FLUID BALANCE AND BAROREFLEX RESPONSE: NEUROMODULATORS, NEURAL CIRCUITS AND SEX CHROMOSOME COMPLEMENT INFLUENCES
(Balance de fluidos corporales y respuesta baroreflexa: Influencias de neuromoduladores, circuitos neurales y complemento cromosómico sexual).

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ABSTRACT
Changes in body water/sodium balance are tightly controlled by the CNS to avoid abnormal cardiovascular function and the development of pathological states. This process of sensory integration takes place in different nuclei, with diverse phenotypes and at different levels of the CNS. Our aim was to study the specific neurochemical groups, their roles, their connections and the associated endocrine responses during body sodium depletion or sodium overload conditions. For this purpose, we combined the immunohistochemical detection of different neurotransmitters, a retrograde transported dye and a marker of neural activity. We also determined the involvement of sex chromosome complement (SCC) in both bradycardic baroreflex response and in brain activity in a sodium depletion model. Our results demonstrated that the activity of serotoninergic and oxytocinergic neurons significantly increases during blood volume expansion, suggesting their involvement in the homeostatic regulatory response. We also observed tonic activity of serotoninergic neurons of the dorsal raphe nucleus (DRN) during the first hours after sodium depletion. The activity then decreased 24 h after sodium depletion and increased after body sodium reestablishment, independently of the sodium concentration of the solution consumed, suggesting that this system is involved in the inhibition of sodium appetite under conditions of satiety. In contrast, the paraventricular and supraoptic oxytocinergic neurons were activated and the oxytocin plasma levels increased only after hypertonic NaCl intake in both depleted and non-depleted animals, suggesting that this system is involved in the processing of hyposmotic signals. Our hodological results provide insight into how different areas, such as the DRN and lateral parabrachial nucleus, form a neural network that regulates body fluid balance, and show the main integratory nuclei involved in the satiety phase of sodium appetite and consequently in the regulation of extracellular volume. Finally, we also demonstrated that the SCC influences bradycardic baroreflex response and modulates brain activity in nuclei closely involved in the regulatory response to RAAS stimulation.

Keywords: Water and electrolyte homeostasis, bradycardic baroreflex response, RAAS, serotonin, oxytocin, sex chromosome complement
1) hypervolemia induced by isotonic or hypertonic blood volume expansion, 2) hyponatremia/hypovolemia induced by sodium depletion that stimulates sodium intake. 3) We also evaluated the sex chromosome complement influences on both bradycardic baroreflex response and brain activity during a body sodium depletion state.

Different nuclei within the brain work together to maintain water and electrolyte homeostasis. Receptive nuclei such as brainstem areas, like the nucleus of the solitary tract (NTS), locus coeruleus (LC), dorsal raphe nucleus (DRN) and lateral parabrachial nucleus (LPBN), receive and integrate the autonomic and sodium balance information. The circumventricular organs (CVOs) of the lamina terminalis (LT), such as the organum vasculosum of the lamina terminalis (OVLT) and the subfornical organ (SFO), participate detecting the natremia/osmolarity and hormone changes in plasma and cerebrospinal fluid. The lack of a blood-brain barrier allows these CVOs to be exposed to modulatory humoral factors, giving them the potential to integrate and modulate the homeostatic response. At the SFO and area postrema (AP) level, angiotensin II (ANG II) is involved in sodium appetite, baroreceptor reflex and blood pressure response, modulating hydroelectrolyte and cardiovascular homeostasis. Furthermore, the extended amygdala nuclei (ExA) and the hypothalamic areas, such as the supraoptic nucleus (SON), paraventricular nucleus (PVN) and median preoptic nucleus (MnPO), are involved in the integration of information, modulating ingestive behavior and renal, endocrine and autonomic responses. We present results relative to serotonergic and oxytocinergic central system involvement and their interrelationship in the neural network of water and electrolyte homeostasis control, as well as the influence of sex chromosome complement on sexually dimorphic bradycardic baroreflex response and its modulatory action on brain activity induced by sodium depletion.

2) CENTRAL NEUROCHEMICAL SYSTEMS AND SEX CHROMOSOME COMPLEMENT EFFECT IN WATER AND ELECTROLYTE HOMEOSTASIS

Serotonergic neurons are located within numerous nuclei in the midbrain and brainstem, and have diffuse projections throughout the CNS. Most of these cells are included within the midline raphe nucleus (Halliday et al., 1995). The DRN is the portion that is commonly involved in the control of sodium appetite and hydroelectrolyte homeostasis (Rouah-Rosilio et al., 1994; Franchini et al., 2002; Olivares et al., 2003; Godino et al., 2005, 2007, 2010, 2013; Margatho et al., 2002, 2007, 2008, 2015). Specifically, central serotonergic system participation in the control of sodium appetite behavior was early demonstrated in our laboratory by Munaro and Chiaraviglio in 1981. In this study, the metabolism of hypothalamic 5-HT was not affected 12-24 h after body sodium depletion, while the levels of both 5-HT and 5-HIAA increased significantly in sodium-depleted rats allowed to drink sodium. Similarly, years later we observed a significant decrease in DRN serotoninergic neuron activity (shown by Fos immunoreactivity) 24 h after sodium depletion and increased activity after sodium intake induced by peritoneal dialysis (Franchini et al., 2002). These studies were then confirmed by several pharmacological and lesion studies (Olivares et al., 2003; Lima et al., 2004; Reis 2007).

In the oxytocinergic system, its cell bodies are located mainly in the PVN and SON, and OT is a neurohormone that has both central and systemic functions. These results in the release of peptide from the projections within the CNS and its secretion from the neurohypophysis into the bloodstream. In addition to its known effects on delivery and lactation in female, there is considerable evidence suggesting that the oxytocinergic system is also involved in natriuresis (Adachi et al., 1995; Verbalis et al., 1991), baroreflex control (Russ and Walker 1994; Higa et al., 2002) and the inhibition of sodium appetite in both sexes (Stricker, 1987; Blackburn et al., 1993; Sticker and Verbalis, 1987, 1996; Amico, 2001; Franchini and Vivas, 1999).

Although there is increasing awareness of sex differences in the pathology of cardiovascular disease, the physiological and pathophysiological cardiovascular mechanisms behind the differences between men and women are still the subject of basic and clinical research. Males and females not only differ in their sex (males born with testes and females with ovaries) but also carry different sex chromosome complements (SCC:XY and XX respectively) and are thus influenced throughout life by different genomes. Although the role of gonadal steroids in sexual dimorphism is undeniable, a growing body of evidence indicates that some sexually dimorphic traits cannot be explained solely as a result of gonadal steroid action, but may also be ascribed to differences in SCC. Exciting new data indicate that X-chromosome inactivation is very far from the ‘all-or-none’ phenomenon that was initially described. Although in female mammals, most genes on one X chromosome are silenced as a result of X-chromosome inactivation, some genes escape X-inactivation and are expressed from both the active and inactive X chromosome. This may thus lead to differences between XX cells and XY cells, potentially contributing to sexually dimorphic traits (Carrel & Willard, 2005; Yang et al., 2006). Thus, genetic and/or hormone pathways may act independently or interact (synergistically/antagonistically) to modulate sexual dimorphic development (Cambiasso et al., 1995; De Vries et al., 2002; Arnold et al., 2009; Arnold & Chen, 2009).
The following brief review of our latest studies shows the sex chromosome complement effect and the involvement of oxytocinergic and serotonergic systems and neural circuitry in the regulation of hydroyelectrolyte homeostasis under different experimental conditions.

3) ALTERATIONS IN WATER AND ELECTROLYTE BALANCE

3.1) Hypervolemia induced by isotonic or hypertonic blood volume expansion: When extracellular volume expansion, such as blood volume expansion (BVE), occurs, several neural, behavioral and hormonal mechanisms work in coordination to inhibit water and salt ingestion and to increase natriuresis and urine flow. In order to restore body fluid balance, thirst and salt appetite are inhibited, renal sympathetic activity and vasopressin (AVP) secretion decrease, and OT and atrial natriuretic peptide (ANP) secretion are stimulated. Although OT secretion can induce natriuresis directly in the kidney, it is mainly induced indirectly by ANP release (Antunes-Rodrigues et al., 2004). With this endocrine response, we also observe at hypothalamic level an increase in the activity of oxytocinergic neurons during isotonic BVE and activated oxytocinergic and vasopressinergic neurons after hypertonic BVE (Table 1; Godino et al., 2005; Antunes-Rodrigues et al., 1992; Haanwinckel et al., 1995).

Table 1.

<table>
<thead>
<tr>
<th>Brain region</th>
<th>BVE 0.15M</th>
<th>Control</th>
<th>BVE 0.3M</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fos/STH positive neurons</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DRN</td>
<td>2.84 ± 0.53*</td>
<td>0.60 ± 0.22</td>
<td>8.00 ± 1.22*</td>
<td>0.01 ± 0.0001</td>
</tr>
<tr>
<td>Fos/OT or AVP positive neurons</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SON (AVP)</td>
<td>3.66 ± 1.77</td>
<td>1.66 ± 1.47</td>
<td>32.60 ± 6.37*</td>
<td>0.01 ± 0.0001</td>
</tr>
<tr>
<td>SON (OT)</td>
<td>15.33 ± 2.48*</td>
<td>4.00 ± 2.12</td>
<td>44.60 ± 7.78*</td>
<td>0.01 ± 0.0001</td>
</tr>
<tr>
<td>PaMM (AVP)</td>
<td>0.67 ± 0.41</td>
<td>2.00 ± 0.71</td>
<td>24.60 ± 6.10</td>
<td>0.50 ± 0.71</td>
</tr>
<tr>
<td>PaMM (OT)</td>
<td>3.66 ± 0.41*</td>
<td>1.66 ± 0.41</td>
<td>22.70 ± 5.90*</td>
<td>1.33 ± 0.41</td>
</tr>
<tr>
<td>PaLM (AVP)</td>
<td>0.67 ± 0.41</td>
<td>1.33 ± 0.82</td>
<td>23.50 ± 2.37*</td>
<td>0.50 ± 0.71</td>
</tr>
<tr>
<td>PaLM (OT)</td>
<td>3.33 ± 0.41*</td>
<td>1.66 ± 0.41</td>
<td>34.00 ± 9.02*</td>
<td>0.50 ± 0.71</td>
</tr>
</tbody>
</table>

Values are means ± SE; *p<0.05 or **p<0.001 versus control group.

As shown previously (Ricksten et al., 1981; Anderson et al., 1986; Petterson et al., 1988), an acute increase in blood volume, as in the present work, induces a series of hemodynamic events, including an increase in central venous pressure, right atrial pressure, central blood volume, peripheral blood volume and cardiac output. On the other hand, BVE decreases heart rate (HR) and total peripheral resistance, while mean arterial blood pressure (MAP) does not change. In our model of expansion, where the high-volume infusion takes place in a short period, we observed an initial decrease in heart rate and also a reduction in mean arterial pressure (Figure 1). This hypotension may be mediated by the pronounced, rapid release of ANP and OT (Figure 1), since both hormones have a vasodilator effect and the plasma concentration is high at 5 min and returns to baseline within 15 min of the BVE (Antunes-Rodrigues et al., 1992; Haanwinckel et al., 1995).
A: Changes from resting levels in MAP and HR in rats that were volume loaded. Values are mean ± S.E.; n=7. * P<0.05 versus basal levels. B: Plasma ANP, OT, and AVP concentrations 5 min after an isotonic BVE in rats. Values are means±S.E.; n=10. * P<0.05 versus control, ** P<0.001 versus control. Figure modified from Godino et al., 2005.

The involvement of the DRN serotonin system in the control of body fluids is evident from pharmacological and lesion studies (Stein et al., 1987; Reis et al., 1989, 1991, 1994; Margatho et al., and 2007). At the same time, neuroanatomical studies indicate the existence of numerous serotonergic projections to forebrain structures involved in the regulation of cardiovascular and water and electrolyte homeostasis (Van de Kar and Lorens, 1979; Sawchenko et al., 1983; Bosler et al., 1988; Lind Ganten, 1990; Petrov et al., 1992). Experiments with agonists and antagonists of 5HT injected into the LPBN show that this system modulates both renal and endocrine responses to BVE (Margatho et al., 2007). Consistent with these results, in vivo and in vitro experiments have demonstrated that the SHT receptor acts directly increasing magnocellular neuron activity, thus modulating OT and AVP neurohypophyseal release (Jorgensen et al., 2003; Ho et al., 2007).

Furthermore, Reis et al. (1994), with lesion experiments and pharmacological SHT depletion in the DRN, observed that both inhibit basal release of ANP and also block the increase of ANP induced by BVE and consequently block the natriuretic response.

We observed that DRN shows a load-dependent volume increase in Fos (a marker of neural activity) and serotonin immunoreactive neurons (Fos-SHT-ir) (Table 1 figure 2). These new findings give neuroanatomical support to several studies that suggest the participation of DRN serotonergic cells in the regulation of the hypervolemic and hypovolemic states induced by BVE and in the inhibition of SA stimulated by SD, respectively (Munaro & Chiaraviglio, 1981; Reis et al., 1994; Rouah-Rosilio et al., 1994; Menani et al., 1996; Franchini et al., 2002; Olivares et al., 2003; Lima et al., 2004). As mentioned previously,
electrolytic or excitotoxic lesions to the DRN induced an acute decrease in ANP secretion both in the basal condition and after volume expansion (Reis et al., 1994), significantly enhancing thirst and the induced sodium appetite (Olivares et al., 2003; Lima et al., 2004). As we said, our Fos studies also showed a significant increase of the number of Fos-ir and Fos-SHT-ir cells in the DRN during spontaneous and induced saline intake, stimulated by sodium depletion. Taking into account the previous and current data, it is reasonable to believe that the serotonergic cells of the DRN may be modulating the ANP release and the ingestive behaviors (sodium appetite and thirst, see below) associated with BVE. Similarly, we also observed a significant reduction in the level of serotonin and its metabolite 5-hydroxyindolacetic acid (5HIAA) in the DRN 15 minutes after BVE, suggesting that these peptides are being released in the areas during the process of extracellular volume regulation.

3.1.1) **LPBN afferent pathways involved in BVE:** The lateral parabrachial nucleus (LPBN), a structure lying dorsolateral to the superior cerebellar peduncle, has been shown to play an important role in the control of body fluid balance (Edwards and Johnson, 1991; Menani et al., 1995, 1996, 1998, 2000; Colombari et al., 1996), and it is connected with hindbrain and forebrain areas that are activated by changes in body fluid volume (Saper and Loewy, 1980; Ciriello et al., 1984; Fulwiler and Saper, 1984; Jhamandas et al., 1996). Recently, Margatho et al. (2007) have shown evidence for serotonergic mechanisms in the LPBN being involved in the control of ANP and OT secretion, as well as in the excretion of sodium, potassium, and water in response to isotonic BVE. In this work, we sought to identify the population group of cells activated after BVE and particularly within the serotonergic clusters of the raphe system that directly project to the LPBN. For this purpose, we combined the detection of a retrograde transported dye, Fluorogold (FG), injected into the LPBN, with analysis of the brain pattern of Fos-ir induced by isotonic BVE.

These data identify the morphofunctional interrelationship between LPBN cells and specific afferent forebrain and brainstem neuronal groups of cells that are activated after isotonic BVE in conscious freely moving rats. Isotonic BVE induced Fos activation in retrogradely labelled cells of the ventral (PaV) portion of the PVN and central ExA structures, specifically the bed nucleus of the stria terminalis laterodorsal subdivision (BNSTLD) and central amygdala (CeA). At brainstem level, we found activated neurons in DRN, LC, and mNTS that project to the LPBN (Margatho et al., 2008) (Figure 3). Together these results are adding new information concerning the specific groups of neurons involved in body volume regulation that have direct or monosynaptic connections with the LPBN, suggesting that they may form a neural network that allows the information arriving at the LPBN to be integrated for the regulatory response to BVE.

We are particularly interested in studying the serotonergic pathways projecting to the LPBN related to the control of body fluid homeostasis. Together, our studies show that serotonergic DRN cells are activated by BVE, and that DRN neurons activated by BVE also project to the LPBN. Our findings are also in agreement with several studies suggesting the participation of a serotonergic circuit in regulation of the hypovolemic state (Reis et al., 1991, 1994). Previous neuroanatomical studies (Petrov et al., 1992) showed the involvement of specific serotonergic neuronal pools within the DRN that project to both the LPBN and the PVN. Thus, taking into account all the data, we propose that the source of the SHT terminals within the LPBN is in the dorsal raphe nucleus serotonergic somas.
3.2) Hyponatremia/hypovolemia induced by sodium depletion stimulating sodium intake: Sodium appetite (SA) is a motivational state that involves significant homeostatic behavior, the seeking out and ingestion of salty substances to compensate sodium losses, and is defined operationally by measuring hypertonic sodium solution consumption under specified experimental conditions. There is a temporal dissociation between sodium depletion (SD) and the appearance of SA behavior. Previous studies from our laboratory have shown that acute SD by peritoneal dialysis (PD) produces a rapid and significant drop in volemia and Na concentration in serum and CSF within 1–4 h after PD. Sodium concentration rises gradually until 20 h later when not only do the animals recover normal blood volume and extracellular Na values (possibly by mobilizing body sodium reservoirs) but also the specific SA becomes evident (Ferreary and Chiaraviglio, 1977; Margatho et al., 2015). Given these times of changes of natremia and sodium appetite, we analyzed if there is a relationship with serotonergic or oxytocinergic neuron activity.

The cerebral structures involved in controlling the excitatory appetitive and inhibitory or satiety phases of sodium intake are likely to be interconnected with each other, constituting a neural network that integrates associated information (Johnson & Thunhorst 2007; Fitzsimons, 1998). Our evidence indicates that salt appetite modulation involves interactions between the circumventricular organs (CVOs) receptive areas and inhibitory hindbrain serotonergic circuits (Badaue-Passos et al., 2007; Godino et al., 2007) or hypothalamic oxytocinergic neurons (Franchini and Vivas, 1999; Godino et al., 2007). That is, for normal salt appetite sensation, and consequently for appropriate salt drinking after sodium depletion, the hyponatremia and the released ANG II should act centrally both to activate brain osmo-sodium and angiotensinergic receptors that stimulate salt appetite, and also to inhibit brain serotonin (5-HT) mechanisms that inhibit sodium appetite, thus removing a “braking” mechanism. The central 5-HT circuits underlying this interaction mainly include bi-directional connections between the CVOs, 5-HT neurons of the DRN and 5-HT terminals within the LPBN where there are 5HT2A/2C receptors (Colombari et al., 1996; Menani et al., 1996, 1998a,b, 2000; Menani and Johnson, 1995; Olivares et al., 2003; Castro et al., 2003; Lima et al., 2004; Tanaka et al., 1998, 2003, 2004; Cavalcante-Lima et al., 2005a,b). Electrolytic and pharmacological (ibotenic acid) lesions of the DRN induced an increase in the intake of hypertonic saline under basal conditions (Olivares et al., 2003). Rats with DRN lesions also developed more intense dipsogenic and natriorexigenic responses after furosemide combined with captopril treatment. These observations made it possible to establish that a deficit in serotonergic tone removed an important inhibitory pathway of sodium appetite and/or satiety marker. This hypothesis was supported by pharmacological studies that suggest the existence of an important inhibitory serotonergic mechanism in the control of sodium intake at LPBN level, because it receives direct serotonergic projections from the DRN (Petrov, 1992). The injection of 5HT antagonists into the LPBN increased hypertonic NaCl intake induced by different models related to ANG II stimulation, whereas the agonists decreased sodium appetite induced by the same treatment (Menani et al., 1996, 1998, 2000).

Following this idea, we studied changes in the activity of 5HT neurons within the DRN and the peripheral renin angiotensin aldosterone system (RAAS) at different times after sodium depletion. We analyzed 2 h (hyponatremic/hypovolemic phase, without sodium appetite), and 24 h (normonatremic/normovolemic and an evident sodium appetite (appetitive phase)) after peritoneal dialysis and during the satiation process of sodium appetite (satiety phase), which develops after the access of the sodium intake test. Our results demonstrated that the pattern of serotonergic neuron activity in the DRN is indirectly related with sodium appetite behavior (Figure 4). During the first hours after sodium depletion induced by peritoneal dialysis (PDO), when sodium appetite is still tonically inhibited, the activities of these cells are maintained like a control group (Margatho et al., 2015). However, 24 h later, when the animals are appetitive for sodium, this activity is reduced in line with the increase in sodium intake (Franchini et al., 2002). Finally, after sodium access in the depleted animals, the activity of DRN-serotonergic neurons increased again (Franchini et al., 2002; Godino et al., 2007) (Figure 4).
During the hypovolemic/hyponatremic state (2 h after PD) and during the sodium appetitive phase (24 h after PD), we also measured the plasma renin activity (PRA) and aldosterone (ALDO) concentration, both of which are part of the renin angiotensin aldosterone system (RAAS) involved in the genesis or induction of thirst and sodium appetite behaviors. We found that both RAAS components significantly increase after PD in relation to control groups, but we found no significant differences between the different times after sodium depletion treatment (2 vs 24 h). This means that changes in plasma sodium concentration, plasma volume or PRA and ALDO concentration are not directly correlated with the onset of SA (table 2, Figure 5; Margatho et al., 2015).

Our study supports the idea that there is an early, specific 5-HT tonic inhibition of SA at LPBN level during the first hour after SD in the hyponatremic/hypovolemic state, since SA was partially released by the administration of the nonselective 5-HT1/2-receptor antagonist, methysergide, into the LPBN. Menani et al. (2000) also demonstrated that the antagonism in the LPBN induced by furosemide or isoproterenol treatment is able to produce a rapid increase in SA. Likewise, a microdialysis study (Tanaka et al., 2004), measuring extracellular levels of 5-HT and its metabolite 5-HIAA in the LPBN, shows that acute SD, induced by combined furosemide-captopril treatment, causes a significant decrease in extracellular 5-HT and 5-HIAA concentration. Moreover, sodium-drinking stimulated by furosemide-captopril treatment produces the opposite effect on 5-HT release in the LPBN (Tanaka et al., 2004).

Our results also indicate that, when an ANG II signal is present, induced by PD (table 2), the local LPBN 5-HT antagonism is enough to induce or release the SA (Figure 5). However, we also observed a partial hypertonic sodium intake release compared with the animals 24 h after PD, suggesting that there are possibly other mechanisms, such as visceral (baroreceptor or mechanoreceptor) or peripheral signals, reaching brainstem areas that might be involved in the inhibition of SA at 2 h PD (Potts et al., 2000). These studies strongly support the idea that the LPBN-5-HT system is in part involved in the inhibition of SA during the temporal dissociation between SD and SA (Figure 5).

**Table 2**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Plasma Na⁺ concentration mEq/l</th>
<th>Plasma protein concentration g/dl</th>
<th>Sodium intake mL/100 gbw</th>
<th>Plasma renin activity ng/ml/h</th>
<th>Plasma aldosterone concentration ng/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD 2 h</td>
<td>135.80 ± 4.77*</td>
<td>9.016 ± 0.26*</td>
<td>0.000 ± 0.20</td>
<td>14.76 ± 1.49*</td>
<td>106.36 ± 8.82*</td>
</tr>
<tr>
<td>PD 24 h</td>
<td>144.11 ± 0.48</td>
<td>7.791 ± 0.16</td>
<td>1.331 ± 0.18*</td>
<td>13.31 ± 1.51*</td>
<td>97.8 ± 9.61*</td>
</tr>
<tr>
<td>CD 2 h</td>
<td>143.88 ± 2.20</td>
<td>7.910 ± 0.26</td>
<td>0.000 ± 0.20</td>
<td>2.94 ± 1.96</td>
<td>8.4 ± 9.6</td>
</tr>
<tr>
<td>CD 24 h</td>
<td>143.70 ± 1.83</td>
<td>7.677 ± 0.21</td>
<td>0.227 ± 0.19</td>
<td>4.55 ± 1.50</td>
<td>8.5 ± 9.61</td>
</tr>
</tbody>
</table>

Values are means± SE; n=5. *P<0.05 significantly different between groups.
Figure 5

A: Cumulative sodium intake at 2h and 24h after PD in rats previously injected with vehicle (VEH) or methysergide (Methy, 4 µg/0.2 µl) into the LPBN. The results are represented by means ± S.E.M (one-way ANOVA repeated measures, Veh, n=6 and Methy n=9). *P<0.05 vs. 2h Veh and + P<0.05 vs. 24 h Veh. B: Photomicrograph of the LPBN section showing the injection site. C: Schematic diagram showing the relationship between sodium appetite and the brainstem serotonergic central circuit after sodium depletion by peritoneal dialysis. Figure modified from Margatho et al., 2015.

We also studied the activity pattern of serotonergic neurons after the satiation process performed during isotonic and hypertonic sodium chloride access. Sodium appetite is typically represented by the avid ingestion of hypertonic NaCl, but the amount of sodium obtained as hypertonic NaCl is also ingested as isotonic NaCl because the rat adjusts the volume ingested according to the sodium concentration in the solution (Tordoff et al., 1991). Thus, recent evidence suggests that each solution (isotonic and hypertonic NaCl) has a different way of regulating the termination of sodium appetite, involving one or two presystemic factors, one related to the volume of ingested fluid (i.e., gastrointestinal distension) and one related to its concentration (i.e., increased osmolality of fluid in the small intestine and adjacent visceral tissue) (Stricker et al., 2007). However, as shown in figure 6, we did not find 5-HT DRN cell activity to be associated with the tonicity of the solution consumed, because the number of Fos-5-HT-ir cells in sodium-depleted (by PD) animals with access to isotonic (PD-0.9%) and hypertonic sodium chloride (PD-2%) animals were similarly increased compared with the control (CD) or non-depleted groups. Nonetheless, both depleted groups reached the same body sodium balance (Table 3). It seems that sodium appetite satiety is finally reached regardless of the mechanism involved, activating the 5-HT neurons in sodium-depleted rats (Figure 6).

Table 3

<table>
<thead>
<tr>
<th>Groups</th>
<th>Na⁺ Exit</th>
<th>Na⁺ enter</th>
<th>Na⁺ Balance mEq/100gr bw</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD 2%</td>
<td>1.16 ± 0.06</td>
<td>0.16 ± 0.029</td>
<td>1.31 ± 0.07</td>
</tr>
<tr>
<td>PD 0.9%</td>
<td>1.13 ± 0.035</td>
<td>0.15 ± 0.031</td>
<td>1.35 ± 0.155</td>
</tr>
</tbody>
</table>

Values are means ±S.E. PD-2%, peritoneally dialyzed animals with access to 2% NaCl. PD-0.9%, peritoneally dialyzed animals with access to 0.9% NaCl.
We also studied the participation of the oxytocinergic system in the inhibition of sodium appetite. Our previous results showed that both the activity of neurons in the oxytocinergic PVN and SON and the release of neurohypophyseal OT to circulation increased after the consumption of hypertonic sodium (NaCl 2%) induced by PD. However, unlike what we saw in the serotonergic system, the activity of these cells and the plasma OT concentration were unchanged in animals sodium-depleted by PD without access to intake testing (24h after PD, appetitive phase) (Franchini and Vivas, 1999). Our double-labeling study (Fos-OT) during the first hours after SD indicated that there is no activation of oxytocinergic neurons at the level of SON and PVN, but this may not explain the initial temporal dissociation observed between sodium depletion and the appearance of SA, at least in this SD model.

In the satiety phase of SA in the oxytocinergic system, in contrast with the 5HT system, we observed that the PD-induced hypertonic sodium intake not only increased the number of OT neurons activated but also the OT plasma levels, whereas PD-stimulated isotonic intake did not produce any changes in OT plasma levels or in OT neuronal activation, suggesting that the satiety process is not being mainly regulated by the OT system (Figure 6). It is important to point out that there was a positive ratio between the number of OT-activated cells and the volume of hypertonic saline consumed, since the animals stimulated to drink, like the PD-2% group, had the greatest number of Fos-OT-ir cells. Likewise, there is evidence that intragastric hypertonic infusion enhances OT plasma levels and oxytocin mRNA expression in the hypothalamic nuclei in euhydrated and dehydrated rats, although this peptide does not increase after isotonic intragastric infusion,
perhaps because it does not cause substantial hypernatremia or a hypertonic state (Huang et al., 2000; Niami et al., 1997; Stricker et al., 2002).

Together, these studies suggest that the OT system is a hypertonicity marker: the OT circuit may be signaling the entry to the body of a hypertonic sodium solution during sodium access, since 2% NaCl loading or ingestion increases OT neuron activity and OT plasma levels. A molecular mechanism supporting this hypothesis was given by Bourque and Qiu’s groups (Qiu et al., 2004; Richard and Bourque 1992; Voisin and Bourque 2002), demonstrating that the SON and PVN magnocellular cells are themselves osmotically and sodium-sensitive due to stretch-inactivated channels located in the body cells.

3.2.1) DRN and LPBN afferent pathways involved in sodium appetite satiation: The cerebral structures involved in the control of water and electrolyte balance are likely to be interconnected with one another, constituting a neural network that integrates associated information (Johnson & Thunhorst, 2007; Fitzsimons, 1998). Our previous evidence indicates that modulation of salt appetite involves interactions between the CVO receptive areas and inhibitory hindbrain serotonergic circuits (Badaue-Passos et al., 2007; Godino et al., 2007, 2010). As mentioned earlier, the central 5-HT circuits underlying the interaction involved in sodium appetite control consist of the CVOs, 5-HT neurons of the DRN and 5-HT terminals within the LPBN (Andrade-Franzé et al. 2010; Colombari et al., 1996; Menani et al., 1996, 1998 a,b, 2000; Menani and Johnson, 1995; Olivares et al., 2003; Castro et al., 2003; Lima et al., 2004; Tanaka et al., 1998, 2003, 2004; Cavalcante-Lima et al., 2005a,b).

Lind (1986) has anatomically demonstrated a neural angiotensin connection originating in the SFO and projecting to the DRN. ANG II injected via the carotid artery, or into the SFO, enhances the electrical activity of SFO neurons that project to the DRN (Tanaka et al., 1998 and 2003). A recent microdialysis study (Tanaka et al., 2003) indicates that ANG II activation of SFO neurons projecting to the DRN results in inhibition of DRN neurons and reduced local 5-HT release in the SFO. This suggests that neurons in the SFO monitor circulating levels of ANG II and send this information to the DRN. A comparable projection from the MnPO to the DRN may play a similar role (Zardetto-Smith et al., 1995).

Our recent connectional studies using retrograde tracers in sodium-depleted rats ingesting salt suggest that structures of the lamina terminalis inform the DRN and LPBN of sodium status or sodium consumption by a descending neural pathway. In this way, cells within the LT may contribute to inhibitory mechanisms involving 5-HT neurons in the DRN and the release of 5HT within the LPBN, which limit the intake of sodium and prevent excess expansion of extracellular volume (Badaue-Passos et al., 2007; Godino et al., 2010). In these morphofunctional studies using the retrograde tracer, fluorogold (FG), with Fos we found significantly increased numbers of Fos-FG double-immunolabeled neurons in the LT and several other brain areas previously involved in the control of water and saline drinking and excretion after fluid depletion (Badaue-Passos et al., 2007; Godino et al., 2010). In these studies, the retrograde tracer was injected into the DRN or the LPBN approximately 10 days before sodium depletion experiments. Subsequently, the rats were sodium depleted and were allowed to rehydrate by drinking water and 2% NaCl.

Increased numbers of double-labeled neurons were found in the OVLT, SFO and the MnPO of the lamina terminalis after the rats drank water and saline in the case of FG injection into the DRN (Badaue-Passos et al., 2007). These results suggest that, during the reestablishment of water and sodium balance, neurons of the LT that are monosynaptically connected with the DRN become significantly stimulated by fluid ingestion. These neurons then send information to the DRN, resulting in modulation of the behavioral response and inhibiting further sodium intake. The number of double-labeled Fos-FG neurons in the LT increased after sodium consumption following sodium depletion. In other experiments using a similar approach, FG was injected into the LPBN (Godino et al., 2010). We observed that specific groups of neurons along the LT, PVN, ExA, insular cortex, NTS and 5HT cells of the DRN are directly connected to the LPBN, and are significantly activated in response to water and sodium ingestion after peritoneal dialysis. During the appetitive phase of sodium appetite, the organism needs to acquire sodium salt from the environment to recover lost sodium and ultimately restore natremia, plasma osmolality and plasma volume. Additionally, with the onset of sodium intake, an inhibitory signal is gradually required to avoid over-ingestion of sodium. This inhibitory signal represents the drive to achieve sodium satiety and is characterized by interruption of the previously motivated salt intake. Control of sodium appetite is attributed in part to serotonergic pathways of the DRN and the LPBN.

3.3) Sex differences in body fluid homeostasis: sex chromosome complement involvement in bradycardic baroreflex and sodium depletion responses: Clinical and basic findings indicate that ANG II differentially modulates hydroelectrolyte and cardiovascular responses in male and female. Furthermore, a growing number of studies have shown that brain RAAS dysfunction is implicated in the development of hypertension, and that males and females do not respond equally to hypertensive treatment with RAAS inhibitors, reflecting a sexually dimorphic

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cardiovascular response to angiotensin (Cox and Bishop, 1991).

The baroreceptor reflex acts as an effective buffer of short-term blood pressure fluctuations and most experimental models of hypertension report a reduction in baroreflex sensitivity and/or resetting of the baroreflex curve toward higher blood pressure. Clinical and experimental evidence demonstrates ANG II bradycardic sex differences among males and females. In male subjects, ANG II produces a significantly lower reflex inhibition of the heart rate (HR) compared with the response induced by PE administration, whereas PE and Ang II exert similar bradycardic baroreflex responses in females (Gandhi et al., 1998; Pamidimukkala et al., 2003).

Although many clinical and experimental studies have analyzed the contribution of the effect of activational gonadal hormones on bradycardic baroreflex control of HR, none has considered the contribution of SCCs isolated from steroid hormonal influence. Taking into account that classic hormonal manipulations have failed to cause sex reversal of the differences observed in ANG II-bradycardic baroreflex response, the aim of our study was to test the hypothesis that sex chromosome complement (XX or XY) modulates sexually dimorphic bradycardic baroreflex response. To that end, we evaluated bradycardic baroreflex responses, related to changes in HR attributed to increases in blood pressure evoked by pressor agents (PE and ANG II), in conscious free-moving mice of the four core genotypes mouse model. This transgenic model, which was kindly provided by Dr. Paul Burgoyne, enables the effects of gonadal sex (testes or ovaries) to be dissociated from sex chromosome complement (XX or XY). It combines a deletion of the testis-determining gene Sry from the Y chromosome (Y) with the subsequent insertion of a Sry transgene onto an autosome.

All individuals possessing the Sry transgene develop testes and have a male external phenotype, regardless of their SCC, while individuals lacking the transgene have ovaries and external female secondary sex characteristics (male and female are defined here according to the gonadal phenotype). Comparing gonadal males and females after gonadectomy can test whether having testes or ovaries causes long-lasting differences in the phenotype (organizational effect), while comparing mice with the same gonadal type but with different SCCs (XX versus XY) makes it possible to determine whether genes residing in the SCC differentially influence sexually dimorphic traits.

As shown in Figure 7, Ang II infusion in GDX-XY male mice induced a blunted bradycardic response compared with the PE response, whereas GDX-XX female, GDX-XX male, and GDX-XY female mice showed the same bradycardic baroreflex response to both PE and ANG II. Mice with XX-SCC but with different gonadal sex (GDX-XX male and GDX-XX female mice) showed the same bradycardic baroreflex response. Moreover, the comparison of female mice with different SCCs (GDX-XX female versus GDX-XY female) showed an attenuated baroreflex response to both pressor agents in GDX-XY female mice. The results indicate that the ANG II bradycardic-baroreflex sexual dimorphic response may be ascribed to differences in sex chromosomes, indicating an XX- sex chromosome complement facilitatory bradycardic-baroreflex control of HR (Caeiro et al., 2011).

Comparative reflex bradycardic baroreflex responses to phenylephrine (PE) and angiotensin II (ANG II) infusion in MF1 gonadectomized (GDX) mice of the four core genotype. Graphs show mean ratio lines relating peak changes in heart rate (HR; delta HR) to increases in blood pressure (BP) induced by both ANG II and PE. *P_0.05, significant differences between the bradycardic response to ANG II and PE infusion; GDX-XY male (n=6), GDX-XX male (n=6), GDX-XY-female (n=5), and GDX-XX-female (n=5) mice. (Reprinted from Caeiro et al., 2011. Hypertension 58: 505–511).
These results in conjunction with Ji et al.’s (2010) data indicate that both the sexually dimorphic ANG II-bradycardic baroreflex and hypertensive responses may be driven primarily by differences in SCC, which suggests that genetic sex differences residing in the sex chromosomes may influence these angiotensinergic sexually dimorphic traits.

**Figure 8**

A - SFO

![Brain pattern of Fos-immunoreactivity (FOS-ir) following acute sodium depletion in subfornical organ (SFO) and area postrema (AP). Panel A (SFO): Mean number of FOS-ir cells in XY male, XX male, XY female, and XX female mice corresponding to sodium depleted (DEP) and control (CON) groups (black and white bars, respectively). Inset: significant interaction of sex chromosome complement (SCC) x treatment factors, *P <0.01 XX-DEP vs. XY-DEP, XY-CON and XX-CON groups. Values are means ± SE, n= 3–5/group. Panel B (AP): Mean number of FOS-ir cells in XY male, XX male, XY female, and XX female mice corresponding to sodium depleted (DEP) and control (CON) groups (black and white bars, respectively). #P ≤ 0.05 XX male-DEP vs. the other DEP and CON groups. Values are means ± SE, n= 3–5/group. (Figure modified from Dadam et al., Am J Physiol Regul Integr Comp Physiol 306: R175–R184, 2014).](image)

We evaluated whether genetic differences within the SCC may differentially modulate the known sexually dimorphic sodium appetite as well as basal or induced brain activity due to physiological stimulation of the renin-angiotensin system by furosemide and low-sodium treatment. As shown in Figure 8, our results indicate that, although differences in sodium intake in male and female mice were driven by organization effects, at the brain level a SCC effect was evident in sodium-depleted mice in two circumventricular organs, the SFO and AP. This suggests a sex chromosome gene participation in the modulation of neural pathways underlying regulatory response to renin-angiotensin stimulation (Dadam et al., 2014).

One of the results of hypotension and hyponatremia induced by sodium depletion is the activation of RAAS. This system manages to compensate the hypotension generated restoring the extracellular volume space and inducing vasoconstriction. Increased plasma ANG II levels stimulate aldosterone secretion, which in turn increases sodium reabsorption by the kidney and also binds the SFO, OVLT, and AP (sensory CVOs). The lack of blood-brain barrier allows these CVOs to be exposed to modulatory humoral factors, giving them the potential to integrate and modulate the homeostatic response (McKinley et al., 2004). At SFO and AP level, ANG II is involved in sodium appetite, baroreceptor reflex, and blood pressure response, modulating hydroelectrolyte and cardiovascular homeostasis. The AP sends projections to a number of neural centers involved in cardiovascular regulation, including the NTS, dorsal vagal complex, the parabrachial nucleus, and rostral ventrolateral medulla, modulating sympathetic-parasympathetic activity, as well as the baroreflex response (Blessing et al., 1987; Shapiro and Miselis, 1985). AP lesion not only attenuates ANG II-mediated hypertention but also abolishes the blunted bradycardic response, since male mice with AP ablation show an ANG II-bradycardic baroreflex response of equal magnitude to that evoked by PE administration (Fink et al., 1987; Xue et al., 2003). Furthermore, studies conducted by Contreras and Stetson (1981) have shown that, while AP lesion in male rats leads to an increase in spontaneous sodium intake, no differences are reported in glucose and KCl solution consumption.

ANGII binds to two G protein-coupled receptor (GPCR) subtypes, AT1R and AT2R, thus exerting opposing effects on the cardiovascular system. AT1R activation leads to elevation of blood pressure due to vasoconstriction and increased sympathetic activity, whereas AT2R stimulation induces vasodilation and natriuresis. Furthermore, Ang (1-7) induces vasodilation via the AT2R receptor or its own receptor, the Mas receptor (MasR), (Kaschina & Unger, 2003; Santos et al., 2003). A significant number of studies indicate that sex differences in ACE activity and AT1R/AT2R expression/sensitivity may account for some of the Ang II-related sex differences associated with...
vasoconstrictor/vasodilator balance of the RAAS (Sullivan, 2008). At the peripheral level (kidney and heart) Sampson et al. (2008) have demonstrated that AT1R expression in males exceeds AT2R expression, and thus vasoconstriction predominates, while the expression of AT2R, MasR, and ACE2 is significantly greater in adult female kidneys than in male kidneys, demonstrating an enhanced vasodilator arm of the renin angiotensin system in female kidneys. Moreover, an enhancement of the vasodilation component of RAAS in females has been described, with the infusion of low doses of Ang II potentially biasing females in the direction of vasodilation and males toward vasoconstriction (Sampson et al., 2008). Xue et al. (2014) have reported sex difference in the sensitizing effects of Ang II, and in the interactions between central estrogen and the RAAS involved in this sensitization process. RT-PCR analyses indicate that estrogen given during induction with a nonpressor dose of Ang II upregulated mRNA expression of the RAAS anti-hypertensive components (ACE2/Ang-(1–7)/MasR), whereas both central estrogen and Ang (1–7) downregulated mRNA expression of RAAS hypertensive components in the lamina terminals.

Taking into account the above data and that two of the components of the vasodilatador arm of the RAAS (AT2 receptor (Agtr2) and ACE2 genes) are located in the X chromosome (De Gasparo et al., 2000), it is tempting to speculate that genes residing in the SCC (which are asymmetrically inherited between males and females) may thus serve as candidate regulators of sexually dimorphic phenotypes (Davies and Wilkinson, 2006). If this is the case, the differential transcription or expression of the vasodilator (ACE/AngII/AT2R and ACE2/Ang-(1-7)/Mas receptor)/vasoconstrictor (ACE/AngII/AT1R) RAAS axis may be responsible for angiotensinergic sex-biased differences. Further investigation is needed, however, to assess the contribution of SCC to AT1 and AT2 receptor expression in these specific brain areas involved in sodium depletion and cardiovascular homeostasis.

4) CONCLUSION

In summary, the current results provide insight into how different areas and neurochemical groups form a neural network that regulates body fluid balance (figure 9). In this network, brainstem and forebrain nuclei would be involved in the reception of peripheral information and in the modulation of the autonomic, endocrine and behavioral responses. Particularly, the LPBN and DRN are integrator nuclei since they receive afferents from hindbrain, hypothalamic, LT and ExA nuclei. The serotonergic system at the DRN level would participate, preventing a sodium overload inhibiting sodium intake in a satiated animal. The oxytocinergic system would report the entry of hypertonic solutions or a BVE. In those situations, OT would be released into the circulation and mediate natriuresis. These areas and systems would work together and may contribute to modulate sodium intake and the endocrine, renal and autonomic responses, to prevent excess volume expansion. Considerable evidence indicates that the LPBN and DRN integrate information reflecting the status of body water and sodium content, as they modulate induced sodium appetite and the endocrine and renal responses observed after BVE. In the present work, we observed that LPBN and DRN were activated during BVE and during sodium intake induced by body sodium depletion. Thus, we used Fluorogold (FG), a retrograde marker, injected into these nuclei to identify thefferent groups of neurons projecting to both nuclei. Our results indicate that LPBN afferents from neural population groups of the PVN, NTS, DRN and ExA, form a neural network that regulates body fluid balance. Similarly, we demonstrated that the DRN receives afferents from the LT nuclei that are activated during the satiety phase of sodium appetite. These results suggest that, in the process of restoring body fluid homeostasis after sodium depletion, neurons of these nuclei projecting to the LPBN and DRN are significantly activated and that these cells may play a role in producing sodium satiety.

Differences between sexes have long been recognized at biochemical, cellular, and physiological levels. For a long time, it was assumed that these differences were completely determined by gonadal hormone actions. However, a large number of studies indicate that the activational and organizational effects of gonadal steroids are not the only ones responsible for such differences. Our results demonstrate that SCC influences bradycardic baroreflex response, showing the contribution of the SCC factor to sex-related differences in the reflex inhibition of HR to PE and Ang II (Caeiro et al., 2011). Moreover, we also
demonstrated that SCC modulates brain activity in nuclei closely involved in the regulatory response to RAAS stimulation, suggesting a sex chromosome gene participation in the modulation of neural pathways underlying cardiovascular and water and electrolyte homeostasis (Dadam et al., 2014).

Understanding in more detail the sex differences between males and females in the regulatory mechanisms underlying physiological differences in water and sodium handling (both at the peripheral and brain levels) may offer important insights into designing improved sex-oriented therapeutic treatments in the future.

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RESUMEN

Alteraciones del balance de agua/sodio corporal y por consiguiente de la volemia son finamente corregidas y controladas a los fines de evitar desórdenes de la función cardiovascular normal y el desarrollo de enfermedades. Para realizar este control el sistema nervioso central (SNC) debe recibir y procesar la información específica que refleje el estatus de agua y sodio corporal. Esta información es integrada a distintos niveles del SNC en núcleos de naturaleza química diversa quienes orquestan las respuestas endocrinas, cardiovasculares, renales y autonómicas que restablecen el equilibrio hidroelectrolítico. Nuestro objetivo fue estudiar los grupos neuroquímicos específicos, sus roles sus conexiones y las respuestas neuroendocrinas asociadas durante un estado de deficiencia de sodio corporal o una condición de sobrecarga. Con este propósito combinamos la detección inmunohistoquímica de distintos neurotransmisores (oxitocina y serotonina), de un marcador retrógrado de vías y de un marcador de actividad neural. Además, determinamos la participación del complemento cromosómico sexual en la respuesta de bradicardia y en la actividad cerebral en un modelo de depleción de sodio. Nuestros resultados demuestran que la actividad de las neuronas serotoninérgicas y oxicinérgicas se incrementa significativamente durante una expansión del volumen sanguíneo o extracelular, sugiriendo que están involucrados en la respuesta homeostática. Además observamos una actividad tónica del las neuronas serotoninérgicas del núcleo dorsal del rafe durante las primeras horas luego de una depleción de sodio. Esta actividad luego disminuye 24 hs después de la depleción de sodio y se incrementa durante el restablecimiento de los niveles de sodio corporal, independientemente de la concentración de sodio de la solución consumida para lograrlo. Esto sugiere que este sistema está involucrado en la inhibición del apetito por el sodio durante el proceso de saciedad. En contraste, las neuronas oxicinérgicas de los núcleos supraóptico y paraventricular hipotalámicos fueron activadas y la concentración plasmática del neuropéptido fue incrementada solo luego del consumo de NaCl hipertónico tanto en los animales depletados como no depletados de sodio, sugiriendo la participación de este sistema en el procesamiento de señales hiperosmóticas. Los resultados hodológicos proveen una clave como dentro de diferentes áreas, como el DRN y el núcleo parabraquial lateral, forman una red neural que regula el balance de los fluidos corporales, y muestran la participación de los principales núcleos integradores durante la fase de saciedad del apetito por el sodio y consecuentemente en la regulación del volumen extracelular. Finalmente, demostramos que el SCC influye en la respuesta baroreflex de bradicardia y modula la actividad cerebral en núcleos involucrados en la respuesta regulatoria a la estimulación del sistema renina-angiotensina-aldosterona (RAAS).

Palabras Claves: Homeostasis de agua y electrolitos, respuesta baroreflex de bradicardia, RAAS, serotonin, oxicocina, complemento cromosómico sexual.