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Research Report

Sodium intake by hyperosmotic rats treated with a $GABA_A$ receptor agonist into the lateral parabrachial nucleus

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ABSTRACT

Inhibitory mechanisms in the lateral parabrachial nucleus (LPBN) and central GABAergic mechanisms are involved in the regulation of water and NaCl intake. Besides increasing fluid depletion-induced sodium intake, the activation of $GABA_A$ receptors with muscimol into the LPBN also induces ingestion of 0.3 M NaCl in normonatremic, euhydrated rats. It has been suggested that inhibitory mechanisms activated by osmotic signals are blocked by $GABA_A$ receptor activation in the LPBN, thereby increasing hypertonic NaCl intake. Therefore, in the present study we investigated the effects of muscimol injected into the LPBN on water and 0.3 M NaCl intake in hyperosmotic cell-dehydrated rats (rats treated with an intragastric load of 2 M NaCl). Male Wistar rats with stainless steel cannulas implanted bilaterally into the LPBN were used. In euhydrated rats, muscimol (0.5 nmol/0.2 μl), bilaterally injected into the LPBN, induced ingestion of 0.3 M NaCl (24.6±7.9 vs. vehicle: 0.5±0.3 ml/180 min) and water (6.3±2.1 vs. vehicle: 0.5±0.3 ml/180 min). One hour after intragastric 2 M NaCl load (2 ml), bilateral injections of muscimol into the LPBN also induced 0.3 M NaCl intake (22.1±5.2 vs. vehicle: 0.9±0.8 ml/210 min) and water intake (16.5±3.6 vs. vehicle: 7.8±1.8 ml/210 min). The $GABA_A$ antagonist bicuculline (0.4 nmol/0.2 μl) into the LPBN reduced the effect of muscimol on 0.3 M NaCl intake (7.1±2.1 ml/210 min). Therefore, the activation of $GABA_A$ receptors in the LPBN induces ingestion of 0.3 M NaCl by hyperosmotic cell-dehydrated rats, suggesting that plasma levels of renin or osmolarity do not affect sodium intake after the blockade of LPBN inhibitory mechanisms with muscimol.

1. Introduction

Recently, important inhibitory mechanisms involved in the control of sodium and water intake have been demonstrated in the lateral parabrachial nucleus (LPBN), a pontine structure located dorso-laterally to the superior cerebellar peduncle (Callera et al., 2005; Colombari et al., 1996; De Luca et al., 2003; Menani et al., 1996, 1998a,b, 2000, 2002; Menani and Johnson, 1995, 1998; Oliveira et al., 2007). The LPBN is reciprocally connected to areas involved in the control of fluid and electrolyte balance, such as area postrema (AP) and medial portion of the nucleus of the solitary tract (mNTS) in the hindbrain, and to

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forebrain areas, such as the paraventricular nucleus of the hypothalamus, central nucleus of amygdala and median preoptic nucleus (Ciriello et al., 1984; Fulwiler and Saper, 1984; Herbert et al., 1990; Jhamandas et al., 1992, 1996; Krukoff et al., 1993; Norgren, 1984; Shapiro and Miselis, 1985). Gustatory and visceral signals important for the control of sodium and/or water intake may be relayed from the AP/mNTS to the LPBN which, in turn, sends inhibitory projections to the forebrain that restrain sodium and water intake (Callera et al., 2005; Colombari et al., 1996; De Luca et al., 2003; Menani et al., 1996, 1998a,b, 2000, 2002; Menani and Johnson, 1995, 1998; Oliveira et al., 2007).

Bilateral LPBN injections of methysergide (serotonin antagonist), DNQX (glutamate antagonist) or α-helical corticotropin-releasing factor$_{9–41}$ (corticotrophin releasing factor antagonist) increase hypertonic NaCl intake and eventually water intake induced the treatment with the diuretic furosemide (FURO) combined with low dose of the angiotensin converting enzyme inhibitor captopril (CAP), while injections of the respective agonists (DOI, AMPA and corticotrophin releasing factor — CRF) produce opposite effects (De Castro e Silva et al., 2006; Menani et al., 1996; Xu et al., 1997). Injections of methysergide into the LPBN also increase sodium intake produced by other stimuli like angiotensin II (ANG II) injected intracerebroventricularly (i.c.v.) or into the subfornical organ, chronic sodium depletion, water deprivation, central cholinergic activation, subcutaneous (sc) injection of isoproterenol (β-adrenergic agonist) or the mineralocorticoid deoxycorticosterone (Colombari et al., 1996; De Gobbi et al., 2000, 2001; Menani et al., 1996, 1998a,b, 2000, 2002; Menani and Johnson, 1995). Increase of FURO+CAP-induced sodium intake is also produced by the blockade of colecystokinin (CCK) receptors with proglumide, the activation of α_2 adrenergic receptors with moxonidine or activation of $GABA_A$ receptors with muscimol into the LPBN (Andrade et al., 2004; Callera et al., 2005; De Gobbi et al., 2001; Menani and Johnson, 1998). Therefore, in the LPBN, neurotransmitters like serotonin (5-HT), glutamate, CRF and CCK activate an inhibitory mechanism, while activation of α_2-adrenergic or GABAergic receptors in the LPBN may block the action of this inhibitory mechanism (Andrade et al., 2004; Callera et al., 2005; De Castro e Silva et al., 2006; De Gobbi et al., 2000, 2001; Menani et al., 1996, 1998a,b, 2000, 2002; Menani and Johnson, 1995, 1998; Oliveira et al., 2007; Xu et al., 1997).

In spite of the increase of FURO+CAP-induced sodium intake produced by the blockade of serotonergic receptors or activation of α_2-adrenergic receptors in the LPBN, the same treatments into the LPBN in normonatremic, euhydrated rats produce no sodium intake. However, the blockade of serotonergic receptors or activation of α_2-adrenergic receptors in the LPBN combined with an increase in plasma osmolarity induces an unexpected ingestion of hypertonic NaCl in addition to water in a two-bottle test (Andrade et al., 2006; De Luca et al., 2003), a clear paradox considering that hypertonic NaCl load usually suppresses sodium appetite (Blackburn et al., 1995; Fitzsimons, 1985). These results suggest that plasma hyperosmolarity or cell-dehydration also signals for sodium intake, not only for water intake. However, in this condition, sodium intake is usually prevented by inhibitory mechanisms that are relayed through the LPBN (Andrade et al., 2006; De Luca et al., 2003). If the LPBN inhibitory mechanisms are deactivated by the blockade of serotonergic receptors or activation of α_2-adrenergic receptors, then the excitatory drive for sodium appetite produced by cell-dehydration allows hypertonic NaCl ingestion (Andrade et al., 2006; De Luca et al., 2003).

In contrast to other treatments in the same area, the activation of $GABA_A$ receptors by muscimol into the LPBN induces hypertonic NaCl intake by normonatremic, euhydrated rats (Callera et al., 2005). GABA is an inhibitory neurotransmitter and, therefore, the effect of muscimol on sodium and water intake is probably the result of the GABAergic disinhibiton. I.e., in the LPBN, the activation of GABAergic receptors inhibit the inhibitory mechanisms, releasing sodium and water intake. Interestingly, the activation of facilitatory mechanisms seems to be not necessary for sodium and water intake produced in this condition. In addition, the increase in plasma osmolality subsequent to sodium ingestion does not appear to prevent further salt intake when GABAergic receptors in the LPBN are activated. I.e., inhibitory signals activated by hypertonic NaCl intake are not able to limit sodium intake after muscimol into the LPBN. It is even possible that without the action of LPBN inhibitory mechanisms, presumably activated by 5-HT, CRF, glutamate or CCK release, increased plasma osmolality may stimulate both water and salt intake after muscimol into the LPBN.

To distinguish between the possibilities raised in explaining the ingestion of hypertonic sodium solution by normonatremic, euhydrated rats after muscimol into the LPBN, it is important to know whether GABAergic activation in the LPBN produces intake of hypertonic NaCl in cell-dehydrated rats like that produced by serotonergic blockade or α_2-adrenergic activation in the LPBN. Therefore, in the present study we investigated the effects of bilateral injections of muscimol alone or combined with the $GABA_A$ receptor antagonist bicuculline into the LPBN on water and 0.3 M NaCl intake, in rats with increased plasma osmolarity, thereby cell-dehydrated, produced by an intragastric load of 2 M NaCl and compared the responses with the effects of muscimol into the LPBN on sodium intake by normonatremic, euhydrated rats. The intragastric load of hypertonic NaCl produces cell dehydration by increasing plasma osmolality and sodium concentration and also reduces plasma renin activity (Pereira et al., 2002).

2. Results

2.1. *Histological analysis*

Fig. 1 shows the typical LPBN injection sites. The injections were centered in the central lateral and dorsal lateral portions of the LPBN (see Fulwiler and Saper, 1984 for definitions of LPBN subnuclei). In some rats, LPBN injections reached the ventral lateral and external lateral portions, as well as the Kölliker–Fuse nucleus. The sites of injections were similar to those that previous studies showed the effects of methysergide, proglumide, moxonidine and muscimol on water and 0.3 M NaCl intake (Andrade et al., 2004, 2006; Callera et al., 2005; De Gobbi et al., 2001; De Luca et al., 2003; Menani et al., 1998a,b, 2000, 2002; Oliveira et al., 2007). In some rats, injections spread to the brachium (superior cerebellar peduncle), or slightly ventral to this structure, reaching the dorsal portions of the medial parabrachial nucleus (MPBN) uni- or bi-laterally. There

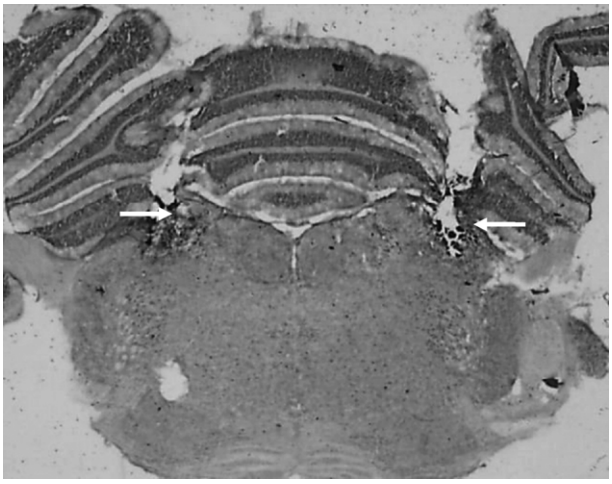

Fig. 1 – Photomicrograph of a brain slice from one rat representative of the groups studied showing the sites of injections into the LPBN (arrows).

was no difference in the effects whether injections were restricted to the LPBN or also spread to brachium and dorsal portions of MPBN.

2.2. *Effects of muscimol injected into the LPBN on water and 0.3 M NaCl intake in normonatremic, euhydrated rats*

In the two-bottle test, bilateral injections of muscimol (0.5 nmol/0.2 μl) into the LPBN in normonatremic, euhydrated rats induced 0.3 M NaCl intake (24.6±7.9 ml/3 h, vs. vehicle: 0.5±0.2 ml/3 h, Figs. 2A, B) and water intake (6.3±2.0 ml/3 h, vs. vehicle: 0.4±0.2 ml/3 h, Figs. 2C, D). ANOVA showed significant differences between treatments with muscimol and vehicle into the LPBN for 0.3 M NaCl intake [$F(1, 10)=7.01$; $P<0.05$] and water intake [$F(1, 10)=5.06$; $P<0.05$] (Fig. 2).

Rats that received saline into the LPBN ingested 0.5±0.2 ml of 0.3 M NaCl and 0.4±0.2 ml of water in 180 min, which yielded a solution of approximately 0.17 M. However, rats that received muscimol into the LPBN ingested 24.6±7.9 ml/180 min of 0.3 M NaCl and 6.3±2.0 ml/180 min of water which results in a hypertonic solution (0.24 M).

2.3. *Effects of the combination of bicuculline and muscimol into the LPBN on water and 0.3 M NaCl intake by cell-dehydrated rats*

In rats previously treated with intragastric load of 2 M NaCl (2 ml/rat), bilateral injections of muscimol (0.5 nmol/0.2 μl) into the LPBN induced 0.3 M NaCl intake (22.1±5.2 vs. vehicle+saline: 0.9±0.8 ml/210 min) and increased water intake (16.5±3.6 ml/210 min, vs. vehicle+saline: 7.8±1.8 ml/210 min) (Fig. 3).

Previous injections of the $GABA_A$ receptor antagonist bicuculline (0.4 nmol/0.2 μl each site) into the LPBN reduced the effects of muscimol on 0.3 M NaCl intake (7.1±2.1 ml/210 min) (Figs. 3A, B). Bicuculline alone or combined with muscimol into the LPBN did not affect water intake induced by intragastric 2 M NaCl (Figs. 3C, D). Although not statistically significant, a tendency to reduce water intake after bicuculline alone into the LPBN occurred in cell-dehydrated rats (Fig. 3C).

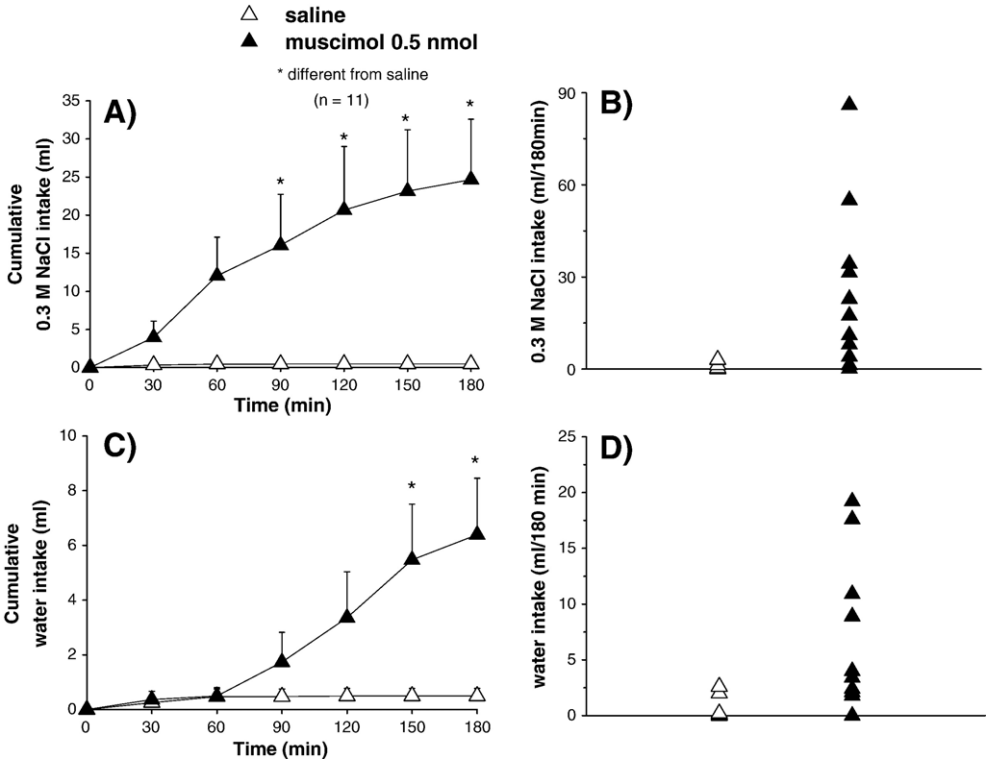

Fig. 2 – (A) Cumulative 0.3 M NaCl intake; (B) Individual 0.3 M NaCl intakes; (C) cumulative water intake; (D) individual water intakes by normonatremic, euhydrated rats that received bilateral injections of muscimol (0.5 nmol/0.2 μl) or saline into the LPBN. A and C values are means±S.E.M.; *n*, number of rats.

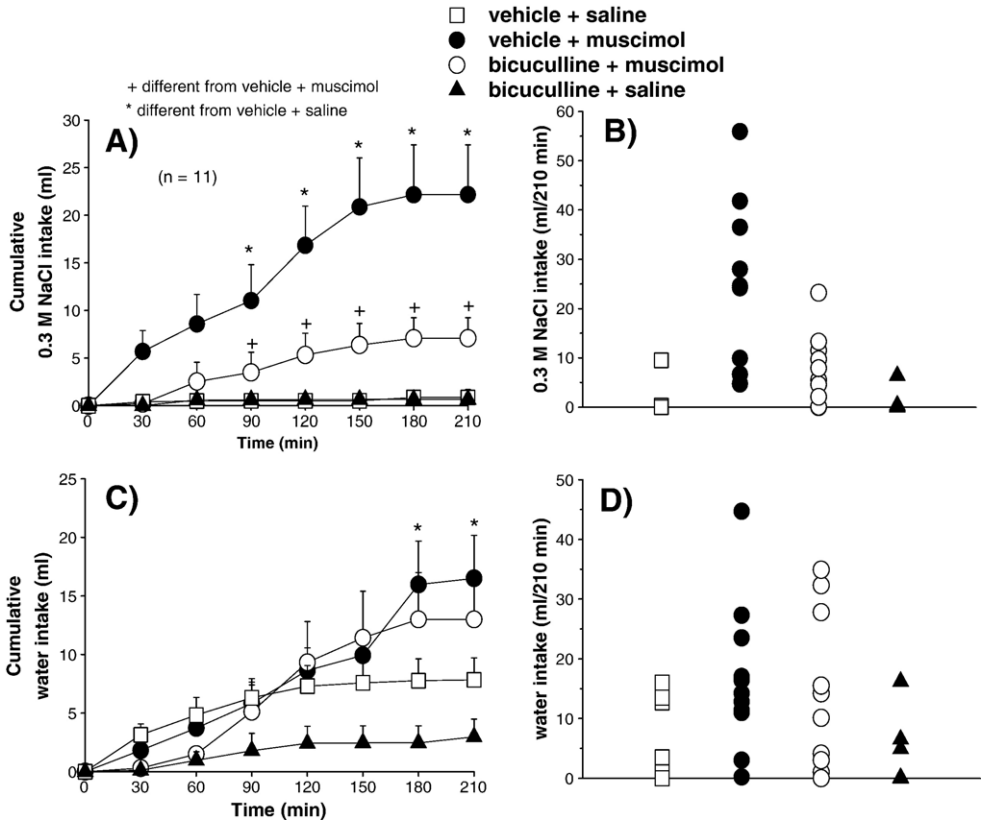

Fig. 3 – (A) Cumulative 0.3 M NaCl intake; (B) individual 0.3 M NaCl intakes; (C) cumulative water intake; (D) individual water intakes by intragastric 2 M NaCl loaded rats that received bilateral injections of bicuculline (0.4 nmol/0.2 μl) or vehicle combined with muscimol (0.5 nmol/0.2 μl) or saline into the LPBN. A and C values are means ± S.E.M.; *n*, number of rats.

ANOVA showed significant differences among treatments for 0.3 M NaCl intake by cell-dehydrated rats that received vehicle or bicuculline combined with saline or muscimol into the LPBN, [$F(3, 30) = 11.89$; $P < 0.001$] and significant interaction between treatments and times for water intake [$F(18, 180) = 3.06$; $P < 0.001$].

Cell-dehydrated rats that received saline into the LPBN ingested 0.9 ± 0.8 ml of 0.3 M NaCl and 7.8 ± 1.8 ml of water in 210 min. Considering the total intake, the result is a hypotonic solution (nearly 0.03 M). Cell-dehydrated rats treated with muscimol into the LPBN ingested 22.1 ± 5.2 ml/210 min of 0.3 M NaCl and 16.5 ± 3.6 ml/120 min of water which results in an almost isotonic solution (0.17 M).

Table 1 – Ingestion of water and 0.3 M NaCl by cell-dehydrated rats treated with vehicle or bicuculline combined with saline or muscimol in sites outside the LPBN (misplaced injections)

Treatments	*n*	0.3 M NaCl intake (ml/210 min)	Water intake (ml/210 min)
Vehicle + saline	13	0.6 ± 0.3	8.8 ± 1.1
Vehicle + muscimol (0.5 nmol)	13	1.8 ± 0.7	8.5 ± 2.1
Bicuculline (0.4 nmol) + muscimol	13	1.7 ± 0.6	7.5 ± 1.2
Bicuculline (0.4 nmol) + saline	13	0.1 ± 0.0	6.7 ± 1.7

Values are means ± S.E.M.; *n*, number of rats.

2.4. *Specificity of injections into the LPBN to produce the effects on water and 0.3 M NaCl intake in cell-dehydrated rats*

The specificity of the LPBN as the site where muscimol produced the effects on water and 0.3 M NaCl intake in cell-dehydrated rats was confirmed by results from rats in which the injections did not reach the LPBN (misplaced injections). Bilateral injections of muscimol (0.5 nmol/0.2 μl), bicuculline (0.4 nmol/0.2 μl) or bicuculline combined with muscimol outside the LPBN ($n = 13$) produced no effect on 0.3 M NaCl nor on water intake (Table 1). ANOVA showed no significant difference among treatments for 0.3 M NaCl intake [$F(3, 36) = 2.47$; $P > 0.05$] or water intake [$F(3, 36) = 1.72$; $P > 0.05$] by cell-dehydrated rats that received injections in sites outside the LPBN (Table 1). Injections outside the LPBN reached different sites; most were dorsal or caudal to the LPBN, though a few were ventral or rostral to the LPBN.

3. Discussion

The present study replicated previous results (Callera et al., 2005) showing that bilateral injections of muscimol into the

LPBN induce 0.3 M NaCl and water intake in normonatremic, euhydrated rats. Similar to normonatremic, euhydrated rats, muscimol into the LPBN also induced hypertonic NaCl intake and increased water intake in rats submitted to an intragastric load of 2 M NaCl. A previous study (Pereira et al., 2002) showed that a 2 M NaCl load induces a 4% increase in plasma sodium and osmolality and reduces plasma renin activity by half without changing blood volume (Pereira et al., 2002). The same study showed that sodium loaded rats also present diuresis and natriuresis (Pereira et al., 2002). Despite the hyperosmolality and reduction of plasma renin activity, the present study shows that animals previously treated with 2 M NaCl ig still ingest 0.3 M NaCl after injections of muscimol into the LPBN, in an amount similar to normonatremic, euhydrated rats given the same treatment in the LPBN. Previous blockade of the $GABA_A$ receptors with bicuculline into the LPBN reduces the effects of muscimol on 0.3 M NaCl intake, supporting the involvement of $GABA_A$ receptors of the LPBN in the control of hypertonic NaCl intake. Results from rats with misplaced injections confirm that muscimol produces effects on water and 0.3 M NaCl intake if injected into the LPBN and not into the surrounding areas. Therefore, the present results show that activation of $GABA_A$ receptors specifically in the LPBN induces ingestion of hypertonic NaCl independent of the level of plasma osmolarity or renin activity.

Bilateral injections of muscimol into the LPBN in normonatremic, euhydrated rats produce a slight increase of arterial pressure without significant change in urinary volume, Na^+ and K^+ (Callera et al., 2005), which suggests that renal or cardiovascular mechanisms are not important for the behavioral effects of muscimol injected into the LPBN. In addition, muscimol into the LPBN did not affect food intake in normonatremic, euhydrated rats (Callera et al., 2005) excluding the possibility that muscimol into the LPBN might have non-specific effects increasing all ingestive behaviors.

The average of fluid (water+0.3 M NaCl) ingested in a two-bottle tests by normonatremic, euhydrated rats treated with muscimol into the LPBN was 30 ml in 3 h. Most of the fluid ingested was 0.3 M NaCl (mean of 24 ml/3 h, vs. 6 ml/3 h of water), which suggests that normonatremic, euhydrated rats prefer hypertonic NaCl. Rats treated with 2 M NaCl ig usually ingest water, not sodium. However, after injections of muscimol into the LPBN, sodium loaded rats ingested 0.3 M NaCl in addition to water, showing again a preference for hypertonic NaCl. The relationship between the ingestion of NaCl and of water by normonatremic, euhydrated rats treated with muscimol into the LPBN suggests that water intake may depend on increases in plasma osmolarity caused by hypertonic NaCl intake. However, in cell-dehydrated rats the relationship was not as obvious because, even without muscimol into the LPBN, these rats ingested significant amounts of water.

The mixture of water and 0.3 M NaCl ingested by normonatremic, euhydrated rats changed from an almost isotonic solution (0.17 M) after vehicle into the LPBN to a hypertonic solution (0.24 M) after muscimol, while the mixture ingested by 2 M NaCl loaded rats changed from a hypotonic solution (~0.03 M or almost only water) after vehicle to an almost isotonic solution (0.17 M) after muscimol into the LPBN. In spite of these differences, in both cases there was a significant change toward an ingestion of more concentrated mixture after muscimol into the LPBN, which suggests increased preference for sodium.

The LPBN is connected to forebrain areas that control fluid and electrolyte balance, such as the paraventricular nucleus of hypothalamus, central nucleus of amygdala and median preoptic nucleus, and to medullary regions, like AP/mNTS that receive visceral and taste information (Ciriello et al., 1984; Fulwiler and Saper, 1984; Herbert et al., 1990; Jhamandas et al., 1992, 1996; Johnson et al., 1999; Krukoff et al., 1993; Norgren, 1984; Shapiro and Miselis, 1985; Spector, 1995). Thus, the LPBN is in an appropriate position to participate in the control of sodium ingestion (Fitzsimons, 1985; Johnson et al., 1999; Menani et al., 1996; Norgren, 1984). Cells in the LPBN are activated by ingestion of NaCl solution by dehydrated rats or by rats that received intragastric load of hypertonic NaCl (Franchini and Vivas, 1999; Kobashi et al., 1993; Yamamoto et al., 1993). The LPBN also receives signals from arterial baroreceptors and volume receptors that ascend from the AP/NTS. Therefore, afferent visceral osmotic and/or volume signals and sodium taste information may be processed and integrated in the LPBN and from there ascend to inhibit forebrain neural circuits subserving salt appetite.

The blockade of different neurotransmitters, like 5-HT, CCK, CRF and glutamate, or the activation of α_2-adrenergic receptors in the LPBN deactivate LPBN inhibitory mechanisms, increasing NaCl intake induced by natrio/dipsogenic stimuli like ANG II/fluid depletion (Andrade et al., 2004; Colombari et al., 1996; De Castro e Silva et al., 2006; De Gobbi et al., 2001; Menani et al., 1996, 1998a,b; Menani and Johnson, 1995, 1998; Xu et al., 1997). However, the same treatments in the LPBN produce no effect on water or hypertonic NaCl intake if not combined with a natrio/dipsogenic stimulus, which might suggest that deactivation of LPBN inhibitory mechanisms alone is not enough to induce water or NaCl intake (Andrade et al., 2004; Colombari et al., 1996; De Gobbi et al., 2001; Menani et al., 1996). In spite of this, serotonergic blockade or α_2-adrenergic activation in the LPBN drives hyperosmotic animals to ingest hypertonic NaCl in addition to the usual ingestion of water (Andrade et al., 2006; De Luca et al., 2003). Similar to serotonergic blockade or α_2-adrenergic activation in the LPBN, another study showed that destruction of central neurons containing atrial natriuretic peptide receptors enhances NaCl intake by hypovolemic rats treated with peripheral hypertonic NaCl or mannitol (Blackburn et al., 1995). To explain the ingestion of NaCl in this condition, Blackburn et al. (1995) proposed that hyperosmolar or hypernatremic solutions might have two opposite effects on NaCl intake: inhibition and stimulation. Usually, inhibition is the predominant effect, while stimulation is revealed when the inhibitory pathways are deactivated, for example after destruction of central neurons containing atrial natriuretic peptide receptors. The ingestion of hypertonic NaCl by hyperosmotic rats submitted to serotonergic blockade or to α_2-adrenergic activation in the LPBN (Andrade et al., 2006; De Luca et al., 2003) is consistent with the idea that signals from osmoreceptors activate circuits subserving sodium intake in parallel to thirst. At the same time, osmoreceptor signals activate LPBN inhibitory mechanisms, restraining sodium intake, at least in hypertonic form, and preventing further cell dehydration (Andrade et al., 2006; De Luca et al., 2003).

The present results show that normonatremic, euhydrated rats or hyperosmotic rats ingested similar amounts of 0.3 M NaCl after bilateral injections of muscimol into the LPBN. This observation means that, after muscimol into the LPBN, sodium intake occurs independent of whether plasma renin levels are normal or reduced. A possible conclusion from these results is that the activation of excitatory mechanisms is not necessary for NaCl intake, which is different from the mechanisms already proposed to explain increased sodium intake after the blockade of LPBN inhibitory mechanisms (Andrade et al., 2004; Colombari et al., 1996; De Gobbi et al., 2000, 2001; Menani et al., 1996, 1998a,b; Menani and Johnson, 1995, 1998; Xu et al., 1997). The reasons for these differences may be related to the level of inhibition produced by each treatment in the LPBN. Blocking the action of one specific neurotransmitter may result in a partial blockade of the inhibitory mechanisms, which may reduce, but not abolish, the action of the inhibitory mechanisms. In this case, ingestion of sodium may occur if excitatory mechanisms were simultaneously activated. Conversely, activation of GABAergic mechanisms may produce a non-specific and widespread inhibition of LPBN inhibitory mechanisms. In this situation, sodium intake occurs independent of excitatory or inhibitory signals for sodium intake.

4. Experimental procedures

4.1. *Animals*

Male Wistar rats weighing 290–310 g were used. The animals were housed in individual stainless steel cages with free access to normal sodium diet (Guabi Rat Chow, Paulinia, SP, Brazil), water and 0.3 M NaCl solution. The position of the bottles containing water and 0.3 M NaCl was rotated daily to avoid place preference. Room temperature was maintained at 23±2 °C and humidity was maintained at 55±10% on a 12:12 light–dark cycle with light onset at 07:30 AM. The procedures followed the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH publications No. 80-23, 1996) and were approved by the Ethical Committee for Animal Care and Use from the School of Dentistry, UNESP, Araçatuba, SP, Brazil. All efforts were made to minimize animal discomfort and the number of animals used.

4.2. *Cerebral cannulas*

Rats were anesthetized with subcutaneous (sc) ketamine (80 mg/kg of body weight) combined with xylazine (7 mg/kg of body weight) and placed in a Kopf stereotaxic instrument. The skull was leveled between bregma and lambda. Stainless steel guide-cannulas (12×0.6 mm o.d.) were implanted bilaterally into the LPBN using the following coordinates: 9.4 mm caudal to bregma, 2.2 mm lateral to the midline, and 3.9 mm below the dura mater. The tips of the cannulas were positioned at a point 2 mm above each LPBN. The cannulas were fixed to the cranium using dental acrylic resin and jeweler screws. A 30-gauge metal obturator filled the cannulas between tests. After the surgery, the rats were allowed to recover for 5 days before starting water and 0.3 M NaCl intake tests.

4.3. *Injections into the LPBN*

Bilateral injections into the LPBN were made using 5-μl Hamilton syringes connected by polyethylene tubing (PE-10) to 30-gauge injection cannulas. At the time of testing, obturators were removed, the injection cannula (2 mm longer than the guide cannula) was carefully inserted into the guide cannula, and manual injection was initiated 15 s later. For bilateral injections, the first injection was performed, the needle then was withdrawn and repositioned on the contra lateral side, and then the second injection was made. The injection volume was 0.2 μl in each side; injections typically required ~30 s/side. The obturators were replaced after the injections and rats were returned to their cages.

4.4. *Water and 0.3 M NaCl intake*

The rats were tested in their home cages. Besides water and food pellets, rats had access to 0.3 M NaCl for at least 5 days before the beginning of the experiments. On the day of the experiment, water, food pellets and 0.3 M NaCl were removed from the cage immediately before the treatments. Water and 0.3 M NaCl were returned to the rats 15 min after the last injection.

Water and 0.3 M NaCl intake was tested in two groups of rats: one group of normonatremic, euhydrated rats and one group of cell-dehydrated rats that received an intragastric (ig) 2 M NaCl load (2 ml/rat). As previously shown, the intragastric 2 M NaCl load produces 4% elevation of plasma osmolality and sodium concentration, a reduction in plasma renin activity and no alteration in plasma volume (Pereira et al., 2002).

The group of normonatremic, euhydrated rats ($n=11$) received only injections of muscimol or saline into the LPBN and 15 min later had access to water and 0.3 M NaCl. In the first experimental session, half of the group received bilateral injections of muscimol (0.5 nmol/0.2 μl) and the other half vehicle (saline) into the LPBN. In the next experimental session the rats received the same treatments in the LPBN in a counterbalanced design. Cumulative water and 0.3 M NaCl intake was measured every 30 min for 180 min using burettes with 0.1 ml divisions adapted for rat drinking. The treatments were separated by >3 days.

The group of cell-dehydrated rats ($n=11$) received an intragastric 2 M NaCl load (2 ml/rat) through a PE-200 polyethylene tubing connected to a 5-ml syringe and were maintained without access to water, food pellets and NaCl solution for 1 h. Thirty minutes after the intragastric load, the rats received bilateral injections of the $GABA_A$ antagonist bicuculline (0.4 nmol/0.2 μl) or vehicle into the LPBN and 15 min later were injected with muscimol (0.5 nmol/0.2 μl) or saline into the LPBN. Cumulative water and 0.3 M NaCl intake was measured every 30 min for 210 min, starting 15 min after injections of muscimol or saline into the LPBN. In each experimental session, the group of rats was divided in two and each half of the group received one of the four treatments in the LPBN: vehicle + saline, vehicle + muscimol, bicuculline + muscimol and bicuculline + saline. The treatments were administered in a randomized order through every test. At the end of the fourth test all rats had received the four treatments, separated by >3 days.

4.5. *Histology*

At the end of the experiments, the animals received bilateral injections of 2% Evans blue solution (0.2 μl/injection site) into the LPBN. They were then deeply anesthetized with thiopental sodium (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, and analyzed by light microscopy to confirm the injection sites in the LPBN.

4.6. *Data analysis*

The results are reported as means ± S.E.M. Water and 0.3 M NaCl intake of normonatremic, euhydrated rats was compared by two-way ANOVA and Newman–Keuls test using treatments and time as factors. Water and 0.3 M NaCl intake of cell-dehydrated rats treated with vehicle or bicuculline combined with saline or muscimol into the LPBN was analyzed by two-way repeated measures ANOVA and Newman–Keuls test using treatments (vehicle + saline, vehicle + muscimol, bicuculline + muscimol, and bicuculline + saline) and time as factors. Differences were considered significant at $P<0.05$. The software used to analyze the data was SigmaStat for Windows, version 2.03 from SSPS Inc.

4.7. *Drugs and solutions*

Muscimol HBr and (+)-bicuculline were purchased from Research Biochemicals Internationals (RBI, Natick, MA, USA). The drugs were dissolved in isotonic saline which served as the vehicle.

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