Respiratory Physiology & Neurobiology 233 (2016) 73–80

Contents lists available at ScienceDirect

Respiratory Physiology & Neurobiology

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

Corticotropin-releasing factor in the locus coeruleus as a modulator of ventilation in rats

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ARTICLE INFO

Article history:
Received 30 June 2016
Received in revised form 7 August 2016
Accepted 16 August 2016
Available online 16 August 2016

Keywords:
Locus coeruleus
CRF receptors
Hypercapnia
Ventilation
Chemoreception

ABSTRACT

The locus coeruleus (LC) is a pontine noradrenergic nucleus that acts as a central chemoreceptor to CO_2/pH and has been implicated in the cognitive aspects of stress responses. This participation is in part mediated by the action of corticotropin-releasing factor (CRF), which when released in these situations increases the firing frequency of LC noradrenergic neurons. Nevertheless, the role of CRF_1 receptors in the LC in breathing and temperature control is unknown. Therefore, we tested the involvement of CRF_1 receptors located in the LC in room air ventilation and the ventilatory response induced by hypercapnia (7% CO_2) in rats. To this end, we injected CRF-R1-selective antagonists (antalarmin-1.2 and 2.4 mmol/0.1 μL or CP-376395-5 nmol/0.1 μL) into the LC of male Wistar rats. Pulmonary ventilation (V_E) and body temperature (Tb, dataloggers) were measured in air, followed by 7% CO_2 in unanesthetized rats. Antalarmin (higher dose) and CP-376395 in the LC caused an increase in V_E during normocapnia and hypercapnia, due to an increase in tidal volume. There were no differences in Tb between groups under normocapnia and hypercapnia. The results suggest that CRF acting on CRF_1 receptors in the LC exerts a tonic inhibitory role in ventilation.

1. Introduction

The locus coeruleus (LC) is an important pontine noradrenergic nucleus involved in the control of physiological and behavioral functions, such as breathing (Erickson and Millhorn, 1984; Oyamada et al., 1998; Fabris et al., 1999; Hilaire et al., 2004; Viemari et al., 2004; Ferreira et al., 2004; Biancardi et al., 2008; De Carvalho et al., 2010; De Souza Moreno et al., 2010) and thermoregulation (Fabris et al., 1999; Almeida et al., 2004; Ravanelli et al., 2007). In this regard, our laboratory has demonstrated that a chemical lesion of 80% of the LC noradrenergic neurons was associated with a 64% decrease in the CO_2 ventilatory response, due to changes in tidal volume, indicating that LC plays an important role in this response in rats (Biancardi et al., 2008).

LC is innervated by fibers that contain several neurotransmitters such as glutamate, gamma-aminobutyric acid (GABA), serotonin, epinephrine, the peptide orexin/hypocretin, and corticotrophin-releasing factor (CRF) (Aston-Jones et al., 1995). We have demonstrated that serotonin (De Souza Moreno et al., 2010), glutamate (Taxini et al., 2013), ATP (Biancardi et al., 2014), and orexin (Vicente et al., 2016) acting on LC neurons modulate the hypercapnic ventilatory response. Regarding CRF, there is an extensive network of CRF afferents onto the LC, which includes the central nucleus of the amygdala (CeA), the bed nucleus of the stria terminalis, the paraventricular nucleus of the hypothalamus, and Barrington's nucleus (Koegler-Muly et al., 1993; Reyes et al., 2014; Valentino et al., 1992, 1994, 1996; Van Bockstaele et al., 1998, 1999). More recently, Pomrenze et al. (2015), using viral delivery of Cre-dependent reporters to identify lateral central amygdala (CeL) CRF neurons, reported robust CRF projections from CeL to the LC. Nevertheless, the role of CRF projections to the LC in breathing control is unknown.

CRF is an important regulator of endocrine, autonomic, immunological, behavioral, and cognitive components of the stress response (Reyes et al., 2014) and acts as a neuromodulator to activate the LC–noradrenergic system in response to certain challenges such as hypotension, hypovolemia, cold, and immobilization (Melia and Duman, 1991; Valentino et al., 1991; Berridge et al., 1993; Smagin et al., 1997; Curtis et al., 2001). CRF axon terminals synapse with LC dendrites, and direct administration of CRF onto LC neurons *in vivo* and *in vitro* produces a long-lasting tonic

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http://dx.doi.org/10.1016/j.resp.2016.08.004
1569-9048/

increase in LC discharge rate (Van Bockstaele et al., 1996, 2001; Curtis et al., 1997; Jedema and Grace, 2004). CRF exerts its actions by acting on two G protein-coupled receptors: CRF_1 and CRF_2 (Hauger and Dautzenberg, 2002; Perrin and Vale, 1999). Sauvage and Steckler (2001) observed a high immunoreactivity of CRF_1 receptors in virtually all LC neurons. Indeed, CRF activation of LC neurons through CRF_1 receptors increases c-fos expression and norepinephrine release in terminal fields (Rassnick et al., 1998; Page and Abercrombie, 1999).

Since CRF stimulates breathing in humans and fetal lamb (Bennet et al., 1990; Schulz and Lehnert, 1996) and reduces the CO_2 threshold for breathing responses in humans (Schulz and Lehnert, 1996), we assessed whether CRF acting on LC CRF_1 receptors is involved in regulating the respiratory and thermal responses under normocapnic and hypercapnic conditions in adult male rats.

2. Materials and methods

2.1. Animals

Experiments were performed on unanesthetized adult male Wistar rats weighing 300–350 g. The animals had free access to water and food and were housed in a temperature-controlled chamber maintained at 24–26 °C (ALE 9902001; Alesco Ltda., Monte Mor, SP, Brazil) with a 12:12 h light:dark cycle (lights on at 7:00 a.m.). The study was conducted in compliance with the guidelines of the National Council for the Control of Animal Experimentation (CONCEA, MCT, Brazil) and with the approval of the local Animal Care and Use Committee (CEUA, FACV-UNESP Jaboticabal; Protocol: 024088/14).

2.2. Drugs and gas mixture

Antalarmin (selective CRF_1 receptor antagonist, Sigma-Aldrich, St. Louis, MO, USA) was used in two concentrations (1.2 and 2.4 mmol/0.1 μL) based on previous studies (Bledsoe et al., 2011; de la Tremblaye et al., 2014) and pilot experiments and was dissolved in 10% dimethyl sulfoxide (DMSO) used as vehicle.

CP-376395 (selective CRF_1 receptor antagonist, Tocris, Ellisville, MO, USA. donated by Dr. Carlos Crestani from Sao Paulo State University) was used in one concentration (5 nmol/0.1 μL) based on previous study (Oliveira et al., 2015) and dissolved in saline (NaCl 0.9%).

The hypercapnic gas mixture (7% CO_2, 21% O_2, balance N_2) was purchased from White Martins Gases Industriais Ltda (Sertãozinho, SP, Brazil).

2.3. Surgeries and microinjection

All surgical procedures were performed under anesthesia with 100 mg/kg of ketamine (Union National Pharmaceutical Chemistry S/A, Embu-Guaçu, SP, Brazil) and 10 mg/kg of xylazine (Laboratories Calier S/A Barcelona, Spain) administered intraperitoneally (I.P.).

The head was shaved, and the skin was sterilized with betadine solution and alcohol. Rats were fixed to a Kopf stereotaxic frame and implanted with a stainless steel guide cannula. The guide cannula (0.7 mm o.d. and 15 mm in length) was implanted 1 mm above the right LC region (distance from lambda: anterior: −3.4 mm; lateral: −1.2 mm and 1.2; and dorsal: −5.8 mm deep from the skull and inclination of vertical stereotaxic bar at 15°) according to the Paxinos and Watson atlas (Paxinos and Watson, 2005). The cannula was attached to the bone using stainless steel screws and acrylic cement. A tight-fitting stylet was kept inside the guide cannula to prevent occlusion. Postoperatively, animals were treated with antibiotic (enrofloxacin, 10 mg/kg, intramuscular) and analgesic (flunixin meglumine, 2.5 mg/kg, subcutaneous) agents. Experiments were performed 7 days postoperatively.

A day before the experiments, the rats underwent a second surgery under ketamine/xylazine anesthesia for the implantation of datalogger (SubCue Dataloggers, Calgary, Canada) into the abdominal cavity through a midline laparotomy to measure the body temperature (Tb). The datalogger was programmed to acquire data every 5 min.

A 5-μL Hamilton syringe and a dental injection needle (Mizzy, 200 μm o.d.) connected to a PE-10 tube was used to perform the microinjections into the LC of unanesthetized rats. The injection needle was 1 mm longer than the guide cannula so that the LC was reached by the needle only at the time of the injection. A volume of 0.1 μL of vehicle or drug solution was injected over a period of 20 s and the needle was removed from the guide cannula after an additional 30 s to avoid reflux. All injections were performed using a microinjector machine (model 310, Stoelting CO., IL, USA). LC includes a region of approx. 840 μm rostrocaudally extending from −10.32 to −9.48 relative to bregma. It is known that once a drug is administered into a nucleus, it diffuses proportionally to its dose and the volume injected. Theoretical calculations by Lipski et al. (1988) estimated that in a 30 nL microinjection, the drug diffused ~325 μm, whereas Mitra et al. (1993) reported that microinjections performed with a volume of 0.1 μL could spread as far as 1 mm. Therefore, in our experiments we used 0.1 μL microinjections to reach a relatively large portion of LC without affecting the nuclei surrounding this area.

2.4. Determination of pulmonary ventilation

Measurements of pulmonary ventilation (V_E) were performed using the whole body plethysmography method as previously described (Bartlett and Tenney, 1970; Biancardi et al., 2008; De Carvalho et al., 2010; Patrone et al., 2014). In brief, freely moving rats were kept in a 5-L chamber ventilated with room air or a hypercapnic gas mixture containing 7% CO_2 (White Martins, Sertãozinho, Brazil) in low ambient noise conditions. The flow rate of the inflow gas into the animal chamber was monitored by a flowmeter (model 822-13-OV1-PV2-V4, Sierra Instruments, Monterey, CA). During measurements, the flow was interrupted, and the chamber was sealed for short periods of time (approximately 2 min); the pressure oscillations due to respiration were monitored by a differential pressure transducer (TSD 160A, Biopac Systems, Santa Barbara, CA). According to a previous study (Gargaglioni et al., 2003) and pilot experiments, the level of O_2 and CO_2 inside the chamber at the end of a 2 min period with the rat breathing inside the box showed virtually no change (0.1% for O_2 and 0.0031% for CO_2). The signals were fed into a differential pressure transducer (DA 100C, Biopac Systems), passed through an analog-to-digital converter, and digitized on a microcomputer equipped with data acquisition software (MP100A-CE, Biopac Systems). The sampling frequency was 1 kHz samples per second. The results were analyzed using the data analysis software Acqknowledge (v. 4.2.3 data acquisition system, Biopac Systems). Tidal volume (V_T) and respiratory frequency (fR) were calculated per breath to estimate ventilation per breath. V_T was calculated by using an appropriate formula (Bartlett and Tenney, 1970). The calibration for volume was obtained during each experiment by injecting the animal chamber with 1 mL of air.

2.5. Experimental protocol

At 7 days after the unilateral implantation of guide cannula, the animals were individually placed in a Plexiglass chamber (5 L) with room temperature maintained at 25 °C and allowed to move freely while the chamber was flushed with humidified air for approximately 30 min and allowed to calm and acclimatize before

measurements. After the animals remained calm (~30 min), control V_E two control measurements of V_E were performed before the microinjection. Subsequently, 0.1 μL of vehicle (10% DMSO or saline) or CRF_1 antagonists (antalarmin – 1.2 and 2.4 mmol/0.1 μL or CP-376395-5 nmol/0.1 μL) was microinjected into the rat LC. Respiratory measurements were performed 5, 10, 15, 20, 30, and 40 min after microinjection under normocapnic conditions. For the hypercapnia groups, after the control V_E measurement, a hypercapnic gas mixture (7% inspired CO_2 in air) was flushed through the chamber for 5 min, and then the rats received 0.1 μL of vehicle or CRF_1 antagonists into the LC, followed by subsequent measurements at 5, 10, 15, 20, and 30 during the addition of 7% CO_2. Finally, the rats were exposed to 50 min of normocapnic recovery and V_E was measured at 50 min. The core body temperature was measured at 5-min intervals throughout the experiment.

2.6. Histology

Upon completion of the experiments, the animals were deeply anesthetized with ketamine and xylasine and perfused intracardially with saline followed by 10% formalin solution. A needle injector (19.6 mm in length) was inserted through the guide cannula and a 0.1-μL microinjection of Evans blue stain was performed. The brain was removed and stored in 10% formalin for at least 2 days. After fixation, the brainstem was embedded in paraffin, sectioned on a microtome (15-μm thick coronal sections), and stained by the Nissl method for light microscopy determination of the region reached by the microinjection according to the Paxinos and Watson atlas (1998). Only rats with a positive site of microinjection into the LC were considered (intra-LC group). Experiments with animals in which the region of microinjection was not within the LC were also analyzed (peri-LC group) to demonstrate the specificity of the site injection.

2.7. Data processing and analysis

The results were reported as mean ± SEM and were tested for normality of deviation (Cramer Von-Mises criterion). Ventilatory parameters were calculated using a period of 2 min of respiratory recordings when the animals were quiet and presenting no body movements. The data recorded when the rats were moving inside the chamber were excluded from analysis since the respiratory activity was contaminated by larger oscillations in the pressure inside the chamber. The normocapnic and hypercapnic conditions were analyzed separately to determine the effect of drug microinjections. The effects of CRF_1 antagonist microinjection on the ventilatory variables and Tb were evaluated by two-way analysis of variance with time and drug as factors and a Bonferroni post hoc test to assess the differences between the groups. P value of <0.05 was considered to be statistically significant.

3. Results

3.1. Histology

Fig. 1 is a representative photomicrograph of the transverse section of rat brainstem through the region of LC injection.

3.2. Effect of intra-LC antalarmin microinjection on VE

In normocapnic conditions, microinjection of 2.4 mmol of antalarmin increased V_E by 20.9% (effect of treatment: $P < 0.0001$; Fig. 2A) by affecting V_T (effect of treatment: $P < 0.0001$). Hypercapnia induced a progressive increase in ventilation in all groups (effect of time: $P < 0.0001$; Fig. 2A); however, microinjection of the lower

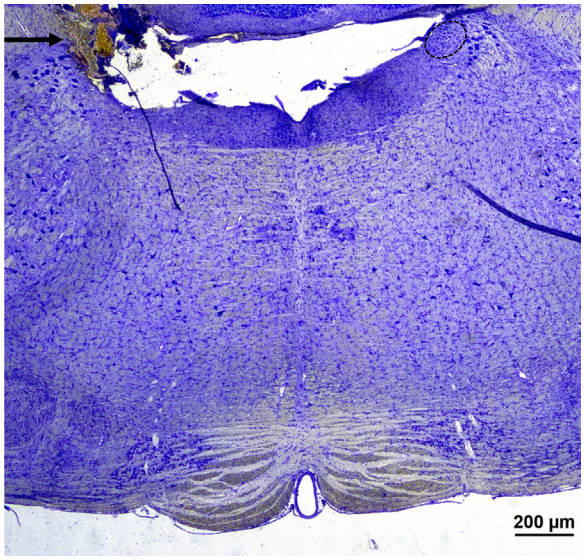

Fig 1. Representative photomicrograph of a coronal section at the pons level, illustrating microinjection of a rat locus coeruleus (LC). Dotted line indicates LC contralateral to the region of microinjection. Arrow indicates the region of microinjection.

and higher dose of antalarmin increased the hypercapnic ventilatory response by 28.3% and 26.6%, respectively, compared to the vehicle and peri-LC groups (effect of treatment: $P < 0.0001$; Fig. 2B). The increased CO_2 ventilatory response was due to enhanced V_T (effect of treatment: $P < 0.0001$; effect of time: $P < 0.0001$; Fig. 2B).

3.3. Effect of intra-LC CP-376395 microinjection on VE

Under room air condition, microinjection of CP-376395 increased V_E by 42.2% compared to the vehicle and peri-LC groups (effect of treatment: $P < 0.0001$; Fig. 3A) by reducing V_T (effect of treatment: $P < 0.0001$; Fig. 3A). Hypercapnia induced an increase in ventilation in all groups (effect of time: $P < 0.0001$; Fig. 3A) and microinjection of CP-376395 enhanced the CO_2-ventilatory response by 33.2% compared to the vehicle and peri-LC groups (effect of treatment: $P < 0.0001$; Fig. 3B) due to an increase in V_T (effect of treatment: $P < 0.0001$; effect of time: $P < 0.0001$; Fig. 3B).

3.4. Effect of intra-LC antalarmin or CP-376395 microinjection on Tb

Under normocapnia and hypercapnia, neither vehicle nor antalarmin and CP-376395 microinjection affected Tb (Fig. 4).

4. Discussion

Several studies have reported on the role of CRF in the LC; however, none has addressed the influence of this neurohormone in the LC on ventilatory and thermal response in unanesthetized rats. Our results indicate that CRF_1 receptors in the LC play an important role in regulating respiration since antagonism with antalarmin and CP-376395 caused a significant increase in ventilation under room air condition and CO_2 exposure. Nevertheless, we found that CRF acting on CRF_1 receptors in the LC does not participate in thermoregulation.

In the present study, antalarmin (at the highest dose) and CP-376395 administration in the LC increased ventilation during normocapnia, due to an increase in tidal volume without affecting the respiratory frequency. These data suggest that CRF acting on CRF_1 receptors in the LC has a tonic inhibitory role in the control

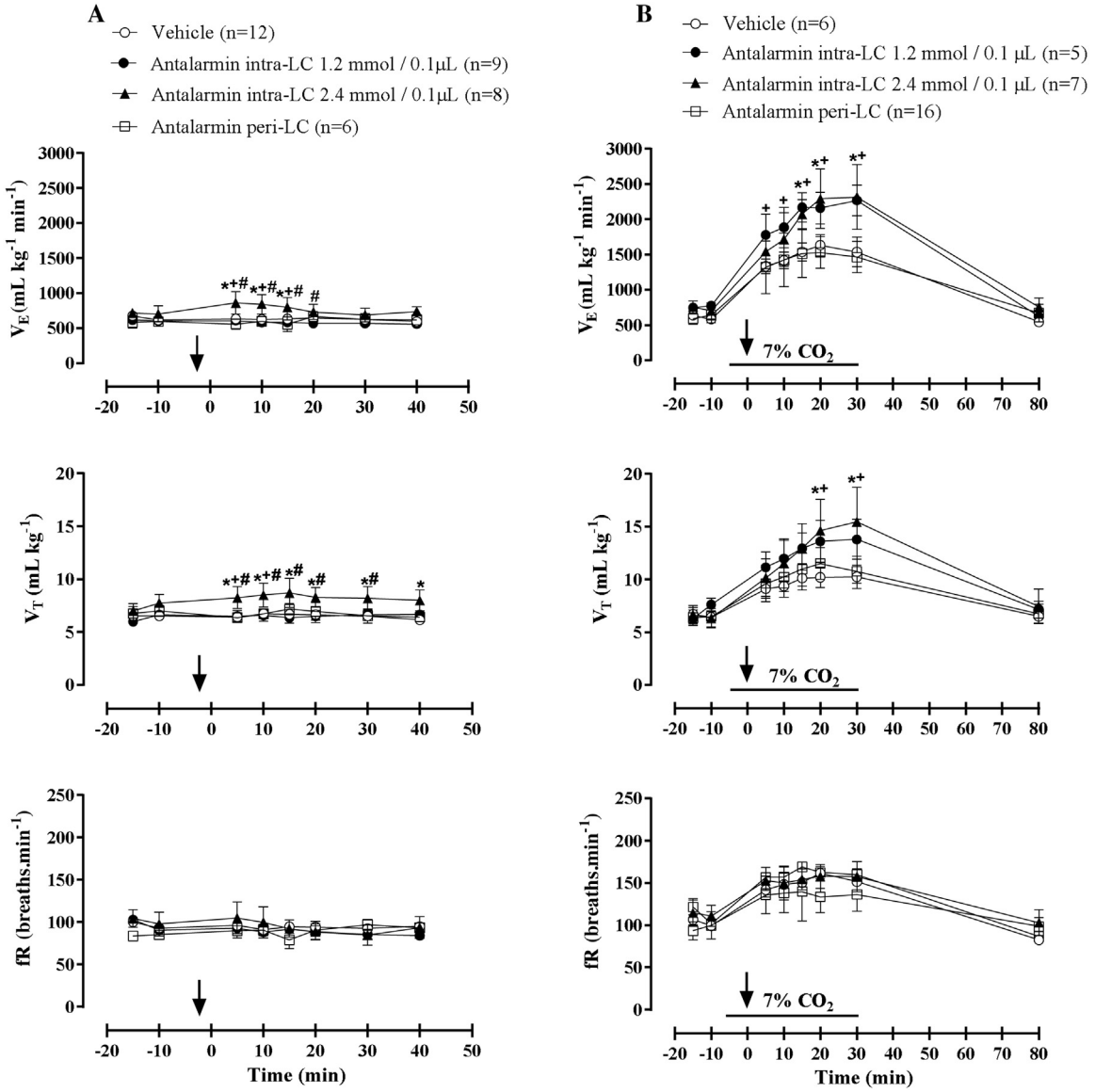

Fig. 2. Effects of intra-LC and peri-LC microinjection of vehicle (DMSO), antalarmin (CRF_1 antagonist, 1.2 and 2.4 mmol/0.1 µL) on tidal volume (V_T), respiratory frequency (fR) and ventilation (V_E) of rats during normocapnia (A) and hypercapnia (B). The arrow indicates the time of the microinjection. *Significant differences between vehicle and antalarmin group. $^+$Significant differences between peri-LC and antalarmin group. $^\#$Significant differences between the two concentrations of antalarmin. Note that during the normocapnia (A) only the highest dose produced an effect and in hypercapnia (B) at 5 and 10 min only the lowest dose produced an effect.

of ventilation. These data differ from those of previous studies that showed that the lesion of noradrenergic neurons of the LC does not affect baseline ventilation (Biancardi et al., 2008; De Carvalho et al., 2010). The effect was higher for CP-376395 (42.2%) compared to antalarmin (20.9%) probably because the affinity of CP-376395 for CRF_1 receptors is higher than antalarmin (Chen et al., 2008)

As the CRF receptors in the LC are considered excitatory for the activity of noradrenergic neurons of the nucleus (Valentino et al., 1983, 1991), administration of the CRF antagonist would result in reduced activity of the LC (Valentino et al., 1991) and consequently, a decrease in noradrenaline release in the areas that protrude LC. Nevertheless, according to Swiergiel (2003), small amounts of CRF_1 antagonist infused directly into the LC can selectively affect the discharge of neurons, depending on the stress or the extra-hypothalamic system that is activated by CRF. Valentino et al. (2001) suggest that different stimuli can activate specific CRF afferents that project to the LC, which end in specific subregions of this nucleus. For example, in hypotensive stress, the central amygdaloid nucleus acts as a primary source for the activation of CRH in the LC (Curtis et al., 2002; Valentino et al., 1991). The LC receives robust CRF-containing projections from the central amygdala and the anxiogenic responses of acute stress depend on the release of CRF from the CeA into the LC neurons (Sun et al., 2015). According to these authors, photostimulation of CeA-LC CRF terminals in the LC may reduce the activity of half of the cells and decrease the activity of some cells. The putative explanation from these divergent actions must be that different LC subpopulations may be activated during different responses. The CRH system in the bed nucleus of the stria terminalis has also been implicated in the coordination of cardiovascular changes associated with stress. CRH injected intracerebroventricularly or directly into the bed nucleus of the stria terminalis results in tachycardiac response that is blocked by β-adrenergic, α-adrenergic, and CRF1 antagonists (Nijsen et al., 2000). Moreover, CRF in the nucleus of the stria medial terminal bed during the stress caused by fear conditioning contributes to the response to cardiac stress, especially by vagal activation, thus causing bradycardia (Nijsen et al., 2001).

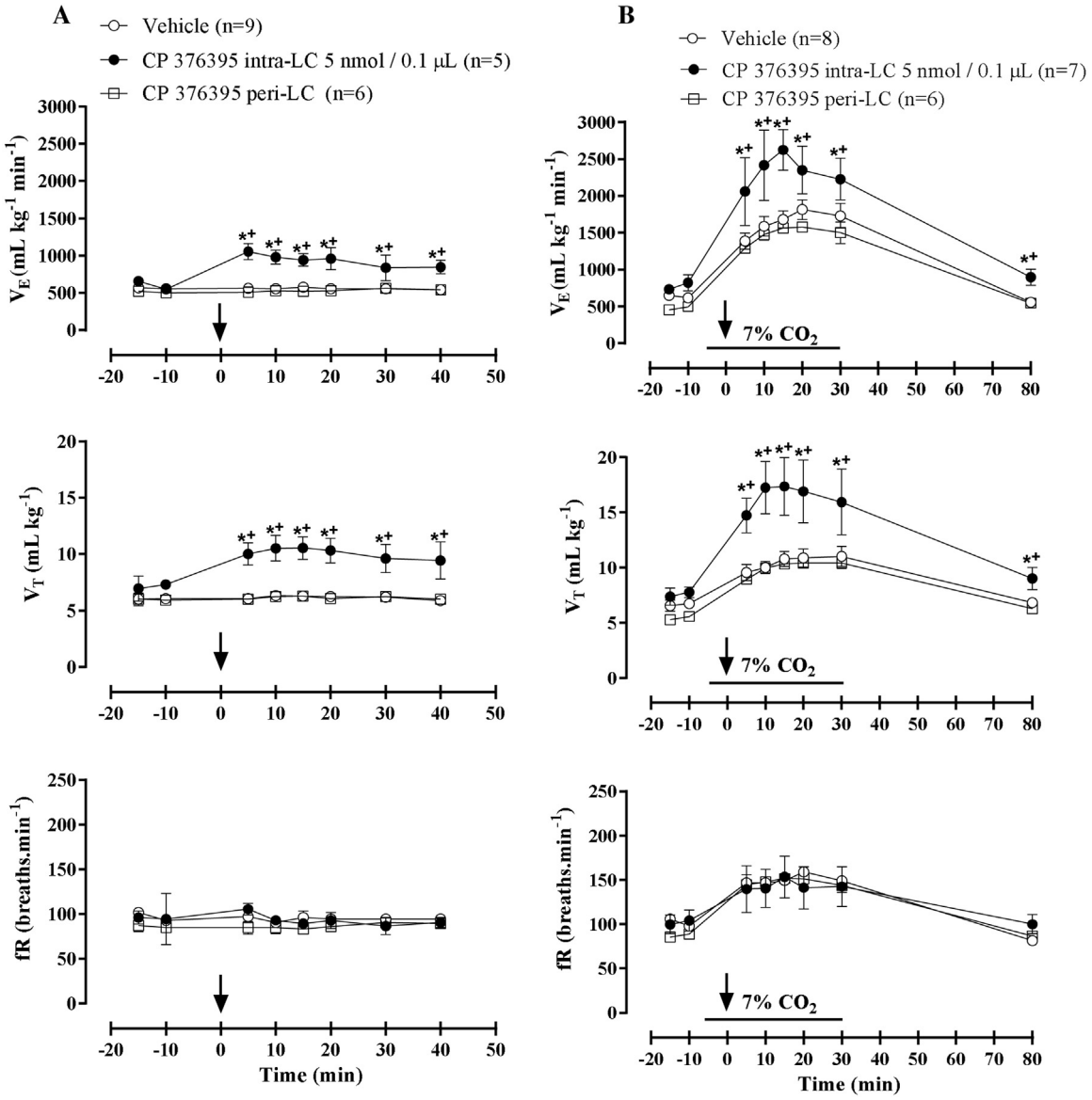

Fig. 3. Effects of intra-LC and peri-LC microinjection of vehicle (saline) and CP-376395 (CRF_1 antagonist, 5 nmol/0.1 μL), on tidal volume (V_T), respiratory frequency (fR) and ventilation (V_E) of rats during normocapnia (A) and hypercapnia (B). The arrow indicates the time of the microinjection. *Significant differences between vehicle and CP-376395 group. +Significant differences between peri-LC and CP-376395 group.

According to studies by Valentino et al. (2001), the CRF-immunoreactive terminals form synaptic specializations on dendrites of LC in rats. These immunoreactive terminals for CRF are co-located with other neuromodulators that modulate the actions of CRF on LC neurons. Among these are glutamate and GABA. CRF is preferably co-located with glutamate at the rostrolateral region of the LC, and CRF may impact on glutamate neurotransmission in the LC via presynaptic or postsynaptic actions. In this context, studies in our laboratory by Taxini et al. (2013) demonstrated that the glutamatergic afferents acting on the ionotropic receptors in the LC exert an inhibitory modulation of ventilatory responses to hypercapnia, similar to the results found in the present study. Another possibility to explain our results is that antalarmin and CP-376395 may be linking to CRF_1 receptors present in GABAergic neurons of the LC and increasing the ventilation in animals. It is known that there are GABAergic neurons in the LC (Iijima and Ohtomo 1988; Iijima et al., 1992; De Carvalho et al., 2010) and they represent about 8% of all neurons present in this nucleus (Iijima and Ohtomo, 1988). In this context, a previous study from our laboratory showed that SP-SAP toxin in the LC damages several GABAergic as well as catecholaminergic neurons (De Carvalho et al., 2010) and leads to reduced inhibition of ventilatory response to CO_2 (30% reduction) compared to lesions with 6-OHDA (64% reduction), which are specific for catecholaminergic neurons. The relationship between CRF and GABAergic neurotransmission system has been reported by Nie et al. (2004) and Herman et al. (2013). According to the latter study, CRF robustly increases GABAergic transmission in the central amygdaloid nucleus of Wistar rats and the treatment with R121919, a CRF_1 antagonist, decreases GABAergic transmission in this area. In addition, Valentino et al. (2001) suggested that some of the actions of CRF on LC activity are through the regulation of local inhibitory GABA circuits, because CRF-immunolabeled axon terminals are frequently opposed to other axon terminals, some of which are GABA immunolabeled.

One cannot rule out the possibility that CRF_1 antagonists in our non-stressed rats could be acting as agonists, increasing the noradrenaline release in the LC neurons, despite the high selectivity of these drugs. In this regard, Jedema and Grace (2004) reported

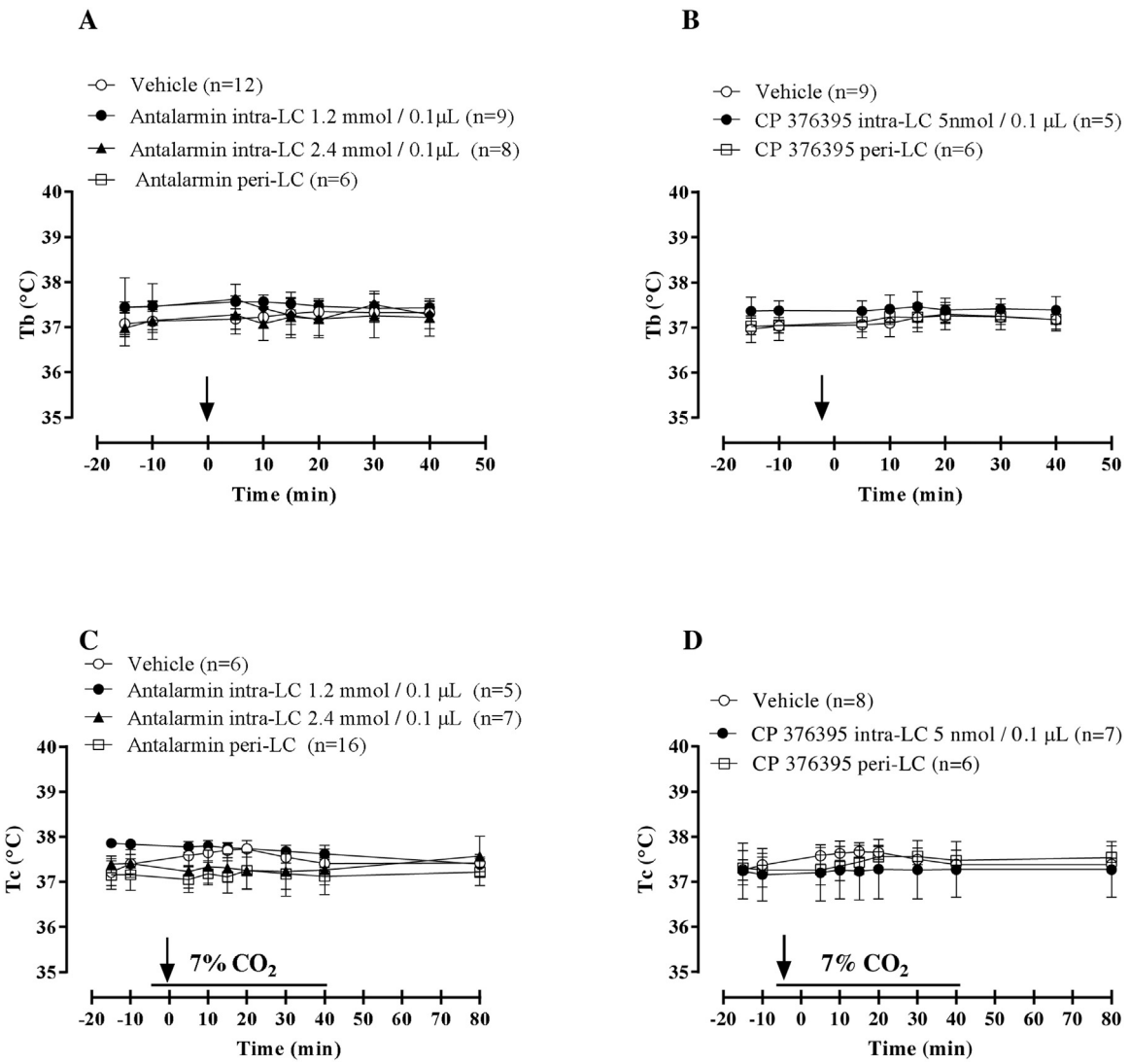

Fig. 4. Effects of intra-LC and peri-LC microinjection of vehicle (DMSO), antalarmin (CRF_1 antagonist, 1.2 and 2.4 mmol/0.1 µL) on body temperature (Tb) of rats during normocapnia (A) and hypercapnia (B). Effects of intra-LC and Peri-LC microinjection of vehicle (saline) and CP-376395 (CRF1 antagonist, 5 nmol/0.1 µL), on Tb of rats during normocapnia (C) and hypercapnia (D).

a significant activation of LC neurons by local or bath application of the antagonist D-Phe-CRH alone. Partial agonist effects of the CRF antagonist, α-helical CRH, have been reported previously *in vitro* (Rainnie et al., 1992; Yu and Shinnick-Gallagher, 1998; Smart et al., 1999) and *in vivo* (Menzaghi et al., 1994; Borsody and Weiss, 1996). In addition, Spina et al. (2000) demonstrated that when astressin, a nonselective CRF hormone antagonist, was injected into non-stressed rats on the elevated plus maze, a tendency toward a reduction in the exploration of the open arms was found at low doses, suggesting a more complex effect on central CRF receptors, possibly on high affinity autoreceptors that control CRF release (Wiersma et al., 1993). We do not believe that this would have occurred in our study, because the doses were used in a similar fashion and were even smaller than those used in previous studies that promoted selective blockade of CRF1 receptors in the central nervous system (Forster et al., 2008; Bledsoe et al., 2011; Liu et al., 2011; Sergio et al., 2014; Oliveira et al., 2015). In a recent study, Sergio et al. (2014), using exactly the same doses used in our study, observed that pretreatment with antalarmin completely blocked the panicogenic effect similar to CRF in the electrical stimulation model of substance dorsal periaqueductal gray (dPAG).

In the present study, hypercapnia induced an increase in ventilation in the vehicle, antalarmin, and CP-376395 groups as a result of increase in V_T and fR. The microinjection of antalarmin and CP-376395 in the LC increased the hypercapnic ventilatory response compared to that in the control animals. Because the lowest dose of antalarmin increased ventilation only during CO_2 exposure and not during normocapnia, our data suggest that CRF acts on CRF_1 receptors in the LC by modulating hypercapnic ventilatory response. It is conceivable that in our study, the increase in ventilatory response to CO_2 after antagonizing CRF_1 receptors can be the result of inhibition of inhibitory interneurons in the LC. Another possibility is that CRF acts directly onto the GABAergic neurons of the LC and then decreases the hypercapnic response.

Regarding the role of LC neurons in thermal control, there is evidence that LC noradrenergic neurons are part of a thermoeffector neuronal pathway that is specifically activated by pyrogens (e.g., PGE2) to induce thermogenesis and produce fever in a subthermoneutral environment (Almeida et al., 2004), but specific lesions of LC noradrenergic neurons do not change the Tb of rats under euthermia in normocapnia or hypercapnia, suggesting that noradrenergic neurons of the LC play no role in Tb regulation under these conditions (Almeida et al., 2004; Biancardi et al., 2008). Buwalda

et al. (1997) observed long-lasting hyperthermia following CRF infusion in rats. CRF appears to mediate thermogenic responses to serotonergic agonists, injury, and cytokines (Rothwell, 1994). In the present study, CRF_1 antagonist injected intra-LC did not affect Tb, indicating that CRF in the LC does not play a role in thermoregulation.

In conclusion, the present results provide evidence that CRF acts on CRF_1 receptors in the LC neurons, plays an important role in the respiratory drive, and exerts an inhibitory modulation effect during normocapnia and hypercapnia, but it does not play a role in the responses of thermoregulation.

Acknowledgements

This research was supported by public funding from *Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP)* and the *Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq)*. This study was part of the activities developed by J.M. Incheglu during her Master at the Joint Graduate Program in Physiological Sciences (PIPGCF) from UFSCar/UNESP. We thank Renata Mello and Euclides Roberto Secato for their excellent technical assistance.

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