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THE LOCUS COERULEUS AND CENTRAL CHEMOSENSITIVITY

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Abstract

The locus coeruleus (LC) lies in the dorsal pons and supplies noradrenergic (NA) input to many regions of the brain, including respiratory control areas. The LC may provide tonic input for basal respiratory drive and is involved in central chemosensitivity since focal acidosis of the region stimulates ventilation and ablation reduces CO_2-induced increased ventilation. The output of LC is modulated by both serotonergic and glutamatergic inputs. A large percentage of LC neurons are intrinsically activated by hypercapnia. This percentage and the magnitude of their response are highest in young neonates and decrease dramatically after postnatal day P10. The cellular bases for intrinsic chemosensitivity of LC neurons are comprised of multiple factors, primary among them being reduced extracellular and intracellular pH, which inhibit inwardly rectifying and voltage-gated K^+ channels, and activate L-type Ca^{2+} channels. Activation of K_{Ca} channels in LC neurons may limit their ultimate response to hypercapnia. Finally, the LC mediates central chemosensitivity and contains pH-sensitive neurons in amphibians, suggesting that the LC has a long-standing phylogenetic role in respiratory control.

Keywords

rodent; amphibian; respiration; chemosensitive signaling; serotonin; glutamate; K channel; hypercapnia; development

1. Introduction

Locus coeruleus (LC) is a well-delineated cluster of noradrenergic neurons located bilaterally adjacent to the fourth ventricle in the pontine region of the brainstem (Dahlström and Fuxe, 1964). It is estimated that ~50% of all the noradrenergic projections in the central nervous system originate in the LC which are directed toward the forebrain, cerebellum, brainstem and spinal cord (Aston-Jones *et al.* 1995; Berridge and Waterhouse, 2003). The LC exhibits arousal-state-dependent activity and it is considered a major wakefulness

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promoting nucleus with activation of the LC resulting in an increase in EEG and signs of alertness (Samuels and Szabadi, 2008)

The LC is implicated in the control of many homeostatic functions including maintenance of attention, motivation, arousal states (Svensson and Thorén, 1979; Bhaskaran and Freed, 1988), sleep (Aston-Jones and Bloom, 1981), circadian regulation of arousal and performance (Aston-Jones *et al*., 2001), cognitive behaviors (for review see Sara, 2009), fever response (Almeida *et al*., 2004), control of breathing (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 Souza-Moreno *et al*., 2010), and cardiovascular function (Sved and Felsten, 1987). This nucleus is involved in some pathophysiological states as well, such as panic disorder (Charney *et al*., 1990; Kaplan, 1992; Sullivan *et al*., 1999; Bailey *et al*., 2003; Griez and Schruers, 2003) and Rett syndrome (RTT) (Taneja *et al*., 2009).

Regarding the regulation of breathing, LC neurons have been shown to be involved in the central respiratory network (Coates *et al.*, 1993; Oyamada *et al*., 1998; Biancardi et al., 2008). Further, CO_2/H^+ sensitive neurons have been identified in the LC (Elam *et al.*, 1981; Pineda and Aghajanian, 1997; Filosa *et al.*, 2002). In fact, LC neurons have been extensively studied with respect to the bases for and mechanisms of chemosensitive signaling (Putnam *et al.*, 2004).

In this review, we will discuss the evidence for involvement of LC in basal respiratory drive as well as central chemosensitivity. We will further consider studies of the basis for cellular CO_2/H^+ sensitivity in chemosensitive LC neurons. Finally, we will examine evidence that shows LC neurons play a role in the control of breathing in amphibians as well as in mammals.

2. LC neurons and basal respiratory drive

In mammals there are some studies demonstrating that LC neurons display a respiratory-related activity, i.e., they have direct access to information about the timing of the respiratory output from the medullary respiratory centers (Oyamada *et al*., 1998, 1999; Andrzejewski *et al*., 2001). Electrical and chemical stimulation applied to the LC attenuates the inspiratory inhibition caused by electrical stimulation at the Bötzinger Complex, suggesting that the LC also plays a role in the modulation of the inspiratory inhibition of Bötzinger Complex stimulation (Wang *et al*., 2004). LC neurons also exert a tonic inhibitory effect on IX respiratory activity in neonatal rat brainstem-spinal cord preparations via alpha2 adrenergic receptor indicating that LC regulates upper-airway expiratory activity as well (Yamanishi *et al*., 2008).

The Phox2a gene is responsible for differentiation of catecholaminergic neurons in restricted areas such as LC (Viemari *et al*., 2004). The inactivation of *Phox2a* leads to the agenesis of the LC proper, but leaves intact all the other noradrenergic centers: the locus subcoeruleus and groups A7, A5, A2, and A1 (Morin *et al.*, 1997; Pattyn *et al*., 2000). Complete *Phox2a* inactivation produces depression of the central respiratory generator and elimination of LC and sensory afferent neurons (Wrobel *et al*., 2007). Elimination of the LC in *Phox2a*$^{-/-}$ mutants is linked to a severe decrease in the breathing frequency (Viemari *et al*. 2004). LC contributes to the adaptation of breathing to physiological needs and according to some studies it provides a tonic excitatory drive that contributes to a normal breathing rate in rats (Guyenet *et al*., 1993; Jodkowski *et al*., 1997; Oyamada *et al*., 1998; Dawid-Milner *et al*., 2001; Li and Nattie 2006) and mice (Shirasawa *et al*., 2000; Hilaire *et al*., 2004). In this context, Hilaire *et al*. (2004) demonstrated that LC noradrenergic neurons provide a tonic excitatory stimulus that maintains breathing frequency and are necessary for the development of a normal respiratory rhythm. Recently, Li and Nattie (2006) showed that

substantial lesions of brainstem catecholaminergic neurons (including LC) slow breathing frequency during air breathing and that this effect is present in both wakefulness and in NREM sleep. Thus, taken together these data suggest that LC noradrenergic neurons provide a tonic drive to breathe. However, Biancardi *et al*. (2008) demonstrated that selective lesion of the LC in adult rats using 6-OHDA (a toxin that selectively eliminates catecholaminergic neurons) did not change basal ventilation and breathing frequency (Fig. 1A), suggesting that noradrenergic neurons located in the LC play no role in respiratory control under resting conditions in adults. Further, injection of a potent toxin conjugate, SP-SAP, in the LC of adult rats for killing neurons expressing the neurokinin-1 (NK-1) receptor did not alter adult breathing under basal conditions (Fig. 1B, Carvalho *et al*., in press). In agreement with this notion, LC unilateral cooling in neonatal sheep did not affect breathing in normoxic normocarbic conditions (Moore *et al*., 1996). The differences between these results and Li and Nattie's study may be due to the fact the elimination of noradrenergic neurons also results in the loss of neurons not located in the LC, since DBH-SAP was injected via the 4th ventricle promoting elimination of other catecholaminergic groups such as A5, A7, C1 and C2. Another possible explanation could be that most of the previous cited studies were performed using anesthetized rats (Guyenet *et al*., 1993; Jodkowski *et al*., 1997; Dawid-Milner *et al*., 2001) or neonatal preparations (Oyamada *et al*., 1998; Shirasawa *et al*., 2000; Hilaire *et al*., 2004) whereas we used unanesthetized and adult animals. Whatever the explanation, in all of our lesion or microinjection studies using animals with manipulations specifically performed in the LC, no difference in basal ventilation has been observed (Biancardi *et al*., 2008; Biancardi *et al*., 2010; de Souza-Moreno, 2010; Carvalho *et al*., *in press*).

3. LC and central chemosensitivity

3.1. Studies in intact animals

3.1.1. Lesion and stimulation studies—Some studies have demonstrated that the c-fos technique can be used to identify neurons involved in the responses elicited by hypercapnia (Haxhiu *et al*., 1996; Teppema *et al*., 1997; Berquin *et al*., 2000). Although neuronal function cannot be inferred from Fos expression, these studies brought new insight into the anatomical distribution of putative intrinsically chemosensitive neurons within chemoreflex pathways (Berquin *et al*., 2000). In mammals, studies under *in vivo* conditions showed that CO_2 stimulation increases the expression of the c-Fos gene in LC neurons (Haxhiu *et al*., 1996; Teppema *et al*., 1997). In addition, extracellular recordings from LC neurons in both neonatal and adult rats showed that they respond to systemic hypercapnia with an increase in spike frequency under *in vivo* conditions (Elam *et al*., 1981). Elam *et al.* (1981) demonstrated a dose-dependent increase in firing frequency of LC neurons in response to hypercapnia (3%–20% CO_2) under *in vivo* conditions, such that the response at ~7% CO_2 would correspond to a ~25% increase in firing frequency. Additionally, using a brain slice preparation, Stunden *et al*. (2001) reported a ~44% increase in firing frequency of LC neurons when solution CO_2 was increased from 5 to 10% and Filosa *et al.* (2002) saw a 93% increase in LC firing rate when solution CO_2 was increased from 5 to 15% CO_2. Thus, the firing response of LC neurons to hypercapnia appears to be dose dependent both in *in vivo* and *in vitro* preparations, although this response also appears to saturate at high levels (between 10–20%) of CO_2 (Pineda and Aghajanian, 1997; Ritucci *et al*., 2005).

One of the first pieces of evidence that demonstrated that LC neurons may function directly as respiratory CO_2/pH chemosensors was reported by Coates *et al*. (1993). In this study, the authors injected acetazolamide, which produces a small and focal acidosis, into various brainstem sites (Coates *et al*., 1993). Focal acidification of LC noradrenergic neurons promotes a large increase in phrenic nerve discharge (37%) in cats. The LC neurons are of particular interest in CO_2 challenge since >80% of these neurons are found to be

chemosensitive, responding to hypercapnia with an increased firing rate (Pineda and Aghajanian, 1997; Oyamada *et al*., 1998; Filosa *et al*., 2002). Recently, Johnson *et al*. (2008) using Prp57 transgenic mice, which express green fluorescent protein (GFP) in the LC, reported that LC neurons exhibited chemosensitivity in culture after pharmacological block of fast excitatory and inhibitory synaptic transmission. According to the authors, more than 85% of GFP-positive LC neurons are stimulated by elevated CO_2/H^+ in culture after pharmacological block of fast excitatory and inhibitory synaptic transmission.

Lesions of catecholaminergic (CA) neurons of the rat brainstem, resulting in approximately 84% of LC-CA neurons being eliminated, significantly decreased the ventilatory response to 7% CO_2, by 28% during sleep and wakefulness, suggesting that brainstem CA neurons participate in central chemoreception *in vivo* both in NREM sleep and wakefulness (Li and Nattie, 2006). These findings suggest that the LC is an important component of the hypercapnic ventilatory response. Recently, Biancardi *et al*. (2008) have investigated the participation of LC noradrenergic neurons in the CO_2-induced drive to breathing. The data indicate that LC noradrenergic neurons modulate the hypercapnic ventilatory response since chemical lesion of this structure reduced the ventilatory response to 7% CO_2, due to a decreased V_T (Biancardi *et al*., 2008). A reduction of approximately 80% of the noradrenergic neurons of LC was associated with an approximately 64% decrease in the ventilatory effect on response to CO_2, indicating once again that the LC exerts a profound the CO_2-induced drive to breathe (Fig. 2). The 64% reduction in the ventilatory response after lesioning only the A6 region compared with the 28% reduction after lesions of noradrenergic (A5, A6, A7) and adrenergic (C1 and C3) neurons (Li and Nattie, 2006) could be due to: 1) differences in the percentage and the magnitude of the response of chemosensitive neurons from various brainstem areas (Putnam *et al*., 2004). It is well known that catecholaminergic cell groups have different brain projections that differentially modulate breathing. Some sites are excitatory to hypercapnic ventilatory response, while others are inhibitory. For instance, C1 adrenergic neurons provide a direct inhibitory input to the great majority of LC noradrenergic neurons (Aston-Jones *et al*., 1992); and 2) LC contains the highest percentage (>80%) of CO_2-activated neurons of any brainstem region (cf. Putnam *et al*., 2004), thus it is expected that a lesion of this site should have a significant effect on the ventilatory response to inspired CO_2, as observed by Biancardi *et al*. (2008).

In summary, these data suggest that catecholaminergic neurons within the LC are involved in the central chemoreceptive response that mediates hyperventilation in response to inspired CO_2 but may not play a significant role in the drive to breathe under normocapnic conditions (see Section 2), at least in adults.

3.1.2. Serotonergic modulation—A number of studies have suggested that 5-HT modulates the firing rate of LC noradrenergic neurons (Pickel *et al*., 1977; Cedarbaum and Aghajanian, 1978; Leger and Descarries, 1978; Haddjeri *et al*., 1997; Kaehler *et al*., 1999; Pudovkina *et al*., 2002; Kim *et al*., 2004) and that LC receives dense serotonergic projections coming mainly from dorsal raphé and pericoerulear 5-HT neurons (Aston-Jones *et al*., 1991; Kaehler *et al*., 1999; Kim *et al*., 2004). Indeed, the dorsal raphé (DR) nucleus exerts effects on LC activity through activation of 5-HT_{1A} and 5-HT_{2A} receptors (Svensson *et al*., 1975; Haddjeri *et al*., 1997).

In a recent study it was reported that inhibition of caudal medullary serotonergic neurons with 8-OH-DPAT had no significant effect on the inhibition of the raphé obscurus ventilatory response to CO_2, but simultaneous and the retrotrapezoid nucleus (RTN) produced an enhanced hypoventilation and a greater reduction of the CO_2 response (Li *et al*., 2006) suggesting that 5-HT neurons modulate breathing by interaction with RTN.

It is possible that 5-HT neurons modify LC output just as they do with RTN neurons. In fact, hypercapnia induced an increase in 5-hydroxyindole-3-acetic acid (5-HIAA) levels and serotonergic turnover (5-HTTIA/5-HT ratio) in the LC (de Souza Moreno *et al*., 2010). In this study, WAY-100635 (a 5-HT_{1A} receptor antagonist), 8-OHDPAT (5-$HT_{1A/7}$ agonist), Ketanserin (5-HT_{2A} antagonist), or DOI (5-HT_{2A} agonist) were microinjected into the LC of conscious, freely behaving rats to determine what 5-HT receptors were involved in the ventilatory response to CO_2. Microinjection of WAY-100635 into the LC decreased the ventilatory response to CO_2, probably through inhibition of NA neurons, while microinjection of Ketanserin increased the ventilatory response to hypercapnia due to an increase in V_T, suggesting that 5-HT acting on 5-HT_2 receptors in the LC exerts an inhibitory modulation on the ventilatory response to hypercapnia. Reinforcing this idea, it was shown that the 5-HT_{2A} receptor agonist DOI microinjected in the LC attenuates the CO_2 drive to breathing.

A possible mechanism through which serotonin (5-HT) modulates the role of the LC in the hypercapnia-induced hyperpnea is shown in Figure 3. According to this model, 5-HT release increases in the LC during hypercapnia and this 5-HT inhibits the LC stimulatory role in the hypercapnic ventilatory response, acting through postsynaptic 5-HT_{2A} receptors in this nucleus. Furthermore, this 5-HT release seems also to be regulated by presynaptic 5-HT_{1A} receptors, a mechanism that may play a role in preventing too great of an inhibition by serotonin of LC neurons during hypercapnia.

3.2. In vitro studies

Considerable progress has been made over the last 10–15 years in understanding the cellular pathways of CO_2/H^+ sensing in LC neurons. We now have a much clearer picture of the development of intrinsic chemosensitivity, the signaling pathways that activate LC neurons in response to elevated CO_2/H^+, and the ion channels that respond to these signals. These studies have been done using *in vitro* approaches with reduced preparations, almost exclusively involving pontine slices, although a recently described preparation with cultured LC neurons (Johnson *et al.*, 2008) and a slice study using voltage-sensitive dyes (Erlichman *et al.*, 2009), should prove to be quite useful for studying chemosensitive signaling. Below we will highlight the understanding that has been gained from the use of *in vitro* preparations.

3.2.1. Intrinsic chemosensitivity—In order to study the signaling of an LC neuron in response to increased CO_2/H^+ we need to assure that we are studying the *intrinsic* response of that neuron, and not the response of some other neuron within the slice that excites the neuron that we are studying through synaptic transmission, either chemical or electrical (gap junctions). There are several approaches to eliminating chemical and electrical synaptic activity and it is virtually impossible to assure, in all but isolated cultured cells, that there is no input from another neuron. However, one technique that has been commonly used (Dean *et al.*, 1990; Richerson, 1995; Oyamada *et al.*, 1998; Wellner-Kienitz and Shams, 1998) is to employ solutions with elevated Mg^{2+} and in some cases reduced Ca^{2+} as well. This blocks the entry of Ca^{2+} pre-synaptically and prevents neurotransmitter release as is shown by the disappearance of post synaptic potentials in the presence of this synaptic block medium (Nichols *et al.*, 2009). This approach has been used in conjunction with carbenoxolone, a blocker of gap junctions to study intrinsic chemosensitivity in LC neurons as well as neurons from the nucleus of the solitary tract (NTS), another chemosensitive area (Dean *et al.*, 2001; Conrad *et al.* 2009; Nichols *et al.*, 2009). The use of blockers of both chemical and electrical synapses to study intrinsic chemosensitivity in neurons from the solitary tract (Conrad *et al.*, 2009) has been described as "…one of the two most thorough analyses of intrinsic neuronal chemosensitivity that I know…" (Leiter, 2009), the other study being

conducted on LC neurons (Hartzler *et al.*, 2007). The results of the latter study will be discussed in the next section.

3.2.2. Development of intrinsic chemosensitivity—In an earlier study it was reported that the firing rate response of LC neurons to hypercapnia was essentially constant from the first postnatal day (P1) until near weaning (P16) (Stunden *et al.*, 2001). These measurements were made without the use of either chemical synaptic blockade or gap junction blocker. Thus, in more recent studies these measurements have been repeated in the presence of synaptic block medium (elevated Mg^{2+}, 11.4 mM, and reduced Ca^{2+}, 0.2 mM) and carbenoxolone (100 μm) (Hartzler *et al.*, 2007; Nichols *et al.*, 2008). Interestingly, under these conditions there is a marked developmental change in the intrinsic chemosensitivity of LC neurons. In LC neurons from neonates younger than postnatal day P10, a high percentage (70–80%) of neurons were chemosensitive (Fig. 4A) and the magnitude of the response, as measured by the chemosensitivity index (CI; Wang and Richerson, 1999), was very high (about 235%) (Fig. 4B), similar to some of the most chemosensitive neurons measured *in vitro* from the medullary raphé (Wang *et al.*, 1998). Neither the percentage of neurons nor the chemosensitivity index were affected by synaptic blockade medium or carbenoxolone in LC neurons from young neonates (<P10) indicating that these neuronal responses to hypercapnia were intrinsic (Hartzler *et al.*, 2007; Nichols *et al.*, 2008). In contrast, in LC neurons from older neonates (>P10) a high percentage of neurons were also activated by hypercapnia but the magnitude of the response was markedly lower (CI of 125%; CI of 100% indicates a nonchemosensitive neuron). As in younger neonates, synaptic blockade medium had very little effect on these values but, remarkably, carbenoxolone dramatically reduced the percentage of intrinsically chemosensitive neurons (around 20%) (Fig. 4A) without affecting the CI (Fig. 4B) (Hartzler *et al.*, 2007; Nichols *et al.*, 2008). These studies indicate that around 10 days after birth, LC neurons undergo a major reduction in chemosensitivity with the percentage of intrinsically chemosensitive neurons and the magnitude of their response both decreasing dramatically. In LC neurons from rats older than P10, gap junctions appear to play a significant role in the response to hypercapnia, with relatively few intrinsic chemosensitive neurons coupled to adjacent nonchemosensitive neurons, resulting in hypercapnia activating a larger proportion of LC neurons than are intrinsically sensitive.

This dramatic reduction in the CO_2 sensitivity of catecholaminergic LC neurons during early development is reminiscent of a similar change in peripheral catecholaminergic cells, rat adrenal chromaffin cells (Muñoz-Cabello *et al.*, 2005). In these cells, hypercapnia induced a marked release of catecholamines in over 60% of the cells from rats younger than P10. However, in adult rats (>P30) only 35% of the cells responded and those that responded had a substantially reduced release of catecholamines (Muñoz-Cabello *et al.*, 2005). These data, together with those from LC neurons, suggest that in very young rats, hypercapnia will induce a substantial systemic catecholamine release as well as major activation of the main noradrenergic center in the brain. We suggest that this may serve as a simple form of ventilatory control in young neonates, increasing ventilation in response to hypercapnia due to catecholamine release both systemically and centrally. We would further suggest that during early development, this system weakens and is replaced by a more nuanced adult form of ventilatory control.

The possibility of a neonatal form of ventilatory control that is distinct from mature, fully developed respiratory control has been suggested previously (Stunden *et al.*, 2001; Putnam *et al.*, 2005) but is controversial (Davis *et al.*, 2006). Stunden *et al.* (2001) reported a marked ventilatory increase to inspired CO_2 that was most clear in very young rats (P0-3) but then waned during the first week of life, with an increase in the ventilatory response to inspired CO_2 re-appearing after age P10. The authors suggested the ventilatory response in

young neonates was due to "neonatal" central chemosensitivity while the response in older rats was due to an "adult" form of central chemosensitivity. During the transition period (around P7 to P10) there was a very low ventilatory response to inspired CO_2. A similar response, although with a much smaller "neonatal" hypercapnic ventilatory increase in ventilation, was seen by Serra *et al.* (2001). Further suggesting a distinct neonatal response to hypercapnia, Wickström *et al.* (2002) showed in very young neonates (P2-4) that inspired CO_2 resulted in a marked increase in respiratory frequency while in older neonates (>P8) this response was reduced and ventilation largely increased due to increased tidal volume. It was once again suggested that central chemosensitivity appears to show marked developmental changes during the first 10 days of life in rats. Finally, the presence of a neonatal form of chemosensitivity early after birth is suggested by the findings of embryonic (~E20) respiratory activity in rats whose frequency is reduced by decreased CO_2 and whose activity is modulated by pontine adrenergic input, although this modulation is inhibitory (Di Pasquale et al., 1992). This activity is similar to activity in young neonates but these authors state that "…the fetal respiratory network might be affected by cell migration, differentiation and death; so the fetal network could be replaced during development by a different one…" (Di Pasquale et al., 1992). In fact, a shift in the respiratory network has been observed in rats between ages P0-1 and P2-4 (Oku et al., 2007). The idea of a shift in the respiratory network early in postnatal development is in essence what we are proposing here with respect to central chemosensitivity, with LC neurons playing a part in the early network ("neonatal" or "fetal") and having a reduced role in the later network ("adult").

There is contradicting evidence suggesting that there is very little hypercapnic ventilatory response in neonates before about P15 (Davis et al., 2006). It is not clear why the results of this study were so different from the early study of Stunden et al. (2001) with respect to the hypercapnic ventilatory response in young neonates. Davis et al. (2006) suggested the difference is due to the normalization of ventilation for body weight (see for example Fig. 9 in Stunden et al., 2001) since they found no hypercapnic ventilatory response in young neonates when increased ventilation in hypercapnia was expressed as a percentage of resting ventilation. This cannot be the explanation of the differences between the two studies, however, since Stunden et al. (2001) also expressed CO_2-induced increased ventilation as a percentage of resting ventilation (see Fig. 10 in Stunden et al., 2001) and still saw a marked hypercapnic ventilatory response in young neonates (P1-P3). Whatever the explanation, the findings of Davis et al. (2006) indicate that it is possible that there is not a neonatal form of ventilatory control. If this is the case the high degree of CO_2 responsiveness of LC neurons from young neonates (Fig. 4) might indicate that LC neurons play some other role in these young rats, such as stimulating arousal or serving as a suffocation alarm during periods of hypercapnia. These issues seem worth addressing more fully in future studies.

3.2.3. Chemosensitive signals—Progress has also been made understanding the cellular signals that allow a neuron to respond to hypercapnia. In theory, when a cell is exposed to hypercapnic acidosis (increased CO_2 at constant HCO_3 with an associated decrease in extracellular pH, pH_o), there are many signals that could affect neuronal excitability and thus firing rate. These signals include the increase of CO_2, the reduction of pH_o, or decreased pH_i. A first approach to differentiating the role of these various signals was studying the firing rate response of LC neurons to various acid challenges, some involving increased CO_2 and others not and some with a decreased pH_o and others not (Filosa *et al.*, 2002). Using such an approach, it was shown that the parameter that best correlated with the increased firing rate was the change in pH_i (Filosa *et al.*, 2002) indicating that a change of intracellular pH plays a major role in chemosensitive signaling in LC neurons.

A problem with this approach is that all acid challenge solutions result in a fall of pH_i so it is difficult to assess the necessity for a change of pH_i in chemosensitive signaling. To address this issue, a technique was developed to study the firing rate response of LC neurons to hypercapnia while clamping pH_i at a constant value (Hartzler *et al.*, 2008). Using this technique, it was shown that hypercapnic acidosis still resulted in an increased firing rate even though pH_i was clamped constant at its initial value (Hartzler *et al.*, 2008). These results clearly indicate that while a change of pH_i can lead to increased firing rate in response to hypercapnia in LC neurons, a change of pH_i is not a necessary signal. However, when pH_i was clamped at its initial value and LC neurons were exposed to isohydric hypercapnic conditions (increased solution CO_2 and HCO_3 such that pH_o is maintained constant at its initial value) their firing rate did not increase (Hartzler and Putnam, unpublished observations). Thus, a decrease in either pH_i or pH_o, but not an increase of CO_2, is capable of increasing the firing rate of LC neurons.

Another response of LC neurons to hypercapnia has been reported. LC neurons exhibit subthreshold rhythmic oscillations which are small oscillating changes in membrane potential believed to be mediated by opening of Ca^{2+} channels (Williams *et al.*, 1984; Williams and Marshall, 1987; Oyamada *et al.*, 1999). The interesting finding was made that the frequency of these oscillations are increased by hypercapnia (Filosa and Putnam, 2003), suggesting that acidosis stimulates these Ca^{2+} channels. This is contrary to the usual observation that Ca^{2+} channels are inhibited by acidosis (Tombaugh and Somjen, 1997; Shah *et al.*, 2001). However, hypercapnia-induced activation of Ca^{2+} channels has been reported in peripheral chemosensitive cells from the carotid body (Summers *et al.*, 2002). This activation of Ca^{2+} channels in carotid body cells appears to be due to activation of soluble adenylyl cyclase (sAC) during hypercapnia by increased intracellular HCO_3^- (Summers *et al.*, 2002), a known potent activator of sAC (Zippin *et al.*, 2001). Thus, hypercapnia not only appears to inhibit K^+ channels in LC neurons but also to activate Ca^{2+} channels, in agreement with the proposed multiple factors model of chemosensitive signaling in LC neurons (Putnam *et al.*, 2004).

The activation of Ca^{2+} channels by hypercapnia in LC neurons raises an intriguing possibility. The increased activity of Ca^{2+} channels in response to hypercapnia should increase intracellular Ca^{2+} and activate Ca-activated K^+ (K_{Ca}) channels. These channels are known to be present in LC neurons (Williams *et al.*, 1984). If these channels are activated during hypercapnic exposure, their activity could serve as a brake on the chemosensitive response of LC neurons. Such an effect has previously been suggested. Aghajanian *et al.* (1983) concluded that "…the influx of calcium into locus coeruleus neurons appears to serve a negative feedback function in the regulation of both spontaneous activity and reactivity to orthodromic stimulation". This could in part explain why the firing rate response to hypercapnia plateaus at higher levels of CO_2 (Pineda and Aghajanian, 1997; Ritucci *et al.*, 2005). If K_{Ca} channels serve such a braking role on the chemosensitive response, inhibiting these channels should increase the firing rate in response to hypercapnia and could offer a novel approach to increasing the gain of central chemosensitivity.

3.2.4. Ion channel targets of chemosensitive signals—Just as the response of LC neurons to hypercapnia involves several signals (including changes of pH_o, pH_i, Ca^{2+} channel activity and Ca_i), emerging evidence suggests that there are several ion channel targets of these signals in chemosensitive LC neurons (for recent review see Putnam, 2010). Numerous channels have been suggested to be sensitive to changes of pH, especially K^+ channels (Putnam *et al.*, 2004). In fact, it has been proposed that multiple pH-sensitive channels with differing pH sensitivities could explain why neurons can be sensitive to pH changes over a broad range (Su *et al.*, 2007).

The first channel to be shown to be involved in the response of LC neurons to hypercapnia was the inwardly rectifying K^+ channel (K_{ir}) (Pineda and Aghajanian, 1997). Although changes of pH_i are known to directly affect K_{ir} channel activity (Zhu *et al.*, 1999; Xu *et al.*, 2000), Pineda and Aghajanian (1997) reported an indirect effect in LC neurons. Decreased pH_i is known to change the charge state of intracellular polyamines, and polyamines are known to inhibit K_{ir} channel activity. Thus, hypercapnia-induced acidification results in K_{ir} channel inhibition in LC neurons (Pineda and Aghajanian, 1997). Several K_{ir} channel subunits are expressed in LC neurons, including Kir1.1, Kir 2.3, Kir4.1 and Kir5.1, the latter two being expressed most strongly (Wu *et al.*, 2004). This is of interest since Kir4.1-5.1 make a heteromeric channel whose pK is in the physiological range (Xu *et al.*, 2000), making these channels highly susceptible to changes of pH.

In accord with the multiple factors model, other K^+ channels in LC neurons have recently also been shown to be inhibited by hypercapnia. An A current (rapidly activating and inactivating voltage-sensitive K^+ channel) is inhibited by a decrease in pH_o (and possible in part by decreased pH_i) in LC neurons (Li and Putnam, 2009; Putnam, 2009; Putnam and Li, 2009). These findings are in agreement with studies showing that 4 aminopyridine, an inhibitor of the A current, significantly reduces, but does not eliminate, the firing rate response of LC neurons to hypercapnia (Martino and Putnam, 2007). It has further been shown that a sustained current that is slowly activated, very slowly inactivated and inhibited by tetraethyl ammonium (probably a delayed-rectifying K^+ channel, K_{dr}) is also inhibited by hypercapnia in LC neurons (Li and Putnam, 2009; Putnam, 2009; Putnam and Li, 2009). Combined with the data discussed above on hypercapnia-activation of L-type Ca^{2+} channels, there is thus evidence for the activity of 4 different channels (K_{ir}, A current, K_{dr}, and L-type Ca^{2+} channels) being affected by hypercapnia in LC neurons. There is likely a relationship between the activation of several signaling pathways and the alteration of the activity of several types of ion channels by hypercapnia in LC neurons.

3.2.5. Somatic vs. dendritic responses to hypercapnia—Neurons are not homogeneous cells. They contain several specialized domains including the axon, dendrites and soma. As we have gained increasing knowledge about the cellular signals and the ion channel targets involved in neuronal responses to hypercapnia, it becomes a significant question to know whether chemosensitivity is mediated largely by events on the dendrites, on the axons or both. This is especially imperative since pH responses to acid challenges differ in dendrites and soma (Schwiening and Willoughby, 2002; Willoughby and Schwiening, 2002) and the distribution of ion channels is often not symmetric in neurons (Korngreen and Sakmann, 2000; Riazanski *et al.*, 2001). Based largely on anatomic data, a number of studies have suggested that chemosensitive signaling might be based on the dendrites in neurons from various chemosensitive regions. Chemosensitive neurons from the ventral medulla send dendritic projections to the ventral surface, suggesting that these dendrites are sampling the solution at the ventral surface (Kawai *et al.*, 1996). A more dramatic example of this is shown by chemosensitive neurons from the retrotrapezoid nucleus, that send dendrites to the marginal layer at the ventral surface which run for several hundred microns along the ventral surface (Mulkey *et al.*, 2004). A variation of these anatomical arguments has been offered for medullary raphé neurons. Bradley *et al.* (2002) have shown that chemosensitive serotonergic neurons have dendritic projections that closely approach large medullary arteries and have suggested that these neurons may be sensing blood CO_2 (Severson *et al.*, 2003).

We are aware of but a single study that directly addresses whether the response to hypercapnia in chemosensitive neurons resides on the soma, on the dendrites, or on both. Ritucci *et al.* (2005) studied the firing rate response of LC neurons in brainstem slices to hypercapnia applied locally by a superfusion pipette. When a large dendrite was exposed to

the hypercapnic solution, there was no increase in neuronal firing rate. In contrast, when the soma (and proximal dendrites) were exposed to hypercapnic solution, LC neuron firing rate increased nearly as much as if the entire slice had been exposed to solution equilibrated with 15% CO_2 (Ritucci *et al.*, 2005). These findings suggest that in LC neurons, at least, the chemosensitive machinery resides within the soma and/or the most proximal portion of the dendrites. This appears to be at odds with the suggested distal chemosensitive signaling suggested by the anatomical studies discussed above. It may well be that chemosensitive neurons from different regions have a different cellular locus for the chemosensitive signaling machinery. Alternatively, as pointed out by Mulkey *et al.* (2004), "…Proximity to the ventral surface does not necessarily in itself confer CO_2 sensitivity…" and it may be that chemosensitive neurons from all regions respond to hypercapnia predominantly based on signaling pathways and ion channels residing close to the soma.

4. LC and chemosensitivity in anuran amphibians

In anurans, González and Smeets (1991) distinguished a large tyrosine-hydroxylase (TH) immunoreactive, but dopamine negative, group of cells at the isthmus region (Fig. 5), which lies at the rostral end of the hindbrain. This isthmic cell group contains noradrenaline (González and Smeets, 1991, 1993) and innervates the spinal cord, cerebellum and telencephalon (Parent, 1975; González and Smeets, 1991; 1993). This area is considered to be homologous to the LC of mammals. This homology is based on its position, noradrenergic content, and projections to both the telencephalon and spinal cord (Marin *et al*., 1996). Since LC is important for the control of breathing and is considered to be a chemosensitive site in mammals, Noronha-de-Souza *et al*. (2006) investigated the participation of this nucleus in the control of breathing and central chemoreception of unanesthetized toads (*Rhinella schneideri*, formerly *Bufo parcnemis*). Initially, morphologic evidence was provided, i.e., the expression of *c-fos* in neurons of the LC after hypercarbic challenge. In this study, an increased inspired CO_2 concentration (5% CO_2) induced Fos-like immunoreactivity in the LC of toads, reinforcing the idea that the LC of amphibians is homologous to the LC of mammals.

In addition, selective chemical lesion of LC catecholaminergic neurons was used to verify the possible involvement of this nucleus in the respiratory responses to CO_2. Similar to mammals, LC chemical lesion with 6-OHDA decreased the hypercarbia-induced hyperventilation (Fig. 6A), largely due to a decreased tidal volume response, but did not affect ventilation under resting conditions (Noronha-de-Souza *et al*., 2006). This finding associated with the fact that the isthmic catecholaminergic cell group of amphibians (where LC is placed) does not contain dopaminergic or adrenergic cell bodies (González and Smeets, 1993), strongly suggests that noradrenergic LC neurons are involved in processing or modulating central chemoreceptor information in amphibians.

To test whether LC neurons are intrinsically pH-sensitive, focal acidification was performed by microinjecting mock CSF with different pH values (7.2, 7.4, 7.6, 7.8 and 8.0) into the area of the LC. Mock CSF perfusion is a well established method for studying the central chemoreceptor drive to breathe (Hitzig and Jackson, 1978; Branco *et al*., 1992). Interestingly, pulmonary ventilation increased after local reduction of the pH (mock CSF of 7.2, 7.4 and 7.6), which suggests that LC may be a chemosensitive site in the CNS of amphibians (Fig. 6B). Future research will be necessary to specify the stimulus or stimuli responsible for LC activation (intracellular pH, extracellular recordings. It will also pH, and/or molecular CO_2) by using electrophysiological be interesting to investigate the role of LC in chemosensitivity during the development of amphibians. Given the functional homology of the LC between amphibians and rodents, we are currently examining the cellular level

chemosensitive properties of LC neurons to determine whether this homology in function is derived from the same neuronal cellular signaling pathways.

5. Conclusions

We have discussed evidence that LC neurons are involved in central chemosensitivity in both mammals and amphibians and that this response is most likely mediated by CO_2/H^+-sensitive neurons within the LC. The development of chemosensitivity within the LC appears to be unusual, with a strong cellular response to CO_2 during the first week of life which subsides after day P10. This pattern suggests an especially important role in central chemosensitivity for the LC in very early periods of development. LC continues to play a role in central chemosensitivity throughout development and into adulthood, but it may also play other roles, such as stimulating arousal, as part of the suffocation alarm system or as an agent in the initiation of panic disorders. A detailed study of the developmental role of LC neurons in central chemosensitivity appears warranted.

We have also reviewed evidence that the output of the LC can be modulated by various neurotransmitters. The role of the neurotransmitters serotonin and glutamate in the regulation of LC neuronal responses to CO_2/H^+ needs to be delineated in detail, especially with regard to the role such modulation may play in state-dependent changes of LC output.

A great deal has been learned about the multiple cellular signaling pathways and ion channel targets involved in the chemosensitive response of LC neurons to CO_2/H^+ but more needs to be learned. It is especially important to understand which ion channel(s) are involved in the response of LC neurons to various chemosensitive signals, how these pathways are modulated by different states of the organism and how the signals and ion channel targets change during development. Recent suggestions for an important role for Ca^{2+} channels and intracellular Ca^{2+} need to be studied fully, especially given the possibility that this pathway may lead to activation of K_{Ca} channels which could serve as a brake on the magnitude of the chemosensitive response of LC neurons. If this is the case, certain respiratory pathologies involving disordered breathing may arise from loss of this braking pathway.

Finally, given the similarities between LC responses in anurans and mammals, it is likely that the chemosensitive function of LC neurons is a basal trait of terrestrial vertebrates. These similarities in LC responses also further support the proposed functional homology of this nucleus in both groups. Detailed studies of the role played by the LC in the control of breathing and the cellular basis of CO_2/H^+-sensing in LC neurons from other vertebrates (especially amphibians) are needed to gain insight into whether or not chemosensitivity is highly conserved in the LC and other putative chemosensitive regions in the brainstem.

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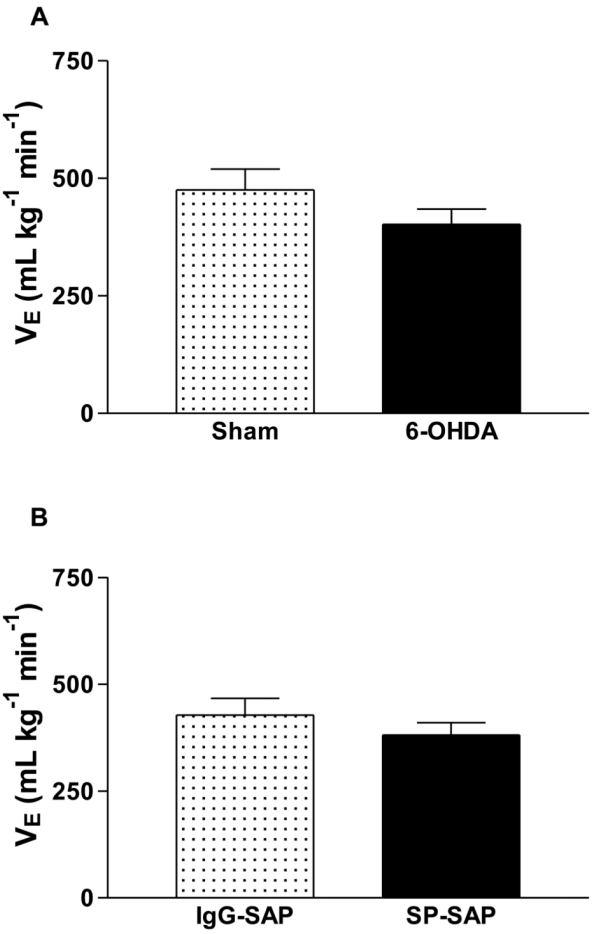

Figure 1.

A. Minute ventilation (V_E) of the sham (n=8) and 6-OHDA rats (n=10) groups during normocapnia (adapted with permission from Biancardi *et al*., 2008). **B.** Minute ventilation (V_E) of the IgG-SAP rats (control, n=9) and SP-SAP (n=8) groups during normocapnia (adapted with permission from Carvalho *et al*., in press). Values are means ± SEM. There is no difference between groups in both A and B.

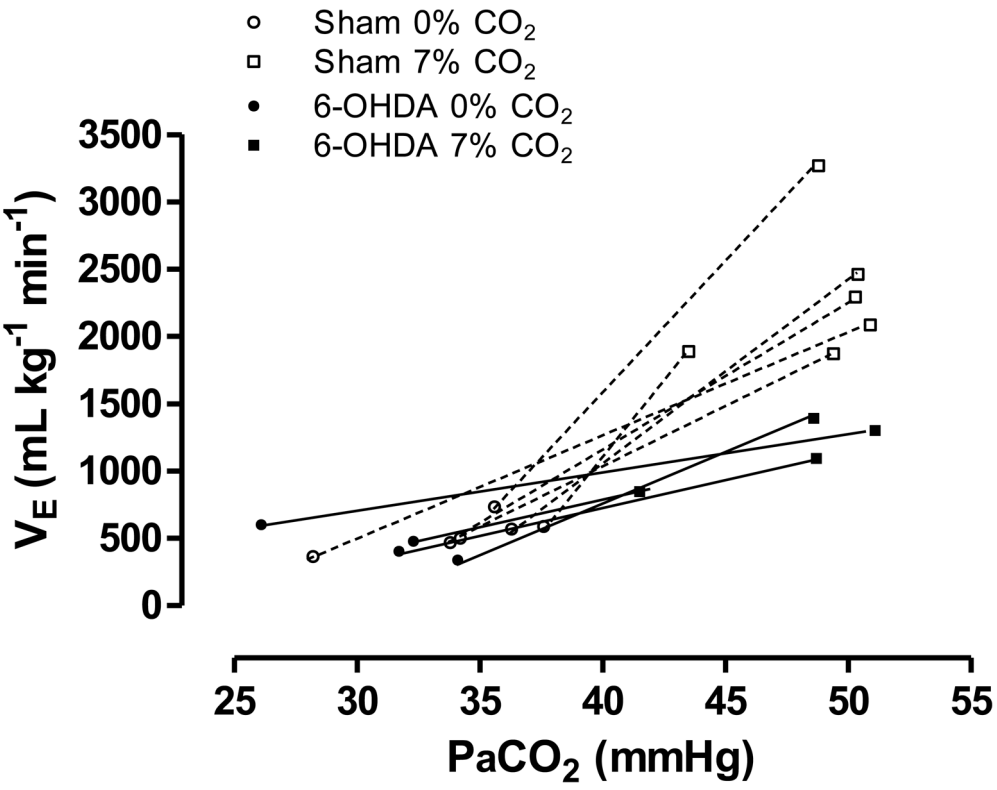

Figure 2.
The relationship between pulmonary ventilation (VE) and CO_2 arterial partial pressure ($PaCO_2$) of the sham and 6-OHDA-lesioned groups exposed to normocapnia (0% CO_2) and hypercapnia (7% CO_2).

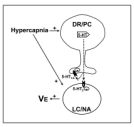

Figure 3.

Schematic diagram showing the proposed mechanism through which serotonin (5-HT) from dorsal raphe (DR) or pericoeruleus region (PC) modulates the LC role in the hypercapnia-induced hyperpnea. Hypercapnia induces an increase in the release of 5-HT in the LC, which acts on postsynaptic 5-HT_{2A} receptors to inhibit noradrenergic (*NA*) neurons and may act on presynaptic 5-HT_{1A} receptors to regulate its own release. Adapted from de Souza Moreno *et al*. (2010).

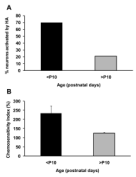

Figure 4.

Intrinsic chemosensitive response to hypercapnic acidosis in LC neurons from the neonatal rat brainstem. Both high Mg^{2+}/low Ca^{2+} and carbenoxolone were used to prevent synaptic communication between neurons. CS responses divided into two age classes: post-natal days P0-9 and P10-19, referenced as <P10 and >P10, respectively. N=23 for <P10, N=24 for >P10. **A.** Percent of neurons activated (increased firing rate) by HA (15% CO_2). **B.** Chemosensitive index (relative magnitude of the chemosensitive response) for those neurons activated by HA. N=16 for <P10, N=5 for >P10. The height of the bars represents the means and the error bars represent one SEM.

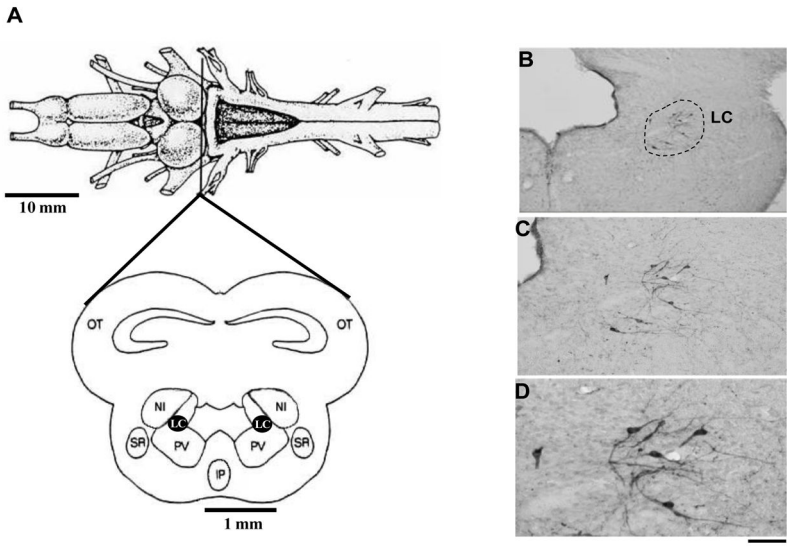

Figure 5.

A. Dorsal view of the anuran brain, indicating the level of midbrain section illustrated below. Adapted from Gargaglioni and Branco (2009). **B, C** and **D.** Photomicrographs of the isthmus region showing the catecholaminergic cell bodies identified by tyrosine hydroxylase immunohistochemical staining of the brains. Detailed (higher magnification) morphology of these neurons is shown in **B** and **C**. Scale bars: 200 μm in *A*, 100 μm in *B*, and 50 μm in *C*. Adapted from Noronha-de-Souza *et al*. (2004). Abbreviations: Aq, Aqueduct of Sylvius; LC, *Locus coeruleus*; NI, nucleus isthmi; Otec, optic tectum. LC, locus coeruleus; IV, fourth ventricle.

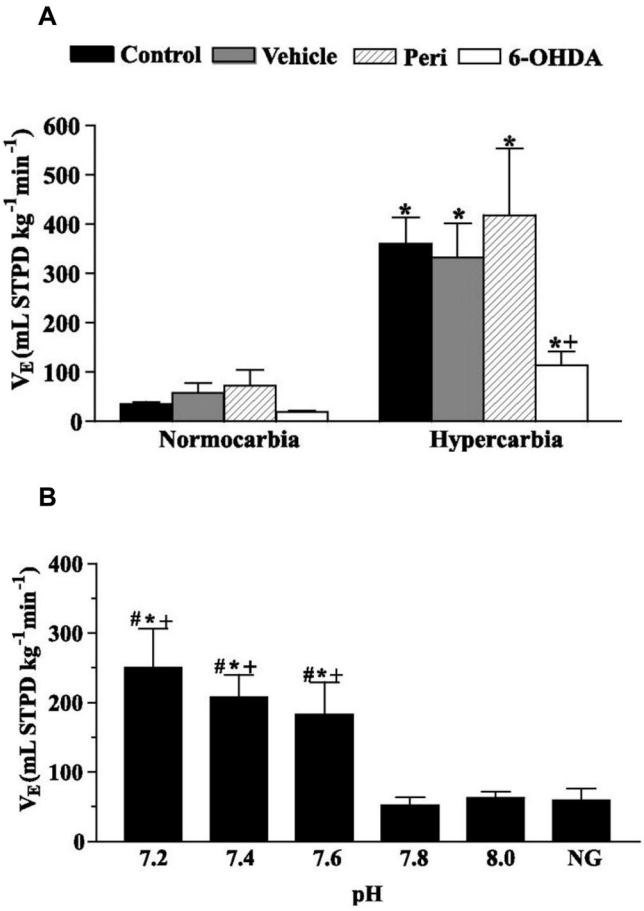

Figure 6.

A. Ventilation (V_E) of the control, vehicle, peri, and 6-OHDA groups exposed to normocarbia and hypercarbia (5% CO_2). * Significant effect of hypercarbia compared with the normocarbic value (paired *t*-test), + Significant differences between 6-OHDA and all other groups during hypercarbia ($P < 0.05$, one-way ANOVA). **B.** V_E after microinjection of mock CSF of pH 7.2, 7.4, 7.6, 7.8 (control pH value), and 8.0. NG means V_E before the injection (no injection group). * Significant difference from pH 7.8 group, # Significant difference from pH 8.0 group; + Significant difference from NG group (one-way ANOVA). Adapted from Noronha-de-Souza *et al*. (2004).