Sodium and water intake are not affected by GABA$_C$ receptor activation in the lateral parabrachial nucleus of sodium-depleted rats

Gean Domingos-Souza[a,*], Cesar Arruda Meschiari[a], Samyra Lopes Buzelle[a],
João Carlos Callera[b], José Antunes-Rodrigues[a]

[a] Department of Physiology, School of Medicine of Ribeirão Preto, University of São Paulo, Ribeirão Preto, SP, Brazil
[b] Department of Basic Sciences, School of Dentistry, UNESP − Universidade Estadual Paulista, Rodovia, Araçatuba, SP, Brazil

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ABSTRACT

The activation of GABAergic receptors, GABA$_A$ and GABA$_B$, in the lateral parabrachial nucleus (LPBN) increases water and sodium intake in satiated and fluid-depleted rats. The present study investigated the presence of the GABA$_C$ receptor in the LPBN, its involvement in water and sodium intake, and its effects on cardiovascular parameters during the acute fluid depletion induced by furosemide combined with captopril (Furo/Cap). One group of male Wistar rats (290–300 g) with bilateral stainless steel LPBN cannulas was used to test the effects of a GABA$_C$ receptor agonist and antagonist on the fluid intake and cardiovascular parameters. We investigated the effects of bilateral LPBN injections of trans-4-aminocrotonic acid (TACA) on the intake of water and 0.3 M NaCl induced by acute fluid depletion (subcutaneous injection of Furo/Cap). c-Fos expression increased ($P<0.05$), suggesting LPBN neuronal activation. The injection of different doses of TACA (0.5, 2.0 and 160 nmol) in the LPBN did not change the sodium or water intake in Furo/Cap-treated rats ($P>0.05$). Treatment with the GABA$_C$ receptor antagonist (Z)-3-[(aminoiminomethyl)thio]prop-2-enoic acid sulfate (ZAPA, 10 nmol) or with ZAPA (10 nmol) plus TACA (160 nmol) did not change the sodium or water intake compared with that for vehicle (saline) ($P>0.05$). Bilateral injections of the GABA$_C$ agonist in the LPBN of Furo/Cap-treated rats did not affect the mean arterial pressure (MAP) or heart rate (HR). The GABA$_C$ receptor expression in the LPBN was confirmed by the presence of a 50 kDa band. Although LPBN neurons might express GABA$_C$ receptors, their activation produced no change in water and sodium intake or in the cardiovascular parameters in the acute fluid depletion rats. Therefore, the GABA$_C$ receptors in the LPBN might not interfere with fluid and blood pressure regulation.

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1. Introduction

Important inhibitory mechanisms that control water and sodium intake have been reported in the lateral parabrachial nucleus (LPBN) (Menani et al., 1996), a pontine structure that lies dorsolateral to the superior cerebellar peduncle. The LPBN is strategically connected to forebrain structures, such as the paraventricular nucleus of the hypothalamus and the amygdala (Feigenspan and Bormann, 1994b). In addition, the LPBN receives projections from the area postrema (AP) and the medial nucleus tractus solitary (mNTS) (Ciriello et al., 1984; Herbert et al., 1990; Jhamandas et al., 1996). These areas are involved in electrolyte balance and cardiovascular responses.

Gamma aminobutyric acid (GABA) is an inhibitory neurotransmitter that is widely distributed in the central nervous system (Bowery et al., 1987). A dense group of GABA-immunoreactive varicosities was reported in the parabrachial (PB) complex/Kolliker fuse nucleus, suggesting a strong GABAergic influence on the neuronal processes in this area, particularly the gustatory and visceral portion of the PB complex (Kobashi and Bradley, 1998).

Three main classes of GABA receptors exist and are termed GABA$_A$, GABA$_B$, and GABA$_C$ receptors (Bormann, 2000). Ionotropic GABA$_A$ receptors (bicuculline-sensitive) are ligand-gated Cl$^-$ channels that form a heteropentameric structure. Metabotropic GABA$_B$ receptors couple to Ca^{2+} and K$^+$ channels via G proteins and are selectively activated by baclofen; these receptors do not respond to known GABA$_A$ receptor modulators, such as barbiturates and benzodiazepines (Bormann, 2000; Bowery, 1989). A third

type of GABA receptor, termed $GABA_C$, has been proposed, and it is distinct from the other known GABA receptors. Ionotropic $GABA_C$ receptors gate Cl^- currents in various parts of the vertebrate brain and are thought to be homo- or hetero-pentamers composed of ρ1, ρ2 and ρ3 subunits (Zhang et al., 2001). $GABA_C$ receptors are insensitive to the selective $GABA_A$ receptor antagonist bicuculline and some $GABA_A$ receptor modulators, and they are not activated by the $GABA_B$ agonist baclofen (Bormann, 2000). The function of $GABA_C$ receptors has mostly been studied in the retina (Feigenspan and Bormann, 1994a). Outside the retina, functional putative $GABA_C$ receptors have been detected in the superior colliculus, amygdala and brainstem (Boller and Schmidt, 2003; Delaney and Sah, 1999; Grabauskas and Bradley, 2001; Milligan et al., 2004). Ionotropic $GABA_C$ receptors are activated by cis-aminocrotonic acid and trans-4-aminocrotonic acid (TACA) and are selectively blocked by (1,2,5,6-tetrahydropyridin-4-yl) methylphosphinic acid and (Z)-3-[(aminoiminomethyl)thio]prop-2-enoic acid sulfate (ZAPA) (Woodward et al., 1992, 1993).

Interestingly, the blockade of LPBN neurons with bilateral injections of the selective $GABA_A$ receptor agonist muscimol increases arterial pressure and induces a high intake of a hypertonic sodium solution and a slight intake of water in rats depleted of fluid by furosemide + captopril (Furo/Cap) (Callera et al., 2005; de Oliveira et al., 2007). In addition, a recent study showed that activation of $GABA_B$ receptors through the administration of baclofen in the LPBN also causes water and sodium intake in fluid replete rats (De Oliveira et al., 2011), suggesting that an LPBN GABAergic mechanism is involved in controlling sodium intake.

Although previous studies showed that the activation of $GABA_A$ and $GABA_B$ receptors in the LPBN causes the intake of water and 0.3 M NaCl solution in fluid-depleted rats and increases 0.3 M NaCl intake in Furo/Cap-treated rats, the possible effects of $GABA_C$ receptor activation in the LPBN on sodium depletion-induced NaCl intake had not been tested. Therefore, in the present study, we investigate the effects of a $GABA_C$ agonist on the sodium and water intake and cardiovascular alterations of rats that were depleted of sodium by Furo/Cap.

2. Materials and methods

2.1. Animals

Male Wistar rats weighing 290–310 g were used. The animals were housed in individual stainless steel cages with free access to food containing a standard level of sodium (Guabi Rat Chow, Paulinia, SP, Brazil), water and 0.3 M NaCl solution. The positions of the bottles containing the water and the 0.3 M NaCl were rotated daily to avoid place preference. The room temperature was maintained at $23 \pm 2 °C$, and the humidity was maintained at $55 \pm 10\%$ with a 12:12 light–dark cycle with light onset at 07:30 AM. The experiments were approved by the local Institutional Animal Research Ethics Committee (process number 1332/2008). All efforts were made to minimize animal discomfort and the number of animals used, and the experiments complied with the recommendations of the Brazilian College of Animal Experimentation (COBEA) and the American National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publications No. 80-23, 1996, USA).

2.2. Drugs

Furosemide (Furo), captopril (Cap) and muscimol HBr were purchased from Sigma (Saint Louis, MO, USA); TACA and ZAPA were purchased from Tocris (Ellisville, MO, USA). Furosemide was dissolved in alkaline saline (pH adjusted to 9.0), and all other drugs

were dissolved in 0.15 M NaCl, which served as the vehicle. Up to our knowledge, this is the first study to use in vivo injection of $GABA_C$ agonist and antagonist in the LPBN, thus, there is no consensus or reports on literature about the dose of these drugs for this use. However, we chose $GABA_C$ receptor agonist and antagonist doses based on previous publications about $GABA_A$ receptors agonists and antagonists (Callera et al., 2005; de Melo e Silva et al., 2013; Kimura et al., 2008) because $GABA_C$ receptors is an ionotropic receptor such as $GABA_A$ receptor. Initially, we used TACA dose similar to muscimol dose (0.5 nmol) (Callera et al., 2005; de Melo e Silva et al., 2013; Kimura et al., 2008). Previous studies that evaluated the agonist profiles of electrophysiological experiments the Xenopus oocytes observed that potent agonists of these receptors were muscimol and TACA, which were approximately equipotent (Hosie and Sattelle, 1996). Muscimol and TACA were the most potent agonists of RDLac homo-oligomers and were full agonists, equipotent with GABA. Moreover, the ZAPA antagonist (Feigenspan et al., 1993) is approximately equipotent with muscimol (agonist) in certain unidentified locust and cockroach neurons (Taylor et al., 1993).

2.3. Brain surgery

Rats were anesthetized with an intraperitoneal (i.p.) injection of ketamine (80 mg/kg of body weight [b.w.]) combined with xylazine (7 mg/kg b.w.) and placed in a stereotaxic instrument (Kopf, USA). The skull was leveled between bregma and lambda. Stainless steel guide cannulas (12×0.6 mm o.d.) were implanted bilaterally with their tips ending 2 mm above the LPBN using the following coordinates: 9.4 mm caudal to bregma, 2.2 mm lateral to the midline, and 3.8 mm below the dura mater. The cannulas were fixed to the cranium using dental acrylic resin and jeweler screws and were filled with 30-gauge metal obturators between tests. After the surgery, the rats received intramuscular injections of the analgesic cetoprophen (1%, 0.03 mL) and a prophylactic dose of the antibiotic penicillin (30,000 IU). The rats were allowed to recover for 5 days before the ingestion tests began, and during this period, they had free access to water, 0.3 M NaCl solution, and food containing 2.7 mg/kg of sodium.

2.4. Injections in the LPBN

Bilateral injections in the LPBN were made using 10 μL Hamilton syringes connected via polyethylene tubing (PE 10) to 30-gauge injection cannulas. At the time of testing, the obturators were removed, and the injection cannula (2 mm longer than the guide cannula) was carefully inserted into the guide cannula. For bilateral injections, the first injection was performed on one side, the needle was removed and repositioned at the contralateral side, and then the second injection was administered. Therefore, injections were performed ~1 min apart. A volume of 0.2 μL was injected in the LPBN at each site. The obturators were reinstalled after the injections, and the rats were returned to their cages.

2.5. Experimental procedures

2.5.1. Water and 0.3 M NaCl intake of Furo/Cap-treated rats

The rats were tested in their home cages. Water and 0.3 M NaCl were provided in burettes with 0.1 mL divisions that were fitted with metal drinking spouts. In one group of rats, water and 0.3 M NaCl intake (two-bottle test) was induced by the treatment with s. c. Furo (10 mg/kg b.w.) plus Cap (5 mg/kg b.w.). The rats received the s.c. Furo/Cap treatment and were returned to their home cages, which lacked water and 0.3 M NaCl solution. One hour later, water and 0.3 M NaCl, but not food, were made available to the animals,

and the cumulative water and sodium intakes were measured every 30 min for 180 min.

Animals were treated with Furo/Cap; afterward, each rat was randomly chosen to receive an LPBN injection of one dose of TACA (0.5, 2.0 and 160 nmol/0.2 μL, $n = 6$ for each dose), muscimol (0.5 nmol/0.2 μL), or vehicle (saline) 15 min before the rats had access to water and 0.3 M NaCl. TACA doses were equal or higher than the muscimol dose which increased water and 0.3 M NaCl intake (0.5, 2.0 and 160 nmol/0.2 μL). The sequence of the treatments in the LPBN in each rat in different tests was randomized, and at the end of the experiments, each rat received all the three treatments. The tests were separated from one another by at least 72 h. A previous study showed that activation of $GABA_A$ receptors with muscimol injected in the LPBN potentiated 0.3 M NaCl intake in Furo/Cap-treated rats (Callera et al., 2005), since Furo/Cap treatment induces such intake (Thunhorst and Johnson, 1994). Muscimol (0.5 nmol/0.2 μL) was injected to ensure that the injection site was in the LPBN (Callera et al., 2005); the correct location was confirmed by the increased water and 0.3 M NaCl intake. Animals with 0.3 M NaCl intake lower than 15 mL after 180 min, were considered not responding the muscimol treatment, and were excluded from the study.

In order to elucidate the effects of $GABA_C$ receptor inhibition on water and 0.3 M NaCl intake of Furo/Cap-treated rats, we used ZAPA, a selective $GABA_C$ receptor antagonist. In addition, to investigate whether $GABA_A$ receptor is activated by TACA, we used ZAPA administration in LPBN, previously. Another group of Furo/Cap-treated rats was tested to determine how an LPBN injection of the combination of ZAPA ($GABA_C$ receptor antagonist) and TACA affected the water and 0.3 M NaCl intake induced by Furo/Cap treatment. On the day of the experiment, food, water and 0.3 M NaCl were removed, and acute depletion of water and sodium was produced by an injection of Furo/Cap, as described above. Bilateral injections of ZAPA (10 nmol/0.2 μL) or saline in the LPBN were performed 15 min before the LPBN injections of the highest dose of TACA (160 nmol/0.2 μL) or saline. During each experimental session, each rat was randomly chosen to receive one of following treatments in the LPBN: saline + saline, saline + TACA, ZAPA + TACA and ZAPA + saline, $n = 10$ for each group. The sequence of the treatments in the LPBN in each rat in different tests was randomized, and at the end of the experiments, each rat received all the four treatments. The cumulative water and 0.3 M NaCl intake were measured every 30 min a 180 min period after the bilateral injections of TACA or saline in the LPBN. A recovery period of at least 72 h was allowed among tests.

All tests began between 13:00 p.m. and 15:00 p.m. The order of treatments was randomized because repeated Furo/Cap injections can enhance stimulated and spontaneous NaCl intake (Menani et al., 1996; Pereira et al., 2010).

2.5.2. Arterial pressure and heart rate recordings

Rats were anesthetized with as described above, and a piece of polyethylene tubing (PE 10 connected to a PE 50) was inserted into the abdominal aorta through the femoral artery. The cannula was tunneled subcutaneously and exteriorized on the back of the rat. Immediately after recovering from the surgery, the rats were moved into individual cages with wood-chip bedding and given free access to food and water for 24 h. Afterward, they were transferred to the cardiovascular recording room. On the next day, food and water were removed, and the arterial catheter was connected to a P23 Db pressure transducer (Statham Gould, Madison, WI, USA) coupled to a preamplifier (model ETH-200 Bridge Bio Amplifier, CB Sciences, Dover, NH, USA) that was connected to a PowerLab computer data acquisition system (PowerLab 8SP, ADInstruments, Colorado Springs, CO, USA) to record the mean arterial pressure (MAP) and heart rate (HR) in

unanesthetized and unrestrained rats. A period of 15–20 min was necessary for stabilization of the MAP and HR readings. Acute fluid depletion was induced in the rats (Furo/Cap treatment); forty-five minutes afterward, one group of rats was tested for the effects of a saline injection ($n = 6$) in the LPBN on MAP and HR, and another group was tested for the effects of TACA (2 or 80 nmol/0.2 μL, $n = 6$ each treatment). The MAP and HR were recorded for the 90 min after the saline or TACA injections in the LPBN. Food, water and NaCl were not available to the rats after the Furo/Cap treatment and during the MAP and HR recordings.

2.6. Histology

At the end of the experiments, the animals were injected bilaterally with 2% Evans blue dye solution (0.2 μL/injection site) in the LPBN. They were then deeply anesthetized with sodium thiopental s.c. (CRISTALIA, Itapira, SP, Brazil, 80 mg/kg b.w.) and perfused transcardially with saline, followed by 10% formalin. The brains were removed, fixed in 10% formalin, frozen, cut coronally into 60 μm sections and stained with Giemsa, and analyzed by light microscopy to confirm the injection sites into the LPBN. Only animals with injections in the LPBN were considered for statistical analysis.

2.7. c-Fos immunocytochemistry

Another group of animals was prepared to determine the effects of Furo/Cap treatment on c-Fos expression. One hour after receiving a Furo/Cap treatment, the animals were anaesthetized with sodium thiopental (80 mg/kg). The heart was exposed, and the animal was perfused via the ascending aorta with ~200 mL of 0.01 M phosphate-buffered saline (PBS; pH 7.6), followed by ~200 mL of 4% paraformaldehyde in 0.1 M PBS. The brain was removed and post-fixed in 4% paraformaldehyde for ~4 h and then placed in 30% sucrose in 0.01 M PBS overnight. Thirty-micrometer coronal sections were cut through the basal forebrain and hindbrain on a cryostat. Sections of the LPBN were rinsed in PBS multiple times and then rinsed in 0.1 M glycine in PBS to remove excess aldehydes. After the PBS washes, the sections were blocked in PBS containing 0.1% Triton X-100 and 1% bovine serum albumin and were incubated with primary antibodies. Sections were stained for c-Fos during an overnight incubation at room temperature with rabbit polyclonal anti c-Fos (SC-52, Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:500 in PBS containing 0.1% Triton X-100 and 1% bovine serum albumin. After the overnight incubation, the sections were rinsed and incubated for 1 h with the appropriate secondary antibodies. To visualize c-Fos, we used a goat anti-rabbit IgG labeled with Alexa Fluor 594 (Molecular Probes, Grand Islands, NY, USA). Next, the slides were rinsed in PBS and coverslipped with Fluoromount-G (Electron Microscopy Sciences, Hat-field, PA, USA). Fos immunoreactivity (Fos-ir) in the bilateral LPBN were counted using 2–3 sections per photomicrograph, according to previous studies. Fos-ir nuclei were quantified using a computerized system including a Zeiss microscope equipped with a DC 200 Leica digital camera attached to a contrast enhancement device. Images were digitized and analyzed using Scion Image PC, based on the NIH 1997 version. Fos-ir cells in each section were counted by setting a size range for cellular nuclei and a threshold level for staining intensity (Andrade et al., 2004; De Gobbi et al., 2008; Margatho et al., 2015). Representative sections at exactly the same level in each group were acquired with the aid of the Adobe Photoshop Image Analysis Program, version 5.5. The counting was done in four animals of each condition and was repeated at least twice on each section analyzed to ensure that the number of profiles obtained was similar. The researcher who conducted the counting of Fos-ir cells

was not aware of the experimental condition (Andrade et al., 2004; De Gobbi et al., 2008; Margatho et al., 2015).

2.8. Detection of GABA_C (ρ2 subunit) by Western blotting

A brain punch biopsy of the LPBN was performed in a cryostat, cut into 900 μM sections for LPBN microdissection, and prepared as previously described (Palkovits and Brownstein, 1988) using an anatomical atlas as a reference (Swanson, 2003). The expression of the GABA_C receptor (ρ2 subunit) (∼50 kDa) in the LPBN was evaluated by Western blotting. The GABA_C protein levels were measured in a brain punch biopsy homogenized in 50 mM Tris HCl buffer (pH 7.4) containing 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 1% SDS, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM sodium orthovanadate, 5 μg/mL aprotinin, 1 mg/mL leupeptin, and 1 mM phenylmethylsulfonyl fluoride at 4 °C. The homogenate was centrifuged at 21,000 × g at 4 °C for 20 min; the supernatant was retained, and the protein content was determined using bovine serum albumin as a standard. An equal volume of sample buffer (20% glycerol (v/v), 125 mM Tris–HCl, 4% SDS, 100 mM dithiothreitol, and 0.02% bromophenol blue; pH 6.8) was added to the supernatant, and the mixture was boiled. Forty micrograms of total protein was separated using SDS-PAGE, transferred to nitrocellulose membranes, and blotted with polyclonal anti-GABA_C R ρ2 (sc-21343 Santa Cruz Biotechnology) antibody at a 1:1000 dilution. Primary antibodies were detected using peroxidase-conjugated secondary antibody (1:2000) and visualized using ECL reagents and an ImageQuant 350 detection system (GE Healthcare, Piscataway, NJ). Ponceau S staining was used as a loading control for the membrane transfer.

2.9. Statistical analysis

The results are reported as the means ± S.E.M. Statistical analysis was performed using two-way analysis of variance (ANOVA) with repeated measures followed by the Student–Newman–Keuls post hoc test to identify significant differences
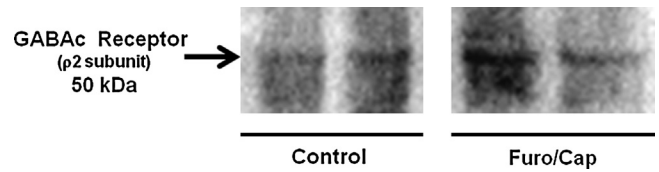
Fig. 1. Representative immunoblots showing the bands corresponding to the GABA_C receptor (ρ2) subunit (∼50 kDa) of non-depleted (control) and Furo/Cap-treated rats.

between groups or the Mann–Whitney test, as appropriated. Differences were considered significant at $P < 0.05$. The software used to analyze the data was SigmaStat for Windows, version 2.03.

3. Results

3.1. Histological analysis

The LPBN injection sites were centered in the central lateral and dorsal lateral portions of the LPBN as defined by Fulwiler and Saper (1984). A representative photomicrograph of the LPBN is shown in the Supplemental data (Supplemental Fig. 1). Injections reaching the ventral lateral and external lateral portions, as well as the Kölliker-Fuse nucleus, were observed in some rats, and the results from these rats were also included in the analysis.

3.1.1. Western blotting
The expression of the GABA_C receptor in the LPBN was confirmed by the presence of a 50 kDa band. A representative immunoblot is shown in Fig. 1.

3.1.2. c-Fos immunoreactivity
Increased expression of the protein c-Fos was observed in the LPBN of the rats with acute fluid depletion (Furo/Cap-treated rats) compared with the non-depleted animals (Fig. 2C, $P < 0.05$). Fig. 2 contains a representative microphotograph showing the c-Fos expression in the LPBN in non-depleted (Fig. 2A) and Furo/Cap-treated rats (Fig. 2B).
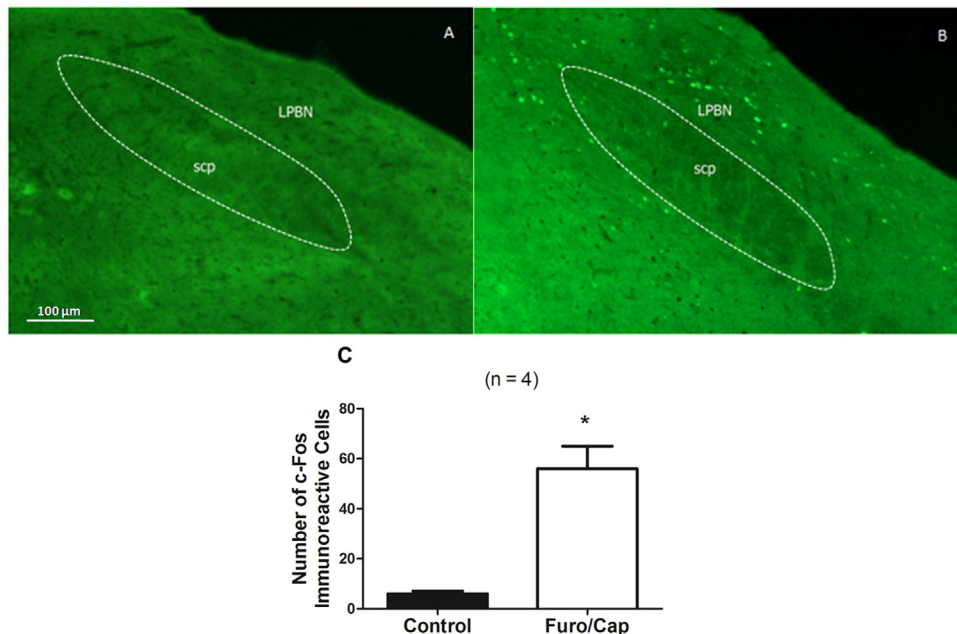
Fig. 2. c-Fos expression in the LPBN. Representative photomicrographs of coronal sections of the LPBN (original magnification 20×). Number of c-Fos-immunoreactive cells in the LPBN of (A) non-depleted (control) or (B) Furo/Cap-treated rats. (C) Quantification of the number of c-Fos-immunoreactive cells. Lateral parabrachial nucleus (LPBN); SCP = superior cerebellar peduncle (brachium conjunctivum). *$P < 0.05$ vs. Control. n = number of rats.

3.1.3. Effects of TACA injections in the LPBN

Experiments were performed to examine whether the effects on sodium and water intake involve GABA$_C$ receptor-mediated mechanisms. Our results showed that injections of different doses of TACA (0.5, 2.0 and 160 nmol) in the LPBN did not change the intake of 0.3 M NaCl intake ($P > 0.05$, Fig. 3A) or water ($P > 0.05$, Fig. 3B) in the Furo/Cap-treated rats ($P > 0.05$, for both comparisons).

3.1.4. Effects of TACA injections in the LPBN on arterial pressure and heart rate

Furo/Cap-treated rats showed no significant difference in MAP (101 ± 4 mmHg) and HR (423 ± 23 bpm) compared with the corresponding values in non-depleted rats (105 ± 6 mmHg and 414 ± 27 bpm, $P < 0.05$, for both comparisons).

Bilateral injections of the lower dose of TACA (2 nmol) in the LPBN of Furo/Cap-treated rats did not affect the MAP ($-0.2 ± 7$ vs. 9.8 ± 6 mmHg (vehicle), $n = 6$, $P > 0.05$) or the HR ($-10.5 ± 16$ vs. $-13.7 ± 23$ bpm (vehicle), $n = 6$, $P > 0.05$, for both comparisons). Injections of the higher dose of TACA (80 nmol) in the LPBN of Furo/Cap-treated rats did not affect the MAP ($-8.0 ± 5.0$ vs. 3.3 ± 6.0 mmHg (vehicle), $n = 6$, $P > 0.05$) or the HR (14.0 ± 29.8 vs. $-6.8 ± 11.2$ bpm (vehicle), $n = 6$, $P > 0.05$). The data represents the mean value at 90 min in record period. Since no effects were observed by TACA treatment on MAP and HR in the previously experiment, we found unlikely ZAPA has any effect on theses parameters. Therefore, ZAPA treatment was not to perform for AP and HR recordings.

### 3.1.5. Effects of TACA and ZAPA injections in the LPBN

To further confirm that GABA$_C$ receptor activation by TACA had no effect on sodium or water intake, the effects of the GABA$_C$ receptor antagonist, ZAPA, were studied. ZAPA (10 nmol) did not change the sodium or water intake compared with the values for the vehicle (saline) injection in the LPBN of Furo/Cap-treated rats (Fig. 4A and B, respectively, $P > 0.05$). Moreover, compared with vehicle (saline), the simultaneous treatment with both ZAPA (10 nmol) and TACA (160 nmol) had no significant effect on the water intake ($P > 0.05$).
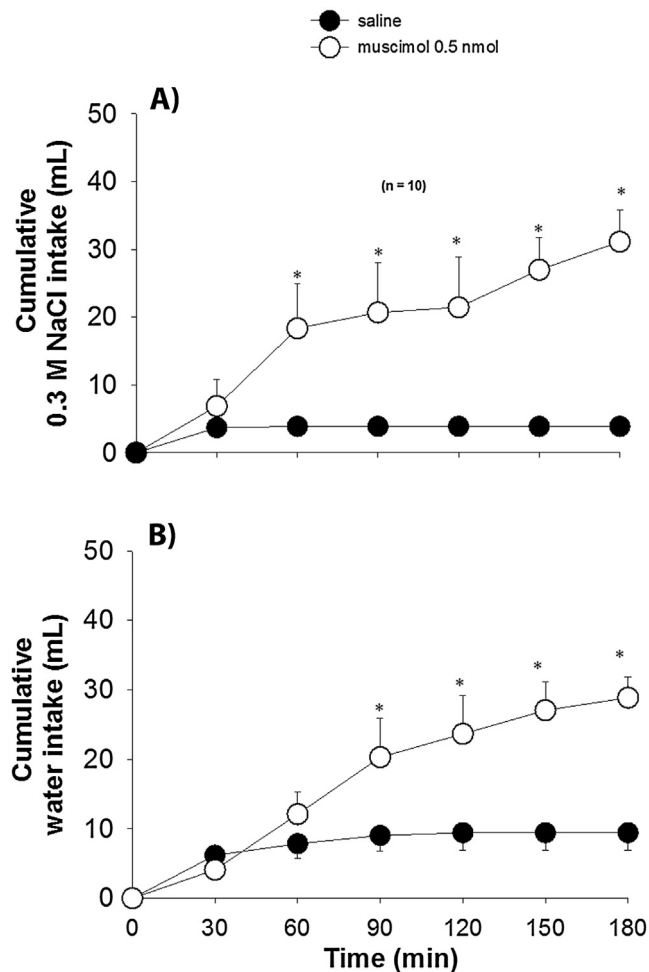
3.1.6. Effects of muscimol injection in the LPBN

The sodium and water intakes were higher in the Furo/Cap-treated rats that received bilateral injections of the GABA$_A$ receptor agonist muscimol (Fig. 5A) than in the rats that received vehicle (saline, Fig. 5B) 60 min after the injections ($P < 0.05$, for all comparison).
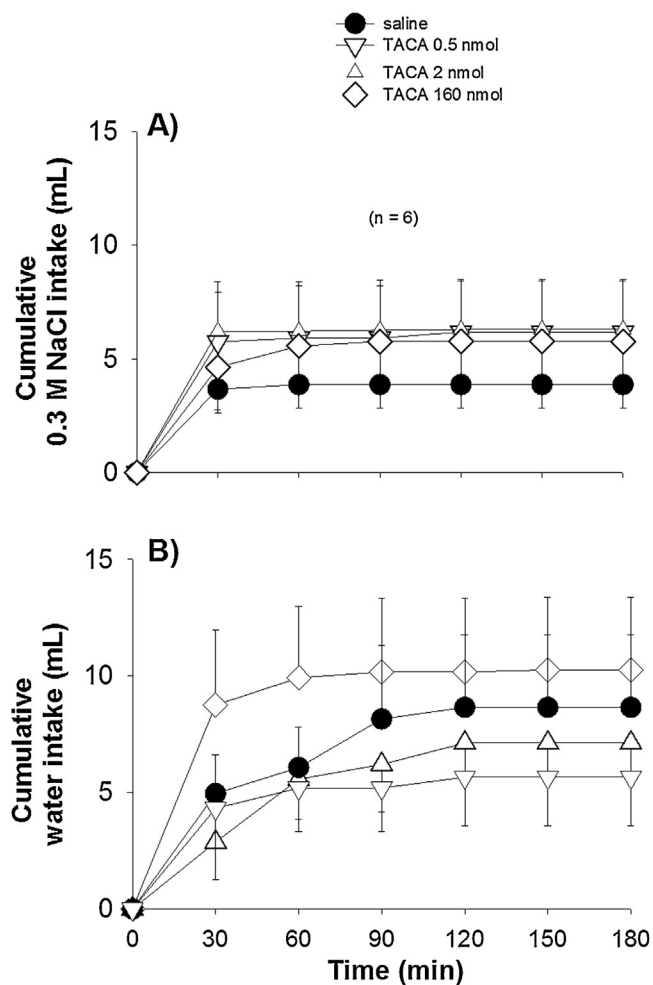
Fig. 3. Effects of TACA on sodium and water intakes. (A) Cumulative 0.3 M NaCl intake; (B) cumulative water intake of Furo/Cap-treated rats that received bilateral injections of TACA (0.5, 2.0 and 160 nmol/0.2 μL) or saline (vehicle) in the LPBN. The results are expressed as the mean ± S.E.M. n = number of rats.
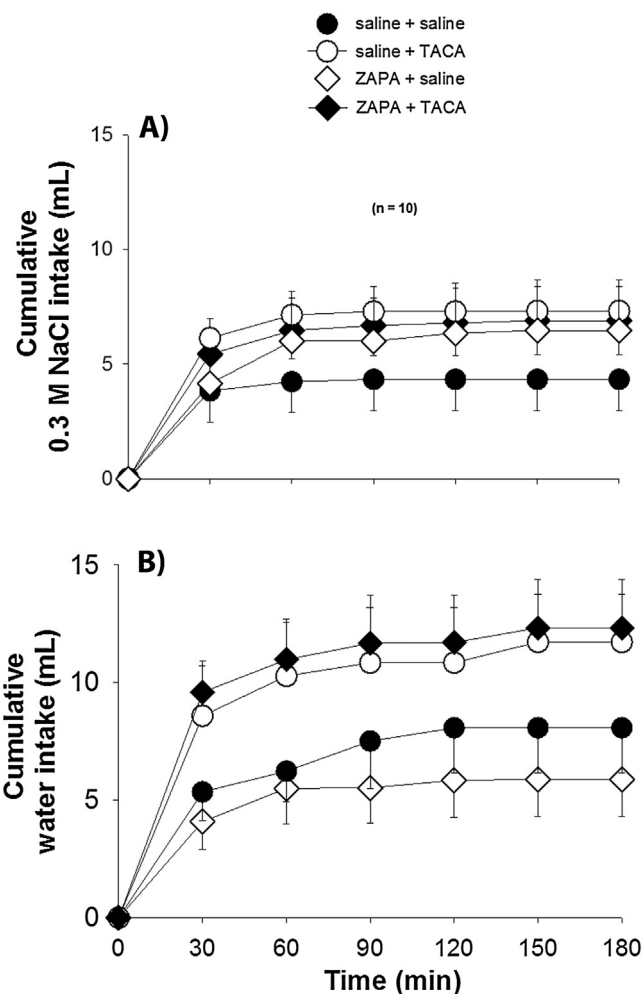
Fig. 4. Effects of ZAPA on sodium and water intakes. (A) Cumulative 0.3 M NaCl intake; (B) cumulative water intake of Furo/Cap-treated rats that received bilateral injections of ZAPA sulfate (10 nmol/0.2 μL) or saline (vehicle) combined with TACA (160 nmol/0.2 μL) or saline (vehicle) in the LPBN. The results are expressed as the mean ± S.E.M. n = number of rats.

Fig. 5. Effects of muscimol on sodium and water intakes. (A) Cumulative 0.3 M NaCl intake; (B) cumulative water intake of Furo/Cap-treated rats that received bilateral injections of muscimol (0.5 nmol/0.2 μL) or saline (vehicle) in the LPBN. The results are expressed as the mean ± S.E.M. n = number of rats. * $P < 0.05$ vs. saline.

4. Discussion

Our results are novel because previous studies did not perform bilateral injections of various doses of selective agonist of GABA$_C$ receptors (Bormann, 2000; Woodward et al., 1992, 1993) in water or sodium intake and hemodynamic parameters in the LPBN. In addition, bilateral injections of TACA in the LPBN produced no change in arterial pressure and heart rate; in contrast, muscimol injections in a previous study increased arterial pressure and the effect persisted for at least 120 min (Callera et al., 2005). The present results also showed that pretreatment of the LPBN with bilateral injections of the GABA$_C$ receptor antagonist ZAPA alone or in combination with TACA at the same site did not affect the water or sodium intake induced by acute fluid depletion.

Our results clearly show increased expression of c-Fos in the LPBN, suggesting neuronal activation in the LPBN after acute sodium depletion, as reported in several studies using Furo/Cap treatments (De Gobbi et al., 2008; Thunhorst et al., 1998). Previous reports suggested that the enhanced c-Fos expression and sodium and water intake observed after Furo/Cap treatment are caused by increases in the renin-angiotensin system activity in circumventricular organs (De Gobbi et al., 2008; Thunhorst et al., 1994, 1998).

GABA receptors are important components of inhibitory circuits in the central nervous system. Usually, they are expressed at different synapses, although in some parts of the central nervous system, such as the spinal cord, colocalizations of different receptors at the same postsynaptic sites have been described (Boue-Grabot et al., 1998; Enz et al., 1995; Park et al., 1999; Rozzo et al., 2002; Zheng et al., 2003). The colocalizations of subunits of GABAA and GABAC receptors has been demonstrated in brain-stem neurons, and the possibility that heteromeric complexes of GABA$_A$ and GABA$_C$ receptor subunits are formed has been suggested (Milligan et al., 2004). In heterologous expression systems, the coassembly of GABA$_C$ and GABA$_A$ receptor subunits has indeed been observed (Ekema et al., 2002; Pan et al., 1989, 2000). GABA receptor transcripts are present in neurons, glia (Lee et al., 2011; Vélez-Fort et al., 2012), and immune cells (Bhat et al., 2010).

A dense plexus of GABA-immunoreactive varicosities have been shown throughout the Parabrachial nucleus (PBN) and Kolliker Fuse. It was also already shown the presence of GABA$_A$ and GABA$_B$ receptors in the LPBN (Callera et al., 2005; De Oliveira et al., 2011). However, have no previous studies showing co-localization of the GABA$_A$, GABA$_B$, or GABA$_C$ receptor in the LBPN.

The LPBN is a pontine area strongly involved with inhibitory mechanisms that control water and NaCl intake. The LPBN is reciprocally connected to forebrain areas implicated in the maintenance of blood pressure and body fluid homeostasis, such as the PBN of the hypothalamus, the central nucleus of the amygdala and the median preoptic nucleus. The LPBN is also richly interconnected with medullary regions, which includes the area postrema (AP) and the medial portion of the nucleus of the solitary tract (mNTS) (Ciriello et al., 1984; Fulwiler and Saper, 1984; Norgren R et al., 1981). Therefore, the LPBN receives taste and visceral signals that ascend from AP/mNTS en route to forebrain areas involved in the control of fluid and electrolyte balance (Menani and Johnson, 1998). GABA$_A$ and GABA$_B$ receptors activation in LPBN induces water and NaCl intake in satiated and depleted rats, and it is amply demonstrated in the literature (Callera et al., 2005; De Oliveira et al., 2011; Menani and Johnson, 1998).

GABA$_C$ receptors are homo-oligomers composed of ρ subunits (Bormann, 2000). The expression of GABA$_C$ receptors is restricted to a few structures and thus differs from the distribution of GABA$_A$ receptors (Feigenspan and Bormann, 1994a). Experiments utilizing reverse transcriptase polymerase chain reaction (PCR) and in situ hybridization showed a predominance of ρ1, ρ2 and ρ3 subunits in rat retina (Feigenspan and Bormann, 1994a). Prior to this study, no reports demonstrated the presence of GABA$_C$ receptors in the LPBN, which is a novel finding of the present study. However, ρ subunits are also expressed in central neurons, including in the amygdala (Delaney and Sah, 1999) and in rostral NTS (Grabauskas and Bradley, 2001; McCall et al., 2002).

An analysis of the distribution of GABA$_A$ receptor subunits in the parabrachial nucleus showed pronounced staining for subunit α1 (Araki et al., 1992) and subunits α1, β1, γ2 and γ3 (Pirker et al., 2000). Therefore, these lines of evidence suggest that the 0.3 M NaCl and water intake in Furo/Cap-treated rats is mediated by GABA$_A$ receptors in the LPBN that contain the functional subunits α1, β1, γ2 and γ3. Electrophysiological studies suggest that different subunits or subunit combinations may mediate different physiological and pharmacological properties (Rudolph et al., 2001; Sieghart, 1995). This report is the first to show GABA$_C$ receptors in the LPBN of rats. Additionally, studies have reported data on the expression of GABA$_C$ receptor subunits in other areas associated with sodium appetite in mammals (Delaney and Sah, 1999).

Studies using PCR and in situ hybridization showed that the ρ1 subunit is highly expressed in neurons of the brainstem but not in the LPBN (Milligan et al., 2004). The present study qualitatively shows the GABA$_C$ receptor expression in the non-depleted and

fluid-depleted LPBN of Furo/Cap-treated rats. Our data suggest that the ρ2 subunit of GABA$_C$ receptors may not have an important role in the ingestion of water or sodium or in the cardiovascular parameters in the Furo/Cap acute depletion model.

TACA is a non-full selective GABA$_C$ receptor agonist, it is also a partial GABA$_A$ receptor agonist. But even in highest dose, TACA did not increase the sodium and water intake in the animals that were depleted by a Furo/Cap treatment, suggesting that GABA$_A$ receptors were not activated by this drug. To confirm this hypothesis, we used a pretreatment with ZAPA, and found that the GABA$_C$ receptor antagonist did not affect the water and sodium intake; thus, the treatment with TACA has no effect on GABA$_A$ receptor activation, even when GABA$_C$ receptors are blocked. Together, these findings indicate that GABA$_C$ receptor activation has no effect on sodium and water intake in rats subjected to acute fluid depletion.

We performed experiments with a GABA$_A$ agonist to compare its effects with those of TACA. In accordance with previous studies (Callera et al., 2005), bilateral injections of muscimol in the LPBN strongly increased the Furo/Cap-induced sodium and water intake (between 60 and 180 min). These results strongly suggest that the injection sites were located in the LPBN, thus these results match to the histological analysis. The involvement of GABA$_A$ receptors of the LPBN in the control of water and sodium intake is supported by previous studies showing that the injections of the GABA$_A$ receptor antagonist bicuculline in the LPBN reduced the water and sodium intake induced by muscimol (Callera et al., 2005; de Oliveira et al., 2007).

5. Conclusions

In conclusion, this study shows the expression of GABA$_C$ receptors in the LPBN of rats and the activation of these receptors may not influence the water and sodium intake or the cardiovascular parameters in the acute depletion Furo/Cap model. Understanding the GABAergic mechanisms involved in the regulation of fluid and electrolyte balance is important, and our study is novel; however, more studies are needed to further elucidate the role of these receptors in the LPBN and other regions of the brainstem associated with water and sodium intake.

Conflict of interest

The authors declare that they have no conflict of interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchemneu.2016.03.001.

References

Andrade, C.A., Barbosa, S.P., De Luca Jr., L.A., Menani, J.V., 2004. Activation of alpha2-adrenergic receptors into the lateral parabrachial nucleus enhances NaCl intake in rats. Neuroscience 129, 25–34.

Araki, T., Kiyama, H., Tohyama, M., 1992. The GABAA receptor gamma 1 subunit is expressed by distinct neuronal populations. Brain Res. Mol. Brain Res. 15, 121–132.

Bhat, R., Axtell, R., et al., 2010. Inhibitory role for GABA in autoimmune inflammation. Proc. Natl. Acad. Sci. U. S. A. 107 (6), 2580–2585.

Boller, M., Schmidt, M., 2003. GABAC receptors in the rat superior colliculus and pretectum participate in synaptic neurotransmission. J. Neurophysiol. 89, 2035–2045.

Bormann, J., 2000. The 'ABC' of GABA receptors. Trends Pharmacol. Sci. 21, 16–19.

Boue-Grabot, E., Roudbaraki, M., Bascles, L., Tramu, G., Bloch, B., Garret, M., 1998. Expression of GABA receptor rho subunits in rat brain. J Neurochem. 70 (3), 899–907.

Bowery, N.G., Hudson, A.L., Price, G.W., 1987. GABAA and GABAB receptor site distribution in the rat central nervous system. Neuroscience 20, 365–383.

Bowery, N., 1989. GABAB receptors and their significance in mammalian pharmacology. Trends Pharmacol. Sci. 10, 401–407.

Callera, J.C., Oliveira, L.B., Barbosa, S.P., Colombari, D.S., De Luca Jr., L.A., Menani, J.V., 2005. GABA(A) receptor activation in the lateral parabrachial nucleus induces water and hypertonic NaCl intake. Neuroscience 134, 725–735.

Ciriello, J., Lawrence, D., Pittman, Q.J., 1984. Electrophysiological identification of neurons in the parabrachial nucleus projecting directly to the hypothalamus in the rat. Brain Res. 322, 388–392.

de Oliveira, L.B., Callera, J.C., De Luca Jr., L.A., Colombari, D.S., Menani, J.V., 2007. GABAergic mechanisms of the lateral parabrachial nucleus on sodium appetite. Brain Res. Bull. 73, 238–247.

De Gobbi, J.I., Menani, J.V., Beltz, T.G., Johnson, R.F., Thunhorst, R.L., Johnson, A.K., 2008. Right atrial stretch alters fore- and hind-brain expression of c-fos and inhibits the rapid onset of salt appetite. J. Physiol. 586, 3719–3729.

De Oliveira, L.B., Kimura, E.H., Callera, J.C., De Luca Jr., L.A., Colombari, D.S., Menani, J.V., 2011. Baclofen into the lateral parabrachial nucleus induces hypertonic sodium chloride and sucrose intake in rats. Neuroscience 183, 160–170.

Delaney, A.J., Sah, P., 1999. GABA receptors inhibited by benzodiazepines mediate fast inhibitory transmission in the central amygdala. J. Neurosci. 19, 9698–9704.

Ekema, G.M., Zheng, W., Lu, L., 2002. Interaction of GABA receptor/channel rho(1) and gamma(2) subunit. Invest. Ophthalmol. Vis. Sci. 43 (7), 2326–2333.

Enz, R., Brandstätter, J.H., Hartveit, E., Wässle, H., Bormann, J., 1995. Expression of GABA receptor rho 1 and rho 2 subunits in the retina and brain of the rat. Eur. J. Neurosci. 7 (7), 1495–1501.

Feigenspan, A., Bormann, J., 1994a. Differential pharmacology of GABAA and GABAC receptors on rat retinal bipolar cells. Eur. J. Pharmacol. 288, 97–104.

Feigenspan, A., Bormann, J., 1994b. Facilitation of GABAergic signaling in the retina by receptors stimulating adenylate cyclase. Proc. Natl. Acad. Sci. U. S. A. 91, 10893–10897.

Fulwiler, C.E., Saper, C.B., 1984. Subnuclear organization of the efferent connections of the parabrachial nucleus in the rat. Brain Res. 319, 229–259.

Grabauskas, G., Bradley, R.M., 2001. Postnatal development of inhibitory synaptic transmission in the rostral nucleus of the solitary tract. J. Neurophysiol. 85, 2203–2212.

Herbert, H., Moga, M.M., Saper, C.B., 1990. Connections of the parabrachial nucleus with the nucleus of the solitary tract and the medullary reticular formation in the rat. J. Comp. Neurol. 293, 540–580.

Jhamandas, J.H., Petrov, T., Harris, K.H., Vu, T., Krukoff, T.L., 1996. Parabrachial nucleus projection to the amygdala in the rat: electrophysiological and anatomical observations. Brain Res. Bull. 39, 115–126.

Kobashi, M., Bradley, R.M., 1998. Effects of GABA on neurons of the gustatory and visceral zones of the parabrachial nucleus in rats. Brain Res. 799, 323–328.

Lee, I.O., Son, J.K., Lim, E.S., Kim, Y.S., 2011. Pharmacology of intracisternal or intrathecal glycine, muscimol, and baclofen in strychnine-induced thermal hyperalgesia of mice. J. Korean Med. Sci. 26 (10), 1371–1377.

Margatho, L.O., Porcari, C.Y., Macchione, A.F., da Silva Souza, G.D., Caeiro, X.E., Antunes-Rodrigues, J., Vivas, L., Godino, A., 2015. Temporal dissociation between sodium depletion and sodium appetite appearance: involvement of inhibitory and stimulatory signals. Neuroscience 297, 78–88.

McCall, M.A., Lukasiewicz, P.D., Gregg, R.G., Peachey, N.S., 2002. Elimination of the rho1 subunit abolishes GABA(C) receptor expression and alters visual processing in the mouse retina. J. Neurosci. 22, 4163–4174.

Menani, J.V., Thunhorst, R.L., Johnson, A.K., 1996. Lateral parabrachial nucleus and serotonergic mechanisms in the control of salt appetite in rats. Am. J. Physiol. 270, R162–168.

Milligan, C.J., Buckley, N.J., Garret, M., Deuchars, J., Deuchars, S.A., 2004. Evidence for inhibition mediated by coassembly of GABAA and GABAC receptor subunits in native central neurons. J. Neurosci. 24, 7241–7250.

Palkovits, M., Brownstein, M.J., 1988. Maps and Guide to Microdissection of the Rat Brain. Elsevier, New York, London.

Pan, Z.Z., Colmers, W.F., Williams, J.T., 1989. 5-HT-mediated synaptic potentials in the dorsal raphe nucleus: interactions with excitatory amino acid and GABA neurotransmission. J. Neurophysiol. 62 (2), 481–486.

Pan, Z.H., Zhang, D., Zhang, X., Lipton, S.A., 2000. Evidence for coassembly of mutant GABAC rho1 with GABAA gamma2S, glycine alpha1 and glycine alpha2 receptor subunits in vitro. Eur. J. Neurosci. 12 (9), 3137–3145.

Park, J.S., Higashi, H., Nagata, K., Yoshimura, M., 1999. Bicuculline-resistant, Cl-dependent GABA response in the rat spinal dorsal horn. Neurosci. Res. 33 (4), 261–268.

Pereira, D.T., Menani, J.V., De Luca Jr., L.A., 2010. FURO/CAP: a protocol for sodium intake sensitization. Physiol. Behav. 99, 472–481.

Pirker, S., Schwarzer, C., Wieselthaler, A., Sieghart, W., Sperk, G., 2000. GABA(A) receptors: immunocytochemical distribution of 13 subunits in the adult rat brain. Neuroscience 101, 815–850.

Rozzo, A., Armellin, M., Franzot, J., Chiaruttini, C., Nistri, A., Tongiorgi, E., 2002. Expression and dendritic mRNA localization of GABAC receptor rho1 and rho2 subunits in developing rat brain and spinal cord. Eur. J. Neurosci. 15 (11), 1747–1758.

Rudolph, U., Crestani, F., Mohler, H., 2001. GABA(A) receptor subtypes: dissecting their pharmacological functions. Trends Pharmacol. Sci. 22, 188–194.

Sieghart, W., 1995. Structure and pharmacology of gamma-aminobutyric acidA receptor subtypes. Pharmacol. Rev. 47, 181–234.

Swanson, L.W., 2003. Brain Architecture: Understanding the Basic Plan. Oxford University Press, Oxford.

Thunhorst, R.L., Johnson, A.K., 1994. Renin-angiotensin, arterial blood pressure, and salt appetite in rats. Am. J. Physiol. 266, R458–465.

Thunhorst, R.L., Morris, M., Johnson, A.K., 1994. Endocrine changes associated with a rapidly developing sodium appetite in rats. Am. J. Physiol. 267, R1168–1173.

Thunhorst, R.L., Xu, Z., Cicha, M.Z., Zardetto-Smith, A.M., Johnson, A.K., 1998. Fos expression in rat brain during depletion-induced thirst and salt appetite. Am. J. Physiol. 274, R1807–1814.

Vélez-Fort, M., Audinat, E., Angulo, M.C., 2012. Central role of GABA in neuron-glia interactions. Neuroscientist 18 (3), 237–250.

Woodward, R.M., Polenzani, L., Miledi, R., 1992. Characterization of bicuculline/baclofen-insensitive gamma-aminobutyric acid receptors expressed in *Xenopus oocytes*. I. Effects of Cl- channel inhibitors. Mol. Pharmacol. 42, 165–173.

Woodward, R.M., Polenzani, L., Miledi, R., 1993. Characterization of bicuculline/baclofen-insensitive (rho-like) gamma-aminobutyric acid receptors expressed in Xenopus oocytes. II. Pharmacology of gamma-aminobutyric acidA and gamma-aminobutyric acidB receptor agonists and antagonists. Mol. Pharmacol. 43, 609–625.

Zhang, D., Pan, Z.H., Awobuluyi, M., Lipton, S.A., 2001. Structure and function of GABA(C) receptors: a comparison of native versus recombinant receptors. Trends Pharmacol. Sci. 22, 121–132.

Zheng, W., Xie, W., Zhang, J., Strong, J.A., Wang, L., Yu, L., Xu, M., Lu, L., 2003. Function of gamma-aminobutyric acid receptor/channel rho 1 subunits in spinal cord. J. Biol. Chem. 278 (48), 48321–48329.