



# Neurochemical and electrical modulation of the locus coeruleus: contribution to CO<sub>2</sub> drive to breathe

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The locus coeruleus (LC) is a dorsal pontine region, situated bilaterally on the floor of the fourth ventricle. It is considered to be the major source of noradrenergic innervation in the brain. These neurons are highly sensitive to CO<sub>2</sub>/pH, and chemical lesions of LC neurons largely attenuate the hypercapnic ventilatory response in unanesthetized adult rats. Developmental dysfunctions in these neurons are linked to pathological conditions such as Rett and sudden infant death syndromes, which can impair the control of the cardio-respiratory system. LC is densely innervated by fibers that contain glutamate, serotonin, and adenosine triphosphate, and these neurotransmitters strongly affect LC activity, including central chemoreflexes. Aside from neurochemical modulation, LC neurons are also strongly electrically coupled, specifically through gap junctions, which play a role in the CO<sub>2</sub> ventilatory response. This article reviews the available data on the role of chemical and electrical neuromodulation of the LC in the control of ventilation.

**Keywords:** rodent, chemosensitive signaling, serotonin, glutamate, hypercapnia, ATP

## INTRODUCTION

Locus coeruleus (LC) is pontine region situated bilaterally on the floor of the fourth ventricle, and produces the majority of noradrenaline (NA) in the forebrain. The LC region is a densely packed nucleus, comprising about 1500 neurons per side (Aston-Jones et al., 1984). The first description of the LC was published by Reil (1809), but the term LC was proposed by the anatomists Wenzel and Wenzel in 1812 (reviewed by Russell, 1955). In addition to NA, the cell bodies also secrete several neuropeptides (Sutin and Jacobowitz, 1991), among which are vasopressin, somatostatin, neuropeptide Y, enkephalin, neurotensin, CRH, and galanin (for review, see Olpe and Steinmann, 1991). This nucleus has been described in a wide variety of animals, such as frogs, birds, rodents, and primates (González and Smeets, 1993).

LC NA neurons project broadly throughout the neuraxis, from spinal cord to neocortex (Swanson and Hartman, 1975; Foote et al., 1983; Berridge and Waterhouse, 2003). In fact, it is estimated that ~50% of all the noradrenergic projections in the central nervous system (CNS) originate in the LC which are directed toward the forebrain, cerebellum, brainstem and spinal cord (Aston-Jones et al., 1995; Berridge and Waterhouse, 2003). LC is the primary source of an extensive, yet regionally specialized, noradrenergic innervation of the forebrain (Berridge and Waterhouse, 2003) and it is considered a major wakefulness promoting nucleus with activation of the LC resulting in an increase in EEG signs of alertness (Samuels and Szabadi, 2008).

The alerting effect of LC activation results from dense excitatory projections to the cerebral cortex, dorsal raphe, pedunculopontine tegmental nucleus and the laterodorsal tegmental nucleus,

and from substantial inhibitory projections to sleep-promoting GABAergic neurons of the basal forebrain and the ventrolateral preoptic area (Samuels and Szabadi, 2008). Additionally, LC also modulates autonomic function through direct output to sympathetic and parasympathetic preganglionic neurons of the intermediolateral cell column of the spinal cord and to projections innervating other autonomic nuclei (Nygren and Olson, 1977; Westlund et al., 1983; Jones and Yang, 1985), among which known examples are the Edinger-Westphal Nucleus (Breen et al., 1983), paraventricular nucleus (Swanson and Sawchenko, 1983; Jones and Yang, 1985), caudal raphe (Hermann et al., 1997), and rostroventrolateral medulla (Van Bockstaele et al., 1989).

LC is innervated by fibers that contain many neurotransmitters such as opiates, glutamate, gamma-aminobutyric acid (GABA), serotonin, epinephrine, and the peptide orexin/hypocretin (Aston-Jones et al., 1995). The nucleus paragigantocellularis lateralis (PGI) is a source for glutamate, GABA, enkephalin, corticotrophin releasing hormone (CRH), and epinephrine. A strongly inhibitory GABA and enkephalin input originates from the dorsomedial rostral medulla, whereas orexin/hypocretin inputs originate in the hypothalamus (Horvath et al., 1999), as do histaminergic inputs (Pollard et al., 1978).

Because of these widespread projections, LC is implicated in the control of many physiological functions, including control of respiration (Oyamada et al., 1998; Fabris et al., 1999; Hilaire et al., 2004; Putnam et al., 2004; Viemari et al., 2004; Biancardi et al., 2008; de Carvalho et al., 2010; de Souza Moreno et al., 2010; Gargaglioni et al., 2010; Biancardi et al., 2014; Patrone et al., 2014) and cardiovascular function (Sved and Felsten, 1987; Yao and

Lawrence, 2005; Biancardi et al., 2014; Patrone et al., 2014). LC is considered a central CO<sub>2</sub>/pH chemoreceptor site in amphibians and mammals (Noronha-de-Souza et al., 2006; Gargaglioni et al., 2010; Santin and Hartzler, 2013) and more than 80% of its neurons are chemosensitive, responding to hypercapnia with an increased firing rate (Pineda and Aghajanian, 1997; Oyamada et al., 1998; Filosa et al., 2002). Local acidification of noradrenergic LC neurons increases respiratory frequency and phrenic nerve discharge in cats (Coates et al., 1993). Lesion of approximately 80% of noradrenergic neurons of LC by using 6-OHDA elicited a large decrease in the response to CO<sub>2</sub>, of approximately 64%, due to a decreased tidal volume (V<sub>T</sub>), indicating that this nucleus exerts a profound effect on the hypercapnic ventilatory response (Biancardi et al., 2008). The objective of this review is to summarize the available data on the role of chemical and electrical neurotransmission in the LC in the control of ventilation during CO<sub>2</sub> challenge.

### ELECTRICAL MODULATION OF THE LC

The traditional concept of cell communication is based on the presumption that the electrical coupling was a “primitive” form of signaling commonly present in invertebrates, since the chemical synapses are characterized as the predominant form of intercellular signaling in the CNS of vertebrates (Naus and Bani-Yaghoob, 1998). However, studies have shown that electrical coupling appears to be an important signaling mechanism for several functions, including cardiorespiratory regulation. In this regard, electrical communication is present in regions of the CNS involved in the generation and modulation of respiratory rhythm, inspiratory motoneuron synchronization, central chemoreception and cardiovascular control (Davidson and Baumgarten, 1988; Nattie, 1999; Solomon et al., 2001; Solomon, 2003; Rash et al., 2007; Pierce et al., 2010). In fact, the presence of gap junctions has been reported in many regions that are considered CO<sub>2</sub>/pH chemosensitive such as the caudal medullary raphe, retrotrapezoid nucleus (RTN), dorsal motor nucleus of the vagus (DMV), nucleus of the solitary tract (NTS), and the LC, thus implying that electrical coupling may constitute an important mechanism for CO<sub>2</sub> drive to breathe (Dean et al., 1997; Rekling and Feldman, 1997; Solomon et al., 2000; Dean et al., 2001, 2002; Solomon and Dean, 2002).

Gap junctions are formed by proteins that create intercellular channels; these transmembrane pores facilitate the direct exchange of small molecules, metabolites, adenosine triphosphate (ATP) and ions between cells in direct contact (Hooper and Subak-Sharpe, 1981; Loewenstein, 1981; Picoli et al., 2012). These channels are composed by a combination of two hemichannels (connexons), each connexon containing a group of six proteins called connexins (Cx). These proteins fold in a hexameric structure creating a hydrophilic channel that connects the cytosol of neighboring cells (Davidson and Baumgarten, 1988; Solomon and Dean, 2002; Picoli et al., 2012).

Cells that communicate via electrical synapses, in most cases, express more than one Cx isoform (Solomon and Dean, 2002), thus each connexon may consist of a single or multiple isoforms of Cxs (homomeric or heteromeric connexons, respectively; Bruzzone et al., 1996; Kumar and Gilula, 1996; Laird, 2006). The

physiological properties of the gap junction depend on the protein isoforms that are present in the connexon (Kumar and Gilula, 1996; Bruzzone and Ressot, 1997), which can explain the differences in sensitivity to intracellular pH (pHi), intracellular calcium and phosphorylation (Goodenough et al., 1996). Among the 15 connexin isoforms identified in the CNS of adult and newborn rats (Beyer et al., 1990; Dermietzel and Spray, 1993; Bruzzone et al., 1996; Dahl, 1996; Condorelli et al., 1998), Cxs 26, 32, and 36 are the most abundantly expressed in neuronal cells (Dermietzel et al., 1989; Belliveau et al., 1991; Belliveau and Naus, 1995; Condorelli et al., 1998; Solomon et al., 2001).

A striking characteristic of the LC neurons is the cell-to-cell coupling via gap junctions (Christie et al., 1989; Christie and Jelinek, 1993; Travagli et al., 1995; Ishimatsu and Williams, 1996; Oyamada et al., 1999; Alvarez-Maubecin et al., 2000; Ballantyne and Scheid, 2000). The cellular coupling facilitates the communication between neurons and it is important to coordinate the firing of the LC neurons, since it seems that the entire nucleus fires simultaneously in response to sensory inputs (Nestler et al., 1999). In fact, this synchronism is important for increase of NA release from LC neurons (Christie et al., 1989; Travagli et al., 1995; Ishimatsu and Williams, 1996; Alvarez-Maubecin et al., 2000). According to Rash et al. (2007), the LC neurons of adult rats express Cx32 and Cx36, while the presence of Cx26, an isoform which is sensitive to CO<sub>2</sub>, is still controversial topic in the field (Alvarez-Maubecin et al., 2000). Previous studies have shown that Cx36 expression in the CNS increases during the first week of life and reduces in adults (Sohl et al., 1998; Belluardo et al., 2000; Solomon, 2003). This Cx participates in the astrocytic and neuronal differentiation during development (Hartfield et al., 2011) and also plays an important role in learning and memory consolidation (Frisch et al., 2005). Regarding Cx32, this isoform is only detected between 5 and 10 days postnatal (Dermietzel et al., 1989; Belliveau et al., 1991; Belliveau and Naus, 1995; Nadarajah et al., 1997; Solomon et al., 2001) and is hypothesized to participate in central CO<sub>2</sub> chemoreception (Solomon et al., 2001). Further studies are needed to clarify the role of Cx36 and Cx32 in the LC.

Oyamada et al. (1998) demonstrated that transversal brainstem slices of newborn rats presented 83% of LC neurons being modulated by the respiratory rhythm and this modulation depends on the activation of an excitatory amino acid pathway. Also there are evidences that LC neurons exhibit spontaneous depolarizations that do not depend on the chemical synaptic transmission (Williams and Marshall, 1987; Dean et al., 2001) and this electrical synchronization is interrupted when a gap junction blocker is administered (Christie et al., 1989; Travagli et al., 1995; Ishimatsu and Williams, 1996; Oyamada et al., 1999; Alvarez-Maubecin et al., 2000; Ballantyne and Scheid, 2000; Andrzejewski et al., 2001; Ballantyne et al., 2004), reinforcing the role of the electrical coupling in the maintenance of endogenous rhythmic activity of LC neurons (Hayashida et al., 2010).

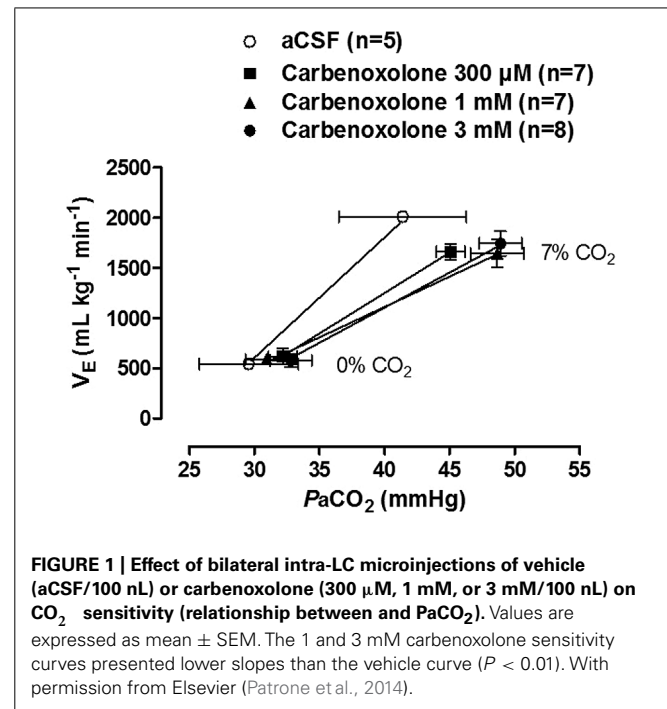
Recently, Nichols et al. (2008) demonstrated using newborn brainstem slices that carbenoxolone (CARB, a gap junction blocker) decreases the percentage of LC neurons that are responsive to CO<sub>2</sub>, as well as the magnitude of this response (chemosensitivity index – CI) during postnatal development. In addition,

Rash et al. (2007) reported that LC neurons are strongly electrically coupled during early post-natal development, suggesting that gap junctions may play an important role in the chemosensitive response of LC neurons during development. However, the function of gap junctions may differ among other chemosensitive nuclei, since the synaptic blockade of NTS neurons by CARB perfusion reduces the ventilatory response to hypercapnia in 7–10 week-old awake rats but not in >10-week-old rats (Parisian et al., 2004). In the RTN, CARB perfusion decreases the ventilatory response to CO<sub>2</sub> in younger animals, but increases the hypercapnic ventilatory response in older animals (Hewitt et al., 2004).

The pattern of development regarding the ventilatory response to CO<sub>2</sub>, present a clear and important change in the ventilatory response to hypercapnia during the neonatal period in rats, especially when comparing young *versus* older animals (Putnam, 2001). The LC of rats younger than 10 days (P10) has a high percentage of chemosensitive neurons (70–80%) and the magnitude of the response to hypercapnia is high (approximately 235%) compared with older neonates (125%; Hartzler et al., 2007). Additionally, the percentage of LC neurons and the CI is not affected by CARB in rats younger than P10 (Hartzler et al., 2007; Nichols et al., 2008). On the other hand, the CO<sub>2</sub> chemosensitive response of LC neurons of older neonates (>P10) is reduced by 20% in the presence of CARB, without affecting the CI, suggesting that the electrical coupling increases the responsiveness to CO<sub>2</sub> of LC neurons in newborn rats (Hartzler et al., 2007; Nichols et al., 2008; Gargaglioli et al., 2010).

A recent study by our group investigated the participation of gap junctions in the CO<sub>2</sub> ventilatory response in unanesthetized adult rats by bilaterally microinjecting CARB into the LC of Wistar rats (Patrone et al., 2014). During normocapnic conditions, the gap junctions have no regulatory role on ventilation, since CARB microinjection did not change the resting ventilation (Patrone et al., 2014). Regarding hypercapnic ventilatory response, our findings corroborate the literature since CARB (1 mM or 3 mM) microinjection into LC neurons resulted in a significant reduction, approximately 24 and 20%, respectively, in the ventilatory response to 7% CO<sub>2</sub>. This result was confirmed by the lower slopes of the 1 and 3 mM CARB CO<sub>2</sub> sensitivity curves compared to the curve for vehicle-injected rats (**Figure 1**). Therefore, our data suggest that gap junctions in the LC are important for modulation of the CO<sub>2</sub> drive to breathe in adult rats.

Recent studies have also addressed whether LC electrical synapses are involved in cardiovascular regulation. Microinjection of CARB in LC did not affect cardiovascular parameters during normocapnia, suggesting that gap junctions in LC neurons are unlikely to play a role in the tonic control of cardiovascular function. However, heart rate decreased after CO<sub>2</sub> exposure in the group treated with 3 mM CARB, indicating a possible role of LC neuronal gap junctions in the regulation of heart rate during CO<sub>2</sub> challenge. Summarizing, electrical synapses in LC neurons, specifically through gap junctions, play a role in the CO<sub>2</sub> drive to breathe and also modulate heart rate under hypercapnic conditions.



## NEUROCHEMICAL MODULATION OF THE LC GLUTAMATE

Glutamate is an endogenous amino acid that acts as a major excitatory neurotransmitter in the mammalian CNS (Ruggiero et al., 2011) and participates in the central generation and transmission of respiratory rhythm (Bonham, 1995). Glutamate receptors are divided into two subtypes, ionotropic and metabotropic. Ionotropic receptors can be further divided in NMDA (N-methyl-D-aspartate) and non-NMDA (AMPA and Kainate). The NMDA receptors bind simultaneously with glutamate and glycine, resulting in the influx of Na<sup>+</sup> and Ca<sup>2+</sup>, whereas the non-NMDA receptors are more rapidly depolarized, causing a Na<sup>+</sup> influx (Bowie, 2008). The metabotropic receptors are G-protein coupled, and they can be divided in mGlu I, II, and III. These receptors appear to be related with presynaptic regulation, and they also modulate the transmission of the respiratory rhythm to phrenic motoneurons; however, they are not involved in respiratory rhythmogenesis (Liu et al., 1990).

Glutamate is a primary excitatory neurotransmitter in the LC (Singewald and Philippu, 1998), and several studies identified different subunits of ionotropic glutamate receptors, with the majority belonging to the non-NMDA category (Sato et al., 1993; Wisden and Seeburg, 1993; Petralia et al., 1994). Experiments using anesthetized rabbits demonstrated that activation of LC neurons with L-glutamate increased the respiratory frequency and discharge rate of the phrenic nerve, while decreasing duration of inspiration and expiration (Li et al., 1992).

The major glutamatergic afferents to the LC come from the lateral nucleus paragigantocellularis (Ennis et al., 1992). Another important source of glutamate inputs are neurons localized in the lateral hypothalamus, which are also responsible for producing orexin (Peyron et al., 1998). A previous study performed by Henny

et al. (2010) demonstrated that a subset of orexinergic terminals have the ability to release glutamate in addition to orexin, and do play a role in postsynaptic targets via glutamatergic synapses, including terminals in the LC. More recently, it was demonstrated that catecholaminergic neurons of the rostral ventrolateral medulla (RVLM), most likely C1 neurons, establish a glutamatergic synapse with LC (Holloway et al., 2013). LC noradrenergic neurons are important for wake-promoting systems; therefore, glutamatergic signaling and LC neurons could be important to the state-dependent control of breathing (Gonzalez and Aston-Jones, 2006).

Recently, we have investigated the role of glutamatergic inputs in the LC modulation of the ventilatory response to hypercapnia in unanesthetized rats by applying Kynurenic Acid (KY, an antagonist at ionotropic glutamate receptors) or  $\alpha$ -methyl-4-carboxyphenylglycine (MCPG, an antagonist at metabotropic glutamate receptors; Taxini et al., 2013). Microinjections of MCPG did not affect cardiorespiratory responses during normocapnia or hypercapnia. Remarkably, microinjection of KY into the LC amplified pulmonary ventilation during 7% CO<sub>2</sub>, indicating that glutamatergic inputs on LC neurons cause an inhibition of the hypercapnia-induced hyperpnea. Our results are comparable to previous evidence in the literature that glutamate acts on ionotropic glutamatergic receptors in the LC and RVLM, thus inhibiting the ventilatory response to hypoxia (Dillon et al., 1991; Ferreira et al., 2004). Despite the fact that glutamate is classically known as an excitatory neurotransmitter in the CNS, there is also evidence that glutamate acts on NMDA receptors exerting a “functionally inhibitory” role, by suppressing circuit-level neural activity through the activation of GABAergic interneurons (Fitzgerald, 2012). Thus, inhibition of NMDA receptors by antagonist drugs may activate GABAergic neurotransmission as observed in rat prefrontal cortex (Homayoun and Moghaddam, 2007). Presumably, the increased ventilatory response to CO<sub>2</sub> after NMDA blockade could be linked to inhibition of inhibitory interneurons presents in the LC, which accounts for approximately 8% of LC neurons (Iijima and Ohtomo, 1988; de Carvalho et al., 2010). A second possible explanation is that the projections from the LC to the central respiratory pattern generator, or directly to the respiratory premotoneurons, may be mediated by LC GABAergic neurons (Figure 2). Therefore, the attenuation of the hypercapnic ventilator response, mediated by LC ionotropic receptors, can be the result of excitatory projections to glycinergic Bötzing (BÖTZ) neurons, which participates in the termination of inspiration (Champagnat et al., 1982; Bonham, 1995; Schreihöfer et al., 1999). Thus, a possible role of LC glutamatergic mechanisms could be in limiting the extent of hypercapnia-induced hyperpnea. However, the interaction between the LC neurons and the glycinergic BÖTZ neurons is still unclear.

With respect to cardiovascular regulation, glutamatergic receptor antagonism did not affect blood pressure and heart rate during normocapnia and hypercapnia, supporting previous findings which demonstrate that the inhibition of LC neurons (Sved and Felsten, 1987; Miyawaki et al., 1991) or electrolytic lesions of this nucleus (Anselmo-Franci et al., 1998) have no effect on cardiovascular parameters.

## SEROTONIN

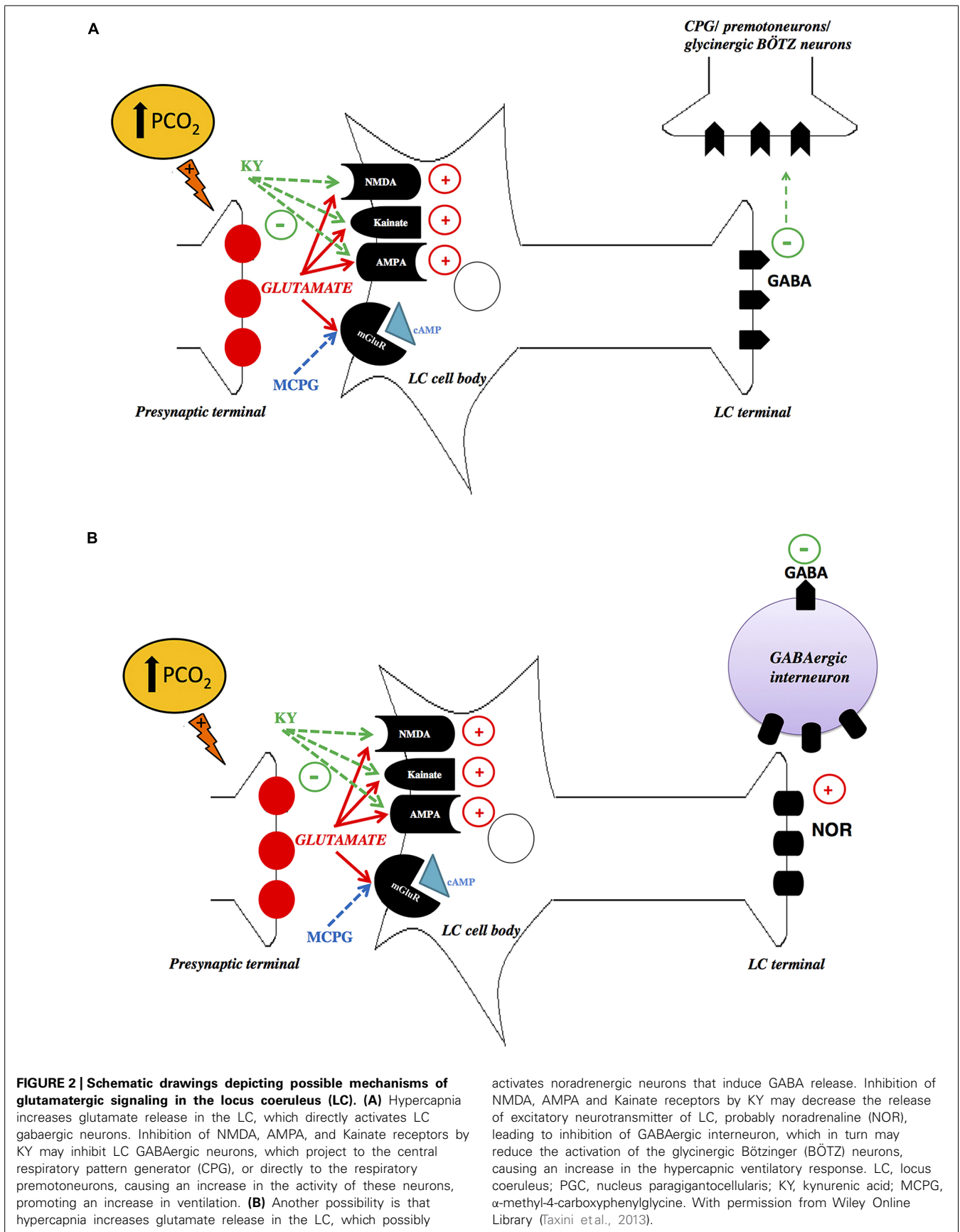
Serotonin (5-hydroxytryptamine, 5-HT) is a neurotransmitter involved in many physiological functions such as the sleep–wake cycle, mood, appetite, and neurovegetative control. Serotonin can also be linked to emotional disorders such as anxiety and depression (Jacobs and Azmitia, 1992; Brown and Gershon, 1993; Jouvet, 1999; Saper et al., 2001; Jones, 2005). Serotonergic neurons are located mainly in the raphe nuclei and project to almost all brain areas and the spinal cord (Törk, 1990; Baumgarten and Göthert, 1997).

Extensive reciprocal projections are reported between raphe and LC neurons (Pasquier et al., 1977; Pickel et al., 1977; Cedarbaum and Aghajanian, 1978; Morgane and Jacobs, 1979; Baraban and Aghajanian, 1981; Vertes and Kocsis, 1994; Luppi et al., 1995; Peyron et al., 1996; Kim et al., 2004). In fact, LC neurons receive dense serotonergic projections coming mostly from dorsal raphe (DR) and pericoerulear 5-HT neurons (Aston-Jones et al., 1991a; Kaehler et al., 1999; Kim et al., 2004), and these projections are important for controlling sleep–wake cycle, vigilance, analgesia, cognition, depression, pain and anxiety (Redmond and Huang, 1979; Segal, 1979; Charney and Redmond, 1983; Uhde et al., 1984; Mokha et al., 1985; Meltzer and Lowy, 1987; Seiver, 1987; Kim et al., 2004). Stimulation of central 5-HT neurons causes reductions in the LC spontaneous and pain-evoked activity (Segal, 1979) and 5-HT, when applied in the LC, attenuates the excitatory responses of this nucleus to sensorial, neurochemical and electrical stimuli (Bobker and Williams, 1989; Aston-Jones et al., 1991a).

Administration of the 5-HT<sub>1A</sub> receptor antagonist WAY-100635 in anesthetized rats suppressed LC activity. Interestingly, this effect can be prevented by lesioning central 5-HT neurons (injection of 5,7 DHT intracerebroventricularly) or by 5-HT<sub>2</sub> receptor antagonism, indicating that the suppressant effect of WAY-100635 on noradrenergic neuron firing is dependent on intact 5-HT neurons and also involves an over-activation of excitatory 5-HT<sub>2</sub> receptors in inhibitory neurons projecting to LC (Haddjeri et al., 1997; Szabo and Blier, 2001).

Recently, we have demonstrated that hypercapnia induces an increase in 5-hydroxyindole-3-acetic acid (5-HIAA) levels and serotonergic turnover (5-HTTIA/5-HT ratio) in the LC of rats (de Souza Moreno et al., 2010). In this study, we also microinjected WAY-100635, Ketanserin (5-HT<sub>2A</sub> antagonist), or DOI (5-HT<sub>2A</sub> agonist) into the LC of unanesthetized rats to verify the role of 5-HT receptors in the CO<sub>2</sub> ventilatory response. Antagonism of 5-HT receptors in the LC had no effect on resting breathing parameters (de Souza Moreno et al., 2010). Microinjection of WAY-100635 into the LC decreased the ventilatory response to CO<sub>2</sub>, possibly due to inhibition of noradrenergic neurons. The reduction of the response to CO<sub>2</sub> by WAY-100635 may be due to its direct antagonism of presynaptic 5-HT<sub>1A</sub> receptors, leading to an increase in 5-HT release and consequential activation of postsynaptic 5-HT<sub>2A</sub> receptors in the LC.

Another possible explanation for our results is that WAY-100635 may decrease the glutamate evoked activation of LC neurons. In this regard, Aston-Jones et al. (1991a) has previously demonstrated that 5-HT decreased glutamate evoked activation



of LC cells using extracellular recordings from single neurons in an anesthetized preparation. Moreover, Bobker and Williams (1989) showed that the receptors mediating this inhibition are the 5-HT<sub>1A</sub> and 5-HT<sub>1B</sub> subtypes, probably located presynaptically in the PGI terminals, since LC receives afferent excitatory glutamatergic inputs from the PGI nucleus (Ennis and Aston-Jones, 1987). Therefore, this interaction between glutamate and 5-HT may play a role in preventing a strong inhibition of LC during hypercapnia.

In fact, microinjections of Ketanserin in the LC increased the hypercapnic hyperpnea, whereas the 5-HT<sub>2A</sub> receptor agonist DOI evoked an opposite effect. Therefore, we suggested that release of 5-HT is increased in the LC during CO<sub>2</sub> challenge and that 5-HT negatively modulates the LC stimulatory role in the hypercapnic ventilatory response, probably acting through postsynaptic 5-HT<sub>2A</sub> receptors or presynaptic 5-HT<sub>2A</sub> in GABAergic terminals. The possible action of 5-HT on GABAergic terminals corroborates previous study that reported that GABA<sub>A</sub> receptor antagonist bicuculline on LC neurons blocked the inhibitory effects of intravenously injected DOI on LC firing (Chiang and Aston-Jones, 1993).

### ATP

ATP is an important intracellular energy source for all cells and is present in all mammalian neurons (Edwards and Gibb, 1993). Burnstock (1972) proposed the concept of purinergic signaling, after the observation that a purine nucleotide could act as a neurotransmitter in non-adrenergic, non-cholinergic (NANC) nerves supplying the gut and urinary bladder. Currently, it is established that ATP acts as a sole neuromodulator or is co-released with other neuromodulators in the peripheral and in the CNS (Abbracchio et al., 2009).

In the CNS, ATP acts on regions involved in cardiovascular and respiratory regulation, including the LC (Thomas and Spyer, 2000; Thomas et al., 2001; Gourine et al., 2002, 2005; Antunes et al., 2005; Yao and Lawrence, 2005). LC neurons are of particular interest as purinergic signaling targets because ATP can act as a neuromodulator on neurons terminating within the LC or as a co-transmitter of recurrent axonal collaterals or dendrites of intrinsic LC neurons (Tschöpl et al., 1992; Nieber et al., 1997; Poelchen et al., 2001). Poelchen et al. (2001) reported for the first time that ATP and NA are co-released from recurrent axon collaterals onto LC neurons producing biphasic synaptic potentials consisting of early depolarizing (eps<sub>p</sub> = excitatory postsynaptic potential) and late hyperpolarizing (ipsp = inhibitory postsynaptic potential) components, respectively.

P2 purinergic receptors are activated by ATP (Burnstock, 1997) and are classified into P2X (ligand-activated cationic channel, ionotropic receptor) and P2Y (G-protein-coupled receptor, metabotropic receptor) subtypes (Abbracchio and Burnstock, 1994; Fredholm et al., 1994; Mateo et al., 1998). The LC contains more P2X receptors, and the P2X population consists mainly of the P2X<sub>2</sub>, P2X<sub>3</sub>, and P2X<sub>6</sub> subtypes (Yao et al., 2000). Although P2X<sub>7</sub> receptors have been found in LC area and it appeared that these receptors are located in astrocytes (Khakpay et al., 2010). A short stimulation of the P2X<sub>7</sub> receptors leads to activation of cationic currents as the other subtypes of P2X receptors although a

repeated or prolonged ATP exposure also opens a large pore which allows the passage of organic cations and dye molecules (Virginio et al., 1999).

ATP evokes the release of NA (Poelchen et al., 2001) and ATP within LC, when acting on presynaptic P2X receptors (Boehm, 1999), and inhibits the release of NA and ATP, when acting on presynaptic P2Y receptors (von Kugelgen et al., 1994, 1995; Poelchen et al., 2001). In LC neurons, activity of NA on postsynaptic  $\alpha$ 2-adrenoceptors has been shown to mediate hyperpolarization (Aghajanian and Vandermaelen, 1982; Poelchen et al., 2001), while activation of both P2X and P2Y receptors by ATP has been shown to depolarize LC neurons (Harms et al., 1992; Shen and North, 1993; Scheibler et al., 2004). Thus, ATP may be released to provide an excitatory counterbalance to the inhibitory noradrenergic drive of these neurons (Illes et al., 1994).

There is evidence that tyrosine hydroxylase (TH) co-localizes with P2X receptors in the LC (Yao et al., 2000) indicating the presence of P2X receptors in LC noradrenergic neurons. Moreover,  $\alpha$ , $\beta$ -meATP (a P2X receptor agonist) excites LC neurons in *in vitro* preparations of the pons (Tschöpl et al., 1992; Shen and North, 1993), increasing the frequency of spontaneous action potentials in LC (Tschöpl et al., 1992).

On the other hand, Kuwahata (2004) demonstrated that ATP produced a hyperpolarizing response or a transient hyperpolarizing response that preceded the depolarization in LC neurons. However, AMP-PNP (adenosine 5'-( $\beta$ , $\gamma$ -imido) triphosphate, which is more resistant to dephosphorylation than ATP, only depolarizes LC neurons. According to Kuwahata (2004) adenosine is the likely source of the ATP-induced hyperpolarizing response in LC neurons.

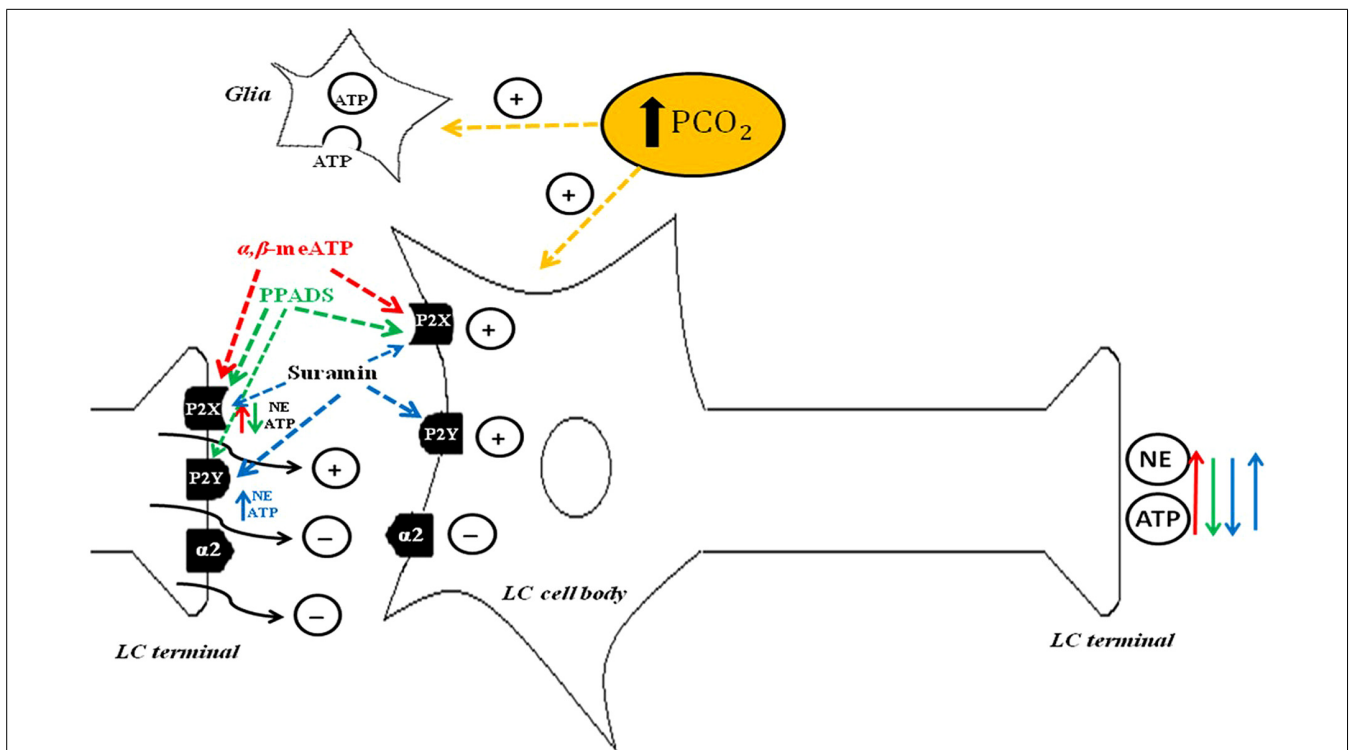
Despite the excitatory effects of ATP in LC neurons, little is known about the physiological consequences of ATP when administered in the LC *in vivo*, with antinociception being an exception (Fukui et al., 2004). A recent study by our group focused on the role of ATP in the cardiorespiratory control through P2 receptor-mediated mechanisms in the LC of unanesthetized rats (Biancardi et al., 2014). In this study we provided the first functional evidence that purinergic neuromodulation within the LC is important for cardiorespiratory control in unanesthetized animals. In normocapnic conditions, ATP release within the LC may occur to maintain respiratory frequency (Biancardi et al., 2014). We observed that PPADS (ATP antagonist with predominant actions on P2X receptors) decreased respiratory frequency, whereas suramin (P2 non-selective antagonist) did not change any of the investigated respiratory variables (Biancardi et al., 2014), when injected into the LC.

Microinjections of ATP and the P2X receptor agonist  $\alpha$ , $\beta$ -meATP into brainstem respiratory nuclei, such as the *nucleus tractus solitarius* (NTS) and RVLM, also cause changes in respiratory amplitude and frequency (Thomas et al., 2001; Antunes et al., 2005). Several studies have shown that hypercapnia triggers ATP release in the RVLM (for review see Gourine et al., 2005). The injection of P2X receptor antagonists in this region reduces the ventilatory response to hypercapnia, indicating that ATP, acting on P2X receptors, plays a critical role in the chemoreception to CO<sub>2</sub>/pH (Thomas and Spyer, 2000; Gourine et al., 2005).

Biancardi et al. (2008) also observed that the ablation of about 80% of LC noradrenergic neurons was associated with a large decrease (64%) in the ventilatory response to increased  $\text{CO}_2$ . More recently, we observed that microinjections of  $\alpha, \beta$ -meATP into the LC promoted a greater increase in ventilation during the hypercapnic challenge (7%  $\text{CO}_2$ ), compared with the controls (Biancardi et al., 2014). This was blocked by pretreatment with PPADS, indicating that  $\alpha, \beta$ -meATP is acting on P2X receptors to evoke that effect. Thus, LC purinergic signaling appears to play a role in the  $\text{CO}_2$  drive to breathe, specifically through the activation of P2 receptors, accelerating the ventilatory response to hypercapnia. This observation was probably due to P2X activation of noradrenergic neurons, triggering further NA and ATP release which in turn activate rhythm-generating circuits and medullary respiratory neurons in one or more of the following brainstem nuclei: RTN, RVLM, prepositus hypoglossi nucleus, and NTS (Aston-Jones et al., 1986, 1991b; Astier et al., 1990; Pieribone and Aston-Jones, 1991; Vulchanova et al., 1997; Llewellyn-Smith and Burnstock, 1998; Kanjhan et al., 1999; Thomas and Spyer, 2000; Yao et al., 2000; Gourine et al., 2003; Lorier et al., 2007, 2008; Huxtable et al., 2009).

Unexpectedly, suramin induced an increase in ventilation during  $\text{CO}_2$  exposure. However, this effect occurred 20 min after the microinjections, while the effect of the P2X agonist appeared only 2 min after microinjections. These observations suggest that suramin may be acting on presynaptic P2Y receptors, which, due to location and metabotropic mechanism of activation, may introduce a delay in the response. Given that these receptors are G-protein coupled (Abbracchio and Burnstock, 1994), the latency to induce neurotransmitter release is prolonged.

Yao and Lawrence (2005) suggested that ATP modulates the cardiovascular system via activation of P2X receptors in the LC of anesthetized rats. Moreover, they hypothesize that purinergic and noradrenergic systems are tonically active within the LC and interact functionally. They also demonstrated that microinjections of a P2X receptor agonist into the LC induced hypotension and bradycardia in anesthetized animals. Conversely, microinjections of P2 receptor antagonists induced hypertension and tachycardia. In another study, Biancardi et al. (2014) evaluated the role of ATP in the modulation of cardiovascular responses to  $\text{CO}_2$  exposure. They observed that both PPADS and suramin induced an increase



**FIGURE 3 | Schematic drawing depicting possible mechanisms of purinergic signaling in the locus coeruleus (LC).** ATP induces depolarization of noradrenergic neurons through P2X and P2Y postsynaptic receptors (Harms et al., 1992; Shen and North, 1993), NE and ATP release through P2X presynaptic receptors (Boehm, 1999) and inhibition of NE and ATP release through P2Y presynaptic receptors (von Kugelgen et al., 1994, 1995; Poelchen et al., 2001).  $\alpha, \beta$ -meATP agonism of P2X presynaptic receptors may promote NE and ATP release, whereas agonism of P2X postsynaptic receptors may activate noradrenergic neurons to further increase NE and ATP release (red arrow, Nieber et al.,

1997). PPADS blocks mainly P2X receptors, which reduces NE and ATP release at LC terminals (green arrow). Suramin blocks P2X and P2Y receptors. The postsynaptic receptors reduce neurotransmitter release (blue down arrow). The presynaptic P2Y receptor subtype increases NE and ATP release, thereby further activating noradrenergic neurons and causing additional NE and ATP release (blue up arrow).  $\text{PCO}_2$  increase in the cerebrospinal fluid activates LC noradrenergic neurons (yellow arrow; Biancardi et al., 2008) and astrocytes (yellow arrow; Alvarez-Maubecin et al., 2000; Spyer et al., 2004) which, in turn, release ATP. With permission from Wiley Online Library (Biancardi et al., 2014).

in mean arterial pressure (MAP) under hypercapnic conditions, although  $\alpha, \beta$ -MeATP did not change cardiovascular parameters in unanesthetized animals. This increase in MAP may be due to a blockade of P2X and P2Y postsynaptic receptors, which may attenuate depolarization of LC noradrenergic neurons, thereby reducing NA release. A decrease in NA, in turn, decreases RVLM inhibition and promotes pressor response.

**Figure 3** depicts proposed LC purinergic neuromodulation mechanisms based on Biancardi et al. (2014) and evidence from Williams et al. (1985), Aghajanian and Wang (1986), Harms et al. (1992), Shen and North (1993), von K ugelgen et al. (1994), von K ugelgen et al. (1995), Nieber et al. (1997), Boehm (1999), Alvarez-Maubecin et al. (2000), Poelchen et al. (2001), Spyer et al. (2004), and Biancardi et al. (2008).

Until recently, there were no studies available in the literature regarding ATP-mediated respiration mechanisms in the LC. However, a recent study from our group (Biancardi et al., 2014) introduced the idea of the participation of purinergic signaling in the LC in the modulation of respiration. Further investigation is needed, nevertheless, to address how hypercapnic challenges modulate purinergic signaling within the LC. An increase in arterial blood PCO<sub>2</sub> triggers immediate ATP release from ventral chemosensory site(s) on the surface of the medulla, and glial cells appear to be the likely source of this ATP release in response to such stimuli (Gourine et al., 2005). The striking abundance of astrocytes in the LC (Alvarez-Maubecin et al., 2000) supports the idea that these cells may play a distinctive role in that nucleus. Based on the reviewed literature, we believe that the field would benefit greatly from further investigation of the possibility that hypercapnic exposure induces ATP release from LC astrocytes, which contribute to modulation of respiratory activity.

## CONCLUSION

We have reviewed evidence that LC neurons exert an important role in hypercapnic ventilatory response, and this response is chemically modulated by 5-HT, glutamate, ATP and electrically controlled by GAP junctions. As it is well known, LC is an important pontine group that has access to respiration-related cell groups in the caudal brainstem, and also modulates the sleep wake-cycle and many other homeostatic function. Therefore, this nucleus can be considered a gateway to adjust respiration to sleep-wake events. In addition, LC has been linked to pathological conditions such as Rett syndrome, where individuals present decreased CO<sub>2</sub> chemosensitivity. This CNS dysfunction is characterized by the loss of function mutations in the X-linked gene encoding in Methyl-CpG-binding protein-2 (MeCP2; Amir et al., 1999). MeCP2 knockout mice presents morphological (decreased soma size), electrical (reduced membrane conductance and strong afterhyperpolarization amplitude), and neurochemical (reduced TH content) alterations of LC neurons (Taneja et al., 2009). However, the neurobiological mechanisms underlying such breathing disorders presented by people with Rett syndrome are still unclear. According to Taneja et al. (2009), LC is a critical site at which loss of MeCP2 results in CNS dysfunction and restoration of normal LC function may attenuate the symptoms of this syndrome. Therefore, a better comprehension of LC and its modulators in breathing control is needed to clarify this issue.

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