# Role of Astrocytes in Central Respiratory Chemoreception

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**Abstract** Astrocytes perform various homeostatic functions in the nervous system beyond that of a supportive or metabolic role for neurons. A growing body of evidence indicates that astrocytes are crucial for central respiratory chemoreception. This review presents a classical overview of respiratory central chemoreception and the new evidence for astrocytes as brainstem sensors in the respiratory response to hypercapnia. We review properties of astrocytes for chemosensory function and for modulation of the respiratory network. We propose that astrocytes not only mediate between $CO_2/H^+$ levels and motor responses, but they also allow for two emergent functions: (1) Amplifying the responses of intrinsic chemosensitive neurons through feedforward signaling via gliotransmitters and; (2) Recruiting non-intrinsically chemosensitive cells thanks to volume spreading of signals (calcium waves and gliotransmitters) to regions distant from the $CO_2/H^+$ sensitive domains. Thus, astrocytes may both increase the intensity of the neuron responses at the chemosensitive sites and recruit of a greater number of respiratory neurons to participate in the response to hypercapnia.

**Keywords** Respiratory rhythm · Central chemoreception · Raphe nuclei · Locus coeruleus nuclei · Retrotrapezoid nuclei · Brainstem · Glia · Gliotransmitters · Astrocytes

## Abbreviations

| | |
|---|---|
| 5-HT | 5-hydroxytryptamine (Serotonin) |
| ACh | Acetylcholine |
| aCSF | Artificial cerebrospinal fluid |
| AMPAR | $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor |
| ANP | Atrial natriuretic peptide |

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| | |
|---|---|
| ATP | Adenosine triphosphate |
| CA | Carbonic anhydrase enzyme |
| CCHS | Central congenital hypoventilation syndrome |
| CNS | Central nervous system |
| CNO | Clozapine-N-oxide |
| $CO_2$ | Carbon dioxide |
| CNQX | 6-cyano-7-nitroquinoxaline-2,3-dione—competitive AMPA/kainate receptor antagonist |
| cNTS | Caudal nucleus tractus solitarius |
| CSF | Cerebrospinal fluid |
| cVLM | Caudal ventrolateral medulla |
| cVRG | Caudal ventral respiratory group |
| Cx | Connexins |
| DRC | Dorsal respiratory columns |
| EPSP | Excitatory postsynaptic potentials |
| GABA | $\gamma$-aminobutyric acid |
| GFAP | Glial fibrillary acidic protein |
| KF | Pontine Kölliker-Fuse nucleus |
| KO | Knock out |
| LC | Locus coeruleus |
| LDT | Laterodorsal tegmental nucleus |
| LPBR | Lateral parabrachial nucleus |
| LTP | Long-term potentiation |
| mRVLM | Medial portion of the rostral ventrolateral medulla |
| MS | Methionine sulfoximine |
| NK1R | Neurokinin 1 receptor |
| NMDA | N-methyl-D-aspartate |
| NMDAR | N-methyl-D-aspartate receptor |
| NO | Nitric oxide |
| NTS | Nucleus tractus solitarius |
| $PaCO_2$ | Partial arterial pressure of carbon dioxide |
| $PCO_2$ | Partial pressure of carbon dioxide |
| $PaO_2$ | Partial arterial pressure of oxygen |
| PF-LHA | Perifornical-lateral hypothalamic area |
| PNS | Peripheral nervous system |
| PPADS | Pyridoxal-phosphate-6-azophenyl-2=,4=-disulfonate |
| PPT | Pedunculopontine tegmental nucleus |
| preBötC | PreBötzinger Complex |
| ORX | Orexin |
| ORX-KO | Prepro-orexin knockout mice |
| RN | Medullary raphe nucleus |
| RPG | Respiratory pattern generator |
| RTN/pFRG | Retrotrapezoid/parafacial respiratory group |
| RVL | Nucleus reticularis rostroventrolateralis |

| | |
|---|---|
| RVLM | Rostral ventrolateral medulla |
| rVRG | Rostral ventral respiratory group |
| SERT | Serotonin transporter |
| SIDS | Sudden infant death syndrome |
| SP | Substance P |
| SSP-SAP | Saporin–substance P conjugate |
| TH | Tyrosine hydroxylase |
| TIRF | Total internal reflection fluorescence |
| TRH | Thyrotropin releasing hormone |
| TRP | Channels Transient receptor potential channels |
| TS-eEPSCs | Tractus solitaries-evoked excitatory postsynaptic currents |
| VLM | Ventrolateral medullary surface |
| VMS | Ventral medullary surface |
| VRC | Ventral respiratory columns |
| VRG | Ventral respiratory group |

## The Respiratory Network

The neural network responsible for generating the respiratory rhythm, the respiratory pattern generator (RPG), is composed of neurons preferentially discharging during inspiration or expiration and distributed along the ventral (VRC) and the dorsal (DRC) respiratory columns (Fig. 1) (Feldman et al. 2003; von Euler 1986). The RPG projects into respiratory motoneurons located at different cranial nerve nuclei (V, VII, IX, X, XII), which innervate muscles controlling airway flow and resistance. In addition, the RPG sends projections and synapses on various spinal cord motoneurons, particularly the phrenic motoneurons (C3–C5), which innervate the diaphragm muscle, and intercostal motoneurons (T1–T10), which innervate intercostal muscles. The RPG imposes on these motoneurons a synchronic and rhythmic activity responsible for generating a sequence of inspiratory, post-inspiratory, and expiratory phases observable in recordings from phrenic, abductor laryngeal, and internal intercostal nerves, respectively (Richter and Spyer 2001). The coordinated activation of these motoneurons results in a sequence of air pressure gradients commanding the inspiratory and expiratory phases of ventilation.

At the RPG, within the VRC, at least two oscillators can be recognized: at the rostral area of the VRC, the pre-inspiratory retrotrapezoid/parafacial respiratory group (RTN/pFRG), arising from *Phox2b* expressing progenitors (Guyenet and Mulkey 2010; Onimaru and Homma 2006; Onimaru et al. 2006, 2009; Stornetta et al. 2006; Wang et al. 2013; Takakura et al. 2014; Dubreuil et al. 2009b; Abbott et al. 2011), and at the caudal portions of the VRC, the inspiratory preBötzinger Complex (preBötC), which is derived from *Dbx1* progenitors and considered

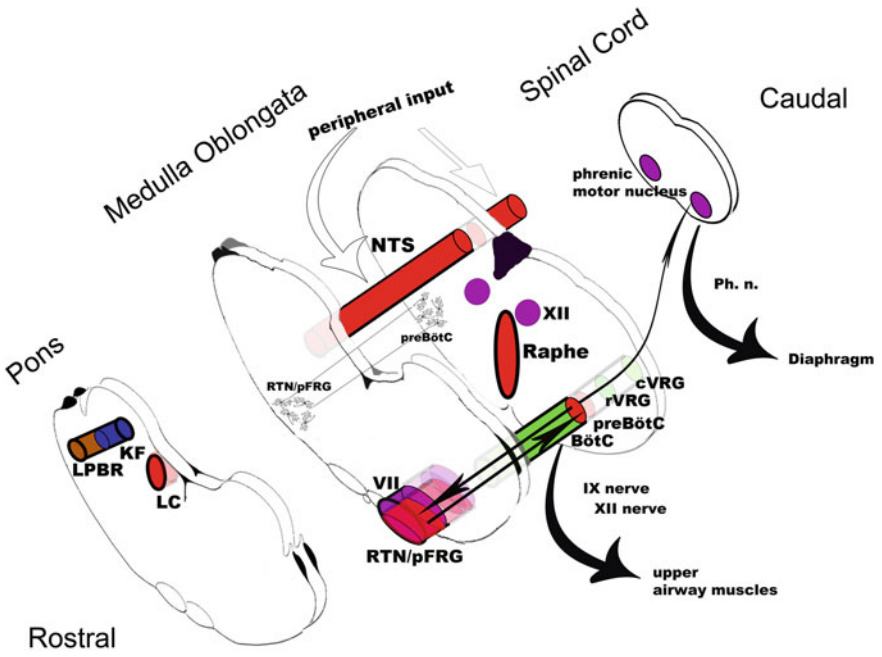


**Fig. 1** Schema of the respiratory neural network and central respiratory chemoreceptors. The dorsal respiratory column (DRC) is represented by the nucleus tractus solitarius (NTS), while the ventral respiratory column (VRC), by the retrotrapezoid/parafacial respiratory group (pFRG), the Bötzinger nucleus, the pre-Bötzinger complex (preBötC), and the rostral and caudal ventral respiratory group (rVRG and cVRG). Input and output to the central respiratory network are indicated with *white* and *black arrows*, respectively; note that the output was represented by respiratory motoneurons localized in cranial and phrenic nuclei. The phrenic nerve (Ph n.) controls the diaphragm muscle, main responsible for generating air pressure gradients during breathing. The main peripheral input is provided by vagal mechanoafferents and peripheral arterial chemoreceptors. Central chemosensitive sites (NTS, preBötc, LC, RTN, raphe) containing cells that sense changes in pH or $PCO_2$ in the interstitial or the cerebrospinal fluid of the brainstem are indicated in *red*. Note that the raphe and LC provide inputs into the respiratory network, while NTS, preBötc, and RTN belong to the respiratory network. KF, pontine Kölliker-Fuse nucleus; LC, locus coeruleus; LPBR, lateral parabrachial nucleus; VII, facial nucleus; XII, hypoglossal nucleus

essential for generating the inspiratory activity (Fig. 1) (Smith et al. 1991; Feldman et al. 2003; Gray et al. 2010).

The RPG receives input from several central nervous system (CNS) structures, including cortex, cerebellum, hypothalamus, and brainstem nuclei, and from the peripheral nervous system (PNS), including vagal mechanosensory afferents and peripheral arterial chemoreceptors (Feldman 1986; von Euler 1986). Peripheral arterial chemoreceptors (carotid and aortic bodies) sense changes in $PaO_2$, $PaCO_2$, pH, osmolarity, temperature, and flow of blood circulating through great arteries (Eyzaguirre et al. 1983). In contrast, central chemoreceptors (Fig. 1 indicated in

red) are activated by changes in pH or $PCO_2$ in the interstitial or the cerebrospinal fluid of the brainstem (Nattie 1999). In fact, $CO_2$- and $H^+$-sensitive neurons are a major source of the tonic input that drives the mammalian respiratory pattern generator (Nattie 1999).

## Central Chemoreception

Central chemoreception can be defined as: "*the detection of* $CO_2$/pH *at sites within the central nervous system and the resultant effects on ventilation*" (Nattie and Li 2010), or in more systemic terms as "*the feedback process whereby changes in the brain* $CO_2$ (*or pH*) *bring about adaptive* (*homeostatic*) *changes in breathing to maintain arterial* $CO_2$ (*or pH*) *near steady-state levels*" (Funk 2010). Central chemoreception is crucial for matching breathing to physiological demands relative to $H^+$ or $CO_2$ elimination. In addition, it appears essential for generating and maintaining the respiratory rhythm (Eugenin 1995), allowing brainstem respiratory neurons to be coordinated and excitable in an optimal manner (Nattie and Li 2012). In fact, $CO_2$- and $H^+$-sensitivities are a major source of the tonic drive that sustains the activity of the RPG (Nattie 1999; Nattie and Li 2012). For example, in *en bloc* preparations from newborn opossum and mice, alkaline superfusion of the brainstem arrests the respiratory rhythm (Eugenin and Nicholls 1997; Infante et al. 2003; Eugenin et al. 2006).

## Localization of Central Chemoreceptors

Various strategies have been used to localize chemosensitive sites in the brain. Detection of c-Fos protein as a marker of neuronal activity revealed that those nuclei in which the number of c-Fos positive neurons increase after exposure to hypercapnia also contain neurons with electrophysiological responses to hypercapnic acidosis (Belegu et al. 1999; Mulkey et al. 2004; Ritucci et al. 2005; Wang and Richerson 1999; Wickstrom et al. 2002; Teppema et al. 1997). In these nuclei we can find neurons that fire in association with or in correlation with the respiratory response to hypercapnia. On the other hand, destruction (Akilesh et al. 1997; Biancardi et al. 2008; da Silva et al. 2011; Dias et al. 2007), genetic ablation (Hodges et al. 2011; Dubreuil et al. 2009b; Ramanantsoa et al. 2011), inactivation, or synaptic inhibition (Nattie and Li 2000; Curran et al. 2001) of specific nuclei reduces the ventilatory response to hypercapnia. More direct evidence of the existence of chemosensitive sites was obtained by focal acidification. Either local application of acetazolamide (Coates et al. 1993), an inhibitor of the enzyme carbonic anhydrase, or reverse microdialysis of artificial cerebrospinal fluid equilibrated with high $CO_2$ within specific CNS areas, increased ventilation (Li et al. 1999; Nattie and Li 2001, 2002a; Li and Nattie 2002; Dias et al. 2008; da Silva et al. 2010; Krause et al. 2009;

Kuwaki et al. 2010; Coates et al. 1993). Notably, as illustrated in Fig. 1 (nuclei in red), central respiratory chemosensitivity was localized to multiple sites, including such nuclei belonging to the RPG as the pFRG/RTN, preBötC, and nucleus tractus solitarius (NTS), and such nuclei or identified regions outside the RPG but projecting into as the medullary raphe (RN), locus coeruleus (LC, A6), ventrolateral medullary surface, hypothalamus, and fastigial nucleus (Ballantyne and Scheid 2001; Coates et al. 1993; Mitchell et al. 1963; Nattie 2001; Oyamada et al. 1998; Wang and Richerson 1999; Li et al. 2006, 2013; Guyenet et al. 2005; Nattie and Li 2006, 2009, 2010; Xu et al. 2001; Xu and Frazier 1995; Martino et al. 2007; Krause et al. 2009). The contribution of specific groups of cells within chemosensitive nuclei were evident from the effects of lesions of neurokinin-1 receptor expressing cells in the RTN, or serotonergic cells in the RN, or catecholaminergic cells in the LC. In all these specific lesions, the $CO_2$ response decreased by 15–30 % during both sleep and wakefulness (Nattie and Li 2008).

## Roles of the RTN and Raphe RN Neurons in Central Chemoreception

### *RTN Neurons*

RTN neurons are glutamatergic, chemosensitive, express the transcription factor Phox2b, provide excitatory projections to other sites in the central respiratory network, and when stimulated activate breathing (Mulkey et al. 2004; Onimaru et al. 2008; Stornetta et al. 2006; Wang et al. 2013; Guyenet and Mulkey 2010; Goridis et al. 2010; Dubreuil et al. 2009b).

Inhibition of RTN neurons by muscimol dialysis or their chemical (kainic acid injection) or electrical destruction reduces basal ventilation and the ventilatory responses to hypercapnia in anesthetized rats (Nattie and Li 1994). More selective lesions restricted to RTN neurons expressing the neurokinin 1 receptor (NK1R), obtained with a saporin–substance P conjugate (SSP-SAP), impairs ventilatory response to hypercapnia in rats (Nattie and Li 2002b). In anesthetized rats, elimination of at least 70 % of $Phox2b^+$ tyrosine hydroxylase negative ($TH^-$) RTN neurons is required for a significant increase of the apnea threshold, but does not affect the sensitivity of the subsequent responses to hypercapnia (Takakura et al. 2008). Allatostatin inhibition of RTN Phox2b-expressing neurons transformed with a lentiviral construct to express the G-protein-coupled Drosophila allatostatin receptor did not affect the basal respiratory activity in unanesthetized, conscious rats (Marina et al. 2010). Nevertheless, allatostatin reduced the amplitude of the phrenic nerve discharge and the $CO_2$-evoked ventilatory responses in anesthetized rats, in in situ preparations, and in conscious rats with denervated or intact peripheral chemoreceptors (by 28 and 60 %, respectively) (Marina et al. 2010; Ramanantsoa et al. 2011).

In contrast, photostimulation of RTN neurons expressing channel rhodopsin-2 under the control of the Phox2-responsive promoter PRSx8, increases ventilation in both anesthetized and conscious animals (Abbott et al. 2009, 2011; Kanbar et al. 2010; Burke et al. 2015).

The human disease called central congenital hypoventilation syndrome (CCHS) shows a spectrum of defects comparable with the ontogenic defects of the autonomic nervous system in Phox2b mutant mice (Brunet and Pattyn 2002; Pattyn et al. 1999). CCHS is a life threatening human disease characterized by hypoventilation periods or apnea during sleep and a variable reduction of ventilatory response to hypercapnia, from moderate to severe. CCHS was attributable to a mutation consisting of a polyalanine expansion in the Phox2b transcription factor (Amiel et al. 2003, 2009). Moreover, genetic generation of a knock-in mouse having the most frequent of the CCHS-mutations, the *Phox2b27Ala* allele, resulted in the selective ablation of glutamatergic neurons in the RTN and a CCHS-like phenotype. These mice showed gasping behavior, cyanosis, disruption of the respiratory chemo reflex at birth and, in contrast to human CCHS patients, they died during the first hours of postnatal life from respiratory failure (Dubreuil et al. 2008, 2009a, b; Goridis et al. 2010; Ramanantsoa et al. 2011).

## *Raphe Nucleus Neurons*

In brainstem slices, $CO_2/H^+$ responsive neurons can be found in the midline Raphe nucleus (RN) (Richerson 1995; Wang et al. 1998). As mentioned above, ventilation increases with focal acidification of the midline RN by microinjection of acetazolamide in anesthetized rats or by reverse microdialysis of acidified cerebrospinal fluid (CSF) in conscious rats or goats (Nattie and Li 2001; Hodges et al. 2004a, b). Inhibition of RN neurons by microdialysis of muscimol (Taylor et al. 2006), by administration of 5-hydroxytryptamine $(5\text{-HT})_{1A}$ autoreceptor agonist (8-OH-DPAT), which inhibits serotonergic neurons, or by microinjections of lidocaine or ibotenic acid significantly decreased the response to hypercapnia in piglets (Messier et al. 2002, 2004; Dreshaj et al. 1998). In the unanesthetized juvenile rat brainstem preparation perfused in situ, $5\text{-HT}_2$ receptor antagonism with ketanserin or $5\text{-HT}_{1A}$ autoreceptor activation with 8-OH-DPAT blunted the respiratory response (Corcoran et al. 2013). In rats, injections of a monoclonal antibody against the serotonin transporter (SERT) conjugated to saporin into the RN specifically killed serotonergic neurons, and as result decreased the average $CO_2$ response (Nattie et al. 2004). In addition, hypercapnic ventilatory response decreased by 50 % in adult knock out (KO) mice (Lmx1bf/f/p and Pet-1 knockout mice) with near complete absence of central 5-HT neurons (Hodges et al. 2008, 2011) or with absence of the 5HT transporter (Li and Nattie 2008). Egr2-null mice have, among others defects, altered serotonergic progeny, low respiratory rate, and severe apneas, dying perinatally due to respiratory insufficiency.

Selective hyperpolarization of Egr2 expressing neurons or 5HT neurons was achieved by clozapine-N-oxide (CNO) activation of the synthetic Gi/o protein–coupled receptor Di expressed selectively on 5-HT neurons using conditional intersectional genetics. Hyperpolarization of Egr2 neurons reduced the ventilatory response by 63 % (Ray et al. 2013). Hyperpolarization restricted to serotonergic neurons reduced the ventilatory chemoreflex in vivo by almost 50 % and reduced the $CO_2$-induced firing rate increase of 5HT neurons in culture (Ray et al. 2011). When Di expression was targeted to a specific subtype of 5HT neuron, the Egr2-Pet1 serotonergic subgroup was found to contribute most to the ventilatory response to hypercapnia and acidosis. Egr2-Pet1 neurons project to other chemosensory areas and show intrinsic chemosensitivity firing in response to a hypercapnic stimulus (Brust et al. 2014).

## Relative Contribution of Chemosensitive Sites to the Overall Response

Determination of the relative contribution of each chemosensory site to the full expression of chemosensitivity has been elusive. Pronounced effects after unilateral chemical or electrolytic lesion of the RTN, NTS, or RN led to the notion that each nucleus provides an essential, indispensable, and singular contribution to the full expression of central chemosensitivity (Berger and Cooney 1982; Nattie and Li 1994). However, these deleterious effects caused by lesion of chemosensitive nucleus were strongly influenced by anesthesia (Nattie and Li 2012; Nattie 2011). In fact, lesion-related impairment of the responses to systemic hypercapnia largely disappeared with recovery of consciousness (Berger and Cooney 1982). Thus, under anesthesia, destruction of the rat RTN reduced the integrated baseline activity of the phrenic nerve and the respiratory response to hypercapnia (Nattie and Li 1994). In contrast, in conscious, unanesthetized rats, similar unilateral lesions of RTN produced minor effects on baseline ventilation and the respiratory response to hypercapnia (Akilesh et al. 1997). In agreement with these results, the magnitude of the ventilatory effects evoked by acidification of chemosensitive areas using reverse microdialysis in conscious, unanesthetized animals was lower than that observed in anesthetized animals (Nattie and Li 2012; Nattie 2011). The reduction in ventilatory effects observed in conscious animals may be explained in part by an enhanced clearance of focal stimulus as a result of an increased cerebral blood flow in unanesthetized mammals.

The relative contributions of chemosensory nuclei in the conscious animal has been studied using either focal inhibition of chemosensitive sites or focal acidification by reverse microdialysis of artificial cerebrospinal fluid (aCSF) equilibrated with high $CO_2$ (Nattie and Li 2009). Assuming that the contributions of chemosensitive sites are independent, the overall respiratory response does not appear to be the result of simple additive interactions of individual contributions

(Nattie and Li 2010). Clear synergisms could be inferred, as for example, observing the ventilatory depression when RTN and caudal RN were simultaneously inhibited (Li et al. 2006). More direct evidence of this synergism was obtained with simultaneous focal acidification of the RTN and caudal RN (Dias et al. 2008). However, unrealistically complex experiments with multiple probes stimulating each chemosensory area individually or several simultaneously during wakefulness and sleep seem to be necessary to fully address this question.

Interestingly, the full expression of central chemoreception also depends on the peripheral chemoreceptor input. In fact, in unanesthetized awake dogs the ventilatory responsiveness to four progressively increasing levels of central hypercapnia depended on the degree of carotid body inhibition or stimulation with respect to basal eupneic conditions (normoxic, normocapnic carotid body perfusion). The increase in carotid body activity via carotid body perfusion with a hypoxic, normocapnic perfusate increased the ventilatory response to hypercarbia by 223 % respect basal conditions. By contrast, silencing of carotid bodies activity with hyperoxic, hypocapnic perfusate reduced the ventilatory response to hypercarbia by 81 %. This interdependence between peripheral and central chemoreception suggests that the whole system of central and peripheral chemosensory structures are functionally interrelated and integrated.

## Central Chemoreception Dependency on Functional State of the Respiratory Network

Special attention should be focused to the fact that contribution of each chemosensitive site to the overall response to hypercapnia depends on the functional state of the respiratory network. Such dependency not only may give account of the differences between conscious and anesthetized animals that are mentioned above, but also of differences in ventilation and ventilatory responses between wakefulness and sleep (Newton et al. 2014). Studies in rats with focal acidosis by reverse microdialysis along the sleep–wake cycle have shown that acidification of the RTN or the perifornical-lateral hypothalamic area (PF-LHA), where orexin neurons are found, or the caudal ventrolateral medulla (cVLM) increased ventilation predominantly in wakefulness (Li and Nattie 2002; Li et al. 2013; da Silva et al. 2010). By contrast, acidification of rostral RN increased ventilation predominantly in sleep (Nattie and Li 2001) while focal acidification of the NTS increased ventilation in both wakefulness and sleep (Nattie and Li 2002a).

Orexin neurons are good candidates to be the link between arousal state and chemoreceptive properties at the brainstem (Nattie and Li 2010, 2012). Orexin neurons are critical for generating wakefulness (Ohno and Sakurai 2008; Sakurai 2014; Alexandre et al. 2013) and controlling breathing (Nakamura et al. 2007; Li et al. 2013; Li and Nattie 2010; Dias et al. 2010; Terada et al. 2008; Dutschmann et al. 2007; Deng et al. 2007; Young et al. 2005b; Toyama et al. 2009). They are

sensitive to $H^+/CO_2$ (Williams et al. 2007; Li et al. 2013; Sunanaga et al. 2009) and their firing rate is maximal during wakefulness (Lee et al. 2005) and minimal during sleep.

As mentioned above, focal acidification of the hypothalamic area containing orexin neurons increased ventilation up to 15 % only in wakefulness but not in sleep (Li et al. 2013). In prepro-orexin knockout mice (ORX-KO) basal ventilation is not affected along the sleep–wake cycle. Neither their ventilatory responses to hypercarbia during sleep period nor their ventilatory responses to hypoxia during wake–sleep cycle when compared with those in wild type mice. However, ORX-KO mice have a ventilatory response to hypercapnia reduced to the half of that in wild type mice during quiet wakefulness. The ventilatory response to hypercapnia was partially restored in ORX-KO mice administered intracerebroventricular with orexin-A or orexin–B, the two orexin subtypes derived from prepro-orexin (Deng et al. 2007).

Such results are compatible with those obtained by dialyzing the rat RTN with SB-334867, orexin receptor-1 antagonist that reduced the hyperventilation caused by hypercapnia by 30 % during wakefulness and 9 % during sleep. A much smaller effect (16 % reduction of hypercapnia-induced hyperventilation) was observed when microdialysis of SB-334867 was performed into rostral RN during wakefulness in dark period and null effect in the ventilatory chemo reflex when administered during sleep (Dias et al. 2010). In addition, almorexant, antagonist of both orexin receptor-1 and orexin receptor-2, administered orally reduced the ventilatory response to hypercapnia by 26 % only in wakefulness during the dark, active period of the diurnal cycle (Li and Nattie 2010). Then, we can conclude that projections of orexin-containing neurons to the RTN and rostral RN contribute, via orexin receptor-1, to the hypercapnic chemoreflex control during wakefulness and to a lesser extent during sleep (Dias et al. 2009). However, a possible role for orexin neurons as a "wakefulness" driver of chemosensitive properties is still uncertain.

## Astrocytes

Astrocytes are not mere intermingled cells of the CNS that outnumber neurons. As already described in Chapter "Glial Cells and Integrity of the Nervous System", they serve multiple functions: structure of the nervous tissue, trophism, metabolic support as for example the lactate shuttle, energy storage in the form of glycogen, ionic and water homeostasis, homeostasis of the synaptic environment buffering the concentration of extracellular potassium and the excess of extracellular neurotransmitters and release of gliotransmitters and neurotransmitters (most of them influencing synaptic strength, Table 1), formation and remodeling of synapses, defense against oxidative stress, scar formation, and tissue repair. Even more, astrocytes are involved in complex processes like neural network plasticity, inflammation, and neurodegeneration (Belanger et al. 2011; Grass et al. 2004; Rodriguez-Arellano et al. 2015).

**Table 1** Substances released by astrocytes

| | Neuroactive substance | Reference |
|---|---|---|
| *Neurotransmitters—neuromodulators* | | |
| Amino acids | L-glutamate | Parpura et al. (1994) and Kimelberg et al. (1990) |
| | L-aspartate | Kimelberg et al. (1990) |
| | Taurine | Kimelberg et al. (1990) |
| | D-serine | Schell et al. (1995) |
| | γ-aminobutyric acid (GABA) | Bowery et al. (1976) |
| Non-amino acids | Dopamine | Chen et al. (2005) |
| | ATP | Guthrie et al. (1999) |
| | Adenosine | Albrecht et al. (1991) |
| | Nitric oxide (NO) | Murphy et al. (1990) |
| | Met-enkephalin | Shinoda et al. (1989) |
| | Somatostatin | Mercure et al. (1996) |
| | Atrial natriuretic peptide (ANP) | Krzan et al. (2003) and Guček et al. (2012) |
| *Metabolic precursors* | | |
| | Lactate | Pellerin and Magistretti (1994) |
| | Glutamine | Yudkoff et al. (1994) |
| | $\alpha$-ketoglutarate | Westergaard et al. (1994) |
| | Malate | Westergaard et al. (1994) |
| | Succinate | Westergaard et al. (1994) |
| *Growth factors* | | |
| | BDNF | Caravagna et al. (2013) |
| | NGF-$\beta$ | Furukawa et al. (1986) |
| | IGF-I | Kadle et al. (1988) |
| | IGF-II | Kadle et al. (1988) |
| *Inflammatory factors* | | |
| | Prostaglandins D2, E2, I2 | Gebicke-Haerter et al. (1988) and Hartung and Toyka (1987) |
| | Thromboxane | Hartung et al. (1988) |
| | IL-1$\beta$ | Corsini et al. (1996) |
| | IL-6 | Wu et al. (2005) |
| | IL-10 | Wu et al. (2005) |
| | TGF-$\beta$ | Constam et al. (1992) |
| | TNF-$\alpha$ | Selmaj et al. (1990) |

There is a remarkable heterogeneity among astrocytes, being their phenotype largely a function of both local anatomy and regional functional demands (Oberheim et al. 2012). They are in intimate contact with most of the structures of the nervous system being largely responsible of its compartmentalization. Astrocytes send end-feet processes that enwrap blood vessels and interact with endothelial cells determining the formation of the blood brain barrier. Astrocytic end-feet processes express, among others, glucose transporters and aquaporin 4. They are involved in the cerebral neurovascular coupling regulating the microvascular flow for matching this to synaptic activity (Iadecola and Nedergaard 2007).

On the other hand, astrocytes send processes that ensheath most synapses. These perisynaptic processes express receptors for cytokines and growth factors. In addition, they express different kind of neurotransmitter receptors, transporters, and ion channels as expected of an active participant in the homeostasis of the synapse. Thus, at the synaptic compartment, astrocytes can sense the synaptic activity by means of neurotransmitter receptors activation (Araque et al. 2014), regulate the levels of neurotransmitters at the synaptic cleft influencing their recapture and release (Hamilton and Attwell 2010), modulate the synaptic transmission through gliotransmitters release, and modulate the neuron excitability by extracellular potassium buffering (Perea et al. 2014).

In hippocampus and cortex from rodent and humans, astrocytes are organized in discrete spatial domains (Oberheim et al. 2012). Each astrocyte extends its processes on a defined territory without important overlap between adjacent astrocytes. On other terms, all cellular structures in a territory (blood vessels, perikarya and synapses) interact with processes from a single astrocyte only (Oberheim et al. 2009). It is estimated that a single spatial domain for a protoplasmic astrocyte in rodent contains 20,000–120,000 synapses, while that in humans contains the extraordinary amount of 270 thousand to 2 million synapses (Oberheim et al. 2009).

A particular feature of astrocytes is that each one of them is coupled to others, through gap junction channels forming an extensive functional syncytium. In hippocampus, each astrocyte forms gap junctions with 11 others astrocytes, in average (Xu et al. 2010). This syncytium offers a route of low electrical resistance for propagation of electronic signaling and ionic currents and for cell-to-cell propagation of second messengers. This syncytium represents a huge sink for buffering the changes in potassium composition of the extracellular space. In addition, this syncytium allows the spreading of calcium waves, which, in humans reach the speed of 37–43 μm/s (Cornell-Bell et al. 1990; Oberheim et al. 2009), into neighboring astrocytes. Thus, astrocytes can be sequentially activated and recruited for performing a common task. Since each astrocytic domain represents an elementary glio-neuronal unit for monitoring the changes in activity of contiguous synapses, the existence of a functional syncytium implies the capability of influencing other astrocytic domains and the spreading of a potential astrocytic response to domains placed far away from an immediate neighborhood. This organization of highly organized and interconnected anatomical domains will allow the recruitment of distant domains, which in turn will influence a larger number of synapses within a neural network. As a consequence, a more intense, and may be, a more synchronized response will arise.

Calcium management of one astrocyte can affect many thousands of excitatory synapses nearby as shown by clamping intracellular $Ca^{2+}$ experiments. In these, clamping of calcium in individual hippocampal astrocytes is made through a whole-cell pipette while an extracellular field excitatory postsynaptic potential (EPSP) recording is done with an extracellular electrode placed either in the immediate vicinity of the clamped astrocyte or in a more distanced CA1 pyramidal cells group. Astrocytic $Ca^{2+}$ clamping blocked long term potentiation

(LTP) induced by tetanic stimulation of Schaeffer collaterals, at nearby, but not far away positions (Henneberger et al. 2010).

Astrocytes are ideally located to sense synapse activity with the perisynaptic processes and metabolic supply from blood vessels with the end feet processes. In fact, they mediate the response consisting in the modification of the local blood flow as function of synaptic or neuronal activity. It has been shown that astrocytes respond to increased neuronal activity by consuming more glucose and producing more lactate, this latter transferred into neighbor neurons as fuel during hyperactivity. As previously mentioned in Chapter "Glial Cells and Integrity of the Nervous System", this is known as the "astrocyte-neuron lactate shuttle" hypothesis (Pellerin et al. 2007).

## Astrocytes in the PreBötzinger Complex (preBötC)

The preBötC is the main generator of the inspiratory activity and a chemosensitive nucleus (Solomon 2003; Solomon et al. 2000). Fluctuations of the extracellular potassium concentrations are induced by the occurrence of rhythmic bursts of action potentials (Richter et al. 1978), which in turn are associated to fluctuation in the neurotransmitter release. Since astrocytes express $K^+$ channels (Kir4.1; KCNJ10), fluctuations in potassium concentrations generates fluctuations in the resting membrane potential, which can induce fluctuations in intracellular calcium concentration in astrocytes. Using whole-cell recordings from astrocytes and two-photon calcium imaging from rhythmic slices, none coupling between respiratory neuronal activity and astrocytic calcium signals was observed. The absence of correlation between respiratory neuronal activity and astrocytic calcium fluctuation (Schnell et al. 2011) indicates that astrocytic release of gliotransmitters is not commanding the respiratory like activity in neurons. Likely, one role of astrocytes in the preBötC is the control of extracellular levels of neurotransmitters and ions, both largely influencing the excitability of respiratory neurons.

## Astrocytes in Central Chemoreception

Over the last two decades, multiple pieces of evidence revealed that astrocytes can contribute to central chemoreception. Such contribution may be accomplished by astrocytes directly playing a role as $H^+/CO_2$ sensors or as part of the mechanisms underlying the cholinergic and glutamatergic hypothesis. Reduction in chemosensitivity of astrocytes may be involved in the pathogenesis of Rett syndrome and may explain the deficit in ventilatory responses to hypercapnia in these patients (Turovsky et al. 2015). Also, it has been proposed that astrocytes can play a modulatory role of the network in charge of the respiratory pattern generation by controlling the extracellular ion and transmitter concentrations (Neusch et al. 2006;

Szoke et al. 2006; Ballanyi et al. 2010; Erlichman and Leiter 2010). Likely, astrocytes in different chemosensitive regions also differ in their contributions to central chemoreception and the mechanisms underlying such contribution.

## Astrocyte Chemosensitivity

As illustrated in Fig. 2, several molecular mechanisms by which astrocytes detect $H^+/CO_2$ have been proposed

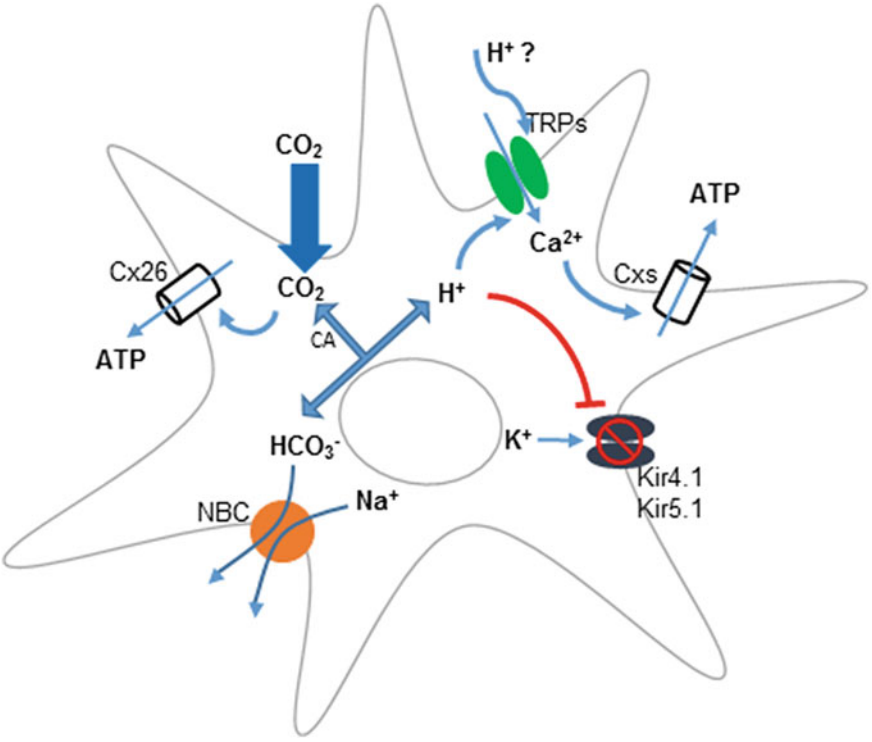


**Fig. 2** Astrocytes may sense acidosis or hypercapnia through different molecular sensors. Inwardly rectifying potassium (Kir) heteromeric channels Kir4.1–Kirk5.1 are inhibited by $CO_2$ resulting in depolarization of astrocytes (Wenker et al. 2010); carbonic anhydrase (CA) enzyme, the $Na^+$-$HCO_3^-$ cotransporter, $Na^+/H^+$ exchanger and the $Na^+$-dependent or $Na^+$-independent $Cl^-/HCO_3^-$ antiporters contribute to pH regulation (Brookes 1997; Baird et al. 1999; Makara et al. 2001; Schmitt et al. 2000; Deitmer and Rose 1996); connexins with a carbamylation motif (Cx26, Cx30, and Cx32), a site for binding $CO_2$ to induce the opening of connexin hemichannels (Meigh et al. 2013) endows cells with $CO_2$-sensitivity and the capacity for releasing ATP as a function of $PCO_2$ at constant extracellular pH (Huckstepp et al. 2010a); TRP channels endows to astrocytes with the ability for responding to hypercapnic but not isocapnic acidosis (Hirata and Oku 2010). It is possible that TRP activation could be given by extracellular acidification (Cui et al. 2011)

(1) Inwardly rectifying potassium (Kir) heteromeric channels Kir4.1–Kirk5.1. These channels contribute to the extracellular potassium regulation and are expressed in brainstem nuclei, including, among others, the LC, the ventrolateral medullary (VLM) area, the RTN, and the NTS (Wu et al. 2004). Kir4.1, and Kir5.1 channel subunits are observed in astrocytic processes contacting the pia mater, blood vessels, and synapses associated to PDZ domains containing syntrophins (Hibino et al. 2004). Depolarization of astrocytes by $CO_2$ would involve inhibition of heteromeric Kir4.1–Kir5.1 channels and contribution of $Na^+$-$HCO_3^-$ cotransporter (Wenker et al. 2010).

(2) Carbonic anhydrase enzyme and, in addition to the $Na^+$-$HCO_3^-$ cotransporter, several other transporters that contribute to pH regulation like the $Na^+/H^+$ exchanger and the $Na^+$-dependent or $Na^+$-independent $Cl^-/HCO_3^-$ antiporters (Brookes 1997; Baird et al. 1999; Makara et al. 2001; Schmitt et al. 2000; Deitmer and Rose 1996).

(3) Connexins presenting a carbamylation motif (Cx26, Cx30, and Cx32), a site for binding $CO_2$ to induce the opening of connexin hemichannels (Meigh et al. 2013) (see Chapter "Physiological Functions of Glial Cell Hemichannels" for further information on hemichannels). In particular, connexin 26 is abundantly expressed at the ventral medullary surface and its $CO_2$ sensitivity is within physiological range having a steep change in conductance centered around 40 mmHg $PCO_2$ (Huckstepp et al. 2010a, b). It is known that heterologous expression of Cx26 endows HeLa cells with $CO_2$-sensitivity and the capacity for releasing adenosine triphosphate (ATP) as a function of $PCO_2$ at constant extracellular pH (Huckstepp et al. 2010a). Accordingly, connexin hemichannel blockers reduce both the ATP release and the ventilatory response induced by hypercapnia in vivo and the ATP release induced by hypercarbia in vitro (Huckstepp et al. 2010b).

(4) Transient receptor potential (TRP) channels endows to astrocytes with the ability for responding to hypercapnic acidosis. This was assayed in enriched glia cells cultures using intracellular calcium- and pH-imaging in addition to perforated patch-clamp methods (Hirata and Oku 2010).

## Astrocyte Involvement in Respiratory Rhythm Modulation

Specific glial metabolic inhibitors have been used to evaluate astrocyte contribution to the ventilatory process. Fluorocitrate or fluoroacetate at low doses, are incorporated selectively by astrocytes and block the tricarboxylic acid (Krebs) cycle by inhibiting the enzyme aconitase. Administration of fluorocitrate into the RTN in either anesthetized mechanically ventilated or conscious adult rats increased the respiratory output (Erlichman et al. 1998; Holleran et al. 2001). This response can be explained on basis of the fluorocitrate-induced ATP and tissue pH decrease.

Inhibition of Krebs cycle reduces ATP levels, which in turn, reduces $Na^+$-$K^+$ATPase activity. Pump inactivation increases the extracellular potassium concentration and, subsequently, depolarizes, among others, chemosensitive neurons. Since chemosensitive neurons also respond to the acidification of the medium, and at the end, as overall result, the respiratory output is increased (Erlichman and Leiter 2010).

In contrast to in vivo experiments, fluoroacetate as well as methionine sulfoximine (MS), an inhibitor of glutamine synthetase, an enzyme present only in astrocytes that catalyzes the synthesis of glutamine from glutamate (see Chapter "Pharmacological Tools to Study the Role of Astrocytes in Neural Network Functions"), reduced the amplitude and frequency of the integrated inspiratory burst recorded from rhythmically active brainstem slices. At a first glance, these results suggest that astrocyte metabolic support or astrocyte functions depend on Krebs cycle and are necessary for the maintenance of the respiratory rhythm (Hulsmann 2000). In brainstem slices, evoked depolarization of the hypoglossal neurons by electrical stimulation of the ventral respiratory column (measured by optical imaging using voltage-sensitive dye) was reduced and delayed after fluoroacetate administration which is compatible with metabolic inhibition of fast synaptic transmission (Hulsmann et al. 2003). Accordingly, after fluoroacetate or MS treatment of brainstem slices, addition of glutamine restored the respiratory rhythm indicating that likely, the respiratory effects of both inhibitors were related, essentially, to impairment of the glutamate neurotransmission. In fact, fluoroacetate also impairs the astrocytic uptake of glutamate and the formation of glutamine (Swanson and Graham 1994)

In vivo administration of MS reduces basal ventilation and the ventilatory response to hypercapnia in conscious neonatal rats (Young et al. 2005a). By contrast, fluorocitrate administered into the RTN in vivo did not affect the respiratory response to acidosis or hypercapnia (Erlichman et al. 1998). Likely, the effects of fluorocitrate-induced reduction in ATP tissue pH oppose and predominate to the impairment in glutamate neurotransmission.

The hypothesis that astrocytes contribute to $H^+/CO_2$ sensitivity concatenates several steps: first, a subset of glial cells is depolarized in response to acidification (Fukuda et al. 1978; Fukuda and Honda 1975; Ritucci et al. 2005). Second, and derived from glial cell depolarization, intracellular $Ca^{2+}$ increases, which is required also for the inter-cellular propagation of calcium waves in glia (Guthrie et al. 1999); third, as a consequence of the intracellular $Ca^{2+}$ increase, ATP is released from astrocytes, likely through connexin hemichannels (Huckstepp et al. 2010b). In fact, electrochemical sensors placed at the ventral medullary surface can detect high levels of ATP ($3.8 \pm 0.9$ μM) during hypercapnia in anesthetized rats (Spyer et al. 2004; Gourine et al. 2005). Activation of glial purinoceptors by ATP can initiate self-propagating calcium waves that are proposed to influence local network excitability (Fiacco and McCarthy 2006). Finally, ATP, or other neuroactive molecules, will activate central chemoreceptor neurons such as those found in RTN/pFG (Spyer et al. 2004; Gourine et al. 2005).

ATP can act by binding to 7 subtypes of ionotropic P2X receptors (P2X1–7Rs) and eight subtypes of metabotropic P2YRs (P2Y1,2,4,6,11–14) (North 2002; Abbracchio et al. 2009).

According to this sequence of events, purinoceptor antagonists should impair the respiratory effects evoked by $CO_2$ stimulation. In fact, reduction and even abolition of ATP induced respiratory responses have been observed in vivo and in vitro (Thomas and Spyer 2000; Gourine et al. 2005; Zwicker et al. 2011; Gourine and Kasparov 2011; Gourine et al. 2010). Hypercapnia induces the release of ATP from the ventral surface of the medulla (Gourine et al. 2005). Further, application of ATP into the most rostral ATP-releasing site, corresponding likely to the retrotrapezoid nucleus, stimulated respiratory output, whereas application of ATP receptor antagonists like PPADS (pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonate) to this area reduced $CO_2$ respiratory responses (Gourine et al. 2005). Hypercapnia-induced ATP-mediated excitation of the respiratory rhythm in rats is likely to involve a potent P2Y1R- activation of the preBötC (Lorier et al. 2007; Gourine et al. 2010). Such P2Y receptor-dependency has also found in vitro RTN neurons (Mulkey et al. 2006). Since pH sensitivity of RTN neurons in bicarbonate-free HEPES medium is not affected after purinergic receptor blockade with PPADS, ATP would play a role of modulator of the activity of pH-sensitive neurons, amplifying their responses to hypercapnia (Mulkey et al. 2006, 2004).

New insights of astrocyte contribution in modulating the function of respiratory neuronal circuits arise from application of molecular and electrophysiological methodology in conjunction with genetically engineered optical stimulation and $Ca^{2+}$ imaging tools. In an elegant work, Gourine et al. (2010) tested the hypothesis that rat astrocytes residing in RTN/pFRG behave as pH sensors, and trigger the respiratory response through the release of ATP. Astrocytes were genetically encoded with a $Ca^{2+}$ indicator associated to the promoter for glial fibrillary acidic protein (GFAP). They could confirm that these astrocytes, but not those from cerebral cortex, responded to physiological decreases in pH with elevations in intracellular $Ca^{2+}$ and ATP release. Accordingly, studies of vesicular fusion using total internal reflection fluorescence (TIRF) microscopy show that 35 % of astrocytes from rat brainstem in dissociated cultures respond to acidification with exocytosis of ATP-containing vesicles. Vesicles were visualized with fluorescent dyes quinacrine, an acridine derivative with very high affinity for ATP, and MANT-ATP, an ATP analogue esterified by the fluorescent methylisatoic acid. Vesicular exocytosis requires intracellular $Ca^{2+}$ signaling and was independent of autocrine ATP actions (Kasymov et al. 2013). By contrast, ATP was necessary to propagate astrocytic $Ca^{2+}$ excitation, since elimination of ATP by the ATP-hydrolyzing enzyme, apyrase, reduced importantly the $CO_2$-evoked astrocytic calcium responses (Gourine et al. 2010). In addition, ATP activating P2Y1Rs excited chemoreceptor neurons leading to the increase in the respiratory rhythm frequency (Gourine et al. 2010). Optogenetic stimulation of astrocytes expressing channelrhodopsin-2 associated under the command of GFAP promoter, resulted in a robust increase in breathing, associated to the increase in intracellular $Ca^{2+}$ in astrocytes. This optogenetic stimulation mimicked the hypercapnia and acidosis induced activation of

chemoreceptor neurons via an ATP-dependent mechanism (Gourine et al. 2010). In agreement with these results, disruption of purinergic signaling decreases $CO_2$ sensitivity of RTN neurons by 25 % (Wenker et al. 2010) as well as gap junction blockers, which decrease $CO_2$-evoked ATP release in the RTN, reduced the whole-animal ventilatory response to $CO_2$ also by 25 % (Huckstepp et al. 2010b). In addition, fluorocitrate-induced depolarization of astrocytes evoked a reversible increase in firing rate of RTN neurons. This increase in neuronal firing rate was abolished by the presence of P2 receptor antagonists (PPADS or suramin) (Wenker et al. 2012) suggesting that a purinergic signaling was a mediator. Purinergic blockade also blunted the hypercapnic ventilatory response in vivo and the firing rate response of RTN neurons to hypercapnic stimulus of slices (10–15 % $CO_2$) (Wenker et al. 2012).

## Regional Differences in Contribution of Astrocytes to Central Chemoreception

As mentioned above, neurons responding to $CO_2$ with increased firing rate can be found, among other sites, at the RTN (Nattie et al. 1993a), RN (Iceman et al. 2013), and the caudal portion of the NTS (Dean et al. 1990; Nichols et al. 2009). Furthermore, focal acidification either by injection of acetazolamide within these three regions in anesthetized cats (Coates et al. 1993) or by microdialysis within these nuclei in unanesthetized awake or sleeping rats (Li et al. 1999; Nattie and Li 2001, 2002a) increases ventilation. Since RTN and RN neurons in culture have intrinsic $CO_2$-pH-sensitivities (Wang et al. 1998; Wang and Richerson 1999; Wang et al. 2013), glia would play a coadjuvant, synergic role in these chemoreceptive nuclei. There is not any study detailing the cytoarchitecture and properties of astrocytes at the different areas of the brainstem. Hitherto, the degree of cell-to-cell interconnections, the extension of astrocyte domains, and the differential expression of receptors, gliotransmitters, are mostly unknown. Since in other regions of the CNS, the population of astrocytes is heterogeneous in shapes and functions (Oberheim et al. 2012), it would not be strange that astrocytes belonging to different chemosensory nuclei at the brainstem differ in their structure and properties. Therefore, it is possible that the mechanisms through which astrocytes interact with the respiratory network at different nuclei could also be different.

As expressed before, data obtained at the RTN suggest the existence of a cascade of events triggered by hypercapnia or acidosis: depolarization of astrocytes, cytoplasmic calcium increase, ATP release, and ATP activation of respiratory neurons. It is worth to remember that this constitute the glial pathway for RTN neurons activation since RTN neurons are chemosensitive themselves. At that respect, the glial pathway appears as intensifier of the RTN neurons response to hypercapnia.

At the NTS and the RN, there is some controversial evidence pointing to the role of ATP as mediator of the response to hypercapnia. P2 receptors are expressed in the NTS and with less intensity, at the RN (Yao et al. 2000).

The administration of ATP or its analogues into the NTS, in awake rats produced cardiorespiratory responses (Antunes et al. 2005; De Paula et al. 2004). On the other hand, the injection of P2 receptor antagonists into NTS reduces the sympatho-excitatory response to peripheral chemoreflex activation (Braga et al. 2007; Boscan et al. 2002). Microinjection of ATP into the raphe magnus reduces the respiratory activity while that into the raphe pallidus increase it in anesthetized and artificially ventilated rats (Cao and Song 2007). The injection of the P2X broad-spectrum antagonist, PPADS, into the rostral medullary raphe blunted the ventilatory response to hypercapnia in conscious rats (da Silva et al. 2012), while this unaffected ventilation when injection was placed into the caudal RN of conscious rats (da Silva et al. 2012) or when it was done into raphe magnus or pallidus in anesthetized rats (Cao and Song 2007).

To test whether an astrocytic ATP-dependent mechanism was involved in central chemoreception at the RN and NTS, ATP antagonists were applied into these nuclei while chemoreflexes were evaluated in vivo as in vitro (Sobrinho et al. 2014). ATP injections into the caudal NTS (cNTS) increased cardiorespiratory activity in anesthetized rats (Sobrinho et al. 2014) confirming results obtained with the rat working heart-brainstem preparation (Antunes et al. 2005). By contrast, the injection of broad range purinergic receptor antagonists like PPADS or suramin into the cNTS did not affect basal ventilation or the ventilatory responses to changes in $CO_2/H^+$ as it does at the RTN (Sobrinho et al. 2014). In the case of RN the results were more negative, because both the injections of ATP or PPADS in anesthetized rats did not affect neither the basal ventilation nor the responsiveness to $H^+/CO_2$ (Sobrinho et al. 2014). Cell-attached NTS neurons recorded from brainstem slices increased their firing rate in response to ATP, while P2 receptors antagonists (PPDAS or suramin) did not modified NTS neurons response to hypercarbia. Likewise, the firing rate of RN neurons were not modified by ATP and their responses to changes in $PCO_2$/pH were unaffected by ATP-receptor blockade (Sobrinho et al. 2014).

Sobrinho et al. (2014) results are unexpected from previous reports indicating the existence of P2 receptors, and the respiratory-related effects of ATP agonist and antagonist injected into the NTS or RN. In fact, it is known that ATP in NTS plays a role in modulating the glutamatergic excitatory transmission as evidenced by the reduction in the amplitude of tractus solitaries-evoked excitatory postsynaptic currents (TS-eEPSCs) by purinergic antagonist (iso-PPADS). The glial cells are the source of ATP released by tractus solitarius electrical stimulation is suggested by the reduction in this TS-eEPSCs induced by the glia toxin, fluoroacetate (Accorsi-Mendonca et al. 2013). Likely, the inconsistency in results may be partly due to methodological differences, for example the use of anesthesia or the use of broad-spectrum antagonists which are weakly effective for blocking specific subset

of P2 receptors. However, it remains possible that astrocytes contribute to the $CO_2/H^+$ responsiveness of cNTS and RN neurons, perhaps by an ATP-independent mechanism.

## Other Gliotransmitters

It is possible that other gliotransmitter, different to ATP, could serve as mediator in NTS or RN. A good candidate is D-serine. D-serine is a D-amino acid synthesized from L-serine by a pyridoxal 5′-phosphate-dependent serine racemase (SR) enzyme, which is present in neurons and astrocytes (Rosenberg et al. 2010; Wolosker 2011). D-serine binds with high affinity to the co-agonist (glycine) site of the N-methyl-D-aspartate (NMDA) glutamate receptor. D-serine effects have not been evaluated in the respiratory network, despite of NMDAR activation increases the respiratory frequency in vivo (Connelly et al. 1992) and in vitro (Greer et al. 1991). Preliminary data from our laboratory indicates that in *en bloc* preparations from neonatal mice, D-serine applied into the superfusion bath increases the respiratory rhythm of neonatal mice (Fig. 3).

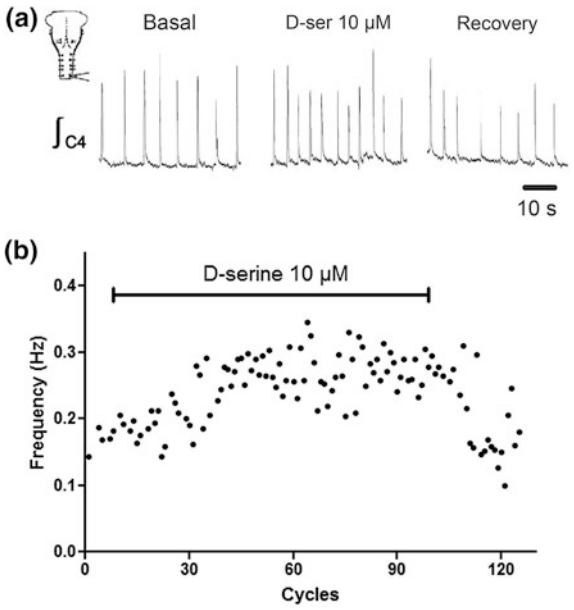


**Fig. 3** Increase of respiratory frequency induced by D-serine. **a** integrated inspiratory burst recorded from C4 ventral root in *en bloc* preparation obtained from CF1 mouse neonate at the third postnatal day before (basal), during, and after (recovery) the superfusion with aCSF containing D-serine 10 µM. **b** Instantaneous respiratory frequency measured cycle-to-cycle before, during (indicated by *horizontal bar*), and after the superfusion with aCSF containing D-serine 10 µM in the preparation from (**a**)

## Astrocytes and Cholinergic-Glutamatergic Hypothesis of Central Chemoreception

Historically, two neurotransmitters have been involved in central chemoreception, acetylcholine and glutamate, what is known as "the cholinergic and glutamate hypothesis of central chemoreception".

*Cholinergic (ACh) hypothesis*: Cholinergic neurons form part of input and output of the respiratory network. They are found at the NTS (Ruggiero et al. 1990; Armstrong et al. 1988; Gotts et al. 2015), the hypoglossal nuclei, facial nuclei, ambiguous nuclei (Kang et al. 2007), within the RN (Tatehata et al. 1987; Ruggiero et al. 1990), the nucleus reticularis rostroventrolateralis (RVL), and the ventral medullary surface (VMS); although cholinergic neurons are also detected in other localizations of the brainstem, like those in the medial portion of the rostral ventrolateral medulla (mRVLM), these would not be involved in cardiorespiratory events (Stornetta et al. 2013). The most important cholinergic inputs to the brainstem are originated from the pedunculopontine tegmental (PPT) and laterodorsal tegmental (LDT) nuclei. These inputs, as well as those provided by the serotonergic RN and the noradrenergic LC may be a clue for understanding pathogenia of respiratory dysfunctions associated to sleep–wake cycle, like sudden infant death syndrome (SIDS).

Muscarine or nicotine applied on the ventral surface of rostral and caudal medulla increase ventilation in anesthetized cats (Dev and Loeschcke 1979a, b). An endogenous cholinergic drive of the respiratory rhythm is revealed with acetylcholinesterase inhibitors (physostigmine, eserine) within rostral and caudal medulla (Dev and Loeschcke 1979a). In part, the respiratory cholinergic drive is exerted on the preBötC where activation of M3 and $\alpha 4\beta 2$ nicotinic receptors increases the frequency of the respiratory rhythm in neonatal rats and mice slices (Shao and Feldman 2005; Shao et al. 2008; Shao and Feldman 2009). A tonic cholinergic respiratory drive in the mouse *en bloc* preparation is revealed by application of atropine, a muscarinic receptor antagonist, which reduces the amplitude and frequency of the respiratory rhythm (Coddou et al. 2009).

That a cholinergic relay may be involved in central chemoreception at the surface of ventral medulla is derived from the fact that acetylcholine-sensitive areas and $H^+$- or $CO_2$-sensitive areas overlapped. In addition, application of cholinergic agonists on these sensitive areas elicits similar patterns of respiratory responses than those evoked by acidic stimulation (Loeschcke 1982; Eugenin and Nicholls 1997). Furthermore, central chemoreception and muscarinic cholinergic neurotransmission are strongly linked (Loeschcke 1982) as indicated by the brainstem distribution of muscarinic receptors (Nattie and Li 1990; Nattie et al. 1994; Mallios et al. 1995). Application of atropine to the rostral and caudal medulla decreases ventilation and, at the same time, reduces importantly the ventilatory response to $CO_2$ (Dev and Loeschcke 1979a; Nattie et al. 1989). Muscarinic blockade also reduces and, sometimes, abolishes the respiratory responses induced by $H^+$ or $CO_2$ in in vitro preparations from neonatal rats (Monteau et al. 1990), newborn opossum (Eugenin

and Nicholls 1997), and neonatal mouse (Coddou et al. 2009). Microinjection of muscarinic M3 antagonist on the rostral ventrolateral medulla (RVLM) has a great efficacy for inhibiting respiratory $CO_2$-evoked response (Nattie and Li 1990). Interestingly, the arcuate nucleus, which is the human homologue of the RVLM, shows decreased muscarinic binding in SIDS infants (Kinney et al. 1995). Such probable reduction of the muscarinic binding in SIDS is compatible with the reduction of the muscarinic contribution to the chemosensory responses in *en bloc* and slices preparations from P0-P3 nicotine-exposed neonates by the prenatal-perinatal nicotine exposure (Coddou et al. 2009; Eugenin et al. 2008).

Unexpected results were obtained when muscarinic receptor knockout (KO) mice were challenged with hypercapnia (3 and 5 % CO2). M1 single KO mice showed normal, while M3 single KO mice showed reduced VT response slope to hypercapnia (Boudinot et al. 2004). Surprisingly, M1/3R or M2/4R double-KO mice showed unaltered chemosensory ventilatory responses (Boudinot et al. 2008). These results are puzzling and will require future research with conditional KO mice to evaluate muscarinic contribution to chemo reflexes in adults in absence of possible compensatory mechanisms exerted during development.

*Glutamate (Glu) hypothesis*: Excitatory glutamate neurotransmission predominates within the mammalian RPG, and the ventral surface of medulla is not an exception. Injection of glutamate into the RVLM increases ventilation in anesthetized cats (Li and Nattie 1995; Nattie and Li 1995). By contrast, microinjection of kynurenic acid, a nonselective glutamate receptor antagonist, or AP5, an NMDA receptor antagonist, or CNQX, a non-NMDA receptor antagonist, into the RVLM region decreased both the amplitude of the integrated phrenic nerve activity and the $CO_2$ sensitivity in a dose-dependent manner in anaesthetized cats (Nattie et al. 1993b). In contrast to in vivo experiments (Connelly et al. 1992), blockade of NMDARs in brainstem slices had a negligible effect on respiratory rhythm (Morgado-Valle and Feldman 2007; Greer et al. 1991), while the blockade of AMPARs completely abolished the rhythm. Similarly, NMDA receptor R1 subunit (NMDAR1) mutant mice were completely unresponsive to NMDA applications and showed a respiratory rhythm almost identical to that of controls. These results indicate that NMDA receptors are not relevant for generating the rhythm and for the development of circuits in charge of it (Funk et al. 1997). As for muscarinic receptors, the effects of glutamate antagonists have not been demonstrated to be specific for chemoreception.

Till now, acetylcholine (ACh) or glutamate (Glu) actions on chemosensitive areas are attributed to direct effects on neurons and a probable contribution of astrocytes in such responses has not been evaluated. Numerous studies demonstrate that astrocytes in different CNS regions express functional neurotransmitter receptors, which allow them to be sensitive to neurotransmitters like ACh and Glu (Perea and Araque 2010; Halassa and Haydon 2010; Ben Achour and Pascual 2010; Paixao and Klein 2010; Attwell et al. 2010; Sidoryk-Wegrzynowicz et al. 2011; Stipursky et al. 2011; Haydon and Carmignoto 2006; Erlichman et al. 2010). It is worth noting that astrocytes in the ventral respiratory group (VRG) express receptors for 5-HT, substance P (SP), and thyrotropin releasing hormone (TRH).

So, projections from chemosensitive RN neurons may modify the activation of astrocytes within the respiratory network (Hartel et al. 2009).

Astrocytes in the respiratory network respond to prevailing neuromodulators with an increase of intracellular calcium concentration (Huxtable et al. 2010; Gourine et al. 2010; Hartel et al. 2009). Besides, astrocytes are also capable of synthesizing and releasing neuro- and glio-transmitters such as ACh, Glu, ATP/adenosine, and D-serine (Haydon and Carmignoto 2006; Hamilton and Attwell 2010; Carmignoto et al. 1998; Araque et al. 2002; Hosli and Hosli 1994b; Hosli et al. 1988). So, theoretically, astrocytes may be involved in mediating or amplifying the ventilatory response to cholinergic and glutamatergic inputs by releasing gliotransmitters able of modifying the activity of the respiratory network. In addition, astrocytes can remove neurotransmitters from the synaptic cleft so they may participate in the control of the synaptic neurotransmitter concentration (Carmignoto et al. 1998; Araque et al. 2002; Hosli and Hosli 1994b; Hosli et al. 1988; Haydon and Carmignoto 2006). For example at the NTS, acidification can depolarize astrocytes by inhibition of both $K^+$ channel current and voltage-sensitive glutamate transporters (Huda et al. 2013). Therefore, as consequence of acidification at the NTS, the inhibition of this glutamate transporter, increases the levels of glutamate at the synaptic cleft affecting the excitatory synaptic transmission (Huda et al. 2013).

In the human infant, about 95 % of the arcuate nucleus neurons (corresponding to the chemosensitive RVLM in cats and rats) are glutamatergic. A large number of astrocytes in the ventral medullary surface express the vesicular glutamate transporter 2 and low levels of 5-$HT_{1A}$ and kainate (GluR5) receptors. So, it is reasonable to propose that astrocytes, which can also express muscarinic and nicotinic receptors (Gahring et al. 2004; Hosli et al. 1994; Hosli and Hosli 1994a, b), may store and release glutamate, possibly in response to stimulation by 5-HT, by ACh, or by glutamate itself (Paterson et al. 2006) affecting, in addition to the inhibition of glutamate uptake, the levels of glutamate at the synaptic cleft.

In addition, astrocytes play an essential role in glutamatergic synapses. Glutamate in the synaptic space is uptaken by astrocytes, converted by them into glutamine, and then transferred as glutamine to the presynaptic terminals for renewal of the glutamate presynaptic pool (Haydon and Carmignoto 2006). In the RPG, most of the excitatory synapses are glutamatergic; interestingly, 5 mM fluoroacetate or 0.1 mM methionine sulfoximine, both glial metabolic toxins, reduce the increase in respiratory frequency induced by ATP in brainstem slices, but they do not affect substance P evoked increase, suggesting that astrocytes contribute to the purinergic drive of the inspiratory rhythm generating network (Huxtable et al. 2010).

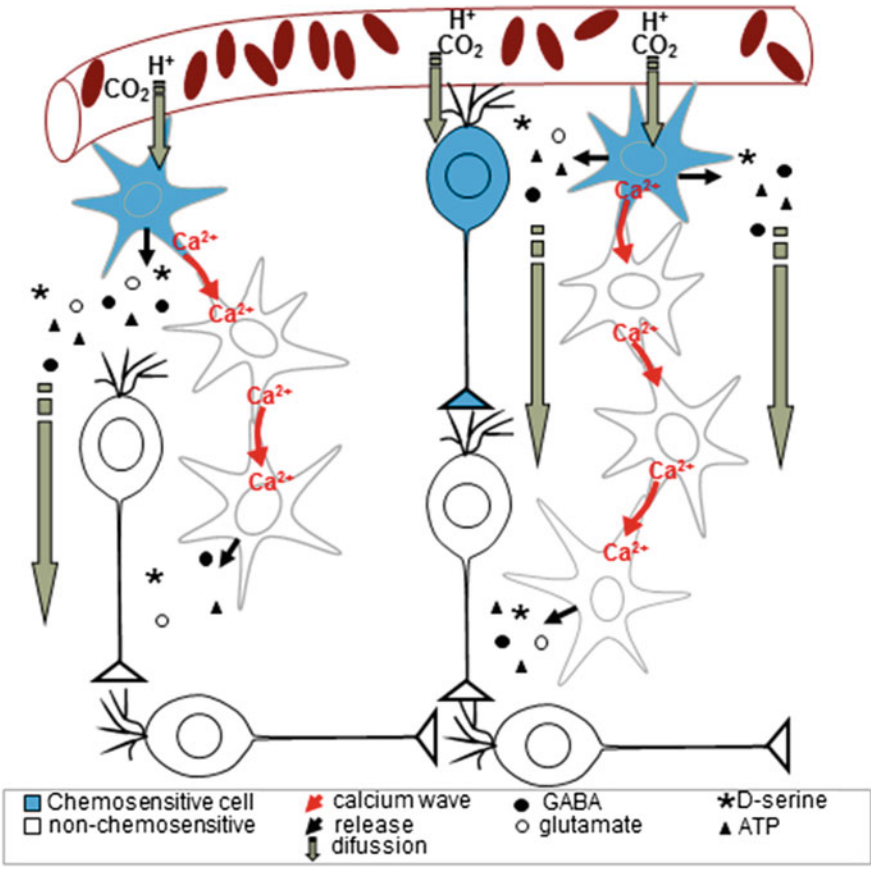


**Fig. 4** Schema of astrocyte contribution to the chemosensory response. Astrocytes enwrapping blood vessels or exposed to the CNS environment are continuously monitoring pH and $PCO_2$. As consequence of their activation (astrocyte depolarization) by acidosis or hypercapnia, there is an increase in intracellular calcium concentration, which may trigger the release, among others, of ACh, Glu, ATP, or D-serine and calcium waves that travel from astrocyte-to-astrocyte influencing the behavior of astrocyte according other astrocyte domains. Thus, the action of gliotransmitters at the local chemosensitive site may enhance the response of chemosensitive cells in the immediate environment. In addition, by volume diffusion of gliotransmitters and by activation of faraway astrocytes influenced by calcium waves more neurons of the respiratory network may be recruited

## Concluding Remarks

Astrocytes have diverse roles in modulation of the respiratory rhythm. These involve controlling neural network excitability through potassium buffering, regulation of synaptic transmitter concentrations via their synthesis, reuptake and release; in particular, at glutamatergic synapses, astrocyte is the source of glutamine, essential for replenish synaptic vesicles of glutamatergic neurons. Respect

to respiratory central chemoreception, astrocytes have the ability of monitoring $PCO_2$ and pH and release gliotransmitters like ATP in the RTN, in response to changes in $CO_2$ and $H^+$ . In addition, they contribute to the regulation of the extracellular pH either by generating acidic substances derived from metabolic coupling (lactate shuttle) leading to amplification of hypercapnic stimulus or through proton buffering (transporters and channels).

On basis to the discussed properties of astrocytes (calcium waves, coupling of astrocytic domains through gap junctions, regulation of neurotransmitters and release of gliotransmitters) we propose that astrocytes may play two emergent roles in central respiratory chemoreception. A first role, as amplifiers of the responses of intrinsic chemosensitive neurons through feedforward signaling via gliotransmitters and a second role as recruiter of non-intrinsic chemosensitive cells thanks to volume spreading of signals (calcium waves and gliotransmitters) to regions far away the $CO_2/H^+$ sensitive domains (Fig. 4).

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