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Angiotensinergic and cholinergic receptors of the subfornical organ mediate sodium intake induced by GABAergic activation of the lateral parabrachial nucleus

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Abstract

Bilateral injections of the GABA_A agonist muscimol into the lateral parabrachial nucleus (LPBN) induce 0.3 M NaCl and water intake in satiated and normovolemic rats, a response reduced by intracerebroventricular (icv) administration of losartan or atropine (angiotensinergic type 1 (AT₁) and cholinergic muscarinic receptor antagonists, respectively). In the present study, we investigated the effects of the injections of losartan or atropine into the subfornical organ (SFO) on 0.3 M NaCl and water intake induced by injections of muscimol into the LPBN. In addition, using intracellular calcium measurement, we also tested the sensitivity of SFO-cultured cells to angiotensin II (ANG II) and carbachol (cholinergic agonist). In male Holtzman rats with cannulas implanted bilaterally into the LPBN and into the SFO, injections of losartan (1 µg/0.1 µl) or atropine (2 nmol/0.1 µl) into the SFO almost abolished 0.3 M NaCl and water intake induced by muscimol (0.5 nmol/0.2 µl) injected into the LPBN. In about 30% of the cultured cells of the SFO, carbachol and ANG II increased intracellular calcium concentration ([Ca²⁺]_i). Three distinct cell populations were found in the SFO, i.e., cells activated by either ANG II (25%) or carbachol (2.6%) or by both stimuli (2.3%). The results suggest that the activation of angiotensinergic and cholinergic mechanisms in the SFO is important for NaCl and water intake induced by the deactivation of LPBN inhibitory mechanisms with muscimol injections. They also show that there are cells in the SFO activated by both angiotensinergic and cholinergic stimuli, perhaps those involved in the responses to muscimol into the LPBN.

Keywords

sodium appetite; parabrachial nucleus; subfornical organ; AT₁ receptor; muscarinic receptor

The lateral parabrachial nucleus (LPBN), a pontine structure located dorsally to the superior cerebellar peduncle (scp), is an important area for the control of sodium and water intake (Ohman and Johnson, 1986, Menani and Johnson, 1995, Ohman and Johnson, 1995, Colombari et al., 1996, Menani et al., 1996, Callera et al., 2005, De Oliveira et al., 2008, De Gobbi et al., 2009). Electrolytic or neurotoxic lesions of the LPBN increase water intake induced by angiotensin II (ANG II) (Ohman and Johnson, 1986, Edwards and Johnson, 1991). Bilateral injections of the serotonergic receptor antagonist methysergide into the LPBN increase 0.3 M NaCl and water intake induced by dipsogenic and natriorexigenic stimuli (Menani and Johnson, 1995, Colombari et al., 1996, Menani et al., 1996, Menani et al., 1998b, De Gobbi et al., 2000, Menani et al., 2002, David et al., 2008). Similar to methysergide, the blockade of cholecystokinin, corticotrophin release factor or glutamate receptors, or activation of α_2 -adrenoceptors in the LPBN increases 0.3 M NaCl intake induced by the combined treatment with subcutaneous (sc) injection of the diuretic furosemide (FURO) and low dose of the angiotensin-converting enzyme inhibitor captopril (CAP) (Menani and Johnson, 1998, De Gobbi et al., 2001, Andrade et al., 2004, De Castro e Silva et al., 2006, De Gobbi et al., 2009, Gasparini et al., 2009). These neurotransmitters could modulate the activity of the LPBN and, consequently, its inhibitory action on the ingestion induced by different facilitatory stimuli.

The importance of the LPBN inhibitory mechanisms for sodium satiety is also demonstrated by studies that have tested the effects of GABAergic activation in the LPBN (Callera et al., 2005, Roncari et al., 2011, Asnar et al., 2013). In satiated and normovolemic rats, bilateral injections of muscimol (GABA_A receptor agonist) into the LPBN induce a strong 0.3 M NaCl and water intake (Callera et al., 2005, Roncari et al., 2011, Asnar et al., 2013). More recent studies showed that 0.3 M NaCl and water intake induced by muscimol injected into the LPBN was reduced by injections of losartan (angiotensinergic type 1 (AT₁) receptor antagonist) or atropine (muscarinic cholinergic receptor antagonist) into the lateral ventricle (LV), suggesting that sodium intake in this condition depends on the blockade of LPBN inhibitory mechanisms by muscimol combined with the activation of angiotensinergic and cholinergic facilitatory mechanisms probably located in the forebrain (Roncari et al., 2011, Asnar et al., 2013).

An important area of the forebrain involved in the control of sodium and water intake is the subfornical organ (SFO), a circumventricular organ located in the lamina terminalis (Simpson and Routtenberg, 1972, 1973, 1974, Simpson et al., 1978, Mangiapane and Simpson, 1980, Johnson and Gross, 1993). The activation of the AT₁ or cholinergic receptors in the SFO induces dipsogenic responses (Routtenberg and Simpson, 1971, Simpson and Routtenberg, 1972, 1973, 1974, Simpson et al., 1978, Mangiapane and Simpson, 1980). In addition, the activation of AT₁ receptors in the SFO by the injection of ANG II directly in this area or by the treatment with FURO + CAP induces vigorous ingestion of 0.3 M NaCl if the serotonergic inhibitory mechanism is blocked by the injection of methysergide into the LPBN, suggesting that an important facilitatory mechanism for sodium intake involving AT₁ receptors is present in the SFO (Colombari et al., 1996, Menani et al., 1998a). Moreover, these studies suggested that the facilitatory mechanisms of the SFO are strongly inhibited by the LPBN mechanisms. Therefore, it is possible that AT₁ receptors of the SFO are part of the facilitatory mechanisms involved in sodium intake

induced by muscimol injected into the LPBN. Although never investigated, it is also possible an involvement of the cholinergic mechanisms of the SFO with sodium intake, particularly sodium intake induced by muscimol into the LPBN, that has been suggested to also depend on central cholinergic mechanisms (Asnar et al., 2013).

In the present study, we investigated the possible participation of the AT₁ and the cholinergic receptors of the SFO on 0.3 M NaCl and water intake induced by injections of muscimol into the LPBN in satiated and normovolemic rats. Additionally, using intracellular calcium measurement, we also tested the sensitivity of SFO-cultured cells to ANG II and the cholinergic agonist carbachol.

EXPERIMENTAL PROCEDURE

Animals

Twenty-six male Holtzman rats weighing 290–310 g were used for *in vivo* experiments. Animals were housed in individual stainless steel cages with free access to normal sodium diet (BioBase Rat Chow, Águas Frias, Brazil), water, and 0.3 M NaCl solution. Room temperature was maintained at 23 ± 2 °C, humidity at $55 \pm 10\%$ and on a 12:12-h light-dark cycle. *In vivo* experimental procedures were approved by Ethical Committee in Animal Use (CEUA) from the School of Dentistry – UNESP. The experimental protocols followed the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication No. 80-23, 1996). For *in vitro* experiments, 11 Sprague-Dawley preweaning pups (18–20 days old) were used (Harlan laboratories, Indianapolis, USA). The mother and pups were housed together in a light-controlled room (12:12-h light-dark cycle) with food (7013 National Institutes of Health-31 modified rat diet) and water provided ad libitum for 4–7 days before they were used. *In vitro* experiments were conducted in accordance with the National Research Council Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals and were approved by the University of Iowa Animal Care and Use Committee.

Brain surgery

Rats were anesthetized with ketamine (80 mg/kg of body weight (b. wt.); Cristália, Itapira, Brazil) combined with xylazine (7 mg/kg of b. wt.; Agener União, Embu-Guaçu, Brazil) intraperitoneally, placed in a stereotaxic instrument (Kopf, Tujunga, CA, USA) with the skull leveled between bregma and lambda. Two stainless steel 23-gauge guide cannulas were bilaterally implanted immediately above the LPBN (coordinates: 9.6 mm caudal to bregma, 2.1 mm lateral to midline, and 3.1 mm below dura mater) and a third cannula was implanted immediately above the SFO (coordinates: 1.2 mm caudal to bregma, on the midline, and 2.7 mm below dura mater). The tips of the guide cannulas were positioned at a point 2 mm above the LPBN and SFO. The guide cannulas were fixed to the cranium using dental acrylic resin and jeweler screws. Between tests, a 30-gauge metal obturator filled the guide cannulas. At the end of the surgery, the animals received an intramuscular injection of antibiotic (benzylpenicillin – 80,000 IUs plus streptomycin – 33 mg; Pentabiótico Veterinário - Pequeno Porte, Fort Dodge Saúde Animal Ltda., Campinas, Brazil) and a sc injection of analgesic/anti-inflammatory (ketoprofen 1% – 0.03 ml/rat; Ketoflex, Mundo

Animal, São Paulo, Brazil). After the surgery, the rats were handled daily and trained for the experimental procedure. Water and 0.3 M NaCl intake tests began after a 5-day period of recovery.

Central injections

Injections into the LPBN and SFO were made using 5 µl Hamilton syringes (Hamilton, Reno, NV, USA) connected by polyethylene tubing (PE-10; Clay Adams, Parsippany, NJ, USA) to a 30-gauge injection cannula. At the time of testing, rats were removed from the cages, metal obturators were removed and the injection cannula (2 mm longer than the guide cannula) was inserted into the guide cannula. Injection volumes into the LPBN and SFO were 0.2 µl each site and 0.1 µl, respectively. The metal obturators were replaced after injections and the rats were placed back into their cages.

Drugs centrally injected

Muscimol HBr, GABA_A receptor agonist (Research Biochemicals International – RBI, Natick, MA, USA) was dissolved in saline and administered in the LPBN at the dose of 0.5 nmol/0.2 µl. Losartan potassium, AT₁ receptor antagonist (Sigma-Aldrich, St Louis, MO, USA) was dissolved in vehicle (phosphate buffer saline – PBS) and administered into the SFO at the dose of 1 µg/0.1 µl. Atropine methyl bromide, muscarinic cholinergic receptor antagonist (Sigma-Aldrich) was dissolved in vehicle and administered into the SFO at the dose of 2 nmol/0.1 µl.

Water and 0.3 M NaCl intake tests

Rats were tested in their home cages. Water and 0.3 M NaCl were provided from burettes with 0.1 ml divisions that were fitted with metal drinking spouts. Rats had no access to food during the tests. A recovery period of at least 2 days was allowed between tests.

Satiated and normovolemic rats received injections of losartan (1 µg/0.1 µl), atropine (2 nmol/0.1 µl) or vehicle (0.1 µl) into the SFO followed immediately by bilateral injections of muscimol (0.5 nmol/0.2 µl) or saline (0.2 µl) into the LPBN. Cumulative intake of 0.3 M NaCl and water was measured every 30 min for 240 min, starting immediately after LPBN injections. The sequence of the treatments into the SFO and LPBN was randomized and each rat received all the four combinations of treatments: (1) vehicle into the SFO + saline into the LPBN, (2) vehicle into the SFO + muscimol into the LPBN, (3) losartan into the SFO + muscimol into the LPBN, (4) atropine into the SFO + muscimol into the LPBN.

Histology

At the end of the last intake test, rats received injections of 2% Evans Blue solution into each site in the same volume used for drug injections. They were then deeply anesthetized with sodium thiopental (80 mg/kg of b. wt.; Cristália) and perfused transcardially with saline followed by 10% formalin. Brains were removed, fixed in 10% formalin, frozen, cut in 50-µm sections, stained with Giemsa stain, and analyzed by light microscopy to confirm the injection sites into the LPBN and SFO.

Cell culture

Cells of the SFO were obtained and cultured for studies. Briefly, rat pups were decapitated, and the head was placed in ice-cold 70% ethanol. The brain was removed immediately and placed in a petri plate with ice-cold cutting solution (220 mM sucrose, 3 mM KCl, 0.2 mM CaCl_2 , 10 mM dextrose, 6 mM MgSO_4 , 1.25 mM NaH_2PO_4 , 26 mM NaHCO_3). A thick coronal slice was made to include tissue from the rostral level of the optic chiasm caudal to the collicular level and a stereo microscope was used to identify the SFO. Isolated SFOs from five or six pups were pooled and transferred to a tube containing Earle's balanced salt solution (Sigma-Aldrich) and dispase I (4 U/2 ml; Roche, Indianapolis, IN, USA) and incubated for 1 h at 37 °C. After three washes in culture medium, a cell suspension was prepared by trituration of the fragments through a fire-polished Pasteur pipette until the tissue fragments were visibly dissociated. Several drops of the cell suspension were plated onto previously precoated (0.1 mg poly-L-lysine/ml; Sigma-Aldrich) round 22-mm coverslips and incubated in a humidified atmosphere (plus 5% CO_2) at 37 °C. Additional culture medium was added after 2–4 h postplating to allow the cells to adhere to the coverslips. The culture medium was Dulbecco's modified Eagle's medium (Sigma-Aldrich) with 10% fetal bovine serum (heat inactivated at 56 °C for 30 min; Sigma-Aldrich) and 1% L-glutamine-penicillin-streptomycin solution (Sigma-Aldrich) added.

Intracellular calcium measurement

Intracellular calcium concentration ($[\text{Ca}^{2+}]_i$) was measured with Fluo-4AM, a fluorescent calcium indicator. Cells were loaded with Fluo-4AM (Invitrogen, Carlsbad, CA, USA) by incubating cells in artificial cerebrospinal fluid (aCSF; 126 mM NaCl, 3 mM KCl, 2 mM CaCl_2 , 10 mM dextrose, 1 mM MgSO_4 , 1.25 mM NaH_2PO_4 , 26 mM NaHCO_3) containing 0.02 mM Fluo-4AM for 60 min at 37 °C. During the incubation, Fluo-4AM is absorbed by cells and hydrolyzed only within living cells to impermeant Fluo-4. For $[\text{Ca}^{2+}]_i$ measurements and pharmacological manipulations, the coverslip containing the incubated cells was mounted in a bath chamber that was attached to the microscope stage. An increase in $[\text{Ca}^{2+}]_i$ produces an increase in Fluo-4 fluorescence intensity with only a little shift in wavelength. Fluorescence intensity was measured using a video microscopic digital image analysis system (FluoView 500 Confocal Laser Scanning Microscope; Olympus, Center Valley, PA, USA) using a 488-nm Argon laser as an excitation source. Experiments were performed in SFO cells after 3 days in culture. The fluorescence intensity was recorded during baseline conditions using aCSF as bath solution. After baseline recording, the bath solution was removed and 0.1 μM ANG II or 100 μM carbachol was diluted into the bath chamber. The fluorescence intensity was recorded one minute after applying the solutions. ANG II and carbachol were dissolved in aCSF and tested in a counterbalanced design with an interval of 10 min between tests. At the end of the experiment, data was analyzed and the fluorescence intensity converted to numerical values. A response to stimulation was discerned as a sudden increase in fluorescence intensity and represented as a percentage change from baseline recording. The percentage of cells sensitive to only ANG II, only carbachol or both was also analyzed. The manipulation of removing and applying bath solution can induce a small change in fluorescence intensity ($2.99 \pm 0.67\%$). Therefore, only cells that presented a change in fluorescence intensity greater than 8.7% (average plus two standard deviations) were considered as being sensitive to the solution applied. Cell viability

was checked at the end of the experiment by applying aCSF containing 50 mM KCl (substituting an equimolar amount of NaCl). All procedures were performed in the dark and at room temperature (24 ± 2 °C).

Statistical analysis

Results are reported as means $\pm$ SEM. For *in vivo* experiments two-way analysis of variance (ANOVA) using treatments and times as factors followed by Student-Newman-Keuls tests was used for comparisons. One-way ANOVA followed by Student-Newman-Keuls was used to analyze $[Ca^{2+}]_i$ measurements data. Differences were considered significant at $p < 0.05$.

RESULT

Histological analysis

Figure 1 shows the typical injection sites into the SFO and LPBN.

The SFO injections were considered properly positioned if the dye was visible only in the parenchyma of the SFO, with no rupture of the ependyma that separates the SFO from the dorsal portion of the third ventricle (III-V). Injections outside the SFO were also analyzed and classified as: (a) injection in the dorsal portion of the III-V, if the ependyma was destroyed and no sign of injections in the SFO parenchyma detected or, (b) injection into the tissue adjacent to the SFO, if the injection site was not into the SFO tissue or in the III-V.

The center of the LPBN injections was located in the central and dorsal portions of the LPBN [see Fulwiler and Saper (Fulwiler and Saper, 1984) for definitions of the LPBN subnuclei]. The sites of the injections into the LPBN in the present study were similar to those of previous studies that showed effects of muscimol into the LPBN on NaCl and water intake (Callera et al., 2005, De Oliveira et al., 2007, Andrade-Franzé et al., 2010a, Roncari et al., 2011, Asnar et al., 2013).

Water and 0.3 M NaCl intake induced by GABAergic activation of the LPBN in rats treated with injection of losartan or atropine into the SFO

Bilateral injections of muscimol (0.5 nmol/0.2 μ l) into the LPBN combined with SFO injection of vehicle in satiated and normovolemic rats induced 0.3 M NaCl (34.5 ± 2.2 ml/4 h, vs. saline into LPBN: 0.4 ± 0.1 ml/4 h) [$F(3,24) = 63.90$; $P < 0.05$] and water intake (12.7 ± 2.4 ml/4 h, vs. saline into LPBN: 0.2 ± 0.1 ml/4 h) [$F(3,24) = 11.42$; $P < 0.05$] (Figure 2). The SFO injections of losartan (1 μ g/0.1 μ l) or atropine (2 nmol/0.1 μ l) reduced 0.3 M NaCl (6.6 ± 1.9 and 8.4 ± 2.2 ml/4 h, respectively) and water intake (4.0 ± 1.0 and 2.5 ± 1.0 ml/4 h, respectively) induced by muscimol injected into the LPBN.

Changes of $[Ca^{2+}]_i$ in cultured SFO cells treated with ANG II or carbachol

Acute application of 0.1 μ M ANG II or 100 μ M carbachol increased Fluo-4 fluorescence intensity in sensitive SFO cells by $44.8 \pm 2.5\%$ and $19.0 \pm 3.4\%$, respectively [$F(2, 280) = 427.63$; $P < 0.05$] (Figure 3). A significant number of SFO cells tested were sensitive to only ANG II (24.9%) (Table 1). A small number of the SFO cells tested were sensitive to only

carbachol (2.6%) or to carbachol and ANG II (2.3%) (Table 1). As a control, cells from the cortex were obtained and cultured. None of the cells tested presented an increase in Fluo-4 fluorescence intensity after acute application of ANG II or carbachol.

Specificity of the SFO and the LPBN as the sites where injections of losartan or atropine and muscimol, respectively, produce the effects described in the present study

The specificity of the SFO as the site of losartan and atropine action was confirmed by results from animals that received injections of muscimol into the LPBN combined with losartan or atropine injection into the III-V or in the tissue surrounding the SFO. Bilateral injections of muscimol (0.5 nmol/0.2 μ l) into the LPBN induced 0.3 M NaCl [$F(3, 21) = 42.55$, $P < 0.05$] and water intake [$F(3, 21) = 24.47$, $P < 0.05$] and the treatment with losartan (1 μ g/0.1 μ l) or atropine (2 nmol/0.1 μ l) injected into the III-V did not affect the ingestion of 0.3 M NaCl or water produced by muscimol injected into the LPBN (Table 2). Muscimol-induced 0.3 M NaCl [$F(3, 15) = 21.48$, $P < 0.05$] and water intake [$F(3, 15) = 86.89$, $P < 0.05$] was also not modified by the injection of losartan (1 μ g/0.1 μ l) or atropine (2 nmol/0.1 μ l) in the tissue surrounding the SFO (Table 3).

Bilateral injections of muscimol (0.5 nmol/0.2 μ l) in sites outside the LPBN combined with SFO injection of vehicle, losartan (1.0 μ g/0.1 μ l) or atropine (2 nmol/0.1 μ l) in satiated and normovolemic rats induced no significant intake of 0.3 M NaCl (0.8 ± 0.1 , 0.4 ± 0.3 and 0.6 ± 0.3 ml/4 h, respectively, $n = 3$) or water intake (1.0 ± 0.3 , 0.5 ± 0.2 and 1.0 ± 0.7 ml/4 h, respectively).

DISCUSSION

The present results show that the blockade of angiotensinergic or cholinergic mechanisms in the SFO almost abolishes NaCl and water intake induced by muscimol injections into the LPBN in satiated and normovolemic rats, suggesting that the activity of these mechanisms in the SFO is necessary for NaCl and water intake to muscimol injected into the LPBN. In addition, *in vitro* studies showed that SFO-cultured cells are activated by the acute application of ANG II or carbachol and can be classified as those sensitive to only ANG II, only carbachol or to both stimuli.

In agreement with earlier findings (Callera et al., 2005, Roncari et al., 2011, Asnar et al., 2013), bilateral injections of muscimol in sites outside the LPBN did not induce NaCl or water intake, confirming again the specificity of the LPBN as the site where muscimol injections induce NaCl and water intake. The specificity of the SFO as the site of losartan and atropine action was also confirmed by the results showing that injections of losartan or atropine into the III-V just below the SFO or in the tissue surrounding the SFO did not affect 0.3 M NaCl and water intake induced by muscimol injected into the LPBN.

It is worth noting that injections of muscimol into the LPBN induce 0.3 M NaCl and water intake in satiated and normovolemic rats, i.e., animals that were not subjected to any treatment that induces the activation of facilitatory mechanisms for NaCl and water intake. However, the present results suggest that the activity of angiotensinergic and cholinergic facilitatory mechanisms in the SFO is essential for the release of NaCl and water intake

when the inhibitory mechanisms are blocked with muscimol injections into the LPBN. These results extend previous studies (Colombari et al., 1996, Menani et al., 1998a, Andrade-Franzé et al., 2010a, Andrade-Franzé et al., 2010b, Roncari et al., 2011, Asnar et al., 2013) that suggested that the activity of forebrain facilitatory mechanisms is necessary for NaCl and water intake to muscimol injections into the LPBN. Therefore, NaCl and water intake induced by LPBN injections of muscimol seems to depend on the combination of the blockade of the inhibitory mechanisms with simultaneous activation of facilitatory mechanisms. These findings suggest that LPBN inhibitory mechanisms are essential to maintain sodium satiety and that angiotensinergic and cholinergic mechanisms in the SFO may stimulate sodium intake if the LPBN inhibitory mechanisms are deactivated even without an extra activation of these facilitatory mechanisms.

Muscimol injections into the LPBN in satiated, normovolemic rats usually induce only a small amount of water intake when only water is available (one-bottle test) (Callera et al., 2005). Therefore, the strong ingestion of water produced by the injections of muscimol into the LPBN when water and 0.3 M NaCl are simultaneously available (two-bottle test) [(Callera et al., 2005, Roncari et al., 2011, Asnar et al., 2013) and present results] is probably a consequence of the increased plasma osmolarity due to excessive ingestion of hypertonic NaCl. Thus, the reason for the reduced ingestion of water after the blockade of SFO angiotensinergic and cholinergic mechanisms in rats treated with muscimol into the LPBN is probably the reduced ingestion of hypertonic NaCl. Nevertheless, considering the importance of central cholinergic mechanisms for water deprivation- or hyperosmolarity-induced water intake (Block and Fisher, 1970, Lee et al., 1996), in the case of central muscarinic blockade a possible direct effect on the reduction of water intake is also possible.

ANG II acting in the SFO produces a robust 0.3 M NaCl intake when serotonergic mechanisms are inhibited by bilateral injections of methysergide into the LPBN and the AT₁ receptor antagonist losartan into the SFO abolishes the increase of NaCl and water intake induced by the injection of methysergide into the LPBN of rats treated with FURO + CAP (Colombari et al., 1996, Menani et al., 1998a). The results of the present study show that SFO injections of losartan reduce NaCl and water intake induced by muscimol injected into the LPBN of satiated, normovolemic rats. As injections of losartan into the III-V or in the tissue surrounding the SFO did not modify muscimol-induced sodium or water intake, it is possible to conclude that NaCl and water intake produced by muscimol injected into the LPBN depends on the activation of SFO AT₁ receptors.

Cholinergic mechanisms of the SFO have been implicated in the control of water intake (Block and Fisher, 1970, Fitts et al., 1985a, b). Injections of cholinergic agonists into the ventricular system induce intense dipsogenic responses and electrolytic lesions of the SFO abolish intracerebroventricular (icv) carbachol-induced water intake (Routtenberg and Simpson, 1971, Simpson and Routtenberg, 1972, 1974). The importance of the cholinergic receptors of the SFO for water intake was also demonstrated by the dipsogenic response to injections of carbachol directly into the SFO (Routtenberg and Simpson, 1971). More recent and the present results have suggested that central cholinergic mechanisms are also involved in the control of sodium intake (Menani et al., 2002, Asnar et al., 2013). Earlier results suggested the opposite, since continuous icv infusion of carbachol induced only thirst and no

sodium intake in spite of a concomitant heavy loss of extracellular sodium (Fitts et al., 1985a, Fitzsimons and Fuller, 1985). Yet, besides inducing water intake, icv injection of carbachol also induces hypertonic NaCl intake if the LPBN inhibitory mechanisms are deactivated (Menani et al., 2002). Moreover, the present results show that injections of atropine into the SFO reduce 0.3 M NaCl and water intake induced by muscimol injected into the LPBN, suggesting that SFO cholinergic receptors are also involved in the control of hypertonic sodium intake in this condition.

The SFO sends projections to several forebrain areas such as the hypothalamic paraventricular nucleus, supraoptic nucleus and amygdala, which are important sites for the integration and control of effector mechanisms (behavioral, hormonal and autonomic) involved on fluid-electrolyte balance and cardiovascular regulation (Li and Ferguson, 1993, Johnson et al., 1996, Johnson and Thunhorst, 1997, Krause et al., 2011). Early studies showed that the SFO is a main central site for ANG II action to induce pressor and dipsogenic responses (Simpson and Routtenberg, 1973, Simpson et al., 1978, Mangiapane and Simpson, 1980). Additionally, the SFO is involved with central cholinergic-induced water intake (Routtenberg and Simpson, 1971, Simpson and Routtenberg, 1972, 1974). The sensitivity of SFO neurons to angiotensinergic and cholinergic stimuli was previously demonstrated by electrophysiological studies (Buranarugsa and Hubbard, 1979, Ferguson and Bains, 1996, Ferguson et al., 1997, Ferguson et al., 2001, Johnson et al., 2001, Ferguson, 2009). Further evidence of the sensitivity of SFO neurons to angiotensinergic and cholinergic stimuli are the present results showing that ANG II and carbachol acutely applied increase $[Ca^{2+}]_i$ in dissociated SFO-cultured cells. From the total of SFO cells tested, about 25% were sensitive to only ANG II, 2.6% were sensitive to only carbachol and 2.3% were sensitive to carbachol and ANG II. Cells of the SFO sensitive to both angiotensinergic and cholinergic stimuli are perhaps those involved in the facilitation of LPBN muscimol-induced sodium and water intake, a response similarly blocked by angiotensinergic or cholinergic antagonist injected into the SFO. More studies are necessary to investigate if cells sensitive to both stimuli are those involved on sodium and water intake to muscimol into the LPBN. However, it is also possible that cholinergic and angiotensinergic sensitive neurons, not necessarily the same neuron, are part of the SFO circuitry involved in the control of sodium and water intake.

In conclusion, the present results show that the activity of angiotensinergic and cholinergic facilitatory mechanisms in the SFO is essential for NaCl and water intake induced by the deactivation of the inhibitory mechanisms with injections of muscimol into the LPBN. This suggests that the activity of facilitatory mechanisms, particularly in the SFO, together with the deactivation of the inhibitory mechanisms, is a condition for strong stimulation of sodium appetite. Considering the presence of different types of cells in the SFO sensitive to ANG II and cholinergic stimuli, future studies are needed to investigate if the AT_1 and cholinergic muscarinic receptors of the SFO implicated in the control of sodium intake are present in the same cell or in different neurons in a local circuit of neurons.

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LIST OF ABBREVIATIONS

aCSF	artificial cerebrospinal fluid
ANG II	angiotensin II
ANOVA	analysis of variance
b. wt	body weight
[Ca²⁺]_i	intracellular calcium concentration
CAP	captopril
FURO	furosemide
icv	intracerebroventricular
III-V	third ventricle
LPBN	lateral parabrachial nucleus
SFO	subfornical organ

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Fig. 1. Photomicrographs of coronal brain sections from an animal representative of the group tested showing (arrows) the typical sites of injections into the (A) SFO and (B) LPBN. scp, superior cerebellar peduncle (delimited by dashed lines in B). Black bar in A and B = 1.0 mm.

Fig. 2.

Cumulative (A) 0.3 M NaCl and (B) water intake in satiated and normovolemic rats that received bilateral injections of muscimol (0.5 nmol/0.2 μ l) or saline into LPBN combined to injection of losartan (1 μ g/0.1 μ l), atropine (2 nmol/0.1 μ l) or vehicle (PBS) into the SFO. Values are reported as means $\pm$ SEM; n = number of animals.

Fig. 3.

(A) Representative Fluo-4 fluorescent images of SFO cells in control condition (left) and after applying ANG II (middle) or carbachol (right). (B) Changes in fluorescence intensity induced by acute application of ANG II or carbachol. Values are reported as means $\pm$ SEM; n = number of cells.

Table 1

Percentage of cultured SFO cells sensitive to acute application of ANG II and/or carbachol.

Stimulus	%	n
Only ANG II	24.9	66
Only carbachol	2.6	7
Both	2.3	6
None	70.2	186

Values are represented as percentage of the total number of cells tested. ANG II (0.1 μ M); carbachol (100 μ M); n = number of cells.

Table 2

Cumulative 0.3 M NaCl and water intake by satiated and normovolemic rats that received bilateral injections of muscimol or saline into the LPBN combined with injection of losartan, atropine or vehicle into the III-V.

Treatment	60 min	120 min	180 min	240 min
0.3 M NaCl intake (ml)				
Vehicle III-V + saline LPBN	0.5 ± 0.4	0.6 ± 0.4	0.6 ± 0.4	0.6 ± 0.4
Vehicle III-V + muscimol LPBN	8.4 ± 4.2*	23.7 ± 3.2*	32.3 ± 2.5*	32.3 ± 2.5*
Losartan III-V + muscimol LPBN	2.7 ± 1.3	19.0 ± 3.1*	27.6 ± 1.6*	27.6 ± 1.6*
Atropine III-V + muscimol LPBN	4.2 ± 1.6	23.0 ± 1.6*	26.6 ± 2.3*	26.6 ± 2.3*
Water intake (ml)				
Vehicle III-V + saline LPBN	0.9 ± 0.4	1.1 ± 0.4	1.2 ± 0.4	1.2 ± 0.4
Vehicle III-V + muscimol LPBN	2.5 ± 0.6	14.2 ± 2.1*	18.8 ± 2.4*	18.9 ± 2.5*
Losartan III-V + muscimol LPBN	1.3 ± 0.6	10.9 ± 2.2*	16.3 ± 2.4*	16.3 ± 2.4*
Atropine III-V + muscimol LPBN	1.1 ± 0.5	12.9 ± 1.4*	15.3 ± 1.6*	15.4 ± 1.6*

Results are reported as means ± SEM;

* Different from vehicle III-V + saline LPBN; n = 8. Muscimol (0.5 nmol/0.2 µl); losartan (1 µg/0.1 µl); atropine (2 nmol/0.1 µl); vehicle (PBS, phosphate buffer saline).

Table 3

Cumulative 0.3 M NaCl and water intake by satiated and normovolemic rats that received bilateral injections of muscimol or saline into the LPBN combined with injection of losartan, atropine or vehicle in the tissue surrounding to the SFO.

Treatment	60 min	120 min	180 min	240 min
0.3 M NaCl intake (ml)				
Vehicle tissue + saline LPBN	1.5 ± 1.3	1.6 ± 1.3	1.7 ± 1.3	1.7 ± 1.3
Vehicle tissue + muscimol LPBN	8.2 ± 4.8 *	32.9 ± 4.3 *	39.1 ± 4.3 *	39.2 ± 4.3 *
Losartan tissue + muscimol LPBN	9.6 ± 7.3	26.0 ± 7.2 *	35.5 ± 5.4 *	35.4 ± 4.2 *
Atropine tissue + muscimol LPBN	10.6 ± 6.1	29.5 ± 3.7 *	35.3 ± 4.2 *	39.9 ± 5.4 *
Water intake (ml)				
Vehicle tissue + saline LPBN	0.8 ± 0.2	0.9 ± 0.2	1.0 ± 0.2	1.0 ± 0.2
Vehicle tissue + muscimol LPBN	1.5 ± 1.0	13.1 ± 1.8 *	23.8 ± 1.2 *	24.9 ± 1.4 *
Losartan tissue + muscimol LPBN	2.4 ± 1.4	13.9 ± 2.6 *	21.5 ± 1.2 *	22.1 ± 1.5 *
Atropine tissue + muscimol LPBN	2.4 ± 1.0	14.3 ± 1.5 *	20.1 ± 1.3 *	23.8 ± 1.9 *

Results are reported as means ± SEM;

* Different from vehicle tissue + saline LPBN; n = 6. Muscimol (0.5 nmol/0.2 µl); losartan (1 µg/0.1 µl); atropine (2 nmol/0.1 µl); vehicle (PBS, phosphate buffer saline).