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**CATEGORY 1B: Research supplies for use in the applicant's home laboratory**

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# Functional antagonism between angiotnensinergic and serotonergic central systems during alterations in the hydroelectrolytic balance.

**Importance of the study:** Numerous studies in animals as well as in humans have shown that both sodium deficiency and excess sodium have adverse health effects. It has been report that chronic deprivation sodium intake causes growth retardation, reproductive deficits and other diseases that can become lethal. On the other hand, we have observed that in most healthy people, eating a lot of salt causes negligible changes in mean arterial pressure, but the same ingestive behavior is a principal risk factor in genetically predisposed to certain diseases or renal deficit, attributed to different pathologies or causes, which do not allow a proper balance of body sodium. Whereupon, study the central control circuit and possible neurochemical systems and mechanisms involved in the maintenance of sodium balance in normal individuals, becomes relevant not only contributing to the general knowledge, but also, when looking for possible alterations or treatments related pathologies.

**Background:** In order to avoid changes in the concentration of sodium and water, mammals have a complex system involving renal and neuroendocrine mechanisms. These systems, continuously monitor and control intra and extracellular compartments fluids. During body **sodium depletion (SD)** occurs hypovolemia / hyponatremia that activate the renin-angiotensin system. This in turn stimulates vascular contraction, secretion of aldosterone and vasopressin (that promote renal reabsorption of sodium and water), and induces thirst and **sodium appetite (SA),** restoring the hydroelectrolyte balance. Angiotensin (AII) interacts with two receptors: AT1 and AT2. The first one is involved in hydroelectrolyte balance (1). Previous studies provide background on the importance of central AT1 in SA stimulation under different experimental protocols to induce sodium appetite (2, 3). The main brain areas involved in the genesis of SA are circumventricular organs (OCVs) of the lamina terminalis (LT): subfornical organ (SFO) and the organ vasculosum of the lamina terminalis. Both structures contain cells which sense changes in osmolality, serum sodium concentration and AII levels. SFO has high levels of AT1 receptors and AII at this level induces thirst and sodium appetite.

Conversely, during **sodium overload (SO)** as well as restoration of body sodium levels after sodium intake induced by SD, inhibitory signals are activated in order to prevent an extracellular volume expansion. Much evidence suggests that neuronal serotonergic (5HT) groups at the dorsal raphe nucleus (DRN) exert inhibitory control during these situations. The 5HT system influence on SA is strongly demonstrated in the literature. Our studies have evaluated the hypothesis that this DRN serotonergic system is one of the mechanisms responsible for satiety of SA signaling. And lesion in DRN in rats showed excessive consumption of hypertonic saline (4,5,6). Furthermore, we suggest that SD by peritoneal dialysis decreases the activity of 5HT neurons in the DRN, while induced sodium consumption increases their activity (evidenced by Fos immunoreactivity) (7.8). Also, we observed by in *vivo* electrophysiological studies, that body SO induced by injection of 2 M NaCl, increasing serum sodium, significantly increases the firing rate of these neurons (9). Taken together, these data indicate that the DRN serotoninergic activity would be modulated by body sodium status, supporting its inhibitory role of SA. However, has not been evaluated its direct interaction with the angiotensinergic system involved in excitatory or appetitive states.

The areas involved in the control of both excitatory and inhibitory states are interconnected. As consequence, the information relates to sodium and water balance is integrated and combed. Our previous results indicate that modulation of sodium balance involves bidirectional interactions between

receptive areas like the OCVs of LT and inhibitory centers such as brainstem serotonergic system. Specifically we observed that neuronal groups from LT project to DRN and lateral parabrachial nucleus (LPBN, where had been seen that 5HT exerts its inhibitory control of SA) and these projections are activated during induced sodium consumption. As an example could be mentioned: humoral changes generated during induced NaCl intake, are detected by LT structures and this information is sent to DRN and LPBN (10,11). Moreover, it has been described angiotensinergic projections from SFO to DRN and bidirectional projection from DRN to SFO (12).

In sum, based on this background, it is possible to *hypothesize* a functional antagonism and interrelationship between the action of the SFO angiotensinergic system and the DRN serotonergic system in the regulation of sodium balance or body sodium status. For this purpose we described anatomically co-localization of serotonin and AT1 in DRN neurons. Lastly, we evaluated whether alterations in sodium balance such as sodium deficiency induced by furosemide combine with a low sodium diet protocol (FURO+LSD) is able to modulate gene expression of AT1 receptors and serotonin synthesis enzyme (TPH2) and 5HT2A/2C (serotonin receptors previously involved in SA inhibition (13)) in the DRN and SFO.

The aim of this work was to study the functional and neuroanatomical relationship between - angiotensinergic and serotonergic central systems (in the SFO and DRN) in the control of sodium balance.

## RESULTS

*Neuroanatomical co-localization of serotonin synthesis enzyme (TPH2) with AT1 receptors in the DRN*

This experiment was performed using confocal microscopy. The dorsal raphe nucleus (DRN) showed AT1 receptors and TPH2 positive cells (Figure 1). Some neurons showed also a co-localization pattern (merge panel). This result allowed us to support physical and neuroantatomical experiments that follow.

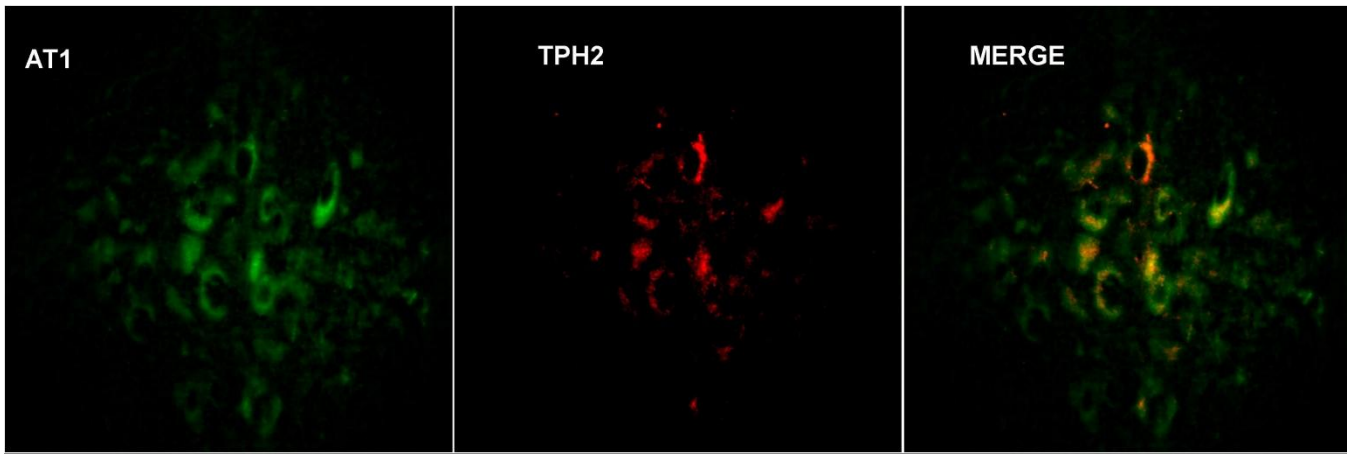


**Figure 1**: Illustrative images (20X) show AT1 receptor (green) and TPH2 (red) immunofluorescence along the dorsal raphe nucleus. In the third panel we show the co localization pattern.

*Plasma measurements 2 h and 24 h after of body sodium depletion induced by FURO+LSD*

Table1: Plasma measurements 2 h and 24 h after of SD

| Groups | Plasma Na+ concentration mEq/l | Plasma Cl- concentration mEq/l | Plasma k+ concentration mEq/l | Plasma protein concentration g/dl | Plasma osmolality mosm/kg $H_2O$ |
|---|---|---|---|---|---|
| SD 2 h | 136,36±3,33* | 73,24000* | 9,58±0,48* | 7.24±0,20+ | 285,20±7,58* |
| SD 24 h | 132, 76±2,01* | 73,50±1,22* | 9,56±0,32* | 6,72±0.14 | 274,8±4,64* |
| CD 2 h | 139,88±1.85 | 88,70±0.65 | 8,78±0.38 | 6,16±0.10 | 296±2.12 |
| CD 24 h | 142,85±0,89 | 90,80±0,76 | 8,98±0,39 | 6,43±0,15 | 300,75±1,65 |

Values are means ±SE; n=5. *P0.05 Significantly different in relation to control groups + P<0.05 Significantly different in relation to others groups.

We performed the plasma electrolytes, proteins and osmolality determinations in order to characterize the sodium depletions protocols. As you can see in the table 1, sodium depletion induced by furosemide and low sodium diet protocol (FURO+LSD) produced a quick and an important drop in the sodium and chloride concentrations and in the osmolality regarding to control animals (CD). Plasma potassium concentration significantly increases after SD in relation to control groups. We also analyzed the time after Furosemide administration was performed. We observed a significant interaction between sodium depletion and time factors in the plasma protein concentration. This result showed an increase in the protein concentration at 2 h after FURO + LSD in relation to others groups, suggesting that the blood volume is reduced immediately after body sodium depletion and it is reestablishing 24 h later without sodium consumption (see Margatho et al., 2015).

Taken into account the functional interrelationship between angiotensinergic and serotonergic systems in the SFO and DRN nuclei, we propose to evaluate whether sodium depletion induced by FURO+LSD modulate gene expression through real time PCR (mRNA) technique.

*Relative mRNA expression levels of AT1, 5HT2C receptors and TPH2 enzyme in the DRN in response to a body sodium depletion*

*TPH2 in DRN*: We analyzed the expression of the main enzyme of conversion of serotonin in the brain, tryptophan hydroxylase 2. The relative TPH2 mRNA expression in the DRN was not modified by the body sodium depletion states (Figure 2).

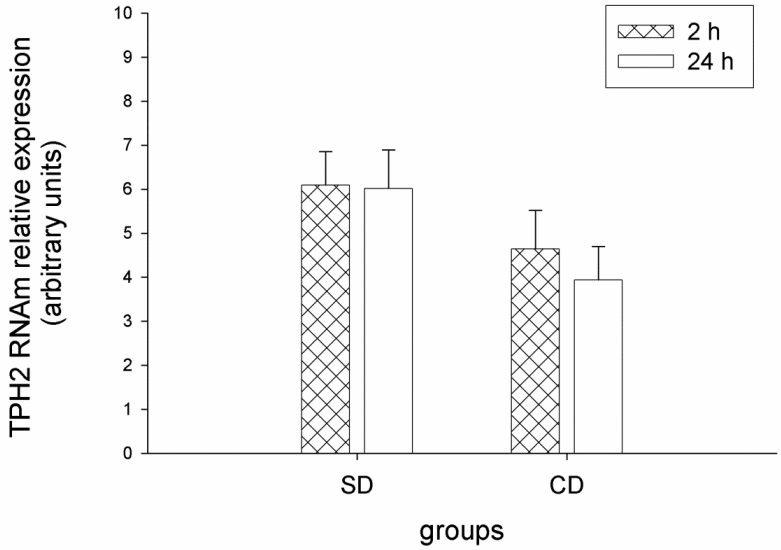


**Figure 2:** Relative mRNA levels of TPH2 from RNA isolated from micropunches of dorsal raphe nucleus (DRN) in sodium depleted animals (SD) and control (CD), 2 h and 24 h after SD induced by FURO +LSD protocol. The samples were measured by quantitative-polymerase chain reaction (qPCR). Values expressed as mean±SE.

*AT1a in DRN.* The relative AT1a mRNA expression in the DRN increased significantly after body sodium depletion (SD) in relation to control or normonatremic groups. However we did not observed any significant differences in relation to time after SD factor (Figure 3).

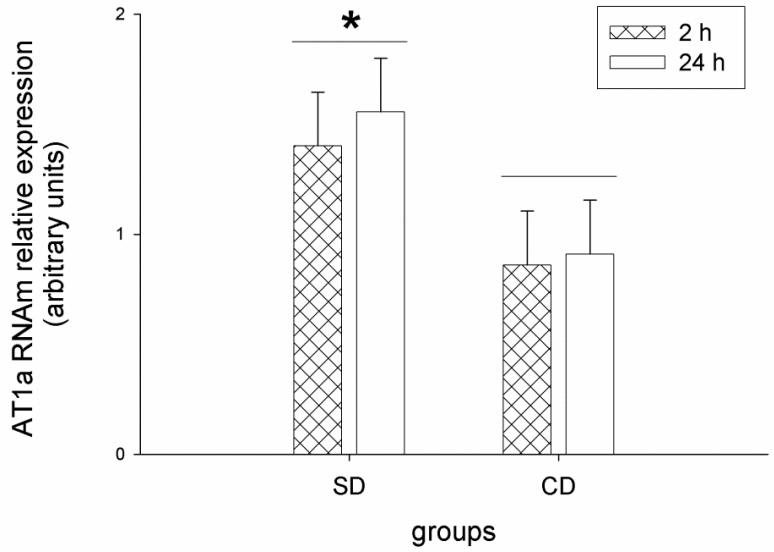


**Figure 3:** Relative mRNA levels of AT1a receptor from RNA isolated from micropunches of dorsal raphe nucleus (DRN) in sodium depleted animals (SD) and control (CD), 2 h and 24 h after SD induced by FURO +LSD protocol. The samples were measured by quantitative-polymerase chain reaction (qPCR). Values expressed as mean±SE, (*) $p<0.05$ Significant differences between SD and CD groups.

*5HT2C in DRN.* As you can see in the figure we observed a significantly increase in sodium depleted animals in the 5HT2C mRNA relative expression along the DRN. However we did not observe any significant differences between the time after FURO+LSD (Figure 4).

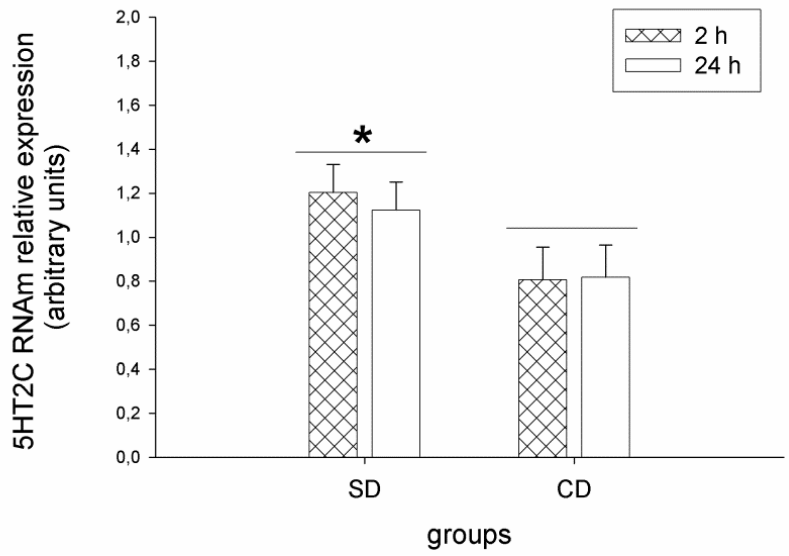


**Figure 4:** Relative mRNA levels of 5HT2C receptor from RNA isolated from micropunches of dorsal raphe nucleus (DRN) in sodium depleted animals (SD) and control (CD), 2 h and 24 h after SD induced by FURO +LSD protocol. The samples were measured by quantitative-polymerase chain reaction (qPCR). Values expressed as mean±SE, (*) p<0.05 Significant differences between SD and CD groups.

*Relative mRNA expression levels of AT1, 5HT2C and 5HT2A receptors in the SFO in response to a body sodium depletion*

*AT1a in SFO*: The SFO sense changes in osmolality, serum sodium concentration and AII levels. We analyzed the expression of AT1a receptors. As you can see in the following figure we observed a significantly increase in the expression of mRNA AT1a receptors during a sodium deficiency state in relation to control no depleted. The time after SD factor and the interaction of both factors did not reach to significant levels. However there is an important tendency (p=0.09) that indicate the possibility that the increases in the mRNA of AT1a expression has a temporal effect and the greatest expression were find 24 h after SD (Figure 5). At this time after FURO+LSD the sodium appetite behavior is evident.

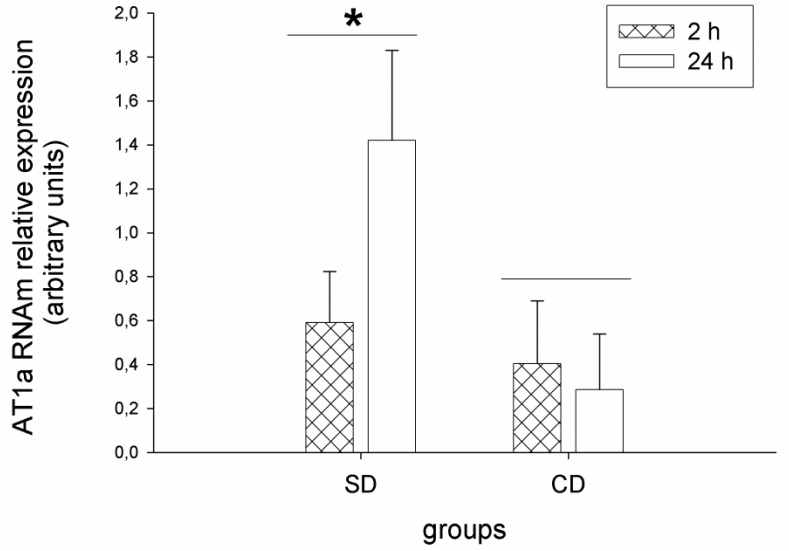


**Figure 5:** Relative mRNA levels of AT1a receptor from RNA isolated from micropunches of subfornical organ (SFO) in sodium depleted animals (SD) and control (CD), 2 h and 24 h after SD induced by FURO +LSD protocol. The samples were measured by quantitative-polymerase chain reaction (qPCR). Values expressed as mean±SE, (*) p<0.05 Significant differences between SD and CD groups.

*5HT2C in SFO*: The figure 6 shown that the mRNA 5HT2C expression is enhanced during a body SD. However we did not observe any significant difference in the time after SD was performed.

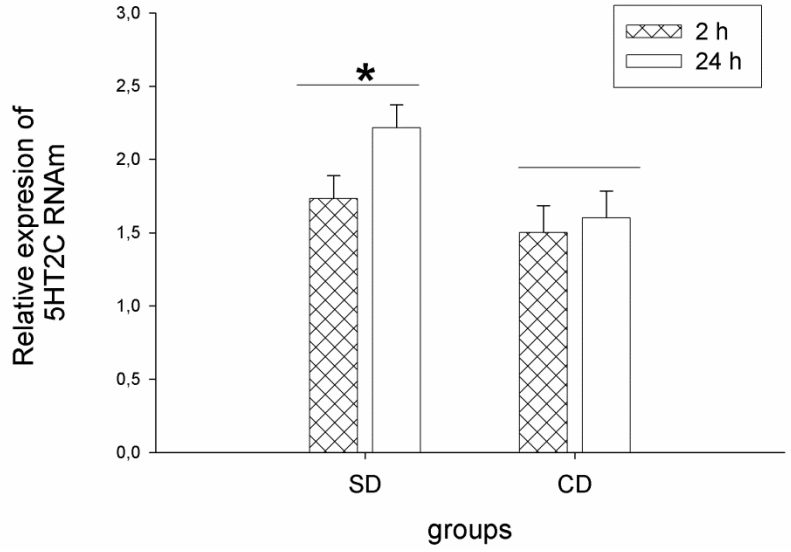


**Figure 6:** Relative mRNA levels of 5HT2C receptor from RNA isolated from micropunches of subfornical organ (SFO) in sodium depleted animals (SD) and control (CD), 2 h and 24 h after SD induced by FURO +LSD protocol. The samples were measured by quantitative-polymerase chain reaction (qPCR). Values expressed as mean±SE, (*) $p<0.05$ Significant differences between SD and CD groups.

*5HT2A in SFO*: Regarding the expression of 5HT2A mRNA we did not observed any significant difference in the factors analyzed (Figure 7).

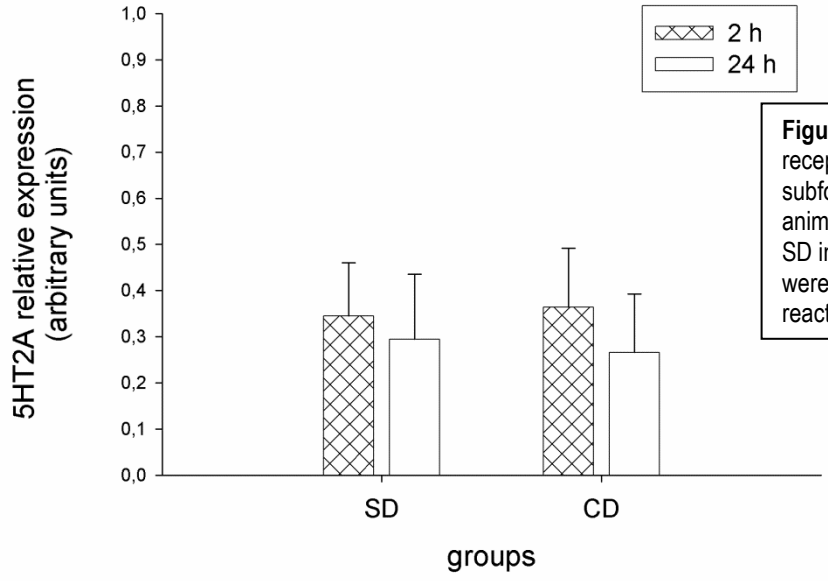


**Figure 7:** Relative mRNA levels of 5HT2A receptor from RNA isolated from micropunches of subforinical organ (SFO) in sodium depleted animals (SD) and control (CD), 2 h and 24 h after SD induced by FURO +LSD protocol. The samples were measured by quantitative-polymerase chain reaction (qPCR). Values expressed as mean±SE.

## SUMMARY

Our present results had shown

1- The neuroanatomical interrelationship between serotonergic neurons and angiotensin type 1 receptors in the DRN.

2-The serotonergic and angiotensinergic central systems have been modulated by body sodium status specifically by a sodium deficiency:

-The mRNA AT1a expression is increased in the SFO and DRN after SD, however at SFO level has a tendency to enhance more 24 h after SD.

-The mRNA 5HT2C expression is augmented by a sodium deficiency independent of the time after FURO+LSD.

-The mRNA TPH2 expression in the DRN and the mRNA 5HT2A expression in the SFO were not been modulated by SD.

-We did not observe an antagonism relationship between the mRNA expression of 5HT2C and AT1a receptors in SFO and DRN nuclei.

**Results communications**

Part of the results obtain in the context of the grant have been recently publish Godino et al., 2013 PlosOne and Margatho et al., Neuroscience 2015, and the data presented in this form it will be presented as a poster the next year.

**The fund was spent on the obtaining the following chemicals and materials:** The funds were used to purchase materials, equipments and supplies necessaries for proposed project. We bought real time-PCR reagents, antibodies for immunohistochemistry reactions and animals for the experiments. It is important to note that supplies in Argentina are more expensive than the same product in the USA, due to shipping and handling costs, insurance, taxes and profits from companies.

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