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NEUROSCIENCE

Mu and kappa opioid receptors of the periaqueductal gray stimulate and inhibit thermogenesis, respectively, during psychological stress in rats

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Abstract The periaqueductal gray matter (PAG) is rich in mu and kappa opioid receptors, and this system is involved in thermoregulation, analgesia, and defensive behaviors. No study approached the involvement of the PAG opioids in body temperature (Tb) regulation during psychological stress such as restraint. Because activation of mu and kappa receptors increases and reduces Tb, respectively, we tested the hypothesis that they exert excitatory and inhibitory modulation, respectively, of the restraint-induced fever in rats. To this end, Tb, heat loss index (HLI, inference for peripheral vasoconstriction/vasodilation), and oxygen consumption (inference for thermogenesis) were monitored in unanesthetized rats, restrained or unrestrained, before and after intra-PAG microinjection of the selective mu opioid receptor antagonist (D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2 cyclic, CTAP; 1 and 10 μg/100 nL) or the selective kappa opioid receptor antagonist (nor-binaltorphimine dihydrochloride, nor-BNI; 1 and 4 μg/100 nL) or saline (100 nL). CTAP and nor-BNI did not change the Tb or HLI of euthermic animals. During restraint, Tb increased (1.0 ± 0.1 °C) in all groups; however, this effect was lower in those animals treated with CTAP and higher in animals treated with nor-BNI. The HLI decreased during restraint and increased after animals were released, but this response was not affected by any treatment. Restraint stress increased oxygen consumption (35.9 ± 3.9% elevation), but this response was diminished by CTAP and overstimulated by nor-BNI. Confirming our hypothesis, the results indicate that the mu and kappa opioid receptors in the PAG of rats play a pyrogenic and antipyretic role, respectively, during fever induced by restraint by affecting the thermogenic but not the heat conservation effector.

Keywords Body temperature · Heat loss index · Oxygen consumption · Opioid receptors · Nor-BNI · CTAP

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Introduction

Body temperature (Tb) affects the body's biochemistry and physiology in such a way that its maintenance becomes especially important. There are certain situations, however, in which changes in Tb are induced by endogenous mechanisms, such as during fever, which is known to be a regulated increase in Tb [45]. In this case, the brain plays an important role in converting the pyrogenic peripheral signals into appropriate adjustments on thermal effector activity (increasing heat conservation and/or production) [50].

Besides inflammatory agents, some stressor stimuli, including manipulation, open-field exposure, new environment exploration, or restraint, can also induce a rise in Tb in several mammalian species [30, 48, 67, 68, 82]. Handling stress and the introduction of a new individual into the same cage have also been demonstrated to induce an increase in preferred Tb in lizards, animals that thermoregulate primarily by behavioral mechanisms [21, 44]. The higher Tb may increase physical and neural performance [16], which can be beneficial for survival during psychogenic stressful situations. In addition,

some data in mammals have indicated that the increase in Tb induced by stress shows similar mechanisms to fever induced by endotoxins [27, 51, 57, 63, 68, 75, 78, 80]. In psychological stress, the brain seems to play a role in both the detection of the stress signal and the activation of appropriate thermoregulatory responses.

Endogenous opioids are possible mediators of the thermal response to psychogenic stress because they are a class of molecules with numerous effects, many of which are associated with stress responses [5] and thermoregulation [4, 9, 17, 34, 35, 77, 91]. Three types of opioid receptors, mu, kappa, and delta [46, 52, 69], were described to be expressed in many brain regions [24, 47, 62, 74]. The mu and kappa receptors seem to have opposite effects on Tb, where mu is involved in Tb increases [8, 18, 38, 39, 81, 92], including the development of endotoxin-induced fever [13, 14, 32], while kappa is related to Tb decreases [22, 81, 92] including the hypoxia-induced anapyrexia, an opposite response to fever [77]. The role of the delta opioid receptors in thermoregulation can involve different effects depending on the agonist or antagonist selectivity [19, 24, 38, 71, 74, 77, 81].

One of the brain regions rich in opioid receptors is the periaqueductal gray matter (PAG) [36, 54] which is involved in autonomic regulation of defensive behaviors [28], nociception, anxiety, analgesia, cardiorespiratory control, and thermoregulation [11, 28, 59, 60, 66, 87, 98]. Intriguingly, most studies about the role of the PAG in behavioral responses to stress do not consider the possible changes induced in Tb. This region contains β-endorphin [31] and expresses a significant density of mu and kappa opioid receptors [36, 54]. Naloxone inhibits the hyperthermia induced by β-endorphin and DAGO (a selective agonist for mu receptors) in the PAG, which suggests the action of mu receptors of the PAG to increase Tb [90]. Besides these facts, PAG is involved in the activation of heat loss (cutaneous vasodilation of the rat tail) [96, 97] and heat production (non-shivering thermogenesis in the brown adipose tissue, BAT) [23] mechanisms. Moreover, there is evidence of functional connections between PAG and hypothalamic regions involved in warmth-defense responses, the median preoptic nucleus (MnPO), and in thermogenesis, the dorsomedial hypothalamus (DMH) [94]. All those data together suggest an important role for the opioid system of PAG in the efferent pathways of autonomic thermoregulation and make this system a possible network for the development of restraint-induced fever. In fact, restraint stress is widely used as a model of unconditioned and unavoidable stress-elicited neuroendocrine, cardiovascular, and thermal responses [27, 75, 80, 84, 85].

Based on the considerations above, we hypothesized that opioid receptors, mu and kappa, in the PAG are involved in the induction and inhibition, respectively, of restraint-induced fever in rats. To this end, we investigated the effect of intra-PAG microinjection of selective antagonists for mu (D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2 cyclic, CTAP) and kappa (nor-binaltorphimine dihydrochloride, nor-BNI) opioid receptors on Tb, as well as on heat production (oxygen consumption) and heat conservation (heat loss index) effector mechanisms in rats subjected to restraint stress.

Materials and methods

Animals

Experiments were performed on adult male Wistar rats weighing 250–280 g. Animals had free access to water and food and were housed in a temperature-controlled chamber at 25 ± 1 °C (model: ALE 9902001; Alesco, Monte Mor, SP, Brazil) with a 12 h:12 h light/dark cycle (lights on at 0630 hours). All the experimental protocols were conducted with the approval of the local animal care committee of the College of Agricultural and Veterinarian Sciences, São Paulo State University (CEUA; protocol no. 007094/13).

Drugs

CTAP (a selective mu opioid receptor antagonist; 1 and 10 μg/100 nL/animal) and nor-BNI (a selective kappa opioid receptor antagonist; 1 and 4 μg/100 nL/animal) were purchased from Sigma-Aldrich (São Paulo, Brazil). Drugs were dissolved in pyrogen-free sterile saline. Doses were chosen on the basis of preliminary experiments and previous reports [13, 74, 77].

Surgery and microinjection

Animals were anesthetized with ketamine (100 mg/kg, intraperitoneal) and xylazine (10 mg/kg, intraperitoneal), fixed in a stereotaxic frame (David Kopf, model 900, Tujunga, CA, USA), and implanted with a stainless steel guide cannula (0.6 mm o.d. and 12 mm in length) 2 mm above the dorsal periaqueductal gray region (dorsomedial and dorsolateral PAG; tower angle 22° to the left; coordinates relative to lambda/antero-posterior +1.1 mm; latero-lateral +1.9 mm; dorso-ventral −3.2 mm from the skull surface; incisor bar −2.5 mm). These coordinates were adapted from Paxinos and Watson atlas [70]. The cannula was attached to the bone with stainless steel screws and acrylic cement. A tight-fitting stylet was kept inside the guide cannula to prevent occlusion. Additionally, animals of all groups were submitted to paramedian laparotomy in order to insert a data logger (SubCue Dataloggers, Calgary, Canada) into the abdomen for body temperature measurements. After surgery, animals were treated with antibiotic (enrofloxacin, 10 mg/kg, intramuscular) and non-steroid anti-inflammatory (flunixin meglumine, 2.5 mg/kg, subcutaneous) agents. Experiments were initiated 1 week after surgeries.

The microinjections were performed in unrestrained animals, a procedure that is routinely employed in our laboratory, using a regular Hamilton syringe (5 μL) and a dental injection needle (Mizzy, 200 μm o.d.) attached by a PE-10 tube. The syringe and the tube were filled with sterile water, with an air bubble separating it from the drug, and the injected volume was evaluated visually by monitoring the movement of the water-air-drug interface. The injection needle was 2 mm longer than the guide cannula so that the PAG can be reached by the needle only at the time of injection. A volume of 100 nL of vehicle or antagonist solution was injected over a period of 30 s, and 30 s more was allowed to elapse before the injection needle was removed from the guide cannula to avoid reflux. All 100 nL injection volumes were precisely administered using a microinjector machine (model 310, Stoelting Co., IL, USA).

Histology

Upon completion of the experiments, animals were deeply anesthetized with ketamine (100 mg/kg, intraperitoneal) and xylazine (10 mg/kg, intraperitoneal) and injected with 100 nL of 2% Evan's Blue solution through the guide cannula. Rats were then perfused through the left cardiac ventricle with saline followed by 10% formalin. The brain was removed and stored in 10% formalin for at least 2 days, 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. Microinjections were considered *intra-PAG* when given directly into the PAG, and *peri-PAG* when located in nuclei surrounding the PAG.

Determination of body temperature

SubCue Dataloggers (Calgary, AT, Canada) were connected to a computer via optic connection and programmed to acquire data every 5 min using the software *SubCue Temperature Datalogger*. At the end of the experiments, data loggers were connected again to the computer and temperature values were corrected according to the specifications of the manufacturer's manual.

Determination of the heat loss index

The skin temperature (Ts) of the tail was measured by means of infrared images using a camera sensitive to such radiation (FLIR SC660, Switzerland). This technique has been commonly used to infer the peripheral blood flow (heat loss/conservation) in rats because the tail is considered a thermal window in these animals [7, 73, 83, 89]. We determined the average of three values of skin temperature for the middle third of the tail length, where temperatures have reduced interference from (1) the body, because the temperature of the base of the tail is higher (influence of Tb), and (2) the environment, because the temperature at the tip of tail is closer to that of the environment [73]. The Tb and Ts were used to infer the heat loss index (HLI) of the animal, which ranges from 0 to 1 (0 indicates maximum vasoconstriction and 1 is maximum vasodilation), and was calculated according to the formula: HLI = (Ts − Ta) / (Tb − Ta), where Ta is the ambient temperature [73].

Determination of oxygen consumption

Metabolic rate was inferred through oxygen consumption measured in an open respirometry system. A continuous flow of air was maintained in the experimental chamber where the inflow and outflow O_2 concentrations were monitored by an O_2 analyzer (Sable Systems International, Inc., Las Vegas, NV, USA) connected to a computer to record and store data. The oxygen consumption ($\dot{V}O_2$) was determined based on the flow rate and gas concentration differences between the inlet (FiO_2) and outlet (FeO_2) of the chamber. Air was pulled into the respirometer at a rate of 1700 mL min^{-1} and passed through an analyzer of water vapor pressure (RH300; Sable Systems, Las Vegas, NV, USA), but only a subsample of this air was pulled through the O_2 analyzer at 185 mL min^{-1} (SS4; Sable Systems, Las Vegas, NV, USA). Each 10 min of recording was composed of 2 min of *baseline*, a sample of incurrent air, and 8 min of excurrent air. Baseline was taken to ensure that O_2 in the air offered to the animal remained constant throughout the experiment and to provide the estimates of F_iO_2. A gas flow distributor (RM8 Intelligent Multiplexer; Sable Systems) was used to control which gas sample (from respirometer or baseline) entered the analyzers. All data were recorded using a data acquisition system (ExpeData v. 1.4.5; Sable Systems), collecting values every second. The O_2 analyzer was calibrated using nitrogen as a zero value and dried air (20.95% O_2) as span. As CO_2 was neither analyzed nor scrubbed, the $\dot{V}O_2$ was calculated using the following equation [53]: $\dot{V}O_2 = [FR_e (F_iO_2 - F_eO_2)] / [1 - F_iO_2 (1 - RQ)]$, where FR_e is the excurrent flow rate, F_iO_2 is the incurrent fractional concentration of oxygen (baseline), F_eO_2 is the excurrent fractional concentration of oxygen, and RQ is the respiratory quotient (considered 0.85). Since the air was not dried during the experiment, FR_e was corrected according to the following formulas: $FR_e = FR_e \times (BP - WVP) / BP$, $F_iO_2 = F_iO_2 \times BP / (BP\text{-}WVP)$, and $F_eO_2 = F_eO_2 \times BP / (BP - WVP)$, where BP is the barometric pressure and WVP is the water vapor pressure. Data are shown in standard conditions of temperature, pressure, and dry air (STPD).

Experimental protocol

The experiments were conducted in unanesthetized animals implanted with a temperature data logger (Tb each 5 min) in the abdominal cavity. Animals were placed in individual cages in the

experimental room (Ta = 25–27 °C) during the afternoon, and the experiments were performed the next morning. Each animal received an intra-PAG microinjection of CTAP, nor-BNI, or saline (vehicle). Animals were randomly divided into two groups: (a) control rats, which received treatments and stayed in their cages without disturbance during the entire experiment, and (b) restraint stress rats, which were placed inside the restraint tube (60 mm o.d.) in their cages 50 min after microinjection (time needed to avoid handling stress before restraint stress), and remained there for 40 min. After the restraint stress, the rats were kept in their cages for a recovery period of 50 min. The time of action of the antagonists is long enough for them to be active for the duration of the experiment [1, 20, 29, 49]. During experiments where animals were not subjected to restraint stress, thermal tail images were captured at 30, 60, 90, and 120 min, and in experiments where animals were subjected to restraint stress, images were captured at 30, 35, 45, 50, 55, 60, 65, 70, 75, 80, 90, 95, 100, 105, 110, and 130 min.

Oxygen consumption was determined in a separate group of animals: (a) control group, which received treatment and remained undisturbed in the respirometer (6.8 L) throughout the experiment, and (b) restraint stress group, which was placed inside the restraint tube after 50 min of microinjection and remained there for 40 min and for an additional 50 min of recovery. The dose of CTAP and nor-BNI (1 μg/100 nL) that affected Tb during restraint in the previous protocol was chosen for the oxygen consumption experiments. Both treatments and conditions were applied in a random fashion.

Statistical analysis

Data are shown as mean ± SEM. The initial Tb, HLI, and $\dot{V}O_2$, i.e., before any microinjection, were compared among all animal groups by one-way ANOVA. The effects of the opioid receptor antagonists, CTAP and nor-BNI, on Tb, HLI, and $\dot{V}O_2$ were analyzed through the analysis of variance (two-way ANOVA) for repeated measures (factors: treatment and time) followed by Tukey's test. In the experiments in which animals were subjected to restraint, the periods of restraint and post-restraint were analyzed separately for evaluating treatment effects. Significant differences were considered for $p < 0.05$.

Results

Microinjection sites in the dorsal PAG are shown in Fig. 1a, and the representative diagrams of microinjection sites are shown in Fig. 1b. The intra-PAG microinjections were located in the dorsolateral and dorsomedial PAG, sites where mu and kappa opioid receptors are known to be expressed [36]. Because of the reduced number of peri-PAG animals injected with each dose of the antagonists and their similar results to saline, the peri-PAG groups in all graphs of Tb, HLI, and $\dot{V}O_2$ were composed of a pool of animals that received both doses of the drugs.

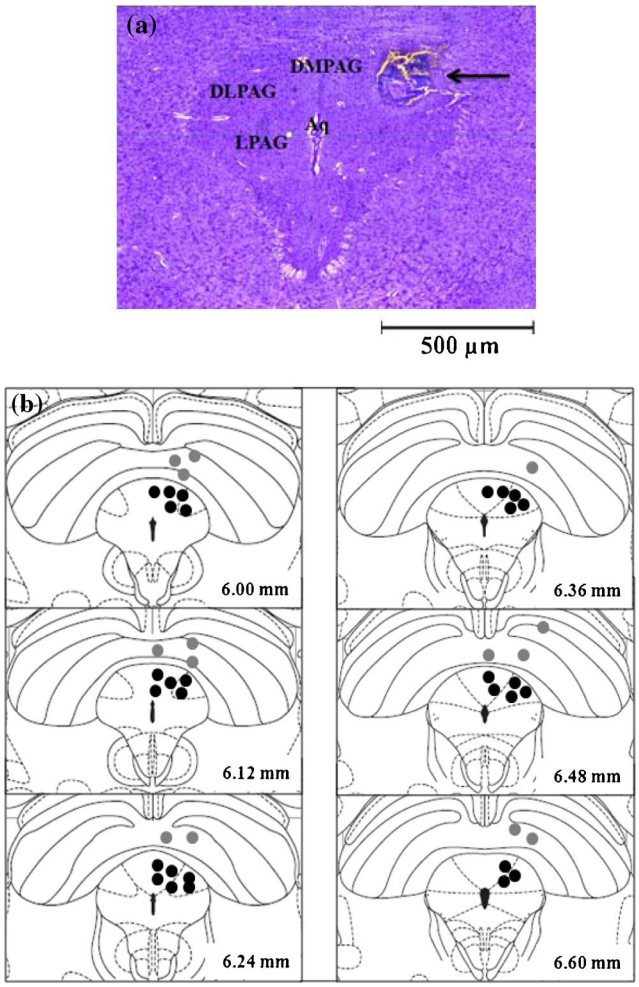

Fig. 1 **a** Photomicrography of the brain of a representative animal showing the typical microinjection site (*arrow*) in the dorsolateral periaqueductal gray matter (*DLPAG*). **b** Representative diagrams showing microinjection sites intra-PAG (*black circles*) and outside-PAG (*gray circles*) at distances (in mm) caudal to the bregma [67]. *DMPAG* dorsomedial PAG, *LPAG* lateral PAG, *Aq* aqueduct

Prior to any treatment, Tb, HLI, and $\dot{V}O_2$ were not different among all the experimental groups ($p = 0.304$, $F_{(6,112)} = 1.217$ for Tb; $p = 0.085$, $F_{(6,112)} = 1.928$ for HLI; and $p = 0.844$, $F_{(4,61)} = 0.348$ for $\dot{V}O_2$). In addition, the pattern of changes in skin temperature of the tail in all groups and treatments (data not shown) was similar to those of HLI.

Effect of intra-PAG microinjection of the selective mu opioid receptor antagonist, CTAP, on the Tb, HLI, and $\dot{V}O_2$ of restrained and unrestrained rats

Saline or the two doses of CTAP microinjected into the PAG did not change the Tb of non-stressed rats (no effect of time: $p = 0.240$, $F_{(3,32)} = 1.244$; no effect of treatment: $p = 0.868$,

$F_{(3,32)} = 0.239$; Fig. 2a). The same lack of treatment effect was observed in HLI (no effect of time: $p = 0.111$, $F_{(3,32)} = 2.057$; no effect of treatment: $p = 0.488$, $F_{(3,32)} = 0.830$; Fig. 2b).

Figure 3 shows the results of microinjection of saline or CTAP in the PAG of rats subjected to restraint stress. Body temperature increased during restraint in both groups (effect of time: $p < 0.001$, $F_{(3,32)} = 97.507$); however, the increase in Tb was significantly lower after intra-PAG microinjection of 1 μg, but not 10 μg, of CTAP (effect of treatment: $p = 0.005$, $F_{(3,32)} = 5.093$; no interaction effect of time and treatment: $p = 0.793$, $F_{(3,32)} = 0.753$; Fig. 3a). The HLI decreased during restraint and then increased shortly after (effect of time: $p < 0.001$, $F_{(3,32)} = 28.715$; Fig. 3b), but no difference among treatments was observed (no effect of treatment: $p = 0.670$, $F_{(3,32)} = 0.523$). Figure 4 depicts the thermal images of the rat tail before, during (10 min) and just after restraint stress in a representative animal injected with saline.

As 1 μg/100 nL of CTAP intra-PAG affected the increase in Tb during restraint, this concentration of CTAP was chosen for the experiments measuring the thermogenic effector during stress. Figure 5a shows that there was no difference in oxygen consumption between CTAP and saline groups in rats that were not subjected to restraint stress (no effect of time: $p = 0.733$, $F_{(2,14)} = 0.557$; no effect of treatment: $p = 0.659$, $F_{(2,14)} = 0.429$). During restraint, however, oxygen consumption increased in both groups (effect of time: $p < 0.001$, $F_{(2,18)} = 26.708$), but CTAP reduced this response (effect of treatment: $p = 0.022$, $F_{(2,18)} = 4.727$; no interaction effect of time and treatment: $p = 0.972$, $F_{(2,18)} = 0.125$; Fig. 5b).

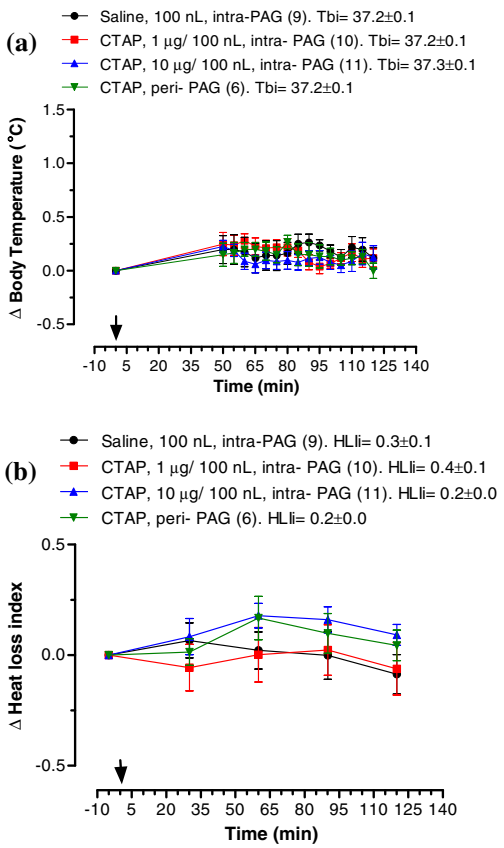

Fig. 2 Effect of microinjection of the mu opioid receptor antagonist CTAP (1 and 10 μg/100 nL/animal) or saline (vehicle; 100 nL) in the periaqueductal gray matter (*PAG*) on body temperature (**a**) and heat loss index (**b**) in animals not subjected to restraint. The *arrow* indicates the time of microinjection. The number of animals in each group is shown in *parentheses*. Tbi (°C) is the mean body temperature before any treatment, and HLIi is the mean heat loss index before any treatment

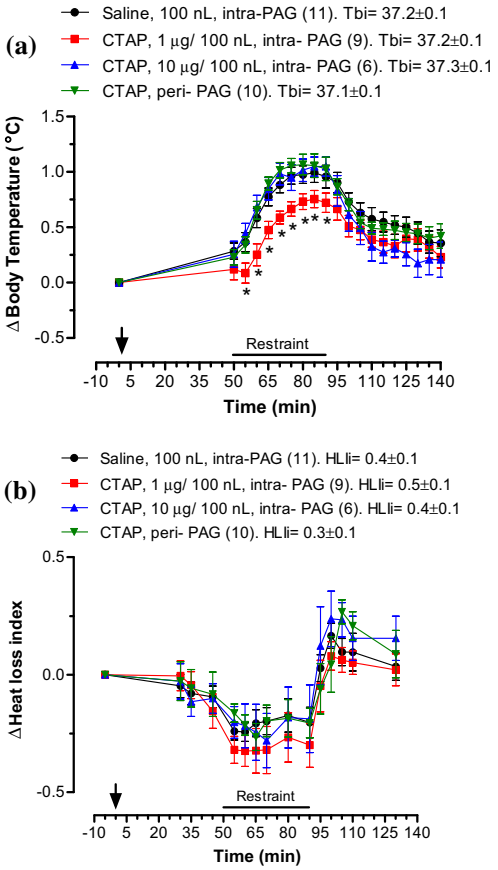

Fig. 3 Effect of microinjection of the mu opioid receptor antagonist CTAP (1 and 10 μg/100 nL/animal) or saline (vehicle; 100 nL) in the periaqueductal gray matter (*PAG*) on body temperature (**a**) and heat loss index (**b**) in animals subjected to restraint. The *arrow* indicates the time of microinjection. The number of animals in each group is shown in *parentheses*. Tbi (°C) is the mean body temperature before any treatment, and HLIi is the mean heat loss index before any treatment. *$p < 0.05$, significant difference from the saline group

Effect of intra-PAG microinjection of the selective kappa opioid receptor antagonist, nor-BNI, on the Tb, HLI, and $\dot{V}O_2$ of restrained and unrestrained rats

Figure 6 shows that treatment with nor-BNI induced no changes in Tb (no effect of time: $p = 0.500$, $F_{(3,29)} = 0.955$; no effect of treatment: $p = 0.676$, $F_{(3,29)} = 0.515$; Fig. 6a) or HLI in non-stressed rats (no effect of time: $p = 0.187$,

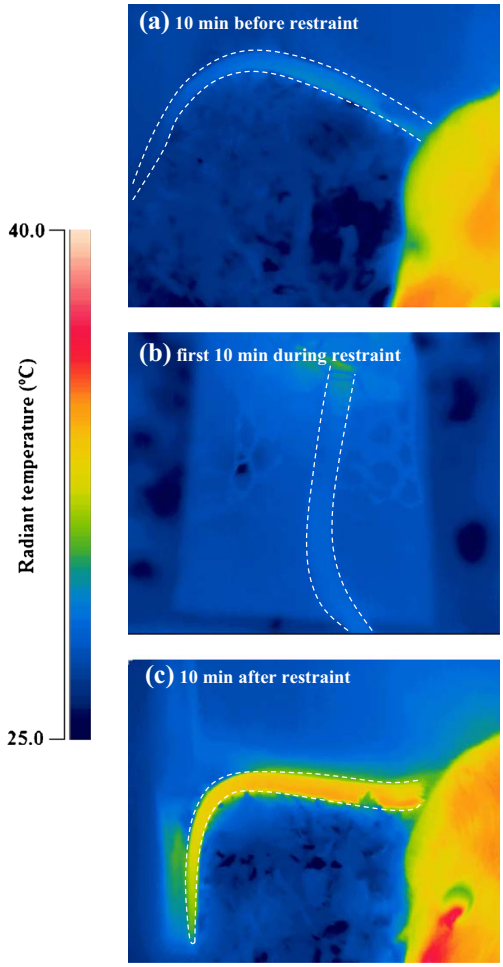

Fig. 4 Thermographic images of the tail of a representative rat in the control group (saline) 10 min before (**a**), during (**b**), and 10 min after (**c**) restraint

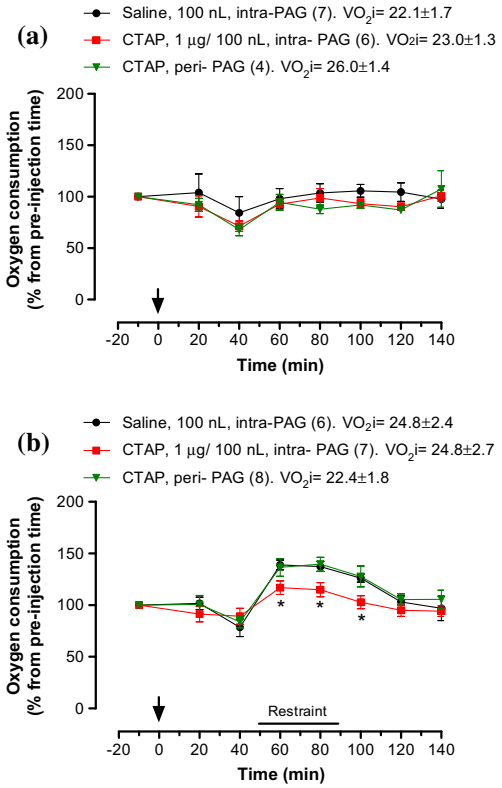

Fig. 5 Effect of microinjection of the mu opioid receptor antagonist CTAP (1 µg/100 nL/ animal) or saline (vehicle; 100 nL) in the periaqueductal gray matter (*PAG*) on oxygen consumption in animals not subjected (**a**) and subjected (**b**) to restraint. The *arrow* indicates the time of microinjection. The number of animals in each group is shown in *parentheses*. VO_2i (mL kg^{-1} min^{-1} STPD) is the mean oxygen consumption before any treatment. *$p < 0.05$, significant difference from the saline group

$F_{(3,29)} = 1.644$; no effect of treatment: $p = 0.333$, $F_{(3,29)} = 1.194$; Fig. 6b). During restraint stress, Tb increased in both saline and nor-BNI groups (effect of time: $p < 0.001$, $F_{(3,30)} = 77.263$; Fig. 7a); however, animals treated with the lower dose of nor-BNI intra-PAG presented a further increase in Tb (effect of treatment: $p = 0.040$, $F_{(3,30)} = 3.128$; no interaction effect of time and treatment: $p = 0.808$, $F_{(3,30)} = 0.739$; Fig. 7a). The HLI increased similarly in all groups after restraint (effect of time: $p < 0.001$, $F_{(3,30)} = 20.993$; no effect of treatment: $p = 0.440$, $F_{(3,30)} = 0.846$; Figs. 4 and 7b).

Figure 8a shows that there was no difference in oxygen consumption between nor-BNI and saline groups in rats that were not subjected to restraint stress (no effect of time: $p = 0.071$, $F_{(2,17)} = 2.122$; no effect of treatment: $p = 0.803$, $F_{(2,17)} = 0.223$). During restraint stress, however, oxygen consumption increased in both groups (effect of time: $p < 0.001$, $F_{(2,19)} = 45.808$), but nor-BNI intensified this response (effect of treatment: $p = 0.037$, $F_{(2,19)} = 3;952$; no interaction effect of time and treatment: $p = 0.267$, $F_{(2,19)} = 1.357$; Fig. 8b).

Discussion

The present results confirm our hypothesis that the mu and kappa opioid receptors, located in the PAG, modulate fever induced by restraint stress in rats, stimulating and inhibiting this response, respectively. They present this action, not by affecting heat conservation but by increasing (mu) and decreasing (kappa) thermogenesis during restraint (Fig. 9). To our knowledge, this is the first demonstration of the thermoregulatory effectors involved in restraint stress, as well as their specific modulations in the brain.

There is enough evidence in the literature on the participation of mu and kappa receptors in stress responses [5] and in the regulation of Tb [2–4, 8, 9, 17, 18, 22, 34, 35, 38, 39, 77, 81, 91, 98]. Our previous data are consistent with the participation of opioid receptors in the preoptic area (POA) in anapyrexia (regulated decrease in Tb, the opposite response of fever) induced by hypoxia; i.e., kappa opioid receptors are involved in the reduction of Tb during hypoxia and mu opioid receptors are important for the return of Tb to the state of post-hypoxia normoxic euthermy [77]. Corroborating those data,

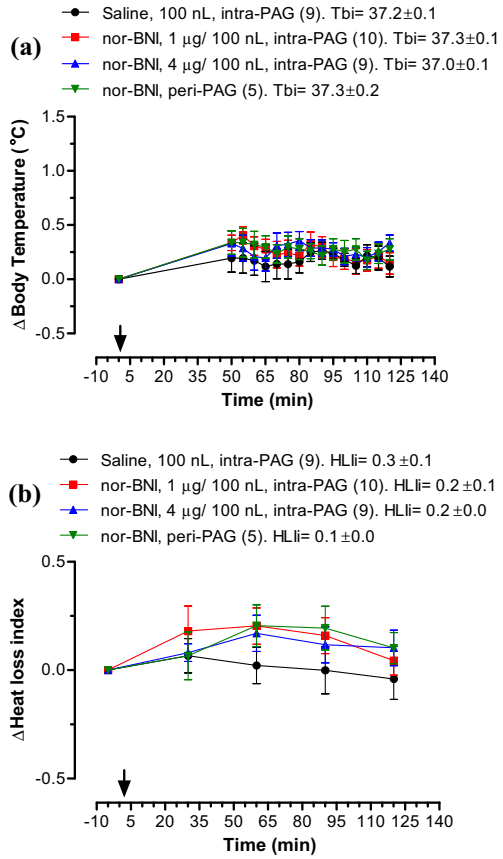

Fig. 6 Effect of microinjection of the kappa opioid receptor antagonist nor-BNI (1 and 4 µg/100 nL/animal) or saline (vehicle; 100 nL) in the periaqueductal gray matter (*PAG*) on body temperature (**a**) and heat loss index (**b**) in animals not subjected to restraint. The *arrow* indicates the time of microinjection. The number of animals in each group is shown in *parentheses*. Tbi (°C) is the mean body temperature before any treatment, and HLIi is the mean heat loss index before any treatment

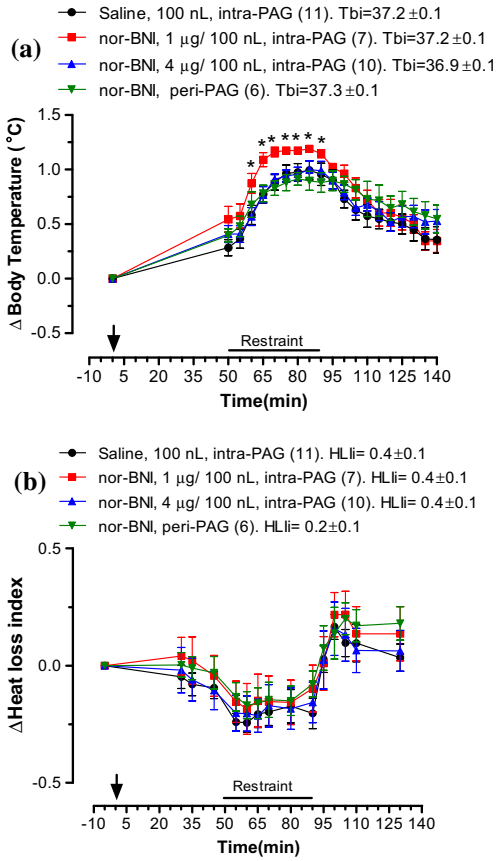

Fig. 7 Effect of microinjection of the kappa opioid receptor antagonist nor-BNI (1 and 4 µg/100 nL/animal) or saline (vehicle; 100 nL) in the periaqueductal gray matter (PAG) on body temperature (a) and heat loss index (b) in animals subjected to restraint. The arrow indicates the time of microinjection. The number of animals in each group is shown in parentheses. Tbi (°C) is mean body temperature before any treatment and HLIi is mean heat loss index before any treatment. *$p < 0.05$ significant difference from the saline group

the microinjection of nor-BNI in the POA of squirrels causes an increase in Tb during hibernation [95], which is a condition also considered a regulated decrease in Tb due to metabolic depression [10]. Moreover, mu opioid receptors, especially in the hypothalamus, have been implicated in the increase in Tb during endotoxin-induced fever [32, 86].

The fever response is characterized by the activation of mechanisms for heat conservation (cutaneous vasoconstriction) and production (thermogenesis) [6, 15]. In our study, during restraint, the HLI decreased (indicating peripheral vasoconstriction) in parallel with the increase in Tb, and at the end of the restraint, the HLI increased (indicating peripheral vasodilation) in parallel with the return of Tb to euthermic values. The treatments with antagonists did not affect tail vasoconstriction/vasodilation during and post-restraint, indicating kappa and mu in the dl/dmPAG are not involved in the modulation of this febrile thermoeffector. These results, however, do not exclude the possible modulation of heat conservation by these receptors in other regions of PAG, such as rostral PAG [94]. For mu receptors, their pyrogenic action in dl/dmPAG seems to be related to the activation of the thermogenic thermoeffector, because CTAP decreased oxygen consumption during restraint, while Tb also decreased. In contrast, for the kappa receptors, the inhibition of thermogenesis seems to be involved in their antipyretic action in the PAG, because nor-BNI increased oxygen consumption during restraint, while Tb increased.

The neural mechanism that links psychological stress to an acute or chronic elevation of Tb is still not fully understood, but the studies by Lkhagvasuren et al. [58] and Kataoka et al. [48] provide evidence that the increase in Tb, at least that which is induced by social defeat stress, involves the participation of the DMH, which receives inputs from preoptic neurons and sends outputs to the rostral medullary raphe (rMR). This neural pathway activates the sympathetic nervous system responsible for stimulating cutaneous vasoconstriction and thermogenesis of BAT [58, 64]. According to the present study, opioid systems in the dorsal PAG may be part of this circuitry involved in thermoregulation during psychological

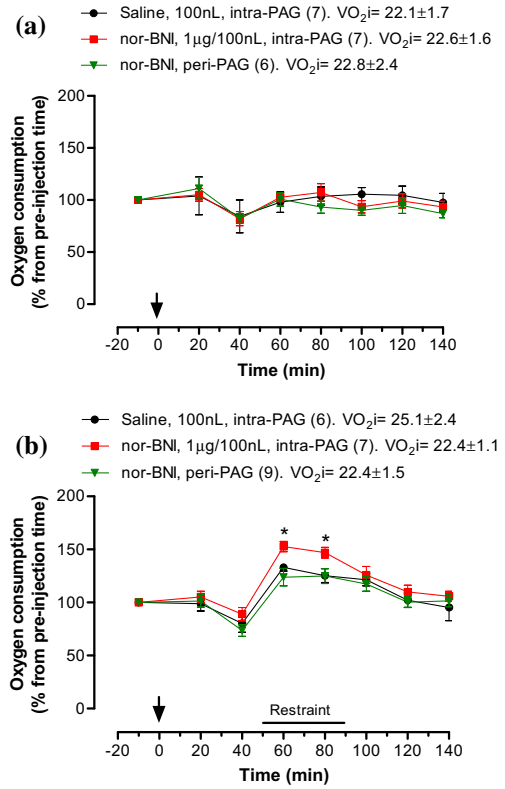

Fig. 8 Effect of microinjection of the kappa opioid receptor antagonist nor-BNI (1 µg/100 nL/animal) or saline (vehicle; 100 nL) in the periaqueductal gray matter (*PAG*) on oxygen consumption in animals not subjected (**a**) and subjected (**b**) to restraint. The *arrow* indicates the time of microinjection. The number of animals in each group is shown in *parentheses*. $\dot{V}O_2i$ (mL kg^{-1} min^{-1} STPD) is the mean oxygen consumption before any treatment. *$p < 0.05$, significant difference from the saline group

stress. There are excitatory connections between DMH and l/dlPAG [25, 26, 94] which seem to be important for thermogenic responses. In turn, the DMH can be activated by acute emotional stimulation and has functional connections with structures involved in fear and stress situations, such as the amygdala [55, 56, 76, 79]. There is also evidence for projections from the PAG to the raphe magnus [43], which has been implicated in analgesia [12] and the activation of non-shivering thermogenesis [48, 64].

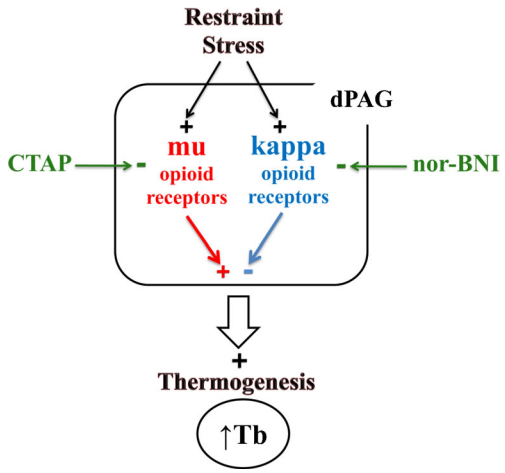

Fig. 9 Diagram showing that the thermogenesis activated during restraint stress is stimulated by mu opioid receptors and inhibited by kappa opioid receptors in the dorsal PAG (*dPAG*). CTAP and nor-BNI antagonizes mu and kappa receptors, respectively. *Plus sign* stimulation; *minus sign* inhibition. See text for details

Approximately 50% of the PAG neurons are GABAergic interneurons [65, 72]. At least for the analgesic effect of opioids on the PAG, it has been demonstrated an inhibition of GABAergic synaptic transmission is controlled by a voltage-dependent potassium conductance as the result of the activation of the mu receptors in the presynaptic terminals [88]. Thus, a suppression of GABA transmission in the PAG by the activation of mu receptors may be a mechanism involved in the activation of BAT thermogenesis mediated by the rMR. In contrast, a predominant suppression of excitatory synapses by a reduction of glutamate transmission in the PAG by kappa receptors may be a mechanism involved in the inhibition of rMR-mediated non-shivering thermogenesis. At least in the nucleus accumbens, the activation of kappa receptors inhibits the release of glutamate along with the entry of calcium [41, 42]. Another possible mechanism for kappa action in the PAG would be via interaction with nitric oxide (NO) neurotransmission, which inhibits neuronal discharge of the dlPAG [37, 61]. There is evidence of colocalization of kappa receptors and NO synthase (NOS) protein in neurons of PAG, which are suggested to be involved in the same intracellular network that controls morphine tolerance and dependence [40]. Moreover, NO is known to potentiate the presynaptic release of GABA in dlPAG, which in turn activates $GABA_B$ receptors in glutamatergic terminals reducing local release of glutamate [93]. Indeed, NO in dlPAG may play a role in inhibiting fever induced by restraint in rats, an issue that is currently under investigation in our laboratory.

Only the lower tested concentrations of the opioid receptor antagonists, CTAP and nor-BNI, had an effect on Tb in the present study. A similar pattern of responses was previously observed by us on Tb changes after hypoxia in rats injected with naltrindole (delta opioid receptor antagonist) in the POA [77] and on hyperventilation induced by hypoxia in rats injected with WAY100635 (5-HT_{1A} receptor antagonist) in the POA [33]. The possible explanation for those effects is that high doses of antagonists may lead to their interaction with different targets. Chen et al. [24] suggested that there is a tonic balance between mu and kappa receptors and that with high doses of a pharmacological agent, it may act as an antagonist or agonist of other receptors. Moreover, Baker and Meert [9] have shown that opioid-induced hypothermia can be modulated by multiple receptors in the opioid system, which have the ability to interact with each other. Therefore, the largest concentrations (higher than 1 µg/100 nL) of the antagonists used in our protocols may have been too high. In fact, the most

effective dose of nor-BNI injected in the POA for inhibiting Tb reduction during hypoxia was 1 μg/100 nL, with a minor effect of a concentration ten times lower [77].

In conclusion, our results are compatible with the idea that mu and kappa opioid receptors of the dorsal PAG play roles as pyrogenic and antipyretic molecules, respectively, during restraint stress-induced fever in rats by affecting the thermogenic, but not the heat conservation, thermoregulatory effector. The existence of those contrasting modulations by endogenous opioids in the PAG brings new insights for the understanding of interactive mechanisms between psychological stress and temperature regulation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethical standards All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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