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Research Report

Effects of acute heat exposure on prosencephalic c-Fos expression in normohydrated, water-deprived and salt-loaded rats

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ABSTRACT

In the present study, the distribution pattern of c-Fos protein immunoreactivity (Fos-IR) in prosencephalic areas of the brain involved in thermoregulatory and osmoregulatory responses was investigated, in rats exposed or not exposed to a hyperthermic environment, under three different conditions: normohydration, dehydration induced by water deprivation and hyperosmolarity induced by an acute intragastric salt load. Normohydrated, water-deprived or salt-loaded male Wistar rats (270±30 g) were submitted or not to acute heat exposure (33 °C for 45 min). A separate group of animals was submitted to the same experimental protocol and had blood samples collected before and after the heating period to measure serum osmolarity and sodium. The brains were processed for c-Fos immunohistochemistry using the avidin–biotin peroxidase method. After analyzing Fos-IR in the brains of animals in the present study, three different types of prosencephalic areas were identified: (1) those that respond to hydrational and to heat conditions, with an interaction between these two factors (PaMP and SON); (2) those that respond to hydrational and to heat conditions, but with no interaction between these factors (MnPO, LSV and OVLT); and (3) those that respond only to hydrational status (SFO and PaLM).

1. Introduction

In homeothermic mammals, dehydration, hyperosmolarity and exposure to hyperthermic environments are conditions that require prompt, adaptive visceral and behavioral responses (Morimoto, 1990). These responses involve a myriad of homeostatic actions that are largely controlled by the central nervous system. Indeed, thirst, acute changes in electrolyte

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Abbreviations: AVP, Arginine vasopressin; AV3V, Anteroventral third ventricle; c-Fos, Protein c-Fos; CSF, Cerebrospinal fluid; DAB, Diaminobenzidine-tetrahydrochloride; Fos-IR, c-Fos-immunoreactive nuclei; LHA, Lateral hypothalamic area; LSV, Lateral septal nuclei, ventral part; OVLT, Organum vasculosum laminae terminalis; MnPO, Median preoptic nucleus; PaLM, Paraventricular hypothalamic nucleus, lateral magnocellular part; PaMP, Paraventricular hypothalamic nucleus, medial parvicellular part; PB, Phosphate buffered; PBS, Phosphate buffered saline; PVN, Paraventricular hypothalamic nucleus; SFO, Subfornical nucleus; SON, Supraoptical nuclei

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excretion, vasomotor and volemic adjustments affecting blood pressure and heart rate regulation, sweating, salivation, and changes in locomotor activity and respiratory performance are coordinated by interconnected areas of the brain (Nagashima et al., 2000).

Dehydration, hyperosmolarity and exposure to hyperthermic environments may occur as isolated conditions and, in these cases, specific corrective responses are normally generated. However, those conditions may be present simultaneously, provoking a complex interplay of homeostatic responses that may trigger both synergistic and conflicting actions (Morimoto et al., 1998).

The hypothalamus, the main thermoregulatory site in the central nervous system, is also related to the control of fluid balance. Indeed, the paraventricular nucleus (PVN) and the supraoptic nucleus (SON) play important neuroendocrine roles in hydroelectrolyte control, while the median preoptic nucleus (MnPO), the subfornical organ (SFO), the organum vasculosum laminae terminalis (OVLT), and the lateral and medial preoptic areas actively participate in the continuous monitoring of plasma osmolarity (Johnson and Thunhorst, 1997; McKinley et al., 1990; Nagashima et al., 2000; Zhang et al., 1997). Several thermoregulatory responses may significantly affect body fluid homeostasis while, on the other hand, body fluid balance may influence thermoregulation. Heating may promote important shifts between fluid compartments, while dehydration increases body temperature, and osmotic changes in the periphery modify the activity of hypothalamic thermosensitive neurons. In addition, cardiovascular responses seems to be modified by thermal conditions and by changes in body fluid status, and changes in blood volume leading to alterations in central venous pressure influence thermoregulation (Morimoto et al., 1998).

There would seem to be a significant functional and anatomical overlap in the osmoregulation and thermoregulatory circuits of the brain. However, information regarding the interplay between these two systems is scanty.

The objective of the present study was to investigate the pattern of the c-Fos protein immunoreactivity (Fos-IR) distribution in prosencephalic areas of the brain involved in thermo- and osmoregulatory responses in rats immediately following acute heat exposure, under three different conditions: normohydration, dehydration induced by water deprivation and hyperosmolarity provoked by an acute intragastric salt load.

2. Results

After analyzing Fos-IR in the brain of animals in the present study, we identified three different types of prosencephalic areas: 1) those that respond to hydration and to heat conditions, with an interaction between these two factors (PaMP and SON); 2) those that respond to hydration and to heat conditions, but with no interaction between these factors (MnPO, LSV and OVLT); and 3) those that respond only to hydration status (SFO and PaLM).

Fig. 1 (Panels A and B) shows the number of c-Fos-immunoreactive nuclei present in the areas that respond to hydration status and to heat and in which there is an interaction between the two (PaMP and SON). Panel A shows Fos-IR in the PaMP following hydration and thermal challenges. In this case, two-way ANOVA indicated significant hydration and thermal main effects and a significant hydration × thermal interaction [$F_{(1,2)}=209.2$, $p<0.05$; $F_{(1,2)}=208.9$, $p<0.05$; $F_{(2,22)}=46.6$, $p<0.05$, respectively]. Panel B shows Fos-IR in the SON in response to hydration and thermal

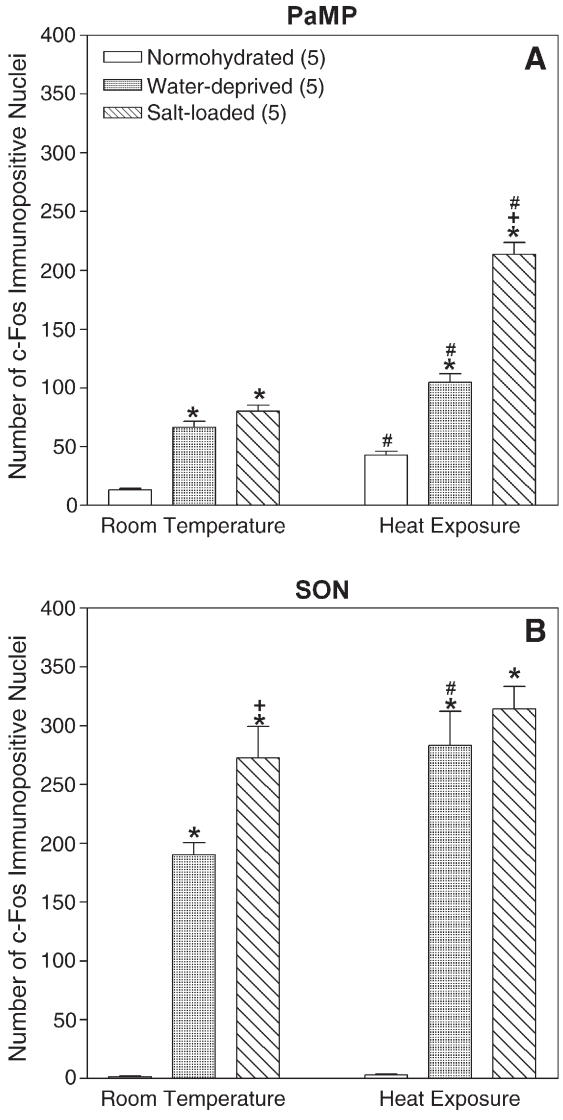


Fig. 1 – Fos-IR (mean ± SEM. number of cell nuclei) in the PaMP—paraventricular hypothalamic nucleus, medial parvicellular part (A) and SON—supraoptic nuclei (B) of normohydrated, water-deprived and salt-loaded animals submitted to heat (33 °C for 45 min) or kept at room temperature (23 °C for 45 min). The statistically significant differences observed by the post-hoc Student–Newman–Keuls test ($p<0.05$): * = significantly different from normohydrated animals in the same temperature condition; # = significantly different from the corresponding hydrational group at room temperature; + = significantly different from the water-deprived group in the same temperature condition.

challenges. Here, two-way ANOVA also showed significant hydration and thermal main effects and a significant hydration × thermal interaction [$F_{(1,2)}=153.17$, $p<0.05$; $F_{(1,2)}=9.80$, $p<0.05$; $F_{(2,22)}=3.57$, $p<0.05$, respectively]. In both the PAMP and the SON, irrespective of thermal conditions, water deprivation and salt load resulted in greater Fos-IR compared to values found in normohydrated rats. Moreover, Fos-IR was higher following heat exposure than when the animal had been maintained at room temperature, irrespective of hydration status. However, post hoc comparison of the interaction revealed differences between the SON and the PaMP. Specifically, Fos-IR was higher in the PaMP of normohydrated, dehydrated and salt-loaded animals submitted to heat compared with the animals maintained at room temperature, while in the SON, Fos-IR increased only in the group of water-deprived animals when submitted to heat.

Fig. 2 (Panels A–C) shows the number of c-Fos-immunoreactive nuclei present in the areas that respond to hydration status and to heat with no interaction between the two (MnPO, LSV and OVLT). Panel A shows Fos-IR in the MnPO after hydration and thermal challenges. In the MnPO, two-way ANOVA showed significant hydration and thermal main effects but no significant hydration × thermal interaction [$F_{(1,2)}=28.8$, $p<0.05$; $F_{(1,2)}=3.58$, $p<0.05$; $F_{(2,22)}=0.92$, ns, respectively]. Panel B shows Fos-IR after hydration and thermal challenges in the LSV. Here, two-way ANOVA showed significant hydration and thermal main effects but no significant hydration × thermal interaction [$F_{(1,2)}=52.19$, $p<0.05$; $F_{(1,2)}=82.74$, $p<0.05$; $F_{(2,22)}=0.35$, ns, respectively]. Panel C shows Fos-IR in the OVLT after hydration and thermal challenges. In this case, two-way ANOVA also showed significant hydration and thermal main effects but no significant hydration × thermal interaction [$F_{(1,2)}=157.5$, $p<0.05$; $F_{(1,2)}=8.31$, $p=0.05$; $F_{(2,22)}=0.21$, ns, respectively]. In each of these areas, irrespective of thermal conditions, salt load resulted in significantly higher Fos-IR than water deprivation, while the normohydrated condition resulted in the lowest levels of Fos-IR. Moreover, irrespective of hydration conditions, heat exposure resulted in higher levels of Fos-IR in the LSV compared to levels in animals maintained at room temperature.

Fig. 3 (Panels A–C) illustrates the number of c-Fos-immunoreactive nuclei present in the lateral magnocellular part of the paraventricular nucleus (PaLM) and in two different areas of the SFO: the peripheral region and the core. Panel A shows Fos-IR expression in the PaLM of the various groups studied. In this case, two-way ANOVA showed a significant

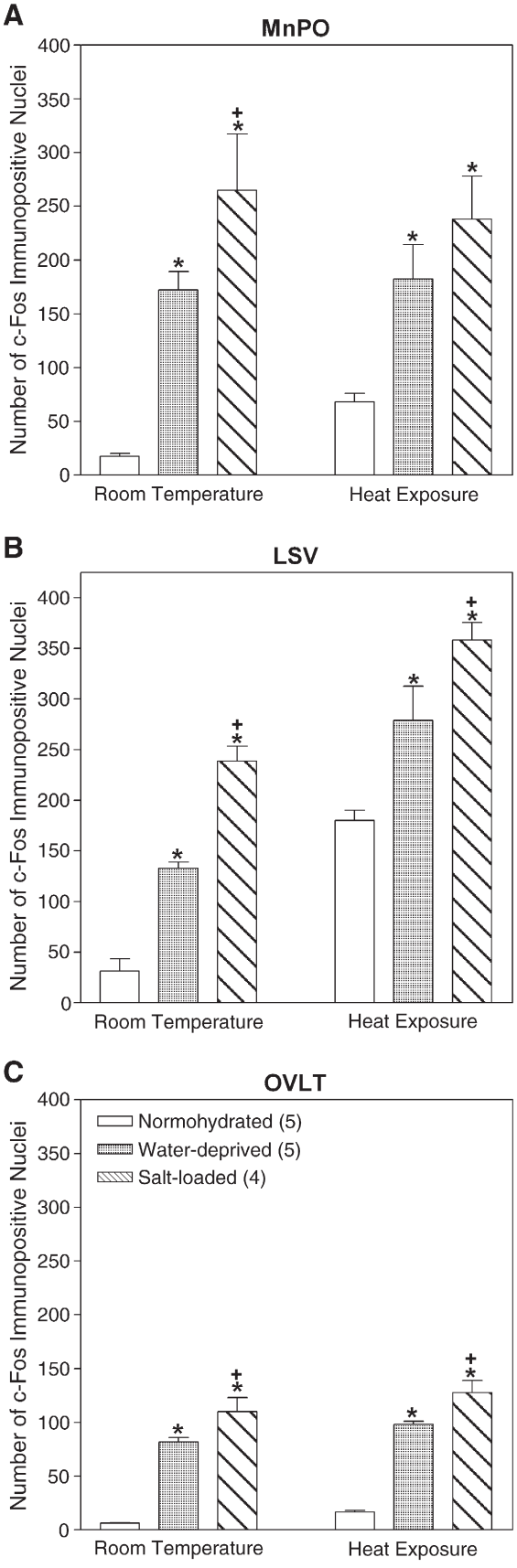
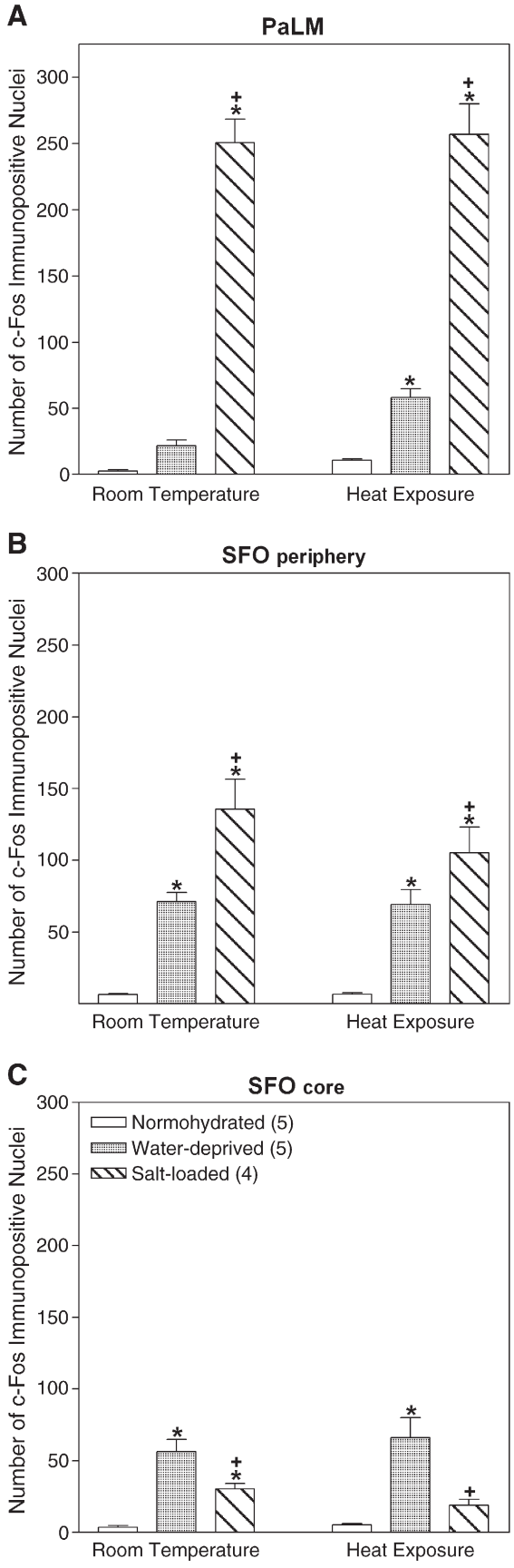


Fig. 2 – Fos-IR (mean ± SEM. number of cell nuclei) in the MnPO-median preoptic nucleus (A), LSV—lateral septal nuclei, ventral part (B) and OVLT—organum vasculosum laminae terminalis (C) of normohydrated, water-deprived and salt-loaded animals submitted to heat (33 °C for 45 min) or kept at room temperature (23 °C for 45 min). The statistically significant differences observed by post-hoc Student–Newman–Keuls test ($p<0.05$): * = significantly different from normohydrated animals in the same temperature condition; + = significantly different from the water-deprived group in the same temperature condition.



hydration main effect but no thermal main effect nor significant hydration × thermal interaction [$F_{(1,2)}=297.76$, $p<0.05$; $F_{(1,2)}=3.86$, ns; $F_{(2,22)}=1.33$, ns, respectively]. Panel B shows Fos-IR in the peripheral region of the SFO after hydration and thermal challenges. Here, two-way ANOVA showed a significant hydration main effect but no thermal main effect nor any significant hydration × thermal interaction [$F_{(1,2)}=53.75$, $p<0.05$; $F_{(1,2)}=1.47$, ns; $F_{(2,22)}=0.33$, ns, respectively]. Panel C shows Fos-IR expression in the core of the SFO of the various groups studied. In this situation, two-way ANOVA again showed a significant hydration main effect but no thermal main effect nor any significant hydration × thermal interaction [$F_{(1,2)}=31.15$, $p<0.05$; $F_{(1,2)}=0.00007$, ns; $F_{(2,22)}=0.93$, ns, respectively]. In the PaLM, and in the periphery and core of the SFO, there was a significant main effect only with respect to hydration. In the PaLM and in the peripheral part of the SFO, salt load resulted in significantly higher levels of Fos-IR compared to water deprivation irrespective of thermal condition, and the normohydrated condition resulted in the lowest levels of Fos-IR. In the core region of the SFO, water deprivation led to significantly higher levels of Fos-IR compared to salt load irrespective of thermal condition, and the normohydrated condition resulted in the lowest levels of Fos-IR.

Photomicrographs exhibiting typical Fos-IR patterns in the PVN, SON and SFO, of normohydrated, water-deprived and salt-loaded animals exposed or not to heat are shown in Fig. 4. Similarly, photomicrographs displaying typical Fos-IR patterns in the MnPO, LSV and OVLT of normohydrated, water-deprived and salt-loaded animals exposed or not to heat, are depicted in Fig. 5.

Table 1 shows the number of c-Fos positive nuclei in the different areas of the brain under two separate conditions (room temperature and following acute heat exposure) in normohydrated animals and in those receiving intragastric isotonic saline solution. In the periphery and in the core of the SFO, neither thermal nor hydration status altered Fos-IR. There was no effect on hydration status in the MnPO, SON, OVLT or PaMP; however, exposure to heat increased Fos-IR in all these areas irrespective of hydrational status; in the PaLM, temperature and hydrational status affected Fos-IR, but there was no interaction. Finally, in the LSV, temperature and hydrational status affected Fos-IR and there also was a temperature-hydrational interaction. In this area, it is important to note that, at room temperature, a significant increase in Fos-IR occurred in animals receiving intragastric isotonic

Fig. 3 – Fos-IR (mean ± SEM. number of cell nuclei) in the PaLM—paraventricular hypothalamic nucleus, lateral magnocellular part (A), SFO—subfornical organ, peripheral part (B) and SFO—subfornical organ, central part (C) of normohydrated, water-deprived and salt-loaded animals submitted to heat (33 °C for 45 min) or kept at room temperature (23 °C for 45 min). The statistically significant differences observed by post-hoc Student–Newman–Keuls test ($p<0.05$): * = significantly different from normohydrated animals in the same temperature condition; + = significantly different from the water-deprived group in the same temperature condition.

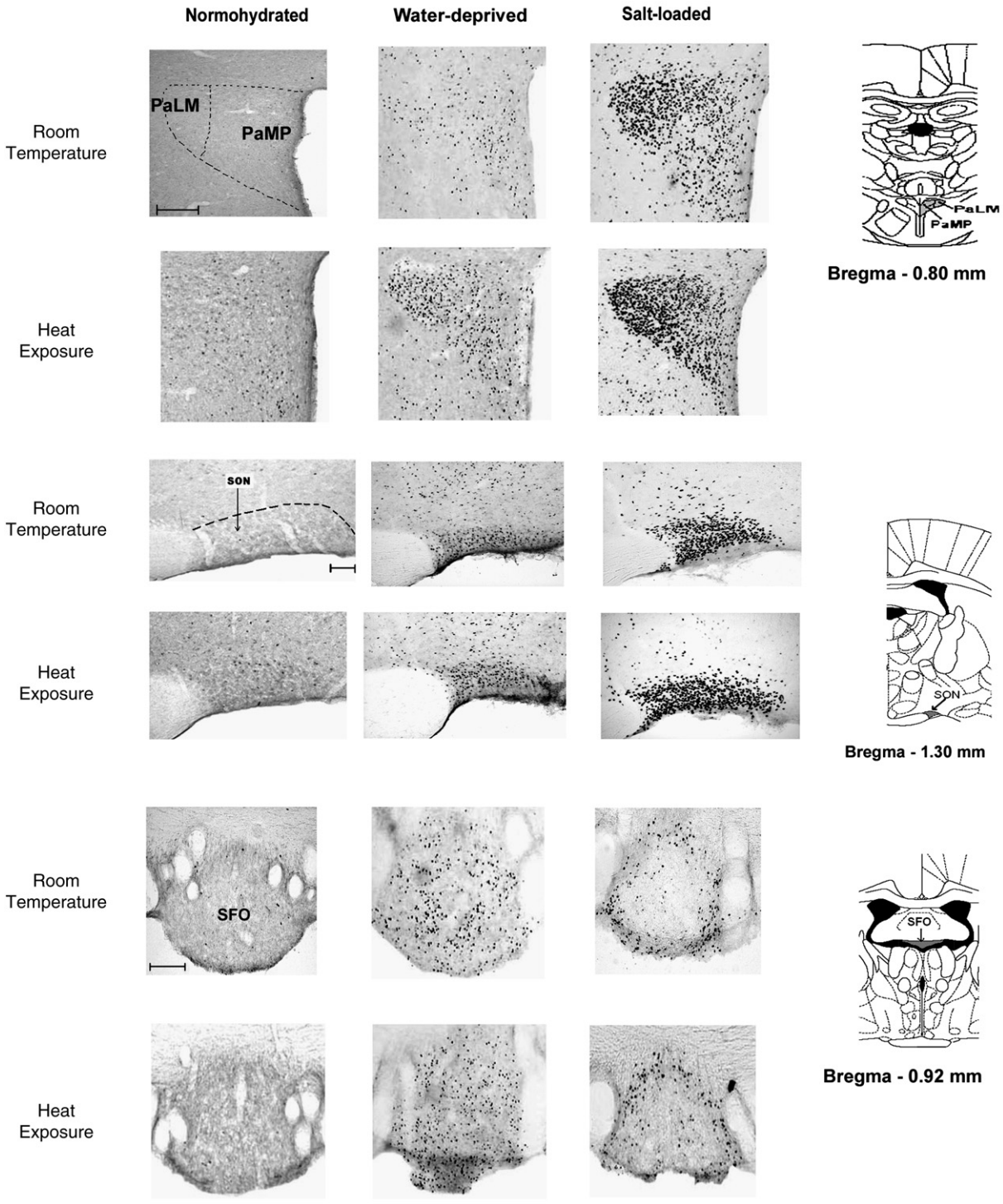


Fig. 4 – Typical photomicrographs of Fos-IR in the paraventricular nuclei (PVN), supraoptic nuclei (SON) and subfornical organ (SFO) of normohydrated, water-deprived and salt-loaded animals submitted to heat (33 °C for 45 min) or kept at room temperature (23 °C for 45 min). Scale bar=500 μm.

saline solution compared to normohydrated controls, while this difference was not found in the animals submitted to heat exposure.

Table 2 summarizes data on body and cage temperatures, serum osmolarity and serum sodium levels in normohydrated, water-deprived and salt-loaded animals submitted or not to acute heat exposure. The data contained in this table show that: (1) compared to normothermic conditions, exposure to heat increased cage temperature, body temperature, serum osmolality and serum sodium levels; (2) water deprivation and the administration of intragastric hypertonic salt load increased serum osmolality and serum sodium levels compared to normohydrated rats in the same thermal conditions. However, the increases found in

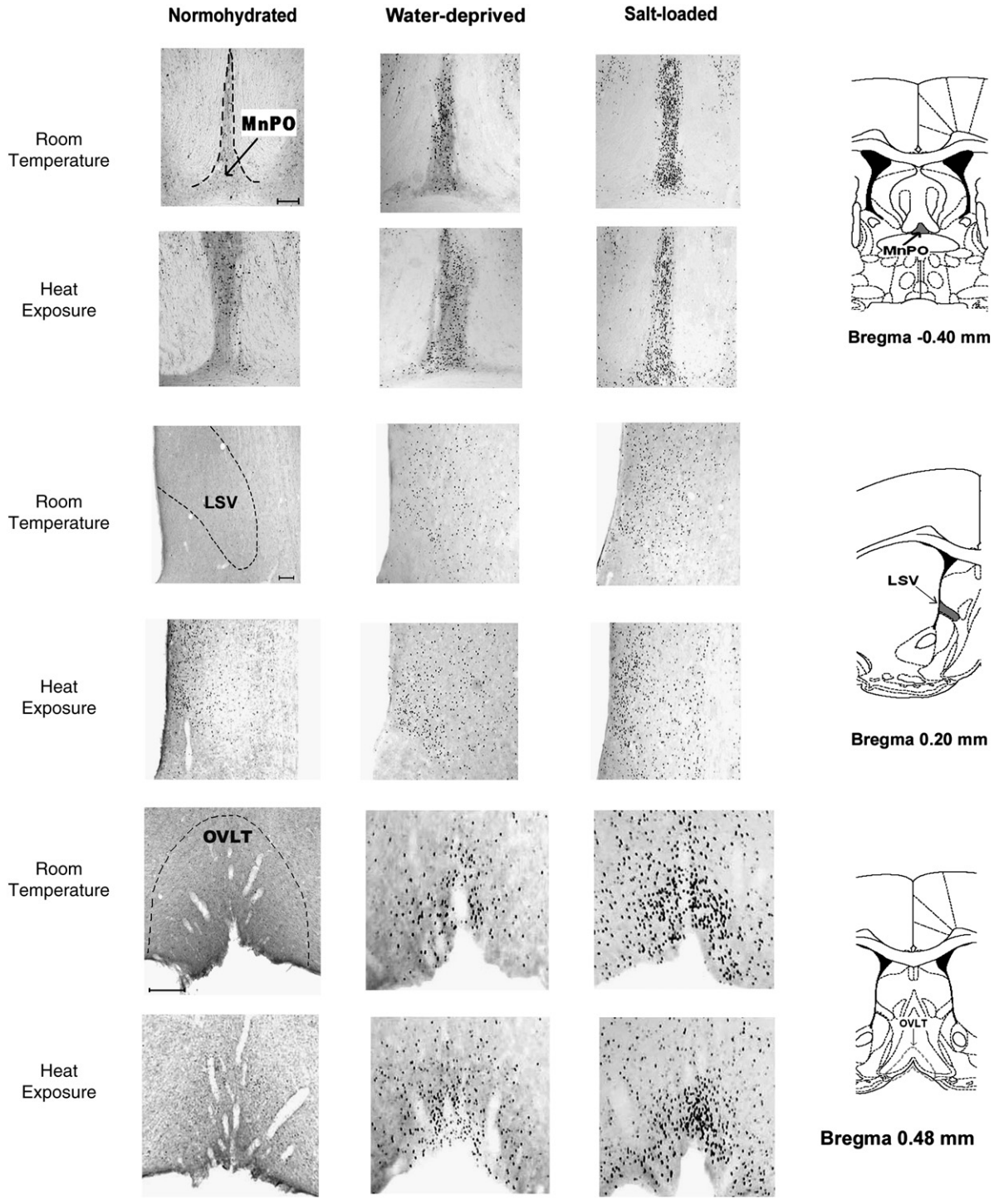


Fig. 5 – Typical photomicrographs of Fos-IR in the median preoptic nucleus (MnPO), lateral septal nuclei, ventral part (LSV) and organum vasculosum laminae terminalis (OVLT) of normohydrated, water-deprived and salt-loaded animals submitted to heat (33 °C for 45 min) or kept at room temperature (23 °C for 45 min). Scale bar = 500 μm.

rats receiving an intragastric hypertonic salt load were greater than those found in water-deprived rats, both in normothermic conditions and following acute heat exposure; (3) under the same thermal conditions, rats receiving an intragastric isotonic saline solution did not differ from normohydrated rats with respect to any of the parameters measured.

3. Discussion

Measurement of brain Fos-IR carried out in the present study clearly shows that, when exposed to changes in thermal and hydration conditions, the prosencephalic regions of rats may be grouped into three different patterns: (1) those that respond

Table 1 – Effects of isotonic saline load and heat exposure (≈33 °C for 45 min) or not (≈24 °C) on the number c-Fos positive nuclei

Area	Hydratation/ Temperature	Normohydrated (5)	Isotonic salt loaded (5)	Two-way ANOVA
MnPO	Normothermia	15.8±2.2	16.6±1.5	Factor A–$F_{(1,1)}$=3.86; ns Factor B–$F_{(1,1)}$=44.5; p=<0.05
	Hyperthermia	68.0±7.9	43.8±8.4	Factor A×Factor B–$F_{(1,16)}$=4.41; ns
LSV	Normothermia	21.4±5.41	76.2± 6.6$^{\#}$	Factor A–$F_{(1,1)}$=10.2; p<0.05 Factor B–$F_{(1,1)}$=233.7; p<0.05
	Hyperthermia	180.2±10.4*	180.2±10.7*	Factor A×Factor B–$F_{(1,16)}$=10.2; p<0.05
SON	Normothermia	1.2±0.6	2.0±0.5	Factor A–F $_{(1,1)}$=2.42; ns Factor B–$F_{(1,1)}$=8.82; p<0.05
	Hyperthermia	3.0±0.1	4.4±0.7	Factor A×Factor B–$F_{(1,16)}$=0.18; ns
SFO core	Normothermia	3.6±1.2	2.8±0.4	Factor A–$F_{(1,1)}$=1.64; ns Factor B–$F_{(1,1)}$=1.64; ns
	Hyperthermia	5.2±1.0	3.6±0.9	Factor A×Factor B–F $_{(1,16)}$=0.183; ns
SFO periphery	Normothermia	6.4±0.7	4.2±0.7	Factor A–$F_{(1,1)}$=9.76; ns Factor B–$F_{(1,1)}$=1.54×10^{-14}; ns
	Hyperthermia	6.6±1.0	4.0±0.4	Factor A×Factor B–F $_{(1,16)}$=0.0678; ns
OVLT	Normothermia	26.2±5.0	21.2±2.8	Factor A–F $_{(1,1)}$=0.49; ns Factor B–$F_{(1,1)}$=25.72; p<0.05
	Hyperthermia	46.0±4.8	59.0±8.5	Factor A×Factor B–F $_{(1,16)}$=2.51; ns
PaLM	Normothermia	2.2±1.2	6.2±1.3	Factor A–F $_{(1,1)}$=5.62; p<0.05 Factor B–$F_{(1,1)}$=37.62; p<0.05
	Hyperthermia	10.8±0.9	12.6±1.4	Factor A×Factor B–$F_{(1,16)}$=0.80; ns
PaMP	Normothermia	12.4±1.3	16.0±2.1	Factor A–$F_{(1,1)}$=0.95; ns Factor B–$F_{(1,1)}$=89.66; p<0.05
	Hyperthermia	43.0±3.3	45.6±4.8	Factor A×Factor B–$F_{(1,16)}$=0.02; ns

The number between parentheses indicates the number of animals used under each condition. In the two-way ANOVA, *Factor A* indicates hydration condition and *Factor B* indicates temperature condition. The statistically significant differences (p<0.05) revealed by the post-hoc Student–Newman–Keuls test are indicated by: *, rats under normothermic condition are compared to rats under hyperthermic condition in the same hydration condition; $^{\#}$, normohydrated rats are compared to rats receiving intragastric isotonic saline in the same thermal condition.

Table 2 – Effects of water-deprivation, salt load and heat exposure (≈33 °C for 45 min) or not (≈24 °C) on body temperature and plasma osmolarity and sodium concentration

	Hydratation/ Temperature	Normohydrated (8)	Water-deprived (8)	Hypertonic salt loaded (8)	Isotonic salt loaded (8)	Two-way ANOVA
Body temperature (°C)	Normothermia	37.5±0.2	37.3±0.2	37.7±0.1	37.7±0.2	Factor A–$F_{(1,3)}$=2.75; p<0.05 Factor B–$F_{(1,3)}$=957.3; p<0.05
	Hyperthermia	40.4±0.1*	41.3±0.1*a	40.8±0.1*b	40.4±0.1*	Factor A×Factor B–$F_{(3,56)}$=8.28; p<0.05
Cage temperature (°C)	Normothermia	24.2±0.1	24.1± 0.1	24.1± 0.1	24.2± 0.1	Factor A–$F_{(1,3)}$=0.857; ns Factor B–$F_{(1,3)}$=17498.1; p<0.05
	Hyperthermia	32.2±0.1	32.1±0.1	32.2±0.1	32.1±0.1	Factor A×Factor B–$F_{(3,56)}$=0.25; ns
Serum osmolarity (mosMo/l)	Normothermia	288.3±0.7	298.1±1.2 *ac*	325.6±0.4 *abc*	290.3±0.9	Factor A–$F_{(1,3)}$=239.45; p<0.05 Factor B–$F_{(1,3)}$=66.45; p<0.05
	Hyperthermia	297.4±2.5*	312.6±1.9**ac*	329.9±1.0**abc*	296.8±2.1*	Factor A×Factor B–$F_{(3,56)}$=4.38; p<0.05
Serum sodium (mEq/l)	Normothermia	134.1±0.4	140.0±0.5 *ac*	143.8±0.3 *abc*	134.4±0.3	Factor A–$F_{(1,3)}$=153.4; p<0.05 Factor B–$F_{(1,3)}$=360.1; p<0.05
	Hyperthermia	141.4±0.4*	145.0±0.4**ac*	146.5±0.5 **abc*	141.5±0.5*	Factor A×Factor B–$F_{(3,56)}$=13.3; p<0.05

The number between parentheses indicates the number of animals used under each condition. In the two-way ANOVA, *Factor A* indicates the hydration condition and *Factor B* indicates the temperature condition. The statistically significant differences (p<0.05) revealed by the post-hoc Student–Newman–Keuls test are indicated by: * comparison between temperature conditions (normothermia×hyperthermia); *a* difference when compared with normohydrated under the same temperature condition; *b* difference when compared with water-deprived under the same temperature condition; *c* indicates significant differences from intragastric isotonic salt load.

to hydration and to heat conditions, with an interaction between these two factors (the PaMP and SON); (2) those that respond to hydration and to heat conditions, but with no interaction between these factors (the MnPO, LSV and OVLT); and (3) those that respond only to hydration status (the SFO and PaLM). These three Fos-IR patterns are discussed separately, as follows.

3.1. *Prosencephalic areas that respond only to hydration status (SFO and PaLM)*

The SFO is a small area in the rostrodorsal part of the third ventricle. Its anatomical localization and high density of capillaries and endothelial fenestrations allow a dynamic relationship between this circumventricular structure and the contents of the cerebrospinal fluid. In addition, it also operates as an interface between the brain and the hemal milieu. The SFO has several afferent and efferent connections with many areas involved in the regulation of hydrosaline balance and blood pressure, such as the OVLT, MnPO, SON and PVN (Phillips et al., 1974; Dellmann and Simpson, 1976; Gutman et al., 1986).

It seems that essential differences do exist between the peripheral and the central parts of the SFO. Indeed, the intraperitoneal injection of hypertonic saline solution leads to an increase in Fos-IR in the peripheral region of the SFO, while hypovolemia enhances Fos-IR in the central part of this structure (Smith and Day, 1995). In addition, studies using double labelling (cFos and AT_{1A} receptors) show that angiotensin II sensitive neurons located in the peripheral region of the SFO are preferentially activated during hypernatremia, while hyponatremia preferentially activates the same type of neuron located in the core of the SFO (Sly et al., 2001; Grob et al., 2004).

Functional specialization would also appear to exist among the subparts of the PVN. The PaLM seems to contain primarily AVP-secreting neurons, while the ring around this sub-region preferentially have neurons that secrete oxytocin (Armstrong, 1995; Sawchenko et al., 1996). In the present paper, in animals submitted or not to heating, salt-load results in a powerful, significant increase in Fos-IR at the PaLM. This may be a consequence of the powerful stimulation of PVN AVP secretion normally induced by hypernatremia.

It is important to note that, in the present study, activation of Fos-IR at the periphery and core of SFO and at the PaLM has the same pattern in animals submitted to heat exposure and in those kept at room temperature. These findings suggest that the SFO and PaLM are regions of the brain that are only involved in the control of hydrosaline balance, remaining unaffected by thermal challenges.

3.2. *Prosencephalic areas that respond to hydration and to heat conditions, but with no interaction between these factors (the MnPO, LSV and OVLT)*

In the MnPO, LSV and OVLT, both thermal and hydration conditions increased Fos-IR, suggesting that these areas are involved in both thermoregulation and body fluid homeostasis. However, the involvement appears to be functionally independent rather than integrative, as demonstrated by the absence of a statistically significant interaction.

In all areas of this group (MnPO, LSV and OVLT), both water deprivation and intragastric salt load were able to increase Fos-IR in animals submitted or not to heat exposure. Under all the conditions studied in this group, salt-load induced an increase in Fos-IR that was significantly higher than that observed in water-deprived animals, the only exception being that salt-loaded and water-deprived rats had similar levels of Fos-IR at the MnPO following heat exposure.

Indeed, the MnPO is sensitive to the actions of angiotensin II (Stocker and Toney, 2005), and plays a pivotal role in the generation of visceral and behavioral thermoregulatory responses (Maruyama et al., 2003; Patronas et al., 1998). The involvement of MnPO in thermoregulatory processes is well known and has been associated with the generation of various behavioral and visceral thermoregulatory responses in rats (Maruyama et al., 2003; Patronas et al., 1998).

The LSV is an area that is normally considered to be involved in the regulation of body fluid homeostasis, participating in many crucial behavioral and visceral responses leading to the correction of hydrosaline disturbances, such as angiotensin II-induced water intake (De Arruda et al., 2003). However, studies on the involvement of the LSV in thermoregulatory responses are sparse. In any case, the lateral septum appears to modulate fever (Blatteis and Sehic, 1997). The findings on LSV presented here are in agreement with data published by other investigators showing that this area is involved in the central perception and integration of thermoregulatory signals (Patronas et al., 1998), and also participates in behavioral responses leading to body fluid homeostasis such as water intake (Abrao Saad et al., 2004).

The OVLT is a circumventricular area that functions as a receptor of bloodborne information. It receives and processes data related to intra- and extracellular volume and blood pressure. The OVLT is the circumventricular structure consistently destroyed in AV3V lesions, a procedure that mediates a series of AVP-dependent responses and drinking. Moreover, hypertonic saline solution has been shown to depolarize OVLT neurons *in vitro*, additional proof of the osmosensitivity of this structure (McKinley et al., 1990, 1999). In addition, the OVLT seems to be involved in the regulation of body temperature during fever and triggers thermoregulatory behavior in rodents (Hubschle et al., 2001; Blatteis and Sehic, 1997).

The data presented here confirm the participation of the MnPO, LSV and OVLT both in thermoregulatory actions and in the control of body fluid homeostasis, and suggest that these areas regulate these events independently.

3.3. *Prosencephalic areas that respond to hydration and to heat conditions, with an interaction between these two factors (the PaMP and the SON)*

At the PaMP and the SON, both thermal and hydration challenges are capable of increasing Fos-IR. Indeed, in these regions, water deprivation and salt-load increased Fos-IR in rats maintained at room temperature and in rats submitted to acute heat exposure. However, there were also differences between these areas.

At the PaMP, under all hydration conditions, Fos-IR after heat exposure was greater than that of rats maintained at room temperature. Under normohydrated conditions, this finding suggests that the area responds to thermal input in the absence of hydration challenges. The greater increase in Fos-IR in response to hydration challenges following heat exposure was not simply additive, and suggests an anatomical and functional overlap between thermo- and fluid-regulatory mechanisms. Following heat exposure, salt load resulted in higher Fos-IR levels than those induced by water deprivation only, suggesting that (a) the combination of hyperthermia with this pronounced hyperosmolality activates the greatest number of neurons and (b) the increase in heat-exposed rats following water deprivation is not the result of a 'ceiling effect'.

The PVN sub-region known as the PaMP contains AVP-secreting neurons in which the CRH is co-localized (Armstrong, 1995). This sub-region of the PVN is associated with the control of body fluid homeostasis in normothermic animals, a very well-known phenomenon (Kiss, 1988, McKinley et al., 1992). It has also been well established that the PVN is involved in thermoregulation by activating heat-loss mechanisms and by inhibiting heat-producing systems (Smith et al., 1998; Sakaguchi et al., 1988). In this study, Fos-IR in water-deprived, salt-loaded animals not submitted to heating was significantly lower than that of water-deprived, salt-loaded animals submitted to acute heat exposure, a finding that confirms the thermoregulatory role of the PVN.

Many of the neurons that use CRH as a neurotransmitter are observed in the PaMP (Kiss et al., 1991). The action of the hypothalamus–pituitary–adrenal axis on salt intake is rather complex and there are indications that ACTH and stress increase salt intake (Weisinger et al., 1978; Denton et al., 1999). However, we have recently demonstrated that, at least in the lateral parabrachial nucleus, CRH potently inhibits sodium intake (De Castro e Silva et al., 2006). Hence, it is not unreasonable to suggest that the high activation of Fos-IR in the PaMP in salt-loaded animals reflects activation of CRH neurons in this region, leading to a decrease in sodium appetite. Therefore, the present data confirm the importance of the PVN as a hypothalamic region controlling both body temperature and hydro-electrolyte parameters, and suggest the prominent participation of one of its sub-regions, the PaMP, in these processes.

The very low level of Fos-IR in the SON expressed by normohydrated animals kept at room temperature is not modified by exposure to heat. This clearly indicates that the SON does not participate in the mechanisms regulating thermoregulatory events in the absence of hydration challenges. Salt load increased Fos-IR in the SON to a significantly higher level than that observed during water deprivation in animals not submitted to heating. This may indicate that additional, corrective circuits are activated in this region when hypernatremia is present. Also, water-deprived animals submitted to acute heat exposure have significantly higher Fos-IR levels in the SON than those observed in water-deprived rats kept at room temperature. This suggests that, in this area, the association of hypernatremia and hypovolemia (a condition that prevails after water deprivation), when further associated with hyperthermia, activates additional circuits that are inactive when water deprivation is not associated with hyperthermia.

Several findings indicate that the SON participates in thermoregulatory events. Indeed, it has recently been suggested that thermal information from the preoptic area sends excitatory signals to the SON (Yoshida et al., 2002), that cholinergic inputs to the supraoptic nucleus increase Fos expression and body temperature in rats (Takahashi et al., 2001a), and that neurons located in the SON increase non-shivering thermogenesis in brown adipose tissue by activating sympathetic pathways (Takahashi et al., 2001b). Therefore, it seems rational to suggest that this type of heat-producing circuitry is inactive in animals undergoing an increase in body temperature due to exposure to a warm environment. It is also reasonable to suggest that the SON does not contain circuits commanding strategic heat loss responses that are normally activated in the course of acute heat exposure. In the PaMP, on the other hand, an increase occurs in the Fos-IR levels of normohydrated animals exposed to heating, as seen above. The existence of heat loss mechanisms activated by the PVN may justify the increase in Fos-IR in the PaMP of normohydrated animals exposed to heating (Smith et al., 1998; Hubschle et al., 2001).

Therefore, the data presented here confirm the participation of the PaMP and the SON in the regulation of body fluid homeostasis and thermoregulation and confirm that a clear interconnection exists between osmo- and thermoregulatory mechanisms in these particular regions.

3.4. *General discussion*

Thermoregulation and body fluid homeostasis are based on interactive and interdependent mechanisms. Thermoregulatory responses may influence body fluid homeostasis while, at the same time, they depend on the nature of pre-existing hydration conditions. Also, it is well known that osmotic factors modulate brain mechanisms involved with thermoregulation. Sweating, a thermoregulatory response, induces hyperosmolarity, leading to cellular dehydration and to a net expansion of plasma volume, at least in the initial phase of thermal dehydration, since the hyperosmotic plasma retains water coming from the intracellular space. On the other hand, plasma hyperosmolarity associated with hypovolemia increases body temperature since it reduces evaporative cooling (Morimoto, 1990). In addition, osmoreceptors that activate thirst-triggering mechanisms and induce vasopressin secretion are involved in the osmotic inhibition of thermoregulatory responses such as sweating or the rate of evaporation (Takamata et al., 1995). Evaporative heat loss in response to hypothalamic heating decreases in dehydrated cats and in cats receiving hypertonic saline infusions (Baker and Doris, 1982a,b; Doris and Baker, 1981). Furthermore, the administration of hypertonic solutions into the third ventricle decreases sweating in heat-stressed monkeys (Owen et al., 1989). Therefore, a clear osmoregulatory modulation of thermoregulatory responses does exist and vice-versa.

Thermoregulatory mechanisms are operated by a myriad of central areas. Indeed, the MnPO, LSV, OVLT, PVN, SON, zona incerta, ventromedial and dorsomedial hypothalamus, parabrachial nucleus, periaqueductal gray matter, amygdala,

suprachiasmatic nucleus and the nucleus of the solitary tract are all involved in thermoregulation. The MnPO is a thermosensitive area that commands vasoactive responses, sweating and salivation. The LSV and the OVLT modulate fever response (Blatteis and Sehic, 1997). Hypothalamic drives controlling sweating travels through the zona incerta to the lower pons and to the medulla oblongata and descend along the spinothalamic tract to reach the intermediolateral cell column in the spinal cord (Saito and Kogure, 1986). The dorsomedial hypothalamus is involved in shivering thermogenesis while the NTS and the ventromedial hypothalamus are related to non-shivering thermogenesis (Thornhill and Halvorson, 1990; Friedman and Wellman, 1987).

Peripheral and central osmoreceptors (OVLT and MnPO) detects changes in plasma or CSF osmolality and convey this information to PVN and SON, helping to regulate thirst and vasopressin release. The SFO and the AV3V region are sensitive to changes in blood borne angiotensin and linked to thirst generation. In addition, many other central areas such as the amygdala, LPBN, NTS and area postrema are involved both in the reception of peripheral information on body fluid status and in the generation of corrective responses to maintain body fluid homeostasis (Johnson and Thunhorst, 1997; Rinaman et al., 1997; Morien et al., 1999).

The interaction between the several brain areas involved in body fluid homeostasis and in thermoregulation has already been demonstrated. Indeed, hyperosmotic stimuli decrease the activity of thermosensitive neurons at the hypothalamic preoptic area, leading to a reduction in sweating when dehydrated animals submitted to heat stress are compared with dehydrated animals kept at room temperature (Hori et al., 1988). Moreover, using an experimental protocol that is similar to that used in the present study based on the expression of Fos-IR in the rat brain, some investigators have shown that the MnPO is an area that is activated separately both by thermal and osmotic stimuli (Patronas et al., 1998), a pattern that was restricted to the PaMP, SON, LSV and MnPO in the present study. In that same study, a significant increase in Fos-IR levels was observed in many of the central areas that responded to heat (OVLT, SFO, MnPO, PVN, SON, LSV and LHA) when heat exposure was combined with dehydration.

It is important to note that in general the data obtained in the present study is in agreement with the study by Patronas et al. (1998). However, we have added new information concerning the association between hyperthermia and hyperosmolarity, an aspect that was not investigated by those authors and that may be important to understand the pathophysiological situations in which heat exposure and hyperosmolarity coexist. It also important to note that, these investigators used longer periods of heat exposure (48 h at 34 °C) and water deprivation (24 h) an experimental condition that may explain some differences in the results obtained by the two studies.

In the present experiments, both hypernatremia and hyperosmolarity were found in water-deprived and salt-loaded rats. All animals submitted to acute heat exposure were significantly hyperthermic, with body temperatures between 40.4 and 41.3 °C. Fluid loss associated with heat exposure is a condition that promotes potentiation of hyperthermia (Barney and Folkerts, 1995) and this may explain why water-deprived rats exposed to heat are significantly more hyperthermic than normohydrated and salt-loaded ones.

Thermal dehydration and dehydration induced by water deprivation represent different phenomena. During water deprivation, the loss of water from cellular and extracellular compartments activates the renin-angiotensin system and stimulates low-pressure atrial baroreceptors. During short periods of thermal dehydration, plasma volume is maintained, the renin-angiotensin system is not activated and plasma hyperosmolarity plays a pivotal role in triggering thirst (Barney, 1997; Barney and West, 1990; Kregel et al., 1990; Rolls et al., 1980). This justifies the experimental models used in this study. Brain responses were studied following short-term heat exposure in normohydrated, dehydrated (water-deprived) and salt-loaded animals exposed or not to heat because these physiological states are distinct and trigger selective corrective mechanisms.

Normohydration, water deprivation and intragastric salt load are distinct physiological situations, and animals in each one of these conditions, submitted or not to acute heat exposure, represent a rather different combination of adaptive and corrective physiological and behavioral responses. However, it is impossible to determine the contribution of hydration and thermal conditions, taken together or individually, in generating the several Fos-IR patterns observed in each of the areas studied using an experimental model that does not phenotypically identify the cell types whose nuclei were Fos-positive.

The procedures required to carry out intragastric salt loading may cause some stress to the animals that could lead to a non-specific, stress-related increase in Fos-IR in some of the areas analyzed in the present study. To rule out this possibility, we compared Fos-IR in all areas in two separate groups of animals: normohydrated rats and those receiving intragastric administration of isotonic saline solution under the same conditions used to induce salt load in the groups of animals receiving intragastric hypertonic saline. As seen in Table 1, the intragastric administration of isotonic saline solution induced no significant increase in Fos-IR in most areas studied as compared to normohydrated rats (a group not submitted to intragastric injection), except in two areas (the LSV and the PaLM) in which Fos-IR increased after this procedure in animals kept at room temperature. Therefore, we cannot exclude the possibility that, in these particular areas and under these circumstances, the increase in Fos-IR may be partially stress-dependent. It is also important to note that the experimental protocol used to induce acute heat exposure may lead to some degree of stress that could alter Fos-IR levels in some brain regions.

In this study, Fos-IR patterns in some prosencephalic areas are described following different experimental protocols. We are aware that analyses of brain Fos-IR in experimental protocols in which no specific stimulation of a selective neurochemical system is made offer particular challenges. In such cases, the demonstration of the presence of Fos-IR in a particular brain area simply indicates that some local neurons have been activated. It does not contribute to the understanding of the neurochemical nature of these particular

nerve cells. Neurons sending stimulatory or inhibitory inputs to other areas and interneurons modulating activation or inhibition of other local neurons may all contribute to the expression of Fos-IR at a given location. For this reason, we chose not to extend our discussion with speculations on the physiological roles played by each of those Fos-positive areas.

In summary, the present study describes the characteristic patterns of Fos-IR in prosencephalic areas in normohydrated, dehydrated and salt-loaded rats submitted or not to acute heat exposure, and demonstrates an anatomical and functional overlap between thermoregulation and body fluid homeostasis in these two areas (the SON and the PaMP).

The clinical association between hyperthermic conditions (fever, exposure to warm environments, exercise) and dehydration is frequent and may involve difficulty in eliciting corrective strategies for some human groups such as infants and children, the elderly, athletes, laborers and military personnel working in the heat (Anderson et al., 1983; Hales and Sakurada, 1998). Combined heat stress and dehydration has been shown to impair aerobic performance (Nevill, 1996), and the association of heat exposure and dehydration leads to progressive increases in both heart rate and core temperature. Furthermore, dehydration occurring during moderately intense, prolonged exercise in the heat reduces blood flow to active muscles, and elevates carbohydrate oxidation and lactate production (Gonzalez-Alonso et al., 1999). For all these reasons, understanding the aspects of the brain that deal with the integration of corrective responses evoked by thermal and hydrosaline challenges is of both clinical and physiological importance.

4. Experimental procedures

4.1. *Animals*

Adult male Wistar rats weighing 270±30 g, were used in this study and were kept under controlled light (lights on from 5 AM to 7 PM) and temperature (22–24 °C) conditions. The animals had free access to water and laboratory chow (Nuvital Nutrientes Ltd., Curitiba, Brazil) in the days prior to the experiments. They were handled daily for 5 days in order to minimize stress during the experimental sessions. The experimental protocols were conducted according to the regulations established by the National Institutes of Health (USA) and were approved by a local committee that analyzes ethical aspects of research in laboratory animals.

4.2. *Experimental design*

Fos-IR was analyzed in the brains of rats under 3 different conditions: normohydration, water-deprivation (rats submitted to an overnight, 14-h period of water deprivation with food available) and after an acute intragastric salt load. The groups of animals submitted to each one of these conditions were subdivided into two separate subgroups: those maintained at room temperature (23±2 °C) and those exposed to a hyperthermic environment (33 °C for 45 min).

4.3. *Intragastric salt load*

Intragastric salt load was achieved by administering 1 ml/100 g of a hypertonic saline solution (1.5 M) via orogastric tubing. The animals were fasted for 14 h (from 6 PM to 8 AM) the night preceding the experiments. They received an intragastric salt load 30 min before exposure to acute heating. This 30-min period after intragastric salt load is sufficient to induce significant increases in serum sodium concentration and osmolarity. The groups of animals receiving the intragastric salt load were also compared to a separate group of animals receiving intragastric isotonic saline solution under the same conditions as the salt-loaded animals.

4.4. *Heating protocol*

The heating protocol used here follows procedures already described elsewhere (Kregel et al., 1990). Briefly, rats were placed in the experimental cages (30×30×50 cm) at the beginning of the experiments after being weighed. A period of 25–30 min was allowed to stabilize body temperature before heating began. Heating (maximum temperature of 33 °C for 45 min) was achieved using a 250 W infrared lamp positioned approximately 40 cm above the animal. A thermistor probe inside the cage monitored the cage temperature. A flexible thermistor probe was inserted 6–7 cm into the colon and taped to the base of the tail. The thermistor probes were connected to a temperature-recording device (Minipa Thermometer, model MT520) that indicated body core temperature (Tc) continuously on a digital display. Control rats kept at room temperature were put in experimental cages during the experimental sessions and were also equipped with a thermistor probe.

4.5. *Measurement of serum sodium levels and osmolarity*

Separate groups of animals (normohydrated, water-deprived and salt-loaded rats, and rats receiving intragastric isotonic saline solution), kept at room temperature, were anesthetized and had blood samples collected from the abdominal aorta for the measurement of serum osmolarity and sodium concentration. These groups were compared to other groups of normohydrated, water-deprived, salt-loaded rats and to rats receiving intragastric isotonic saline solution from which blood samples were collected from the abdominal aorta immediately after exposure to heating. After centrifugation, serum was used for the measurement of osmolarity by determining freezing point (Osmette Precision System, model 2007, MA USA). Sodium concentration was measured by flame photometry (Micronal, model B262, SP, Brazil).

4.6. *Animal perfusion and histology*

In all groups studied, the animals were anesthetized (thionembutal, 50 mg/kg, i.p.) immediately after the experimental sessions (heating or maintenance at room temperature) and transcardially perfused with 400 ml of phosphate buffered saline (PBS) 0.1 M (pH 7.4) followed by 4% paraformaldehyde (pH 7.4). After these procedures, the brains were removed and stored overnight in the same fixative at 4 °C and then submerged in 30% sucrose solution for at least 2 days. The

prosencephalic regions of the brain were serially sectioned at 40 μm in a cryostat.

4.7. *Immunohistochemistry*

The free floating sections were rinsed three times for 5 min in 0.01 M phosphate buffer (PB). After this initial washing step, tissue sections were incubated in PB containing 1% hydrogen peroxide for 15 min to block endogenous peroxidase activity. Sections were then washed a further three times in PB and incubated in PB containing 5% normal goat serum for 1 h. Next, this solution was replaced by PB with 0.3% Triton X-100 (Sigma Co., St. Louis, MO, USA) and Fos primary antisera diluted 1:4000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at room temperature. The sections were washed a further three times with PB and incubated with a biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA, USA) diluted 1:400 in PB for 1 h at room temperature. Again, the sections were rinsed three times with PB and finally incubated at room temperature for 60 min with avidin-biotinylated horseradish peroxidase complex (Avidin–Biotin Complex-Kit, Vector, USA) diluted 1:200 in PB. After a final three washes, sections were incubated in a PB solution containing 0.02% 3,3′-diaminobenzidine-tetrahydrochloride (DAB; Sigma Co., St. Louis, MO, USA), 0.08% nickel sulfate and 0.0002% hydrogen peroxide for 10 min. The enzymatic peroxidase reaction was stopped by rinsing the sections with PB. Immunohistochemistry was carried out simultaneously on the brains of experimental and control animals.

Following these procedures, the brain sections were mounted on gelatin-coated glass slides and allowed to dry overnight, soaked for 20 min in 100% xylene and cover-slipped with Permount (Fischer Scientific International Inc., NJ, USA).

A preliminary qualitative analysis identified the prosencephalic brain regions in which c-Fos expression was detected under the various conditions studied. In these areas, the number of Fos-positive nuclei was counted under microscopy using a computerized image analysis system (Image-Pro Plus, Media Cybernetic, Inc., Silver Spring, MD, USA). Fos-IR was measured in one section of each area of interest; in each case, the section which appeared to have the greatest number of Fos-positive nuclei. In the case of the PVN, Fos-positive nuclei were counted in two different parts: the lateral magnocellular and the medial parvicellular. Also, Fos-IR was counted in two different parts of the SFO: in the core and in the peripheral region. The system was calibrated to ignore background staining. Cells containing a nuclear brown-black reaction product were considered positive for Fos immunoreactivity. Neuroanatomical sites were identified with the help of Paxinos and Watson's Atlas (1998).

4.8. *Statistical analysis*

A computer software package (SigmaStat for Windows, Jandel Scientific, San Rafael-CA, USA) was used to carry out two-way analyses of variance for each area studied, using hydration and thermal conditions as factors, followed by the post-hoc Student–Newman–Keuls test for comparison of each group. When interaction between the factors (hydration and thermal) was not present, only intragroup comparison was performed (hydration). The data are presented as mean ± SEM Fos-IR cells for each particular area studied. The groups were considered significantly different when $p<0.05$; non significant differences are indicated as *ns*.

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