Respiratory Physiology & Neurobiology 265 (2019) 3–8

Contents lists available at ScienceDirect

Respiratory Physiology & Neurobiology

journal homepage: www.elsevier.com/locate/resphysiol

Astrocyte networks modulate respiration – *sniffing glue*

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ARTICLE INFO

Keywords:
Astrocytes
Respiration
Neural and neuronal network
Chemosensitivity

ABSTRACT

The role of astrocytes in the modulation of breathing has emerged. Within the two major respiratory control centers, the inspiration generating preBötzinger Complex and the chemosensitive parafacial respiratory group/retrotrapezoid nucleus, rhythmically active astrocytes have been discovered. These are connected in glial subnetworks that intermingle with the neuronal network. Furthermore, astrocytes modify overall respiratory network behavior through gliotransmitter release, especially during hypoxic and hypercapnic stress. Here, we review some recent discoveries regarding astrocyte-neuronal interactions on a cellular as well as neural network level including the novel gliotransmitter PGE2.

1. Astrocytes and respiration

Since their discovery in 1895 (Lenhossék, 1895), astrocytes have not been given the attention they deserve, with less than 10% of scientific publications in neuroscience up until 2010 investigating them (Mathey et al., 2010). However, during the last decade, the role of astrocytes in shaping neuronal signaling has come to the forefront of neuroscience research. Their role in vital neural functions ranging from homeostasis maintenance to signal modulation are discovered each year (Verkhratsky et al., 2015; Parpura et al., 2012; Araque et al., 1999; Sheikhbahaei et al., 2018a; Farmer and Murai, 2017).

Astrocytes exhibit Ca^{2+} mediated excitability (Verkhratsky et al., 1998) due to the presence of Ca^{2+} pumps and Ca^{2+} release channels in the endoplasmic reticulum membrane (Verkhratsky et al., 2012). They also express various metabotropic receptors coupled to the inositol 1,4,5-triphosphate ($InsP_3$) pathway, which induces a release of Ca^{2+} from the endoplasmic reticulum (Finkbeiner, 1993) and exocytosis of transmitters to the extracellular space (Verkhratsky et al., 1998; Kettenmann and Verkhratsky, 2016; Parpura and Verkhratsky, 2012; Parpura and Zorec, 2010; Turovsky et al., 2016; Montero and Orellana, 2015; Malarkey and Parpura, 2008; Parpura et al., 1994). Astrocytes may also signal among themselves through gap junction-mediated Ca^{2+} waves (Parpura et al., 2012; Verkhratsky et al., 2012). These signaling systems organize astrocytes into glial networks in the brain and brainstem (Funk et al., 2015). Furthermore, recent findings suggest that several functionally distinct astrocytic subtypes exist (Farmer and Murai, 2017; Matyash and Kettenmann, 2010). Astrocytes also play an important role in the respiratory system (Erlichman et al., 1985). Glia-specific toxins depress breathing *in vivo* (Young et al., 2005) as well as preBötzinger Complex (preBötC) activity *in vitro* (Huxtable et al., 2010). Recently, preBötC astrocytes were found to be morphologically more complex compared to other brainstem astrocytes (Sheikhbahaei et al., 2018b). This review aims to summarize what is known about the role of astrocytes in respiratory rhythm generation and modulation, and also present some novel findings regarding the interaction between neuronal and astrocytic networks.

2. Rhythmic astrocytes in the preBötC

In the preBötC, astrocytes can be divided into active and inactive subpopulations, depending on the presence of rhythmic behavior. We recently utilized our newly developed organotypic slice culture system (Forsberg et al., 2016, 2017a) together with Ca^{2+} time-lapse imaging to investigate astrocytes in the preBötC (Forsberg et al., 2017b). We imaged calcium activity in slice cultures containing the preBötC after 7 days in vitro using the calcium dye Fura-2. During live imaging, we identified rhythmic Ca^{2+} oscillations in 13 ± 7% of the preBötC astrocytes (Forsberg et al., 2017b). However, the experimental setup did not allow us to detect whether this rhythmicity was synchronized with the respiratory-related motor rhythm. Throughout this review we will refer to astrocytes that maintain oscillations in either changes in Ca^{2+} concentration or membrane potential as rhythmic astrocytes. It is important to keep in mind that this rhythmicity of single cells is not necessarily directly linked to the generation of the respiratory rhythm, i.e. breathing activity.

Rhythmic, inwardly directed currents entrained to preBötC neuron population discharges were detected in 10% of preBötC astrocytes (Grass et al., 2004). Another study, by Okada et al., also found

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https://doi.org/10.1016/j.resp.2018.06.015
Received 15 February 2018; Received in revised form 17 June 2018; Accepted 29 June 2018
Available online 30 June 2018
1569-9048/

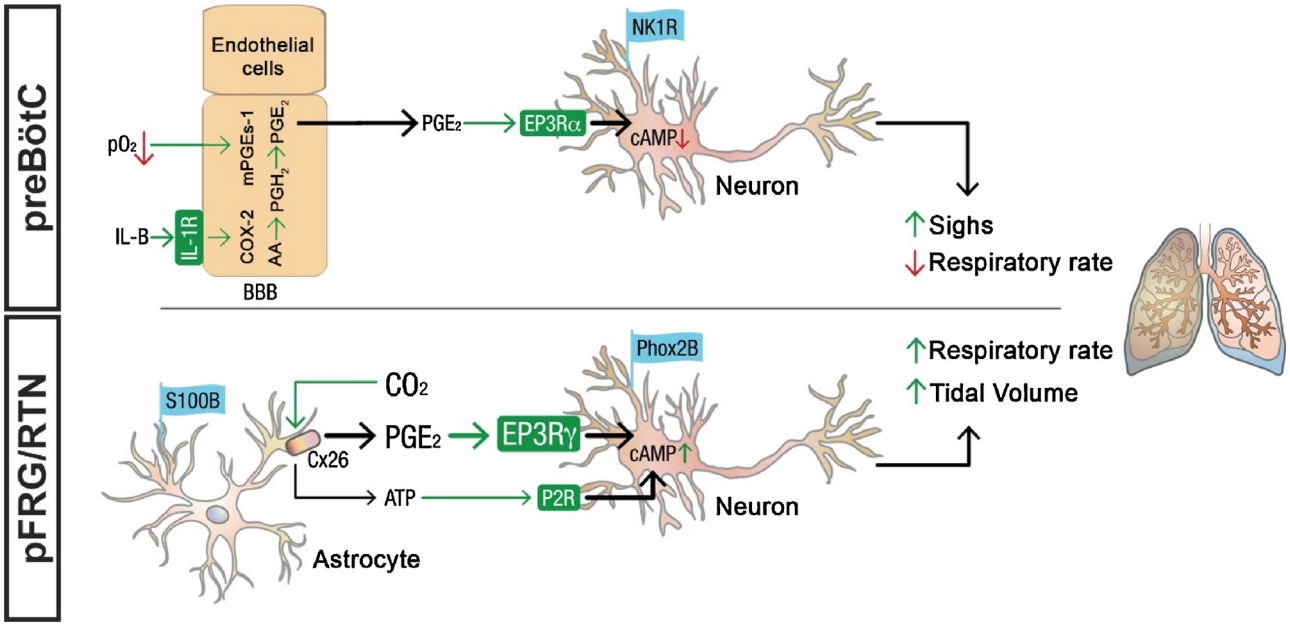

Fig. 1. Model of how PGE_2 modulates respiration through actions on the brainstem central pattern generators. Systemic inflammation, through IL-1β, and hypoxia, induces the production of PGE_2 in blood-brain barrier (BBB) endothelial cells (Hofstetter et al., 2007). PGE_2 subsequently induces respiratory depression and increases sigh activity via the inhibitory G-protein coupled receptor EP3Rα in the preBötC. In the pFRG/RTN, PGE_2 plays a role in the response to elevated pCO_2. CO_2 directly modulates Cx26 hemichannels, leading to ATP release. Cx26 also releases PGE_2 from astrocytes. PGE_2 then increases respiratory activity via the stimulatory G-protein coupled receptor EP3Rγ on pFRG/RTN neurons. Thus, inflammation, hypoxia, and hypercapnia alter respiratory neural network and motor output and breathing activity through distinct effects of PGE_2 in the pFRG/RTN and the preBötC, respectively. Elevated PGE_2 levels, as observed during ongoing inflammation and at birth, may decrease the central pattern generators' ability to respond to hypoxic and hypercapnic events. Therefore, an infant's ability to adjust its breathing and arouse when pCO_2 levels increase is limited. This may have fatal consequences.
Adapted from (Forsberg et al., 2016; Forsberg, 2017).

rhythmically active astrocytes in the preBötC (Okada et al., 2012). These astrocytes retained rhythmic Ca^{2+} rises even after blockade of neuronal activity using tetrodotoxin (Okada et al., 2012). Together these data indicate that astrocytes have a role in the central pattern generators of the brainstem (Fig. 1).

Three subpopulations of astrocytes can be identified based on potassium currents (Grass et al., 2004). The first group consists of passive astrocytes. The second group is defined by astrocytes with an initial transient outward current during voltage steps. The third group consists of astrocytes with an outwardly rectifying current, a lack of inward currents, and absence of neurotransmitter reuptake or K^+ buffering (Grass et al., 2004). Passive astrocytes express different glutamate transporters at high levels (Grass et al., 2004). Blockage of these transporters decreases the signal amplitude of active astrocytes. Furthermore, glutamate can induce Ca^{2+} signals in preBötC astrocytes (Hartel et al., 2009, 2007), likely through activation of metabotropic receptors (Schnell et al., 2011).

Further investigation of the active astrocytes by Schnell et al. could not find any synchronization between astrocytic Ca^{2+} signals and overall preBötC rhythmicity. Neither did they find a correlation with single neuronal discharges. Thus, neuronal activity could not trigger Ca^{2+} signals in preBötC astrocytes in their study (Schnell et al., 2011). In contrast, Okada et al. did report a correlation between rhythmic astrocytes and neuronal respiratory-related signals (Okada et al., 2012). They found that 10% of the astrocytes displayed Ca^{2+} rises approximately 2 s before neuronal inspiratory signals. Thus, a subpopulation of the astrocytes displayed a synchronized pre-inspiratory activity. As Schnell et al. (2011) and Okada et al. (2012) presented opposing results, the data set from Schnell et al. (2011) was reanalyzed in a third study (Oku et al., 2016). This identified rhythmically active astrocytes in the preBötC with a low oscillating frequency (Oku et al., 2016), similar to what we and others have described (Forsberg et al., 2017b; Grass et al., 2004; Okada et al., 2012; Hartel et al., 2009; Schnell et al., 2011). Interestingly, the reanalysis of the data displayed a correlation between astrocyte oscillation and the preBötC respiratory-related rhythm, when phase-shifts between -4 and +4 s were allowed. About 10% of the astrocytes showed correlated, although irregularly occurring, pre-inspiratory signals (Oku et al., 2016), as initially suggested (Okada et al., 2012). We recently found that a subset of the rhythmically active astrocytes, showed correlated Ca^{2+} signaling peaks where every second astrocytic peak were timed with a neuronal peak (see Fig. 2a in (25)). Thus, there is evidence for a synchronization between astrocytic and neuronal activity in the preBötC (Hulsmann et al., 2000). However, at present, no studies have investigated whether phase-locking between astrocyte signaling and respiratory output occurs. Future studies that monitor astrocytic rhythmicity in parallel with recordings of respiratory motor output both *in vivo* and *in vitro* are needed.

3. The astrocytic network

We recently investigated the astrocytic component in the respiratory regions on a multi-cellular network level (Forsberg et al., 2017b), utilizing our newly developed organotypic slice cultures (Forsberg et al., 2016, 2017a). In organotypic cultures, the neural tissue can be maintained for several weeks, allowing functional circuits to be studied and manipulated over time (Gogolla et al., 2006; Gahwiler et al., 1997; Yamada and Cukierman, 2007; Preynat-Seauve et al., 2009; Phillips et al., 2018). The technique is well established and has mainly been used for hippocampal slices (Gahwiler et al., 1997), which compared to the brainstem slice, contains a far less endogenously active network.

As mentioned, we show a correlation between astrocytic and neuronal activity (Forsberg et al., 2017b), similar to that observed previously (Okada et al., 2012; Oku et al., 2016). Furthermore, we identified the spatial distribution and organization of the preBötC network

in the organotypic slice cultures (Forsberg et al., 2016, 2017b). This identified three separate, but interconnected, subnetworks. One glial and one neuronal network, as well as a glia-neuron network joining the two cell populations. These networks were all organized in a small-world network structure (Watts and Strogatz, 1998). Interestingly, both astrocytes and neurons displayed a similar amount of connections to their corresponding cell-types. Clustering was also similar when comparing neurons and astrocytes. Thus, our novel data shows that the glial network shares many structural features with its neuronal counterpart, investigated more extensively previously (Forsberg et al., 2016; Hartelt et al., 2008).

4. The role of the rhythmic astrocytes

To decipher the exact role of rhythmic astrocytes in respiratory control further investigation is required. Inhibition of astrocytes with methionine sulfoximine depresses breathing *in vivo* (Young et al., 2005), and glial inhibitors (e.g. fluorocitrate, fluoroacetate, and methionine sulfoximine) decrease preBötC activity *in vitro* (Erlichman et al., 1985; Huxtable et al., 2010; Hulsmann et al., 2000). *In silico*, an astrocytic network with a low oscillation frequency may modulate inspiratory pacemaker neuron activity by neurotransmitter release (Oku et al., 2016). The model also indicates the presence of suppressive feedback from the neurons (Oku et al., 2016). *In vitro*, optogenetic stimulation of astrocytes in the preBötC elicited single or burst firing of neurons in close proximity of the astrocytes (Okada et al., 2012). We utilized another approach, where transgenic mice ectopically expressed the G-protein coupled MrgA1 receptor in astrocytes of the brain. Application of the receptor ligand *in vitro*, induced an increase in Ca^{2+} oscillation frequency of active astrocytes, which was sustained for more than 1 h. Notably, preBötC neuron frequency was not increased by astrocytic stimulation (Forsberg et al., 2017b). In contrast, activation of a G_q-coupled Designer Receptor Exclusively Activated by Designer Drug ($DREADD_{Gq}$) *in vivo* resulted in phospholipase C-mediated vesicular release of ATP and potentially other gliotransmitters from astrocytes, which increased respiratory-related activity in the preBötC (Sheikhbahaei et al., 2018a). Furthermore, blockage of this vesicular-dependent release in preBötC astrocytes reduced breathing frequency *in vivo* and sigh behavior (Sheikhbahaei et al., 2018a). Similarly, astrocyte stimulation in our mouse model *in vivo* promotes sigh behavior (unpublished data). Additionally, blockage of astrocytic vesicular release also reduced the exercise capacity of rats (Sheikhbahaei et al., 2018a). Thus, there is emerging evidence that astrocytes are involved in the generation and essential for the modulation of breathing activity. However, whether astrocytes are a fundamental component in the respiratory rhythm-generating machinery remains to be determined.

5. Astrocytes in the pFRG/RTN

Several studies have investigated chemosensitivity in astrocytes of the parafacial respiratory group/retrotrapezoid nucleus (pFRG/RTN) (Turovsky et al., 2016, 2015; Erlichman et al., 2010; Gourine et al., 2010; Huckstepp et al., 2010). However, to the best of our knowledge, the role of astrocytes in the network structure of the pFRG/RTN have only been examined by us so far (Forsberg et al., 2016, 2017b). Our novel data depicts that, similar to the preBötC, the cells of the pFRG/RTN are organized in a small-world network structure (Forsberg et al., 2016, 2017b). As in the preBötC, we found three individual subnetworks; one astrocytic and one neuronal as well as connections between the two. Interestingly, the astrocytic network constituted a larger proportion of the total network in the pFRG/RTN compared to the astrocytic proportion of the preBötC network. The pFRG/RTN network also contained more astrocyte-neuron connections than the preBötC (Forsberg et al., 2017b). The measured correlation does not imply causality and does not provide any detailed information on the signaling between the cells. The different proportions may reflect a different role of astrocytes in the two regions. This could be related to the role of astrocytes in chemosensitivity within the pFRG/RTN, which would require more substantial communication between the cell types (Ben Haim and Rowitch, 2017). However, astrocytic vesicular release plays a role in chemosensitivity within the preBötC as well (Sheikhbahaei et al., 2018a). Activation of astrocytes through the transgenically expressed MrgA1 receptor increased not only astrocytic oscillation frequency, but also the neuronal frequency. Thus, astrocytes can modulate respiratory network activity in the pFRG/RTN, which corroborates the involvement of astrocytes in chemosensitivity through purinergic and prostaglandin signaling (Forsberg et al., 2017b; Erlichman et al., 2010, 2010; Gourine et al., 2010; Huckstepp et al., 2010; Angelova et al., 2015; Gourine and Kasparov, 2011).

6. Astrocytes and chemosensitivity

We do not yet to fully understand the mechanisms behind the central hypercapnic response (Funk et al., 2015; Guyenet et al., 2013). Several brainstem regions are involved, including the pFRG/RTN (Onimaru and Dutschmann, 2012; Mellen and Thoby-Brisson, 2012), the raphe nucleus (Mulkey et al., 2007), NTS (Dean and Putnam, 2010) and the preBötC (Sheikhbahaei et al., 2018a). The pFRG/RTN is the main central chemosensitive region, but the importance of serotonergic input from the raphe nucleus is debated (Teran et al., 2014).

The involvement, and potentially crucial role, of astrocytes in the hypercapnic response have become more evident throughout the last decade (Erlichman et al., 2010; Gourine et al., 2010; Beltran-Castillo et al., 2017). Respiratory activity is increased (similar to the hypercapnic response) after stimulation of pFRG/RTN astrocytes (Erlichman et al., 1985; Gourine et al., 2010). Furthermore, patients with Rett syndrome exhibit a reduced response to increased pCO_2 levels, likely due to a dysfunction in astrocytic methyl-CpG binding protein 2 (MeCP2). Mice lacking MeCP2 are used as a model for Rett syndrome and have reduced CO_2 sensitivity. Notably, re-introduction of the MeCP2 gene selectively in astrocytes rescues the normal respiratory phenotype. Thus, a major part of the blunted hypercapnic response is due to the inability of astrocytes to react to changes in pCO_2 and pH (Turovsky et al., 2015).

Even slight acidification is followed by an increase of Ca^{2+} concentration in astrocytes. This leads to vesicle-dependent ATP release (Gourine et al., 2010; Onimaru and Dutschmann, 2012; Wenker et al., 2010), and subsequent activation of purinergic receptors on pre-inspiratory neurons within the preBötC (Gourine et al., 2010; Huckstepp et al., 2010). Moreover, ATP may also be released through the opening of Cx hemichannels (Huckstepp and Dale, 2011). Some Cxs (Cx26, Cx30 and Cx32) are directly modified by CO_2 (Reyes et al., 2014; Meigh et al., 2013): CO_2 induces the formation of carbamate bridges in the Cx26 protein structure, inducing an open state (Meigh et al., 2013). Brainstem astrocytes may also release D-serine and glutamate simultaneously in response to high CO_2 levels (Beltran-Castillo et al., 2017). These transmitters then act on NMDA receptors to drive the respiratory rhythm. In contrast to the ATP release in the pFRG/RTN, D-serine was found to be released from astrocytes within the raphe nucleus and the ventral respiratory column (Beltran-Castillo et al., 2017). In addition to ATP and potentially D-serine, we have demonstrated that the inflammatory molecule prostaglandin E_2 (PGE_2) is released by both hypercapnic challenge and stimulation of astrocytes (Forsberg et al., 2016, 2017b). PGE_2 release was not seen during inhibition of gap junctions (Forsberg et al., 2016). Notably, the hypercapnic response was reduced after activation of pFRG/RTN astrocytes. This suggests that PGE_2 and probably other gliotransmitter stores were depleted, and may explain the effect of inflammation on the hypercapnic response (Siljehav et al., 2015, 1985).

Astrocytes are also involved in the responsiveness to low oxygen levels–hypoxia. The first phase of the hypoxic response involves increased breathing and is mainly due to activation of the carotid bodes.

The second depressive phase, however, is of central origin (Rajani et al., 2017) and involves signaling with adenosine (Runold et al., 1989; Herlenius et al., 2002; Johansson et al., 2001) and PGE2 (Hofstetter et al., 2007; Siljehav et al., 2012). During development, the extent of depression during the second phase declines (Moss, 2000).

Although the importance of a central oxygen sensor is debated (Angelova et al., 2015; Gourine and Funk, 1985), the respiratory networks of the brainstem are sensitive to hypoxia (Thoby-Brisson and Ramirez, 2000; Ramirez et al., 1998; Pena et al., 2004; Marina et al., 2015). Furthermore, astrocytes seem to play an important role (Angelova et al., 2015; Gourine and Kasparov, 2011; Gourine and Funk, 1985; Marina et al., 2015; Gourine, 2005; Gourine et al., 2005). ATP signaling is critical. The hypoxic response is abolished after denervation of peripheral oxygen sensors and blocking of purinergic signaling (Angelova et al., 2015; Rajani et al., 2017). preBötC astrocytes release ATP, which increases respiratory activity during hypoxia (Rajani et al., 2017), putatively counteracting the depressive effects of hypoxia (Rajani et al., 2017; Marina et al., 2016). Hypoxic conditions trigger an increase in intracellular Ca^{2+} levels due to decreased mitochondrial respiration, mitochondrial depolarization and release of inositol-triphosphate (IP_3) (Angelova et al., 2015). The rise in Ca^{2+} levels induce a vesicular ATP-release (Angelova et al., 2015), similar to what occurs during pH-dependent hypercapnia (Gourine et al., 2010). However, ATP rapidly degrades into adenosine, facilitating the depressive phase in the neonate (Rajani et al., 2017; Herlenius et al., 2002; Johansson et al., 2001; Herlenius and Lagercrantz, 1999; Herlenius et al., 1997). In addition, hypoxia induces the production of and release of PGE_2 (Hofstetter et al., 2007) that also depresses breathing in both rodent and human neonates.

7. Chemosensitivity and inflammation

PGE_2 and its signaling pathway via the EP3 receptor is an important link between respiration, chemosensitivity, and inflammation (Hofstetter et al., 2007; Siljehav et al., 2012, 2015). Since EP3Rs are found on astrocytes (Forsberg et al., 2016), PGE_2 could exert an autocrine effect during the hypercapnic response to emphasize it. Exogenous PGE_2 released from endothelial cells during systemic inflammation (Hofstetter et al., 2007) could therefore activate pFRG/RTN astrocytes. This would potentially deplete the gliotransmitter stores resulting in the blunted hypercapnic response observed *in vitro* (Forsberg et al., 2017b).

Furthermore, astrocytic activation by PGE_2 might further explain the stimulatory effect of PGE_2 in the pFRG/RTN (Forsberg et al., 2016). The PGE_2 pathway could have important clinical implications. An inappropriate central control of respiration is an important mechanism in the pathogenesis of apnea of infancy (Katz-Salamon, 2004). Infection, with associated release of PGE_2 in the vicinity of brainstem respiration related regions, increases the incidence of apnea and hypopneic events in preterm infants (Hofstetter et al., 2007; Herlenius, 2011). Thus, infection and inflammation is associated with altered cardiorespiratory control in infants and the pathogenesis of apnea includes inflammatory pathways (Siljehav et al., 2015).

The mechanism underlying PGE_2 release from astrocytes remains to be investigated. CO_2 affects intracellular pH and drives bicarbonate and sodium ions into the cell. This triggers a Ca^{2+} influx (Turovsky et al., 2016) and subsequently stimulates cell activity. In parallel, CO_2 can directly modify Cx26 (Huckstepp et al., 2010; Meigh et al., 2013). Moreover, gap junction inhibition both inhibits the PGE_2 release during hypercapnia and reduces the hypercapnic response.

Inflammation is connected to respiration-related diseases such as apnea and sudden infant death syndrome (Siljehav et al., 2015, 2012; Herlenius, 2011; Idborg et al., 2014; Huxtable et al., 2011; Koch et al., 2015). There is thus increasing interest in the potential role(s) of microglia in modulation of breathing. It was recently shown that these immune cells can modulate respiration and affect the ability to autoresuscitate. However, similar effects were seen both after inhibition and activation of microglia, raising some concern on their specific role (Lorea-Hernandez et al., 2016). Microglia also seem to be involved in the response to hypoxia. Sustained hypoxia increases microglial activity in the brainstem while the hypoxic response is attenuated. Further, inhibition of microglia prevented the attenuation of the hypoxic response during sustained hypoxia (MacFarlane et al., 2016).

8. Conclusions

The evidence for astrocytes being involved in the modulation of breathing activity and rhythm, especially during hypoxic and hypercapnic challenges, is strong. However, how, and to what extent requires further investigation. There is several studies pointing towards signaling pathways via Ca^{2+} and gliotransmitter release through vesicles (Sheikhbahaei et al., 2018a; Kettenmann and Verkhratsky, 2016; Gourine et al., 2010; Beltran-Castillo et al., 2017). Furthermore, opening of gap junction hemichannels such as connexins and pannexins are also likely pathways for gliotransmitter release (Forsberg et al., 2017b; Huckstepp et al., 2010; Meigh et al., 2013). Both these signaling systems interconnects astrocytes and neurons to form functional networks in the brainstem (Forsberg et al., 2017b).

Furthermore, astrocytes link the inflammatory system and respiratory control. Inflammation is well-known to affect breathing in newborn babies (Siljehav et al., 2015) and affect the respiratory adjustments during both hypoxia and hypercapnia *in vivo* (Siljehav et al., 1985; Hofstetter et al., 2007; Siljehav et al., 2012). The recent discovery that PGE_2 is released from astrocytes during hypercapnia (Forsberg et al., 2016, 2017b), suggest a mechanism by which inflammation may disturb the modulation of breathing.

It is important to remember that a number of studies have been performed on neonatal animals. Thus, how the astrocytic part change during development is yet unknown. Astrocytes may play a greater role to early in development, increasing plasticity (Grass et al., 2004). Later in development, a more robust neuron-dominated network may emerge to provide stability.

Astrocytes can modulate respiratory network activity (Sheikhbahaei et al., 2018a; Erlichman et al., 1985; Young et al., 2005; Huxtable et al., 2010; Forsberg et al., 2017b; Erlichman et al., 2010). However, whether they are necessary for respiratory rhythm generation remains to be elucidated. Future experiments where astrocyte stimulation and inhibition is performed during recording of respiratory-related motor output *in vitro* and breathing *in vivo* is needed. This would determine if phase-locking and entrainment by astrocyte signals exists and if this is important for the respiratory drive.

Acknowledgments

We thank Torkel Mattesson and Evangelia Tserga for technical assistance. This study was supported by the Swedish Research Council, the Stockholm County Council, the Karolinska Institutet, and grants from the Swedish Brain Foundation, M & M Wallenberg, Fraenkel, Axel Tielman, Freemasons Children's House and Swedish National Heart and Lung Foundations.

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