Neuroscience 298 (2015) 120–136

EARLY FREE ACCESS TO HYPERTONIC NACL SOLUTION INDUCES A LONG-TERM EFFECT ON DRINKING, BRAIN CELL ACTIVITY AND GENE EXPRESSION OF ADULT RAT OFFSPRING

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Abstract—Exposure to an altered osmotic environment during a pre/postnatal period can differentially program the fluid intake and excretion pattern profile in a way that persists until adulthood. However, knowledge about the programing effects on the underlying brain neurochemical circuits of thirst and hydroelectrolyte balance, and its relation with behavioral outputs, is limited. We evaluated whether early voluntary intake of hypertonic NaCl solution may program adult offspring fluid balance, plasma vasopressin, neural activity, and brain vasopressin and angiotensinergic receptor type 1a (AT1a)-receptor gene expression. The manipulation (M) period covered dams from 1 week before conception until offspring turned 1-month-old. The experimental groups were (i) Free access to hypertonic NaCl solution (0.45 M NaCl), food (0.18% NaCl) and water [M-Na]; and (ii) Free access to food and water only [M-Ctrol]. Male offspring (2-month-old) were subjected to iv infusion (0.15 ml/min) of hypertonic (1.5 M NaCl), isotonic (0.15 M NaCl) or sham infusion during 20 min. Cumulative water intake (140 min) and drinking latency to the first lick were recorded from the start of the infusion. Our results indicate that, after systemic sodium overload, the M-Na group had increased water intake, and diminished neuronal activity (Fos-immunoreactivity) in the subfornical organ (SFO) and nucleus of the solitary tract. They also showed reduced relative vasopressin (AVP)-mRNA and AT1a-mRNA expression at the supraoptic nucleus and SFO, respectively. The data indicate that the availability of a rich source of sodium during the pre/postnatal period induces a long-term effect on drinking, neural activity, and brain gene expression implicated in the control of hydroelectrolyte balance.

Key words: pre/postnatal programing, AVP and AT1a mRNA relative gene expression, *c-fos*, subfornical organ, nucleus of the solitary tract, vasopressin.

INTRODUCTION

Development in an altered osmotic environment during a sensitive prenatal and postnatal period can differentially program fluid intake patterns in animals and humans that persist even until adulthood (Mouw et al., 1978; Contreras and Kosten, 1983; Contreras and Ryan, 1990; Nicolaidis et al., 1990; Crystal and Bernstein, 1995; Galaverna et al., 1995; Stein et al., 1996; Leshem, 1998, 2009; Argüelles et al., 2000; Katovich et al., 2001; Wang et al., 2003; Curtis et al., 2004; Perillan et al., 2004; Shirazki et al., 2007; Mecawi et al., 2010; Wu et al., 2011; Zhang et al., 2011). However, knowledge is limited about the effects of prenatal and postnatal programing on underlying brain circuit activity or gene expression mediating the neuroendocrine and behavioral osmoregulatory responses (Roitman et al., 2002; Clark and Bernstein, 2006; McBride et al., 2006; Na et al., 2007).

Our recent results indicate that maternal voluntary ingestion of hypertonic NaCl solution during pregnancy and lactation until one week post-weaning alters the offspring's central osmoregulatory mechanisms in adulthood, modulating water and sodium intake after Furosemide-sodium depletion and also the cell activity of brain nuclei involved in the control of hydroelectrolyte

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Abbreviations: 5-HT, serotonin; ab, antibody; ABP, arterial blood pressure; ANOVA, analyses of variance; AP, area postrema; AT1a, angiotensinergic receptor type 1a; AVP, vasopressin; BNST, bed nucleus of the *stria terminalis*; BW, body weight; CeA, central lateral amygdaloid nucleus; CVOs, circumventricular organs; DAB, diaminobenzidine hydrochloride; DRN, dorsal raphe nucleus; HP, hypertonic sodium solution; ISO, isotonic sodium solution; LPBN, lateral parabrachial nucleus; LT, lamina terminalis; M, manipulation; MnPO, median preoptic nucleus; MnPOd, dorsal subdivisions of MnPO; MnPOv, ventral subdivisions of MnPO; NHS, normal horse serum; NTS, nucleus of the solitary tract; OT, oxytocin; OVLT, organum vasculosum of the lamina terminalis; PaLM, lateral magnocellular subdivision of paraventricular nucleus; PaV, parvocellular ventral subdivision of paraventricular nucleus; PB, phosphate buffer; PD, postnatal day; PVN, paraventricular nucleus; RT, retrotranscription; SFO, subfornical organ; SON, supraoptic nucleus.

http://dx.doi.org/10.1016/j.neuroscience.2015.04.004
0306-4522/

balance (Macchione et al., 2012). As the main effect of this imprinting model was on offspring water balance, altering water intake and vasopressinergic system activity, we now tested the hypothesis that this pre/postnatal availability of a rich source of sodium may program osmoregulatory mechanisms in response to sodium overload.

Many studies have demonstrated developmental plasticity changes in the osmosensitive mechanisms that alter, for example, the osmotic threshold for vasopressin (AVP) release or water drinking in offspring exposed to an increase in maternal hypernatremia (Ramirez et al., 2002; Desai et al., 2003; Ross et al., 2005) or maternal high sodium diet (Contreras and Kosten, 1983; Curtis et al., 2004). However, it is important to note that, in these studies, the animals were subjected to obligatory high sodium intake, while the present report investigates the effect of early hypertonic NaCl consumption as free choice, eliminating the stress provoked by dehydration-induced anorexia and possible chronic renal alterations (Balbi et al., 2008; Machado et al., 2008; Sánchez et al., 2008; Mecawi et al., 2010).

The aim of the present study was to evaluate whether early access to hypertonic NaCl intake may program adult offspring fluid balance, neural activity, and brain AVP and angiotensinergic receptor type 1a (AT1a)-receptor gene expression. Hence, the behavioral profile of water intake, the brain activity of specific areas implicated in the control of salt and water homeostasis, as well as AVP-mRNA and AT1a-mRNA relative gene expression in the supraoptic nucleus (SON) and subfornical organ (SFO), respectively, were analyzed after hypertonic NaCl infusion in adult offspring. Urinary excretion parameters and plasma AVP concentration were also analyzed.

EXPERIMENTAL PROCEDURES

Animals

Thirty Wistar-derived female rats, born and reared in the vivarium of the Instituto Ferreyra (INIMEC-CONICET-UNC, Córdoba, Argentina), weighing 220–250 g, 70–75 days old and non-littermates, were individually housed in standard holding chambers (40 × 40 × 70 cm). Room lights were on for 12 h/day, beginning at 08:00 am, and temperature was controlled at 23 °C ± 1. Animal handling and experimental procedures were approved by the Animal Care and Use Committee of our institute, and the National Institutes of Health (NIH) Guidelines were followed.

The protocol was executed according to Macchione et al. (2012), briefly summarized as follows: 7 days before mating, female rats were randomly divided in two groups to receive the appropriate manipulation (M): one group without manipulation [M-Ctrol group] had free access to tap water and standard commercial diet (Cargill Inc. Argentina, containing approx. 0.18% NaCl) and the other group, in addition to tap water and commercial diet, had voluntary access to a hypertonic NaCl solution (0.45 M NaCl) [M-Na group]. After a week of adaptation, one couple per cage was placed for mating in the same standard holding chamber until found sperm-positive, maintaining the hypertonic NaCl solution access in the M-Na group. When pregnancy was confirmed (1–5 days), males were removed. Pregnant rats were maintained in the same holding chamber.

Within 24 h after birth, litters were culled to nine pups, retaining both males and females in each litter. Litters with fewer than six pups were not included. Dams continued to receive their respective conditions of manipulation until pups were weaned at postnatal days 21–22 (PD21–22). After weaning, only male pups continued to the experiments, and these received the same conditions as their dam until reaching 1 month old (PD28). As the pups probably started drinking at about PD18, they therefore had approximately 2 weeks access to high salt solution. From then on, males of both experimental conditions were kept in standard conditions of water and food until 2 months of age (PD60). To avoid litter-specific effects, no more than three males per litter were used for the same condition. As in our previous study (Macchione et al., 2012), we decided to analyze only the males' intake in the present study since we were aware of the sexual dimorphism of thirst and sodium appetite (Stricker et al., 1991; Chow et al., 1992; Dadam et al., 2014), and also how estrogen level changes influence female fluid intake (Dalmasso et al., 2011).

Experiment 1.1: Water intake induced by iv infusion of hypertonic NaCl solution in adult offspring. Adult males (⩾PD60, 11–15 animals/experimental group) from both groups were anaesthetised and were implanted with intravenous (iv) cannulas via the femoral vein, as detailed in Section 'Femoral vein cannulation'. Once the animals recovered from anesthesia, they were placed in individual metabolic cages with *ad libitum* access to commercial food and distilled water. Body weight at surgery (BW-1), percentage of weight lost as a result of surgery (% BW lost) as well as overnight water intake and latency at test were recorded. Furthermore, the state of health and hydration were determined and the opening of the catheter was checked with a small infusion (∼0.1 ml) of isotonic saline. Subsequently, the animals were connected to an infusion pump (SyringePump, NE1800) in their individual cages and the iv infusion was started 5 min later at a rate of 0.15 ml/min. Animals were infused with a hypertonic (1.5 M NaCl) or isotonic (0.15 M NaCl) NaCl solution for 20 min (Ho et al., 2007). Latency to drink and water intake were evaluated during the 20-min infusion and 120 min thereafter (140 min). Another infusion control group was included that received the same protocol (surgery, fluid access and handling) but with sham infusions (Sham group).

Experiment 1.2: Urinary excretion in response to iv infusion of hypertonic NaCl solution in adult offspring. In the same animals used in Experiment 1.1, the urine excreted was collected during the infusion period and also during the water intake test (140 total minutes). Samples were immediately centrifuged (4 °C, 3000 rpm, 20 min) and 1 ml of the supernatant was collected and

kept at −20 °C until use. Urinary osmolality was analyzed by vapor pressure osmometry (VAPRO 5520), and electrolyte concentration ($[Na^+]$, $[K^+]$, $[Cl^-]$) was determined using an Ion Selective Electrode (Hitachi Modular P + ISE. Roche Diagnostic, Mannheim, Germany). The same parameters were analyzed in the simulated infusion animals (Sham group).

Experiment 2: Neuronal activity in response to iv infusion of hypertonic NaCl solution in adult offspring

Twenty-four adult males from the M-Ctrol and M-Na groups (3–5 animals/experimental group) were anaesthetised and underwent iv cannulation surgery via the femoral vein (see section 'Femoral vein cannulation'). They were infused with hypertonic (1.5 M NaCl) or isotonic (0.15 M NaCl) NaCl solution for 20 min (0.15 ml/min), as described in Experiment 1.1 (see section 'Experiment 1.1'). It should be clarified that animals in this experiment did not have access to the water intake test. Sixty minutes after the infusion was stopped, animals were anaesthetised and brains were collected for the immunohistochemical detection of Fos in combination with AVP (Fos/AVP-ir), OT (Fos/OT-ir) or serotonin (5-HT) (Fos/5HT-ir), see Section 2.6 for more details. Simulated un-infused animals (Sham group) were also analyzed.

Experiment 3: Relative mRNA expression levels of AVP in the SON and angiotensinergic receptor AT1a in the SFO in response to iv infusion of hypertonic NaCl solution in adult offspring

Adult males from both groups (M-Ctrol and M-Na, 3–5 animals/experimental group) were treated as explained in section 'Animals'. Sixty minutes after the infusion ended, animals were euthanized by decapitation. Brains were collected and frozen in dry ice in RNAse-free conditions, and immediately stored at −70 °C for AT1a and AVP mRNA determinations by qPCR, as described in section 'Microdissection, total RNA isolation and qPCR assay'.

Experiment 4: Determination of plasma AVP concentration in response to iv infusion of hypertonic NaCl solution in adult offspring

The animals used for Experiment 3 were employed to collect trunk blood in EDTA tubes (2 mg/ml), 60 min after the infusion ended. The samples were immediately centrifuged (4 °C, 3000 rpm, 20′) and plasma collected and stored at −70 °C until determination of plasma AVP concentration by RIA (see section 'Determination of plasma AVP concentration by RIA' for more details).

Femoral vein cannulation. Adult males were anaesthetised with ketamine–xylazine (1.25 ml/kg., sc) for cannulation surgery as described in Caeiro and Vivas (2008). The right leg area was shaved and a horizontal incision made. A polyethylene PE-10 catheter (0.025″ OD-0.011″ ID, Clay Adams, Parsippany, New Jersey, USA) with heparin–saline (2 IU/ml) was implanted in the left femoral vein, welded to a PE-50 (0.039″ OD-0.023″ ID, Clay Adams, Parsippany, New Jersey, USA). The catheter led subcutaneously to an exit between the shoulder blades and connected to a modified needle, in order to prevent catheter movement, and sealed. After the completion of the different experimental protocols, the correct implantation of the catheter was verified visually.

Immunohistochemistry. Animals were anaesthetised with 6% chloral hydrate (0.6 ml/100 g bw, ip) and perfused transcardially with ~100 ml of normal saline solution followed by ~400 ml of 4% paraformaldehyde (Sigma–Aldrich, USA) in 0.1 M phosphate buffer (PB, pH 7.2). The brains were removed, fixed overnight in the perfusion solution, and stored at 4 °C in PB containing 30% sucrose. Free-floating 40-μm coronal sections were cut, using a freezing microtome. Immediately before immunostaining, sections were placed in a mixture of 10% H_2O_2 and 10% methanol during 60 min. Next, they were incubated in 10% normal horse serum (NHS-Gibco, Auckland, New Zealand) in PB for 1 h to block non-specific binding sites. The free-floating sections were incubated overnight at room temperature with anti c-Fos antibody (ab) raised in rabbits against a synthetic 14-amino acid sequence corresponding to residues 4–17 of human Fos (Ab-5, Oncogene Science, Manhasset, NY, USA). The incubated solution had the anti c-Fos ab diluted 1:10,000 in a solution of PB containing 2% NHS (Gibco, Auckland, New Zealand) and 0.3% Triton X-100 (Sigma Chemical Co., St. Louis, MO, USA). After being washed in PB, Fos-ir was detected using a standard avidin–biotin peroxidase protocol. Free-floating sections were incubated in biotin-labeled anti-rabbit immunoglobulin (Jackson Immunoresearch Laboratories, West Grove, PA, USA, dilution 1:500 in 1% NHS-PB) and avidin–biotin peroxidase complex (Vector Laboratories Inc., Burlingame, CA, USA, dilution 1:200 in 1% NHS-PB) for 1 h each, at room temperature. The peroxidase label was detected using diaminobenzidine hydrochloride (DAB; Sigma Chemical Co., St. Louis, MO, USA) intensified with 1% cobalt chloride and 1% nickel ammonium sulfate. This method produces a blue–black nuclear reaction product.

The Fos-ir staining was followed by double-labeling procedures described in Franchini and Vivas (1999), Godino et al. (2005). Hypothalamic Fos-labeled sections were processed for immunocytochemical localization of AVP or oxytocin (OT), and the brainstem sections for immunocytochemical localization of 5-HT. In all cases, sections were incubated for 72 h at 4 °C in a PB solution containing 2% NHS (Gibco, Auckland, New Zealand) and 0.3% Triton X-100 (Sigma Chemical Co., St. Louis, MO, USA) with corresponding primary antibodies as follows: polyclonal rabbit anti-AVP ab (Chemicon International Inc., Temecula, California, USA) and polyclonal rabbit anti-5HT ab (ImmunoStar, Melbourne, Victoria, Australia) both diluted to 1:10,000 and polyclonal rabbit anti-OT ab (Peninsula Laboratories, San Carlos, CA,

USA) diluted to 1:25,000. After incubation, sections were rinsed and incubated in biotin-labeled anti-rabbit immunoglobulin (Jackson Immunoresearch Laboratories, West Grove, PA, USA, dilution 1:500 in 1% NHS-PB) and avidin–biotin peroxidase complex (Vector Laboratories Inc., Burlingame, CA, USA, dilution 1:200 in 1% NHS-PB). Cytoplasmic label was detected with unintensified DAB (Sigma Chemical Co., St. Louis, MO, USA) to produce a brown reaction product. Finally, the free-floating sections were mounted on gelatinised slides (with Albrecht's gelatine, 1.5% gelatine-80% alcohol), air-dried overnight, dehydrated and cleared in xylene, and placed under a coverslip with DPX mounting medium (Fluka, Buchs, Switzerland).

Cytoarchitectural and quantitative analysis. The brain nuclei evidencing Fos-ir were identified and delimited on the basis of the plates from the rat brain atlas (Paxinos and Watson, 2007). Fos-ir neurons of nuclei were counted at only one level because of the neuroanatomical and neurofunctional differentiation found within the antero-posterior axis. Distance from the bregma of the corresponding plates is as follows: lateral magnocellular (PaLM) and ventral (PaV) subdivisions of paraventricular nucleus (PVN) −1.80 mm, SON −1.3 mm, central lateral amygdaloid nucleus (CeA) −2.8 mm, bed nucleus of the *stria terminalis* (BNST) and median preoptic nucleus −ventral and dorsal subdivisions (MnPOv and MnPOd) −0.40 mm, SFO −0.92 mm, *organum vasculosum* of the *lamina terminalis* (OVLT) −0.20 mm, dorsal raphe nucleus (DRN) −8.00 mm, lateral parabrachial nucleus (LPBN) −9.3 mm and nucleus of the solitary tract adjacent to area postrema (NTS/AP) −13.68 mm.

Fos-ir nuclei were quantified using a computerized system that included a Zeiss microscope equipped with a DC 200 Leica digital video camera attached to a contrast enhancement device. Video images were digitised and analyzed using Image J PC software, 1.42q version. Fos-ir cells in each section were counted by setting a size range for cellular nuclei (in pixels) and threshold level for staining intensity. Representative sections in all the groups were acquired under exactly the same conditions, with the aid of the Adobe Photoshop Image Analysis Program CS2, version 9. When representative sections for every case were not found, it was preferred to discard the case for this nucleus. The counting was repeated at least twice on each section analyzed, to ensure that the number of profiles obtained was similar. The investigator who conducted the Fos-ir cells counting was blinded to the experimental groups. Because one section of each nucleus was quantified, no corrections were necessary to avoid double counting. The double-labeled cells (Fos/AVP-ir; Fos/OT-ir; Fos/5HT-ir) were hand counted.

Microdissection, total RNA isolation and qPCR assay. Thick coronal sections (1320 μm) from coordinates −0.80 mm to −2.12 mm relative to bregma (Paxinos and Watson, 2007) from plate 21 to plate 26 were obtained in a freezing microtome. A 2-mm and 1.5-mm diameter stainless steel punch needle was used to collect tissue samples of SON and SFO, respectively and these were immediately transferred to a microtube containing 1 ml of Trizol (Invitrogen, Buenos Aires, Argentina). Tissue samples were homogenized with the aid of a 30G-syringe and total RNA was obtained according to the manufacturer's protocol. All procedures were performed on ice.

The homogenates were incubated for 5 min at room temperature and then 0.2 ml of ice chloroform (Cicarelli) was added. The samples were vigorously shaken for 15 s and centrifuged at 12,000*g* for 15 min at 4 °C. The upper aqueous phase was transferred to another clean tube and 0.5 ml of ice isopropanol (Cicarelli) was added. Again, the samples were shaken vigorously and stored overnight at −20 °C for RNA decantation. The next day, the samples were centrifuged at 12,000*g* for 10 min at 4 °C and supernatants were discarded. Pellets were washed with 75% ethanol in RNAse-free water and centrifuged at 12,000*g* for 15 min at 4 °C. Supernatants were discarded and each pellet was suspended in 15 μl of RNAse-free water and solubilized in a hybridisation oven at 60 °C for 10 min. The isolated total RNA was treated with DNAse I (Ambion, Buenos Aires, Argentina, 1 UI) for 20 min at 37 °C according to the protocol provided by the manufacturer, followed by quantification through absorbance at 260 nm and 280 nm performed on a Perkin Elmer Lambda 25 UV/VIS. In addition, the integrity and quality of the total RNA extracted were verified in 1.2% agarose gel (Biodynamics, Buenos Aires, Argentina) with 4 μl of ethidium bromide (10 mg/ml, Promega). Samples were run on an electrophoresis cube with power supply (BioRad, Buenos Aires, Argentina) for 60 min at 90 mV at constant amperage. Bands corresponding to 18S and 28S ribosomal subunits were visualized with a high performance ultraviolet transilluminator.

For the retrotranscription (RT), 1 μg of total RNA was added to 20 μl reaction mixture containing buffer (5× M-MLV Reaction Buffer, Promega), ribonuclease inhibitor (Invitrogen, Buenos Aires, Argentina, 40 IU), dNTPs (Fermentas, Buenos Aires, Argentina, 1 mM), random hexamers (Biodynamics, Buenos Aires, Argentina, 1 μg) and M-MLV reverse transcriptase (Promega, 100 IU). The RT was carried out at 37 °C for 60 min followed by 5 min at 95 °C in a Multigene thermocycler (Labnet International, Inc.). cDNA were stored at −20 °C until quantification by qPCR. In each RNA-extraction, a reaction tube without RNA was run as a negative control.

Quantification by qPCR was performed using a QuantiTect SYBR Green Real-Time PCR 1× Kit (QIAGEN, Buenos Aires, Argentina, GmbH), oligonucleotide (Forw/Rev; 10 pmol) and cDNA (100 ng) at a final reaction volume of 20 μl, in duplicate. Real-time amplification reaction was carried out on a real-time PCR Systems Stratgene QPR Mx3005P (Agilent Technologies, Buenos Aires, Argentina). Three pairs of oligonucleotides (Invitrogen, Buenos Aires, Argentina), specific for each gene of interest, 18S, AT1a and AVP, were used. Table 1 shows the oligonucleotide sequences, the number of base pairs and product length.

Table 1. Oligonucleotide sequences used in the qPCR experiment

Target gene		Sequence	Oligonucleotide length	Product length (bp)
18S	Forward	5′-CCATCCAATCGCTAGTAG-3′	18	
	Reverse	5′-GTAACCCGTTGAACCCCA-3′	18	151
AVP	Forward	5′-CGA GTG TCG AGA GGG TTT TT-3′	20	
	Reverse	5′-AGC CAG CTG TAC CAG CCT AA-3′	20	109
AT1a	Forward	5′-AACCCTCTGTTCTACGGC-3′	18	
	Reverse	5′-ACCTGTCACTCCACCTCA-3′	18	194

Amplification conditions comprised a pre-incubation phase at 95 °C for 15 min followed by 40 cycles of cDNA amplification phases: 95 °C × 30″ to denaturation; 55 °C × 1′ to annealing and 72 °C × 30″ to elongation. The quantitative differences were obtained by calculating the relative expression between the gene of interest and the constitutive gene or housekeeping (18S) using the mathematical model described by (Pfaffl, 2001). The efficiency of each oligonucleotide was required and these were optimal and nearly 100% in all cases. As a control sample, an arbitrary M-Ctrol/Sham was chosen which referred to the rest of the samples. In parallel, a negative control was attached in each qPCR run.

Determination of plasma AVP concentration by RIA. For extracting plasma hormone, 1 ml of plasma was placed in 2 ml ice-cold acetone (Merck) followed by stirring for 30 min and centrifugation (2500 rpm, 25 min, 4 °C). The supernatant was decanted into a tube containing 2 ml of petroleum ether (Reagen) and maintained under stirring at room temperature for 2 min. The supernatant (petroleum ether) was discarded by aspiration and the remaining liquid (acetone) containing AVP was lyophilized.

At the time of assay, the lyophilised product was resuspended in 250 µl of buffer for protein quantification by the Bradford method (BioRad, Buenos Aires, Argentina) and the samples were immediately diluted 1:20,000 to carry out the RIA. All procedures were performed on ice. To determine extraction efficiency, ~25,000 cpm of peptide labeled with I^{125} was added to 1 ml of plasma and subjected to the extraction described above. The supernatants and precipitates generated in each extraction were analyzed in a gamma counter and the extraction process efficiency was calculated with the radioactivity values obtained. The extraction was considered effective when the percentage of labeled hormone recovery (AVP) was over 90% (procedure described previously by Robertson et al. (1973)). All assays were performed in duplicate. Assay sensitivity was 0.9 pg/ml and intra- and inter-assay coefficients of variation were 7.7% and 11.9%, respectively.

Statistical analysis. All data are expressed as mean ± SE. All variables were analyzed by appropriate analyses of variance (ANOVA) with repeated measures (time) when applicable. Both main effects and interactions were considered to be statistically significant at $p < 0.05$. Analyses were performed using Statistica version 8.0, (Stat-Soft Inc., Tulsa, OK, USA).

Experimental design for each ANOVA was:

- 1-way ANOVA for BWs, percentage of BW loss, overnight water intake and drinking latency [Exp. 1.1; Factor: M]. Because only the infusion of hypertonic NaCl solution induces an active drinking behavior, the drinking latency was analyzed just in the M-Ctrol/HP and M-Na/HP groups.
- 2-way ANOVA for excretion-parameters: urine volume, osmolality, electrolyte concentration and total sodium excreted [Exp. 1.2, Factors: M × Solution]. Immunohistochemical analysis (of Fos-ir, Fos/AVP-ir, Fos/OT-ir, Fos/5HT-ir [Exp. 2], relative mRNA levels [Exp. 3] and plasma AVP concentration [Exp. 4] also were conducted by a 2-way ANOVA [Factors: M × Solution].
- A 3-way ANOVA with repeated measures for induced water intake after iv infusion of hypertonic NaCl solution [Exp.1.1; Factors: M × Solution × time]. It is important to note that, although Fig. 1 was constructed with cumulative data, the 3-way ANOVA used non-cumulative data.

Post hoc comparisons were performed using Least Significant Difference (LSD) tests as follow-ups to identify significant differences between groups.

RESULTS

Experiment 1.1: Water intake induced by iv infusion of hypertonic NaCl solution in adult offspring

As shown in Fig. 1, the data revealed a significant increase in induced-water intake of the M-Na group compared to the M-Ctrol group. This increase was evident from 15 min after ending the infusion and persisted until the end of the intake test. The statistical analyses were performed with a 3-way ANOVA (factors: M × Solution × time) with repeated measures, and we indicate below all the significant differences found:

- Significant main effect of solution (HP vs. isotonic sodium solution (ISO)) [$F_{1,47} = 40.247$, $p < 0.0001$].
- Significant main effect of M [$F_{1,47} = 6.2296$, $p < 0.0161$].
- Significant interaction of M and Solution [$F_{1,47} = 7.2866$, $p < 0.0096$] and between time × Solution [$F_{8,376} = 3.8034$, $p < 0.0003$].

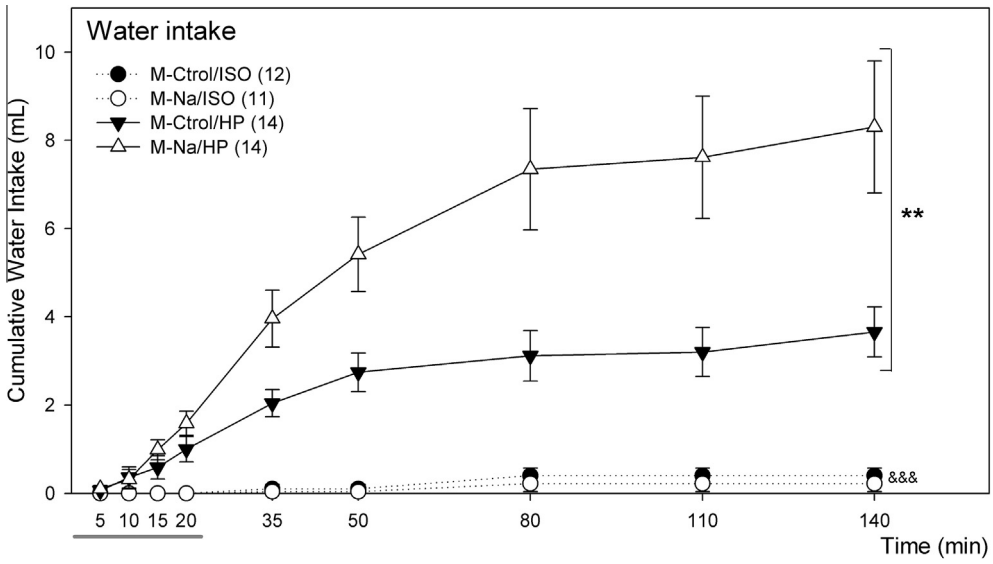

Fig. 1. Cumulative water intake during the 20-min iv infusion (0.15 ml/min) of a hypertonic (1.5 M NaCl: HP) or isotonic (0.15 M NaCl: ISO) solution and for 120 min afterward. Values expressed as Mean ± SE, (*n*). **Significant differences between M-Na vs. M-Ctrol, $p < 0.01$. &&&Significant differences between ISO and HP solutions, $p < 0.001$. Gray bar indicates infusion period (20 min).

Although in the M-Na group infused with HP solution there was a tendency for an increase in water intake during the test, this difference was not statistically significant [interaction of M × Solution × time: $F_{8,376} = 1.4577$, $p = 0.1712$].

Water intake of animals that received a simulated infusion was also evaluated and surgery per se was seen to have no effect. Water intake was nil in sham-infused animals during the 180 min period (data not shown). No significant differences were observed in the drinking latency during the intake test (M-Ctrol: 10.64 ± 1.77 min and M-Na: 9.93 ± 1.22 min), the BW at surgery (M-Ctrol: 288.13 ± 13.56 g and M-Na: 299.33 ± 11.83 g), the BW loss after surgery (M-Ctrol: 2.64 ± 0.64% and M-Na: 2.07 ± 0.54%) or overnight water drinking before NaCl infusions (M-Ctrol: 37.01 ± 1.67 ml and M-Na: 35.90 ± 1.74 ml).

Experiment 1.2: Urinary excretion pattern in response to iv infusion of hypertonic NaCl solution in adult offspring

As expected, our data showed that HP-infused animals had an increase in urine volume (ml) and total sodium excretion (meq). Furthermore, a higher concentration of sodium and chlorine (mM) in urine compared to ISO-infused animals was found (Table 2). The opposite effect was found in potassium concentration, with lower values of this electrolyte in HP-infused than in ISO-infused animals. However, there were no significant effects of M and no interactions (M × Solution).

Table 2 shows a significant main effect of the Solution factor on: urine volume, [Na^+], [K^+], [Cl^-] and total sodium excretion; [$F_{1,47} = 117.32$, $p < 0.001$, $F_{1,49} = 77.47$, $p < 0.001$; $F_{1,46} = 37.00$, $p < 0.001$; $F_{1,49} = 31.82$, $p < 0.001$, $F_{1,46} = 125.42$, $p < 0.001$, respectively]. Finally, there was no effect of the factors or interaction between them in relation to osmolality (mosm/kg H_2O, Table 2). Urine volume, osmolality and electrolytes excreted in animals with simulated infusion were not determined because urine volumes during the experiment were insignificant.

Experiment 2: Neuronal activity in response to iv infusion of hypertonic NaCl solution in adult offspring

The number of Fos immunoreactive neurons (Fos-ir) in response to the different infused solutions (HP–ISO–Sham) was measured. In all cases, data were analyzed by a 2-way ANOVA (factors: M × Solution).

Table 2. Urinary excretion parameters in adult manipulated offspring after iv infusion (0.15 ml/min) of isotonic (ISO) or hypertonic (HP) NaCl solution

Solution infused	Isotonic		Hypertonic (***)	
M group	M-Ctrol	M-Na	M-Ctrol	M-Na
Urine Volume (ml/140 min)	4.21 ± 0.69 (12)	5.00 ± 0.94 (11)	19.11 ± 1.64*** (14)	20.07 ± 1.64*** (14)
Na^+ (mM)	58.37 ± 8.13 (12)	62.27 ± 7.84 (11)	186.56 ± 14.42*** (14)	165.38 ± 15.74*** (14)
K^+ (mM)	64.52 ± 10.70 (12)	62.25 ± 10.08 (11)	25.03 ± 3.73 *** (14)	24.04 ± 4.01*** (14)
Cl^- (mM)	74.64 ± 11.68 (12)	91.47 ± 13.37 (11)	198.43 ± 20.88*** (14)	173.04 ± 20.04*** (14)
Osmolality (mosm/kg H_2O)	547.70 ± 51.39 (12)	513.00 ± 36.11 (11)	538.86 ± 15.99 (14)	506.72 ± 24.14 (14)
Total Na excreted (meq)	0.28 ± 0.08 (12)	0.36 ± 0.09 (10)	3.44 ± 0.34*** (14)	3.10 ± 0.30*** (14)

The urine was collected during the infusion period and also during the water intake test (20+ 120 total minutes). Values are expressed as mean ± SE, (*n*). *** Significant differences between solutions ISO vs. HP in urine volume, Na^+, K^+ and Cl^- concentrations; $p < 0.001$.

Lamina terminalis (LT). In the LT-nuclei, infusion of hypertonic NaCl solution increased the number of Fos-immunoreactive neurons in the OVLT and in both subdivisions (dorsal and ventral) of the MnPO [main effect Solution in OVLT, MnPO dorsal and MnPO ventral: $F_{2,16} = 102.46$, $p < 0.001$; $F_{2,17} = 39.98$, $p < 0.001$ and $F_{2,18} = 42.72$, $p < 0.001$, respectively]. *A posteriori* comparisons showed significant differences between the HP group and the ISO and Sham groups. There were no significant differences or interactions for the M groups (Fig. 2).

SFO. The analysis revealed that although there was an increase in the number of Fos-ir neurons induced by hypertonic NaCl infusion, manipulated animals (M-Na) showed a comparatively lower number of activated neurons than the M-Ctrol/HP group (Figs. 2 and 3c–f). A main effect of both M and Solution was found [$F_{1,15} = 8.53$, $p < 0.0105$; $F_{2,15} = 195.35$, $p < 0.001$, respectively] as well as an interaction between these factors [$F_{2,15} = 9.26$, $p < 0.0024$]. *A posteriori* comparisons showed a significant difference in the number of Fos-ir neurons in the HP group compared to the ISO and Sham groups, regardless of pre/postnatal manipulation and also in the number of Fos-ir neurons of M-Ctrol vs M-Na groups infused with hypertonic NaCl solution. (Figs. 2 and 3).

Hypothalamic SON and PVN. In these nuclei, not only was the number of Fos-immunoreactive cells quantified, but also the number of vasopressinergic and oxytocinergic activated neurons, through double immunostaining (Fos/AVP-ir and Fos/OT-ir) in response to the different solutions infused (Sham, ISO or HP).

An increased number of both vasopressinergic and oxytocinergic Fos-ir neurons was found in response to HP infusion in SON, and also in the lateral magnocellular subdivision (PaLM) of the PVN (Fig. 4). Thus, a main effect of Solution was found in the number of Fos-ir, Fos/AVP-ir and Fos/OT-ir within the SON: [$F_{2,18} = 90.77$, $p < 0.001$; $F_{2,18} = 40.09$, $p < 0.001$ and $F_{2,18} = 47.33$, $p < 0.001$] and also within the PaLM [$F_{2,16} = 197.59$, $p < 0.001$; $F_{2,16} = 153.88$, $p < 0.001$; $F_{2,16} = 73.43$, $p < 0.001$]. *A posteriori* comparisons in both nuclei showed significant differences between the HP and the ISO and Sham groups; however there were no significant differences or an interaction for the M groups.

In the parvocellular ventral subdivision (PaV) of PVN, significant differences were found between the infused solutions [main effect of Solution factor: $F_{2,17} = 14.11$; $p < 0.0002$]. Pairwise comparisons showed significant differences between HP and ISO groups and also between ISO and Sham groups. However, no significant differences or interaction were found for the M groups (Fig. 4a).

Extended amygdala. The number of Fos-immunoreactive neurons was studied in two subdivisions of the Central Extended amygdala: CeA and the laterodorsal subnuclei of the BNST in response to different infused solutions (HP, ISO or Sham). In both subdivisions of this complex, an increased number of Fos-ir neurons was found in response to infused HP

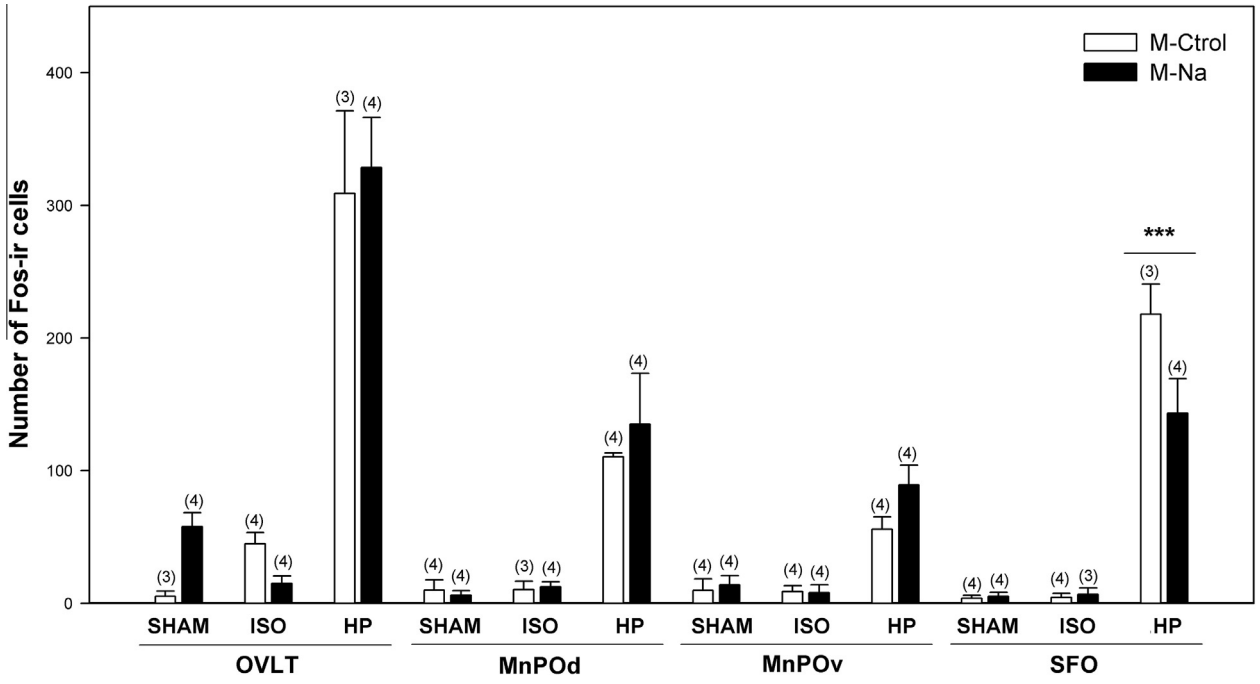

Fig. 2. Average number of Fos-immunoreactive (Fos-ir) cells in the *lamina terminalis* nuclei (OVLT, MnPO -ventral and dorsal- and SFO) of both M groups, after iv hypertonic (HP), isotonic (ISO) or simulated (Sham) NaCl infusion. Values expressed as Mean ± SE, (*n*). Significant differences between HP group vs. ISO and Sham groups are not represented in the figure. ***Significant differences between M-Ctrol-HP vs. M-Na/HP, $p < 0.001$. SFO = subfornical organ; OVLT = *organum vasculosum* of the *lamina terminalis*; MnPO = median preoptic nucleus.

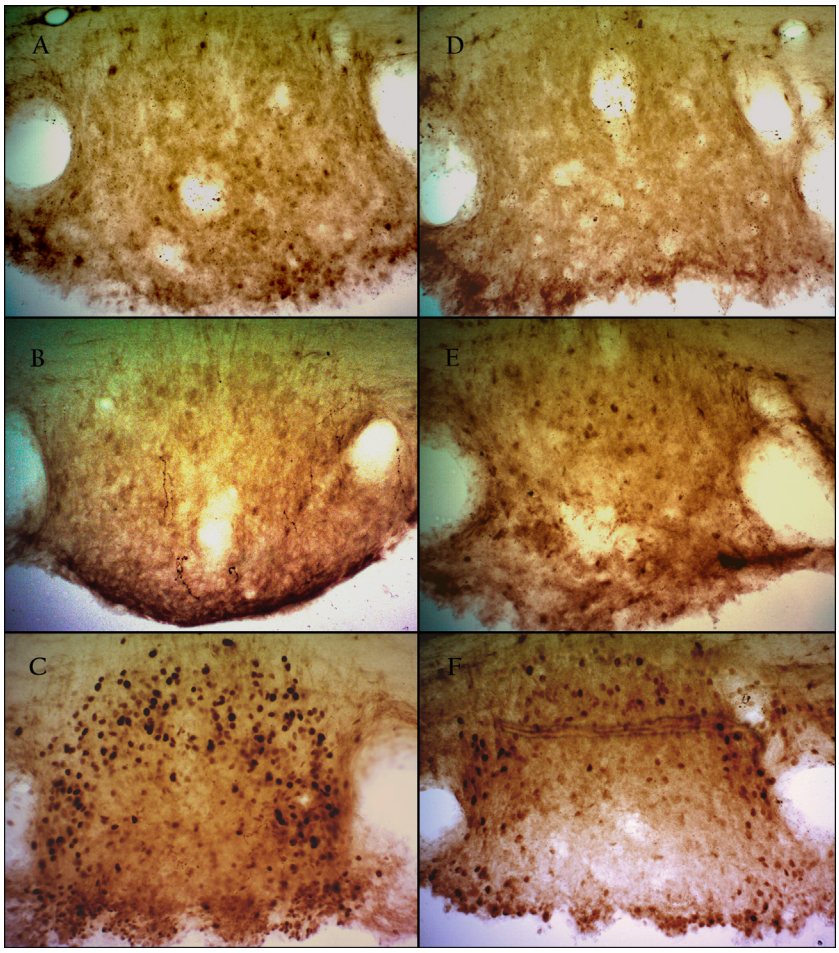

Fig. 3. Photomicrographs showing the pattern of Fos-immunoreactivity in the subfornical organ (SFO) of M-Ctrol (A, B, C) and M-Na (D, E, F) animals after simulated infusion (SHAM: A, D) or iv infusion of isotonic (ISO: B, E) or hypertonic (HP: C, F) NaCl solutions.

solution [main effect of Solution factor at BNST-$F_{2,17}$ = 39.21; $p < 0.001$ and at CeA-$F_{2,17}$ = 67.81; $p < 0.001$]. Pairwise comparisons showed significant differences between HP and ISO or Sham. However, no significant differences or interaction were found for the M groups (Table 3).

Brainstem nuclei. The number of Fos-ir neurons in the LPB, NTS, AP and DRN was quantified, as well as the number of active serotonergic neurons in the DRN, through double immunostaining (Fos/5HT-ir) in response to different infused solutions (HP, ISO or Sham).

LPBN. In the central subdivision of the LPB (LPBc), no effect of M or Solution or their interaction was found. In the external subdivision of the LPBN (LPBe), there was only a main effect of Solution [$F_{2,15}$ = 6.93, $p < 0.0074$]. *A posteriori* comparisons showed a significant increase in the number of Fos-ir neurons in HP-animals vs. ISO and Sham groups only (Fig. 5).

NTS and AP. In the AP, the same effect as obtained in the LPBe subdivision was found, due to the infusion of hypertonic sodium solution (HP) [$F_{2,14}$ = 94.26; $p < 0.001$]. *A posteriori* comparisons showed a significant increase in the number of Fos-ir neurons in the AP of the HP group compared to the ISO and Sham groups. There was no significant difference of M and Solution (Fig. 5).

NTS analysis showed a main effect of Solution, with an increased number of Fos-ir neurons in response to the infusion of HP [$F_{2,14}$ = 40.30, $p < 0.001$]. In addition, despite the main effect of the M factor not reaching statistically significant levels, there was a significant interaction between M and Solution factors [$F_{2,14}$ = 5.49; $p < 0.0174$]. *A posteriori* comparisons showed significant differences between M-Na/HP vs. M-Ctrol/HP, similar to those found in the SFO. As shown in Fig. 6c–f, M-Na animals infused with HP had a smaller number of activated cells (Fos-ir positive) in the NTS than M-Ctrol animals infused with the same solution (Figs. 5 and 6).

DRN. No significant differences or main effect (M and Solution) and no interaction were found, in the number of active neurons (Fos-ir) and also in the number of active serotonergic neurons (Fos/5HT-ir). This suggests that the manipulation did not induce changes in the serotonergic system and there also seems to be no effect of the infusion of a hypertonic NaCl solution, at

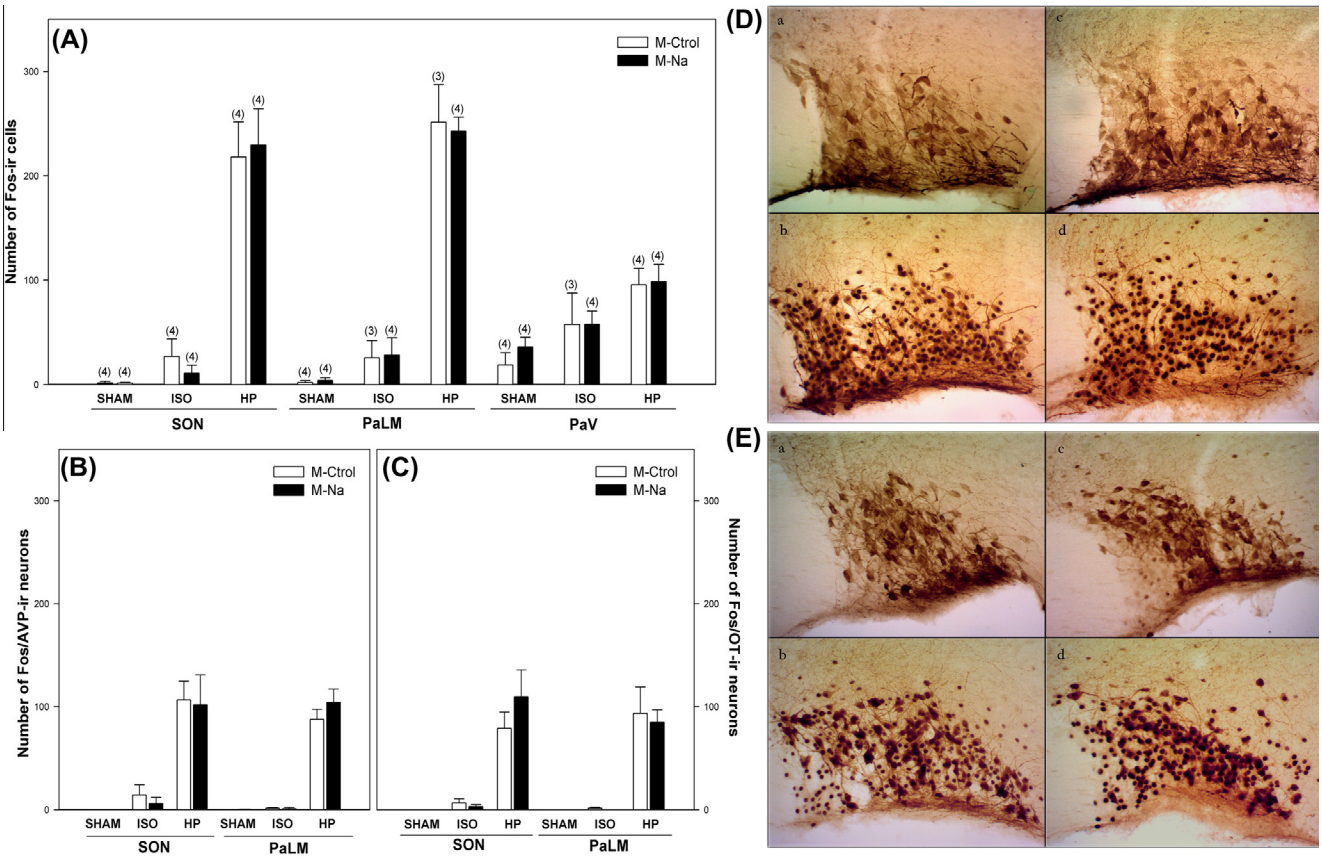

Fig. 4. Average number of Fos-ir (A), Fos/AVP-ir (B) and Fos/OT-ir (C) cells in the supraoptic nucleus (SON), and paraventricular hypothalamic nucleus along the lateral magnocellular and ventral subdivisions (PaLM and PaV, respectively) after iv hypertonic (HP), isotonic (ISO) or simulated (Sham) NaCl solution infusion. Values expressed as Mean ± SE, (*n*). Significant differences between HP group vs. ISO and Sham groups are not represented in the figure. Photomicrographs showing the pattern of Fos/AVP-ir (D) and Fos/OT-ir (E) in the supraoptic nucleus (SON) of M-Ctrol (a, c) and M-Na (b, d) animals after iv infusion of isotonic (ISO: a, b) or hypertonic (HP: c, d) NaCl solutions.

Table 3. Number of Fos-ir cells in the BNST and CeA after hypertonic (HP), isotonic (ISO) or simulated (Sham) sodium solution iv infusion in adult manipulated offspring

M group	Solution infused	BNST	CeA
M-Ctrol	Sham	5.50 ± 4.63 (4)	3.75 ± 1.66 (4)
	ISO	8.00 ± 3.92 (4)	17.75 ± 6.71 (4)
	HP	49.25 ± 8.52*** (4)	81.25 ± 13.92*** (4)
M-Na	Sham	12.00 ± 7.56 (4)	7.25 ± 2.08 (4)
	ISO	7.33 ± 4.71 (3)	20.33 ± 12.13 (3)
	HP	51.75 ± 6.89*** (4)	105.25 ± 13.39*** (4)

Values are expressed as mean ± SE, (*n*). *** Significant differences between HP vs.ISO and Sham groups, $p < 0.001$.

least in these conditions of rate, infused volume and NaCl concentration (Table 4).

Experiment 3: Relative mRNA levels of AVP in SON and angiotensinergic receptor subtype AT1a in SFO in response to iv infusion of hypertonic NaCl solution in adult offspring

AT1a in SFO. The relative AT1a mRNA expression in the SFO increased significantly in M-Ctrol animals infused with isotonic and hypertonic solution vs. those treated with sham infusion (Fig. 7a). Moreover, in M-Ctrol animals the expected increase in relative AT1a mRNA expression in response to HP solution was found; however, the M-Na group showed an unaltered AT1a-relative mRNA expression level in response to HP or any other infused solutions.

A main effect of the Solution [$F_{2,14} = 3.91$, $p < 0.0448$] as well as a main effect of the M [$F_{1,14} = 6.15$, $p < 0.0265$] was found. Likewise, an interaction was found between M and Solution [$F_{2,14} = 4.36$, $p < 0.0338$].

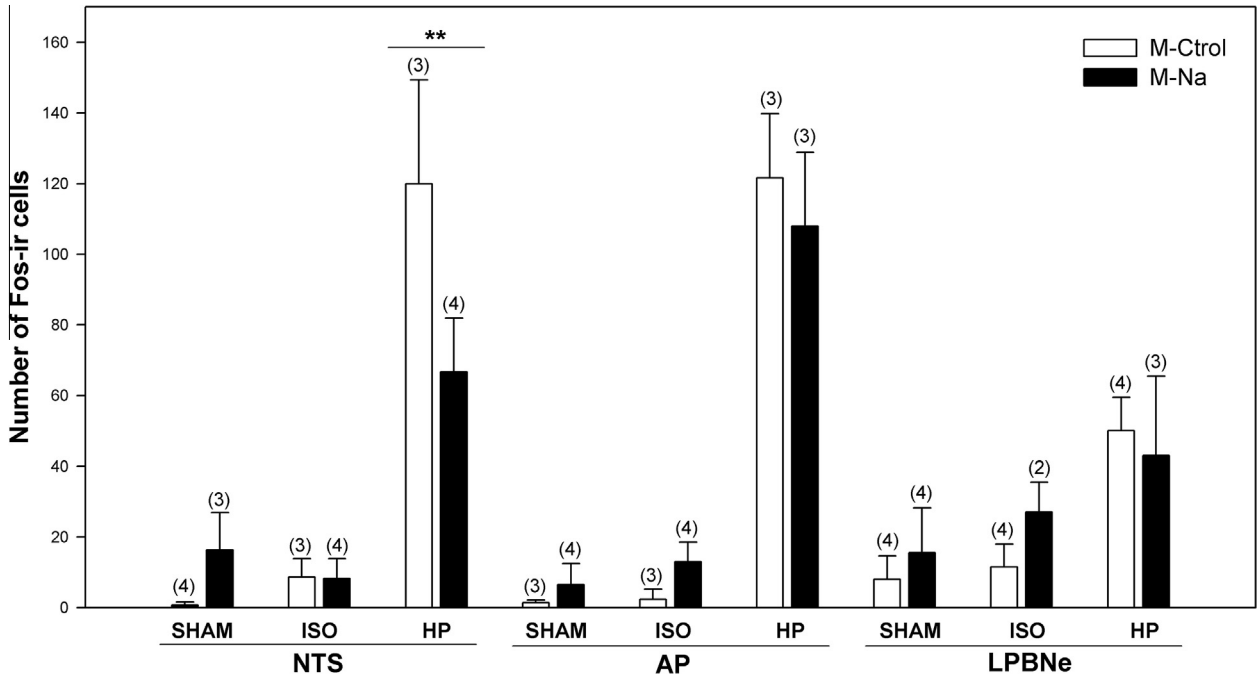

Fig. 5. Average number of Fos-ir cells in NTS, AP and central (LPBC) or external (LPBE) subdivisions of lateral parabrachial nucleus after iv hypertonic (HP), isotonic (ISO) or simulated (Sham) NaCl solution infusion. Values expressed as Mean ± SE, (*n*). Significant differences between HP group vs. ISO and Sham groups are not represented in the figure. **Significant differences between M-Ctrol/HP group in relation to M-Na/HP, $p < 0.01$.

AVP in SON. When performing the quantification of AVP-relative mRNA expression at the SON, the results showed a significant increase in the relative AVP mRNA expression in M-Ctrol descendants when they were infused with hypertonic NaCl solution in relation to isotonic or sham-infused animals. However, this significant increase in levels of relative AVP mRNA expression in response to HP infusion was not found in M-Na offspring (Fig. 7b). Thus, animals with early experience of a sodium rich environment (M-Na) showed no adjustment of relative AVP mRNA expression in response to a sodium overload. The analysis showed a main effect of the M factor [$F_{1,19} = 5.33$, $p < 0.0324$] as well as an interaction between the M × Solution factors [$F_{2,19} = 3.83$, $p < 0.0399$].

Experiment 4: Determination of plasma AVP concentration in response to iv infusion of hypertonic NaCl solution in adult offspring

As expected, our data show a significant increase in the amount of AVP released in blood in response to hypertonic NaCl solution infusion, regardless of M conditions [Fig. 8; main effect of Solution factor $F_{2,31} = 83.95$, $p < 0.001$]. However, no main effect of M factor or interaction between the factors was found.

DISCUSSION

We analyzed the effect of maternal and post-weaning voluntary intake of salt on the osmoregulatory response to sodium in behavior, brain activity and expression of certain key genes (AVP, AT1a) involved in hydroelectrolyte balance regulation in adult rat offspring. The changes found at cellular and molecular levels are noteworthy.

Our data show evidence of neuroadaptive changes in response to sodium overload in adult offspring of dams with free access to hypertonic NaCl solution during the prenatal and early postnatal period. It is important to clarify that, although this imprinting model did not affect plasma sodium concentration, osmolality or protein concentration in dams, despite their enhanced sodium intake (Macchione et al., 2012), their pups at weaning (postnatal day 21) showed an increased plasma sodium concentration and osmolality, indicating that the offspring was certainly subject to chronic sodium overload. The absence of differences in those parameters in dams might be explained by the efficiency of maternal fluid homeostatic systems compensating for variations caused by the voluntary ingestion of hypertonic NaCl solution. On the other hand, the increase observed in plasma sodium concentration and osmolality of M-Na pups suggests that, even though systems involved in salt and water homeostasis are functionally mature at weaning, perhaps they are still not efficient enough to maintain hydromineral balance in response to chronic exposure to hypertonic NaCl solution.

The present protocol does not enable us to distinguish whether the changes observed later in life are due to maternal intake during pregnancy or during lactation, or by offspring voluntary consumption until PD28. However, our previous unpublished data showed, for example, that an animal's access to hypertonic sodium chloride after weaning and until adulthood did not

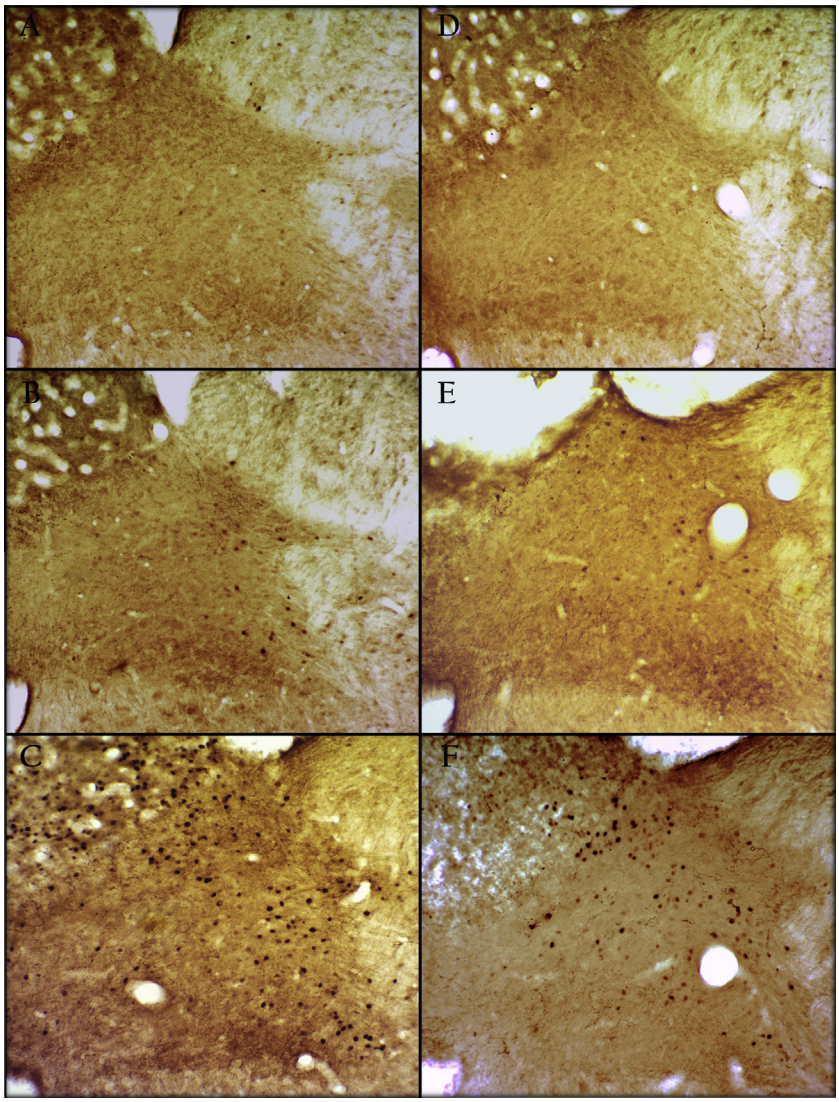

Fig. 6. Photomicrographs showing the pattern of Fos-immunoreactivity in the nucleus of the solitary tract (NTS) of M-Ctrol (A, B, C) and M-Na (D, E, F) animals after simulated infusion (SHAM: A, D) or iv infusion of isotonic (ISO: B, E) or hypertonic (HP: C, F) NaCl solutions.

Table 4. Number of Fos-ir cells and Fos/5HT-ir neurons in the DRN after hypertonic (HP), isotonic (ISO) or simulated (Sham) sodium solution iv infusion in adult manipulated offspring

M group	Solution infused	Fos-ir cells	Fos/5HT-ir neurons
M-Ctrol	Sham	68.00 ± 5.03 (3)	24.33 ± 2.03 (3)
	ISO	94.00 ± 15.00 (4)	26.75 ± 5.25 (4)
	HP	70.75 ± 7.25 (4)	25.75 ± 3.07 (4)
M-Na	Sham	96.20 ± 23.78 (5)	33.60 ± 7.70 (5)
	ISO	65.25 ± 12.67 (4)	23.00 ± 3.70 (4)
	HP	59.66 ± 7.31 (3)	18.00 ± 5.51 (3)

Values are expressed as mean ± SE, (*n*). No significant differences between groups were found.

induce any significant difference in water and sodium ingestion after different fluid challenges, demonstrating that this period at least may be not enough to induce any programing. Further studies are necessary to discriminate the precise stage responsible for the changes observed later in life.

This chronic stimulation during a critical developmental period may involve plasticity changes in the fluid volume/osmosensitive mechanisms, which may alter the brain activity and or gene expression threshold in the SON and in two nuclei, the SFO and NTS, which are the main entrance to the central nervous system of

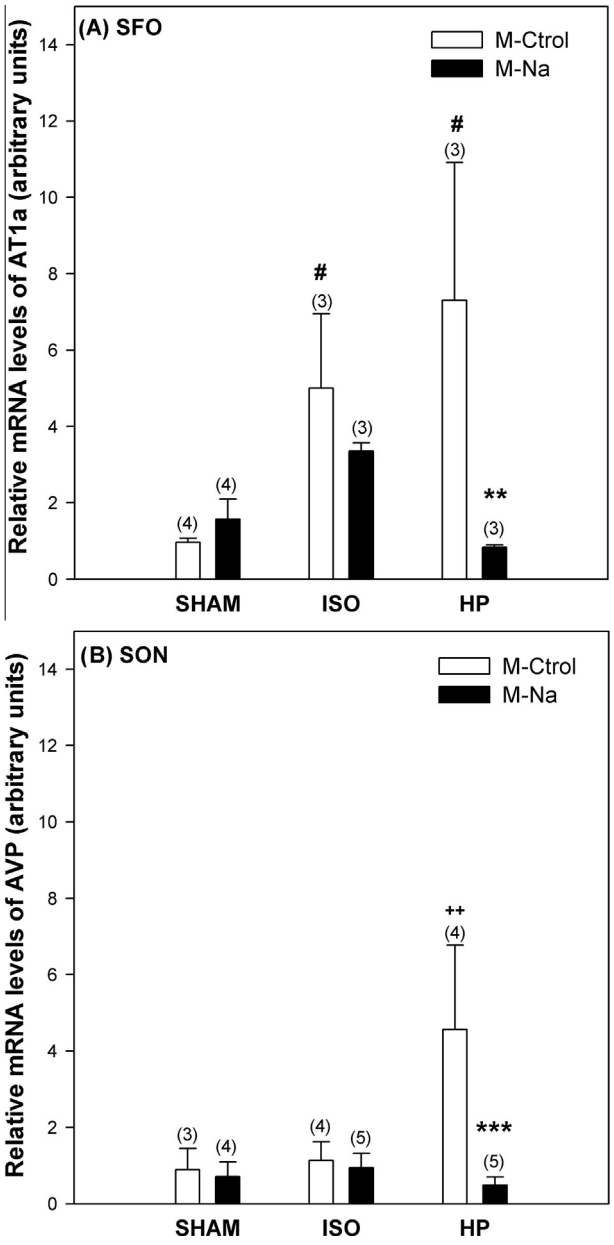

Fig. 7. Relative mRNA levels of AT1a (A) or AVP (B) from RNA isolated from micropunches of subfornical organ (SFO; A) and supraoptic nucleus (SON; B) in M-Ctrol or M-Na adult offspring after iv hypertonic (HP), isotonic (ISO) or simulated (Sham) NaCl solution infusion. The samples were measured by quantitative-polymerase chain reaction (qPCR). Values expressed as mean ± SE, (*n*). (#) Significant differences between HP and ISO groups vs. Sham group in M-Ctrol animals, $p < 0.05$. (++) Significant differences between HP vs. ISO and Sham in M-Ctrol group, $p < 0.01$. **Significant differences between M-Ctrol vs. M-Na in animals infused with HP solution, $p < 0.01$. ***Significant differences between M-Ctrol vs. M-Na within animals infused with HP solution, $p < 0.001$.

the appropriate stimulation (coming from baroreceptors, sodium-osmosensors and volume sensors) (Badauê-Passos et al., 2007; Vivas et al., 2013). Particularly considering that early experience of sodium overload may induce long-lasting changes in the baroreceptor threshold of stimulation and thus in arterial blood pressure (ABP) regulatory responses, the apparently contradictory results regarding enhanced drinking and diminished brain activity and gene expression in those nuclei (SFO/NTS) may be explained. As shown in Fig. 9, sodium overload may induce changes in ABP that affect water intake and the activity of different central nuclei; however, ABP may be differently affected by the infusion of hypertonic NaCl in M-Ctrol and M-Na rats, thus influencing water drinking, the activity of particular nuclei and also AVP/AT1 mRNA expression in different ways. The control group would thus have a comparatively greater increase in ABP in response to hypertonic saline infusion, which would cause greater inhibition of thirst, since it is already established that an increase in ABP inhibits thirst (Robinson and Evered, 1987; Stocker et al., 2001). Besides, the differential brain nuclei activity observed in M-Na rats specifically along the NTS and SFO is not surprising, since these two nuclei are mainly involved in cardiovascular/osmotic responses to a body sodium overload (Hochstenbach and Ciriello, 1994). Although we did not measure ABP during iv infusion of hypertonic NaCl, previously published data using similar procedures showed that infusions of 1.4 M NaCl for 10 min (at 5.5 ml/kg; total volume approximately 1.65 ml (Hochstenbach and Ciriello, 1994) or 1 M NaCl for 2 h (at 2 ml/h; total volume: 4 ml (Stocker et al., 2001); induced an increase in ABP by approximately 2–10 mmHg, respectively, returning to baseline values at the end of the infusion or when water access was allowed. In our model, a 1.5 M NaCl solution was infused for 20 min at a rate of 0.15 ml/min, resulting in an injected volume (3 ml), intermediate in terms of the other two studies, of a more concentrated NaCl solution; it is thus possible that a transient increase in ABP occurred during this infusion, which is given simultaneously with the drinking test. Therefore, taking into account previous evidence showing that: 1 – An increase in ABP inhibits thirst stimulated by the infusion of hypertonic NaCl and angiotensin II (Stocker et al., 2001); 2 – Sinoaortic-denervated rats ingested more water than control rats after increases in plasma osmolality induced by iv infusion of hypertonic NaCl (1 M NaCl, for 2 h at 2 ml/h), or when infused with different doses of angiotensin II (Stocker et al., 2002), it is possible to speculate on the existence in the present imprinting model of long-lasting changes in the baroreceptor threshold of stimulation and/or the regulatory responses modulated by ABP, such as drinking, AVP and AT1 *de novo* syntheses, and the activity of key brain nuclei involved in ABP regulation.

Another possibility would be that hyperosmolality may induce a similar increase in ABP in both groups but trigger responses of different intensity in M-Ctrol and M-Na rats, with the latter being more tolerant to the stimulation based on their previous experience of early and chronic sodium exposure. In this case, the chronic experience may induce a long-lasting enhancement of the firing threshold that would result in a lower number of activated cells in the SFO and NTS after salt loading, but at the same time, once the pathways are activated, this may trigger a more efficient or potentiated behavioral response, as shown by the increased water intake of the M-Na group.

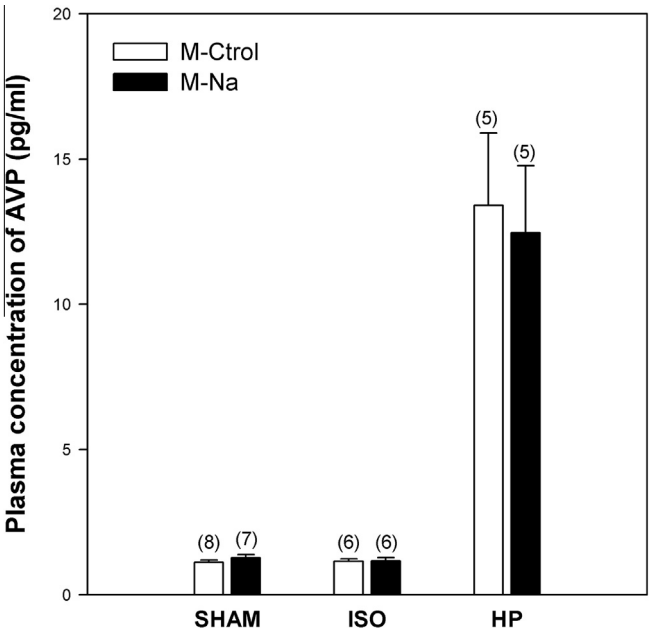

Fig. 8. Plasma AVP in M-Ctrol and M-Na groups after iv hypertonic (HP), isotonic (ISO) or Sham NaCl solution infusion. Values expressed as mean ± SE, (*n*). Significant differences between HP vs. ISO and Sham groups are not represented in the figure.

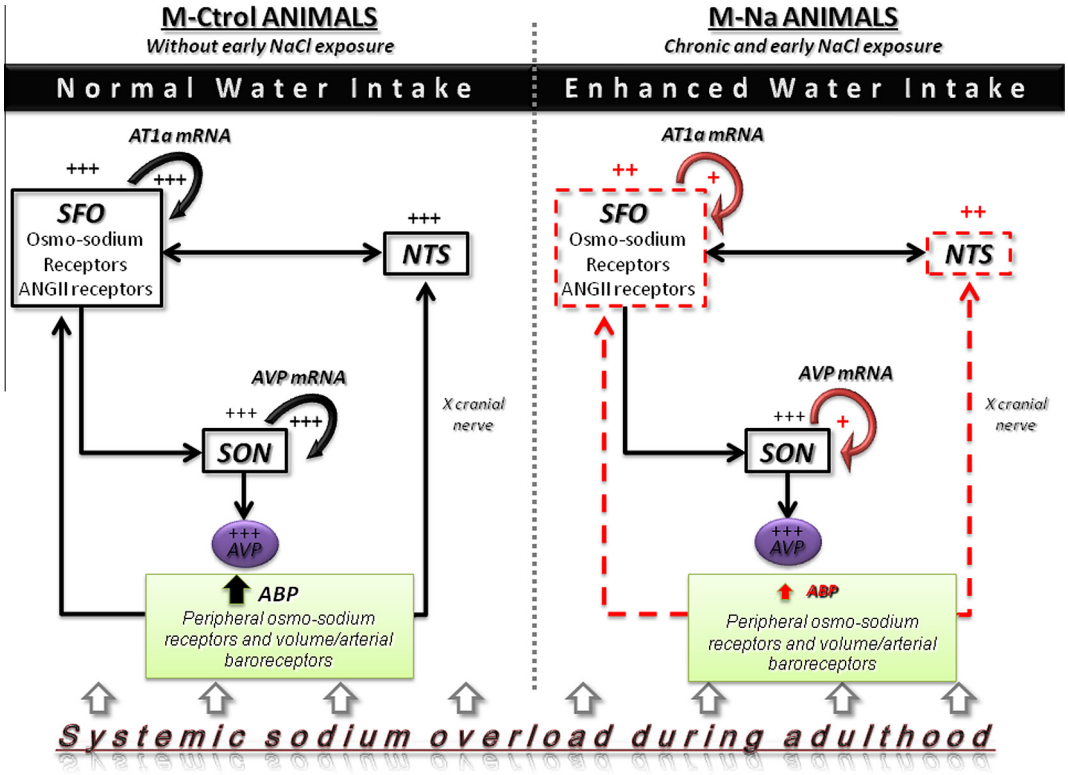

Fig. 9. Scheme that summarizes the different brain areas and pathways that might be more vulnerable to the programing effect of sodium, altering the normal way of attaining water and electrolyte homeostasis. Red and/or dotted lines indicate hypothetical pathways and/or mechanisms subjected to neuroadaptive changes. SFO: subfornical organ; NTS: nucleus of the solitary tract; SON: supraoptic nucleus; AVP: vasopressin; ABP: arterial blood pressure; AT1a: angiotensinergic AT1a-receptor. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Many studies, such as those on water deprivation (Sanvitto et al., 1997; Barth and Gerstberger, 1999), sodium depletion (Charron et al., 2002), and sodium overload (Sandberg et al., 1994; Chen et al., 2003), have shown that AT1R gene expression is affected by body sodium status levels, and by its own agonist (Wei et al., 2009). Salt loading (5 days of 2% NaCl consumption) induces an upregulation of AT1a mRNA expression in several hypothalamic nuclei, and this was clearly indicated in the SFO (Chen et al., 2003). In our model, the control-manipulated animals show the expected upregulation of AT1a mRNA expression within the SFO after intravenous infusion of hypertonic NaCl; however, sodium-exposed animals showed essentially no osmotic/pressure response, which may also be associated with long-lasting changes in the peripheral and central baroreceptor/osmoreceptor threshold of stimulation of these animals.

The present results also demonstrate that pre/postnatal sodium exposure abolishes the expected increase of AVP mRNA expression in offspring in response to sodium overload (see Fig. 7). Once again, these results may indicate plasticity changes in baro/osmosensitive mechanisms that may alter the pressure/osmotic threshold for AVP synthesis, although we were not able to see changes in plasma AVP release or neural activity (as shown by double Fos/AVP-ir). The observed increase in plasma AVP concentration may be due to the osmotic-induced release of AVP already stored in the neurohypophysis; however, the mechanisms that trigger the AVP *de novo* syntheses may be imprinted, which implies susceptibility to this programing. One possibility is epigenetic programing, which is a phenomenon that usually occurs in the early stages of development. Epigenetic mechanisms may alter gene expression through protein–DNA interactions without changing the genetic code (Tsankova et al., 2007), and classic examples of epigenetic phenomena modulating chromatin access of the transcriptional machinery (and gene expression) are DNA methylation/demethylation, histone N-terminal tails, covalent addition or removal of acetyl, phosphate, methyl, and other radicals (Zentner and Henikoff, 2013). It has been shown, for example, that early-life stress in mice leads to high corticosterone plasma levels and paraventricular AVP mRNA expression, and is related to AVP gene hypomethylation in adulthood (Murgatroyd et al., 2009). In sum, the osmotic-induced plasma AVP released may be equal in both groups, but the imprinted animals may have a delayed or diminished AVP gene expression associated with AVP gene hypermethylation.

Previous studies have demonstrated that the plasma osmolality threshold for AVP release is increased in offspring exposed *in utero* to an increase in maternal hypernatremia in response to maternal water restriction, indicating an altered set point for systemic osmolality (Desai et al., 2003). In the same model, the authors also demonstrated that neonatal lambs had higher pituitary AVP content but lower hypothalamic AVP gene expression (Ramirez et al., 2002). Their results and model, although different to ours, in general terms also showed a discrepancy between plasma AVP released from the pituitary, and lower or absent AVP synthesis in SON, in response to salt loading. The authors postulated that maternal and fetal plasma hypertonicity initially stimulated fetal pituitary AVP release and hypothalamic AVP gene expression, but then this activation resulted in reduced pituitary AVP, increased AVP mRNA and the activation of AVP synthesis. After a long period of hypertonicity, the foetus acclimatized to the hypertonic condition and returned to basal AVP secretion rates. Although AVP secretion returned to basal levels, the increased AVP synthesis resulted in excess pituitary AVP storage and, ultimately, feedback inhibition of hypothalamic mRNA synthesis.

Other recent work has also shown how early-life stress, such as maternal deprivation, provokes an increase during adulthood in the relative abundance of AVP mRNA along the PVN and SON and altered plasma AVP concentration dynamics during water deprivation (Renard et al., 2010; Zhang et al., 2012), while the attenuated response to stress during adulthood observed in the same model also involves vasopressinergic neuroadaptive changes (Renard et al., 2010; Banihashemi et al., 2011). Thus, different types of stressors (osmotic or psychological) during early life may change the vasopressinergic responses to different AVP-induced stimuli during adulthood, suggesting vasopressinergic system vulnerability.

The reduced activity observed within the NTS in the present model, not only after sodium overload but also after sodium depletion (Macchione et al., 2012), can also be interpreted as reduced osmotic stress-induced noradrenergic signaling from the NTS in the M-Na group, as has been previously shown in repeated brief maternal separation, another perinatal imprinting model of stress (Banihashemi et al., 2011). Thus, early-life experience of osmotic or psychological stress may attenuate adult stress-induced Fos activation within the NTS, which may result in reduced autonomic responses to stress.

In summary, it is possible to speculate about the occurrence of neuroadaptive changes in the firing threshold of neurons and sensors that monitor adjustments in blood pressure and osmolality/sodium concentration due to pre/postnatal environmental modifications. As outlined in Fig. 9, hypervolemia and hypernatremia induced by a body sodium overload stimulate central and peripheral osmo-sodium receptors, volume/arterial baroreceptors and central vasopressinergic/renin–angiotensin systems. These stimuli act mainly through the sensory circumventricular organs (CVOs) and the NTS to activate brain neural pathways that regulate ABP, AVP release, sympathetic nerve activity and water ingestion. Different brain areas and pathways may be more vulnerable to the pre/postnatal programer effect of sodium (as shown by dotted and/or red lines, Fig. 9), resulting in intrinsic changes at different levels: cellular, molecular, and systems (i.e., vasopressinergic, angiotensinergic), altering normal functioning or the way of attaining water and electrolyte homeostasis. The programed systems, circuits and/or cells would generate a new performance

activity, which would be positive or negative depending on the circumstances to which they are subjected. In the present experimental conditions, the peripheral sodium overload during adulthood would induce a comparatively lower ABP increase in M-Na animals (with early and chronic sodium exposure), which provokes enhanced water intake and reduced activity/gene expression in two particular nuclei (SFO and NTS) that are the main window of key stimulation coming from the baro-/volume- and osmo-sensors.

Knowledge from CVOs lesion studies suggests, for example, that one function of the SFO is to provide a quick response to a rapid rise in plasma osmolality, since lesioned rats took longer (>15 min) to begin drinking (Starbuck and Fitts, 1998). Besides, a SFO lesion in rats was more effective than a lesion of the OVLT in reducing the drinking response to intragastric hyperosmotic NaCl, and a combined SFO and OVLT lesion produced the same deficit as a SFO lesion alone (Fitts et al., 2004) indicating that the SFO is a key site for this sort of stimulation. It should also be noted that a previous study has suggested that the changes found in SFO activity (shown as Fos-ir) reflect the neural plasticity responsible for the expression of enhanced sodium intake with repeated depletions (Na et al., 2007).

With regard to NTS involvement in the responses affected by pre/postnatal programing in this study, there is important electrophysiological evidence showing that the majority of the hypertonic saline-responsive units of this nucleus receive baroreceptor inputs, indicating the existence of neurons capable of activating a circuit responsible for correcting blood pressure and body fluid disturbances (Hochstenbach and Ciriello, 1994).

Taking into account that imprinting in the osmoregulatory system by early access to hypertonic NaCl solution causes changes in drinking, brain cell activity and brain gene expression in response to sodium overload, it is important that health professionals be made aware of the need for caution during the pregnancy and postnatal stages. For example, a careful, close monitoring of the mother's fluid and electrolyte balance, and of feeding, weight and urination frequency in the neonate is important to prevent the development of severe dehydration and hypernatremia, and pathology-related sodium overload in the future (Crystal and Bernstein, 1995, 1998; Stein et al., 1996; Leshem, 1998; Shirazki et al., 2007; Kusuma et al., 2009).

From an evolutionary perspective, the primary aim of this neuroadaptive process would be to maximize the adaptive capacity of the animal in direct relation to its present environment (Gluckman and Hanson, 2004). However, if environmental conditions vary from the ontogenetic stage to adulthood, the adaptive capacity of this process could well become negative, resulting in disadvantage to the animal.

Acknowledgments—The authors thank Dr. Jose Antunes-Rodrigues from the School of Medicine of Ribeirão Preto, University of São Paulo, for vasopressin plasma concentration measurements.

Funding acknowledgments: This research was supported by public funding from Consejo Nacional de Investigación Científica y Técnica (CONICET), Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), Secretaría de Ciencia y Técnica (SECyT) and Ministerio de Ciencia y Técnica de Córdoba (MINCyT). A.F.M. holds a fellowship from CONICET.

REFERENCES

Argüelles J, Brime JI, López-Sela P, Perillán C, Vijande M (2000) Adult offspring long-term effects of high salt and water intake during pregnancy. Horm Behav 37:156–162.

Badauê-Passos D, Godino A, Johnson A, Vivas L, Antunes-Rodrigues J (2007) Dorsal raphe nuclei integrate allostatic information evoked by depletion-induced sodium ingestion. Exp Neurol 206:86–94.

Balbi APC, Marin ECS, Francescato HDC, Costa RS, Coimbra TM (2008) MAPK and angiotensin II receptor in kidney of newborn rats from losartan-treated dams. Pediatr Nephrol 23:1433–1444.

Banihashemi L, O'Neill EJ, Rinaman L (2011) Central neural responses to restraint stress are altered in rats with an early life history of repeated brief maternal separation. Neuroscience 192:413–428.

Barth SW, Gerstberger R (1999) Differential regulation of angiotensinogen and AT1A receptor mRNA within the rat subfornical organ during dehydration. Brain Res Mol Brain Res 64:151–164.

Caeiro XE, Vivas L (2008) Beta-endorphin in the median preoptic nucleus modulates the pressor response induced by subcutaneous hypertonic sodium chloride. Exp Neurol 210:59–66.

Charron G, Laforest S, Gagnon C, Drolet G, Mouginot D (2002) Acute sodium deficit triggers plasticity of the brain angiotensin type 1 receptors. FASEB J 16:610–612.

Chen Y, da Rocha MJA, Morris M (2003) Osmotic regulation of angiotensin AT1 receptor subtypes in mouse brain. Brain Res 965:35–44.

Chow SY, Sakai RR, Witcher JA, Adler NT, Epstein AN (1992) Sex and sodium intake in the rat. Behav Neurosci 106:172–180.

Clark JJ, Bernstein IL (2006) Sensitization of salt appetite is associated with increased "wanting" but not "liking" of a salt reward in the sodium-deplete rat. Behav Neurosci 120:206–210.

Contreras RJ, Kosten T (1983) Prenatal and early postnatal sodium chloride intake modifies the solution preferences of adult rats. J Nutr 113:1051–1062.

Contreras RJ, Ryan KW (1990) Perinatal exposure to a high NaCl diet increases the NaCl intake of adult rats. Physiol Behav 47:507–512.

Crystal SR, Bernstein IL (1995) Morning sickness: impact on offspring salt preference. Appetite 25:231–240.

Crystal SR, Bernstein IL (1998) Infant salt preference and mother's morning sickness. Appetite 30:297–307.

Curtis KS, Krause EG, Wong DL, Contreras RJ (2004) Gestational and early postnatal dietary NaCl levels affect NaCl intake, but not stimulated water intake, by adult rats. Am J Physiol Regul Integr Comp Physiol 286:R1043–R1050.

Dadam FM, Caeiro XE, Cisternas CD, Macchione AF, Cambiasso MJ, Vivas L (2014) Effect of sex chromosome complement on sodium appetite and Fos-immunoreactivity induced by sodium depletion. Am J Physiol Regul Integr Comp Physiol 306:R175–R184.

Dalmasso C, Amigone JL, Vivas L (2011) Serotonergic system involvement in the inhibitory action of estrogen on induced sodium appetite in female rats. Physiol Behav 104:398–407.

Desai M, Guerra C, Wang S, Ross MG (2003) Programing of hypertonicity in neonatal lambs: resetting of the threshold for vasopressin secretion. Endocrinology 144:4332–4337.

Fitts DA, Freece JA, Van Bebber JE, Zierath DK, Bassett JE (2004) Effects of forebrain circumventricular organ ablation on drinking or

salt appetite after sodium depletion or hypernatremia. Am J Physiol Regul Integr Comp Physiol 287:R1325–R1334.
Franchini LF, Vivas L (1999) Distribution of Fos immunoreactivity in rat brain after sodium consumption induced by peritoneal dialysis distribution of Fos immunoreactivity in rat brain after sodium consumption induced by peritoneal dialysis. Am J Physiol Regul Integr Comp Physiol 276:R1180–R1187.
Galaverna O, Nicolaïdis S, Yao SZ, Sakai RR, Epstein AN (1995) Endocrine consequences of prenatal sodium depletion prepare rats for high need-free NaCl intake in adulthood. Am J Physiol 269:R578–R583.
Gluckman PD, Hanson MA (2004) Living with the past: evolution, development, and patterns of disease. Science 305:1733–1736.
Godino A, Giusti-Paiva A, Antunes-Rodrigues J, Vivas L (2005) Neurochemical brain groups activated after an isotonic blood volume expansion in rats. Neuroscience 133:493–505.
Ho JM, Zierath DK, Savos AV, Femiano DJ, Bassett JE, McKinley MJ, Fitts DA (2007) Differential effects of intravenous hyperosmotic solutes on drinking latency and c-Fos expression in the circumventricular organs and hypothalamus of the rat. Am J Physiol Regul Integr Comp Physiol 292:R1690–R1698.
Hochstenbach SL, Ciriello J (1994) Effects of plasma hypernatremia on nucleus tractus solitarius neurons. Am J Physiol 266: R1916–R1921.
Katovich MJ, Aerni JD, Cespedes AT, Rowland NE (2001) Perinatal dietary NaCl level: effect on angiotensin-induced thermal and dipsogenic responses in adult rats. Physiol Behav 72:621–627.
Kusuma S, Agrawal SK, Kumar P, Narang A, Prasad R (2009) Hydration status of exclusively and partially breastfed near-term newborns in the first week of life. J Hum Lact 25:280–286.
Leshem M (1998) Salt preference in adolescence is predicted by common prenatal and infantile mineralofluid loss. Physiol Behav 63:699–704.
Leshem M (2009) Biobehavior of the human love of salt. Neurosci Biobehav Rev 33:1–17.
Macchione AF, Caeiro XE, Godino A, Amigone JL, Antunes-Rodrigues J, Vivas L (2012) Availability of a rich source of sodium during the perinatal period programs the fluid balance restoration pattern in adult offspring. Physiol Behav 105: 1035–1044.
Machado FG, Poppi EPB, Fanelli C, Malheiros DMAC, Zatz R, Fujihara CK (2008) AT1 blockade during lactation as a model of chronic nephropathy: mechanisms of renal injury. Am J Physiol Renal Physiol 294:F1345–F1353.
McBride SM, Culver B, Flynn FW (2006) Prenatal and early postnatal dietary sodium restriction sensitizes the adult rat to amphetamines. Am J Physiol Regul Integr Comp Physiol 291:R1192–R1199.
Mecawi AS, Araujo IG, Rocha FF, Coimbra TM, Antunes-Rodrigues J, Reis LC (2010) Ontogenetic role of angiotensin-converting enzyme in rats: thirst and sodium appetite evaluation. Physiol Behav 99:118–124.
Mouw DR, Vander AJ, Wagner J (1978) Effects of prenatal and early postnatal sodium deprivation on subsequent adult thirst and salt preference in rats. Am J Physiol 234:F59–F63.
Murgatroyd C, Patchev AV, Wu Y, Micale V, Bockmühl Y, Fischer D, Holsboer F, Wotjak CT, Almeida OFX, Spengler D (2009) Dynamic DNA methylation programs persistent adverse effects of early-life stress. Nat Neurosci 12:1559–1566.
Na ES, Morris MJ, Johnson RF, Beltz TG, Johnson AK (2007) The neural substrates of enhanced salt appetite after repeated sodium depletions. Brain Res 1171:104–110.
Nicolaidis S, Galaverna O, Metzler CH (1990) Extracellular dehydration during pregnancy increases salt appetite of offspring. Am J Physiol 258:R281–R283.
Paxinos G, Watson C (2007) The rat brain in stereotaxic coordinates: hard cover edition. Academic Press.
Perillan C, Costales M, Diaz F, Vijande M, Argüelles J (2004) Thirst changes in offspring of hyperreninemic rat dams. Pharmacol Biochem Behav 79:709–713.
Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:e45.
Ramirez BA, Wang S, Kallichanda N, Ross MG (2002) Chronic in utero plasma hyperosmolality alters hypothalamic arginine vasopressin synthesis and pituitary arginine vasopressin content in newborn lambs. Am J Obstet Gynecol 187:191–196.
Renard GM, Rivarola MA, Suárez MM (2010) Gender-dependent effects of early maternal separation and variable chronic stress on vasopressinergic activity and glucocorticoid receptor expression in adult rats. Dev Neurosci 32:71–80.
Robertson GL, Mahr EA, Athar S, Sinha T (1973) Development and clinical application of a new method for the radioimmunoassay of arginine vasopressin in human plasma. J Clin Invest 52:2340–2352.
Robinson MM, Evered MD (1987) Pressor action of intravenous angiotensin II reduces drinking response in rats. Am J Physiol 252:R754–R759.
Roitman MF, Na E, Anderson G, Jones TA, Bernstein IL (2002) Induction of a salt appetite alters dendritic morphology in nucleus accumbens and sensitizes rats to amphetamine. J Neurosci 22. RC225 (1–5).
Ross MG, Desai M, Guerra C, Wang S (2005) Prenatal programing of hypernatremia and hypertension in neonatal lambs. Am J Physiol Regul Integr Comp Physiol 288:R97–R103.
Sánchez SI, Seltzer AM, Fuentes LB, Forneris ML, Ciuffo GM (2008) Inhibition of angiotensin II receptors during pregnancy induces malformations in developing rat kidney. Eur J Pharmacol 588:114–123.
Sandberg K, Ji H, Catt KJ (1994) Regulation of angiotensin II receptors in rat brain during dietary sodium changes. Hypertension 23:I137–I141.
Sanvitto GL, Jöhren O, Häuser W, Saavedra JM (1997) Water deprivation upregulates ANG II AT1 binding and mRNA in rat subfornical organ and anterior pituitary. Am J Physiol 273: E156–E163.
Shirazki A, Weintraub Z, Reich D, Gershon E, Leshem M (2007) Lowest neonatal serum sodium predicts sodium intake in low birth weight children. Am J Physiol Regul Integr Comp Physiol 292:R1683–R1689.
Starbuck EM, Fitts DA (1998) Effects of SFO lesion or captopril on drinking induced by intragastric hypertonic saline. Brain Res 795:37–43.
Stein LJ, Cowart BJ, Epstein AN, Pilot LJ, Laskin CR, Beauchamp GK (1996) Increased liking for salty foods in adolescents exposed during infancy to a chloride-deficient feeding formula. Appetite 27:65–77.
Stocker SD, Stricker EM, Sved AF (2001) Acute hypertension inhibits thirst stimulated by ANG II, hyperosmolality, or hypovolemia in rats. Am J Physiol Regul Integr Comp Physiol 280:R214–R224.
Stocker SD, Stricker EM, Sved AF (2002) Arterial baroreceptors mediate the inhibitory effect of acute increases in arterial blood pressure on thirst. Am J Physiol Regul Integr Comp Physiol 282:R1718–R1729.
Stricker EM, Thiels E, Verbalis JG (1991) Sodium appetite in rats after prolonged dietary sodium deprivation: a sexually dimorphic phenomenon. Am J Physiol 260:R1082–R1088.
Tsankova N, Renthal W, Kumar A, Nestler EJ (2007) Epigenetic regulation in psychiatric disorders. Nat Rev Neurosci 8:355–367.
Vivas L, Godino A, Dalmasso C, Caeiro XE, Macchione AF, Cambiasso MJ (2013) Neurochemical circuits subserving fluid balance and baroreflex. A role for serotonin, oxytocin, and gonadal steroids. In Neurobiology of body fluids homeostasis: transduction and integration (De Luca LA, Johnson AK, Menani J V, eds), pp. 141–166.
Wang S, Chen J, Kallichanda N, Azim A, Calvario G, Ross M (2003) Prolonged prenatal hypernatremia alters neuroendocrine and electrolyte homeostasis in neonatal sheep. Exp Biol Med 228:41–45.
Wei S-G, Yu Y, Zhang Z-H, Felder RB (2009) Angiotensin II upregulates hypothalamic AT1 receptor expression in rats via

the mitogen-activated protein kinase pathway. Am J Physiol Heart Circ Physiol 296:H1425–H1433.
Wu L, Mao C, Liu Y, Shi A, Xu F, Zhang L, Xu Z (2011) Altered dipsogenic responses and expression of angiotensin receptors in the offspring exposed to prenatal high sucrose. Peptides 32:104–111.
Zentner GE, Henikoff S (2013) Regulation of nucleosome dynamics by histone modifications. Nat Struct Mol Biol 20:259–266.
Zhang H, Fan Y, Xia F, Geng C, Mao C, Jiang S, He R, Zhang L, Xu Z (2011) Prenatal water deprivation alters brain angiotensin system and dipsogenic changes in the offspring. Brain Res 1382: 128–136.
Zhang L, Hernández VS, Liu B, Medina MP, Nava-Kopp AT, Irles C, Morales M (2012) Hypothalamic vasopressin system regulation by maternal separation: its impact on anxiety in rats. Neuroscience 215:135–148.

(Accepted 3 April 2015)
(Available online 12 April 2015)