Melanin-concentrating hormone regulates the hypercapnic chemoreflex by acting in the lateral hypothalamic area

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Abstract
Melanin-concentrating hormone (MCH) is a hypothalamic neuropeptide involved in a broad range of homeostatic functions including regulation of the hypercapnic chemoreflex. We evaluated whether MCH modulates the hypercapnic ventilatory response by acting in the lateral hypothalamic area (LHA) and/or in the locus coeruleus (LC). Here, we measured pulmonary ventilation ($V_E$), body temperature, electroencephalogram (EEG) and electromyogram (EMG) of unanaesthetized adult male Wistar rats before and after microinjection of MCH (0.4 mM) or MCH receptor 1 (MCH1-R) antagonist (SNAP-94847; 63 mM) into the LHA and LC, in room air and 7% CO2 conditions during wakefulness and sleep in the dark and light periods. MCH intra-LHA caused a decreased CO2 ventilatory response during wakefulness and sleep in the light period, while SNAP-94847 intra-LHA increased this response, during wakefulness in the light period. In the LC, MCH or the MCH1-R antagonist caused no change in the hypercapnic ventilatory response. Our results suggest that MCH, in the LHA, exerts an inhibitory modulation of the hypercapnic ventilatory response during the light-inactive period in rats.

KEYWORDS
central chemoreception, hypercapnia, MCH

1 INTRODUCTION

Melanin-concentrating hormone (MCH) is a 19-amino-acid peptide in mammals, synthesized from a precursor molecule, prepro-MCH (pMCH) in neurons whose cell bodies are located mainly in the lateral hypothalamic area (LHA), and to a lesser degree, in the adjacent incerto-hypothalamic area (Adamantidis & de Lecea, 2008; Bittencourt, 2011; Bittencourt et al., 1992; Vaughan et al., 1989). MCH receptors have been found throughout the entire central nervous system (CNS) (Lembo et al., 1999; Schlumberger et al., 2002) and are divided into two types, MCH1-R and MCH2-R, but MCH2-R is expressed only in humans, not in rodents (Chung, Saito & Civelli, 2009; Sailer et al., 2001). Studies of MCH receptor signalling have demonstrated that there is an activation of several intracellular signalling pathways by coupling to $G_{i\alpha}$, $G_{q\alpha}$ and $G_{o\alpha}$ proteins (Chung, Saito & Civelli, 2009). The fact that MCH receptors couple to different G proteins indicates that different second messenger responses can be elicited according to the cellular environment (Saito et al., 1999), and this may contribute to the diverse physiological processes regulated by MCH, such as regulation of energy homeostasis (Marsh et al., 2002), cardiovascular functions (Messina & Overton, 2007), sleep–wake cycle (Ono & Yamanaka, 2017; Torterolo et al., 2015), thermoregulation...
(Glick et al., 2009), control of food intake and weight loss (Della-Zuana et al., 2002), respiratory control (Badami et al., 2010), and the hypercapnic chemoreflex (Li et al., 2014).

Regarding the central chemoreflex, it has been demonstrated that down-regulation of MCH in the CNS by knocking down the MCH precursor (pMCH) caused an enhanced hypercapnic chemoreflex in rats only in wakefulness (Li, Nattie & Li, 2014), which suggests that MCH acts as a suppressor of the hypercapnic ventilatory response during wakefulness. However, the mechanisms involved have not been elucidated so far. Since MCH neurons send projections to some CO₂ chemosensitive regions in the CNS such as the locus coeruleus (LC) (Bittencourt et al., 1992; Skofitsch et al., 1985), one hypothesis is that the role of MCH in the central hypercapnic chemoreflex is due to its interaction with chemosensitive neurons at a distance. In addition, the existence of reciprocal innervation has been demonstrated between MCH and the nearby orexinergic neurons (OX) (Deng et al., 2007; Sunanaga et al., 2009; Williams et al., 2007), which like LC noradrenergic neurons are considered chemosensitive to CO₂/pH, according to the classification of Kuwaki et al. (2010). Therefore, another possibility would be that the role of MCH in modulating the hypercapnic chemoreflex is by means of an interaction between MCH neurons and the CO₂/pH-chemosensitive OX neurons in the LHA.

Experimental evidence indicates that MCH and OX are not expressed in the same neurons in LHA; however, they establish reciprocal synaptic relationships with each other (Guan et al., 2002), while the orexins are predominantly excitatory neuropeptides (Burdakov et al., 2013; Li & Nattie, 2010), MCH is inhibitory (van den Pol et al., 2004; Wu et al., 2009). Consequently, OX and MCH neurons are antagonistic in many of their physiological functions, including in respiratory control (Li, Nattie & Li, 2014). Thus, the mechanism by which MCH neurons modulate the central chemoreflex could be due to their inhibitory effect on chemosensitive neurons that express MCH1-R, either in the LHA region itself (Lembo et al., 1999; Saito et al., 2001), where synaptic interactions occur with OX neurons (Guan et al., 2002), or at a distance, acting in other chemosensitive nuclei such as the LC.

Among the chemosensitive nuclei, LC can be chosen as a possible target for MCH neurons in the modulation of the central chemoreflex as the neurons in this region receive dense projections from MCH neurons (Yoon & Lee, 2013), express the MCH1-R (Lembo et al., 1999; Saito et al., 2001; Ye et al., 2018), and the noradrenergic neurons in this region are CO₂/pH chemosensitive with an expressive role in hypercapnic drive to breathe (Biancardi et al., 2008; Gargaglioni et al., 2010). Moreover, as MCH neurons, the LC noradrenergic neurons participate in the regulation of the sleep–wake cycle (Aston-Jones & Bloom, 1981; Berridge, Schmeichel & España et al., 2012; Berridge & Waterhouse, 2003).

Concerning the involvement of MCH in the control of the sleep–wake cycle, it has been suggested that MCH neurons may promote sleep, especially REM sleep (Tsunematsu et al., 2014; Vetrivelan et al., 2016). The role of MCHergic neurons in the induction of sleep may be related to the inhibitory effect of MCH on OX neurons, which are known to be critical for maintaining wakefulness (Li, Nattie & Li, 2014; Sakurai et al., 2010; Torterolo et al., 2011). Moreover, it has been demonstrated that REM sleep occurrence is facilitated by the deactivation of LC noradrenergic neurons by MCH (Monti et al., 2015). Accordingly, the interaction between MCH and OX neurons, as well as between MCH and LC noradrenergic neurons seems to contribute to the regulation of the sleep–wake cycle (Ono & Yamanaka, 2017). For instance, it is known that the central hypercapnic chemoreflex is modulated by the sleep–wake cycle (Burke et al., 2015) and chemosensitive sites interact differently in sleep and wakefulness (Nattie & Li, 2010). The interaction between MCH and LHA and/or LC could, therefore, contribute to the modulation of the central chemoreflex in a sleep–wake cycle-dependent manner. Interestingly, MCH levels in the cerebrospinal fluid (CSF) of rats (Pelluru et al., 2013) and in hypothalamic sections of mice (Gericcs et al., 2017) are higher in the inactive period (light phase) compared to the active period (dark phase), but whether the mechanisms by which MCH acts in the central chemoreflex depend on the light–dark cycle is uncertain.

According to previous studies, both LHA and LC are involved in thermoregulation (Morrison, 2016; Ravanelli & Branco, 2008; Takahashi el al., 2001). Considering the participation of the MCHergic system in energy balance, it has been shown that the loss of MCH signalling in MCH knockout mice results in increased body temperature, oxygen consumption, heart rate and mean arterial pressure (Takase et al., 2014). Furthermore, it has been shown that mice without MCH1-R expression have an increased average basal body temperature, especially during the light period (Ahnaou et al., 2011), suggesting that the MCH neuropeptide may participate in the regulation of body temperature. In addition, mice centrally treated with MCH for 14 days presented a decrease in body temperature and energy expenditure (Glick et al., 2009). Since studies have demonstrated that hypercapnia causes a decrease in body temperature and metabolic rate in rats (Gautier et al., 1989; Gautier et al., 1993; Vicente et al., 2016) and MCH is involved in thermoregulation, these findings together point to a possible involvement of MCH in body temperature control during high levels of CO₂.

New Findings

- **What is the central question of this study?**
  Melanin-concentrating hormone (MCH) suppresses the hypercapnic chemoreflex: what is the mechanism by which this effect is produced?

- **What is the main finding and its importance?**
  MCH acting in the lateral hypothalamic area but not in the locus coeruleus in rats, in the light period, attenuates the hypercapnic chemoreflex. The data provide new insight into the role of MCH in the modulation of the hypercapnic ventilatory response.
Therefore, we hypothesized that MCH neurons modulate the hypercapnic chemoreflex due to their inhibitory effect on chemosensitive neurons that express MCH1-R, either in the LHA region itself or/and by modulating other chemosensitive regions such as the locus coeruleus, and that this mechanism is dependent on the sleep–wake and the light–dark cycle.

2 | METHODS

2.1 | Ethical approval

This study was carried out under the ethics principles and regulations on animal experimentation required by Experimental Physiology, as described in the editorial by Grundy (2015). Besides, all experimental procedures used in the present study were also in accordance with the guidelines of the National Council for the Control of Animal Experimentation (CONCEA, MCT, Brazil), with the approval of the Animal Care and Use Committee for the Institute of Biosciences at Botucatu, Brazil (CEUA – IBB, UNESP, Botucatu, SP, protocol no. 1104).

2.2 | Animals

Adult male Wistar rats (270–350 g) were obtained from the Central Animal Facility of UNESP – Botucatu. The animals had free access to water and food and were housed in a light- and temperature-controlled room (23–25°C), and with a 12:12 h light–dark cycle (lights on at 07.00 h for the light period group, and at 15.00 h for the dark period group). All experiments were carried out between 09.00 and 15.00 h. The animals in the dark group were adapted to the partially inverted cycle for at least 2 weeks before the experimental protocol. To help the experimenters’ view, a red light was used during all the procedures performed with animals in the dark period. In addition, during the experiments, a black cloth was placed around the experimental chamber to prevent light from entering.

2.3 | Surgery

Animals were submitted to general anaesthesia by intramuscular administration of ketamine (100 mg kg$^{-1}$) and xylazine (15 mg kg$^{-1}$). Depth of anaesthesia was assessed by the loss of movement, loss of blink reflex, muscle relaxation and loss of withdrawal responses to stimulation. The head and a portion of the abdomen were shaved, and the skin was sterilized with betadine solution and alcohol. A temperature-recording capsule, programmed to take a reading every 5 min (Subcuve Datalogger, Calgary, Alberta, Canada), was introduced into the abdominal cavity for body temperature measurements. After that, the rats were fixed in a Kopf stereotoxic frame and implanted with a stainless-steel guide cannula (0.7 mm o.d. and 17 mm in length to LHA and 16 mm in length to LC) 1 mm above the LHA region, bilaterally, or the LC region, unilaterally. The coordinates for the cannula placement in the LHA were 2.7 mm caudal to bregma, 1.2 mm lateral to midline and 7.4 mm below the bone surface. For LC surgeries, the coordinates were 3.4 mm caudal to lambda, 1.3 mm lateral to midline and 7.4 mm below the bone surface, with an inclination of vertical stereotaxic bar at 15°. These coordinates were adapted from Paxinos and Watson (2014) and based on previous studies (Rodrigues et al., 2019, 2020; Silva et al., 2017). Different animals were used for LHA and LC cannulation. The guide cannula was secured with cranioplastic cement. Three EEG electrodes were implanted into the skull and a pair of EMG electrodes was inserted deep into the neck muscle. The incision was closed, the animals received antibiotic (0.3 ml of benzyl-penicillin, 1,200,000 U, intramuscular) and analgesic (flunixin meglumine, 2.5 mg kg$^{-1}$, subcutaneously) agents, and were allowed to recover for 7 days.

2.4 | Microinjection

Microinjections of MCH (0.4 mM; Phoenix Pharmaceuticals, Burlingame, CA, USA), as well as MCH1-R antagonist (SNAP-94847; 63 mM; Sigma, St Louis, MO, USA) or their respective vehicles (sterile saline for MCH and artificial CSF + 5% dimethyl sulfoxide for the MCH1-R antagonist) were performed using a 5-μl Hamilton syringe (Hamilton, Reno, NV, USA) and a dental injection needle (28 gauge) connected to a PE-10 tube. The microinjected volume was 100 nl, which was injected over a period of 30 s. An additional period of 30 s was allowed before the injection needle was removed from the guide cannula to avoid reflux. Prior to microinjection, the top of the plethysmograph chamber was opened and the animals, still kept inside the chamber, were handled gently in order to insert the needle injector into the guide cannula. Once the needle was in the correct position, injections were made manually, without any manipulation or restriction of the rats. This whole injection procedure usually did not cause significant behavioural changes in the animals. The doses used were based on previous studies (Brown et al., 2007; Ye et al., 2018).

2.5 | Pulmonary ventilation recordings

Ventilation ($\dot{V}_E$) measurements were obtained by the whole-body plethysmograph method as previously described (Bartlett & Tenney, 1970; Dias et al., 2009). In brief, freely moving rats were placed in a 5-litre Plexiglas chamber, flushed with humidified room air (or hypercapnic mixture, when required by the protocol) at a rate of 2 l min$^{-1}$. The outlet flow was connected to a regulated vacuum system to pull 2 l min$^{-1}$ from the chamber, allowing the continuous recording of the ventilation of the animals. The pressure oscillations caused by the animals’ breathing were monitored by a pressure transducer (TSD 160A, Biopac Systems, Santa Barbara, CA, USA) connected to the chamber. The pressure signals were amplified, filtered and recorded on a microcomputer equipped with data acquisition software (MP150WSW, Biopac Systems). By analysing the breathing events, data on respiratory frequency ($f_R$) and tidal volume ($V_T$) were obtained (Lab Chart Pro data analysis software, ADInstruments, Bella Vista NSW, Australia). $V_T$ was calculated using an appropriate formula (Malan, 1973), based on the technique described by Drorbaugh and...
Rodrigues ET AL.

2.6 Determination of vigilance state

The EEG and EMG electrodes, inserted into the plastic holder, were connected to an insulated and shielded cable, which was attached to an electrical swivel to allow the rats to move freely inside the chamber. A four-channel amplifier was then connected to the opposite end of the swivel. The signals from the skull and neck muscle were amplified (10,000x for EEG signals and 2000x for EMG signals) and band-pass filtered (low and high cut-off: 10 and 500 Hz for EMG signals and 0.3 and 50 Hz for EEG signals, respectively). A computer equipped with a Biopac acquisition system (MP150WSW, Biopac Systems) was used to acquire and record the signals (sample rate: 2 kHz). The data were separated into two groups: wakefulness and NREM sleep, which were determined by analysis of EEG and EMG recordings using LabChart 8 software (ADInstruments). Periods of REM sleep were not common in our experiments, and when they occurred, were very brief. Therefore, ventilatory recordings that occurred during REM sleep, or when the sleep state was indeterminate, were excluded from our analysis.

2.7 Histology

Upon completion of the experiments, the animals were deeply anaesthetized with sodium thiopental (60 mg kg\(^{-1}\), I.P.); the needle injector was inserted through the guide cannula, and a 100 nl microinjection of Evans blue was performed, and then they were perfused transcardially with 300 ml of phosphate buffer, followed by 300 ml of 4% paraformaldehyde solution. The perfusion was performed using a peristaltic perfusion pump (Masterflex, Cole Parmer International, Vernon Hills, IL, USA), adjusted to a flow of 30 ml min\(^{-1}\). The brain was then removed and post-fixed overnight in a 4% paraformaldehyde solution. After fixation, the brains were cryoprotected by immersion in a 30% sucrose solution for 72 h. The brains were then frozen, cut into 40-μm-thick coronal sections with a Reichert–Jung cryostat (Leica Microsystems, Wetzlar Germany), and stained by the Nissl method for light microscopy. The anatomical region of microinjection was determined according to the atlas of Paxinos and Watson (2014). Only rats with the tip of the microinjection located within the LHA or LC were considered.

2.8 Experimental protocol

Seven days after the surgery, the animals were gently handled, and the EEG and EMG electrode cables were connected. The animals were then placed in a 5-litre plethysmograph chamber and were allowed to move freely while room air was flushed through the chamber. The acclimatization period lasted 40–60 min. Afterwards, the experimental protocol began. Under room air conditions, \(V_e\), EEG and EMG were recorded continuously, and body temperature measurements were taken at 5 min intervals for 30 min. At the end of this period, the rats received the microinjection of MCH or MCH1-R antagonist or their vehicle solutions bilaterally into the LHA or unilaterally into the LC. The rats were maintained in normocapnia for a period of 10 min after the injection, the measurements were repeated and then the inspired air was switched to a hypercapnic gas mixture, containing 7% CO\(_2\) and 21% O\(_2\), balance N\(_2\), and the measurements were taken for an additional 40 min (from 10 to 50 min after injection). This protocol was performed in a group of rats during the light period and in another group during the dark period. Each rat received only one microinjection and was submitted to only one experimental protocol. For all the experiments, the chamber temperature was maintained between 23 and 25°C.

2.9 Statistical and data analyses

For the statistical analysis we used Prism version 6 (GraphPad Software, San Diego, CA, USA). The variances in body temperature and ventilatory responses to hypercapnia were analysed among groups by two-way ANOVA, followed by Bonferroni’s multiple comparisons test for post hoc comparisons. Data were expressed as means ± standard deviation (SD). Differences were considered significant when \(P < 0.05\).

Analyses of all parameters, including breathing parameters, were performed throughout the experimental period. In the graphs, the time points correspond to the average of measurements taken over a certain period, as follows: (i) Basal: mean ± SD of measurements taken during the period before microinjection; (ii) 10 min: mean ± SD of measurements taken during the 10 min of normocapnia, after the microinjection; (iii) 20 min: mean ± SD of measurements taken throughout 20 min of hypercapnia; (iv) 30 min: mean ± SD of measurements taken from 20 to 30 min of hypercapnia; (v) 40 min: mean ± SD of measurements taken from 30 to 40 min of hypercapnia.

3 RESULTS

3.1 Microinjection location

Figure 1 shows representative stained brain sections of the different groups, indicating the placement of the tip of the microinjection bilaterally within the LHA (Figure 1a) and unilaterally within the LC (Figure 1b).

3.2 Effect of MCH microinjection intra-LHA on ventilation in normocapnia and hypercapnia in rats, during wakefulness and sleep, in the light and dark period

In Figure 2, we show the effects of microinjection of MCH (0.4 mM) or vehicle into the LHA on the baseline breathing and the hypercapnic ventilatory response in the light period. In room air conditions,
**FIGURE 1** Anatomical location of the microinjection. Photomicrographs of a coronal section of a rat brain, representative of the groups, showing a typical site of microinjection in the lateral hypothalamic area (LHA; black arrow) (a) and in the locus coeruleus (LC; black arrow) (b). The dotted circle indicates the LC, contralateral to the microinjection region. 3V, third ventricle; 4V, fourth ventricle.

**FIGURE 2** Hypercapnic ventilatory response is decreased by MCH in the LHA, in rats during wakefulness and sleep, in the light period. Effect of MCH (red) or vehicle solution (blue) microinjected in the LHA, on tidal volume ($V_T$), respiratory frequency ($f_R$) and pulmonary ventilation ($V_E$) of rats exposed to normocapnia and hypercapnia (7% CO$_2$) during wakefulness (a) and NREM sleep (b) in the light period. The black bar indicates the hypercapnia period. *$P = 0.0428$, **$P = 0.0001$, *$P = 0.0027$, $^+$ $P = 0.0282$, ++ $P = 0.0047$, ## $P = 0.0029$, MCH vs. sterile saline.
FIGURE 3  Hypercapnic ventilatory response does not change by MCH in the LHA, in rats during wakefulness and sleep, in the dark period. Effect of MCH microinjection (red) or vehicle solution (blue) microinjected in the LHA on tidal volume ($V_t$), respiratory frequency ($f_R$) and ventilation ($\dot{V}_E$) of rats exposed to normocapnia and hypercapnia (7% CO$_2$) during wakefulness (a) and NREM sleep (b) in the dark period. The black bar indicates the hypercapnic period.

Microinjection of MCH had no effect on $\dot{V}_E$ during light period. However, as observed, MCH in the LHA decreased the hypercapnic ventilatory response throughout the hypercapnic period. At 20 min, $\dot{V}_E$ was decreased by 27% ($\dot{V}_E = 1923 \pm 384$ (n = 7) vs. 2645 $\pm$ 293 ml kg$^{-1}$ min$^{-1}$ (n = 6); $P = 0.0001$, two-way ANOVA) due to decreased $f_R$ ($f_R = 176 \pm 21$ (n = 7) vs. 198 $\pm$ 10 breaths min$^{-1}$ (n = 6); $P = 0.0428$, two-way ANOVA) (Figure 2a) while during sleep (Figure 2b) $\dot{V}_E$ was decreased by 16%, compared to the control group ($\dot{V}_E = 2142 \pm 315$ (n = 5) vs. 2007 $\pm$ 339 ml kg$^{-1}$ min$^{-1}$ (n = 5)).

In the dark period, as shown in Figure 3, the MCH microinjection did not alter the $\dot{V}_E$ under normocapnia and hypercapnia either during wakefulness ($\dot{V}_E$ hypercapnia, 20 min $= 2473 \pm 233$ (n = 6) vs. 2352 $\pm$ 315 ml kg$^{-1}$ min$^{-1}$ (n = 6)) or during sleep ($\dot{V}_E$ hypercapnia $= 2323 \pm 236$ (n = 5) vs. 2159 $\pm$ 284 ml kg$^{-1}$ min$^{-1}$ (n = 5)).

3.3 | Effect of MCH1-R antagonist (SNAP-94847) in the LHA on ventilation in normocapnia and hypercapnia in rats, during wakefulness and sleep, in the light period

In Figure 4, we show the effects of MCH1-R antagonist (SNAP-94847, 63 mM), or vehicle microinjected into the LHA on the baseline breathing and the hypercapnic ventilatory response in the light period. As observed, SNAP-94847 in the LHA did not change the baseline breathing but increased the hypercapnic ventilatory response by 18% during wakefulness ($\dot{V}_E = 2590 \pm 252$ (n = 6) vs. 1955 $\pm$ 329 ml kg$^{-1}$ min$^{-1}$ (n = 6); $P = 0.043$, two-way ANOVA). No effect was observed during sleep compared to the control group in the light period ($\dot{V}_E$ hypercapnia $= 2142 \pm 315$ (n = 5) vs. 207 $\pm$ 339 ml kg$^{-1}$ min$^{-1}$ (n = 5)).

3.4 | Effect of MCH microinjection intra-LC on ventilation in normocapnia and hypercapnia in rats, during wakefulness and sleep, in the light and dark period

In Figure 5, we show the effect of the MCH (0.4 mM) or vehicle microinjected into the LC on the hypercapnic ventilatory response in the light period. As observed, the MCH microinjection did not change $\dot{V}_E$ in normocapnic conditions and had no effect in the hypercapnic ventilatory response compared with the vehicle, during wakefulness ($\dot{V}_E$ hypercapnia 20 min $= 1735 \pm 158$ (n = 6) vs. 1925 $\pm$ 255 ml kg$^{-1}$ min$^{-1}$ (n = 5)) or sleep ($\dot{V}_E$ hypercapnia $= 1688 \pm 242$ (n = 6) vs. 1661 $\pm$ 288 ml kg$^{-1}$ min$^{-1}$ (n = 5)).

Similarly, as shown in Figure 6, during the dark period MCH microinjection (0.4 mM) into the LC did not change the normocapnic
**FIGURE 4** Hypercapnic ventilatory response is increased by MCH1-R antagonist in the LHA, in awake and sleeping rats, during the light period. Effect of SNAP-94847 microinjection (red) or vehicle solution (blue) into the LHA on tidal volume (VT), respiratory frequency (fR) and pulmonary ventilation (VE) of rats exposed to normocapnia and hypercapnia (7% CO₂) during wakefulness (a) and during NREM sleep (b) in the light period. The black bar indicates the hypercapnia period. *P = 0.043, SNAP-94847 vs. sterile saline.

**FIGURE 5** MCH into the LC does not change the hypercapnic ventilatory response in rats during the light period. Effect of MCH microinjection (red) or vehicle (blue) solution into the LC on tidal volume (VT), respiratory frequency (fR) and ventilation (VE) of rats exposed to normocapnia and hypercapnia (7% CO₂) during wakefulness (a) and NREM sleep (b) in the light period. The black bar indicates the hypercapnia period.
FIGURE 6  MCH into the LC does not change the hypercapnic ventilatory response in rats during the dark period. Effect of MCH microinjection (red) or vehicle (blue) into the LC on tidal volume ($V_T$), respiratory frequency ($f_R$) and ventilation ($\dot{V}E$) of rats exposed to hypercapnia (7% CO$_2$) during wakefulness (a) and NREM sleep (b) in the dark period. The black bar indicates the hypercapnia period.

breathing and did not alter the ventilatory response to CO$_2$, compared with the control group, either during wakefulness ($\dot{V}E$ hypercapnia 20 min = $1905 \pm 296$ (n = 5) vs. $1885 \pm 312$ ml kg$^{-1}$ min$^{-1}$ (n = 6)) or sleep ($\dot{V}E$ hypercapnia = $1698 \pm 329$ (n = 5) vs. $1688 \pm 218$ ml kg$^{-1}$ min$^{-1}$ (n = 6)).

3.5  Effect of MCH1-R antagonist (SNAP-94847) in the LC on ventilation in normocapnia and hypercapnia in rats, during wakefulness and sleep, in the light period

In Figure 7, we show the effects of MCH1-R antagonist (SNAP-94847, 63 mM) or vehicle microinjected into the LC on the baseline breathing and the hypercapnic ventilatory response in the light period. As observed, SNAP-94847 in the LC did not change the baseline breathing and had no effect in the hypercapnic ventilatory response either during wakefulness ($\dot{V}E$ hypercapnia 20 min = $1613 \pm 322$ (n = 6) vs. $1775 \pm 259$ ml kg$^{-1}$ min$^{-1}$ (n = 6)) or sleep ($\dot{V}E$ hypercapnia = $1599 \pm 273$ (n = 6) vs. $1645 \pm 113$ ml kg$^{-1}$ min$^{-1}$ (n = 6)).

3.6  Body temperature

All the treatments into LHA or LC caused no effect on body temperature in normocapnic or hypercapnic conditions, compared with the vehicle, either in the light or in the dark period (Table 1).

4  DISCUSSION

The present study investigated the hypothesis that the MCH system modulates the hypercapnic ventilatory response through its action in the LHA and/or in the LC in unanaesthetized rats and that this role is dependent on the sleep-wake and light-dark cycles. According to our results, we can suggest that MCH, via MCH1 receptors in LHA, exerts an inhibitory effect on the CO$_2$ chemoreflex in the light (inactive) period since the bilateral MCH microinjection intra-LHA significantly decreased the ventilatory response to CO$_2$ in rats during both wakefulness and sleep in the light period but not in the dark period and the bilateral microinjection of SNAP-94847 (MCH1-R antagonist) significantly increased the ventilatory response to CO$_2$ in rats during wakefulness in the light period of the diurnal cycle. Indeed, the participation of MCH in the hypercapnic chemoreflex does not depend on the interaction of MCH neurons with the chemosensitive neurons of the LC as MCH or MCH1-R antagonist microinjection intra-LC did not change the ventilatory response to CO$_2$.

Recently, Li et al. (2014) showed the first evidence of the involvement of MCH in the central hypercapnic chemoreflex, using prepo-MCH knockdown rats. They observed an exacerbated
FIGURE 7 Hypercapnic ventilatory response is not altered by MCH1-R antagonist in the LC, in awake and sleeping rats, during the light period. Effect of SNAP-94847 microinjection (red) or vehicle solution (blue) into the LC on tidal volume ($V_T$), respiratory frequency ($f_R$) and pulmonary ventilation ($\dot{V}E$) of rats exposed to normocapnia and hypercapnia (7% CO$_2$) during wakefulness (a) and during NREM sleep (b) in the light period. The black bar indicates the hypercapnia period.

TABLE 1 MCH and SNAP-9487 intra-LHA and intra-LC caused no effect on body temperature

| Group                     | Body temperature (°C) | Room air | Room air post-microinjection | 7% CO$_2$ |
|---------------------------|-----------------------|----------|------------------------------|-----------|
| Light phase               |                       |          |                              |           |
| MCH intra LHA (n = 7)     | 37.4 ± 0.4            | 37.9 ± 0.6 | 37.7 ± 0.5                  |           |
| Vehicle intra LHA (n = 6) | 37.4 ± 0.3            | 38.2 ± 0.5 | 38.3 ± 0.6                  |           |
| SNAP-94847 intra LHA (n = 6) | 37 ± 0.3          | 36.9 ± 0.4 | 37.6 ± 0.5                  |           |
| Vehicle intra LHA (n = 5) | 36.9 ± 0.2            | 36.8 ± 0.2 | 37.4 ± 0.4                  |           |
| MCH intra LC (n = 6)      | 37.1 ± 0.2            | 36.8 ± 0.3 | 36.6 ± 0.4                  |           |
| Vehicle intra LC (n = 5)  | 37.3 ± 0.7            | 37.1 ± 0.9 | 36.8 ± 0.7                  |           |
| SNAP-94847 intra LC (n = 6) | 37 ± 0.1           | 37 ± 0.2   | 36.8 ± 0.1                  |           |
| Vehicle intra LC (n = 6)  | 37.2 ± 0.1            | 37.2 ± 0.1 | 37 ± 0.2                    |           |
| Dark phase                |                       |          |                              |           |
| MCH intra LHA (n = 6)     | 37.4 ± 0.5            | 37.7 ± 0.4 | 37.6 ± 0.6                  |           |
| Vehicle-intra LHA (n = 6) | 37.4 ± 0.1            | 38.1 ± 0.6 | 37.9 ± 0.6                  |           |
| MCH intra LC (n = 6)      | 37.2 ± 0.4            | 37.2 ± 0.3 | 36.7 ± 0.7                  |           |
| Vehicle intra LC (n = 5)  | 37.1 ± 0.1            | 37.2 ± 0.2 | 36.8 ± 0.3                  |           |

Body temperature (°C) of rats under normocapnic and hypercapnic conditions before and after microinjection of MCH or SNAP-94847 intra-LHA and intra-LC and their respective vehicle solutions in the light and dark period. Values are shown as means ± SD.
hypocapnic ventilatory response in these animals compared to the control group (Li et al., 2014), suggesting that MCH plays an inhibitory role in the central hypocapnic chemoreflex, but the mechanisms were still unclear. MCH neurons establish synaptic connections with chemosensitive sites, such as the LHA itself, and at a distance, with the LC, the medullary raphe and the nucleus of the solitary tract (NTS) (Adamantidis & de Lecea, 2008; Diniz & Bittencourt, 2017; Skofitsch, Jacobowitz & Zamir, 1985). Thus, one plausible hypothesis would be that the role of MCH in the inhibitory modulation of the central chemoreflex could be due to an inhibitory influence of MCH on CO₂ chemosensitive neurons, reducing the hypocapnic ventilatory response. We speculated that this mechanism would involve, in part, the activation of MCH₁-R in the LHA. Our data corroborate this hypothesis since the bilateral microinjection of MCH in the LHA caused a decrease in $V_\text{E}$ under hypercapnia (7% CO₂) during both wakefulness and sleep, but only in the light period (Figure 2) and the microinjection of the MCH₁-R antagonist (SNAP-94847) caused an increase in $V_\text{E}$ under hypercapnia during the light period (Figure 4).

Therefore, we suggest that MCH acting on MCH₁-R in the LHA exerts an inhibitory modulation in the central chemoreflex in the light period of the diurnal cycle, possibly through its action in orexinergic (OX) neurons, which are recognized as CO₂/pH chemosensitive (Deng et al., 2007; Sunanaga et al., 2009; Williams et al., 2007).

An accumulating body of evidence indicates that the OX and MCH neurons, within the LHA, establish reciprocal synaptic relationships, but while orexin’s effects are predominantly excitatory (Burdakov, Karnani & Gonzalez, 2013), the effects of MCH are inhibitory (Van Den Pol et al., 2004; Wu et al., 2009). The interaction between MCH and OX neurons seems to be extremely complex. It has been shown that MCH neurons are densely innervated by OX neurons, and a post-synaptic excitatory effect of orexins A and B on MCH neurons has been described (van den Pol et al., 2004). In contrast, optogenetic stimulation of OX neurons of hypothalamic mouse slices caused an inhibition of the action potential firing in most MCH neurons (Apergis-Schoute et al., 2015). This inhibitory effect of orexinergic neurons on the activity of MCH neurons appears to be caused by the release of dynorphin, a neuromodulator also synthesized by orexinergic neurons, while the effect of orexin per se on the activity of MCH neurons appears to be excitatory (Li & van den Pol, 2006). On the other hand, MCH appears to inhibit OX neurons. In this context, MCH receptor-1 knockout (MCHR1 KO) mice have an enhanced efficacy of the glutamatergic synapses in the orexinergic neurons and exhibit a facilitation of action potential triggering induced by orexin-A, which suggests that MCH has an inhibitory effect on the activity of OX neurons (Rao et al., 2008). It has been suggested that the inhibitory action of MCH cells counter and fine-tune the excitatory actions of the OX neurons (Li & van den Pol, 2006). Based on this, it seems reasonable to us to suggest a possible mechanism, shown in Figure 8, in which the decreased hypocapnic ventilatory response observed with the microinjection of MCH and the potentiation of the CO₂ chemoreflex with the microinjection of the MCH₁-R antagonist in the LHA could be due to an inhibitory effect of MCH on the activity of OX neurons.

\textbf{FIGURE 8} Schematic illustration of a putative mechanism underlying the involvement of MCH signalling in the LHA, in the modulation of the central hypocapnic chemoreflex. OX neurons are chemosensitive to CO₂/pH changes and stimulate the central pattern generator (CPG) and/or other chemosensitive sites. We propose a model in which, in the light period, OX neurons, when activated by CO₂/pH (1) release OX that excites the nearby MCH neurons (2), which in turn release MCH that has an inhibitory effect on the OX neurons and on the hypocapnic ventilatory response (3). Dynorphin is coexpressed in OX neurons and can inhibit MCH neurons through κ-opioid receptors (KOR) (4).

The activity of the MCH system is considered dependent on the sleep-wake cycle. It is known that these neurons are more active during NREM and REM sleep and practically silent during waking (Hassani et al., 2009). Despite its recognized role in promoting sleep (Monti et al., 2015), and the greater activity of MCH neurons during sleep, the injection of MCH in the LHA reduced the hypocapnic ventilatory response not only during sleep but also during wakefulness, in the light phase.

Evidence suggests that there are diurnal variations in the activity of the MCHergic system, regardless of the variations throughout the sleep-wake cycle. For example, recent evidence showed that in CSF of rats (Pelluru, Konadhode & Shiromani, 2013) and in hypothalamic slices of mice (Gericke et al., 2017), MCH levels are higher in the inactive period (light phase) compared to the active period (dark phase) of rodents. In addition, a previous study showed that in slices of the frontal cortex and hippocampus of control rats, levels of MCH₁-R expression are lower in ZT0 (beginning of the light phase, at 07.00 h) compared to ZT8 (end of the light phase, at 15.00 h), suggesting that expression of MCH₁-R varies throughout the day (Dias Abdo Agamme et al., 2015). Moreover, it is important to point out that the involvement of LHA in the central hypocapnic chemoreflex appears to be vigilance-state- and diurnal-cycle-dependent (Dias, Li & Nattie, 2010; Li et al., 2013). For instance, it has been shown that: (i) the blockade of the orexin-1 receptor within the retrotrapezoid nucleus inhibited the hypocapnic ventilatory response predominantly during wakefulness (Dias, Li & Nattie, 2009); (ii) the inhibition of the orexin-1 receptor in the medullary raphe and locus coeruleus reduced the...
CO₂ ventilatory response in wakefulness, only in the dark phase (Dias, Li & Nattie, 2010; Vicente et al., 2016); (iii) acidification of the LHA region enhanced breathing in awake rats (Li, Li & Nattie, 2013); (iv) oral administration of an orexin receptor antagonist attenuated the CO₂ response during wakefulness in the dark phase (Li & Nattie, 2010); (v) ATP in the LHA increased the hypercapnic ventilatory response only during wakefulness in the dark phase (da Silva et al., 2018); and (vi) MCH1-R knockout mice showed an increase in wakefulness due to a decrease in NREM sleep in the light and dark phase and an increase in mean basal body temperature only in the light phase (Ahnaou et al., 2011). This highlights the importance of assessing, in our study, the breathing responses not only during sleep and wakefulness but also during the light and the dark phases. Supporting this, here we showed that MCH, in the LHA, decreased the hypercapnic ventilatory response in awake and sleeping rats, in the light period, but not in the dark. The fact that MCH on LHA did not result in effects during the dark phase seems, at first glance, to be intriguing, since in this phase the MCH neurons are less active and, therefore, one would expect that the inhibitory effect of the MCH injection on the hypercapnic ventilatory response would be more prominent in the dark phase. Different mechanisms could explain the lack of MCH’s effect in this phase. These mechanisms could involve the high excitability of orexinergic neurons in the dark-active phase. A prevalence of excitatory innervation to OX neurons during the dark phase has been demonstrated (Laperchia et al., 2017). Therefore, it is possible that MCH inhibition does not have much of an overall effect on OX neurons at this stage of the diurnal cycle.

To determine if MCH signalling in the LC also plays a role in the modulation of the central chemoreflex, we also tested the effects of MCH microinjection into the LC on baseline breathing and the ventilatory response to CO₂ in unanaesthetized rats. Surprisingly, microinjection of MCH into the LC had no effect in the ventilation of the rats in normocapnia and in the hypercapnic ventilatory response during wakefulness and sleep in both light and dark period (Figures 5 and 6). In addition, microinjection of the MCH1-R antagonist SNAP-94847 in the LC did not change the hypercapnic ventilatory response of the rats (Figure 7). These data suggest that the MCHergic signalling in the LC does not participate in the central chemoreflex. However, since the MCH injection was unilateral, we cannot discard the possibility that contralateral LC compensation is occurring. Nevertheless, a comparison between the group of animals that received MCH bilaterally in the LHA and the group injected unilaterally showed no difference in its hypercapnic ventilatory responses (data not shown). The unilateral group, as with the bilateral group, had a reduction in the hypercapnic ventilatory response compared with the control group, demonstrating that there was no compensation on the contralateral LHA.

Although MCH projections to LC have been considered crucial for sleep–wake cycle control (Hassani, Lee & Jones, 2009; Monti et al., 2015), here we provide evidence that these projections to LC do not have a role in the modulation of the central hypercapnic chemoreflex. The results of the present study point to the heterogeneity of function of LC neurons. This interpretation is consistent with previous neuro-anatomical data showing that subpopulations of LC noradrenergic cells innervate specific brain regions (Loughlin et al., 1986; Schwarz & Luo, 2015). Moreover, it has been shown that LC neurons exhibit distinct firing patterns for the generation of different behaviours (Schwarz & Luo, 2015).

It is important to point out that MCH projections and MCH1-R are also found in other brain sites related to the activation or modulation of the central hypercapnic chemoreflex, for example, the NTS, dorsal and medullary raphe nucleus and the diaphragm motoneurons (Badami et al., 2010; Bittencourt et al., 1992; Saito et al., 2001). Therefore, we cannot rule out the possible involvement of MCH signalling to other nuclei in the modulation of the hypercapnic chemoreflex. It is possible that MCH neurons can modulate the hypercapnic ventilatory responses not only indirectly, altering the neuronal activity in other chemoreceptor sites, but also directly, regulating the phrenic motor neuron activity. Future studies are necessary to investigate the role of these other pathways in the context of the role of MCH central chemoreflex modulation.

LHA and LC are believed to be involved in thermoregulation (Almeida et al., 2004; Morrison, 2016; Ravanelli & Branco, 2008; Takahashi et al., 2001). Moreover, it has been shown that mice lacking the MCH1-R had a higher mean basal body temperature, mainly during the light period (Ahnaou et al., 2011). Therefore, we also investigated whether MCH acting on LHA or LC, participates in the modulation of body temperature. However, MCH microinjection in the LHA or LC did not alter the body temperature compared with the respective control groups, either in the light or in the dark period, suggesting that MCH acting on MCH1-R located in the LHA or in the LC does not participate in the control of body temperature in rats in the light and dark periods of the diurnal cycle.

In conclusion, the present study provides new data on the role of MCH in the modulation of the hypercapnic chemoreflex. Taken together, we suggest that the MCH signalling in the LHA, but not in the LC, exerts an inhibitory modulation of the central chemoreflex and this role seems to be dependent on the light–dark cycle, occurring exclusively in the light-inactive period in unanaesthetized rats.

**AUTHOR CONTRIBUTIONS**

Concept/design: Laisa T. C. Rodrigues and Mirela B. Dias. Acquisition, analysis/interpretation of data: Laisa T. C. Rodrigues, Luis Gustavo A. Patrone, Mirela B. Dias and Luciane H. Gargaglioni. Drafting/revision of the manuscript: Laisa T. C. Rodrigues, Luis Gustavo A. Patrone, Mirela B. Dias and Luciane H. Gargaglioni. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

**COMPETING INTERESTS**

None.
Bartlett, D., & Tenney, S. M. (1970). Control of breathing in experimental v1.

DATA AVAILABILITY STATEMENT
Data are available on https://doi.org/10.6084/m9.figshare.20453979.

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**SUPPORTING INFORMATION**

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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