Midbrain Structures and Control of Ventilation in Amphibians

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Abstract Despite recent advances, the mechanisms of neurorespiratory control in anuran amphibians are far from understood. Among key brainstem structures believed to play a major role in the ventilatory control of amphibians is the nucleus isthmi (NI) and *Locus coeruleus* (LC). It has been suggested that the NI acts to inhibit hypoxic and hypercarbic drives to breathing. The putative mediators for these responses are glutamate and nitric oxide. As to the LC, it has now been reported that this nucleus is a CO_2-sensitive receptor site in amphibians, which mediates the ventilatory response to hypercapnia. This chapter reviews the available data on the role of the NI and LC in the control of ventilation in amphibians.

1 Introduction

Amphibians and mammals share several common features of control of breathing. In both classes, the rhythmogenic and pattern-forming elements are particularly adapted to approach the demands determined by the environment: behavior, metabolic needs, and breathing mechanisms. Studies on amphibians document an involvement of the chemical drive to breathing and the receptors (for review see Gargaglioni and Milsom 2007). Hypoxic ventilatory responses are elicited by peripheral arterial chemoreceptors (Van Vliet and West 1992), whereas hypercarbic ventilatory responses are predominantly mediated by central chemoreceptors that are sensitive to increasing H^+ concentrations in the extracellular fluid that surrounds the fourth ventricle (Smatresk and Smits 1991; Branco et al. 1992; Reid et al. 2000). Recently, we have provided evidence that *Locus coeruleus* (LC) is a chemosensitive area of the central nervous system. To our knowledge there is no data available on the role of LC in hypoxia-induced hyperventilation in amphibians. Thus, this seems

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to be a ripe topic still to be assessed. We will discuss the role of LC as a key site involved in the control of breathing, more specifically to the ventilatory responses to hypercarpnia, later in this chapter.

Studies in tadpoles and adult amphibians indicate that the endogenous respiratory rhythm is generated within the medulla, as observed in mammals (McLean et al. 1995; Reid et al. 2000; Torgerson et al. 1998; Wilson et al. 2002). Therefore, a possible homology between the amphibian lung rhythm generator and the mammalian pre-Bötzinger area has been proposed, and this region may be phylogenetically conserved as a neural structure that permitted development of air breathing in bimodal respiration (Torgerson et al. 1998).

Despite these similarities, the breathing patterns of the two classes are different. Mammals and birds maintain homeostasis of arterial blood gases by rhythmic and continuous breathing. Some teleost fish also ventilate continuously, especially at high temperatures or during hypoxic conditions (Soncini and Glass 2000). Conversely, amphibians, reptiles, and most air-breathing fish display an intermittent pattern of aerial respiration, characterized by lung ventilations that occur in single events or are grouped into episodes of several breaths separated by nonventilatory periods (apnea) of variable duration (Milsom 1991). Mammals may breathe episodically when their metabolic needs are low, e.g., during hibernation, whereas amphibians and reptiles may also breathe continuously (if the respiratory drive is elevated), which suggests a common mechanism for breathing in vertebrates (cf. Kinkead et al. 1997). Episodic breathing occurs even when the respiratory drive is constant, i.e., without fluctuations of arterial blood gases or chemoreceptor input. Therefore, it seems that this pattern is an intrinsic property of the central respiratory control system that is independent of oscillations of arterial blood gases (Kinkead 1997).

Oka (1958a, b) found that the clustering of the breaths into distinct episodes could be completely eliminated only by a transection behind the optic lobes (i.e., behind the midbrain), just in front of the cerebellum. This suggested that a more rostral site was essential for the creation of breathing episodes, and subsequent studies employing in vitro (Reid et al. 2000) and in situ preparations (Gargaglioni et al. 2007) identified areas in caudal half of the midbrain of the bullfrog (*Rana catesbeiana*) that appear responsible for the production of episodic breathing patterns (Milsom et al. 1999; Reid et al. 2000; Gargaglioni et al. 2007).

Kinkead et al. (1997) suggested that the nucleus isthmi (NI), a mesencephalic structure located in the amphibian brain between the roof of the midbrain and the cerebellum, might be such a structure (Fig. 1). Thus, structures within the midbrain of anurans may both provide a tonic drive to breathing and produce specific breathing patterns. In addition, Kinkead et al. (1997) proposed that, from a neuroanatomical perspective, the NI could be compared to the pontine respiratory group, which in mammals contributes to the control of the breathing pattern in a fashion that mimics the effects of pulmonary vagal feedback.

The NI was first described in anurans by Gaupp in 1897 (cf. Wang 2003), and has now been described for all vertebrates, except for cyclostomes and mammals (Hoffmann 1973). Some studies have shown that the NI is a visual center that

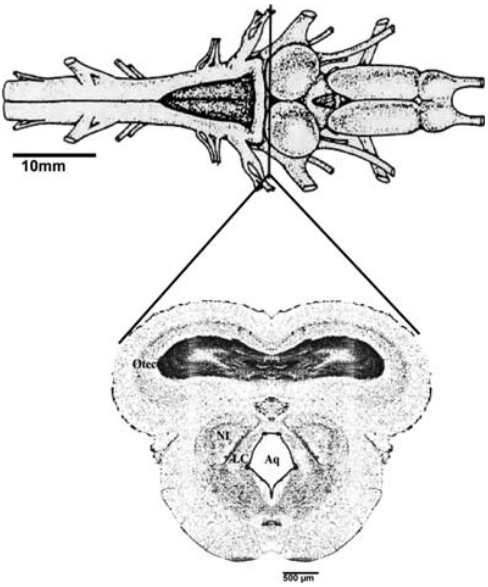

Fig. 1 Dorsal view of the anuran brain, indicating the level of midbrain section illustrated bellow. Abbreviations: *Aq* Aqueduct of Sylvius. *LC Locus coeruleus*, *NI* nucleus isthmi, *Otec* optic tectum

receives information from the optic tectum but is not an auditory center, as previously suggested, since no auditory responses were recorded from amphibian NI (cf. Wang 2003). In anuran amphibians, the NI consists of a cortex and a medulla. While the cortex is rich in cells, the medulla shows scattered cells and numerous nerve fibers (Larsell 1924). The dendrites of NI neurons generally lie in the horizontal plane and extend towards the medulla. The NI consists of approximately 8,000 neurons in frogs and 4,800 neurons in toads (Wang 2003). Interestingly, the NI differentiates during metamorphosis, a period that marks the initiation of pulmonary ventilation in bullfrogs (Burggren and Infantino 1994) and the transfer of central chemoreceptive influence from gill to lung ventilatory control (Torgerson et al. 2001). This evidence is consistent with the notion that the NI plays a role in neurorespiratory control in amphibians. Below we address studies on the involvement of the NI in the control of breathing in amphibians, based on different experimental approaches.

2 Lesion Studies

2.1 Basal Respiratory Drive

As previously mentioned, studies by Oka (1958a, b) showed that transection at the level of the NI removes episodic breathing. However, it was not until 1997 that Kinkead et al. (1997) investigated the role of the NI in anuran ventilatory control.

The authors reported that, following microinjection of kainic acid into the NI (1.5 h prior to the experiments), the respiratory motor output in the Vth and Xth cranial nerves of bullfrogs often shifted from an episodic to evenly spaced pattern. Breathing episodes were, however, still observed in some animals after microinjections of kainic acid, and the reduction in eucapnic ventilation was proportional to the reduction of chemosensitivity. Therefore, it was suggested that the NI is not directly responsible for turning breathing episodes on and off, but that it provides a tonic excitatory input to the respiratory centers. The participation of the NI in the control of breathing in unanesthetized toads (*Chaunus schneideri*, formerly known as *Bufo paracnemis*) has also been tested by performing an electrolytic lesion in this area (Gargaglioni and Branco 2000). The episodic breathing pattern was not eliminated following electrolytic lesion, in agreement with the study by Kinkead. In our study, however, we did not observe a reduction in the resting pulmonary ventilation as demonstrated by Kinkead et al. (1997). It is well-known that both electrolytic and kainic lesions present technical limitations. Electrolytic lesions cause damage both to cell bodies and axons of passage, and kainic acid can result in lesions at sites distant from the injection (Maglóczky and Freund 1995). Moreover, the literature reports that kainic acid stimulates glutamatergic synapses for a few hours after its administration, an event followed by gliosis and neuronal lesions of the glutamatergic neurons (Winn 1991). To address this issue, we conducted chemical lesions (ibotenic acid) in the NI and performed the experiments 3 days later, when chemical lesions were expected to have occurred. According to Jarrard (1989), ibotenic acid appears to be the excitotoxin of choice when specific lesions of neurons that do not affect fibers of passage are required. In our study, we used the method of Klüver-Barrera (Kiernan 1990) to evaluate the intensity of perikarya damage. Histochemical analysis of neuronal cell components in the midbrain tissue lesioned with ibotenic acid revealed that this neurotoxin exclusively destroyed neuronal somata, while neuronal axons and dendrites were preserved. We were then able to further prove that neither cell bodies nor fibers of passage, in fact, do not participate in the resting control of ventilation (Gargaglioni et al. 2002), as previously shown with electrolytic lesions (Gargaglioni and Branco 2000).

Lesions of NI cell bodies did not affect the instantaneous breathing frequency (Kinkead et al. 1997; Gargaglioni and Branco 2000; Gargaglioni et al. 2002), which has been used as a reliable indicator of the endogenous respiratory rhythm in intermittent breathers. Thus the available data are consistent with the notion that the NI does not participate in the generation of the respiratory rhythm. In conclusion, the NI seems not to be involved in the production of episodic breathing in anuran amphibians, but it does participate in the ventilatory response to hypoxia and hypercarbia, as discussed below.

2.2 Responses to Hypoxia

It is now well established that the hyperventilation induced by hypoxia in adult anurans primarily results from activation of peripheral chemoreceptors, located in the aortic arch and carotid labyrinth (Van Vliet and West 1992). Chemoreceptors may,

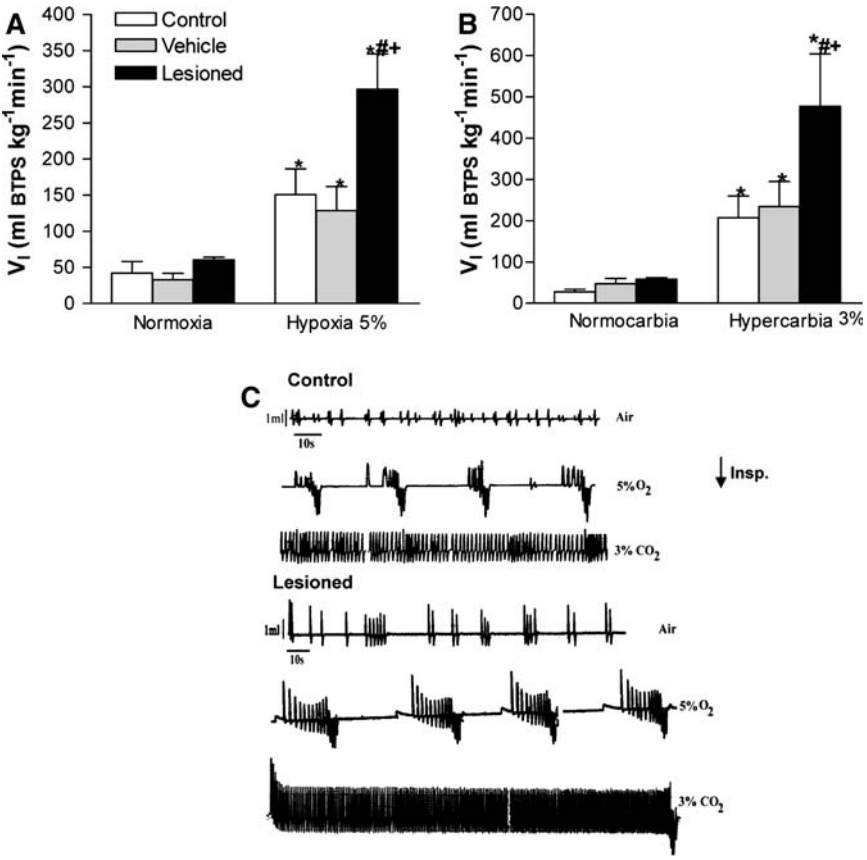

Fig. 2 **a** Ventilation (V_I) of the control, vehicle and ibotenic acid lesioned groups during normoxia and hypoxia (5% O_2). **b** Ventilation (V_I) of the control, vehicle and ibotenic acid lesioned groups during normocarbia and hypercarbia (3% CO_2). **c** Pulmonary ventilation recordings obtained for the control and ibotenic acid lesioned groups during air, hypoxia (5% O_2).and hypercarbia (3% CO_2). *Asterisk* indicates a significant effect of hypoxia or hypercarbia compared to normoxic/normocarbic value, *hash* indicates a significant difference between the control and lesioned groups, *plus* indicates a significant difference between the vehicle and lesioned groups. (Adapted from Gargaglioni et al. 2002)

however, be present on the pulmocutaneous trunk (Hoffmann and de Souza 1982). The peripheral arterial chemoreceptors are analogous, and perhaps homologous, to the chemoreceptors of mammals (Van Vliet and West 1992).

Studies from our laboratory (Gargaglioni and Branco 2000, 2004; Gargaglioni et al. 2002) established that both electrolytic and chemical lesions of the NI cause an increased ventilatory response to hypoxia (Fig. 2), which suggests that NI inhibits hypoxic ventilatory response in toads.

As a key point, the increased ventilation during hypoxia in NI-lesioned toads is consistent with previous studies on our laboratory rats. This shows that both electrolytic lesion of the Locus coeruleus (a noradrenergic pontine nucleus) (Fabris et al. 1999) and chemical lesion of the pontine Nucleus raphe magnus (NRM, a serotoninergic cell group) (Gargaglioni et al. 2003) also caused an elevated respiratory response to hypoxia, due to increases in tidal volume. This suggests an interesting parallel between LC, NRM and NI in ventilatory control during hypoxic conditions.

2.3 Responses to Hypercarbia

Central respiratory CO_2 chemoreceptors have clearly been documented in adult anuran amphibians and are thought to be distributed throughout the medulla, surrounding the fourth ventricle (Smatresk and Smits 1991 and Branco et al. 1992). In intact anaesthetized toads, the central chemoreceptors contribute about 80% of the hypercapnic respiratory drive (Branco et al. 1992), suggesting a dominant role in the ventilatory acid–base regulation. In mammals, these receptors were once thought to be located only on the surface of the ventral medulla but seem to be distributed more widely. Recently, sites have been identified in the ventrolateral medulla, nucleus of the solitary tract, ventral respiratory group, locus coeruleus, caudal medullary raphe, and fastigial nucleus of the cerebellum (for a review, see Nattie 2001). However, it remains uncertain if the same is true for amphibians. In addition to central chemoreceptors, olfactory receptors sensitive to CO_2 powerfully inhibit breathing in unanesthetized bullfrogs (Coates and Ballam 1990). Moreover, in adult anurans, pulmonary stretch receptors are also CO_2-sensitive, since their firing rates decrease in response to elevated CO_2 levels (Milsom and Jones 1977).

In bullfrogs, the NI differentiates during metamorphosis in which the transition to pulmonary ventilation occurs (Burggren and Infantino 1994). At this point, the central chemoreceptors assume control of lung ventilation (Torgerson et al. 2001; Wilson et al. 2002). These changes suggest that the NI may be involved in hypercarbic drive to breathing. In agreement with this notion, Kinkead et al. (1997) and Gargaglioni et al. (2002) have established that CO_2 chemosensitivity is indeed altered following NI lesions. In our study (Gargaglioni et al. 2002), we found that toads with chemical NI lesions show an enhanced ventilatory response to hypercarbia (3% inspired CO_2). On the other hand, Kinkead et al. (1997), demonstrated that microinjection of kainic acid into the NI attenuates the increase in fictive breathing induced by hypercapnia. We believe that these differences may reside in the fact that kainic acid is known to stimulate glutamatergic synapses a few hours after its administration, representing a long-lasting excitatory effect (Watanabe et al. 1987). Since Kinkead et al. (1997) performed the experiments only 1.5 h after the administration of kainic acid, NI neurons may have been activated, rather than chemically lesioned. Another possible explanation is that ibotenic acid produces more selective and limited lesions, compared to kainic acid. According to Guldin and Markowitsch (1982), ibotenic acid is to be preferred relative to kainic acid, when local neuronal

lesions are desired. Therefore, we used ibotenic acid; the production of more selective lesions may promote a more consistent understanding of the role played by the NI in ventilatory control.

3 Putative Mediators Within the NI

As stated above, the NI inhibits pulmonary ventilation in toads exposed to hypoxic and hypercarbic conditions. This indicates that NI may be considered an important site for the control of breathing, although the mediators/modulators involved are poorly understood. We have studied two putative mediators, glutamate and nitric oxide (NO), based on previous data showing that this nucleus contains glutamate receptors and expresses nitric oxide synthase (Wang et al. 1995; Muñoz et al. 1996), and that both neurotransmitters/neuromodulators (glutamate and NO) are important in the control of ventilation (Haxhiu et al. 1995; Gozal et al. 1996).

3.1 Glutamate

L-glutamate receptors are known to be present in the NI (Wang et al. 1995). Such receptors can be divided into two major classes, ionotropic and metabotropic receptors. Ionotropic L-glutamate receptors are ligand-gated ion channels. They can be classified into three types, named after their most selective agonists, NMDA (*N*-methyl-D-aspartate), kainic acid, and DL-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA). In contrast to the ionotropic glutamate receptors, the metabotropic glutamate receptors are G protein-coupled receptors that modulate second messenger systems.

A growing number of studies report a participation of L-glutamate in the control of breathing. In rats submitted to hypoxia, inhibition of L-glutamate receptors by the NMDA antagonist, MK-801, reduces the magnitude of the hyperventilatory response to hypoxia, whether the antagonist is applied directly to the ventral surface of the medulla (Ang et al. 1992) or administered systemically (Soto-Arape et al. 1995). As to the CO_2 response, Harris and Milsom (2001) found that intracerebroventricular injection of MK-801 to the ground squirrel during hypercapnia caused an increase in the respiratory frequency and a decrease in tidal volume. In amphibians, L-glutamate is known to be one of the neurotransmitters in the rostral brainstem involved in the control of ventilation in *Rana catesbeiana* (McLean et al. 1995). Therefore, we investigated the role of glutamatergic neurotransmission in the NI on the hypoxic or hypercarbic drives to breathing in amphibians (Gargaglioni and Branco 2003). In this context, we microinjected the L-glutamate receptor antagonist, kynurenic acid, into the NI, before exposing the toads to hypoxia and hypercarbia. This drug antagonizes all ionotropic L-glutamate receptor types (Birch et al. 1988).

Microinjection of kynurenic acid into the NI had no effect on normal breathing, which indicates that L-glutamate plays no role under resting conditions. This blocker, however, increased the ventilatory response to hypoxia (7% and 5% inspired O_2) and hypercarbia (3% inspired CO_2), due to an increased tidal volume. Taken together, these data indicate that L-glutamate exerts an inhibitory influence on hyperventilation induced by hypoxia and hypercarbia. L-glutamate has been referred to as the primary central excitatory neurotransmitter involved in the control of ventilation, and we found an increased ventilatory response to hypoxia and hypercarbia after kynurenic acid microinjection. Similar results were observed by Dillon et al. (1991), who reported that microinjection of kynurenic acid into the rostral ventrolateral medulla of rats caused an increase in the ventilatory response to hypoxia and hypercapnia. In addition, rats microinjected with KYN in the locus coeruleus also exhibit an increased ventilatory response to hypoxia, which results from an increased tidal volume (Ferreira et al. 2004). However, the hyperventilation observed in Dillon's study was due to a significant increase in respiratory frequency and in tidal diaphragm activity. On the other hand, our results differ from those obtained by Ohtake et al. (1998), who performed intravenous administration of the NMDA receptor antagonist MK-801 in rats. They suggest that L-glutamate may not be involved in the ventilatory response to hypercapnia. These contradictory results may be related to the route of administration of the antagonists, in as much as systemic drug administration usually elicits different results from those obtained from the central drug administration. Furthermore, MK-801 antagonizes only NMDA receptors, whereas kynurenic acid blocks both NMDA and non-NMDA receptors. Therefore, it is possible that the hypercapnic ventilatory response may involve non-NMDA receptors. Future studies are needed in order to clarify this matter. To summarize, data indicate that the glutamatergic receptors in the NI do not participate in the control of ventilation during normoxia, but that they keep the level of hyperventilation to a minimum during hypoxia and hypercarbia.

3.2 Nitric Oxide

Since the discovery of the biological actions of NO, this gas has been found involved in several functions (Jaffrey and Snyder 1995). NO is a free radical gas that mediates important physiological regulatory events in cell regulation, cell–cell communication and signaling, functioning as an intracellular messenger, neurotransmitter, and hormone (Murad 1999). It is synthesized from L-arginine by a family of enzymes, the NO synthases (NOS) (Garthwaite and Boulton 1995), of which three types have been identified: the neuronal (nNOS), the endothelial (eNOS) and the inducible (iNOS) forms (Bredt et al. 1991). The importance of NO can be demonstrated by inhibiting its effect (Rees et al. 1990), using L-arginine analogs, such as L-NAME a nonselective inhibitor of NOS that acts on both the constitutive and inducible isoforms of the enzymes.

Recently, it became clear that NOS is expressed in the isthmic region of amphibians (Muñoz et al. 1996). Although NO synthases were originally identified in mammalian tissues, there is evidence that NO may be a neuronal messenger of early phylogenetic origin and conserved through the evolution (Muñoz et al. 1996). Accordingly, a substantial number of studies have indicated that NO is involved in a wide variety of central and peripheral processes in vertebrates and invertebrates (cf. Garthwaite and Boulton 1995). Consistently, a recent study (Hedrick et al. 1998) suggested the importance of endogenous NO for the neurotransmission and/or neuromodulation of the respiratory drive to breathing in the bullfrog brainstem.

Based on these facts, we examined the role of the NO pathway in the hypoxic and hypercarbic ventilatory responses in the NI of toads by microinjecting L-NAME (Gargaglioni and Branco 2001). With regard to the effect of NO in hypoxia-induced hyperventilation, microinjection of L-NAME into the NI elicited an increased response to 5% inspired O_2, due to an increase in tidal volume. These data suggest that NO may also mediate the inhibitory effect of the NI in the ventilatory response to hypoxia, as previously demonstrated for glutamate. In mammals, NO has been shown to play an important role by mediating central hypoxic ventilatory reflexes (Haxhiu et al. 1995; Gozal et al. 1996). Previous studies by Gozal et al. (1996) demonstrated that systemic administration of L-NAME induces markedly reduced ventilatory responses to hypoxia. Likewise, Haxhiu et al. (1995) documented that oxygen deprivation leads to activation of the NO pathway in the central nervous system, contributing to hypoxia-induced increase in ventilation. The authors suggested that NO may inhibit inhibitory pathways which, together with excitatory pathways, are triggered by hypoxia. In agreement with these findings, studies from our laboratory have shown that central NO plays a major role in the ventilatory response to hypoxia, since i.c.v. injection of L-NAME abolished the ventilatory response to hypoxia in conscious rats (Fabris et al. 1999). On the other hand, more recent studies by Fabris et al. (2000) and Nucci et al. (2004) demonstrated that microinjection of L-NAME into the LC and NRM, respectively, caused an increase in the ventilatory response to hypoxia, which suggests that in these pontine structures NO acts as an inhibitory neurotransmitter during hypoxia-induced hyperventilation. From these studies it is evident that NO is an important messenger molecule in peripheral and central neuronal structures, which are associated with the control of breathing during hypoxia. Moreover, NO may mediate both excitatory and inhibitory components of hypoxic ventilatory responses.

With regard to the response to CO_2, microinjection of L-NAME into the NI causes a significant increase in ventilatory response to hypercarbia, due to a higher tidal volume. Therefore, the data available support the notion that NO in the NI may exert an inhibitory influence on the integration of the CO_2-drive to breathing. Compared to hypoxia, the effects of NO in the ventilatory response to CO_2 are poorly understood. A previous study (Teppema et al. 1997) reported that the ventilatory CO_2 sensitivity in the peripheral and central chemoreflex loops is depressed after intravenous administration of the NOS inhibitor (N^{ω}-nitro-L-arginine [L-NNA]) in anesthetized cats. More recently, Barros and Branco (1998) demonstrated that the ventilatory response to hypercapnia does not change by administering

L-NNA to rats. In addition, these authors observed an unusual ventilatory breathing pattern 2 h after NOS inhibition. This pattern consisted of episodes of many breaths, separated by episodes of few breaths, similar to the patterns of some ectothermic vertebrates. This indicates that this respiratory control pattern is highly conserved in vertebrates, and that NO plays a role in normal respiratory function in rats.

We may therefore conclude that NO has no role in the NI under resting conditions, but that it exerts an inhibitory modulation on the hypoxic and hypercarbic drives to breathe, which acts on the tidal volume. The present observations, along with other studies on the presence of NO synthase in amphibians (Muñoz et al. 1996), indicate a considerable degree of phylogenetic conservation of the NO pathway amongst vertebrates.

4 Locus Coeruleus

As mentioned above, the first description of the *Locus coeruleus* (LC) was published by Reil (1809) but the term *Locus coeruleus* was proposed by the anatomists Wenzel and Wenzel (1812), reviewed by Russel (1955). The LC nucleus is a well-delineated cluster of noradrenaline neurons (A6 cell group), located adjacent to the fourth ventricle in the pontine brainstem (Dahlström and Fuxe 1964). Apart from noradrenaline the cell bodies also contain several neuropeptides (Sutin and Jacobowitz 1991). It has been described in a large number of animals, ranging from frogs and birds to primates. In the rat, each LC consists of about 1,500 densely packed neurones, all of which are immunoreactive for tyrosine hydroxylase, while LC provides noradrenergic projections to the forebrain, cerebellum, brainstem, and spinal cord.

It is estimated that ∼50% of all the noradrenergic projections in the central nervous system originate in the LC (Aston-Jones et al. 1995; Berridge and Waterhouse 2003). Consequently, LC is implicated in the control of many homeostatic functions including the maintenance of attention, motivation and arousal states (Svensson and Thorén 1979; Bhaskaran and Freed 1988), sleep (Aston-Jones and Bloom 1981), fever response (Almeida et al. 2004; Ravanelli et al. 2007), control of breathing (Erickson and Millhorn 1984; Guyenet et al. 2005; Oyamada et al. 1998; Dawid-Milner et al. 2001; Hilaire et al. 2004; Viemari et al. 2004) and cardiovascular function (Sved and Felsten 1987; Ward and Darlington 1987; Miyawaki et al. 1993; Murase et al. 1993; Anselmo-Franci et al. 1998).

In anurans, González and Smeets (1991) distinguished a large tyrosine-hydroxylase (TH) immunoreactive, but dopamine negative, group of cells at the isthmus region, which lies at the rostral end of the hindbrain (Fig. 1). This isthmic cell group contains noradrenaline (González and Smeets 1991, 1993, 1994) and innervates the spinal cord, cerebellum and telencephalon (Parent 1975; Tohyama et al. 1975; González and Smeets 1991, 1993, 1994). This area is considered to be homologous to the LC of mammals, and this homology is based on its position, noradrenergic content, and projections to both the telencephalon and spinal cord (González and Smeets 1993; González et al. 1994; Marin et al. 1996). Different from rats, the

LC in *Rana* is constituted by 100–140 TH immunoreactive cells (TH-ir) on each side (Marin et al. 1996). Despite considerable efforts and recent advances in studies of respiratory control in amphibians, the role of LC is not completely understood. In this section we present some data on the involvement of LC in the control of breathing in anuran amphibians.

4.1 Basal Respiratory Drive

In mammals, some studies indicate 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). Moreover, LC contributes to the adaptation of adult breathing to physiological needs, and provides a tonic excitatory drive that contributes to a normal breathing rate in rats (Guyenet et al. 1993; Oyamada et al. 1998; Dawid-Milner et al. 2001; Li and Nattie 2006) and mice (Shirasawan et al. 2000; Hilaire et al. 2004). In this context, Hilaire et al. (2004) pointed out 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. Taken together these data may indicate that LC noradrenergic neurons provide a tonic drive to breathe. However, recent data from our laboratory demonstrated that selective lesion of the LC using 6-OHDA (a toxin that selectively eliminates catecholaminergic neurons) did not change basal ventilation (Biancardi et al. 2008), which suggests that noradrenergic neurons are located in the LC play no role in respiratory control under resting conditions.

Recently, Gargaglioni et al. (2007) showed that electrical stimulation believed to be in the vicinity of the LC of frogs (*Rana catesbeiana*) caused an increase in respiratory frequency, whereas the chemical stimulations (glutamate) had no effect. These differences may be due to the fact that electrical stimulation excites both fibers of passage and cell bodies, whereas chemical stimulation activates only cell bodies. Additionally, we have tested the participation of noradrenergic LC neurons in the control of breathing in unanesthetized toads (*Chaunus schneideri*) by using 6-OHDA lesion (Noronha-de-Souza et al. 2006). The LC lesion did not change the pulmonary ventilation of toads under resting conditions. Moreover, lesions of LC noradrenergic neurons did not affect the instantaneous breathing frequency, which has been used as a reliable indicator of the endogenous respiratory rhythm in intermittent breathers. This suggests that the LC neurons do not participate in the generation of the respiratory rhythm.

4.2 Responses to Hypercapnia

Several studies on mammals have demonstrated that c-Fos techniques can be used to identify neurons involved in 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 c-Fos gene in LC neurons (Haxhiu et al. 1996; Teppema et al. 1997). In addition, extracellular recordings from LC neurones in both neonatal and adult rat show that they respond to systemic hypercapnia with an increase in spike frequency under in vivo conditions (Elam et al. 1981; Stunden et al. 2001). Local acidification of noradrenergic neurons of LC increases respiratory frequency and phrenic nerve discharge in cats (Coates et al. 1993).

The LC neurons are of particular interest in CO_2 challenge since >80% of 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, Li and Nattie (2006) lesioned the catecholaminergic (CA) neurons of the rat brainstem and found that the ventilatory response to 7% CO_2 was significantly decreased in sleep and wakefulness, suggesting that brainstem CA neurons participate in central chemoreception in vivo during both NREM sleep and wakefulness. In their study, approximately 84% of LC–CA neurons were eliminated, indicating that LC is an important site for hypercapnic ventilatory response. Recently, we have investigated the participation of LC noradrenergic neurons in relation to the CO_2 drive to breathe. Our data indicate that LC noradrenergic neurons modulate the hypercapnic ventilatory drive, since chemical lesion of this structure reduced the hypercapnic ventilatory response, due to a decreased V_T (Biancardi et al. 2008). We found that a reduction of approximately 80% of noradrenergic neurons of LC was associated with a large decrease in the response to CO_2 of approximately 64%, indicating that this nucleus exerts an important influence on CO_2-drive to breathing.

Until recently, there were no data available for the role of LC in the central chemoreception of amphibians. Therefore, we have investigated if LC is a CO_2/H^+ chemoreceptor site in anuran amphibians. Initially, we provided morphologic evidence, i.e., the expression of *c-fos* in neurons of the LC after hypercarbic challenge. The LC is one of the brainstem cell groups thought to be involved in physiological responses to hypercarbia, since a marked increase in *c-fos* positive cells in this nucleus was induced after 3 h of exposure to a hypercarbic gas mixture (Noronha-de-Souza et al. 2006).

In addition, we have used selective chemical lesion of LC catecholaminergic neurons to verify a possible involvement of this nucleus in the respiratory responses to hypercarbia. Our data show that chemical lesions of the LC with 6-OHDA resulted in an attenuation of hypercarbia-induced hyperventilation (Fig. 3), whereas this effect was absent 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

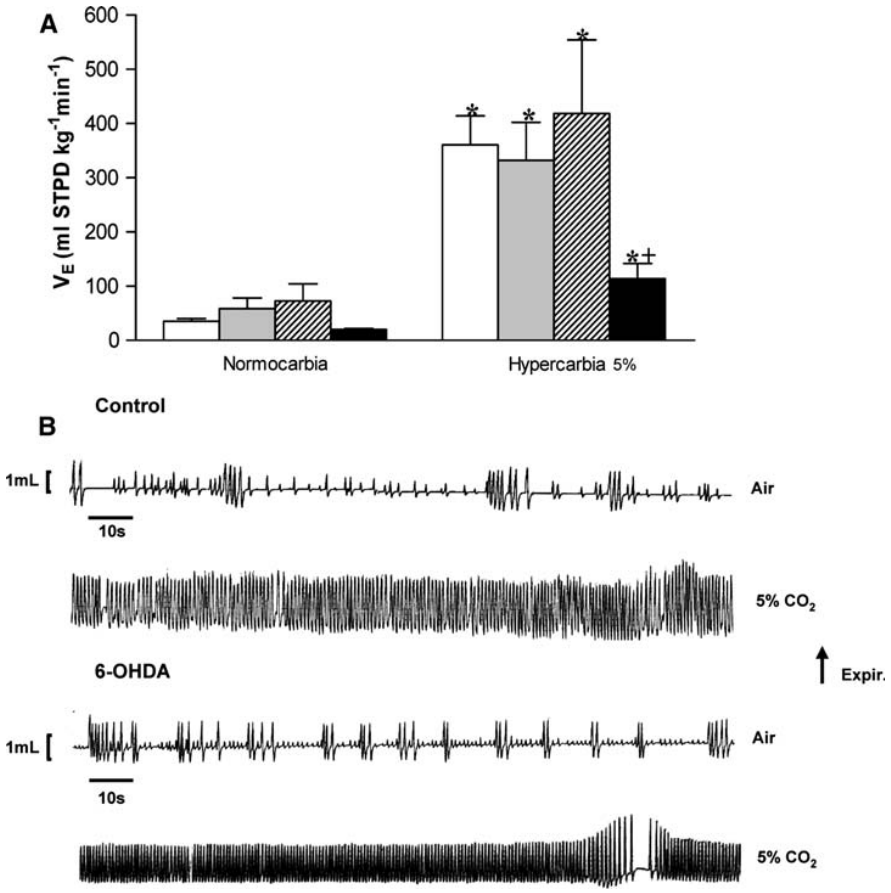

Fig. 3 **a** Ventilation (V_E) of the control, vehicle, peri and 6-OHDA groups exposed to normocarbia and hypercarbia (5% CO_2). **b** Pulmonary ventilation recordings obtained for the control, vehicle, peri and 6-OHDA groups during normocarbia and hypercarbia (5% CO_2). *Asterisk* indicates a significant effect of hypercarbia compared to the normocarbic value, *plus* indicates significant differences between 6-OHDA and all other groups during hypercarbia. (Adapted from Noronha-de-Souza et al. 2006)

bodies (González and Smeets 1993), strongly suggests that noradrenergic LC neurons are involved in processing or modulating central chemoreceptor information in amphibians.

We further investigated whether LC neurons are intrinsically pH-sensitive. To test this hypothesis we performed local acidification by microinjecting mock CSF with different pH values (7.2, 7.4, 7.6, 7.8 and 8.0). Mock CSF perfusion is a well-established method for studying the central chemoreceptor drive to breathe (Hitzig and Jackson 1978; Hitzig et al. 1985; Branco et al. 1992; Sanchez et al. 2001).

We performed local acidification of the LC by microinjection of mock CSF with different pH values. Notably, pulmonary ventilation increased after local reduction of the pH (mock CSF of 7.2, 7.4 and 7.6), which suggests that LC is a chemosensitive site in the CNS of amphibians.

Richerson (1999) and Putnam et al. (2004) proposed two essential criteria that a central chemoreceptor neuron must possess: (1) it must respond to changes in CO_2 that occur under nonpathological conditions in vivo, and this response must be due to mechanisms intrinsic to the specific cell; (2) it must have the capability of increasing respiratory output in response to an increase in CO_2, which could be accomplished if the neuron is part of the respiratory network or projects to respiratory neurons. The data of Noronha-de-Souza et al. (2006) fit the first criterion since lesions of the LC resulted in an attenuation of the hypercarbia-induced hyperventilation, and local tissue acidosis increased the ventilation of the toads. With regard to the second criterion, it is well known that the LC of mammals projects to respiratory neurons such as ventral medullary and solitary tract nuclei (Van Bockstaele et al. 1998, 1999). There is neuroanatomical evidence suggesting that LC of amphibians is homologous to the LC of mammals primarily on the basis of its position, noradrenergic content, and projections to brainstem structures (González and Smeets 1993; González et al. 1994; Marin et al. 1996). Furthermore, under in vivo conditions, in mammals, LC neurons appear to be activated by systemic hypercapnia, as judged by increased expression of *c-fos* gene product (Haxhiu et al. 1996; Teppema et al. 1997). The c-*fos* expression technique has been extensively used as a marker of neuronal activity, induced by a number of stimuli including hypercarbia. We found that an increased inspired-CO_2 concentration (5% CO_2) induces Fos-like immunoreactivity in the LC of toads, reinforcing the idea that the LC of amphibians is homologous to the LC of mammals. Further analysis is needed to understand the specific functional connections of the LC with the neuronal circuitry involved in the control of respiration in amphibians, but we can speculate that the LC of amphibians might be a central chemoreceptor site.

5 Final Remarks and Perspectives

The understanding of the neurorespiratory control mechanisms in amphibians has recently advanced considerably. The generation of episodic breathing is an intriguing topic. This pattern is currently accepted as an intrinsic property of the central respiratory control system. It was previously suggested that the NI might be responsible for generating the episodic breathing in anurans (Kinkead et al. 1997). Nevertheless, manipulation of the NI (by lesion or drug microinjection) failed to eliminate the episodic pattern, which suggests that the NI is not directly responsible for turning breathing episodes on and off (Kinkead et al. 1997; Gargaglioni and Branco 2000, 2001, 2003; Gargaglioni et al. 2002). Nonetheless, the accumulated data suggest that the NI acts to inhibit hypoxic and hypercarbic drives to breathe by restricting increases in tidal volume. The inhibitory role of the NI is similar to that of certain

pontine sites (LC and NRM) in rats (Fabris et al. 1999; Nucci et al 2004). The LC and NRM of mammals and the NI of amphibians are not homologous, but the analogous effects of these lesions are remarkable. There may be a number of putative mediators for these responses, but so far only glutamate and nitric oxide have been described.

Since we found an alteration in tidal volume after NI lesion and microinjection of drugs, it is possible that NI acts as a relay site for pulmonary stretch receptor information. According to Wang et al. (1999), these receptors are sensitive to lung volume, and provide a feedback for lung inflation, which limits tidal volume. In general, activation of stretch receptors suppresses inspiration and enhances expiration (Hering-Breuer reflex). Additionally, a recent study by Reid et al. (2000) showed that pulmonary stretch receptor feedback has a crucial role in the integration of CO_2 chemosensory information during hypercapnia in *Bufo marinus*. The NI appears to provide an inhibitory input to respiratory sites, which limits breathing amplitude when the respiratory drive is elevated. This is supported by McLean et al. (1995) who verified that microinjection of excitatory and inhibitory amino acids in isolated adult frog brainstem influenced the frequency and amplitude of lung-burst activity. Further experiments are necessary to investigate the relationship between brainstem modulating regions and the NI.

The interaction between L-glutamate and NO in the NI is also an important topic for future studies. In this context, we observed that the effect of L-NAME on ventilation resembled the effect observed following kynurenic acid microinjection into the NI, suggesting a possible interaction between L-glutamate and NO. In fact, it has been reported that activation of brain L-glutamate leads to synthesis and release of NO (Garthwaite et al. 1989). Previous studies demonstrated that NO formation mediates L-glutamate-induced cGMP accumulation (Bredt and Snyder 1989) and NMDA-evoked neurotransmitter release in the brain (Montague et al. 1994). L-glutamate released from a presynaptic terminal, acting on NMDA receptors, promotes an increase in intracellular calcium that can stimulate constitutive NOS, generating NO. The NO formed diffuses to the presynaptic terminal, where it stimulates guanylate cyclase and elevates cyclic GMP concentration, leading to further increase in the release of L-glutamate and augmentation of synaptic transmission (Garthwaite 1991).

Relatively less is known about LC. A recent study supports the notion that the noradrenergic neurons of toads are a site of central chemoreception (Noronha-de-Souza et al. 2006). Based on these data, we can suggest that the presence of widespread central chemoreceptor sites evolved early, since they are present in amphibians. Very probably, the transition from water to air breathing was associated with demands for a more flexible and sensitive CO_2 control system, which brought the development of multiple central sites for CO_2/H^+ detection. Our findings emphasize further the similarities between anuran and mammalian LC, and support the proposed homology of this nucleus in both groups.

In summary, midbrain structures such as NI and LC are essential in the mediation of CO_2 drive to breathing. Despite of recent significant advances in this field, further studies are urgent in an attempt to shed light on the neurochemical

mechanisms responsible for these vital responses to environmental stress. For instance, the putative role of LC mediating the hypoxic ventilatory responses is still pending.

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