Brain Structure and Function (2019) 224:2775–2786
https://doi.org/10.1007/s00429-019-01933-6

ORIGINAL ARTICLE

The time-course of thermoregulatory responses during treadmill running is associated with running duration-dependent hypothalamic neuronal activation in rats

Paulo M. A. Lima[1] · Helton O. Campos[1] · Daniela R. C. Fóscolo[1] · Raphael E. Szawka[1] · Samuel P. Wanner[2] · Cândido C. Coimbra[1]

Received: 14 November 2018 / Accepted: 31 July 2019 / Published online: 9 August 2019

Abstract

This study evaluated the hypothalamic neuronal activation during exercise and investigated whether this activation is related to heat storage and exercise duration. Rats were subjected to a treadmill running that was interrupted at three different moments: (1) at the early phase, when minimal heat dissipation occurred due to tail vasoconstriction and the tail skin temperature (T_{skin}) reached its nadir; (2) at the steady-state phase, when both the T_{skin} and core body temperature (T_{core}) plateaued at a high level (~20 min); and (3) at fatigue, when T_{core} and T_{skin} were still elevated. c-Fos expression in the medial and ventromedial preoptic areas (mPOA and vmPOA), median preoptic nucleus (MnPO), paraventricular and supraoptic nucleus (PVN and SON), and septohypothalamic nucleus (SHy) was determined. Exercise increased the expression of c-Fos in all brain areas, but with different activation patterns of activation. c-Fos expression in the SHy and vmPOA was similar in all exercising groups, while in the mPOA, MnPO, and PVN, c-Fos expression gradually increased during exercise. Increased c-Fos in the SON was only evident after 20 min of exercise. Neuronal activation in the mPOA, MnPO, PVN, and SON was positively correlated with both exercise duration and heat storage. Our findings indicate that with the exception of SON, the brain areas analyzed are recruited following small changes in T_{core} (~0.5 °C), while the SON is recruited only when T_{core} reaches higher values (greater than 1.0 °C increase). c-Fos expression in the PVN, mPOA, MnPO, and SON is also influenced by exercise duration, which does not occur in the SHy and vmPOA.

Keywords c-Fos · Fatigue · Heat · Hypothalamus · Temperature · Physical exercise

Introduction

Muscle contractions associated with physical exercise provide mechanical and metabolic stimuli sufficiently strong enough to modify the energetic state of an organism, which responds to these stimuli by promoting multiple but very specific alterations that allow the maintenance of body homeostasis (Coyle 2000). Regarding the thermoregulatory responses, the greater metabolic stimulus provided by exercise augments heat production, leading to increases in the core body temperature (T_{core}) that are sensed by the central and visceral thermoreceptors; this sensory information is processed by the hypothalamus and preoptic area (POA) to trigger appropriate effector responses, such as sweating (or salivation in rats) and increased skin blood flow to dissipate the body heat (Gleeson 1998).

The physiological responses induced by exercise are largely dependent on the type, intensity, and duration of the physical exertion. For example, the increase in T_{core} induced by a moderate-intensity treadmill running in temperate environments results from a temporary imbalance between the rate of metabolic heat production and the rate of cutaneous heat loss during the early stage of the physical exercise (Webb 1995). In response to exercise initiation, blood flow is redirected from splanchnic, renal, and cutaneous beds to

✉ Cândido C. Coimbra
coimbrac@icb.ufmg.br

[1] Department of Physiology and Biophysics, Institute of Biological Sciences, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627, Belo Horizonte, Minas Gerais 31270-901, Brazil

[2] Exercise Physiology Laboratory, School of Physical Education, Physiotherapy and Occupational Therapy, Universidade Federal de Minas Gerais, Belo Horizonte, MG 31270-901, Brazil

the active skeletal musculature (Rowell 1974). In particular, the sympathetic outflow to the cutaneous vessels in the non-hairy skin decreases heat loss and consequently, T_{core} increases rapidly (Wanner et al. 2015). T_{core} continues to increase until the threshold for cutaneous vasodilation is reached, when heat loss starts to increase exponentially; thereafter, T_{core} plateaus at a high level that is sustained until exercise cessation (Gleeson 1998; Lacerda et al. 2005; Wanner et al. 2015; Webb 1995).

Although the thermoregulatory responses to an exercise performed under temperate conditions are well described, the neural pathways modulating the dynamic recruitment of thermoeffector activity are far from being elucidated. In this context, studies with continuous recordings of the T_{core}, tail skin temperature (T_{skin}), and oxygen consumption in the rats subjected to treadmill running have yielded information regarding the role of different neurotransmitters in the control of these exercise-induced thermoregulatory responses (Balthazar et al. 2010; Hasegawa et al. 2008; Lacerda et al. 2005, 2006; Leite et al. 2006, 2007; Soares et al. 2003, 2004).

In addition to the identification of neurotransmitters, the description of the activated brain areas is another important step to elucidate the neural circuitries involved in running-induced thermoregulatory responses. An important tool to achieve this goal is the measurement of the expression of c-Fos protein, which is a nuclear phosphoprotein product of the immediate early gene *c-fos* that has been extensively used as a marker of neuronal activation (Hoffman et al. 1993; Kovacs 1998). Using this tool, a number of studies have evaluated the recruitment of hypothalamic and brainstem areas involved in neuroendocrine control, fluid balance, and cardiorespiratory functions (Barna et al. 2012; Iwamoto et al. 1996; Nunez et al. 2012; Saito and Soya 2004; Soya et al. 2007; Tomita and Takayama 2008; Yanagita et al. 2007). However, fewer studies have investigated the association between neuronal activation and exercise thermoregulation. More recently, we showed that the central nitric oxide transmission is important for activating the paraventricular nucleus of the hypothalamus (PVN), which in turn, modulates thermoregulatory responses, enabling the maintenance of physical performance (Lima et al. 2014). A key limitation of the above-mentioned studies is that c-Fos expression was compared between the beginning and end of an exercise and therefore, provided an overall measure of neuronal activation in selected brain areas throughout the exercise period. Thus, no study has investigated the temporal pattern of this neuronal activation, which may represent a more mechanistic approach to understand the central regulation of exercise thermoregulation.

Here, we investigated the temporal pattern of neuronal activation in hypothalamic areas associated with thermoregulation and stress responses in rats subjected to treadmill running. In addition, this study verified whether the time-course of thermoregulatory responses during treadmill running is associated with the running duration-dependent hypothalamic neuronal activation. Considering that c-Fos technique has low temporal resolution (Kovacs 2008), the expression was assessed at three different moments of the treadmill running to achieve our purposes: (1) in the early phase, when the T_{core} increases rapidly due to the imbalance between the rates of heat production and heat loss; (2) at the beginning of steady-state phase, when T_{core} and T_{skin} reach a plateau; and (3) when the animals no longer tolerate the physical exertion (at fatigue).

Materials and methods

Ethics statement

All experimental procedures were approved by the Ethics Commission on the Use of Animals of the Universidade Federal de Minas Gerais (protocol number 241/2016) and were conducted in accordance with the regulations of the National Council for the Animal Experimentation (CONCEA/Brazil).

Animals and experimental design

Male Wistar rats ($n = 29$) weighing 250–350 g were housed in individual cages at room temperature 23 ± 1 °C under 12-h light/12-h dark cycles and had free access to water and rat chow. The rats were familiarized with exercise on a motor-driven treadmill (Model LE8706, Letica Scientific Instruments) by subjecting them to 5 min, daily running sessions at a constant speed of 10 m min^{-1} on 4 consecutive days. The treadmill inclination was set at 5% during all familiarization sessions and experimental trials. The purpose of these preliminary exercise sessions was to show the animals in which direction they should run and to prevent the manipulation and exposure to a novel environment from influencing both the T_{core} and neuronal activation. Levels of electrical stimulation were determined according to the tolerance of each rat and this stimulation produced a discomfort without injuring the animal. On the fifth day, the rats were subjected to an incremental-speed exercise to determine their maximal aerobic running speed (S_{max}). The rats started running at 10 m min^{-1} and the treadmill speed was increased by 1 m min^{-1} every 3 min until they were fatigued. Fatigue was defined as the point at which the rats were no longer able to keep pace with the treadmill for 10 s.

The incremental exercise was followed by anesthesia with a cocktail containing ketamine (80 mg kg^{-1}; *i.p.*) and xylazine (10 mg kg^{-1}; *i.p.*), a temperature sensor (ER-4000; G2 E-Mitter; 15.5 mm × 6.5 mm, 1.1 mg; Mini Mitter Company Inc.) was implanted in the abdominal cavity through

a small incision in the linea alba of the rectus abdominis. This sensor allowed the measurement of T_{core} by telemetry and was sutured to the rectus abdominis to prevent its movement inside the abdomen. Following implantation, the abdominal muscle and skin were sutured and then the rats received a single dose of an analgesic (flunixin meglumine 1.0 mg kg^{-1}; *s.c.*) and different antibiotics (including 48,000 IU kg^{-1}; *i.m.* benzyl penicillin, Fort Dodge Animal Health).

All animals were allowed to recover for at least 4 days before being subjected to one of the exercise or resting trials. All experiments were performed at a room temperature of 23 ± 1 °C, between 8:00 and 12:00.

Experimental trials

On the day of the experiments, the rats were weighed to ensure that they had recovered from surgery and exceeded the preoperative weight. The rats were left undisturbed for 60 min in their home cages until the T_{core} achieved resting and stable values of approximately 37.0 ± 0.5 °C for at least 20 min. After stabilization of the T_{core} values, the rats were transferred from their home cage to the motor-driven treadmill, and a thermocouple (series 409-B, Yellow Springs Instruments) was taped to the lateral surface of the tail, at 1 cm from its base, to measure the T_{skin}. The abdominal temperature was measured every 5 s and used as the T_{core} value and the T_{skin} was measured every 30 s and considered as an index of cutaneous heat loss.

The animals were subjected to a constant-speed running exercise at 70% of the S_{max} attained during the incremental test. This exercise intensity was chosen to ensure that (1) all rats could run more than 20 min; (2) fatigue would not coincide temporally with the beginning of the steady-state phase; and (3) a marked increase in T_{core} high enough to activate cutaneous heat dissipation to be observed.

The exercise duration was determined in accordance with the group to which each rat was assigned: (1) NADIR: the exercise was interrupted immediately after the T_{skin} reached its lowest value; (2) 20 MIN: the exercise was interrupted at the twentieth min of running, when the T_{core} and T_{skin} plateaued at a high level; (3) FATIGUE: the exercise was interrupted when the rats fatigued. As a control group (REST), the animals were kept in their home cages for additional 60 min after observation of stable T_{core} values.

Ninety minutes after the interruption of running (in the exercising groups) or the rest period, the rats were anesthetized with ketamine and xylazine and were transcardially perfused with 40 mL of heparinized 0.01 M phosphate-buffered saline (PBS), followed by 400 mL of 4% paraformaldehyde (PFA) diluted in 0.2 M phosphate buffer (pH 7.4). The brains were removed for subsequent immunohistochemical analysis. As reviewed by Kovács, the maximal level of c-Fos protein occurs between 1 and 3 h and then it gradually disappears from the cell nucleus by 4–6 h after an exposure to a stressful condition (Kovacs 2008); thus, the moment at which we collected the brains was within the timeframe corresponding to the maximal c-Fos expression induced by exercise.

Immunohistochemistry

After perfusion, the brains were removed and postfixed in 4% PFA for 2 h at 4 °C. Thereafter, the brains were stored in cold 30% sucrose for 72 h (until the brains sank into the solution), frozen in isopentane 99% (C_5H_{12}) at − 50 °C, and then stored at − 80 °C until sectioning in a cryostat. Forty-µm coronal sections were cut in the cryostat and stored in a cryoprotectant solution at − 20 °C until the immunohistochemistry assay.

The brain sections were subjected to dual immunoperoxidase staining, nuclear c-Fos (to determine neuronal activation) and cytoplasmic arginine vasopressin (AVP; to identify neurons in the PVN and supraoptic nucleus, SON). Tissues were removed from the cryoprotectant solution and washed with 0.01 M PBS. Unless otherwise mentioned, all solutions were prepared in PBS and sections were washed with PBS between all the steps. The sections were sequentially incubated with 0.1 M glycine, 1% hydrogen peroxide, 0.4% Triton X-100 and 2% bovine serum albumin (BSA). Next, the sections were incubated with a primary antibody directed against c-Fos (Ab5 rabbit polyclonal antibody; Calbiochem, EMD Chemicals) at 1:10,000 in PBS containing 0.3% Triton X-100 and 1% BSA (all antibodies were diluted in the same solution) for 40 h at 4 °C, with a biotinylated universal horse IgG (Vectastain Elite ABC kit, Vector Laboratories) at 1:600 for 2 h at room temperature, and with avidin-biotinylated horseradish peroxidase complex (Vectastain Elite ABC kit, Vector Laboratories) at 1:400 for 1 h. The black reaction product was made visible for peroxidase activity in a solution of nickel sulfate (25 mg/mL), 3,3-diaminobenzidine-HCl (0.2 mg mL^{-1}), and 0.03% H_2O_2 in 0.175 M acetate buffer. The reaction was stopped by washing the sections with acetate buffer followed by washing with PBS. To visualize cytoplasmic AVP, the sections were then incubated with a second primary antibody directed against AVP (PS4-ATCCRL 1799 NIH, Bethesda, USA) at 1:5,000 for 40 h at 4 °C. The same staining procedure as described for c-Fos was performed, except the procedure for staining visualization. AVP staining was visualized from the brown reaction product in the cytoplasm of neurons, after incubating the sections in a solution of 3,3-diaminobenzidine-HCl (0.2 mg/mL) and 0.03% H_2O_2 in 0.05 M Tris–HCl buffer. AVP staining was used to facilitate the visualization of the areas corresponding to the PVN and SON in the brain sections; however, we could not delimitate each AVP-expressing neuron

with precision (a marked brown staining covered the PVN and SON areas) and therefore, we could not count the number of AVP-expressing neurons activated by exercise. The sections were mounted onto gelatin-coated slides, air-dried, rinsed in a graded ethanol series, cleared in xylene and coverslipped with Entellan® (Merck).

Cell counts and quantification

The number of c-Fos-immunoreactive (ir) cells was counted by an experimenter without previous knowledge of the experimental group. To determine that a cell is indeed a c-Fos-ir cell, the nucleus was required to be stained black. The number of c-Fos-ir neurons was quantified bilaterally in at least two sections in anterior–posterior coordinates between −0.24 and −0.36 mm from bregma for the septohypothalamic nucleus (SHy), medial preoptic area (mPOA), and ventromedial preoptic area (vmPOA); −0.96 and −1.20 mm from bregma for the SON; −1.72 and −1.92 mm from bregma for the PVN; and one section between −0.00 and −0.12 mm from bregma for median preoptic nucleus (MnPO), according to the rat brain atlas (Paxinos and Watson 2007). Boxes (width 390 µm; length 310 µm) were drawn to delimit the area in which the neurons were counted.

Calculation

Heat storage (HS, cal) was calculated as HS = [body weight (g)] × [specific heat of body tissues ($c = 0.826$ cal g^{-1} °C^{-1})] × [change in T_{core} (°C)].

Statistical analysis

The data are reported as the mean ± standard errors of the mean (SEM). The differences in thermoregulatory responses (i.e., T_{core} and T_{skin}) were compared across time points and between experimental trials using two-way analyses of variance (ANOVAs) followed by the post hoc Student Newman–Keuls test. One-way ANOVAs were used to compare the body weight, running speed, running duration, final T_{core} and T_{skin}, HS, and c-Fos expression between groups. These ANOVAs were followed by the post hoc Tukey test, except for the ANOVA related to c-Fos expression, which was followed by the post hoc Student Newman–Keuls test. All correlations were assessed using the Pearson's correlation coefficient. The above-mentioned correlations were classified according to Evans's criteria as follows: 0.00–0.19, very weak; 0.20–0.39, weak; 0.40–0.59, moderate; 0.60–0.79, strong; and > 0.80, very strong (Evans 1996). The significance level was set at $\alpha < 0.05$.

Results

Initially, it is important to note that the S_{max} attained by the rats of different groups during the incremental exercise was not different, indicating that these animals presented similar aerobic capacity. Therefore, the speed at which the rats ran during the constant exercise, which corresponded to 70% of S_{max}, was not different between groups (Table 1). The body weight was also not different between groups; however, the running duration was higher in the FATIGUE group than in the NADIR and 20 MIN groups. In addition, this parameter was also higher in the 20 MIN group than in the NADIR group (Table 1).

As illustrated in Fig. 1a, the treadmill running at 70% of S_{max} induced a clear hyperthermic response. T_{core} increased rapidly during the early phase of the exercise (i.e., dynamic phase of thermoregulation) and became different from baseline values within 5–6 min of running. This rapid increase of T_{core} persisted until approximately 20 min (time chosen for exercise interruption in the 20 MIN group), when T_{core} plateaued at a high level that was sustained until the rats fatigued (i.e., steady-state phase of thermoregulation during exercise).

The exercise-induced changes in T_{skin} had different dynamics relative to the changes in T_{core} (Fig. 1b). T_{skin} initially decreased in all exercising groups, reaching a nadir within 3–5 min of running (NADIR: 24.65 ± 0.42 °C; 20 MIN: 24.72 ± 0.48 °C; FATIGUE: 25.46 ± 0.37 °C). Thereafter, T_{skin} increased at 9 and 8 min after the exercise had been initiated in the 20 MIN and FATIGUE groups, respectively, indicating that cutaneous heat loss was activated. Similar to T_{core}, T_{skin} continued to increase until approximately 20 min of running and then was maintained at a stable high

Table 1 Body weight, running speed and running duration performed by rats during the submaximal exercise at 70% of the maximal speed

	REST ($n = 6$)	NADIR ($n = 8$)	20 MIN ($n = 8$)	FATIGUE ($n = 7$)
Body weight (g)	308.2 ± 12.7	286.4 ± 12.5	268.7 ± 5.7	287.0 ± 14.7
Running speed (m min^{-1})	NA	16.5 ± 0.6	17.3 ± 0.7	17.1 ± 0.5
Running duration (min)	NA	6.1 ± 0.4	20.0 ± 0.0[a]	95.1 ± 13.9[ab]

The data are expressed as the mean ± SEM. Statistical significance: $p < 0.05$: a, compared with the NADIR; b, compared with the 20 MIN

NA not applicable

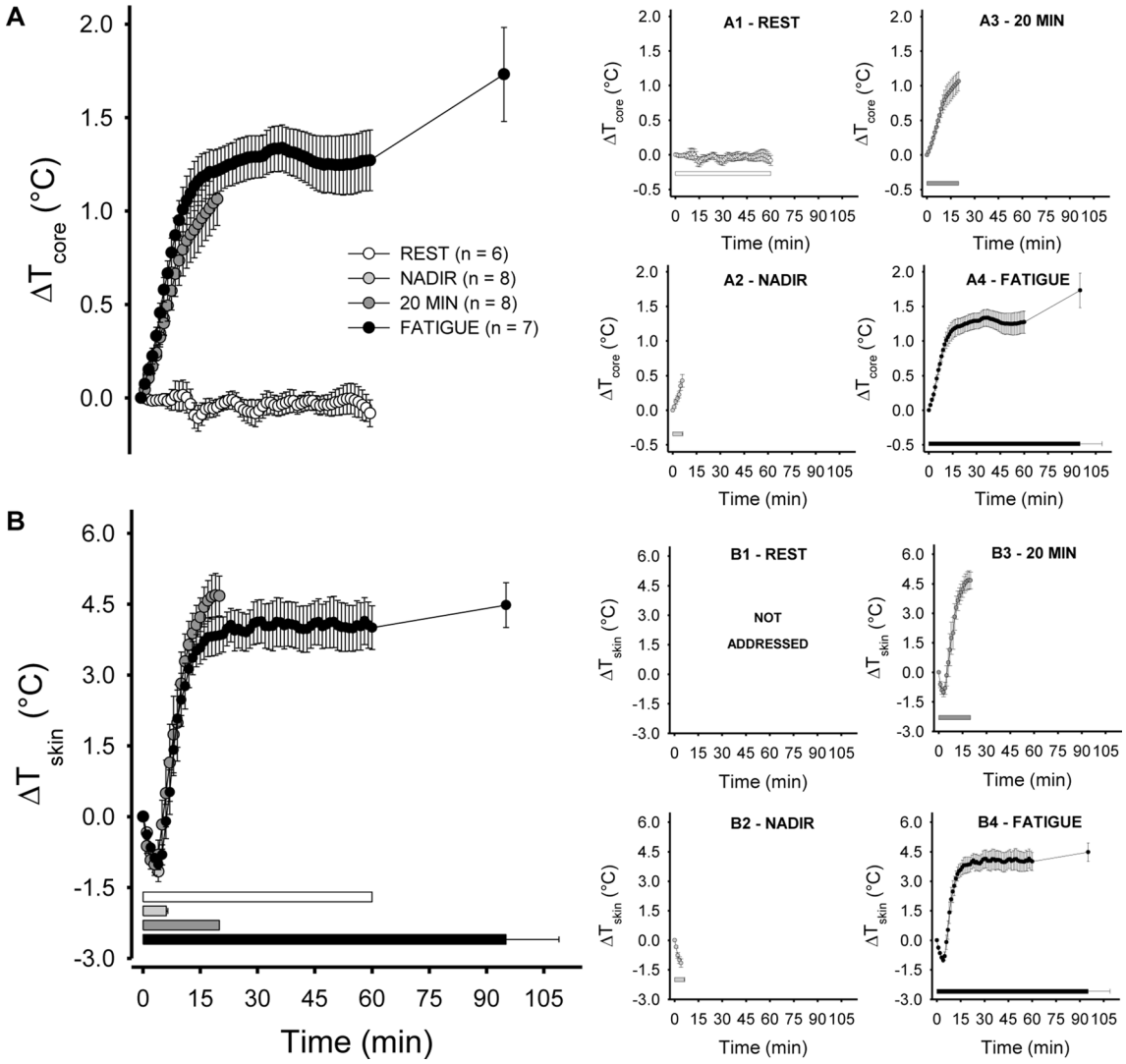

Fig. 1 Effect of submaximal running exercise (at 70% of S_{max}) on the changes in core temperature (ΔT_{core}, **a**) and tail skin temperature (ΔT_{skin}, **b**) at three different moments of exercise (i.e., NADIR, 20 MIN, and FATIGUE). Running duration is indicated by the horizontal bars at the bottom in **b**. Data are expressed as the mean ± SEM

level until the rats fatigued (20 MIN: 30.40 ± 0.29 °C vs. FATIGUE: 30.95 ± 0.52 °C; $p = 0.25$) (Fig. 1b).

As illustrated in Fig. 2a, T_{core} at the end of the experimental trials was not different between the 20 MIN (38.01 ± 0.26 °C) and FATIGUE (38.60 ± 0.27 °C) groups, but these groups presented T_{core} values higher than those of REST group (37.14 ± 0.09 °C; $p < 0.05$ and $p < 0.001$, respectively). Moreover, final T_{core} was higher in the FATIGUE than in the NADIR group (37.47 ± 0.11 °C; $p < 0.01$). In contrast, the final T_{core} in the NADIR group was not different from the final T_{core} in the REST group ($p = 0.70$). Also, no differences were observed between the 20 MIN and NADIR groups.

Similar responses were observed in HS (Fig. 2b). The 20 MIN (240.9 ± 36.7 cal) and FATIGUE (409.9 ± 62.9 cal) groups showed higher HS than the REST group (− 8.1 ± 14.1 cal $p < 0.001$), although this difference was not observed between the NADIR group (88.9 ± 18.9 cal) and the REST group ($p = 0.33$). In addition, the 20 MIN and FATIGUE groups accumulated more heat than the NADIR group ($p < 0.01$ and $p < 0.001$, respectively). Finally, HS was higher in the FATIGUE group than in the 20 MIN group ($p = 0.05$).

The overall thermal strain induced by the treadmill running was dependent on the effort duration, as evidenced by the positive and strong correlations between the different running durations and the final T_{core} ($r = 0.701$ $p < 0.001$) or HS ($r = 0.765$ $p < 0.001$).

The c-Fos expression allowed the assessment of neuronal activation induced by submaximal exercise in six

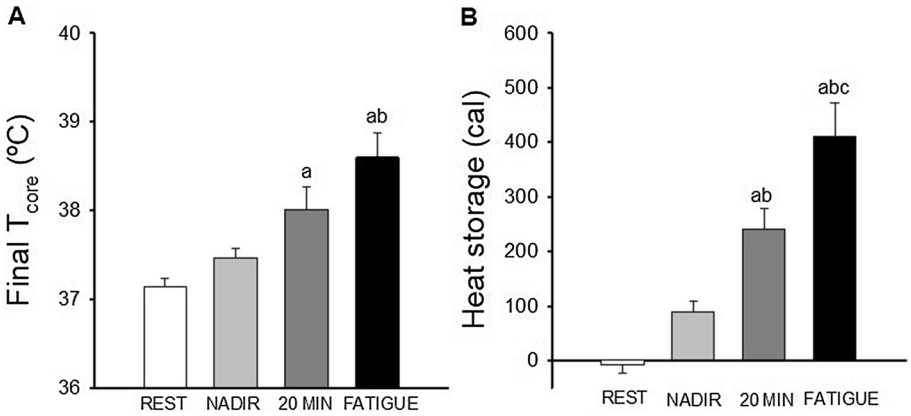

Fig. 2 Effect of moderate-intensity running with different durations (i.e., NADIR, 20 MIN, and FATIGUE) on the final T_{core} (**a**) and heat storage (**b**). Data are expressed as the mean ± SEM. Statistical significance: $p < 0.05$. a, compared with the REST; b, compared with the NADIR; c, compared with the 20 MIN

hypothalamic areas (i.e., SHy, mPOA, vmPOA, MnPO, PVN, and SON; Figs. 3 and 4) involved with the autonomic control of thermoregulatory and endocrine functions.

In the SHy neurons, c-Fos expression was higher in all three groups subjected to treadmill running than in the REST group; the expression was increased by 445% ($p < 0.01$), 613% ($p < 0.01$), and 505% ($p < 0.01$) in the NADIR, 20 MIN, and FATIGUE groups, respectively. No differences were observed between these three exercising groups. As observed for the SHy neurons, physical exercise induced a similar pattern of activation of the vmPOA neurons at the three different moments during exercise that were evaluated (NADIR: 500% increase; 20 MIN: 486% increase; FATIGUE: 465% increase; $p < 0.001$ for the three comparisons; all of these percentages and *p*-values are relative to the REST groups).

Regarding the activation of mPOA and MnPO neurons, a progressive increase in c-Fos expression was observed as exercise was prolonged. The NADIR group showed higher neuronal activation than the REST group (mPOA: 1067%; MnPO: 266%, $p < 0.05$). In addition, c-Fos expression in the mPOA in both the 20 MIN and FATIGUE groups was higher than in the NADIR group (76% and 95%, respectively; $p < 0.05$). In the MnPO, the c-Fos expression in the FATIGUE group was higher than both the NADIR and 20 MIN groups (85% and 54%, respectively; $p < 0.05$).

In the PVN neurons, c-Fos expression was higher in all three groups subjected to treadmill running than in the REST group (NADIR: 389% increase $p < 0.01$; 20 MIN: 680% increase $p < 0.001$; FATIGUE: 793% increase $p < 0.001$; all of these percentages and p-values are relative to the REST group). Both the 20 MIN and FATIGUE groups displayed higher neuronal activation than the NADIR group (159% $p < 0.05$ and 183% $p < 0.01$, respectively). However, no differences in c-Fos expression were observed in the PVN neurons between the rats of the 20 MIN and FATIGUE groups ($p = 0.38$).

Interestingly, the SON was the only brain nucleus that did not show any increase in neuronal activation in the NADIR group when compared to the REST group ($p = 0.33$). However, increased c-Fos expression was observed in the 20 MIN group relative to the REST group (147% $p < 0.05$), while the FATIGUE group showed a higher neuronal activation when compared with the other three groups (288% increase vs. REST $p < 0.001$; 157% increase vs. NADIR $p < 0.001$; 57% increase vs. 20 MIN $p < 0.01$).

Next, Pearson's correlations were performed to verify whether the pattern of neuronal activation in the selected brain areas/nuclei was associated with running duration or HS (Table 2). The neuronal activation in the mPOA, MnPO, PVN, and SON was significantly and positively correlated with both the running duration and HS. In contrast, the activation of the SHy neurons showed a significant positive correlation only with HS, whereas the number of activated neurons in the vmPOA did not show significant correlations with either of the two parameters.

Discussion

The present results indicate that submaximal treadmill running performed at temperate conditions activates neuronal populations in different hypothalamic nuclei/areas involved in autonomic regulation. The nuclei/areas investigated showed different temporal patterns of neuronal activation. More specifically, the neuronal activation in the MnPO, mPOA, PVN, and SON correlated with both the running duration and HS, the activation in the SHy correlated only with HS, whereas the activation in the vmPOA did not correlate with either of the two parameters. It is noteworthy that the strength of correlations suggests that the POA nuclei are mainly involved with thermoregulatory responses; the SON with running duration; while, the PVN responds similarly to both stimuli. Although the observed neuronal activation in the vmPOA and SHy was not associated with exercise duration or the thermoregulatory response, a maximal neuronal recruitment in these areas seems to be induced even across the small variations of T_{core} (i.e., low HS) or the short

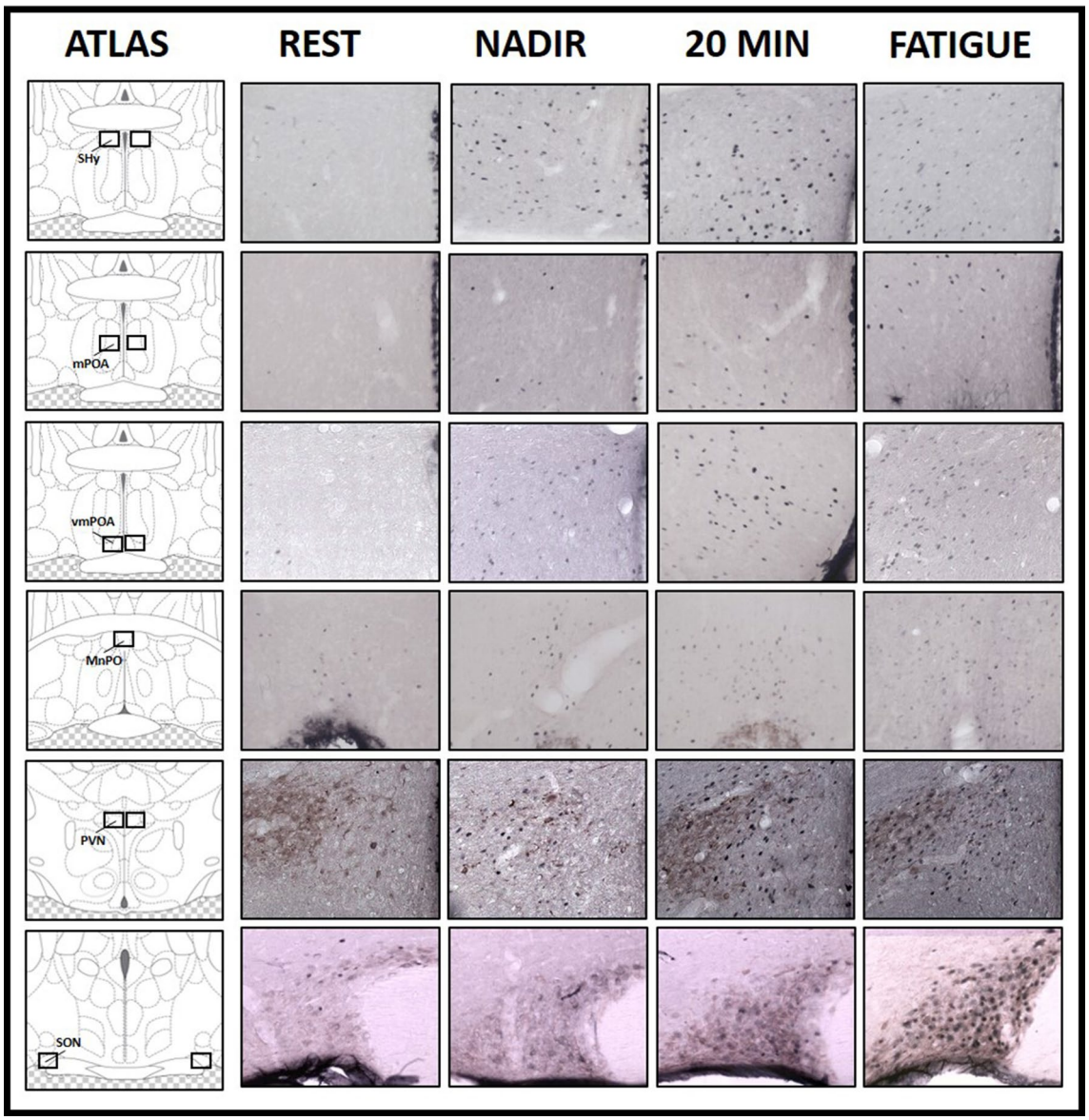

Fig. 3 Photomicrographs of coronal brain sections showing c-Fos immunoreactive cells (dark gray-black nuclei) in the septohypothalamic nucleus (SHy), medial preoptic area (mPOA), ventromedial preoptic area (vmPOA), median preoptic nucleus (MnPO), paraventricular nucleus of hypothalamus (PVN), and supraoptic nucleus (SON), after 60 min of rest (REST), and at different moments of treadmill running (i.e., NADIR, 20 MIN, and FATIGUE)

duration of exercise. Nevertheless, the information presented in the last two sentences should be analyzed with caution, because correlations do not allow the establishment of a causal relationship between the parameters evaluated.

Although previous studies have already reported that several brain areas, such as the PVN, SON, mPOA, suprachiasmatic nucleus, anterior hypothalamus, and arcuate nucleus show increased c-Fos expression after treadmill running (Nunez et al. 2012; Saito and Soya 2004; Soya et al. 2007; Yanagita et al. 2007), the present investigation is the first to verify the influence of running duration and/or thermal load on neuronal recruitment induced by exercise during different phases of the thermoregulatory response (i.e., dynamic and steady-state phases). Thus, the novelty of the present study consists of determining a temporal pattern of the hypothalamic neuronal recruitment by a given submaximal exercise intensity and determining the association between this neuronal recruitment with the running duration or changes in thermoregulation.

The experimental design used in the present study does not allow us to precisely identify which factor has a larger contribution than the other with regard to regulating neuronal activity; taking our data into consideration, we can only provide some speculation, which is presented in the

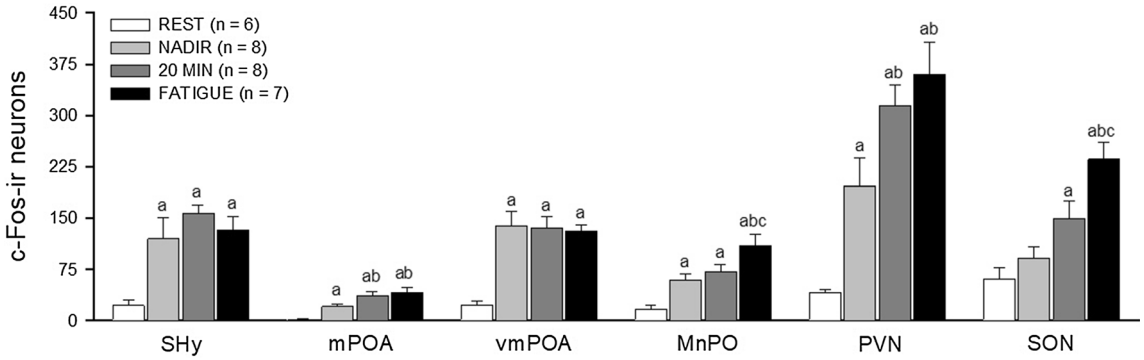

Fig. 4 Number of immunoreactive neurons per coronal section in different brain nuclei/areas of rats after the resting period (REST) and at different moments of treadmill running (i.e., NADIR, 20 MIN, and FATIGUE). The data are expressed as the mean ± SEM. Statistical significance: $p < 0.05$. a, compared with the REST; b, compared with the NADIR; c, compared with the 20 MIN

Table 2 Correlation between the running duration or heat storage and c-Fos expression in selected brain nuclei/areas

Brain region	Running duration (min)			Heat storage (cal)		
	Pearson's coefficient (r)	p value	Classification according to Evan's criteria	Pearson's coefficient (r)	p value	Classification according to Evan's criteria
SHy	0.206	0.28	Weak	0.438	< 0.05	Moderate
mPOA	0.585	< 0.001	Moderate	0.431	< 0.05	Moderate
vmPOA	0.295	0.12	Weak	0.287	0.13	Weak
MnPO	0.593	< 0.001	Moderate	0.633	< 0.001	Strong
PVN	0.606	< 0.001	Strong	0.606	< 0.001	Strong
SON	0.739	< 0.001	Strong	0.542	< 0.01	Moderate

first paragraph of the discussion. To answer this question, it would be interesting to also conduct passive (without exercise) heat exposure experiments, evaluate the neuronal recruitment in these areas at different time points of exposure and then compare with exercise-induced neuronal activation. The data yielded from heat exposure would allow the identification of areas that respond basically to thermal stimulus, without being influenced by increased muscle metabolism and locomotor activity. For example, a previous investigation has shown that mice exposed to 38.5 °C for 60 min became hyperthermic and presented c-Fos expression in the MnPO, mPOA, PVN, and SON (Harikai et al. 2003), thus suggesting that most of the areas investigated in the present study can be activated by passive heat exposure-driven thermal stimuli.

All exercising groups showed greater neuronal activation in the PVN compared to the REST group, with this activation being pronounced in the beginning of the steady-state phase and maintained until fatigue. In addition, c-Fos expression in the PVN was positively correlated with both the running duration and HS, suggesting that neuronal recruitment can be related to the maintenance of physical exercise or the thermoregulatory strain. The higher c-Fos expression in the NADIR group than in the REST group suggests that the exercise stimulus by itself induces neuronal activation in the PVN, because the running time until the nadir was not sufficiently long to induce high levels of T_{core} and represented only 6% of the total running duration. As exercise continued and hyperthermia increased, neuronal activation also increased. The fact that T_{core} and neuronal activation in the PVN plateaued after 20 min indicates that thermoregulatory factors become more determinant for activation relative to the exercise duration.

The PVN is a major integrative site within the brain for autonomic function and modulates endocrine, and sympathetic responses elicited by a variety of stimuli, including physical exercise and thermal stress (Badoer 2010; Cham and Badoer 2008b; Chen et al. 2008; Lima et al. 2014; Nunez et al. 2012; Saito and Soya 2004; Soya et al. 2007; Swanson and Sawchenko 1980; Yanagita et al. 2007). It has been shown that PVN neurons are activated by exercise to regulate the following responses: release of corticotrophin-releasing hormone, adrenocorticotropic hormone, and AVP, stimulation of adrenaline production, transient hyperosmolality and increased blood glucose and lactate concentrations. Therefore, our results reinforce the notion that exercise is a stimulus that activates the hypothalamus–pituitary–adrenal axis as an adaptive response that assists the metabolic

regulation during physical exertion (Soya et al. 2007). The activation of PVN neurons may also be related to the magnitude of hyperthermia-induced by exercise because this nucleus contains thermosensitive neurons (Inenaga et al. 1987). Additionally, some studies have shown that elevation in T_{core} resulting from passive hyperthermia strongly activates neurons in the PVN (Bachtell et al. 2003; Bratincsak and Palkovits 2004; Harikai et al. 2003; McKitrick 2000; Yoshida et al. 2002).

The role of the PVN in cardiovascular adjustments recruited by hyperthermia is well established. Exposure to a hot environment elicits reflex responses that promote heat loss, including increases in heart rate, respiration rate, vasodilation of the skin vasculature, vasoconstriction of the visceral vasculature, sweating in humans, and salivary secretion in rodents. All of these responses are involved with cardiovascular responses which are mediated by the central nervous system through the regulation of sympathetic nerve activity (Cham and Badoer 2008b; Chen et al. 2008; Kanosue et al. 1986, 1994; Kazuyuki et al. 1998; Kregel et al. 1988; Morrison and Nakamura 2011; Nagashima et al. 2000; Owens et al. 2002; Romanovsky 2007). PVN neurons also send projections to the pressor region of the rostral ventrolateral medulla, a critical autonomic region that projects directly to the sympathetic preganglionic motor neurons in the intermediolateral cell column of the thoracolumbar spinal cord which regulates the sympathetic nerve activity (Cham and Badoer 2008a; Dampney 2004; Guyenet et al. 1989; Leite et al. 2012). Evidence indicates that the inhibition of neuronal function in the PVN abolishes the classic blood flow redistribution in response to hyperthermia (Cham and Badoer 2008b; Chen et al. 2008).

The c-Fos expression in the SON also increased gradually and was, therefore, dependent on running duration and on exercise-induced HS. However, unlike the PVN, neuronal activation in the SON was not observed in the early phase of exercise (NADIR group). This response can be related to a greater involvement of this nucleus in fluid balance regulation than in thermoregulation. During exercise, plasma osmolality usually increases due to water shift from the vascular space to the interstitial and intracellular spaces resulting from increased blood pressure and due to evaporation of body fluids to dissipate heat (Cheuvront et al. 2003; Nunez et al. 2012). The neuroendocrine system, which is regulated by the PVN and SON neurons, plays an important role in adjusting the water–electrolyte balance. Thus, it is likely that neuronal activation in the SON is more evident at the steady-state phase of exercise, when T_{core} reaches values that are high enough to change the water–electrolyte balance.

The hypothesis above is supported by recent studies in which AVP-neurons in the SON were activated by exercise (Saito and Soya 2004). In addition, SON activation is only reported in animals subjected to exercise protocols that increase water intake, hematocrit, plasma osmolality, and plasma concentrations of proteins (Nunez et al. 2012). In this context, 30 min of running at a speed below the lactate threshold did not affect the number of c-Fos immunoreactive cells in the PVN and SON, including AVP-neurons, whereas running at a speed above the lactate threshold increased the number of c-Fos immunoreactive cells and plasma osmolality (Saito and Soya 2004; Soya et al. 2007). During heat exposure, simultaneously with the activation of evaporative heat loss, the plasma concentration of AVP increases, leading to water conservation (Doris 1982; Jasnic et al. 2013). Under these experimental conditions, c-Fos expression in the SON was increased when heat exposure induced both hyperthermia and dehydration in mice, but not when exposure caused only hyperthermia (Harikai et al. 2003).

We evaluated neuronal activation in three different areas/nuclei of the POA, namely, the vmPOA, mPOA, and MnPO. The increased activation in the vmPOA was similar for the three exercising groups, suggesting that neuronal recruitment in this area occurs at the maximal extent even after small changes in T_{core} (such as those observed in the NADIR group) and does not depend on the duration of the stressor stimulus. This hypothesis is supported by the lack of association between vmPOA activation and either HS or running duration. On the other hand, the neuronal activation in the mPOA and MnPO was higher in the FATIGUE group than in the NADIR group, and positive correlations were observed between the neuronal activation in these areas with both the exercise duration and HS. The similar pattern of neuronal activation of the mPOA and MnPO is corroborated by previous functional mapping studies showing connectivity between these POA neurons. The GABA-ergic neurons in the MnPO receive projections of neurons from the lateral parabrachial nucleus (Nakamura and Morrison 2008) and project to the warm-sensitive GABA-ergic neurons in the mPOA (Nakamura and Morrison 2008).

The POA in the hypothalamus occupies a crucial position among the brain structures participating in autonomic thermoregulation (Boulant 2000; Gordon and Heath 1986; Kobayashi 1986; Nakayama 1985). Previous studies have shown that rats exposed to a warm environment present higher neuronal activation in several nuclei/areas of the POA (Bachtell et al. 2003; Bratincsak and Palkovits 2004; Harikai et al. 2003; Kiyohara et al. 1995; McKitrick 2000; Scammell et al. 1993; Yoshida et al. 2002). As the POA neurons are also activated by exercise (Soya et al. 2007), it has been suggested that this activation induces thermoregulatory adjustments during exercise, contributing to the maintenance of thermal homeostasis. Toward this end, the POA warming elicits skin vasodilation, which is the major autonomic effector that favors non-evaporative heat loss in rats (Kanosue et al. 1994).

A similarly high level of neuronal activation was observed in the SHy, with this activation not different between the exercising groups and following a similar pattern as that observed for the vmPOA neurons. Moreover, a positive correlation between c-Fos expression in the SHy was only found with HS, but not with exercise duration. These results suggest that SHy activation is mainly related to the thermal load rather than the maintenance of physical exercise. Functions related to the SHy have been little studied and are poorly understood. However, there is previous evidence implicating the participation of this nucleus in thermoregulatory responses; a high number of c-Fos-immunoreactive neurons in the SHy was observed following heat stress (Bratincsak and Palkovits 2004).

The neuronal activation patterns revealed by the present findings may underlie some physiological responses that are dependent on exercise duration and heat storage, including the sequential recruitment of thermoeffectors and/or accessory responses that support increased heat dissipation during exercise. As recently reviewed, thermoeffectors are usually recruited in a certain order (Romanovsky 2018); for example, the recruitment of physiological effectors involved in heat defense occurs, at least in humans, as follows: skin vasodilation, sweating, and panting. Moreover, greater heat storage, which is commonly observed in prolonged exercises, may be associated with dehydration and marked cardiovascular responses, thus increasing neuronal activation in the areas investigated. In this sense, c-Fos expression in the MnPO, mPOA, PVN, and SON was observed only when mice were exposed for 60 min to 38.5 °C, but not to 34.0 °C (Harikai et al. 2003). Interestingly, the exposure to 38.5 °C was associated with greater dehydration levels and hyperthermia than the exposure to 34.0 °C (Harikai et al. 2003), which may explain the activation of additional hypothalamic neurons.

In summary, our results indicate that moderate intensity treadmill running induces neuronal activation in brain areas related to autonomic control, including those related to the regulation of body temperature, metabolism, vasomotor tonus, and osmotic balance. Small exercise-induced increases in T_{core} (i.e. low HS), even in a short time frame, result in high neuronal activation in the POA, SHy, and PVN. However, as exercise is continued and the hyperthermic response is increased, the SON, an important center implicated in osmotic balance becomes recruited. In general, neuronal recruitment in the MnPO, mPOA, PVN, and SON, appears to increase gradually as exercise is prolonged, leading to greater hyperthermia, whereas, in the SHy and vmPOA, neuronal recruitment seems to rely primarily on the initial T_{core} changes and does not depend on the duration of the stressor stimulus.

Acknowledgements The authors thank Harold Gainer, PhD, for providing AVP antibody. This study was supported by grants from CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), FAPEMIG (Fundação de Amparo à Pesquisa do Estado de Minas Gerais), and PRPQ-UFMG (Pró-Reitoria de Pesquisa da Universidade Federal de Minas Gerais).

References

Bachtell RK, Tsivkovskaia NO, Ryabinin AE (2003) Identification of temperature-sensitive neural circuits in mice using c-Fos expression mapping. Brain Res 960:157–164

Badoer E (2010) Role of the hypothalamic PVN in the regulation of renal sympathetic nerve activity and blood flow during hyperthermia and in heart failure. Am J Physiol Renal Physiol 298:F839–F846. https://doi.org/10.1152/ajprenal.00734.2009

Balthazar CH, Leite LH, Ribeiro RM, Soares DD, Coimbra CC (2010) Effects of blockade of central dopamine D1 and D2 receptors on thermoregulation, metabolic rate and running performance. Pharmacol Rep 62:54–61

Barna BF, Takakura AC, Moreira TS (2012) Pontomedullary and hypothalamic distribution of Fos-like immunoreactive neurons after acute exercise in rats. Neuroscience 212:120–130. https://doi.org/10.1016/j.neuroscience.2012.03.039

Boulant JA (2000) Role of the preoptic-anterior hypothalamus in thermoregulation and fever. Clin Infect Dis 31(Suppl 5):S157–S161. https://doi.org/10.1086/317521

Bratincsak A, Palkovits M (2004) Activation of brain areas in rat following warm and cold ambient exposure. Neuroscience 127:385–397. https://doi.org/10.1016/j.neuroscience.2004.05.016

Cham JL, Badoer E (2008a) Exposure to a hot environment can activate rostral ventrolateral medulla-projecting neurones in the hypothalamic paraventricular nucleus in conscious rats. Exp Physiol 93:64–74. https://doi.org/10.1113/expphysiol.2007.039560

Cham JL, Badoer E (2008b) Hypothalamic paraventricular nucleus is critical for renal vasoconstriction elicited by elevations in body temperature. Am J Physiol Renal Physiol 294:F309–F315. https://doi.org/10.1152/ajprenal.00488.2007

Chen F, Dworak M, Wang Y, Cham JL, Badoer E (2008) Role of the hypothalamic PVN in the reflex reduction in mesenteric blood flow elicited by hyperthermia. Am J Physiol Regul Integr Comp Physiol 295:R1874–R1881. https://doi.org/10.1152/ajpregu.90384.2008

Cheuvront SN, Carter R 3rd, Sawka MN (2003) Fluid balance and endurance exercise performance. Curr Sports Med Rep 2:202–208

Coyle EF (2000) Physical activity as a metabolic stressor. Am J Clin Nutr 72:512S–520S

Dampney R (2004) Medullary pathways regulating sympathetic outflow: the need for more lateral thinking. Am J Physiol Regul Integr Comp Physiol 286:R446–R448. https://doi.org/10.1152/ajpregu.00696.2003

Doris PA (1982) Vasopressin and the regulation of evaporative water loss and body temperature in the cat. Brain Res 251:127–136

Evans JD (1996) Straightfoward statistics for the behavioral sciences. Brooks/Cole Publishing Company, Pacific

Gleeson M (1998) Temperature regulation during exercise. Int J Sports Med 19(Suppl 2):S96–S99. https://doi.org/10.1055/s-2007-971967

Gordon CJ, Heath JE (1986) Integration and central processing in temperature regulation. Annu Rev Physiol 48:595–612. https://doi.org/10.1146/annurev.ph.48.030186.003115

Guyenet PG, Haselton JR, Sun MK (1989) Sympathoexcitatory neurons of the rostroventrolateral medulla and the origin of the sympathetic vasomotor tone. Prog Brain Res 81:105–116

Harikai N, Tomogane K, Sugawara T, Tashiro S (2003) Differences in hypothalamic Fos expressions between two heat stress conditions in conscious mice. Brain Res Bull 61:617–626

Hasegawa H, Piacentini MF, Sarre S, Michotte Y, Ishiwata T, Meeusen R (2008) Influence of brain catecholamines on the development of fatigue in exercising rats in the heat. J Physiol 586:141–149. https://doi.org/10.1113/jphysiol.2007.142190

Hoffman GE, Smith MS, Verbalis JG (1993) c-Fos and related immediate early gene products as markers of activity in neuroendocrine systems. Front Neuroendocrinol 14:173–213. https://doi.org/10.1006/frne.1993.1006

Inenaga K, Osaka T, Yamashita H (1987) Thermosensitivity of neurons in the paraventricular nucleus of the rat slice preparation. Brain Res 424:126–132

Iwamoto GA, Wappel SM, Fox GM, Buetow KA, Waldrop TG (1996) Identification of diencephalic and brainstem cardiorespiratory areas activated during exercise. Brain Res 726:109–122

Jasnic N, Djordjevic J, Vujovic P, Lakic I, Djurasevic S, Cvijic G (2013) The effect of vasopressin 1b receptor (V1bR) blockade on HPA axis activity in rats exposed to acute heat stress. J Exp Biol 216:2302–2307. https://doi.org/10.1242/jeb.082842

Kanosue K, Matsuo R, Tanaka H, Nakayama T (1986) Effect of body temperature on salivary reflexes in rats. J Auton Nerv Syst 16:233–237

Kanosue K, Yanase-Fujiwara M, Hosono T (1994) Hypothalamic network for thermoregulatory vasomotor control. Am J Physiol 267:R283–R288

Kazuyuki K, Hosono T, Zhang YH, Chen XM (1998) Neuronal networks controlling thermoregulatory effectors. Prog Brain Res 115:49–62

Kiyohara T, Miyata S, Nakamura T, Shido O, Nakashima T, Shibata M (1995) Differences in Fos expression in the rat brains between cold and warm ambient exposures. Brain Res Bull 38:193–201

Kobayashi S (1986) Warm- and cold-sensitive neurons inactive at normal core temperature in rat hypothalamic slices. Brain Res 362:132–139

Kovacs KJ (1998) c-Fos as a transcription factor: a stressful (re)view from a functional map. Neurochem Int 33:287–297

Kovacs KJ (2008) Measurement of immediate-early gene activation-c-fos and beyond. J Neuroendocrinol 20:665–672. https://doi.org/10.1111/j.1365-2826.2008.01734.x

Kregel KC, Wall PT, Gisolfi CV (1988) Peripheral vascular responses to hyperthermia in the rat. J Appl Physiol (1985) 64:2582–2588

Lacerda AC, Marubayashi U, Coimbra CC (2005) Nitric oxide pathway is an important modulator of heat loss in rats during exercise. Brain Res Bull 67:110–116. https://doi.org/10.1016/j.brainresbull.2005.06.002

Lacerda AC, Marubayashi U, Balthazar CH, Coimbra CC (2006) Evidence that brain nitric oxide inhibition increases metabolic cost of exercise, reducing running performance in rats. Neurosci Lett 393:260–263. https://doi.org/10.1016/j.neulet.2005.09.076

Leite LH, Lacerda AC, Marubayashi U, Coimbra CC (2006) Central angiotensin AT1-receptor blockade affects thermoregulation and running performance in rats. Am J Physiol Regul Integr Comp Physiol 291:R603–R607. https://doi.org/10.1152/ajpregu.00038.2006

Leite LH, Lacerda AC, Balthazar CH, Marubayashi U, Coimbra CC (2007) Central AT(1) receptor blockade increases metabolic cost during exercise reducing mechanical efficiency and running performance in rats. Neuropeptides 41:189–194. https://doi.org/10.1016/j.npep.2007.01.002

Leite LH, Zheng H, Coimbra CC, Patel KP (2012) Contribution of the paraventricular nucleus in autonomic adjustments to heat stress. Exp Biol Med (Maywood) 237:570–577. https://doi.org/10.1258/ebm.2011.011286

Lima PM, Santiago HP, Szawka RE, Coimbra CC (2014) Central blockade of nitric oxide transmission impairs exercise-induced neuronal activation in the PVN and reduces physical performance. Brain Res Bull 108:80–87. https://doi.org/10.1016/j.brainresbull.2014.09.002

McKitrick DJ (2000) Expression of fos in the hypothalamus of rats exposed to warm and cold temperatures. Brain Res Bull 53:307–315

Morrison SF, Nakamura K (2011) Central neural pathways for thermoregulation. Front Biosci (Landmark Ed) 16:74–104

Nagashima K, Nakai S, Tanaka M, Kanosue K (2000) Neuronal circuitries involved in thermoregulation. Auton Neurosci 85:18–25. https://doi.org/10.1016/S1566-0702(00)00216-2

Nakamura K, Morrison SF (2008) A thermosensory pathway that controls body temperature. Nat Neurosci 11:62–71. https://doi.org/10.1038/nn2027

Nakayama T (1985) Thermosensitive neurons in the brain. Jpn J Physiol 35:375–389

Nunez P, Diaz B, Perillan C, Vijande M, Arguelles J (2012) Progressive training effects on neuronal hypothalamic activation in the rat. Neurosci Lett 517:113–117. https://doi.org/10.1016/j.neulet.2012.04.039

Owens NC, Ootsuka Y, Kanosue K, McAllen RM (2002) Thermoregulatory control of sympathetic fibres supplying the rat's tail. J Physiol 543:849–858

Paxinos G, Watson C (2007) The rat brain in stereotaxis coordinates. Academic Press, San Diego

Romanovsky AA (2007) Thermoregulation: some concepts have changed. Functional architecture of the thermoregulatory system. Am J Physiol Regul Integr Comp Physiol 292:R37–R46. https://doi.org/10.1152/ajpregu.00668.2006

Romanovsky AA (2018) The thermoregulation system and how it works. Handb Clin Neurol 156:3–43. https://doi.org/10.1016/B978-0-444-63912-7.00001-1

Rowell LB (1974) Human cardiovascular adjustments to exercise and thermal stress. Physiol Rev 54:75–159. https://doi.org/10.1152/physrev.1974.54.1.75

Saito T, Soya H (2004) Delineation of responsive AVP-containing neurons to running stress in the hypothalamus. Am J Physiol Regul Integr Comp Physiol 286:R484–R490. https://doi.org/10.1152/ajpregu.00453.2003

Scammell TE, Price KJ, Sagar SM (1993) Hyperthermia induces c-fos expression in the preoptic area. Brain Res 618:303–307

Soares DD, Lima NR, Coimbra CC, Marubayashi U (2003) Evidence that tryptophan reduces mechanical efficiency and running performance in rats. Pharmacol Biochem Behav 74:357–362

Soares DD, Lima NR, Coimbra CC, Marubayashi U (2004) Intracerebroventricular tryptophan increases heating and heat storage rate in exercising rats. Pharmacol Biochem Behav 78:255–261. https://doi.org/10.1016/j.pbb.2004.03.015

Soya H et al (2007) Threshold-like pattern of neuronal activation in the hypothalamus during treadmill running: establishment of a minimum running stress (MRS) rat model. Neurosci Res 58:341–348. https://doi.org/10.1016/j.neures.2007.04.004

Swanson LW, Sawchenko PE (1980) Paraventricular nucleus: a site for the integration of neuroendocrine and autonomic mechanisms. Neuroendocrinology 31:410–417

Tomita K, Takayama K (2008) Effects of treadmill running exercise on neuronal expression of c-Fos protein in the medulla oblongata after unilateral phrenicotomy in Wistar rats. Neurosci Lett 440:327–330. https://doi.org/10.1016/j.neulet.2008.05.109

Wanner SP et al (2015) Thermoregulatory responses in exercising rats: methodological aspects and relevance to human

physiology. Temperature (Austin) 2:457–475. https://doi.org/10.1080/23328940.2015.1119615
Webb P (1995) The physiology of heat regulation. Am J Physiol 268:R838–R850
Yanagita S, Amemiya S, Suzuki S, Kita I (2007) Effects of spontaneous and forced running on activation of hypothalamic corticotropin-releasing hormone neurons in rats. Life Sci 80:356–363. https://doi.org/10.1016/j.lfs.2006.09.027
Yoshida K, Maruyama M, Hosono T, Nagashima K, Fukuda Y, Gerstberger R, Kanosue K (2002) Fos expression induced by warming the preoptic area in rats. Brain Res 933:109–117