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Importance of the central nucleus of the amygdala on sodium intake caused by deactivation of lateral parabrachial nucleus

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ABSTRACT

The lateral parabrachial nucleus (LPBN) and the central nucleus of the amygdala (CeA) are important central areas for the control of sodium appetite. In the present study, we investigated the importance of the facilitatory mechanisms of the CeA on NaCl and water intake produced by the deactivation of LPBN inhibitory mechanisms. Male Holtzman rats ($n=7$–14) with stainless steel cannulas implanted bilaterally in the CeA and LPBN were used. Bilateral injections of moxonidine (α_2-adrenoceptor/imidazoline agonist, 0.5 nmol/0.2 μl) into the LPBN increased furosemide+captopril-induced 0.3 M NaCl (29.7±7.2, vs. vehicle: 4.4±1.6 ml/2 h) and water intake (26.4±6.7, vs. vehicle: 8.2±1.6 ml/2 h). The $GABA_A$ agonist muscimol (0.25 nmol/0.2 μl) injected bilaterally into the CeA abolished the effects of moxonidine into the LPBN on 0.3 M NaCl (2.8±1.6 ml/2 h) and water intake (3.3±2.3 ml/2 h). Euhydrated rats treated with muscimol (0.5 nmol/0.2 μl) into the LPBN also ingested 0.3 M NaCl (19.1±6.4 ml/4 h) and water (8.8±3.2 ml/4 h). Muscimol (0.5 nmol/0.2 μl) into the CeA also abolished 0.3 M NaCl (0.1±0.04 ml/4 h) and water intake (0.1±0.02 ml/4 h) in euhydrated treated with muscimol into the LPBN. The present results show that neuronal deactivation of the CeA abolishes NaCl intake produced by the blockade of LPBN inhibitory mechanisms, suggesting an interaction between facilitatory mechanisms of the CeA and inhibitory mechanisms of the LPBN in the control of NaCl intake.

Abbreviations: ANG II, angiotensin II; CAP, captopril; CeA, central nucleus of amygdala; FURO, furosemide; GABA, γ-aminobutyric acid; LPBN, lateral parabrachial nucleus; NTS, nucleus of the solitary tract; s.c., subcutaneously

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1. Introduction

The lateral parabrachial nucleus (LPBN), a pontine structure that lies dorsolaterally to the superior cerebellar peduncle, is connected with several brain areas that belong to a circuit subserving the control of sodium appetite and thirst like the nucleus of the solitary tract (NTS), area postrema (AP), paraventricular nucleus of the hypothalamus (PVN), central nucleus of amygdala (CeA) and median preoptic nucleus (MnPO) (Ciriello et al., 1984; Fulwiler and Saper, 1984; Herbert et al. 1990; Jhamandas et al., 1992, 1996; Krukoff et al., 1993; Lança and van der Kooy, 1985; Norgren, 1981).

The LPBN strongly inhibits hypertonic NaCl intake, an influence which is hypothesized to prevent excessive solute intake (Andrade-Franze et al., 2010a, 2010b; Andrade et al., 2004, 2011; Callera et al., 2005; De Oliveira et al., 2008; Gasparini et al., 2015a; Menani et al., 2014; Roncari et al., 2014). Signals that influence water and NaCl intake like those from arterial baroreceptors, cardiopulmonary receptors, gustatory receptors and other visceral receptors that reach the NTS ascend to the LPBN (Norgren, 1981; Lança and van der Kooy, 1985; Ciriello et al., 1984; Fulwiler and Saper, 1984; Herbert et al. 1990; Jhamandas et al., 1992, 1996). These signals may modulate the activity of LPBN inhibitory mechanisms by releasing different neurotransmitters like serotonin, cholecystokinin, corticotrophin-releasing factor (CRF) and glutamate which increase the inhibitory action, whereas others like GABA, opioids, ATP and noradrenaline reduce the inhibitory action (Andrade et al., 2004, 2011; Callera et al., 2005; De Gobbi et al., 2009, Gasparini et al., 2009, De Oliveira et al., 2007, 2008, 2011; Menezes et al., 2011, 2014; Roncari et al., 2014). The deactivation of the inhibitory mechanisms by changing the activity of specific neurotransmitters/receptors in the LPBN increases hypertonic NaCl and/or water intake induced by different dipsogenic or natriorexigenic stimuli like angiotensin II (ANG II), sodium depletion, water deprivation, central cholinergic activation or even osmoreceptor activation (Menani and Johnson, 1995, 1998; Menani et al., 1996, 2002, 2014; De Luca et al., 2003; Andrade et al., 2004, 2006; De Gobbi et al., 2009; Gasparini et al., 2015b). In addition, the neuronal deactivation with bilateral injection of the $GABA_A$ agonist muscimol into the LPBN stimulates hypertonic NaCl intake by euhydrated rats (Callera et al., 2005; De Oliveira et al., 2007).

Bilateral electrolytic lesions of the CeA abolish daily 0.5 M NaCl intake and sodium appetite induced by 24 h of sodium depletion, subcutaneous (s.c.) deoxycorticosterone (DOCA) or yohimbine (α_2-adrenoceptor agonist) or by intracerebroventricular (i.c.v.) injections of renin (Covian et al., 1975; Galaverna et al., 1992; Zardetto-Smith et al., 1994), suggesting that contrary to the LPBN, important facilitatory mechanisms for the control of sodium intake are present in the CeA. Damage to the CeA also abolishes the increased water and 0.3 M NaCl intake produced by bilateral injections of the α_2-adrenoceptor agonist moxonidine into the LPBN of rats treated with subcutaneous (s.c.) injections of the diuretic furosemide (FURO) combined with low dose of captopril (CAP) s.c. (Andrade-Franze et al., 2010b). The FURO+CAP is a treatment that induces hypovolemia, mild hypotension and acute NaCl and water intake dependent on central production of angiotensin II (ANG II) (Andrade et al., 2004; Fitts and Masson, 1989; Gasparini et al., 2009; Thunhorst and Johnson, 1994). Furthermore, bilateral electrolytic lesions of the CeA abolish water and 0.3 M NaCl intake produced by bilateral injections of the $GABA_A$ agonist muscimol into the LPBN in normovolemic and euhydrated rats (Andrade-Franze et al., 2010a). These results suggest that the integrity of the CeA is essential for sodium intake that results from the deactivation of the LPBN inhibitory mechanisms (Andrade-Franze et al., 2010a, 2010b).

Although the integrity of the CeA is certainly important for the increase of sodium intake that results from the deactivation of LPBN inhibitory mechanisms (Andrade-Franze et al., 2010a, 2010b), the effects of electrolytic lesions might result from non-specific destruction of fibers of passage and not of neuronal cell bodies. Therefore, the objective of the present study was to find out if the local neuronal activity in the CeA is important for the increased water and NaCl intake that results from deactivation of the LPBN inhibitory mechanisms. For this purpose, the activity of CeA neurons was blocked with injections of muscimol into the CeA. The injections into the CeA were combined with injections of either muscimol or moxonidine into the LPBN to deactivate the inhibitory mechanisms in the LPBN.

2. Results

2.1. *Histological analysis*

Fig. 1A shows the typical bilateral injection sites in the CeA. The CeA injection sites were located lateraly to the tip of the optic tract, above the basomedial amygdaloid nucleus and medial to the basolateral amygdaloid nucleus. The sites of the injections in the present study were similar to those that previous studies showed the effects of lesions of the CeA on NaCl intake (Andrade-Franze et al., 2010a, 2010b).

Fig. 1B shows the typical bilateral injection sites in the LPBN. The LPBN injection sites were centered in the central lateral and dorsal lateral portions of the LPBN (see Fulwiler and Saper, 1984, for definitions of LPBN subnuclei). The sites of the injections in the present study were similar to those that previous studies showed the effects of muscimol or moxonidine injected into the LPBN on NaCl and water intake (Andrade-Franze et al., 2010a, 2010b; Andrade et al., 2004, 2006; Callera et al., 2005).

2.2. *FURO+CAP-induced water and 0.3 M NaCl intake in rats treated with bilateral injections of muscimol into the CeA combined with moxonidine into the LPBN*

ANOVA showed differences between treatments for 0.3 M NaCl [$F(3,18)=10.0$; $p<0.05$] ($n=7$) (Fig. 2A) and water intake, [$F(3,18)=9.1$; $p<0.05$] (Fig. 2B).

In rats treated with saline into the CeA, bilateral injections of moxonidine (0.5 nmol/0.2 μl) into the LPBN increased FURO+CAP-induced 0.3 M NaCl and water intake. Bilateral injections of muscimol (0.25 nmol/0.2 μl) into the CeA abolished the increase in FURO+CAP-induced 0.3 M NaCl and water

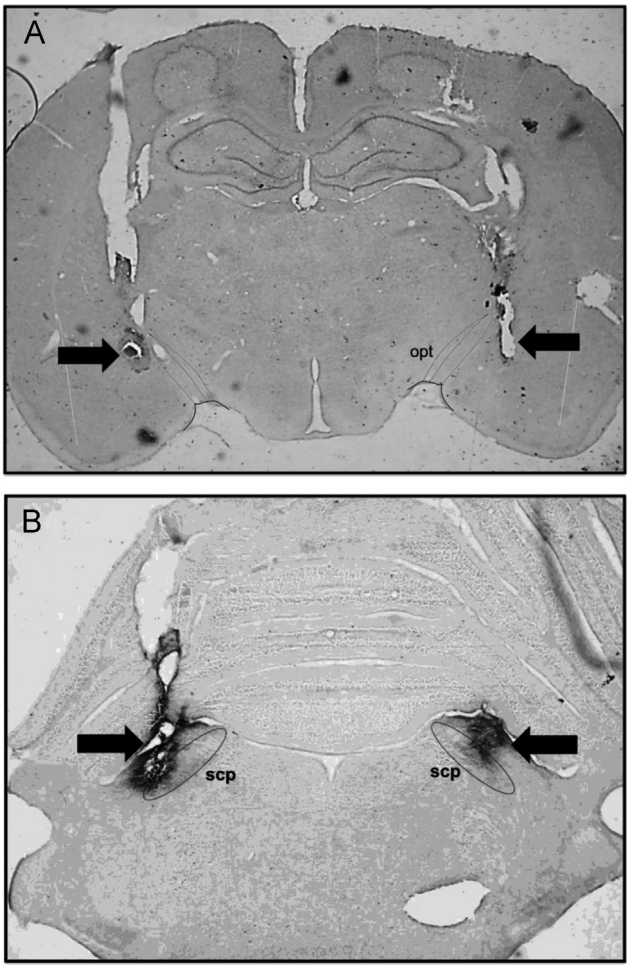

Fig. 1 – Photomicrographs of coronal sections of a brain from one rat representative of those tested showing (arrows) (A) the sites of bilateral injections into the CeA and (B) the sites of bilateral injections into the LPBN. (opt-optic tract; scp-superior cerebellar peduncle).

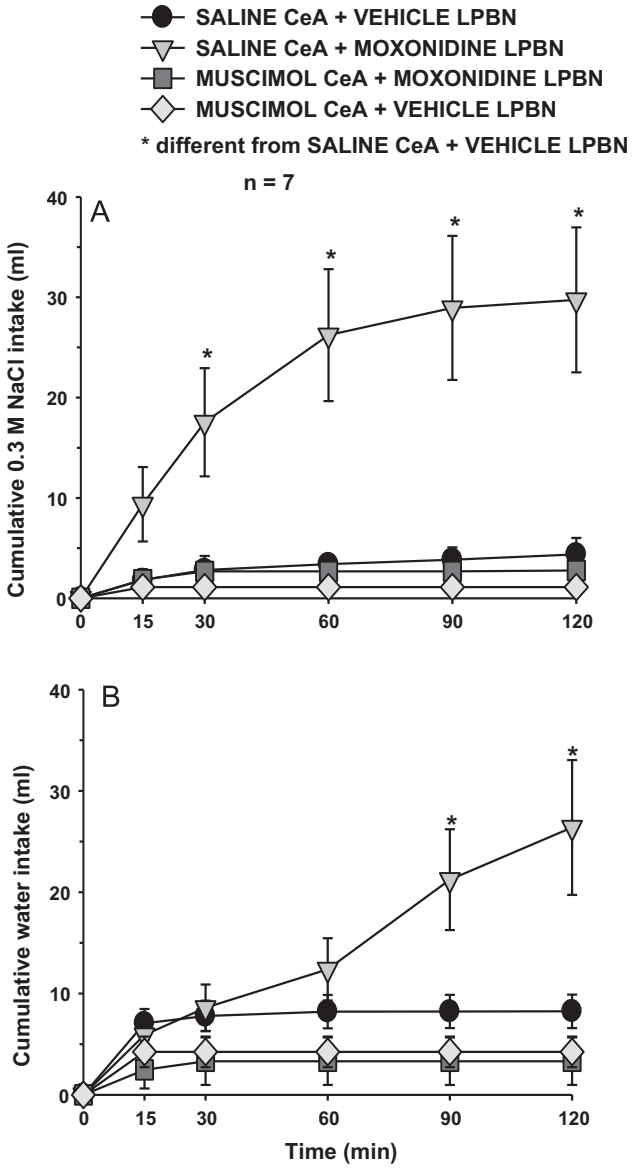

Fig. 2 – (A) Cumulative intake of 0.3 M NaCl and (B) cumulative intake of water by the treatment with FURO+CAP sc in rats that received bilateral injections of muscimol (0.25 nmol/0.2 μl) or saline into the CeA combined with bilateral injections of moxonidine (0.5 nmol/0.2 μl) or vehicle into the LPBN. The results are expressed as means ± SEM, n = number of animals.

intake produced by the treatment with bilateral injections of moxonidine into the LPBN (Fig. 2). Muscimol injected into CeA in rats treated with vehicle into LPBN did not significantly modify 0.3 M NaCl or water induced by FURO+CAP.

2.3. *Water and 0.3 M NaCl intake by euhydrated rats treated with muscimol injections simultaneously into the LPBN and CeA*

ANOVA showed difference between treatments for 0.3 M NaCl [$F(3,30)=9.22$; $p<0.05$] ($n=11$) (Fig. 3A) and water intake [$F(3,30)=6.61$; $p<0.05$] (Fig. 3B).

Euhydrated rats treated with bilateral injections of muscimol (0.5 nmol/0.2 μl) into the LPBN combined with saline injections into the CeA ingested marked amount of 0.3 M NaCl and water (Fig. 3). Muscimol (0.5 nmol/0.2 μl) injected bilaterally into the CeA abolished 0.3 M NaCl and water intake in rats treated with muscimol injected bilaterally into the LPBN (Fig. 3). Muscimol injections into the CeA combined with saline injections into the LPBN did not affect 0.3 M NaCl or water intake in euhydrated rats.

2.4. *Water and 0.3 M NaCl intake by rats that received injections unilaterally, bilaterally or partially outside the LPBN and CeA*

Rats with injections not correctly placed bilaterally into the CeA or LPBN (injections made unilaterally, bilaterally or partially outside the LPBN or CeA) were grouped as rats with injections outside those areas and the results of these rats were analyzed and presented on Tables 1 and 2.

Bilateral injections of moxonidine (0.5 nmol/0.2 μl) into the LPBN combined with injections of saline outside the CeA increased FURO+CAP-induced water and 0.3 M NaCl. Injections of muscimol (0.25 nmol/0.2 μl) outside the CeA only

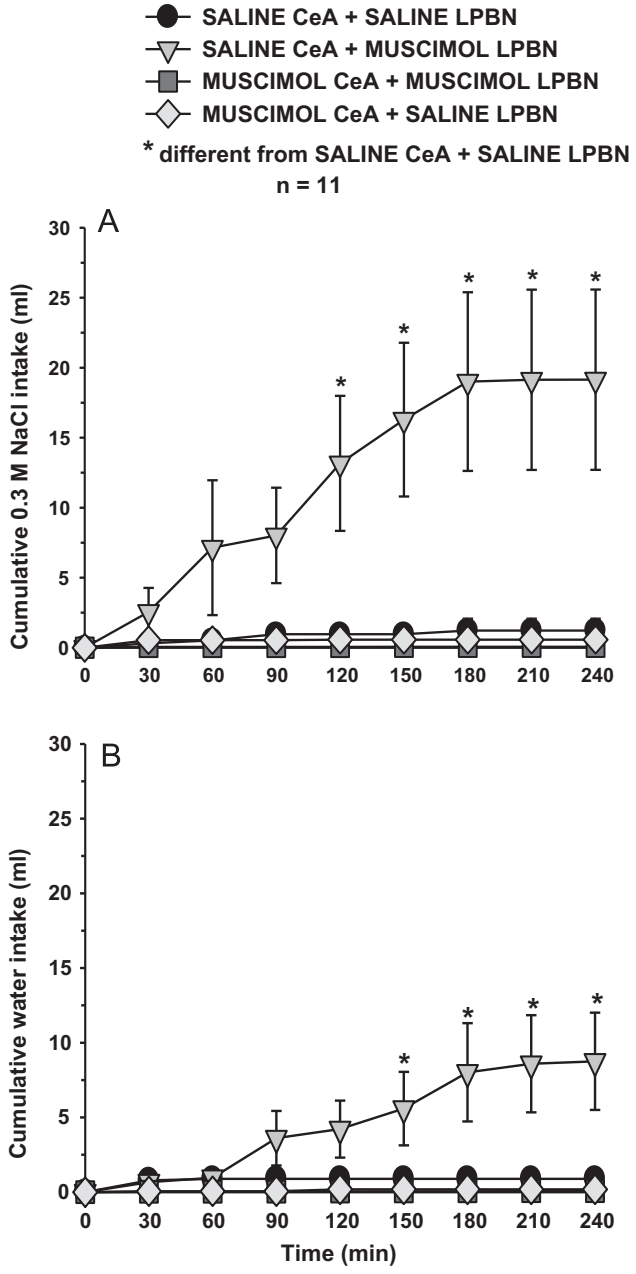

Fig. 3 – (A) Cumulative intake of 0.3 M NaCl and (B) cumulative intake of water by euhydrated rats that received bilateral injections of muscimol (0.5 nmol/0.2 μl) or saline into the CeA combined with bilateral injections of muscimol (0.5 nmol/0.2 μl) or saline into the LPBN. The results are expressed as means ± SEM, *n* = number of animals.

partially reduced water and 0.3 M NaCl in rats treated with moxonidine into the LPBN (Table 1), an effect different from that of rats treated with muscimol bilaterally into the CeA combined with moxonidine into the LPBN in which the ingestion was almost abolished (2.8 ± 1.6 ml/2 h). These results suggest that bilateral inhibition of the CeA neurons is necessary for complete inhibition of sodium intake produced by the injections of moxonidine into the LPBN in rats treated with FURO+CAP, which reinforces the importance of the CeA facilitatory mechanisms for sodium intake in this condition.

Injections of muscimol (0.5 nmol/0.2 μl) outside the LPBN combined with saline or muscimol (0.5 nmol/0.2 μl) outside the CeA produced no significant ingestion of 0.3 M NaCl or water in euhydrated rats (Table 2), which demonstrates the specificity of LPBN as the site where muscimol injections produce effects on water and NaCl intake.

Rats treated with partial or bilateral injections of muscimol (0.5 nmol/0.2 μl) into the LPBN combined with saline outside the CeA ingested a low amount of 0.3 M NaCl, which was reduced when rats were treated with muscimol outside

Table 1 – Water and 0.3 M NaCl intake induced by the treatment with FURO+CAP s.c. in rats that received injections of muscimol or saline unilaterally, bilaterally or partially outside the CeA combined with bilateral injections of moxonidine or vehicle into the LPBN.

Treatment	0.3 M NaCl *intake* (ml/2 h)	*Water intake* (ml/2 h)
Saline out CeA+vehicle LPBN	4.0 ± 1.0	12.4 ± 1.0
Saline out CeA+moxonidine LPBN	35.5 ± 2.8*	26.8 ± 1.6*
Muscimol out CeA+vehicle LPBN	1.5 ± 0.3	4.5 ± 1.4
Muscimol out CeA+moxonidine LPBN	14.0 ± 5.5*	7.3 ± 2.5*

The results are expressed as means ± SEM, *n* = 10. Muscimol (0.25 nmol/0.2 μl); moxonidine (0.5 nmol/0.2 μl); out CeA: unilateral, bilateral or partial injection outside the CeA.
* Different from saline out CeA+vehicle LPBN; $p < 0.05$.

Table 2 – Water and 0.3 M NaCl intake by euhydrated rats that received injections of muscimol or saline unilaterally, bilaterally or partially outside the CeA combined with injections of muscimol or saline into or outside the LPBN.

Treatment	0.3 M NaCl *intake* (ml/4 h)	*Water intake* (ml/4 h)
Saline out CeA+saline out LPBN	0.07 ± 0.04	0.04 ± 0.02
Saline out CeA+muscimol out LPBN	0.3 ± 0.2	1.0 ± 0.6
Muscimol out CeA+saline out LPBN	0.1 ± 0.1	0.9 ± 0.5
Muscimol out CeA+muscimol out LPBN	0.2 ± 0.2	0.5 ± 0.4
Saline out CeA+saline LPBN	0.07 ± 0.03	0.5 ± 0.2
Saline out CeA+muscimol LPBN	8.0 ± 3.7*	3.6 ± 1.4*
Muscimol out CeA+saline LPBN	0.07 ± 0.02	0.1 ± 0.1
Muscimol out CeA+muscimol LPBN	0.1 ± 0.1	0.07 ± 0.02

The results are expressed as means ± SEM, *n* = 7–14. Muscimol (0.5 nmol/0.2 μl); out CeA or LPBN: unilateral, bilateral or partial injection outside the CeA or LPBN.
* Different from saline out CeA+saline LPBN; $p < 0.05$.

Table 3 – Summary of the effects of the combination of treatments into the CeA and LPBN on 0.3 M NaCl intake in euhydrated rats or rats treated with FURO+CAP.

Treatment	CeA	LPBN	0.3 M NaCl intake
Euhydrated rats	Saline	Muscimol	↑
Euhydrated rats	Muscimol	Muscimol	No intake
FURO+CAP	Saline	Moxonidine	↑
FURO+CAP	Muscimol	Moxonidine	No intake

the CeA (Table 2). These results suggest that perhaps a partial inhibition of the facilitatory mechanisms of the CeA is enough to affect the ingestion of sodium in this condition. It is also not possible to exclude an inhibitory action of muscimol at the dose of 0.5 nmol in other nuclei of the amygdala adjacent to the CeA.

3. Discussion

The results show that bilateral injections of muscimol into the CeA abolished water and 0.3 M NaCl intake induced by bilateral injections of muscimol into the LPBN in euhydrated rats or by injections of moxonidine into the LPBN in FURO+-CAP-treated rats (Table 3). The present results with the blockade the CeA neuronal activity are similar to previous results (Andrade-Franze et al., 2010a, 2010b) with electrolytic lesions of the CeA and suggest that the activity of the facilitatory mechanisms of the CeA is essential for sodium intake in response to the deactivation of the LPBN inhibitory mechanisms.

The injections of muscimol into the CeA (present results) similar to electrolytic lesions of the CeA (Andrade-Franze et al., 2010a) produced a tendency to reduce FURO+CAP-induced 0.3 M NaCl intake in rats treated with vehicle into the LPBN. The same treatments in the CeA reduced 0.3 M NaCl intake produced by 24 h of sodium depletion (furosemide followed by 24 h sodium deficient diet) (Galaverna et al., 1992; Wang et al., 2012), which suggests that the facilitatory mechanisms of the CeA are involved in mediating sodium intake in sodium-depleted rats. The low amount of 0.3 M NaCl ingested and the variability of 0.3 M NaCl intake by rats treated with FURO+CAP may explain the absence of significant reduction of 0.3 M NaCl intake in FURO+CAP treated rats that received muscimol into the CeA.

Bilateral injections of muscimol (0.5 nmol/0.2 μl) into the LPBN only slightly increased water intake when rats had only water available (one-bottle test) (Callera et al., 2005). Bilateral injections of moxonidine (0.5 nmol/0.2 μl) into the LPBN also produced no effect on FURO+CAP-induced water intake if only water was available for rats to drink (Andrade et al., 2004). Moreover, in the present work, the increase in 0.3 M NaCl intake preceded water intake in response to both treatments. Therefore, the marked ingestion of water in rats treated with muscimol or moxonidine into the LPBN combined with saline into the CeA is probably a consequence of the increased plasma osmolarity due to the simultaneous excessive ingestion of hypertonic NaCl. In this case, the reduced ingestion of water caused by the injections of muscimol into the CeA is probably due to the reduced ingestion of hypertonic NaCl, similar to what was proposed previously for electrolytic lesions of the CeA (Galaverna et al., 1992; Wang et al., 2012; Zardetto-Smith et al., 1994). The inactivation of the CeA with injections of muscimol increases the exploratory behavior in rats (Moreira et al., 2007), which suggests that the effects of muscimol injections into the CeA on water and sodium intake are not due to an impairment of motor activity. Bilateral injections of muscimol into the CeA also did not change food intake in 24 h food deprived-rats (unpublished data), which suggests that muscimol into the CeA does not inhibit all motivated behaviors. In addition, bilateral injections of moxonidine (0.5 nmol/0.2 μl) into the LPBN produce no change in the ingestion of 0.06 M sucrose or in food deprivation-induced food intake (Andrade et al., 2004, 2007), suggesting that moxonidine into the LPBN does not increase behavioral responses in general.

The anatomical specificity of the LPBN and CeA as the central sites where moxonidine and/or muscimol injections produce effects on water and/or NaCl intake was demonstrated by previous studies (Andrade et al., 2004, Callera et al., 2005; Wang et al., 2012). The present results show that muscimol injected outside of the CeA at the dose of 0.25 nmol did not abolish NaCl intake in rats treated with moxonidine into the LPBN, whereas at the dose of 0.5 nmol it blocked NaCl intake in rats treated with muscimol into the LPBN. The results with injections of muscimol (0.25 nmol) outside or partially outside the target sites suggest that bilateral inhibition of the CeA neurons is necessary for complete inhibition of sodium intake. This reinforces the importance of the CeA facilitatory mechanisms for sodium intake in this condition. On the other hand, the effects of muscimol (0.5 nmol) injected outside the CeA might suggest that not only the neuronal activity of the CeA is important for the sodium intake. Thus, it is not possible to exclude an inhibitory action of muscimol (0.5 nmol) in other nuclei of the amygdala adjacent to the CeA.

The CeA has reciprocal direct connections with the parabrachial nucleus (PBN) (Jhamandas et al., 1996; Norgren, 1995). Visceral and gustatory signals that ascend to the NTS, and are important to control sodium intake, make a second relay in the PBN, prior to projecting to the CeA (Flynn et al., 1991; Geerling and Loewy, 2006; Johnson and Thunhorst, 1997; Mungarndee et al., 2006). In addition, the CeA is also connected with the lamina terminalis which is the primary site of ANG II acting in the brain (Fitzsimons, 1998; Johnson and Thunhorst, 1997; McKinley et al., 2001). Therefore, CeA may receive signals of extracellular dehydration produced by ANG II acting in the lamina terminalis, and electrolytic lesions of the CeA impair ANG II-induced hypertonic NaCl intake (Galaverna et al., 1992; Zardetto-Smith et al., 1994). Similar to FURO+CAP, water and 0.3 M NaCl intake produced by muscimol injected into the LPBN depends on angiotensinergic activation in the forebrain, particularly in the lamina terminalis (Andrade et al., 2004; Fitts and Masson, 1989; Gasparini et al., 2009; Roncari et al., 2011, 2014; Thunhorst and Johnson, 1994). One possibility is that inhibitory and facilitatory signals relaying in the LPBN and lamina terminalis, respectively, converge to the CeA and the predominant activation or inhibition of this area determines sodium

appetite or not. Disrupting the neuronal activity in the CeA with muscimol reduces or blocks signal output and, therefore, sodium intake is suppressed.

In conclusion, the present results show that disrupting the neuronal activity in the CeA abolishes NaCl intake produced by the blockade of LPBN inhibitory mechanisms, suggesting an interaction between the facilitatory mechanisms of the CeA and the inhibitory mechanisms of the LPBN in the control of NaCl intake.

4. Experimental procedure

4.1. Animals

Male Holtzman rats (total of 49 rats) bred in the Unesp facility at Araraquara – SP, Brazil, weighing 250 to 270 g at the beginning of the tests, were used. The animals were housed in individual stainless steel cages with free access to normal 0.5–1.0% sodium diet (Guabi Rat Chow, Paulinia, SP, Brazil), water and 0.3 M NaCl solution. Rats were maintained at a temperature of 23±2 °C, humidity of 55±10% and on a 12-h light/dark cycle with light onset at 7:00 AM. All the experimental procedures were approved by Ethical Committee in Animal Use (CEUA) from Dentistry School of Araraquara – UNESP (Proc. CEUA nr. 35/2010). 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). Efforts were made to reduce animal discomfort with analgesic medication and the number of animals used by testing the same rat four times.

4.2. Brain surgery

Rats were anesthetized with ketamine (80 mg/kg of body weight; Cristalia, Itapira, SP, Brazil) combined with xylazine (7 mg/kg of body weight; Agener União, Embu-Guaçu, SP, Brazil) intraperitoneally, placed in a stereotaxic instrument (Kopf, Tujunga, CA, USA) and had the skull leveled between bregma and lambda. Bilateral stainless steel cannulas (0.6 mm o.d.) were implanted dorsally to the LPBN and CeA using the following coordinates: 9.2 mm caudal to bregma, 2.1 mm lateral to the midline and 4.2 mm below the dura mater for the LPBN and 2.2 mm caudal to bregma, 4.0 mm lateral to the midline and 4.5 mm below the dura mater for the CeA (Paxinos and Watson, 2004).The tips of the cannulas were positioned 2 mm above the LPBN and the CeA. The cannulas were fixed to the cranium using dental acrylic resin and jeweler screws. A metal obturator (0.3 mm o.d.) filled the cannulas between tests. At the end of the surgery, the animals received an intramuscular injection of antibiotic (Pentabiótico Veterinário – Pequeno Porte, Fort Dodge Saúde Animal Ltda., 0.2 ml/rat) and a subcutaneous injection of the analgesic Ketoflex (ketoprofen 1%, 0.03 ml/rat). The experimental procedures started five days after the surgery. Rats were tested in their home cages.

4.3. Drugs

Moxonidine hydrochloride (α_2-adrenoceptor/imidazoline agonist, 0.5 nmol/0.2 μl) was dissolved in a mix of propylene glycol and water 2:1 (vehicle). Muscimol HBr ($GABA_A$ receptor agonist, 0.25 or 0.5 nmol/0.2 μl) was dissolved in saline. Furosemide (10 mg/kg of body weight) was dissolved in alkaline saline (pH adjusted to 9.0 with NaOH). Captopril (5 mg/kg of body weight) was dissolved in saline. All drugs were purchased from Sigma-Aldrich Chem., St Louis, MO, USA.

4.4. Injections into the LPBN and CeA

Injections into the LPBN and CeA were made using 5-μl Hamilton syringes (Hamilton, Reno, NV, USA) connected by polyethylene tubing (PE-10) to injection needle (0.3 mm o.d.). Starting one day after cerebral surgery, rats were handled daily and trained for the procedure of central injections. At time of testing, rats were removed from the cages and restrained by a hand on a table. Obturators were removed and the injection needles (2 mm longer than the guide cannulas) introduced in the brain. The injection volume into the LPBN and CeA was 0.2 μl each site. The obturators were replaced after injections, and the rats placed back into the cages.

4.5. Water and 0.3 M NaCl intake induced by FURO+CAP in rats treated with moxonidine into the LPBN combined with muscimol into the CeA

Rats ($n=17$) received injections of FURO (10 mg/kg of body weight)+CAP (5 mg/kg of body weight) s.c. and were returned to their home cages in the absence of food, water and NaCl. Forty-five min after FURO+CAP, rats received bilateral injections of moxonidine (0.5 nmol/0.2 μl) or vehicle into the LPBN, and of muscimol (0.25 nmol/0.2 μl) or saline into the CeA; and 15 min later they had access to water and 0.3 M NaCl. Water and 0.3 M NaCl were provided from burets with 0.1-ml divisions that were fitted with metal drinking spouts. Cumulative water and 0.3 M NaCl intake was measured at 15, 30, 60, 90 and 120 min starting 1 h after FURO+CAP treatment (intake test). In each intake test, of a total of four, the rats were divided in two groups and each group received one of the following combination of treatments: saline (CeA)+vehicle (LPBN); muscimol (CeA)+vehicle (LPBN); saline (CeA)+moxonidine (LPBN); muscimol (CeA)+moxonidine (LPBN). The sequence of these treatments was randomized in each group across different tests. At the end of the four tests, each rat received all the four combination of treatments. The interval between two tests was 3 days.

4.6. Water and 0.3 M NaCl intake by euhydrated rats treated with bilateral injections of muscimol into the LPBN and CeA

The blockade of the neuronal activity with injections of muscimol into the LPBN causes strong ingestion of 0.3 M NaCl in euhydrated rats that received no additional treatment (Callera et al., 2005, Andrade-Franzé et al., 2010b; Roncari et al., 2011, 2014; Asnar et al., 2013). Therefore, the

objective of this protocol was to test if the blockade of the neuronal activity in the CeA with injections of muscimol would affect 0.3 M NaCl caused by deactivation of LPBN inhibitory mechanisms with muscimol injected into the LPBN in euhydrated rats.

Euhydrated rats ($n=32$) received first bilateral injections of muscimol (0.5 nmol/0.2 μl) or saline into the LPBN, and immediately after into the CeA. After returning to their home cages, rats had water and 0.3 M NaCl available in the absence of food. Water and 0.3 M NaCl were provided from burets with 0.1-ml divisions that were fitted with metal drinking spouts. Cumulative water and 0.3 M NaCl intake was measured at every 30 min during 240 min (intake test). In each intake test, of a total of four, the rats were divided in two groups and each group received one of the following combination of treatments: saline (CeA)+saline (LPBN); muscimol (CeA)+saline (LPBN); saline (CeA)+muscimol (LPBN); muscimol (CeA)+muscimol (LPBN). The sequence of these treatments was randomized in each group across different tests. At the end of four tests, each rat received all the four combination of treatments. The interval between two tests was 2 days.

4.7. *Histology*

At the end of ingestive behavior tests, rats received bilateral injections of 2% Evans blue solution (0.2 μl) into the LPBN and CeA. They were then deeply anesthetized with sodium thiopental (80 mg/kg of body weight) and perfused transcardially with saline followed by 10% formalin. The brains were removed, fixed in 10% formalin, frozen, cut in 50 μm sections, stained with Giemsa stain (Iñiguez et al., 1985) and analyzed by light microscopy to confirm the injection sites into the LPBN and into the CeA.

4.8. *Statistical analysis*

The results are reported as means ± S.E.M. Two way repeated measures analysis of variance (ANOVA) using treatments and times as within-subjects factors followed by Newman–Keuls tests was used for comparisons. Differences were considered significant at $P<0.05$.

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