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Glutamate metabotropic receptors in the lateral hypothalamus/perifornical area reduce the CO_2 chemoreflex

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ABSTRACT

It has been shown that the lateral hypothalamus/perifornical area (LH/PFA) exerts an important role on arousal-state variations of the central chemoreflex, but the mechanisms that underlie LH/PFA chemoreception are poorly understood. Here we asked whether glutamate inputs on metabotropic receptors in the LH/PFA modulate the hypercapnic ventilatory response. We studied the effects of microinjection of a glutamate metabotropic receptor (mGluR) antagonist ((+)-α-Methyl-4-carboxyphenylglycine; MCPG; 100 mM) and a selective Group II/III mGluR antagonist ((2*S*)-2-Amino-2-[(1*S*,2*S*)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid; LY341495; 5 mM) into the LH/PFA of conscious rats on ventilation in room air and in 7% CO_2, during wakefulness and sleep, in the dark and light periods of the diurnal cycle. Microinjection of MCPG and LY341495 increased the hypercapnic ventilatory response in both the light and the dark period during wakefulness, but not during sleep, ($p < 0.001$). Our data suggest that glutamate, acting on Group II/III metabotropic receptors in the LH/PFA, exerts an inhibitory modulation of the hypercapnic ventilatory response in awake rats.

1. Introduction

Central chemoreceptors in the brain detect CO_2/pH changes, promoting respiratory adjustments that are necessary to correct these alterations, contributing to the maintenance of acid-base balance and, consequently, homeostasis. Evidence indicates that central chemoreceptors are widely distributed throughout the central nervous system, and include the orexinergic neurons of the lateral hypothalamus and perifornical area (LH/PFA) (Ben-Shiang Deng et al., 2007; Dias et al., 2010; Kuwaki et al., 2010; Sunanaga et al., 2009; Williams et al., 2007).

The CO_2 chemosensitivity of the LH/PFA has been shown to be dependent on sleep-wake states (B.S. Deng et al., 2007; Dias et al., 2010, 2009; Li et al., 2013). In this context, it was demonstrated that focal acidification of the LH/PFA, by microdialysis of acidic artificial cerebrospinal fluid (aCSF), increased ventilation during wakefulness, but not during sleep (Li et al., 2013). The arousal state-dependent aspect of the role that the LH/PFA plays in central chemoreception is in accordance with the activity of the putative chemosensitive neurons of this region, the orexin/hypocretin neurons, which are synchronized with the sleep-wake cycle, firing more frequently during wakefulness (Lee et al., 2005; Mileykovskiy et al., 2005). In fact, the importance of the orexinergic system on the maintenance of arousal is well established (Gestreau et al., 2008). Further, the role of the LH/PFA in central chemoreception varies not only with arousal state, but also with the diurnal cycle, being predominant during the dark/active period (Dias et al., 2010; Nattie and Li, 2010), which is congruent with the diurnal variation of orexin levels in rat cerebrospinal fluid, where higher values are observed during the dark period compared with the light period (Desarnaud et al., 2004).

Several neurotransmitters and neuromodulators have been suggested to influence orexinergic neuron activity. Studies have demonstrated the existence of glutamatergic projections to the LH/PFA and the expression of glutamate receptors in this region (Ozhan et al., 2001; van den Pol and Trombley, 1993). Evidence suggests that the source of glutamatergic inputs to the orexin neurons may be the basal forebrain, the lateral parabrachial nucleus, the LH/PFA itself, and many other brain areas that have not yet been identified (Eyigor et al., 2012a; Henny and Jones, 2006; Li et al., 2002b; Ohno and Sakurai, 2008).

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Glutamate is suggested to affect orexin neurons and to modulate the orexinergic function in the regulation of wakefulness and feeding (Eyigor et al., 2012b; Rosin et al., 2003). Glutamate is known to provide mainly excitatory inputs on the orexinergic system (Eyigor et al., 2012b), but inhibitory modulation has also been demonstrated, which appears to involve the activation of metabotropic receptors (Acuna-goycolea et al., 2004). In fact, metabotropic glutamate receptors in LH/PFA maintain tonic presynaptic inhibition at excitatory (and inhibitory) synapses (Kuzmiski et al., 2009; Kuzmiski and Bains, 2010). In this regard, the group III of metabotropic glutamate receptor has been shown to trigger presynaptic glutamate release onto orexin cells modulating the output of the orexinergic arousal system (Acuna-Goycolea et al., 2004). Nevertheless, it is still unknown whether this glutamatergic modulation in the LH/PFA, via metabotropic receptors, is also involved in the diurnal modulation of the ventilatory response to the central chemoreflex. Thus, we investigated the glutamatergic regulation, through metabotropic receptors on the LH/PFA, on the hypercapnic ventilatory responses in conscious rats during dark and light periods of the diurnal cycle.

2. Material and methods

2.1. Animals

Experiments were performed on male Wistar rats, weighing 250–320 g. The animals were maintained in a light- and temperature-controlled room (23 ± 2 °C), under a 12:12 h light/dark cycle (lights on at 2:00 p.m. for the dark period group, and 7:00 a.m. for the light period group) and with free access to food and water. The experimental protocols were performed between 9:00 a.m. and 1:00 p.m.

The methods described here were within the guidelines of the National Council for the Control of Animal Experimentation (CONCEA, MCT, Brazil) and were approved by the Animal Care and Use Committee for the Institute of Biosciences at Botucatu, Brazil (CEUA - IBB, UNESP, Botucatu campus; protocol n°. 597). All animal experiments complied with the ARRIVE guidelines and were carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines.

2.2. Surgery

Animals were anaesthetized intraperitoneally with ketamine (100 mg/kg) and xylazine (15 mg/kg), the head and a portion of the abdomen were shaved and the skin was sterilized with betadine solution and alcohol. The rats were then fixed to a Kopf stereotaxic frame and implanted with a guide cannula (CMA11, Microdialysis AB, Stockholm, Sweden) 1 mm above the LH/PFA region. The coordinates for the cannula placement were roughly 2.7 mm caudal and 1.4 mm lateral from lambda, and 7.4 mm below the surface of the skull (Paxinos and Watson, 2005). For EEG recordings, three EEG electrodes were screwed into the skull (2 mm to the right of the bregma suture; 2 mm to the right of lambda; and 1 mm anterior to lambda), and to record EMG, a pair of EMG electrodes was inserted deep into the dorsal cervical neck muscle. The electrode wires were fed into a plastic pedestal, which was covered with a dust cap (Plastics One Inc.). The guide cannula and the plastic pedestal containing the electrode wires were then mounted on and attached to the bone with acrylic cement.

Additionally, rats were submitted to a paramedian laparotomy, and a temperature datalogger was implanted inside the abdominal cavity for body temperature measurements. Each datalogger was programmed to take a reading every 5 min (SubCue Dataloggers, Calgary, AB, Canada). At the end of the surgery, rats received 0.2 mL (1,200,000 U) of benzyl-penicillin intramuscularly, and the animals were transferred to individual cages to recover for 7 days.

2.3. Microinjection

For microinjection into the LH/PFA, a dental injection needle (28 gauge) was connected via a polyethylene tube (PE-10) to a 10 µL Hamilton syringe (Hamilton, Reno, NV). The microinjected volume was 100 ηL, with a duration of approximately 30 s. The metabotropic glutamate receptor antagonist, MCPG (100 mM; Sigma Chemical, St. Louis, MO, USA), and the Group II/III metabotropic glutamate receptor antagonist, LY341495 (5 mM; Sigma Chemical, St. Louis, MO, USA), were dissolved in aCSF and pH was adjusted to 7.4. The concentrations of these drugs were selected based on previous studies and pilot experiments (de Paula and Branco, 2005; Ferreira et al., 2004). 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 behavioral changes in the animals.

2.4. Ventilation recordings

Ventilation ($\dot{V}_E$) recordings were obtained by the whole-body plethysmograph technique, as described previously (Bartlett and Tenney, 1970). Before the beginning of the experiment, the plethysmograph was calibrated with 1 mL injections of room air into the chamber (5 L). After this procedure, the animals were placed inside the chamber ventilated with room air. A pressure transducer (TSD 160 A, Biopac Systems, Santa Barbara, CA), connected to the plethysmograph chamber, received the respiratory pressure oscillations, which then passed through an analog-to-digital converter and were digitized on a microcomputer equipped with data acquisition software (MP150WSW, Biopac Systems). During the recordings, the flow was interrupted and the chamber was sealed for short periods of time (approximately 2 min). For the hypercapnia protocols, after the microinjection procedure, the gas inside the chamber was replaced by a hypercapnic gas mixture containing 7% CO_2 and 21% O_2, balanced with N_2 **(White** Martins, Sertãozinho, Brazil). The mixture of the gases and the inflow rate (2 L/min) were maintained by a gas mixer (Pegas 4000 F, Columbus Instruments, Columbus, OH, USA).

After the experiment, breathing events were individually selected using the Lab Chart Pro software (AD Instruments, Australia). Tidal volume (V_T) was calculated using the formula described by from Drorbaugh and Fenn (1955):

$V_T = V_K \times (P_T / P_K) \times Tb \times (P_B - P_C) / Tb \times (P_B - P_C) - T_A \times (P_B - P_R)$

where P_T is the pressure deflection associated with each V_T, P_K is the pressure deflection associated with the injection of the calibration volume (V_K), T_A is the air temperature in the animal chamber, P_B is the barometric pressure, P_C is the water vapor pressure in the animal chamber, Tb is the body temperature, and P_R is the vapor pressure of water at Tb. The $\dot{V}_E$ was calculated as the product of the f_R and the V_T. $\dot{V}_E$ and V_T are presented under conditions of ambient barometric pressure, at Tb and saturated with water vapor (BTPS). The P_C was calculated indirectly using an appropriate table (Dejours, 1981). Body temperature (Tb) was measured by an i.p.-implanted temperature datalogger, and the temperatures in the room and inside the chamber were measured using a thermosensor (model HH801B; Spectris, Sao Paulo, SP, Brazil).

2.5. Determination of vigilance state

The EEG and EMG electrodes, inserted into the plastic pedestal, were connected to an insulated and shielded cable, which, in turn, 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 skull and neck muscle were

amplified (10,000x for EEG signals and 2,000x 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). Arousal state was determined by analysis of EEG and EMG recordings using the Labchart8 software.

2.6. Anatomical analysis

Upon completion of the experiments, the animals were deeply anesthetized and a 100-ηL microinjection of Evan's Blue was performed through the guide cannula. The rats were perfused transcardially with 300 mL of phosphate buffer (PB), followed by 300 mL of 4% paraformaldehyde solution. The perfusion was performed with the aid of a peristaltic perfusion pump (Masterflex®, Cole Parmer International, Vernon Hills, IL, USA), adjusted to a flow of 30 mL/min. The brain was then removed and post-fixed overnight in a 4% paraformaldehyde solution. After fixation, the brain was cryoprotected by immersion in a 30% sucrose solution for 48 h. The brains were then frozen, cut into 40-µm-thick coronal sections with a Reichert–Jung cryostat (Leica, Germany), and the slices were mounted directly onto microscope slides to be further stained by the Nissl method for light microscopy. The anatomic region of microinjection was determined using the (Paxinos and Watson, 2005). Only rats with the site of microinjection in the LH/PFA were considered.

2.7. 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 into a 5-L 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, ventilation and body temperature measurements were taken at 5 min intervals for 30 min. EEG and EMG were recorded continuously. Then, the rats received the microinjection of MCPG, LY341495 or vehicle unilaterally into the LH/PFA. Subsequently, the inspired air was switched to a hypercapnic gas mixture, containing 7% CO_2 and 21% O_2, balanced with N_2, and the measurements were made for an additional 40 min. This protocol was performed in a group of rats during the light period, and in another

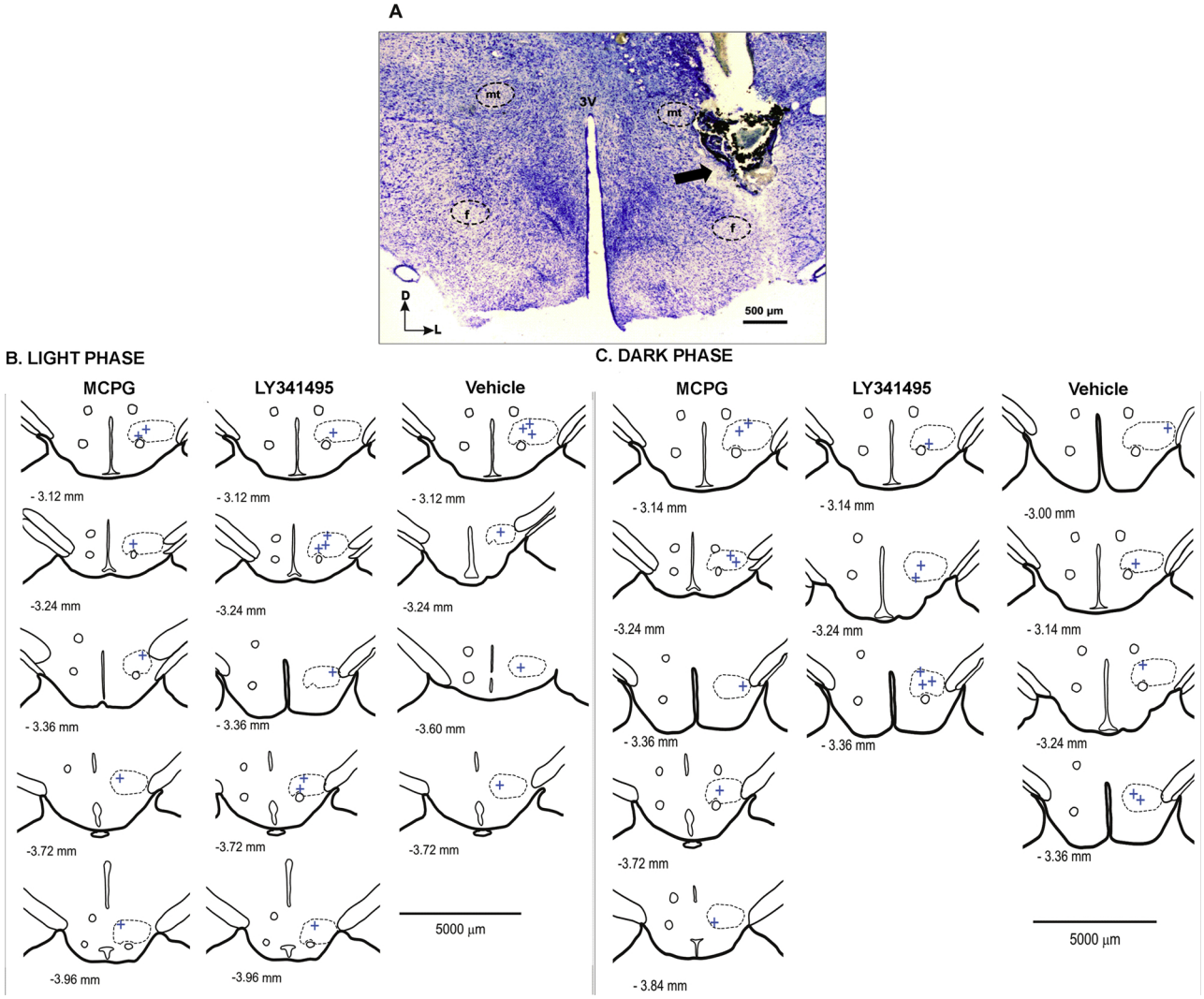

Fig. 1. Anatomical location of the microinjection. (A) Photomicrograph of a coronal section of the brain of a rat, representative of the groups, showing a typical site of microinjection in the lateral hypothalamus/perifornical area (LH/PFA; black arrow). Schematized anatomical cross-sections show in (B) the location of the microinjection for animals of the light phase group, receiving MCPG (left, N = 6), LY341495 (middle, N = 8) and vehicle (right, N = 6); and (C) the microinjection site in the dark phase group receiving MCPG (left, N = 7), LY341495 (middle, N = 6) and vehicle (right, N = 5). The cross symbols represent the locations of microinjections in the LH/PFA region (dotted line areas). The numbers below the figures refer to millimeters caudal to Bregma.

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 24–25 °C.

2.8. Statistical analyses

Values are reported as means ± SEM. The variances in body temperature and ventilatory responses to hypercapnia were analyzed among groups by two-way ANOVA, followed by Bonferroni's test for post-hoc comparisons. The significance level was set at $p < 0.05$. The statistical analyses were performed using a software program (GraphPad Prism 5).

3. Results

3.1. Microinjection location

A representative photomicrograph of a typical microinjection site in the LH/PFA is shown in Fig. 1A. A schematic cross-section series of the hypothalamus demonstrating the microinjection sites, from rostral to caudal, are shown in Fig. 1B and 1C.

3.2. The effects of microinjection of MCPG and LY341495 into the LH/PFA on the ventilatory response to hypercapnia during wakefulness and sleep, in the light period

In Fig. 2, we show the effects of the microinjection of 100 mM MCPG, 5 mM LY341495 or vehicle into the LH/PFA on the hypercapnic ventilatory response in the light period. As observed, MCPG in the LH/PFA exacerbated the hypercapnic ventilatory response by 39%, compared to the control group ($p < 0.01$), while LY341495 caused a 57% increase in the CO_2 ventilatory response ($p < 0.001$) during wakefulness. These effects were due to a significant increase in V_T ($p < 0.01$). The CO_2 response did not change in the animals who received the microinjection of either MCPG or LY341495 outside of the LH/PFA (data not shown).

MCPG had no effect on the ventilatory response to 7% CO_2 during

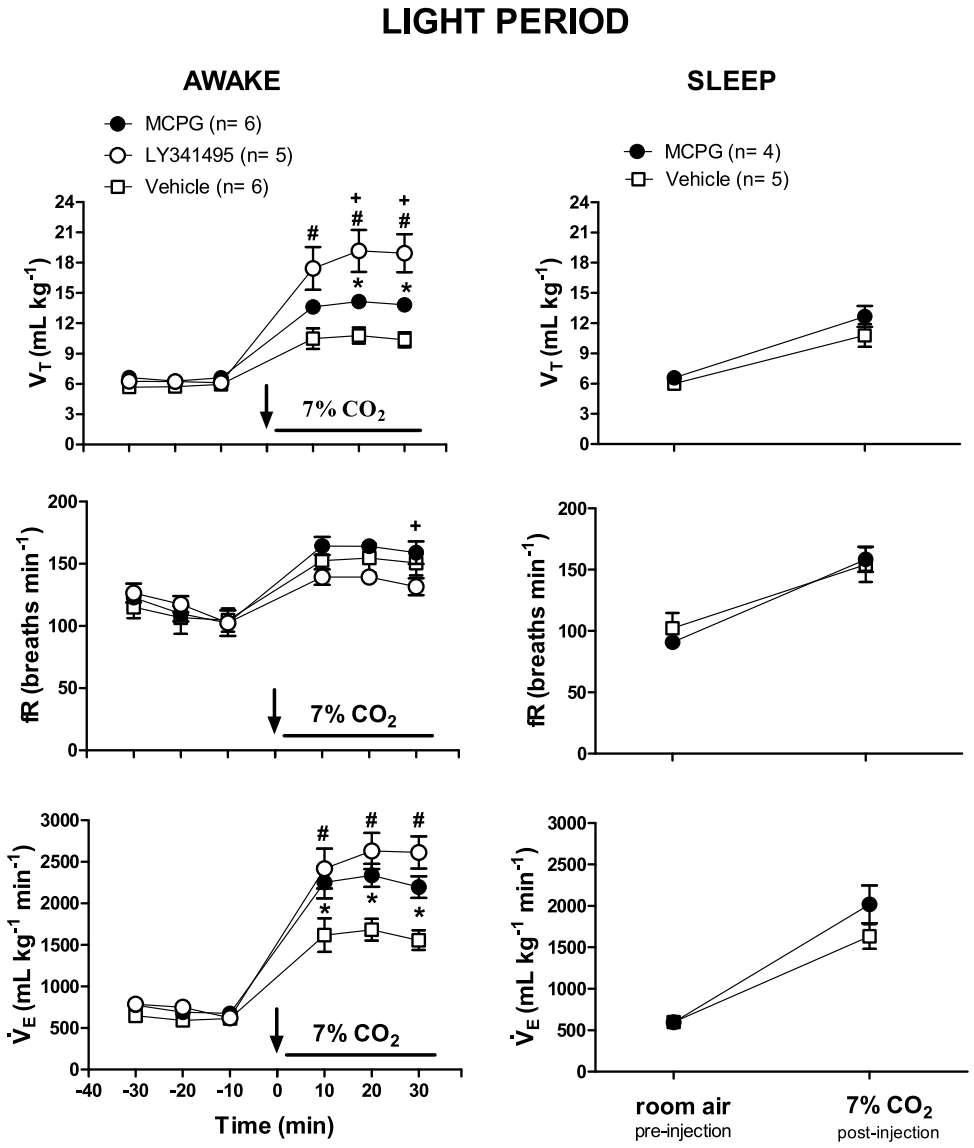

Fig. 2. Hypercapnic ventilatory response is augmented by MCPG and LY341495 in awake rats during the light phase. Effect of microinjection of MCPG, LY341495 or vehicle solution into the LH/PFA on tidal volume (V_T), respiratory frequency (f_R) and ventilation ($\dot{V}_E$) of rats exposed to hypercapnia (7% CO_2) during wakefulness (A) and NREM sleep (B) in the light period. The arrow indicates the time of injection. Values are expressed as mean ± S.E.M. *$p < 0.05$, MCPG *versus* aCSF; $^{+}p < 0.01$, MCPG *versus* LY341495; $^{\#}p < 0.001$, LY341495 *versus* aCSF.

NREM sleep, while LY341495 microinjection increased the alertness of the rats in such a way that periods of sleep were not common and, when they occurred, were very short. Therefore, it was not possible to study the effects of LY341495 injected into the LH/PFA on the ventilatory response to hypercapnia during sleep.

3.3. The effects of microinjection of MCPG and LY341495 into the LH/PFA on the ventilatory response to hypercapnia during wakefulness and sleep, in the dark period

As observed in Fig. 3, during the dark period, MCPG exacerbated the hypercapnic ventilatory response by 29% during wakefulness, but not during NREM sleep, compared to the control group ($p < 0.01$). Similarly, LY341495 affected the CO_2 ventilatory response in awake rats, increasing the $\dot{V}_E$ by 44% under hypercapnia ($p < 0.001$).

3.4. The effects of microinjection of MCPG and LY341495 into the LH/PFA on ventilation under normocapnia during wakefulness and sleep

To confirm that the changes in ventilation observed with MCPG and LY341495 during hypercapnia were caused by an effect on CO_2 ventilatory response mechanisms, rather than any effect on baseline breathing, we tested the effects of these drugs under normocapnic conditions. As shown in Fig. 4, the antagonism of glutamate metabotropic receptors, and the specific Group II/III antagonism, did not alter the respiratory parameters of the rats during either wakefulness or sleep. These experiments were performed during the light period of the diurnal cycle. Considering that the effect of MCPG and LY341495 on ventilation under hypercapnia was robust in the light period and no effect was observed under normocapnia, this protocol was not repeated in the dark period.

3.5. Body temperature

Fig. 5 shows the effect of injection of MCPG, LY341495 or vehicle into the LH/PFA on body temperature in room air and under hypercapnia. The treatment with MCPG did not affect Tb during the normocapnic condition, and did not change the Tb response to hypercapnia, in either the dark or the light period. However, microinjection of LY341495 caused a robust decrease in Tb ($p < 0.001$)

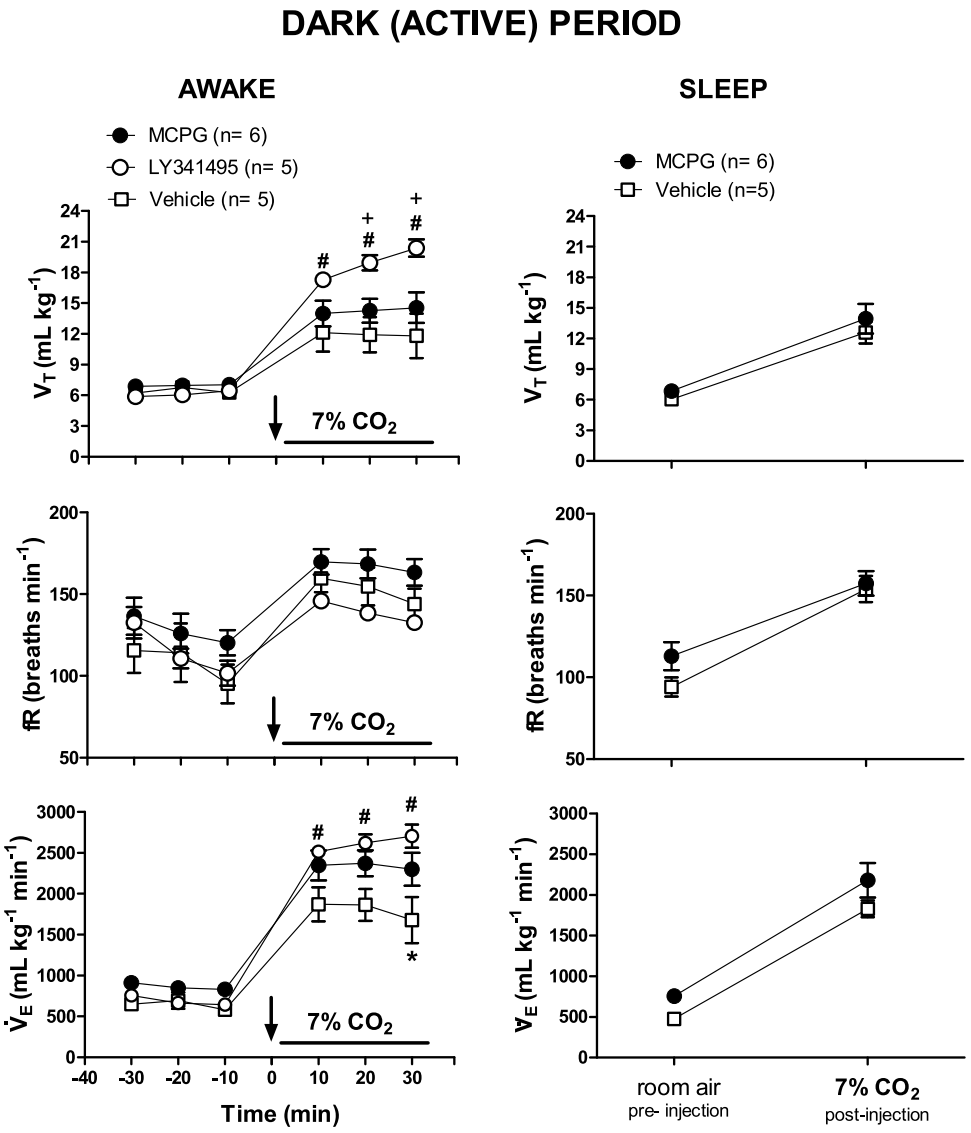

Fig. 3. Hypercapnic ventilatory response is augmented by MCPG and LY341495 in awake rats during the dark phase. Effect of microinjection of MCPG, LY341495 or vehicle solution into the LH/PFA on tidal volume (V_T), respiratory frequency (f_R) and ventilation ($\dot{V}_E$) of rats under 7% CO_2 exposure during wakefulness (A) and NREM sleep (B) in the dark period. The arrow indicates the time of injection. Values are expressed as mean ± S.E.M. *$p < 0.05$, MCPG *versus* aCSF; $^{+}p < 0.01$, MCPG *versus* LY341495; $^{\#}p < 0.01$, LY341495 *versus* aCSF.

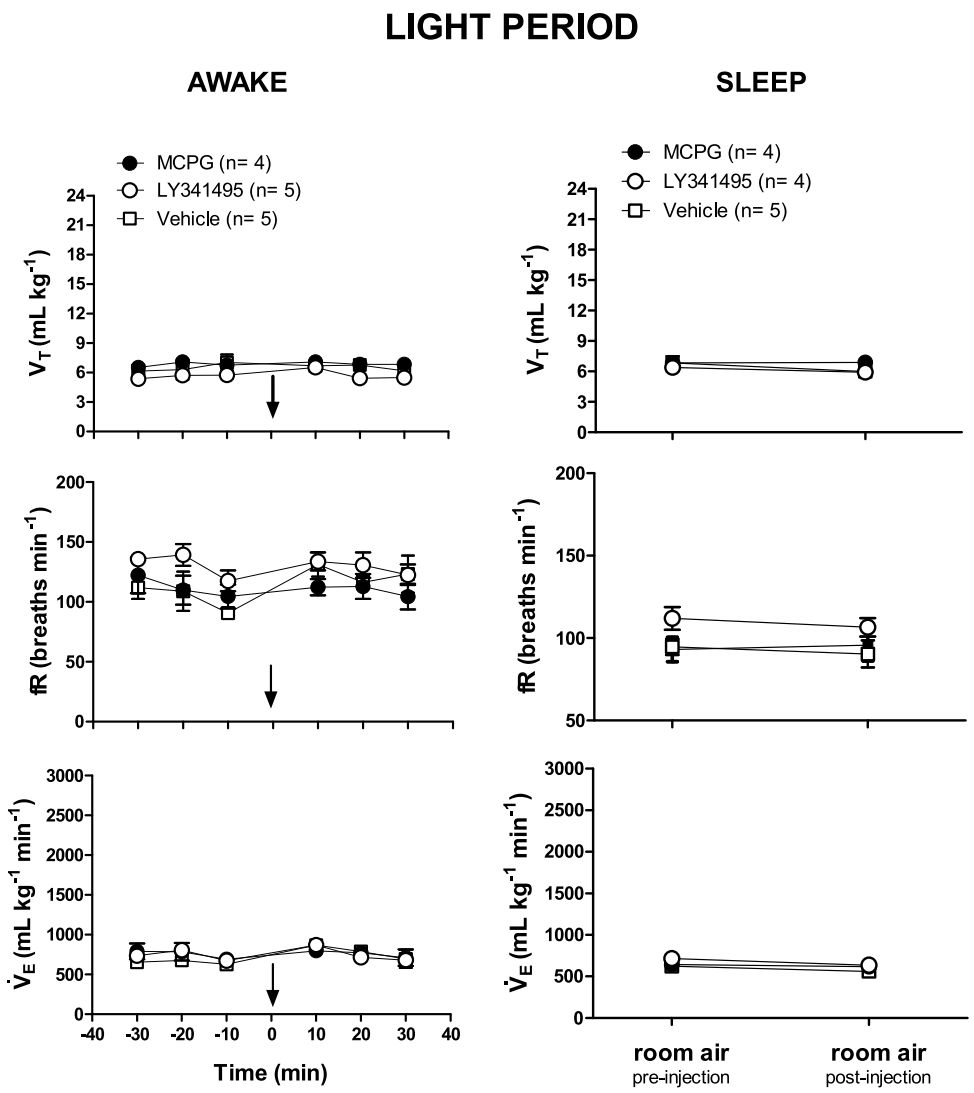

Fig. 4. MCPG and LY341495 microinjected into the LH/PFA do not change ventilation under normocapnia. Effect of microinjection of MCPG, LY341495 or vehicle solution into the LH/PFA on tidal volume (V_T), respiratory frequency (f_R) and ventilation ($\dot{V}_E$) of rats in wakefulness (A) and sleep (B) under normocapnia. The arrow, in the left panel, indicates the time of injection. Values are expressed as mean ± S.E.M.

when the rats were under hypercapnia during both the light and dark periods. In a separate group of animals, who received the injection of the LY341495 outside of LH/PFA, the Tb did not drop during hypercapnia (data not shown).

Fig. 6 shows the relationship between Tb and $\dot{V}E$ in the rats treated with LY341495, under hypercapnia in the light (Fig. 6A) and dark period (Fig. 6B).

4. Discussion

The main objective of this study was to investigate the possible role of metabotropic glutamatergic receptors, localized in the LH/PFA, in the modulation of the hypercapnic ventilatory response during wakefulness and sleep, in the light and dark periods of the diurnal cycle. According to our results, we can suggest that glutamate, acting at the metabotropic receptors (mGluRs) in the LH/PFA, performs an inhibitory modulation of the central chemoreflex during wakefulness, since the antagonism of these receptors through microinjection of MCPG (nonspecific antagonist of mGluRs) and LY341495 (specific antagonist of mGluRs of Groups II and III) increased the hypercapnic ventilatory response of rats while they were awake.

Glutamate is referred to as the main excitatory amino acid neurotransmitter in the hypothalamus (Eyigor et al., 2012a; van den Pol et al., 1990). In the lateral hypothalamus, the presence of glutamate, the expression of its receptors and a broad number of its functions have been demonstrated (Eyigor et al., 2012b; van den Pol and Trombley, 1993). The CO_2 chemosensitive neurons of the LH/PFA, the orexin neurons, receive glutamatergic inputs (Eyigor et al., 2012b; Henny and Jones, 2006) which are suggested to influence orexin neuron activity (Li et al., 2002a). Although the main effect of glutamatergic synapses on orexin neurons appears to be excitation (Li et al., 2002a) the activation of mGluRs has been shown to attenuate the excitation of orexin neurons (Acuna-goycolea et al., 2004). Nevertheless, whether the glutamatergic neurotransmission through mGluRs on the LH/PFA affects the hypercapnic ventilatory response was not known.

To examine the possible role of mGluRs in the LH/PFA in the hypercapnic ventilatory response, a nonspecific antagonist of mGluRs, MCPG, was microinjected into the LH/PFA. MCPG elicited a significantly increased hypercapnic ventilatory response in awake rats, but not during sleep, in the light and dark phases, suggesting that glutamate, acting on mGluRs in the LH/PFA, exerts an inhibitory modulation of the central chemoreflex, and that this function may be dependent on the sleep-wake cycle. The glutamatergic metabotropic receptors are divided into three groups: Group I, consisting of mGluR1 and mGluR5;

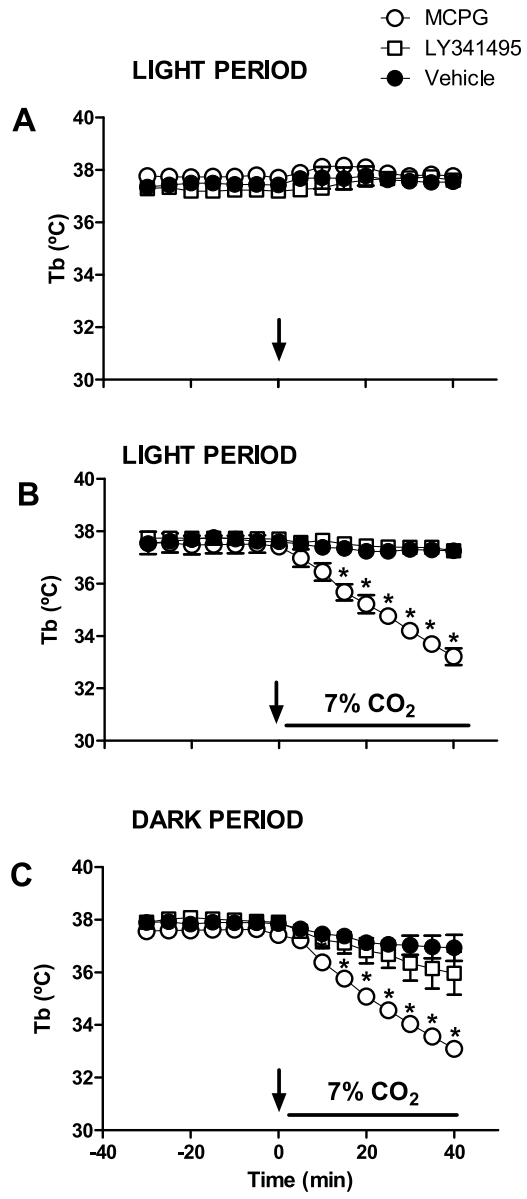

Fig. 5. LY341495 decreased Tb under hypercapnia during the light and dark phases. (A) The effect of microinjection of MCPG (n = 4), LY341495 (n = 5) or vehicle solution (n = 5) into the LH/PFA on body temperature of rats in room air. **(B)** The effect of microinjection of MCPG (n = 6), LY341495 (n = 5) or vehicle solution (n = 6) into the LH/PFA on body temperature under 7% CO_2 hypercapnic conditions during the light period. **(C)** The effect of microinjection of MCPG (n = 6), LY341495 (n = 5) or vehicle solution (n = 5) into the LH/PFA on body temperature under hypercapnia 7% CO_2 during the dark period. The arrow indicates the time of injection. Values are expressed as mean ± S.E.M. *p < 0.001, LY341495 *versus* aCSF and MCPG.

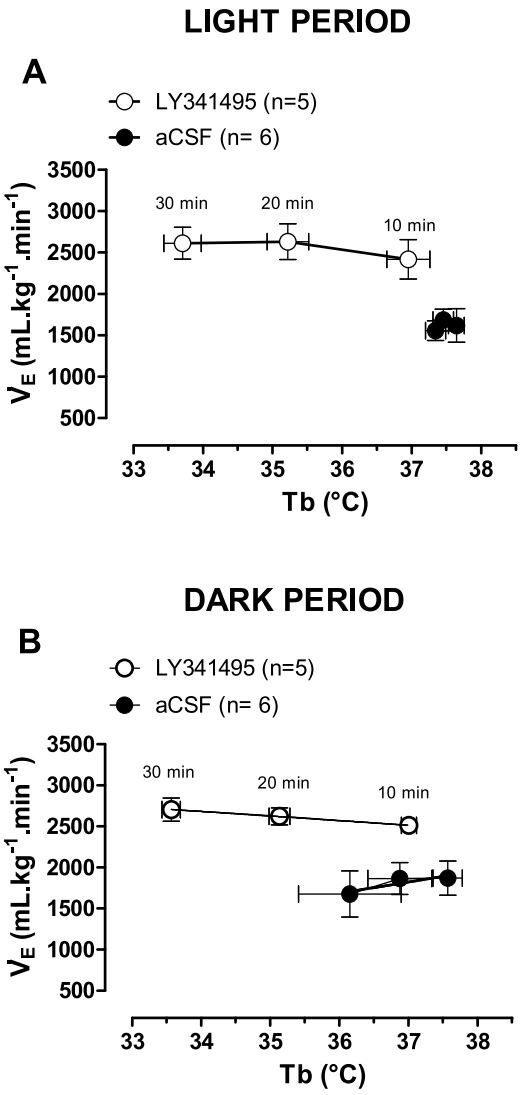

Fig. 6. The increase in the hypercapnic ventilatory response is not related to the decrease in body temperature. (A) Relationship between pulmonary ventilation ($\dot{V}_E$, data from Fig. 2) and body temperature (Tb, data from Fig. 5B) in rats treated with LY341495 or aCSF into the LH/PFA, under 7% CO_2 exposure, in the light period. **(B)** Relationship between pulmonary ventilation ($\dot{V}_E$, data from Fig. 3) and body temperature (Tb, data from Fig. 5C) in rats treated with LY341495 or aCSF into the LH/PFA, under 7% CO_2 exposure, in the dark period. The time corresponding to 10, 20 and 30 min of hypercapnia exposure are indicated in the LY341495 group data points.

Group II, consisting of mGluR2 and mGluR3; and Group III, comprising of mGluR4, mGluR6, mGluR7 and mGluR8. Group I mGluRs are coupled to Gq and stimulate the phospholipase C pathway. Conversely, Group II and group III mGluRs are coupled to Gi, which prevents the formation of cyclic adenosine 3′5′-monophosphate (cAMP) (Conn and Pin, 1997). Group II and III mGluRs are expressed both presynaptically and postsynaptically but they predominate in presynaptic elements, where their activation can decrease glutamate and other neurotransmitters transmitter release (Anwyl, 1999; Cartmell and Schoepp, 2000). In the LH/PFA, the predominant location of group II mGluR has not been described but group III mGluRs are believed to be localized primarily in presynaptic terminals in this area. It has been suggested, according to data from whole-cell voltage- and current-clamp recording in mouse hypothalamic slices, that group III mGluRs are probably located on both glutamate and GABA presynaptic axons that innervate the hypocretin neurons. I was demonstrated that the activation of group III mGluRs resulted in a reduction of synaptic activity recorded in hypocretin neurons by a presynaptic mechanism which envolved the selective inhibition of glutamate release, but not of GABA, mediated by group III mGluRs (Acuna-goycolea et al., 2004).

Since the receptors of Groups II and III are of inhibitory action (Blumcke et al., 1996), we raised the possibility that, when injected into the LH/PFA, the effect of MCPG on the hypercapnic ventilatory response could be due to the blockade of metabotropic receptors of Groups II and III. To test this hypothesis, we performed, in another experimental group, the microinjection of a selective antagonist for Group II/III mGluRs, LY341495. With the microinjection of LY341495, we observed a large increase in the ventilatory response to CO_2 during

wakefulness, but not during sleep, both in the light phase and in the dark phase, suggesting that the inhibitory modulation of the central chemoreflex exerted by glutamatergic neurotransmission in the LH/PFA possibly involves the Group II/III mGluRs.

Although the effects of MCPG and LY341495 were observed both in the light period and in the dark period, a greater effect occurred in the light period. In the dark period, microinjection of MCPG increased the hypercapnic ventilatory response by 29%, whereas in the light period, the increase was 39%. LY341495, on the other hand, increased the ventilatory response to CO_2 in the dark period by 44%, and in the light period, by 57%. In view of such results, it is reasonable to assume that glutamatergic inhibitory modulation in the LH/PFA may occur through the activation of Group II/III mGluRs expressed on orexinergic neurons, which have a recognized chemosensitive function (Li and Nattie, 2010; Williams et al., 2007). A higher inhibition during the light period is consistent with the lower activity of the orexinergic neurons in this phase of the diurnal cycle (Desarnaud et al., 2004), and with the lower chemosensitive activity of this region in the light period (Dias et al., 2010; Li et al., 2013).

The inhibition of the hypercapnic chemoreflex exerted by mGluRs might have a regulatory role, providing a negative feedback of the central chemoreflex. The fact that this effect was observed only during wakefulness may be related to the state-dependent control of central chemoreception. Moreover, it is well known that during sleep, the respiratory system is particularly susceptible to instability due to, among other factors, the absence of two important stimuli for breathing: the voluntary control and the effect of alertness, the so-called “wakefulness drive to breathe” (Krimsky and Leiter, 2005). Thus, some negative feedback mechanisms of the central chemoreflex probably operate just during wakefulness. If these mechanisms acted during sleep in the same way as during wakefulness, the instability of breathing control would be even greater, which could increase the incidence of sleep-related respiratory disease.

Neither MCPG nor LY341495 affected respiratory parameters under normocapnic conditions, compared with the microinjection of vehicle. The treated group showed no change in baseline ventilation compared to the control group, suggesting that glutamate, acting on mGluRs in the LH/PFA, does not affect the tonic drive to breathe.

It is known that hypercapnia, as well as hypoxia, can result in a decrease in body temperature, but the mechanisms involved are not very well known (Barros and Branco, 1998; Saiki and Mortola, 1996). One hypothesis is that when there is an acute hypercapnic exposure, acidosis plays an inhibitory role in metabolism, increases heat loss due to vasodilation of the blood vessels of the skin, and changes the concentrations of serotonin and norepinephrine in the hypothalamus, causing a decrease in body temperature (Schaefer et al., 1975). However, in rats, the temperature drop during hypercapnia is not as common as it is with hypoxia, as it seems to depend on the level of hypercapnia employed and the intensity of hyperventilation. We observed, in the control group, that hypercapnia did not alter the body temperature of the animals as observed in previous studies (Biancardi et al., 2008; de Carvalho et al., 2010) however, the group treated with LY341495, but not with MCPG, had a notable decrease in body temperature, both in the light phase and in the dark phase. There are three possible explanations for this result: First, since the injection of LY341495 into the LH/PFA caused intense hyperventilation, this may have caused a great loss of heat, resulting in hypothermia. Second, the observed effects on body temperature with the microinjection of LY341495 but not MCPG could be due to the specificity of LY341495 compound, which is highly selective for metabotropic receptors of groups II and III. MCPG, on the other hand, affects all groups of the glutamate metabotropic receptors - Group I, consisting of mGluR1 and mGluR5, Group II and Group III (Schoepp et al., 1999). While Group I exerts excitatory effects via IP3/Ca^{2+} signal transduction, Groups II/III have inhibitory effects via inhibitory G-protein activation (Niswender and Conn, 2010). Another reasonable hypothesis that deserves investigation is the possibility that the observed decreased body temperature could be a result of the effect of LY341495 on melanin concentrating hormone (MCH) neurons, since MCH-expressing neurons are intermingled with orexinergic neurons in the LH/PFA, and are reported to affect energy balance and to lower body temperature (Glick et al., 2009).

We analyzed the relationship between $\dot{V}_E$ and Tb during hypercapnia in the LY341495 groups, to confirm that the increase of the CO_2 response, with the antagonism of the Group II/III mGluRs, did not occur as a result of the fall of Tb in these groups. As observed in Fig. 6, the increase in the ventilatory response to CO_2 occurred from the onset of hypercapnia when the temperature drop was not maximal yet. In addition, as the animals continue to decrease body temperature, the ventilatory response remains essentially the same, which suggests that the effect of Group II/III mGluRs antagonist on the ventilatory response to CO_2 is not dependent on its effect on body temperature.

In our experiments, microinjection of LY341495 increased the alertness of the rats and, as a result, periods of sleep did not occur after the injection. There is a possible explanation for this. Treatment with LY341495 possibly resulted in an augmented excitation of orexinergic neurons by inhibiting the inhibitory mechanisms mediated by Group II/III mGluRs. It is well known that orexin neurons stimulate arousal; the increase in activation of these neurons could explain why the rats did not sleep after microinjection of the Group II/III antagonist.

Our data emphasize the role of the LH/PFA in modulating the hypercapnic ventilatory response in a state-dependent manner, and allows us to suggest that glutamate, acting via Group II/III mGluRs, exerts an inhibitory modulation of the ventilatory response to hypercapnia during wakefulness, but not during sleep.

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Conflict of interest

The authors declare that they have no conflict of interest.

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