rhythmicity within optimal operational ranges – i.e. a range whereby the behaviour is more suitable to the demands of the organism at the given moment in time.

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