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# Inhibition of nNOS in the paraventricular nucleus of hypothalamus decreases exercise-induced hyperthermia

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ABSTRACT

The paraventricular nucleus of the hypothalamus (PVN) is an important site for autonomic control, which integrates thermoregulation centers and sympathetic outflow to thermoeffector organs. PVN neurons express the neuronal isoform of nitric oxide synthase (nNOS) whose expression is locally upregulated by physical exercise. Thus, the aim of the present study was to evaluate the role of nNOS in the PVN in the exercise-induced hyperthermia. Seven days after surgery, male Wistar rats received bilateral intra-PVN microinjections of the selective nNOS inhibitor Nw-Propyl-L-Arginine (NPLA) or vehicle (saline) and were submitted to an acute progressive exercise session on a treadmill until fatigue. Abdominal and tail skin temperature ($T_{abd}$ and $T_{tail}$, respectively) were measured, and the threshold (*Hthr*; °C) and sensitivity (*Hsen*) for heat dissipation calculated. Performance variables were also collected. During the progressive exercise protocol, all animals displayed an increase in the $T_{abd}$. However, compared to vehicle group, the microinjection of NPLA in the PVN attenuated the exercise-induced hyperthermia. There was no difference in $T_{tail}$ or *Hthr* between NPLA and control rats. In contrast, *Hsen* was increased in the NPLA group compared to vehicle. In addition, heat storage was lower in NPLA-treated animals. Despite the temperature differences, inhibition of nNOS in the PVN did not affect running performance on the treadmill. These results suggest that nitrergic signaling within the PVN, under nNOS activation, drives the increase of body temperature, being necessary for the proper thermal regulatory mechanisms during progressive exercise-induced hyperthermia.

## 1. Introduction

The core body temperature of mammals is tightly regulated within narrow limits by a precise, well-coordinated balance between the rates of heat production and heat loss (Romanovsky et al., 2009; Tan and Knight, 2018a; Wanner et al., 2015b; Webb, 1995). However, in specify conditions, such as physical exercise, body temperature deviate from these narrow limits (Wanner et al., 2015b). Muscle contractions associated with physical exercise provide mechanical and metabolic stimuli sufficient to modify the energetic state of an organism (Coyle, 2000). Part of the chemical energy required to muscle contraction is converted in mechanical work, while the major part (~70–80%) is converted in heat (Brooks et al., 1984; Coyle, 2000). The exercise-induced increase in body temperature results from a temporary imbalance between the above-mentioned rates, with the rate of heat production increasing faster than the rate of cutaneous heat loss (Gleeson, 1998; Webb, 1995). In rodents, tail skin vasodilatation plays an important role in heat dissipation during running, as the skin has dense vascularization and displays high surface area and volume ratio (Romanovsky et al., 2002). The homeostatic control of body temperature depends on the central integrating centers. The thermal information inputs from the thermosensors of the skin, viscera, and central nervous system (CNS) compose the afferent limb of thermoregulation. The signals from peripheral afferents are transmitted through the spinal cord to the thermoregulatory centers, the main one being the preoptic area (POA), where thermal information is integrated and the internal temperature is constantly regulated through the efferent pathways (Boulant, 2000; Morrison and Nakamura, 2011; Romanovsky et al., 2009; Tan and Knight, 2018b). The

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POA neurons are connected with other hypothalamic areas, including important neural projections to the paraventricular nucleus of hypothalamus (PVN) (Nagashima, 2006; Romanovsky, 2007). The PVN is an important integrative structure and a well-known site for autonomic and neuroendocrine control (Swanson and Kuypers, 1980; Ranson et al., 1998; Coote, 1995). Moreover, thermoregulation is also included in the diversity of PVN physiologic functions. Inenaga and cols firstly demonstrated the presence of thermosensitive neurons in the PVN, using extracellular recordings in the rat hypothalamic slice preparation (Inenaga et al., 1987). Additional evidence was collected using Fos immunohistochemistry to map thermosensitive cell groups in rats under acute thermal experiment, which confirmed PVN neuronal activation by warm exposure (Cham et al., 2006; Palkovits and Bratincsa, 2004). Furthermore, the warm-activated PVN neurons project directly to the lateral intermediate column of the spinal cord (Cham et al., 2006), modulating the sympathetic activity of thermoregulatory effectors such as the brown adipose tissue, salivary gland, and cutaneous vasculature of the tail skin (Cham and Badoer, 2008; Leite et al., 2012; Madden and Morrison, 2009; Smith et al., 1998). A number of evidence points to the effects of central nitric oxide (NO) on thermoregulation during exercise (Lacerda et al., 2006, 2005; Lima et al., 2014; Wanner et al., 2015b). The nitric oxide synthase (NOS), including the neuronal isoform (nNOS), is known to be expressed in the PVN, where endogenous NO modulates autonomic responses (Busnardo et al., 2019, 2013; Leite et al., 2012; Raquel et al., 2018; Zhang et al., 1997), including those related to thermoregulation (Monda et al., 1995; Zhang and Patel, 1998). In this context, the global effects of central NO on thermoregulation during exercise have been demonstrated (Lacerda et al., 2006, 2005; Lima et al., 2014; Wanner et al., 2015a). The intracerebroventricular (icv) injection of the non-selective NOS inhibitor, L-NAME, decreased heat dissipation, thereby increasing heat accumulation, in rats during exercise (Lacerda et al., 2006, 2005). Accordingly, intracerebroventricular injection of the NO precursor L-arginine increased heat dissipation, thus attenuating the increase in body temperature and improving exercise tolerance in rats (Wanner et al., 2015a). Although these experiments are not site-specific, they suggest that central NO increases heat dissipation during exercise to avoid an overshoot in body temperature. However, the role of endogenous NO within specific brain areas in thermoregulation remains poorly understood. Considering the evidence in the literature that the PVN is part of the central thermoregulatory pathway, express NOS and is activated during exercise, we investigated whether and how the local nitrergic modulation mediated by nNOS activation within the PVN is involved in the exercise-induced thermoregulatory responses. Therefore, the objective of the present study was to evaluate the role of nNOS in the PVN in the thermoregulatory responses to acute progressive running exercise in rats.

## 2. Material and methods

### 2.1. Animals and ethics statement

Adult male Wistar rats (n = 22) weighing 270–300 g were housed in collective cages in the Animal facility of the Department of Physiology and Biophysics (ICB/UFMG) at room temperature 24 ± 1 °C and under a 12 h light/dark cycle. Housing conditions included free access to water and food. All experimental procedures were carried out in compliance with the National Committee for Animal Care and Use (CONCEA), Guide for Care and Use of Laboratory Animals (2011) and were approved by the Institutional Ethics Committee on the Use of Animals at the Federal University of Minas Gerais (CEUA/UFMG; protocol number 421/2018).

### 2.2. Familiarization to treadmill running

Previously to the surgical procedures and to the progressive running tests, rats were familiarized to exercise on the motor-driven treadmill (AVS Projects, São Carlos, SP, Brazil) for 5 consecutive days by running at an initial speed of 10 m/min, with an increase of 1 m/min each two and half minutes (at 5% inclination). In each day of the familiarization period the animals underwent a 5 min-habituation session, with an increment of 1 m/min a day, hence in the last day (5th day) the final speed of 15 m/min was reached. The purpose of this 5-day preliminary exercise was to avoid a stress-related increase of the abdominal temperature on the experiment day, because of the manipulation and the exposure to an unknown environment (Wanner et al., 2007). Electrical stimulation was fixed in 0.2 mA and produced only a small discomfort without any injury.

### 2.3. Surgical procedures

Two days after the end of treadmill familiarization, the surgical procedures took place. Animals were anesthetized with an intraperitoneal injection (i.p.) of ketamine (80 mg/kg) and xylazine (10 mg/kg). A small ventral midline incision of the abdominal muscle was made to implant a temperature sensor (ER-4000; G2 E-Mitter; 15.5 mm × 6.5 mm, 1.1 mg; Mini Mitter Company Inc.) into the abdominal cavity. The sensor was attached to the musculature to prevent its movement inside the abdomen. Following implantation, the abdominal muscle and skin were sutured and animals were fixed in a stereotaxic apparatus (Bonther, Ribeirão Preto, SP, Brazil). The head was shaved, sterilized and skull was surgically exposed, following by a small hole made with a dental drill (Beltec Micromotores, LB2000, SP, Brazil). In order to reach the PVN, the stainless-steel guide cannulas (26 G) were bilaterally positioned at 1.6 mm posterior to the bregma, centered over the sagittal sinus and 6.8 mm ventral to the skull. These coordinates were obtained from the stereotaxic atlas (Paxinos and Watson, 2007). The microinjection needle was 0.4 mm longer than the guide cannula so that the final depth of the injection needle was 7.2 mm below the skull. A tight-fitting rigid steel wire was kept inside the guide cannula to prevent its occlusion. Guide cannulas were fixed to the skull with a metal screw and dental cement (Dental Vip, SP, Brazil). After the surgery, animals received analgesic (flunixin meglumine 2.5 mg/kg; subcutaneous [s.c.]) and antibiotic (Pentabiotico, Fontoura-Wyeth, SP, Brazil, 80.000 UI; intramuscular [i.m.]). The experiments were carried out seven days after surgery.

### 2.4. Drug and microinjection into the PVN

The selective nNOS inhibitor Nw-Propyl-L-Arginine (NPLA, TOCRIS, Westwoods Business Park Ellisville, Missouri, USA) was dissolved in sterile pyrogen-free saline (0.9% NaCl). The dose used was 0.4 nmol/100nL and has been selected based on pilot experiments and previous reports in the literature using intra-PVN NPLA (Busnardo et al., 2009, 2010, 2019; Raquel et al., 2018). As demonstrated in previous studies, this dose did not affect baseline values of tail skin temperature, arterial pressure, heart rate and corticosterone levels (Busnardo et al., 2019, Busnardo et al., 2010). A lower dose (0.04 nmol/100 nL) was also applied and, additionally, a group of animals composed a second control SHAM group, i.e., animals underwent the same surgical procedures but did not receive intra-PVN injections. Bilateral PVN microinjections of vehicle or NPLA were performed using a 1 μL syringe (Hamilton, Reno Nevada, USA) connected to a microinjection needle (33G) through a PE-10 polyethylene tubing. The volume of microinjection was 100 nL per PVN side. The microinjection needle was 0.4 mm longer than the guide cannula. Drug was prepared before the experiments and stored at − 20 °C, and at the experimental day was thawed just before the microinjections.

### 2.5. Experimental protocol

Animals recovered for seven days after surgery, prior to the acute exercise protocol. The protocol consisted in a single bout of acute progressive exercise on a treadmill. Initially, the animals were gently

handled, the injection needle positioned and bilateral drug (or vehicle) microinjections were performed into the PVN. Upon microinjection completion, the needle remained into the guide cannula for 30 s to avoid reflux. Next, the animal was placed on the treadmill to start the progressive exercise protocol. The initial speed was set at 10 m/min, with an increase of 1 m/min every 3 min until fatigue. Fatigue consisted in the moment that the rat was unable to maintain physical performance, remaining for more than 10 s in the electrical grid (Lacerda et al., 2005). The electrical stimulation was used only during the familiarization sessions and was not needed and disabled during the experimental protocols. Abdominal temperature ($T_{abd}$) was measured throughout the protocol every minute from the temperature sensor implanted in the abdominal cavity using a telemetry system (ER-4000; G2 E-Mitter; Mini Mitter Company Inc.). A thermocouple (Yellow Springs Instruments, 4600 Precision Thermometer) was fixed to the lateral surface of the tail skin, 1 cm from the base of the tail to measure the skin tail temperature ($T_{tail}$). The $T_{tail}$ was obtained each minute throughout the protocol. All experimental protocols were carried out during the light-phase cycle. Previous evidence from our group has demonstrated that although exercise capacity can vary in the light vs dark period, the neuronal activation in the PVN induced by progressive running exercise is similar and independent of the light-dark period (Machado et al., 2016a, 2016b).

### 2.6. Histological verification

After completion of the experiments, the rats were euthanized by an overdose of anesthesia (ketamine: 240 mg/kg, i.p. and xylazine: 31.5 mg/kg, i.p.). An injection of Evan's blue (the same volume of drug or vehicle) was made in the PVN. Upon confirmation of the animal's death, its brain was carefully removed for the histological verification of the microinjection sites, that were identified by observation of the injected dye along with the mechanical marks at the injection sites. After removal, the brains were fixed for 48 h in 4% paraformaldehyde at 4 °C, and then transferred to 30% sucrose (in 0.01 M PBS, pH 7.4) for 72 h at 4 °C. Next, they were frozen in isopentane at − 50 °C and sectioned in 40 μm slices. Coronal sections containing the PVN were collected, mounted onto glass slides, and stained with neutral red. Photomicrographs of the slides containing the cannula tracing and the microinjection sites were taken. Only the animals with a positive intra-PVN microinjection (vehicle and drug) were considered in the experimental groups.

### 2.7. Measurements and calculations

$T_{abd}$ was measured using a telemetry system (ER-4000; G2 E-Mitter; Mini Mitter Company Inc.). The sensor signals were transmitted (radio frequencies waves) to a receiver plate (model ER-4000 energizer/receiver, Respironics INC. Company. Mini) positioned next and lateral to the treadmill. The recording was obtained in a specific software (Vital View; Mini-Mitter, OR, USA). During the exercise, the dry ambient temperature inside the treadmill was maintained at 25 °C with the aid of an air conditioning system (Komeco, Palhoça, Brazil). The temperature of 25 °C corresponds to the low end of the thermoneutral zone of rats resting on a treadmill, as evidenced by a heat loss index (Romanovsky et al., 2002) ranging from 0.20 to 0.25 under these experimental conditions (Malheiros-Lima et al., 2018).

The workload (J), an exercise performance index, was calculated as [body weight (kg)] *x* [gravity acceleration ($m{\cdot}s^{-2}$)] *x* [treadmill speed (m.min-1)] *x* [senθ (treadmill inclination)] *x* [time of exercise (min)] (Drummond et al., 2019). The thermal threshold for heat dissipation (*Hthr*; °C) and sensitivity for heat dissipation (*Hsen;* arbitrary units) were calculated using a method based on that described by Cheuvront et al. (2009) (Cheuvront et al., 2009) and used in human (Machado-Moreira et al., 2015) and rat (Drummond et al., 2019; Rabelo et al., 2019) studies. Briefly, the $T_{tail}$ was plotted against $T_{abd}$, and the threshold for activation of tail heat loss was identified visually in the plotted curve (i.e., $T_{abd}$ at the initiation of the rapid increase in $T_{tail}$). Next, the data before and after this threshold were separated. To describe the relationship between $T_{tail}$ and $T_{abd}$, linear regression analyses were performed for data before and after the threshold. The intersection of the regression lines was used to determine the *Hthr*. The *Hsen* was defined as the regression slope of the five points that followed the threshold and corresponded to the steepest part of the rising curve (Drummond et al., 2020). The thermoregulatory efficiency was calculated by the ratio between the heat accumulation and the work performed (lower values indicate greater efficiency) (Gomes et al., 2019). The heat storage (cal) was calculated as [abdominal temperature variation (°C)] *x* [body weight (g)] *x* [specific heat of the animal's tissues (0.826 cal/g.°C)].

### 2.8. Statistical analysis

The results are expressed as mean ± SEM. The Shapiro-Wilk test was used to verify the normality of the data. Two-way ANOVA for repeated measures was used for the comparison of $T_{abd}$ and $T_{tail}$ curves between groups (vehicle vs drug) and exercise time points (every minute, in the time interval that all animals were running). Tukey's post hoc multiple comparison test was used. Body mass, maximum and minimum temperatures, *Hthr* and *Hsen* were evaluated using Student *t*-test. Values of $p < 0.05$ were considered significant.

## 3. Results

Fig. 1 A shows a photomicrography of a coronal section of one representative animal that received bilateral microinjections of NPLA into the PVN. A schematic figure also shows the intra-PVN microinjection sites in the rostro-caudal extent (Fig. 1B, C, D), depicting the location of injection needles for each animal included in this study (NPLA, n = 10; vehicle, n = 12). Microinjections occurred over the length of the PVN region, mainly its medial region, at − 1.8 mm posterior to the bregma.

### 3.1. Selective inhibition of nNOS in the PVN attenuates the increase in $T_{abd}$, but does not change $T_{tail}$ in progressive exercise

The Two-way ANOVA for repeated measures revealed the existence of an interaction among group (NPLA or Vehicle) and time points for $T_{abd}$ ($F = 4.89$; $p < 0.001$) but not for $T_{tail}$ ($F = 1.20$; $p = 0.17$). More specific comparisons are presented in the following paragraphs. During acute progressive exercise, the $T_{abd}$ of all animals showed the typical dynamic increase, i.e., exercise-induced hyperthermia. However, animals treated with the nNOS inhibitor NPLA (0.4 nmol) in the PVN showed an attenuation of exercise-induced increase in $T_{abd}$ at the final stage (max. $T_{abd}$), compared to the vehicle group (38.28 ± 0.22 °C vs. 39.35 ± 0.16 °C, $p = 0.005$; Fig. 2A and C). No difference in $T_{abd}$ between NPLA and vehicle-injected rats was observed in the beginning of the exercise protocol (Fig. 2B). Moreover, experiments were also carried out in rats injected with lower dose (0.04 nmol) of NPLA and without drug (sham group). The thermal responses of sham group were not different of the vehicle group. Similar to sham group, microinjection of a lower dose of NPLA in the PVN did not produce significant effect when compared to control vehicle group [$T_{abd}$ max: 38.24 ± 0.17, 38.75 ± 0.3 and 39.35 ± 0.16 °C; sham, lower dose and vehicle groups, respectively].

Regarding $T_{tail}$, no difference was observed between groups throughout the exercise protocol (Fig. 3A). The initial $T_{tail}$ was 27.86 ± 0.77 °C in NPLA group (0.4 nmol) and 27.94 ± 0.23 °C in vehicle group (Fig. 3B). The minimal (26.33 ± 0.83 and 26.89 ± 0.25 °C; NPLA vs vehicle; Fig. 3C) and maximum $T_{tail}$ (32.60 ± 0.55 and 33.33 ± 0.34 °C; NPLA vs vehicle; Fig. 3D) values were also similar between the experimental groups. Likewise, the $T_{tail}$ values obtained with lower dose (0.04 nmol) of NPLA and without drug (sham group) were not different from vehicle group.

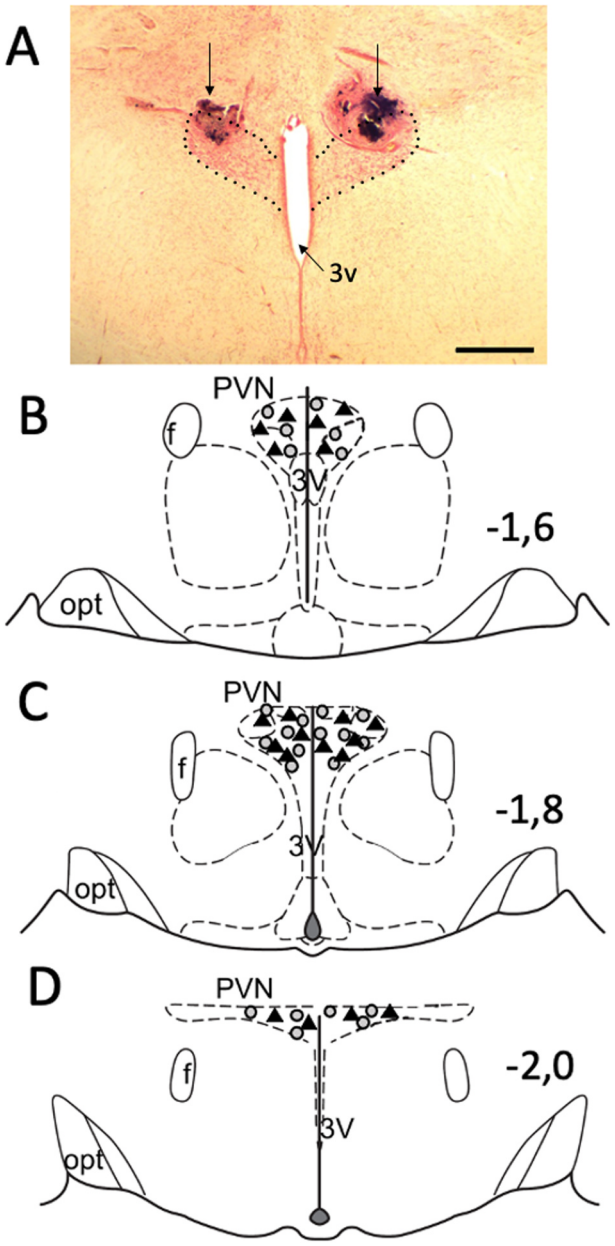


**Fig. 1.** NPLA microinjections in the PVN. Panel A shows a photomicrograph of a coronal section displaying a bilateral microinjection in the PVN of a NPLA-injected rat. The PVN region is depicted by the trace line, the downside arrows indicate the sites of injections and the arrow pointing to midline shows the 3 V. Panels B, C and D show schematic representations of the microinjection sites. On the right side of each plate, the rostro-caudal location in relation to the bregma is shown. The filled triangles represent the locations of the NPLA microinjections (0.4 nmol/100nL; n = 10). The circles represent the locations of the vehicle microinjections (0.15 M NaCl; n = 12). Coordinates to reach the PVN were from Paxinos and Watoson (2007). 3 V: third ventricle; f: fornix; opt: optical tract. Scale bar = 500 micrometers.

### 3.2. Microinjection of NPLA in the PVN does not change the $H_{thr}$ but increases the $H_{sen}$ in progressive exercise

From the relationship between $T_{abd}$ and $T_{tail}$ (Fig. 4A), two variables were calculated: i) *Hthr*, which expresses the $T_{abd}$ at which heat dissipation from the tail skin starts (i.e., at this $T_{abd}$, a steep increase in the $T_{tail}$ occurs), and ii) *Hsen*, which is given by $T_{abd}$-$T_{tail}$ curve slope. There was no difference in *Hthr* between NPLA and vehicle groups (37.63 ± 0.17 °C vs. 37.74 ± 0.16 °C; Fig. 4B). Nevertheless, PVN treatment with NPLA increased the *Hsen* (leftward shift), when compared to the vehicle group (13.74 ± 1.45 vs. 8.84 ± 0.91, $p < 0.05$; Fig. 4C). For both, *Hthr* and *Hsen*, no differences among groups were observed with lower dose (0.04 nmol) of NPLA, without drug (sham group) and vehicle group, respectively [*Hthr:* 37.11 ± 0.21; 37.47 ± 0.11 and 37.74 ± 0.16 °C; *Hsen*: 9.64 ± 1.64; 12.26 ± 1.72 and 8.84 ± 0.91].

### 3.3. Inhibition of nNOS in the PVN attenuates heat storage and improves thermoregulatory efficiency in progressive exercise

The heat storage was lower in animals treated with NPLA in the PVN, when compared to the vehicle group (335.14 ± 39.44 cal vs. 569.61 ± 51.63 cal, $p = 0.002$; Fig. 5A). Besides, thermoregulatory efficiency was improved in the NPLA group (1.66 ± 0.22 cal/J vs. 2.53 ± 0.15 cal/J, $p = 0.004$; Fig. 5B).

### 3.4. Inhibition of nNOS in the PVN does not affect running performance on the treadmill

The exercise performance in the progressive protocol was assessed by the quantification of exercise duration (running time; min), maximum speed (m/min) and workload (J). The microinjection of NPLA in the PVN did not change the performance on the treadmill, and none of these variables were different between groups. The running time was 36.50 ± 2.18 min in the NPLA group and 37.91 ± 1.85 min in animals treated with vehicle. In a similar manner, the maximum speed reached was 21.80 ± 0.70 m/min in the NPLA group and 22.33 ± 0.60 m/min in the vehicle group. The workload, which depends on the exercise time and running speed, was also not different between the NPLA and vehicle groups, with values of 212.57 ± 18.92 J vs. 231.20 ± 23.27 J, respectively.

## 4. Discussion

The results of the present study suggest a participation of NO produced by nNOS in the PVN in thermoregulation during acute progressive exercise. The inhibition of the nNOS enzyme in the PVN resulted in an attenuation of exercise-induced hyperthermia, without changing the tail skin temperature. In addition to the evidence showing the involvement of PVN in thermal adjustment to exposure to warm environment (Inenaga et al., 1987; Leite et al., 2012), the role of PVN in thermoregulation during exercise has also been demonstrated in the literature. Previous studies by our group showed that PVN neurons are activated during exercise (Drummond et al., 2020; Lima et al., 2019), corroborating other studies that evaluated activation of neurons in this hypothalamic nucleus evoked by acute running exercise (Barna et al., 2012; Nuñez et al., 2012; Saito and Soya, 2004; Soya et al., 2007; Yanagita et al., 2007). Our group also began previously to elucidate the hypothalamic sites in which nitrergic mechanisms act to regulate body temperature during exercise. Lima et al. (2014) microinjected the nonspecific NOS inhibitor L-NAME into the right lateral ventricle of rats and observed an attenuated exercise-induced expression of c-fos in the PVN, reduced the ability to dissipate heat through the tail skin and decreased running performance (Lima et al., 2014). Similarly, L-NAME injected into the right lateral ventricle of rats caused a marked increase in body temperature during exercise and the animals stopped the exercise earlier than control animals (Lacerda et al., 2005). However, these studies used non-selective NOS inhibitors globally administered in the brain i.e., intracerebroventricular (i.c.v.) injections. These experimental differences may explain the discrepancy from data obtained in the present study in relation to those mentioned above. Indeed, we observed that selective nNOS blockade in the PVN attenuated the increase in abdominal temperature during exercise, an opposite finding in relation to that evoked by i.c.v. injection of non-selective NOS inhibitors (Lacerda et al., 2005). Of note, i.c.v. microinjections affect a number of hypothalamic regions

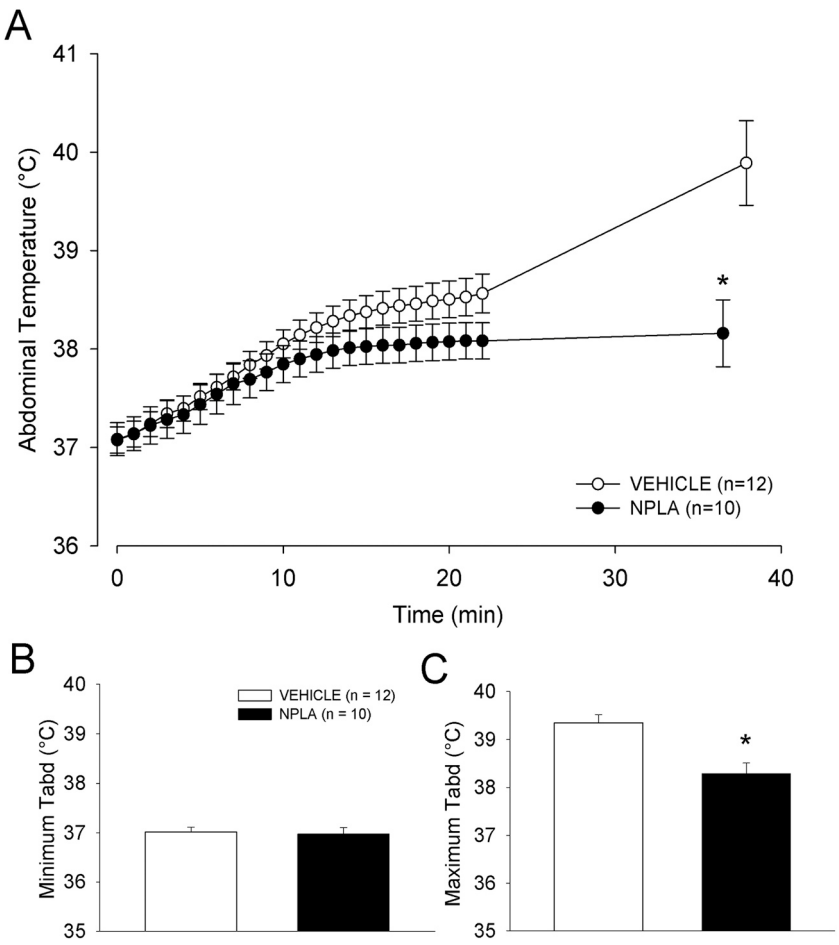


**Fig. 2.** Abdominal temperature during the acute exercise protocol (A). The time in the x-axis represents the duration of exercise. Minimum abdominal temperature (B) and maximum abdominal temperature (C) of the animals in the vehicle and NPLA groups submitted to the progressive exercise test. Data expressed as mean ± SEM. *$p < 0.05$ vs. vehicle. $T_{abd}$: abdominal temperature.

other than the PVN. Thus, the present study was the first to perform a selective, PVN-specific inhibition of nNOS to evaluate the endogenous NO role in the thermoregulation during an acute exercise session. Our findings reveal that nNOS inhibition in the PVN decreased the hyperthermia elicited by the acute progressive running exercise. The thermoregulatory responses of rats during running exercise on treadmill can be divided into two distinct phases, an initial dynamic rapid phase, followed by a slow increase and steady-state phase. At the beginning of an exercise session, the heat production abruptly increases, which results from an augmented metabolic rate in the working muscles. Additionally, the $T_{tail}$ decreases during the first minutes of exercise, suggesting the occurrence of tail skin vasoconstriction. Thus, the higher metabolic heat production is not immediately counteracted by a higher rate of heat loss. Consequently, the core temperature rapidly increases in response to the initiation of exercise (Lacerda et al., 2005, 2006; Wanner et al., 2015a, 2015b; Wilson et al., 1978). After the initial moments of exercise, the $T_{tail}$ begins to markedly increase until it reaches a plateau that is sustained until physical exertion is interrupted. This increase in Ttail indicates that cutaneous vasodilation mechanisms are activated and leads to the second phase of thermal balance during exercise. In this phase, the rates of heat production and heat loss tend to stabilize at similar levels. Thereafter, the core temperature will be maintained at a high level or will slightly increase until exercise cessation (Lacerda et al., 2005, 2006; Wanner et al., 2015a, 2015b; Wilson et al., 1978). The present study shows that intra-PVN nNOS inhibition increased the sensitivity for heat dissipation (*Hsen)*. Taken together, the results of the present study suggest that NO mechanisms in the PVN play a predominant role in the slow (or steady-state) phase, with minimal, if any, effects in the initial dynamic phase, and this has been similarly reported by previous studies (Lacerda et al., 2005, 2006). No difference was observed in the *Hthr*, which is the abdominal temperature at which heat dissipation mechanisms are activated. However, as mentioned, the speed at which heat loss occurs, which is translated by the variation in $T_{tail}$ in function of $T_{abd}$, defined as *Hsen*, was higher in the NPLA group. This group displayed a leftward shift of the sensitivity curve for heat dissipation, showing that with a smaller variation in $T_{abd}$, the $T_{tail}$ increased faster. Besides, NPLA rats had a lower rate of heat accumulation. The sensitivity for increased heat dissipation can interfere with the rapid heat gain during the mechanical work of the progressive exercise, which may have interfered with the thermal response of these animals (Wanner et al., 2015b). Thus, the attenuation of the $T_{abd}$ rise during exercise by intra-PVN NPLA can be at least partially explained by the increase of the sensitivity to heat dissipation.In this context, the role of brown adipose tissue (BAT) should also be considered, although its activity during acute exercise remains elusive. As well known, like exercise, BAT is a thermogenic tissue involved in heat production. Since both, an increase in BAT activity and exercise performance can raise core body temperature, it is not intuitive, at first glance, to think that the thermogenic role of BAT would increase during acute exercise. However, considering that BAT is mainly regulated by the sympathetic nervous system and that sympathetic outflow is increased during exercise, it is possible that acute exercise could stimulate BAT via increased sympathetic activation. During acute exercise performance, as far as we know, scarce evidence shows direct BAT's activity that could contribute

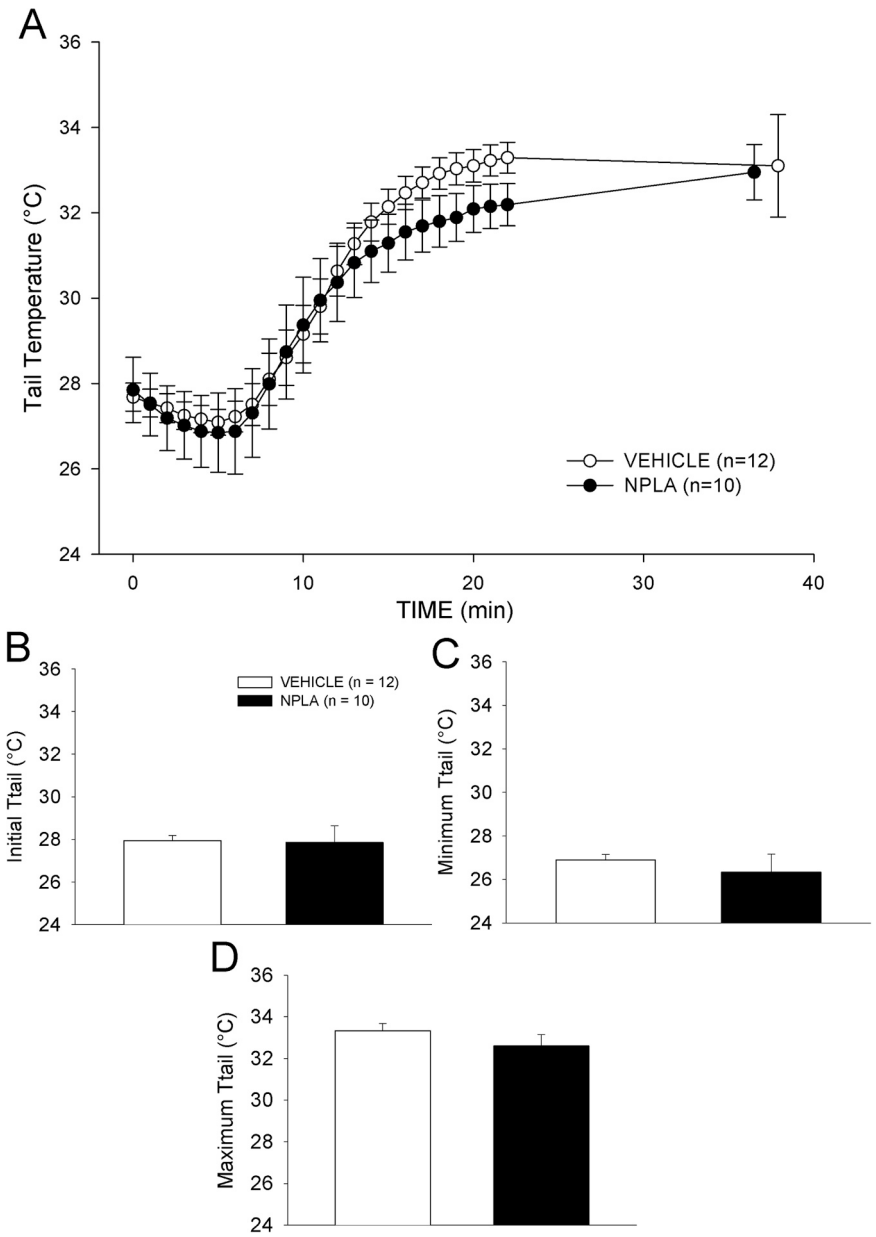


**Fig. 3.** Tail skin temperature during the acute exercise protocol (A). The time in the x-axis represents the duration of exercise. The initial (B), minimum (C) and maximum (D) tail skin temperature. Data expressed as mean ± SEM. $T_{tail}$: tail skin temperature.

to the exercise-induced hyperthermia. In this context, Shibata and Nagasaka (1987) recorded BAT and core temperature in running rats and demonstrated that untrained animals had a higher increase in BAT temperature compared to core temperature during running exercise (13 m/min for 10 min). In contrast, trained/habituated animals did not show BAT temperature increase (running at 20 m/min for 30 min). This evidence suggests that BAT could have a small contribution to exercise hyperthermia in untrained rats. However, given the differences between the experimental conditions, this evidence remains difficult to reconcile with our present data. Furthermore, mechanical efficiency could also affect heat production during exercise, and hence the $T_{abd}$. During exercise, ~20–30% of the energy expended can be used for external work, whereas the remaining is dissipated as heat (Brooks et al., 1984). Our group has already demonstrated that the inhibition of the NO pathway in the CNS (by i.c.v. injection of L-NAME) affected metabolic rate adjustment induced by exercise. The L-NAME-treated rats showed a ~15% higher metabolic cost than controls during steady-state exercise. This was closely associated with the decrease in the mechanical efficiency observed in L-NAME-treated rats (Lacerda et al., 2006). Another possible path for heat dissipation is the panting behavior, used by countless species of birds and mammals (Mortola and Frappell, 2000; Mortola and Maskrey, 2011). Panting change the breathing pattern by increasing respiratory rate, and it is a form of evaporative heat loss by the respiratory system (Meyer et al., 1989; Mortola and Maskrey, 2011). Therefore, in hyperthermia, ventilation is adjusted to respond to homeostatic demand signaled in the medullary and pontine nuclei, and influenced by integrating hypothalamic structures of the thermoregulatory system such as POA and PVN (Bradley et al., 1974; Mortola and Maskrey, 2011; Von Euler et al., 1970). Evidence in humans showed that the breathing pattern during exercise in warm changed to a tachypneic pattern (more rapid and less deep) when compared to the same exercise performed in normothermia (Geerling et al., 2010; Martin et al., 1979; Petersen and Vejby-Christensen, 1973). Rats show an increase in respiratory drive in hyperthermia (Boden et al., 2000a), that is mediated by the POA (Boden et al., 2000b). Considering the connection between the POA and PVN, the latter can be related to respiratory evaporative heat

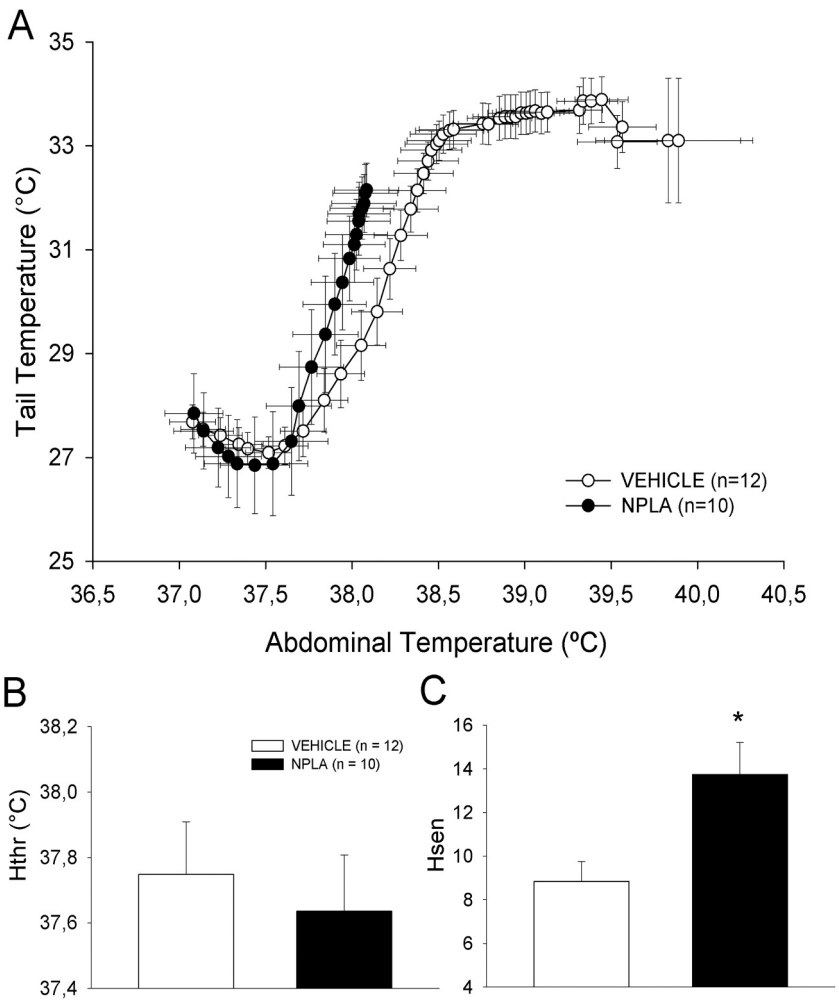


**Fig. 4.** Relationship between abdominal temperature and tail skin temperature (A), thermal threshold for heat dissipation (B) and sensitivity for heat dissipation (C) during progressive exercise. Data expressed as mean ± SEM. *p < 0.05 vs. vehicle. *Hthr*: thermal threshold for heat dissipation. *Hsen*: sensitivity for heat dissipation.

loss, a possible route for heat dissipation. As a matter of fact, the neural substrate for this mechanism is existent since the PVN contains connection with brainstem nuclei involved in the neural control of breathing (Geerling et al., 2010; Kc et al., 2002; Kc and Dick, 2010; Yeh et al., 1997). However, the present study did not measure any respiratory variable to explore this mechanism, and hence this is hypothetical and elusive. Nonetheless, it remains as an interesting topic to be further investigated.Previous report in the literature have shown that the inhibition of nNOS in the PVN with NPLA caused a deleterious effect in the autonomic control, with an increase in the arterial pressure and heart rate in both sedentary and trained animals (Raquel et al., 2018). These results suggest a role of the PVN nitrergic pathway in autonomic modulation reducing sympathetic autonomic outflow (Li et al., 2004; Zhang and Patel, 1998). In contrast, Busnardo and collaborators (2019) used an acute restraint stress model with selective inhibition (NPLA) of nNOS intra-PVN and observed an attenuated increase in the cardiovascular variables (blood pressure and heart rate), suggesting a sympatoexcitatory action of NO released from nNOS in the PVN during aversive threats (Busnardo et al., 2019). In the present study, cardiovascular and/or autonomic variables were not directly measured and it is possible that altered autonomic responses underly part of the observed effects of nNOS inhibition in the PVN, causing a decreased exercise-induced hyperthermia.Another factor to consider is that PVN has a heterogeneous neuronal population. Pre-autonomic parvocellular neurons, which control the sympathetic nervous system (Ranson et al., 1998), are concentrated in the medial portion of the nucleus, while neuroendocrine parvocellular and magnocellular neurons are responsible for the production of oxytocin, vasopressin and corticotrophin (Kuypers and Maisky, 1975; Martins et al., 2005; Swanson and Kuypers, 1980). It is known that acute exercise increases the expression of c-fos in corticotrophinergic neurons in the PVN (Otsuka et al., 2016). Although the NPLA microinjection site varied in the rostro-caudal PVN extent, the volume of the drug injected bilaterally diffuse over the extension of the nucleus, reaching both pre-autonomic and neuroendocrine regions. Thus, the effects of nNOS inhibition in the PVN certainly comprise autonomic and neuroendocrine neuronal population.

In summary, the selective inhibition of nNOS (and its consequent effect in the endogenous NO release) with a hypothalamic site-specific NPLA microinjection i.e., in the PVN, attenuated the hyperthermia during acute exercise, providing evidence that the nNOS-NO pathway in the PVN is important to achieve proper thermal responses during treadmill acute progressive exercise in rats, acting as a facilitatory modulator of exercise-induced hyperthermia.

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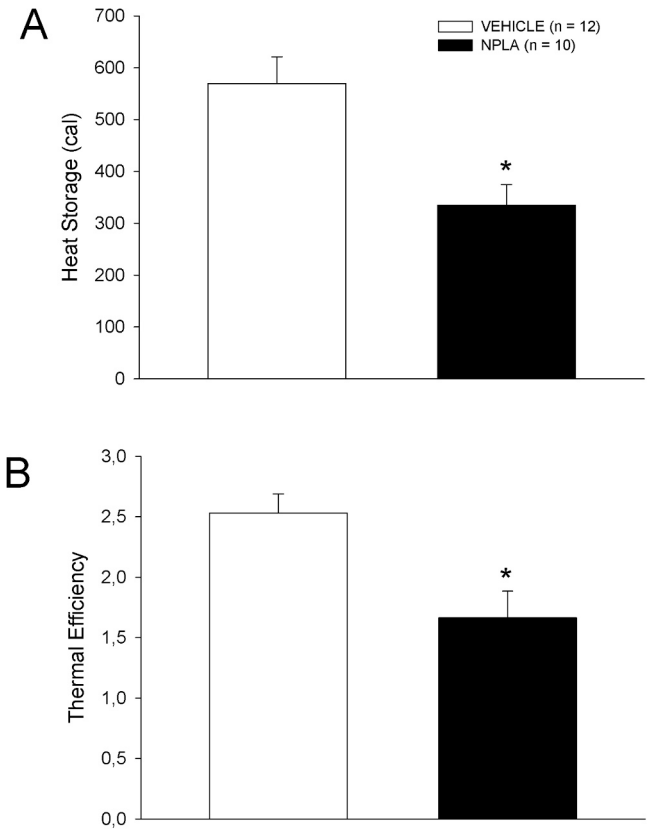


**Fig. 5.** Heat storage (A) and thermoregulatory efficiency (B) during progressive exercise in animals in the vehicle and NPLA group. Data expressed as mean ± SEM. In (A), *p < 0.05 vs. vehicle. In (B), *p < 0.05 vs. vehicle.

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## Author contributions

**BL Nunan** contributed to acquisition, analysis, interpretation of data and manuscript drafting and revision. **LR Drummond and QT Rodrigues** contributed to data acquisition, analysis and manuscript revision. **CC Crestani** contributed to data analysis and manuscript revision. **CC Coimbra and GSF da Silva** contributed to acquisition and interpretation of data, conception, study design, drafting and manuscript revision. All authors revised and approved the final version of the manuscript.

## Data statement

All data supporting the findings of this study are available in the result section and in the Figs. 1 to 5. Additional research data can be shared upon request.

## Competing Interests

The authors declare no conflicts of interest/competing interests.

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