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

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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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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