Water deprivation increases Fos expression in hypothalamic corticotropin-releasing factor neurons induced by right atrial distension in awake rats

Mauricio Benedetti, Rodrigo Rorato, Margaret Castro, Benedito H. Machado, Jose Antunes-Rodrigues, and Lucila L. K. Elias

Departments of 1Internal Medicine and 2Physiology, School of Medicine of Ribeirao Preto, University of Sao Paulo, Sao Paulo, Brazil

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Water deprivation increases Fos expression in hypothalamic corticotropin-releasing factor neurons induced by right atrial distension in awake rats. Am J Physiol Regul Integr Comp Physiol 295: R1706–R1712, 2008. First published September 10, 2008.—Atrial mechanoreceptors, sensitive to stretch, contribute to regulating heart rate and intravascular volume. The information from those receptors reaches the nucleus tractus solitarius and then the paraventricular nucleus (PVN), known to have a crucial role in the regulation of cardiovascular function. Neurons in the PVN synthesize CRF, AVP, and oxytocin (OT). Stimulation of atrial mechanoreceptors was performed in awake rats implanted with a balloon at the junction of the superior vena cava and right atrium. Plasma ACTH, AVP, and OT concentrations and Fos, CRF, AVP, and OT immunolabeling in the PVN were determined after balloon inflation in hydrated and water-deprived rats. The distension of the balloon increased the plasma ACTH concentrations, which were higher in water-deprived than in hydrated rats (P < 0.05). In addition, the distension in the water-deprived group decreased plasma AVP concentrations (P < 0.05), compared with the respective control group. The distension increased the number of Fos- and double-labeled Fos/CRF neurons in the parvocellular PVN, which was higher in the water-deprived than in the hydrated group (P < 0.01). There was no difference in the Fos expression in magnocellular PVN neurons after distension in hydrated and water-deprived groups, compared with respective controls. In conclusion, parvocellular CRF neurons showed an increase of Fos expression induced by stimulation of right atrial mechanoreceptors, suggesting that CRF participates in the cardiovascular reflex adjustments elicited by volume loading. Activation of CRF neurons in the PVN by cardiovascular reflex is affected by osmotic stimulation.

bridge in dogs (9), and it was also described in other species, including humans (10, 12, 34, 62).

The atrial mechanoreceptors are located at the venous-atrial junction of the heart, and it is continuously signaling the central venous volume to the brain. Through vagal afferent projections, the information from the atrial mechanoreceptors reaches the nucleus tractus solitarius (NTS) in the brain stem, and then the paraventricular nucleus (PVN) of the hypothalamus (4, 17). A specific subset of parvocellular neurons in the PVN, with direct projections to sympathetic preganglionic neurons in the spinal cord, has been implicated in basal and reflex control of the sympathetic nervous system (6, 16, 38, 46, 51). Previous studies have indicated that PVN tonically influences blood pressure and heart rate through activation of sympathetic nerve discharge, and this effect is modulated by a tonic GABA-ergic inhibition (1, 2, 15, 39). Selective activation of atrial mechanoreceptors has been described in response to right atrial distension by inflation of a small balloon placed at the superior vena cava-right atrium junction, without obstruction of blood flow to the heart (37). Using this method, others have described an activation of parvocellular neurons (21, 47) and an inhibition of magnocellular neurons in the PVN (29).

In the parvocellular subdivision of the PVN, vasopressin- and oxytocin-expressing neurons have been suggested to be involved in the control of cardiac and renal sympathetic outflow and the subsequent increase in heart rate, urine flow, and natriuresis, following the stimulation of volume receptors (17). In addition to vasopressin and oxytocin, parvocellular neurons in the PVN also synthesize CRF, which has also been shown to activate the sympathetic outflow and the cardiovascular function (23).

CRF is the main activator of the HPA axis (61). It is well established that CRF plays a key role in the stress-induced response other than activating HPA axis, including activation of the locus coeruleus, sympathetic nervous system, and the adrenal medulla (27). In fact, it was demonstrated that CRF antagonist reduces the stress-induced ACTH and tachycardia responses, confirming that endogenous CRF participates in the stress-induced adaptive responses (41). However, there are no data on the involvement of CRF neuron in the cardiovascular adjustments in response to activation of atrial mechanoreceptors. Therefore, this study aimed to investigate the activity of CRF neurons in the PVN, following right atrial distension. To ascertain the interplay between CRF, vasopressin, and oxytocin neurons in the cardiovascular adjustments induced by stimula-

Changes in blood volume or arterial and venous pressure lead to activation of physiological responses to restore cardiovascular homeostasis. These responses involve regulatory adjustments that are detected by arterial baroreceptors and cardiopulmonary mechanoreceptors (19, 53). Hemorrhage produces unloading of arterial baroreceptors that initiate a reflex response to increase the sympathetic vasomotor activity and vasopressin secretion (19, 52). On the other side, blood volume expansion activates atrial mechanoreceptors, with an increase of heart rate, inhibition of renal sympathetic nerve activity, and a decrease of vasopressin release (5, 30). The reflex tachycardia in response to hyperemia was first described by Bain...
tion of atrial mechanoreceptors, the experimental protocols were undertaken in awake hydrated and water-deprived rats.

MATERIAL AND METHODS

Male Wistar rats weighing 250–280 g were individually housed in a controlled environment with a light-dark cycle of 12:12-h light-dark cycle (light on at 6:00 AM and light off at 6:00 PM). Animals were given at least 7 days to acclimate to the housing quarters and had free access to standard rat food and water, except in the water deprivation protocol. All procedures were carried out between 8:00 and 11:00 AM. All the experimental protocols were approved by the Ethical Committee for Animal Use of the School of Medicine of Ribeirão Preto, University of São Paulo (protocol number 037/2005).

Experimental Protocols

After the acclimatation, rats were anesthetized with 2.5% tribromoethanol (TBE; Aldrich, Milwaukee, WI; 1 ml/100 g body wt ip) and had a Silastic cannula (ID 0.51 mm, OD 0.94 mm; Dow Corning, Auburn, MI), with an inflatable balloon at the end, inserted in the right jugular vein, and advanced to the right superior vena cava and right atria junction (37). Inflation of a balloon placed in the superior vena cava does not affect venous return in rats, since blood from the head drains through the left jugular vein, which drains into the inferior vena cava (37). After surgery, the rats were allowed to recover for 3 days.

Arterial pressure and heart rate recordings. In this protocol, 3 days before the experiments, hydrated rats had the balloon placed at the vena cava and right atria junction. On the day before the experiments, rats were anesthetized with TBE, and a catheter was inserted into the abdominal aorta through the femoral artery [polyethylene (PE)-10 connected to PE-50 tubing; Clay Adams, Parsippany, NJ] for the arterial pressure measurement. The catheters were tunneled subcutaneously and exteriorized through the back of the neck. On the next day, the arterial catheter was connected to a pressure transducer (model CDX III, Cobe Laboratories, Lakewood, CO), which was connected to a physiological recorder (Narcotrace 80, Narco Biosystems, Austin, TX). Heart rate (HR) was derived from the interval between arterial pressure pulses with a biotachometer coupler (Narco Bio-Systems, model 7302). The arterial catheter used for recording median arterial pressure (MAP) and HR was suspended, enabling animals to move freely. The cardiovascular parameters were recorded in conscious freely moving rats. MAP and HR were recorded after 15 min of resting (Basal), during the distension of the balloon for 1 min using saline (50 μl), and for at least 5 min after the end of the distension.

Effect of right atrial distension on plasma ACTH, vasopressin, and oxytocin concentrations in hydrated and water-deprived rats. In this protocol, freely moving rats were assigned into two groups: 1) rats with a balloon, but not subjected to inflation procedure, and 2) rats subjected to right atrial distension for 1 min, using saline (50 μl) for balloon inflation, and decapitated 5 min after the end of distension. Another set of rats was subjected to the same procedures, after 24 h of water deprivation. Blood samples were obtained by decapitation for determination of plasma ACTH, vasopressin, and oxytocin concentration.

Effect of right atrial distension on the Fos/CRF, Fos/AVP, and Fos/OT immunoreactivity in hydrated and water-deprived rats. In this protocol, freely moving rats were subjected or not to the inflation-deflation procedure. We observed a slight increase of Fos expression in the PVN after single balloon inflation during 1 min. Because this stimulus is very subtle, a repeated inflation-and-deflation procedure was used, as described by Pyner and Coote (46). This procedure consisted of 1-min inflation following 2-min deflation, and it was repeated five times. Two hours after the last inflation, rats were anesthetized with an overdose of TBE and transcardially perfused with 200 ml of saline, followed by 400 ml of 4% paraformaldehyde in 0.1 M phosphate buffer. Brain was collected, postfixed in 4% paraformaldehyde for 4 h, placed in PBS containing 30% sucrose and stored at 4°C. Another set of rats was subjected to 24 h of water deprivation and thereafter was subjected to the same inflation-deflation procedure, transcardiac perfusion, and brain tissue collection.

At the end of each experiment, the chest was opened, and the position of the tip of cannula was verified. Each balloon was inflated with 50 μl of saline, as used during the experiments. To be included in the analysis, the correctly placed balloon was shown to stretch the vena cava and right atrium junction. Rats with incorrect balloon placement or rats with a balloon that did not inflate were not included in the study.

Radioimmunoassays

Plasma ACTH, AVP, and OT levels were measured by double antibody-specific RIAs, as previously described (22, 30). The assay sensitivity, intra-assay, and interassay coefficients of variations were 10 pg/ml, 6.3% and 14% for ACTH; 0.9 pg/ml, 7.7% and 11.9% for AVP; and 0.9 pg/ml, 7.0% and 12.6% for OT.

Immunohistochemistry

Free-floating frozen brain coronal sections (30 μm) were prepared using a microtome. The sections from the brain were stained for Fos, AVP, OT, and CRF immunoreactivity. The staining procedures were based on the double-labeling procedure described in Godino et al. (26). Briefly, free-floating sections were incubated overnight at room temperature with a rabbit anti-Fos antibody (Ab-5; Oncogene Science, Manhasset, NY), diluted 1:10,000 in PB containing 2% normal goat serum (Gibco, Auckland, NZ), and 0.3% Triton X-100 (Sigma Chemical, St. Louis, MO). The sections were then washed with PB and incubated with biotin-labeled goat anti-rabbit IgG and the avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA; 1:200 in 1% NHS-PB) for 1 h at room temperature. The peroxidase label was detected using diaminobenzidine hydrochloride (DAB; Sigma Chemical, St. Louis, MO), intensified with 1% cobalt chloride and 1% nickel ammonium sulfate. This method produces a blue-black nuclear reaction product. The Fos-labeled sections, also processed for OT, AVP, or CRF were incubated for 48 h at 4°C with their corresponding antibodies (Peninsula Laboratories, San Carlos, CA): rabbit anti-OT antibody (1:25,000), rabbit anti-AVP antibody (1:25,000), or anti-CRF (1:10,000). After incubation, the sections were rinsed and incubated with biotinylated goat anti-rabbit IgG and avidin-biotin-peroxidase complex. Cytoplasmic OT, AVP, and CRF labeling was detected using DAB as a chromogen, without intensification with nickel sulfate, which produces a brown reaction product.

Fos, vasopressin, oxytocin, and CRF, immunoreactivity was counted at medial (PaMP) and posterior (PaPo) parvocellular subdivisions of the PVN and lateral (PaLM) magnocellular subdivisions of the PVN, according to Paxinos and Watson (44) (PaMP and PaLM: −1.80 mm; PaPo: −2.12 mm from bregma). Neurons double labeled with Fos/AVP, Fos/OT or Fos/CRF were counted unilaterally in two sections, with the aid of a computerized system that includes a Leica microscope equipped with a DC 200 Leica digital camera attached to a contrast enhancement device. Images were digitized and analyzed using Leica IM50 ver. 4.0, under a ×40 objective. Fos-positive cells, as indicated by the black staining, were identified when the nuclear structure demonstrated a clear immunoreactivity compared with the background level. Cytoplasmic CRF, OT, and AVP labeling (brown) was counted only if it had a clearly labeled cell body surrounding a nucleus. Counting of immunolabeled cells was obtained as a mean of positive labeling from two sections from each animal. The visual counting of neurons was performed in five to seven animals of each experimental condition, by two participants blind to the experimental protocols.
RESULTS

Arterial Pressure and Heart Rate Recordings

There was no difference in the MAP obtained in basal condition, during, and after the end of right atrial distension (Table 1). On the other hand, there was a significant increase of HR during the balloon distension, compared with basal condition ($P < 0.001$). There was no difference in the HR obtained during and after the right atrial distension.

Effect of Right Atrial Distension on Plasma ACTH, AVP, and OT Concentrations in Water-Deprived Rats

Water-deprived rats with no balloon inflation ($n = 8–11$) showed an increase of plasma ACTH, AVP, and OT concentrations ($P < 0.05$) (Fig. 1), compared with hydrated controls ($n = 6–11$). The distension of the balloon increased plasma ACTH concentrations, which were higher in water-deprived rats ($n = 7$) compared with hydrated rats ($n = 7$) ($P < 0.05$). In addition, the distension of the balloon in the water-deprived group ($n = 7$) decreased plasma AVP concentrations ($P < 0.05$), compared with the respective control group ($n = 11$). Plasma oxytocin concentrations in water-deprived rats ($n = 8$) were not different between controls ($n = 11$) and after the distension of the balloon.

Effect of Right Atrial Distension on Fos, CRF, AVP, and OT Expression in the Hypothalamus in Hydrated and Water-Deprived Rats

A single-balloon distension during 1 min did not significantly change the number of Fos-expressing neurons in the PaMP (control: $6.5 \pm 1.1$, distension: $9.6 \pm 1.6$; $n = 10$) and PaPo (control: $6.6 \pm 1.5$, distension: $11 \pm 2$; $n = 10$). Because single-balloon inflation is a very subtle stimulus, we used a repeated inflation-deflation procedure to evaluate Fos expression, as described in MATERIALS AND METHODS.

Among rats with the uninflated balloon (control), water deprivation increased Fos expression in the parvocellular ($n = 5$) and magnocellular ($n = 6$) PVN neurons. In hydrated rats, the repeated distension of the balloon increased the number of Fos and Fos/CRF-immunoreactive neurons in the medial ($n = 6$) and posterior ($n = 5$) parvocellular subdivisions of the PVN ($P < 0.02$) (Figs. 2 and 3). The repeated distension of the balloon promoted a higher increase of Fos- and Fos/CRF-immunoreactive neurons in the medial and posterior parvocellular subdivisions of the PVN in the water-deprived group ($P < 0.02$), than in the hydrated group ($P < 0.001$).

DISCUSSION

In the present study, we observed that stimulation of right atrial volume receptors, by distension of a balloon placed at the vena cava and right atrial junction, increased the Fos/CRF double labeling in the parvocellular PVN neurons, which was enhanced by water deprivation. There was no change in Fos/AVP and Fos/OT double labeling in magnocellular PVN neurons, after right atrial distension, either in hydrated or dehydrated conditions. However, vasopressin secretion, induced by water deprivation, was decreased by cardiovascular neural reflex, mediated by atrial mechanoreceptors.

In different species, stretching of the vena cava and right atrium junction, as used in the present study, was shown to stimulate cardiac receptors, which results in a reflex increase in heart rate, known as Bainbridge reflex (33, 36). It has been suggested that Bainbridge reflex is activated to reduce cardiac preload under volume loading to reduce the risk of excessive dilation and failure of the heart (9, 10).

We observed that balloon inflation increased Fos expression in neurons in the parvocellular subdivisions of the PVN. This result is consistent with previous reports showing the activation of PVN neurons following the stimulation of cardiac mechanoreceptors, through hypervolemia (18, 45) or inflation of balloon placed at the vena cava and right atrial junction in anesthetized rats (47) and nonanesthetized rats (32). The key role of PVN in the neuroendocrine and autonomic regulation has been well demonstrated (55, 58, 59). Several physiological studies have demonstrated that the PVN is involved in the control of sympathetic nervous activity and blood pressure (17, 31, 47, 64). The present study demonstrates that, within the PVN, CRF neurons are one of the specific subset of neurons in this nucleus that showed an increase of Fos immunoreactivity, following stimulation of cardiac mechanoreceptors. CRF neurons located in the medial parvocellular PVN project to the external layer of the median eminence (40), where CRF is released into the hypophysial-portal circulation to stimulate ACTH release from corticotrophs in the anterior pituitary. In fact, we observed an increase of plasma ACTH concentrations after balloon inflation. The role of atrial receptors in the control of ACTH release was previously demonstrated by Baertschi et al. (7). By using sinusoidal volume changes applied to the
atrium, these authors showed an increase of plasma ACTH concentrations after right atrial pulsation.

PVN neurons have been shown to innervate central nervous system areas involved with the reflex regulation of cardiovascular function, such as intermediolateral cell column of the thoraco-lumbar spinal cord that contains the sympathetic preganglionic motoneurons, where sympathetic preganglionic neurons send projections to the heart and the kidney (19, 57). It was also shown that PVN also sends projections to the rostral ventrolateral medulla (RVLM), a crucial area implicated in the generation and modulation of the sympathetic activity to the cardiovascular system (6, 19). In fact, Kantzides et al. (32) demonstrated that stimulation of cardiac mechanoreceptors, elicited by balloon inflation as used in the present study, activated midcaudal PVN neurons that send projections to the RVLM. We also observed an increase of Fos/CRF expression in the posterior parvocellular PVN. It has been shown that CRF participates in the modulation of sympathetic outflow (13, 24, 43). Peripheral or central administration of CRF has been shown to induce a fall in blood pressure and reflex increase in heart rate in different species, including humans (43). CRF was also shown to alter baroreflex function through inhibition of cardiac vagal outflow (25). Likewise, Nijsen et al. (41) demonstrated that endogenous CRF contributes to the increase of heart rate induced by conditioned stress. CRF-induced elevations of heart rate are suggested to be mediated by enhanced cardiac sympathetic outflow and decreased baroreceptor-induced activation of cardiac vagal motor neurons (25). Therefore, the present results showing an increase of Fos/CRF expression in the PVN, in response to stimulation of cardiac mechanoreceptors, corroborate the CRF effects on modulation of autonomic cardiovascular responses.

We observed that water deprivation increased the number of Fos-immunoreactive neurons in the parvocellular PVN, indicating that these neurons participate in the adaptive response to osmotic challenge. This result is consistent with data from Arnhold et al. (3), showing an increase of Fos expression in parvocellular CRF neurons in the PVN after water restriction. It has been shown that water deprivation increases sympathetic outflow, contributing to the maintenance of blood pressure (50). Osmosensitive inputs from circumventricular organs to PVN have been suggested to contribute to the excitatory...
cardiovascular and sympathetic effects of increased central osmolality (60). Indeed, it was also demonstrated that autonomic parvocellular PVN neurons projecting to the spinal cord and rostral ventrolateral medulla are activated during water deprivation (54). It should be pointed out that besides increasing plasma osmolality, water deprivation also induces fluid deficit in extracellular fluid compartments, which activates renin-angiotensin system (11, 28). ANG II has been shown to increase CRF mRNA expression (56); therefore, in the present model, ANG II might also contribute to the increase of CRF neuron activity. In addition, spinal cord-projecting neurons of the PVN receive excitatory projections from the subfornical organ (8, 42), a brain site with a high density of angiotensin receptor (20), making conceivable the possibility that the angiotensin system may contribute to the increase of Fos/CRF staining in parvocellular PVN neurons induced by cardiovascular reflex in the presence of osmotic stimulation. These data suggested that parvocellular PVN contributes to the elevated sympathetic nervous activity and arterial blood pressure control, in response to water deprivation. We also observed that the number of Fos-immunoreactive neurons in the parvocellular PVN was higher after atrial distension in water-deprived rats, compared with hydrated rats, suggesting that under conditions of water deprivation, the synaptic transmission in the pathways activated by atrial mechanoreceptor stimulation is facilitated.

Right atrial distension did not change the Fos expression in the magnocellular oxytocin and vasopressin neurons in the PVN, both in hydrated and water-deprived rats. The absence of changes in Fos expression suggests that these neurons were inhibited or may not be affected by the balloon inflation. It was previously demonstrated that right atrial distension decreases the activity of AVP neurons in the supraoptic nucleus, without affecting OT neurons (29). The present data showing the decrease of plasma AVP concentration with no changes in plasma OT concentrations, following the balloon inflation in the water-deprived group, are in agreement with previous electrophysiological studies. Therefore, our results suggest that AVP secretion during dehydration is influenced by cardiovascular reflex, mediated by volume receptors. It was also previously demonstrated that blood volume expansion induced by short (49) or prolonged (48) saline infusion activates oxytocinergic neurons in the SON and PVN. The difference of those previous data from the findings of the present study may be related to the subtle stimulation of cardiac mechanoreceptors induced by the balloon inflation in the present study, compared with the slow expansion produced by volume infusion.

In summary, the present study demonstrated that there is an increase of double-labeled Fos/CRF parvocellular PVN neurons induced by stimulation of right atrial mechanoreceptors, indicating that CRF participates in the cardiovascular reflex responses elicited by volume loading. In addition, we demon-

### Table 2. Number of total CRF neurons and the percentage of Fos/CRF double immunolabeled cells in the PaMP and PaPo PVN of hydrated and water-deprived groups without (control) and with atrial distension

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<tr>
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<th>PaMP</th>
<th>PaPo</th>
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<tr>
<td></td>
<td>CRF Neurons</td>
<td>% of Fos/CRF Neurons</td>
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<tr>
<td><strong>Hydrated</strong></td>
<td></td>
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<tr>
<td>Control (n = 5)</td>
<td>101±6.3</td>
<td>1.9±0.3</td>
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<tr>
<td>Atrial distension (n = 6)</td>
<td>100±4.3</td>
<td>6.7±0.6*</td>
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<tr>
<td><strong>Water Deprived</strong></td>
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<tr>
<td>Control (n = 5)</td>
<td>100.4±3.1</td>
<td>2.5±0.5</td>
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<tr>
<td>Atrial distension (n = 6)</td>
<td>100.9±1.7</td>
<td>13±1.8*+</td>
</tr>
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Data are expressed as means ± SE. PaMP, medial parvocellular; PaPo, posterior parvocellular; PVN, paraventricular nucleus; CRF, corticotropin-releasing factor. *P < 0.02 vs. respective control group; +P < 0.001 vs. hydrated group with atrial distension.
stated that parvocellular PVN neurons are recruited by cardiovascular reflex, and this activity is affected by water deprivation.

**Perspectives and Significance**

We found that Fos/CRF expression is increased after atrial cardiovascular reflex induced by stimulation of right atrial mechanoreceptors. On the basis of the results of Fos staining, it could be speculated that CRF neurons in the PVN participate in the cardiovascular reflex responses to challenges produced by volume expansion, and this participation is enhanced under conditions of water deprivation. The present data reinforce the cardiovascular effects of CRF (43) and should provoke further study into elucidating the precise mechanisms and pathways involved in the CRF neuron recruitment in response to stimulation of atrial mechanoreceptors. The possible role of CRF neurons in the PVN on the cardiovascular reflex adjustments in blood volume expansion in pathophysiological conditions, such as heart failure and renal dysfunction, remains to be investigated. Finally, elevated CRF levels in the cerebrospinal fluid in depression and stress disorders (35) and altered central regulation of atrial mechanoreceptors. On the basis of the results of Fos staining, it could be speculated that CRF neurons in the PVN participate in the cardiovascular reflex responses to challenges produced by volume expansion, and this participation is enhanced under conditions of water deprivation. The present data reinforce the cardiovascular effects of CRF (43) and should provoke further study into elucidating the precise mechanisms and pathways involved in the CRF neuron recruitment in response to stimulation of atrial mechanoreceptors. The possible role of CRF neurons in the PVN on the cardiovascular reflex adjustments in blood volume expansion in pathophysiological conditions, such as heart failure and renal dysfunction, remains to be investigated. Finally, elevated CRF levels in the cerebrospinal fluid in depression and stress disorders (35) and altered central autonomic regulation underlying the increased cardiac mortality have been described in major depressive disorder (14, 63). Therefore, the translation of our results into the clinical perspectives is an important issue to be explored in the next few years.

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