Research Report

Purinergic mechanisms of lateral parabrachial nucleus facilitate sodium depletion-induced NaCl intake

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ABSTRACT

Purinergic receptors are present in the lateral parabrachial nucleus (LPBN), a pontine structure involved in the control of sodium intake. In the present study, we investigated the effects of α,β-methyleneadenosine 5′-triphosphate (α,β-methylene ATP, selective P2X purinergic agonist) alone or combined with pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS, P2X purinergic antagonist) or suramin (non-selective P2 purinergic antagonist) injected into the LPBN on sodium depletion-induced 1.8% NaCl intake. Male Holtzman rats with stainless steel cannulas implanted into the LPBN were used. Sodium depletion was induced by treating rats with the diuretic furosemide (20 mg/kg of body weight) followed by 24 h of sodium-deficient diet. Bilateral injections of α,β-methylene ATP (2.0 and 4.0 nmol/0.2 μl) into the LPBN increased sodium depletion-induced 1.8% NaCl intake (25.3 ± 0.8 and 26.5 ± 0.9 ml/120 min, respectively, vs. saline: 15.2 ± 1.3 ml/120 min). PPADS (4 nmol/0.2 μl) alone into the LPBN did not change 1.8% NaCl intake, however, pretreatment with PPADS into the LPBN abolished the effects of α,β-methylene ATP on 1.8% NaCl intake (16.9 ± 0.9 ml/120 min). Suramin (2.0 nmol/0.2 μl) alone into the LPBN reduced sodium depletion-induced 1.8% NaCl intake (5.7 ± 1.9 ml/120 min, vs. saline: 15.5 ± 1.1 ml/120 min), without changing 2% sucrose intake or 24 h water deprivation-induced water intake. The combination of suramin and α,β-methylene ATP into the LPBN produced no change of 1.8% NaCl intake (15.2 ± 1.2 ml/120 min). The results suggest that purinergic P2 receptor activation in the LPBN facilitates NaCl intake, probably by restraining LPBN mechanisms that inhibit sodium intake.

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1. Introduction

Important inhibitory mechanisms in the control of water, and particularly NaCl, intake are located in the lateral parabrachial nucleus (LPBN), a pontine structure that lies dorsolateral to the superior cerebellar peduncle (Edwards and Johnson, 1991; Menani and Johnson, 1995; Colombari et al., 1996; Menani et al., 1996, 1998a,b, 2000).

Early studies showed that bilateral injections of methysergide, a serotonergic receptor antagonist, into the LPBN increased water and 1.8% NaCl intake induced by angiotensin II (ANG II) administered either intracerebroventricularly (i.c.v) or into the subfornical organ (SFO) (Colombari et al., 1996; Menani et al., 1996). Methysergide injected bilaterally into the LPBN also increased NaCl intake induced by subcutaneous (s.c.) injection of the diuretic, furosemide (FURO), in combination with a low dose of the angiotensin converting enzyme inhibitor, captopril, whereas 2,5-dimetoxy-4-iodoamphetamine hydrobromide (DOI) (a serotonergic 5-HT$_{2A/2C}$ receptor agonist) into the LPBN reduced NaCl intake induced by FURO+captopril (Menani et al., 1996). In addition to serotonin, cholecystokinin (CCK) injected into LPBN inhibited NaCl and water intake (Menani and Johnson, 1998). These studies suggested that signals that inhibit sodium intake are integrated in the LPBN, and involve the release of serotonin and CCK in this area.

In addition to serotonin and CCK, glutamate, corticotrophin releasing factor (CRF), noradrenaline, GABAergic and opioid receptors in the LPBN are involved in the control of sodium intake (Menani et al., 1996, 1998a,b, 2000; De Gobbi et al., 2000, 2009; Fratucci De Gobbi et al., 2001, Andrade et al., 2004, 2006; Callera et al., 2005; De Castro e Silva et al., 2006; De Oliveira et al., 2008; Andrade-Franzé et al., 2010; Gasparini et al., 2009). Some of these neurotransmitters, like serotonin, CCK, glutamate and CRF, act in the LPBN to inhibit sodium and water intake, whereas noradrenaline, GABAergic and opioid agonists act in the LPBN to facilitate sodium intake. All these studies suggest that facilitation or inhibition of sodium intake is probably related to activation or suppression of inhibitory LPBN mechanisms.

Adenosine-5'-triphosphate (ATP) was first recognized as extracellular signaling molecule and neurotransmitter/neuromodulator by Burnstock (1972). ATP binds to two classes of purinergic receptors: the ionotropic P2X and the metabotropic P2Y receptors (Ralevic and Burnstock, 1998). Several functional studies have suggested that ATP and purinergic receptors participate in central pathways involved in cardiorespiratory and thermal regulation (Ergene et al., 1994; Barraco et al., 1996; Phillis et al., 1997; Scislo et al., 1997, 1998; Gourine et al., 2002, 2003, 2004, 2005; de Paula et al., 2004; Antunes et al., 2005a,b). Purinergic receptors are present in central areas involved in the control of fluid–electrolyte balance, particularly in the LPBN (Yao et al., 2000); however, to our knowledge, the involvement of ATP or purinergic receptors in the control of thirst or sodium appetite has not been investigated.

Considering the importance of LPBN inhibitory mechanisms for the control of water and NaCl intake and the existence of purinergic receptors in the LPBN, in the present study we investigated the effects of bilateral injections of a non-selective P2 purinergic receptor antagonist suramin or a selective P2X purinergic receptor antagonist pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) and the P2X purinergic receptor agonist α,β-methyleneadenosine 5′-triphosphate (α,β-methylene ATP) alone or combined into the LPBN on sodium depletion-induced 1.8% NaCl intake.

2. Results

2.1. Histological analysis

Fig. 1 is a photomicrograph of a transverse section of the brainstem of one rat, representative of the groups tested, showing the typical bilateral injections into 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 LPBN injection sites in the present study were similar to those from previous studies showing the effects of serotonergic or cholecystokinergic antagonists and gabaergic or opioid agonists on sodium intake (Menani et al., 1996; Menani and Johnson, 1998; De Gobbi et al., 2000; Fratucci De Gobbi et al., 2001; Callera et al., 2005; De Oliveira et al., 2008).

From the total of 85 animals tested, 49 rats had bilateral injections correctly placed into LPBN. Misplaced injections that reached tissue surrounding the LPBN were located mainly dorsal or ventral to LPBN and some of them medial to the LPBN. Results from rats with misplaced injections of suramin or α,β-methylene ATP were also analyzed and reported to confirm the specificity of the LPBN as the site of injections that produce the effects on sodium intake.

2.2. Water and 1.8% NaCl intake by satiated or sodium depleted rats treated with bilateral injections of α,β-methylene ATP into the LPBN

2.2.1. Sodium depleted rats
ANOVA showed significant differences on sodium depletion-induced 1.8% NaCl intake comparing rats treated with bilateral injections of different doses of α,β-methylene ATP or saline into the LPBN [F(3,24)=13.39; p<0.001] (Fig. 2A).

Bilateral injections of the highest dose of α,β-methylene ATP (4.0 nmol/0.2 μl each site) into the LPBN increased sodium
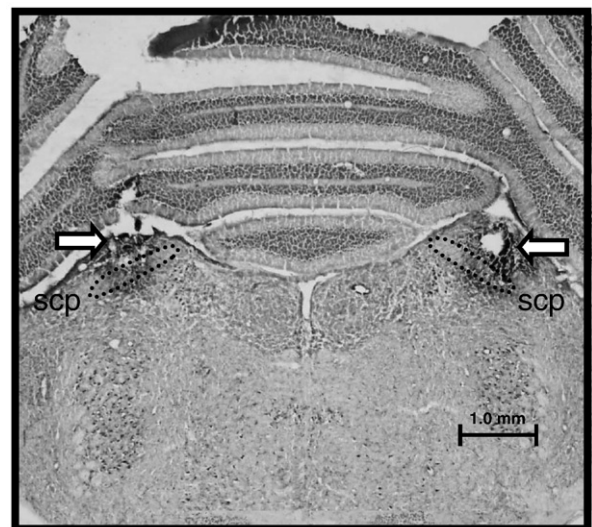

Fig. 1 – Photomicrograph showing the sites of injections into the LPBN (arrows). scp, superior cerebellar peduncle (outlined).
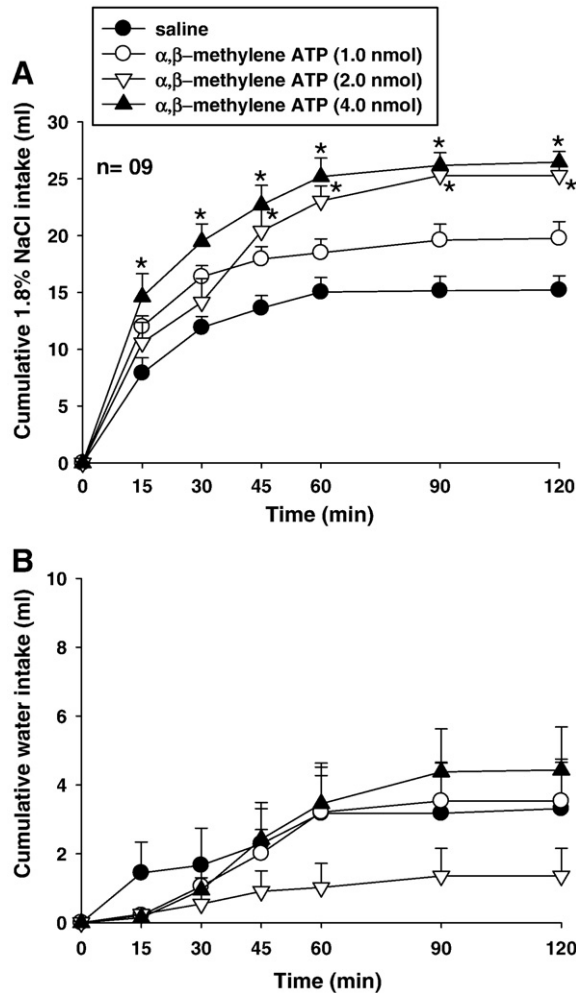
Fig. 2 – (A) Cumulative 1.8% NaCl intake and **(B)** cumulative water intake induced by 24 h of sodium depletion in rats that received bilateral injections of α,β-methylene ATP (1.0, 2.0 or 4.0 nmol/0.2 μl) or saline into the LPBN. Results are expressed as means±S.E.M.; n, number of rats. *Significantly different from saline.

depletion-induced 1.8% NaCl intake from 15 to 120 min of the test with p values ranging from $p < 0.01$ at 15 min to $p < 0.001$ from 45 to 120 min (Newman–Keuls post hoc test) (Fig. 2A). The injections of the intermediate dose of α,β-methylene ATP (2.0 nmol/0.2 μl each site) into the LPBN increased sodium depletion-induced 1.8% NaCl intake from 45 to 120 min of test with p values ranging from $p < 0.005$ (at 45 min) to $p < 0.001$ (from 60 to 120 min, Newman–Keuls post hoc test) (Fig. 2A). Bilateral injections of the lowest dose of α,β-methylene ATP (1.0 nmol/0.2 μl each site) into the LPBN did not change sodium depletion-induced 1.8% NaCl intake (Fig. 2A).

Injections of α,β-methylene ATP (1.0, 2.0 and 4.0 nmol/0.2 μl) produced no effect on water intake (3.5±1.1, 1.3±0.8, 4.4±1.2 ml/120 min, respectively, vs. vehicle: 3.3±1.4 ml/120 min) [$F(3,24) = 1.56$; $p > 0.05$] (Fig. 2B).

2.2.2. Sodium replete rats

Bilateral injections of α,β-methylene ATP (2.0 nmol/0.2 μl each site) into the LPBN in sodium replete rats produced no effect

on 1.8% NaCl (1.3±0.9 vs. saline: 0.8±0.4 ml/120 min, $n=6$) or water intake (0.2±0.1 vs. saline: 0.6±0.3 ml/120 min).

2.3. Water and 1.8% NaCl intake by sodium depleted rats treated with bilateral injections α,β-methylene ATP combined with PPADS, suramin or saline into the LPBN

2.3.1. Water and 1.8% NaCl intake by rats treated with α,β-methylene ATP after pretreatment with PPADS or saline into the LPBN

ANOVA showed significant differences on sodium depletion-induced 1.8% NaCl intake comparing rats treated with bilateral injections of α,β-methylene ATP (2.0 nmol/0.2 μl each site) or saline after pretreatment with PPADS (4 nmol/0.2 μl) or saline into the LPBN [$F(3,27) = 10.97$; $p < 0.001$] (Fig. 3A).

Bilateral injections of α,β-methylene ATP (2.0 nmol/0.2 μl each site) after pretreatment with saline into the LPBN increased sodium depletion-induced 1.8% NaCl intake from 30 to 120 min of the test with p values ranging from $p < 0.05$ at

Fig. 3 – (A) Cumulative 1.8% NaCl intake and **(B)** cumulative water intake induced by 24 h sodium depletion in rats that received bilateral injections of PPADS (4.0 nmol/0.2 μl) or saline+α,β-methylene ATP (2.0 nmol/0.2 μl) or saline into the LPBN. Results are expressed as means±S.E.M.; n, number of rats. *Significantly different from all the other treatments into the LPBN.

30 min to $p < 0.005$ at 90 and 120 min (Newman–Keuls post hoc test) (Fig. 3A).

Bilateral injections of PPADS (4 nmol/0.2 μl) + saline into the LPBN did not change 1.8% NaCl intake ($p \geq 0.1$ at any of the times studied, Newman–Keuls post hoc test) (Fig. 3A). PPADS (4 nmol/0.2 μl) + α,β-methylene ATP (2.0 nmol/0.2 μl) bilaterally injected into the LPBN abolished the effects of α,β-methylene ATP in 1.8% NaCl intake by rats treated with FURO (Fig. 3A). For all the times tested, sodium depletion-induced 1.8% NaCl intake after PPADS + α,β-methylene ATP into the LPBN was not different from control test with saline injections into the LPBN ($p > 0.1$, Newman–Keuls post hoc test) (Fig. 3A). However, sodium depletion-induced 1.8% NaCl intake after PPADS + α,β-methylene ATP into the LPBN was significantly different from the intake after saline combined with α,β-methylene ATP injections into the LPBN for all the times tested, with p values ranging from $p < 0.05$ at 15 min to $p < 0.001$ from 30 to 120 min (Newman–Keuls post hoc test) (Fig. 3A).

Injections of α,β-methylene ATP or PPADS alone or combined into the LPBN produced no effect on water intake by sodium depleted rats [$F_{(3,27)} = 0.13$; $p > 0.05$] (Fig. 3B).

2.3.2.   Water and 1.8% NaCl intake by rats treated with α,β-methylene ATP after pretreatment with suramin or saline into the LPBN

ANOVA showed significant differences on sodium depletion-induced 1.8% NaCl intake comparing rats treated with bilateral injections of α,β-methylene ATP (2.0 nmol/0.2 μl each site) or saline after pretreatment with suramin (2 nmol/0.2 μl) or saline into the LPBN [$F_{(3,24)} = 35.47$; $p < 0.001$] (Fig. 4A).

Bilateral injections of α,β-methylene ATP (2.0 nmol/0.2 μl each site) after pretreatment with saline into the LPBN increased sodium depletion-induced 1.8% NaCl intake from 30 to 120 min of the test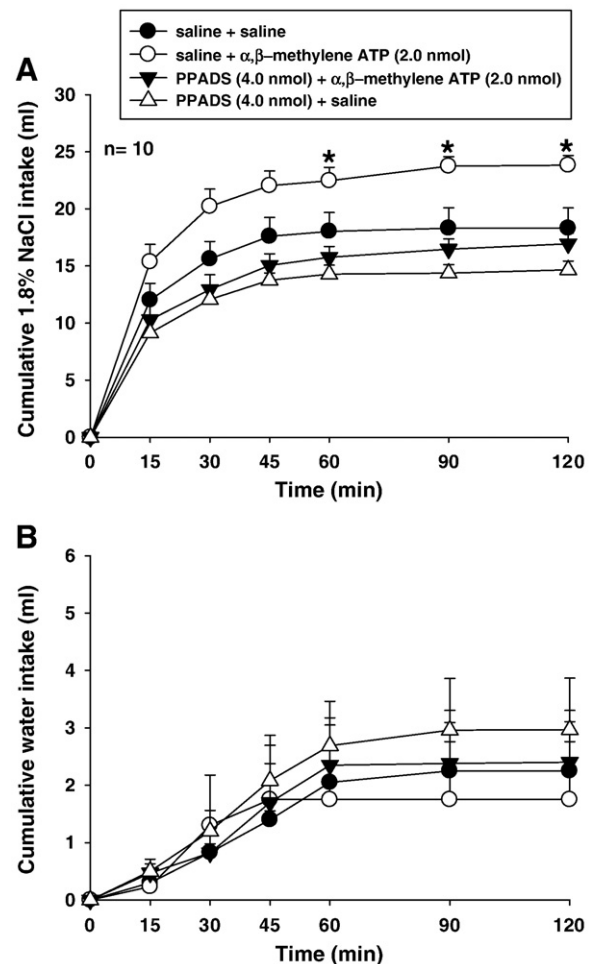 with p values ranging from $p < 0.05$ at 30 min to $p < 0.001$ from 45 to 120 min (Newman–Keuls post hoc test) (Fig. 4A). In contrast, bilateral injections of suramin (2 nmol/0.2 μl) + saline into the LPBN decreased sodium depletion-induced 1.8% NaCl intake from 15 to 120 min of the test ($p < 0.001$ for all the times, Newman–Keuls post hoc test) (Fig. 4A).

Unlike bilateral injections of suramin or α,β-methylene ATP + saline into the LPBN, the combination of suramin and α,β-methylene ATP into the LPBN produced no change in 1.8% NaCl intake by rats treated with FURO (Fig. 4A). For all the times tested, sodium depletion-induced 1.8% NaCl intake after suramin + α,β-methylene ATP into the LPBN was not different from control test with saline injections into the LPBN ($p > 0.5$ for all times, Newman–Keuls post hoc test) (Fig. 4A). However, sodium depletion-induced 1.8% NaCl intake after suramin + α,β-methylene ATP into the LPBN was significantly different from 1.8% NaCl intake after saline + α,β-methylene ATP injections into the LPBN from 30 to 120 min of the test, with p values ranging from $p < 0.05$ at 30 min to $p < 0.001$ from 45 to 120 min (Newman–Keuls post hoc test) (Fig. 4A). Sodium depletion-induced 1.8% NaCl intake after combining suramin and α,β-methylene ATP into the LPBN was also significantly different from 1.8% NaCl intake after saline + suramin injections into the LPBN from 15 to 120 min of test ($p < 0.001$ for all the times, Newman–Keuls post hoc test) (Fig. 4A).
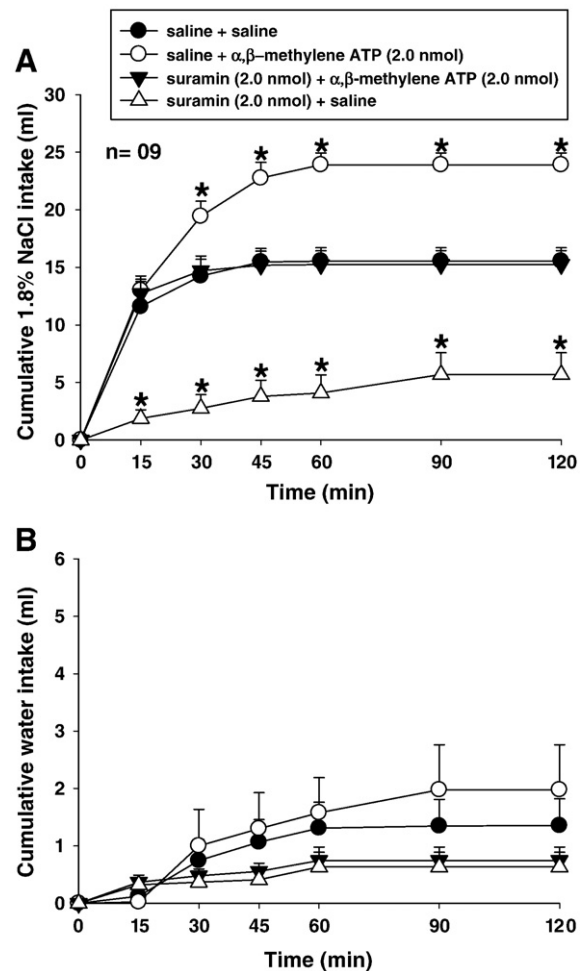

Fig. 4 – (A) Cumulative 1.8% NaCl intake and (B) cumulative water intake induced by 24 h sodium depletion in rats that received bilateral injections of suramin (2.0 nmol/0.2 μl) or saline + α,β-methylene ATP (2.0 nmol/0.2 μl) or saline into the LPBN. Results are expressed as means ± S.E.M.; n, number of rats. *Significantly different from saline + saline or from suramin + α,β-methylene ATP.

Injections of α,β-methylene ATP or suramin alone or combined into the LPBN produced no significant effect on water intake by sodium depleted rats [$F_{(3,24)} = 1.88$; $p > 0.05$] (Fig. 4B).

2.4.   Water or 2% sucrose intake by rats treated with bilateral injections of suramin into the LPBN

2.4.1.   Water and 2% sucrose intake by satiated rats treated with suramin into the LPBN

Bilateral injections of suramin (2.0 nmol/0.2 μl each site) into the LPBN increased 2% sucrose intake (7.1 ± 1.3 vs. saline: 5.3 ± 0.8 ml/90 min) as suggested by the significant interaction between treatments and times [$F_{(5,35)} = 4.42$; $p < 0.05$] (Fig. 5A); however, injections of suramin into the LPBN produced no effect on water intake (0.3 ± 0.1 vs. saline: 0.1 ± 0.1 ml/120 min) [$F_{(1,7)} = 1.42$; $p > 0.05$] (Fig. 5B).
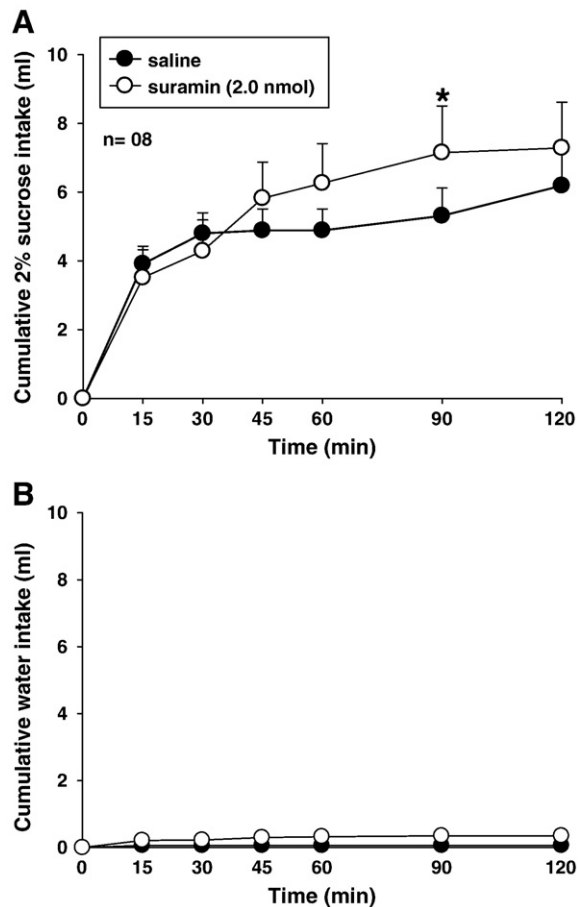
Fig. 5 – (A) Cumulative 2.0% sucrose intake and (B) cumulative water intake in rats that received bilateral injections of suramin (2.0 nmol/0.2 μl) or saline into the LPBN. Results are expressed as mean ± S.E.M.; *n*, number of rats. *Significantly different from saline.

2.4.2. Ingestion of 2% sucrose by 24 h food deprived rats treated with suramin into the LPBN

Bilateral injections of suramin (2.0 nmol/0.2 μl each site) into the LPBN produced no change in 2% sucrose intake by 24 h food deprived rats [$F(1,5) = 5.7; p > 0.05$] (Table 1).

2.4.3. Water intake by 24 h water deprived rats treated with suramin into the LPBN

Bilateral injections of suramin (2.0 nmol/0.2 μl each site) into the LPBN produced no change on 24 h of water deprivation-induced water intake [$F(1,6) = 0.37; p > 0.05$] (Table 2).

2.5. Water and 1.8% NaCl intake by sodium depleted rats that received injections of α,β-methylene ATP or suramin in sites outside of the LPBN

To confirm that the LPBN is the site in which injections of α,β-methylene ATP (2.0 nmol/0.2 μl) or suramin (2.0 nmol/0.2 μl) produced effects on sodium depletion-induced 1.8% NaCl intake, results from rats with misplaced injections (dorsal, ventral or medial to the LPBN) were also analyzed.

Bilateral injections of α,β-methylene ATP (2.0 nmol/0.2 μl) or suramin (2.0 nmol/0.2 μl) in sites outside of the LPBN

Table 1 – Cumulative 2% sucrose intake induced by 24 h of food deprivation in rats treated with bilateral injections of suramin or saline into LPBN.

LPBN treatment	2% sucrose intake (ml)			
	30 min	60 min	90 min	120 min
Saline	6.5 ± 0.8	11.7 ± 1.2	14.6 ± 1.8	17.0 ± 2.7
Suramin	8.7 ± 1.3	14.3 ± 1.7	17.1 ± 2.2	20.2 ± 3.8

Values are means ± S.E.M. *n* = 06 rats. The dose of suramin was 2.0 nmol/0.2 μl.

produced no change in 1.8% NaCl [$F(1,7) = 2.44; p > 0.05$] and [$F(1,7) = 1.01; p > 0.05$], respectively or in water intake [$F(1,7) = 1.30; p > 0.05$] and [$F(1,7) = 3.26; p > 0.05$], respectively (Table 3).

3. Discussion

The present data show that bilateral injections of the P2X purinergic receptor agonist (α,β-methylene ATP) into the LPBN increase sodium depletion-induced NaCl intake. Injections of the selective P2X antagonist, PPADS, alone had no effect on sodium intake, however, it abolished the increase of sodium intake produced by α,β-methylene ATP, suggesting that α,β-methylene ATP may act on P2X purinergic receptors in the LPBN to facilitate sodium depletion-induced sodium intake. Unlike PPADS, the non-selective P2 antagonist, suramin, injected alone into the LPBN reduced sodium depletion-induced sodium intake, which suggests that purinergic P2 receptors in the LPBN are part of the pathways activated by sodium depletion to induce sodium intake. Unexpectedly, the combination of suramin and α,β-methylene ATP in the LPBN produced no change in sodium depletion-induced sodium intake, which suggests that each one acts on different receptors, producing opposite effects that, together, result in no net change in sodium intake. Injections of suramin or α,β-methylene ATP in sites outside the LPBN produced no effect on sodium depletion-induced NaCl intake, which confirms the specificity of LPBN as the site of injections that produced the effects on NaCl intake. The ingestion of hypertonic sodium by sodium depleted rats usually drives rats to ingest a small and variable amount of water and this ingestion of water was not affected by treatments with agonist or antagonists of purinergic P2 receptors in the LPBN. Suramin into the LPBN slightly increased 2% sucrose intake and did not affect food deprivation-induced 2% sucrose intake or water deprivation-

Table 2 – Cumulative water intake induced by 24 h of water deprivation in rats that received bilateral injections of suramin or saline into LPBN.

LPBN treatment	Water intake (ml)			
	30 min	60 min	90 min	120 min
Saline	9.1 ± 2.1	13.1 ± 1.9	14.7 ± 2.2	15.0 ± 2.1
Suramin	10.8 ± 1.3	14.9 ± 1.9	16.2 ± 1.8	16.7 ± 1.8

Values are means ± S.E.M. *n* = 07 rats. The dose of suramin was 2.0 nmol/0.2 μl.

Table 3 – Water and 1.8% NaCl intake by sodium depleted rats that received bilateral injections of suramin, α,β-methylene ATP or saline in sites outside the LPBN.

LPBN treatment	1.8% NaCl intake (ml/120 min)	Water intake (ml/120 min)
Saline	16.9±1.0	4.1±1.4
Suramin	16.0±1.4	1.0±0.6
Saline	18.9±1.5	2.6±1.2
α,β-methylene ATP	16.8±1.1	0.9±0.4

Values are means±S.E.M. $n=08$ rats/group. The dose of suramin was 2.0 nmol/0.2 μl and the dose of α,β-methylene ATP was 2.0 nmol/0.2 μl.

induced water intake, suggesting that the inhibition of sodium intake by suramin into the LPBN is not due to non-specific inhibition of ingestive behaviors.

Evidence for the involvement of LPBN in the control of water intake arose by studies showing that electrolytic or chemical (ibotenic acid) lesions of the LPBN increased ANG II-induced water intake (Ohman and Johnson, 1986, 1989; Johnson and Edwards, 1990; Edwards and Johnson, 1991). Similar to these results from LPBN-lesioned rats, it was also shown that bilateral injections of lidocaine or methysergide into the LPBN also increased ANG II-induced water intake (Menani and Johnson, 1995). Early studies also showed that bilateral injections of methysergide into the LPBN increased NaCl intake induced by different stimuli and that proglumide (a CCK receptor antagonist) into the LPBN increased hypertonic NaCl intake induced by i.c.v. ANG II or FURO+captopril s. c. (Menani et al., 1996, 1998a, 2000; Menani and Johnson, 1998; De Gobbi et al., 2000). In addition to serotonin and CCK, glutamate and CRF, acting in the LPBN, inhibit sodium and water intake, whereas GABAergic, opioid and adrenergic agonists acting in the LPBN facilitate sodium intake (Menani et al., 1996, 1998a,b, 2000; De Gobbi et al., 2000, 2009; Fratucci De Gobbi et al., 2001; Andrade et al., 2004, 2006; Callera et al., 2005; De Castro e Silva et al., 2005; De Oliveira et al., 2008; Gasparini et al., 2009; Andrade-Franzé et al., 2010). Therefore, all these studies suggest that inhibition or facilitation of sodium and occasionally water intake by different neurotransmitters in the LPBN is probably related to activation or deactivation of LPBN inhibitory mechanisms, respectively. The present results suggest that activation of P2 purinergic receptors in the LPBN facilitate sodium depletion-induced hypertonic NaCl intake. Therefore, similar to GABAergic, opioid or adrenergic activation in the LPBN, P2 purinergic receptor activation in the LPBN facilitates sodium intake by likely deactivating LPBN inhibitory mechanisms.

Functional studies have suggested the involvement of purinergic mechanisms in the control of cardio-respiratory and thermal regulation (Ergene et al., 1994; Barraco et al., 1996; Phillis et al., 1997; Scislo et al., 1997, 1998; Gourine et al., 2002, 2003, 2004, 2005; de Paula et al., 2004; Antunes et al., 2005a,b). The present study is the first evidence showing the involvement of central purinergic mechanisms in the control of fluid–electrolyte balance and, more specifically, of NaCl intake. Similar to the blockade of serotoninergic and cholecystokinergic mechanisms in the LPBN, activation of purinergic P2X

receptors in the LPBN increased 1.8% NaCl intake by sodium depleted rats; however, the same dose of α,β-methylene ATP injected into the LPBN produced no change in 1.8% NaCl intake by sodium replete rats. Therefore, the present results clearly show that purinergic mechanisms in the LPBN facilitate sodium ingestion induced by the activation of an excitatory mechanism like those activated by sodium depletion. Results showed no evidence that activation of purinergic P2X receptors in the LPBN may affect sodium or water intake by satiated rats, however, only one dose of α,β-methylene ATP was tested in satiated rats. Therefore, more studies testing the effects of higher doses of α,β-methylene ATP injected into the LPBN in satiated rats are necessary to confirm this suggestion.

Injections of PPADS into the LPBN at the same dose that blocked the effects of α,β-methylene ATP produced no change in NaCl intake induced by sodium depletion. Therefore, although P2X receptor activation in the LPBN facilitates sodium depletion-induced NaCl intake, it seems that the activation of these receptors is not necessary for sodium ingestion by sodium depleted rats. In contrast to PPADS, suramin, a non-selective P2 purinergic antagonist into the LPBN almost abolished sodium depletion-induced NaCl intake, suggesting that activation of purinergic receptors in the LPBN is essential for NaCl intake by sodium depleted rats. More specifically, sodium appetite arises only if purinergic mechanisms are activated. In addition, a specific subpopulation of P2X receptors may block inhibitory mechanisms, thereby further increasing salt intake.

Suramin or α,β-methylene ATP injection into the LPBN produced opposite effects on NaCl intake but, when combined, they produced no effect. Considering that suramin might block purinergic P2X and P2Y receptors (Ralevic and Burnstock, 1998), no effect of α,β-methylene ATP was expected after suramin. However, injections of α,β-methylene ATP reduced the effects of suramin in the LPBN, which suggests that α,β-methylene ATP was still acting and produced effects opposite to that of suramin. Thus, no change in sodium intake was observed. Although suramin is a non-specific antagonist for P2X and P2Y receptors, it has been suggested that suramin may not block P2X4 and P2X6 receptor subtypes (Ralevic and Burnstock, 1998), which might be activated by α,β-methylene ATP to facilitate sodium intake and oppose the effects of suramin. Further studies testing the effects of agonists and antagonists for different purinergic receptors in the LPBN are necessary to investigate this possibility.

Although the present results clearly show that purinergic mechanisms in the LPBN are involved in the control of sodium intake, it is important to consider that they probably do not act alone and may interact with other neurotransmitters in the LPBN to control this behavior. Studies have suggested that different neurotransmitters like serotonin, CCK, glutamate, CRF, noradrenaline, GABA and opioids modulate sodium and water intake in the LPBN (Colombari et al., 1996; Menani et al., 1996, 1998a; Menani and Johnson, 1998; De Gobbi et al., 2000, 2009; Andrade et al., 2004, 2006; Callera et al., 2005; de Oliveira et al., 2007, 2008; Gasparini et al., 2009). ATP may act as a cotransmitter with noradrenaline and may increase the release of noradrenaline and GABA (Burnstock, 1986, 2007; Espallergues et al., 2007). In fact, similar to α,β-methylene ATP, noradrenaline and GABA in the LPBN facilitate NaCl intake.

Therefore, without excluding the possibility of interactions with other neurotransmitters, purinergic receptor activation in the LPBN might facilitate NaCl intake by increasing the release of noradrenaline or GABA.

The LPBN is connected with the area postrema (AP) and nucleus of the solitary tract (NTS) (Norgren, 1981; Shapiro and Miselis, 1985). The NTS receives signals from arterial baroreceptors and cardiopulmonary, gustatory and other visceral receptors, whereas the AP, an area that lacks a blood-brain barrier, may also receive humoral signals important in the control sodium and water intake (Norgren, 1981; Johnson and Thunhorst, 1997). From the AP/NTS these signals may reach the LPBN and there they activate inhibitory mechanisms for sodium and water intake. The present results suggest that during sodium depletion, activation of purinergic receptors in the LPBN, alone or in conjunction with other neurotransmitters like noradrenaline or GABA, attenuates the effect of these inhibitory mechanisms and, therefore, facilitates NaCl intake. However, more studies are necessary to investigate possible interactions between purinergic and the other mechanisms of the LPBN involved in the control of NaCl intake, as well as inputs to the LPBN that activate these mechanisms.

4. Experimental procedures

4.1. Animals

Male Holtzman 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, Paulínia, SP, Brazil), water and 1.8% NaCl solution. Room temperature was maintained at $23 \pm 2\ °C$ and humidity at $55 \pm 10\%$ with a 12-h light/dark cycle with light onset at 7:30 AM. The experimental protocols used in the present study were approved by the Ethical Committee for Animal Care and Use from the Dentistry School of Araraquara, UNESP Brazil (Proc. CEEA no. 03/2008) and they followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication no. 80-23, 1996, USA). All efforts were made to minimize animal discomfort and the number of animals used.

4.2. Cerebral cannulas

Rats were anesthetized with ketamine (80 mg/kg of body weight, Cristália, Itapira, SP, Brazil) combined with xylazine (7 mg/kg of body weight, Agener União, Embu-Guaçu, SP, Brazil) and placed in a Kopf stereotaxic instrument. The skull was leveled between bregma and lambda. Bilateral stainless steel 23-gauge cannulas were implanted to target the LPBN using the following coordinates: 9.4 mm caudal to bregma, 2.1 mm lateral to the midline, and 4.2 mm below the dura mater (Paxinos and Watson, 1997). 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. The rats were allowed to recover 6 days before drug injections into the LPBN.

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 time of testing, obturators were removed and the injection needle (2 mm longer than the guide cannulas) was introduced in the brain. All the injections into the LPBN were 0.2 μl for each site and performed over a period of 1 min, with 1 additional min allowed to elapse before the injection needle was removed from the guide cannula to avoid reflux. The movement of an air bubble inside the PE 10 polyethylene tubing connected to the syringe confirmed drug flow. The obturators were replaced after injection, and the rats were placed back into the cage.

4.4. Drugs

Furosemide (FURO, 20 mg/kg of body weight, Sigma Chem., St. Louis, MO, USA) dissolved in alkaline saline (pH adjusted to 9.0 with NaOH) was administered s.c. Pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS, 4.0 nmol/0.2 μl, a P2X purinergic receptor antagonist), suramin (2.0 nmol/0.2 μl, a non-selective P2 purinergic receptor antagonist) and α,β-methyleneadenosine 5'-triphosphate (α,β-methylene ATP, 1.0, 2.0 and 4.0 nmol/0.2 μl, a P2X purinergic receptor agonist) from Sigma Chemical, St. Louis, MO were administered into the LPBN. Suramin, PPADS and α,β-methylene ATP were dissolved in isotonic saline. Doses of drugs injected into the LPBN were based on a previous study (de Paula et al., 2004).

4.5. Histology

At the end of the tests, the animals received bilateral injections of 2% Evans Blue dye solution (0.2 μl) into the LPBN. 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, and analyzed by light microscopy to confirm the injection sites in the LPBN.

4.6. Statistical analysis

The results are reported as means $\pm$ S.E.M. Two-way analysis of variance (ANOVA) with repeated measures for both factors (treatments and times), followed by Newman–Keuls post hoc test was used to analyze the results, except 1.8% NaCl and water intake by rats with injections outside the LPBN which were analyzed by one-way ANOVA. Differences were considered significant at $p < 0.05$. Statistical analysis was performed using Sigma Plot 11 from Systat Software, Inc.

4.7. Experimental protocols

4.7.1. Water and 1.8% NaCl intake by satiated or sodium depleted rats treated with bilateral injections of α,β-methylene ATP into the LPBN

4.7.1.1. *Sodium depleted rats.* Food, water and 1.8% NaCl were removed and the cages were rinsed with water. Rats were

treated with a s.c. injection of FURO (20 mg/kg of body weight) followed by free access to water and sodium-deficient food (powdered corn meal; 0.001% sodium and 0.33% potassium) for 24 h (sodium depletion). After this period, water and sodium-deficient food were removed from the cages and rats received injections of drugs into the LPBN. Ten minutes later, rats were given water and 1.8% NaCl in 0.1-ml graduated glass burettes fitted with stainless steel spouts. Cumulative water and 1.8% NaCl intakes were recorded at 15, 30, 60, 90 and 120 min.

Treatment with FURO and sodium-deficient diet produced losses of 1.5 to 2.0 mEq of sodium per rat in 24 h, which induces a consistent intake of hypertonic sodium solutions (De Luca et al., 1992; Jalowiec, 1974; Rowland and Fregly, 1992; Sakai et al., 1989).

To study the effects of different doses of α,β-methylene ATP (1.0, 2.0 and 4.0 nmol/0.2 μl) into the LPBN, one group of rats was submitted to four tests. In each test, the group of rats was divided in two, and each half received a different drug treatment into the LPBN (saline or one of the three doses of α,β-methylene ATP). The sequence of drug treatments was randomized; all animals received all four treatments. The interval between tests was 72 h.

4.7.1.2. *Sodium replete rats.*   To test if injections of α,β-methylene ATP into the LPBN of sodium replete rats would affect water and 1.8% NaCl intake, another group of rats not treated with FURO received bilateral injections of α,β-methylene ATP (2.0 nmol/0.2 μl) or saline into the LPBN and 10 min later rats were given water and 1.8% NaCl. Cumulative water and 1.8% NaCl intake was measured at 15, 30, 60, 90, and 120 min. This group of rats was submitted to two tests. In the first test, half of the group received bilateral injections of α,β-methylene ATP into the LPBN and the other half received injections of saline into the LPBN. In the next test, rats received the same treatments into the LPBN in a counter-balanced design. The interval between the two tests was 48 h.

4.7.2.   *Water and 1.8% NaCl intake by sodium depleted rats treated with bilateral injections α,β-methylene ATP or saline after pretreatment with PPADS, suramin or saline into the LPBN*

4.7.2.1. *Water and 1.8% NaCl intake by rats treated with α,β-methylene ATP or saline after pretreatment with PPADS or saline into the LPBN.*   In a group of rats submitted to sodium depletion as described above (Section 4.7.1a), PPADS (4 nmol/0.2 μl) or saline was bilaterally injected into the LPBN 15 min prior to injections of α,β-methylene ATP (2 nmol/0.2 μl) or saline into the LPBN. Therefore, this group of rats received four combinations of treatments into the LPBN: saline+saline; saline+α,β-methylene ATP, PPADS+α,β-methylene ATP and PPADS+saline. In each test, the group of rats was divided in two and each half of the group received one of the four combinations indicated above. The sequence was randomized; all animals received all four treatments. The interval between tests was 72 h.

4.7.2.2. *Water and 1.8% NaCl intake by rats treated with α,β-methylene ATP or saline after pretreatment with suramin or saline into the LPBN.*   In another group of rats submitted to sodium depletion as described above (Section 4.7.1a), suramin

(2 nmol/0.2 μl) or saline was bilaterally injected into the LPBN 15 min prior to injections of α,β-methylene ATP (2 nmol/0.2 μl) or saline. This group of rats was also submitted to four tests, following the same protocol described above, except that suramin instead of PPADS was injected into the LPBN. The interval between tests was 72 h.

4.7.3.   *Water or 2% sucrose intake by rats treated with bilateral injections of suramin into the LPBN*
To confirm that the inhibition of sodium depletion-induced 1.8% NaCl intake by suramin into the LPBN is not due to non-specific inhibition of all ingestive behaviors, ad libitum 2% sucrose intake, food deprivation induced 2% sucrose intake or water deprivation induced water intake were tested after injections of suramin into the LPBN.

4.7.3.1. *Water and sucrose intake by satiated rats treated with suramin into the LPBN.*   A group of rats with ad libitum access to food and water had also access to 2% sucrose for 2 h every day for 1 week. After this period of training, suramin (2.0 nmol/0.2 μl) or saline was injected bilaterally into the LPBN, 10 min before rats were given 2% sucrose solution. Cumulative water and 2% sucrose solution intake was measured at each 15 min for 2 h. This group of rats was submitted to two tests. In the first test, half of the group received bilateral injections of suramin into the LPBN and the other half received injections of saline into the LPBN. In the next test, rats received the same treatments into the LPBN in a counterbalanced design. The interval between the two tests was 48 h.

4.7.3.2. *Food deprivation-induced 2% sucrose intake by rats treated with suramin into the LPBN.*   Another group of rats had food removed from the cage, whereas water was available. Twenty-four hours after starting food deprivation, the animals received suramin (2.0 nmol/0.2 μl) or saline into the LPBN. Ten minutes after the injections, rats had access to 2% sucrose. Cumulative 2% sucrose intake was measured at each 30 min for 2 h in the absence of food. This group of rats was also submitted to two tests, following the same counter-balanced design described previously to test sucrose intake by satiated rats. The interval between the two tests was 72 h.

4.7.3.3. *Water intake induced by 24 h of water deprivation in rats treated with suramin into the LPBN.*   Another group of rats had only food pellets available for 24 h. After this period, food was removed and suramin (2.0 nmol/0.2 μl) or saline was injected into the LPBN 10 min before access to water. Cumulative water intake was measured at each 30 min for 2 h in the absence of food. This group of rats was also submitted to two tests, following the same counterbalanced design described previously for sucrose intake test. The interval between the two tests was 72 h.

Acknowledgments

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