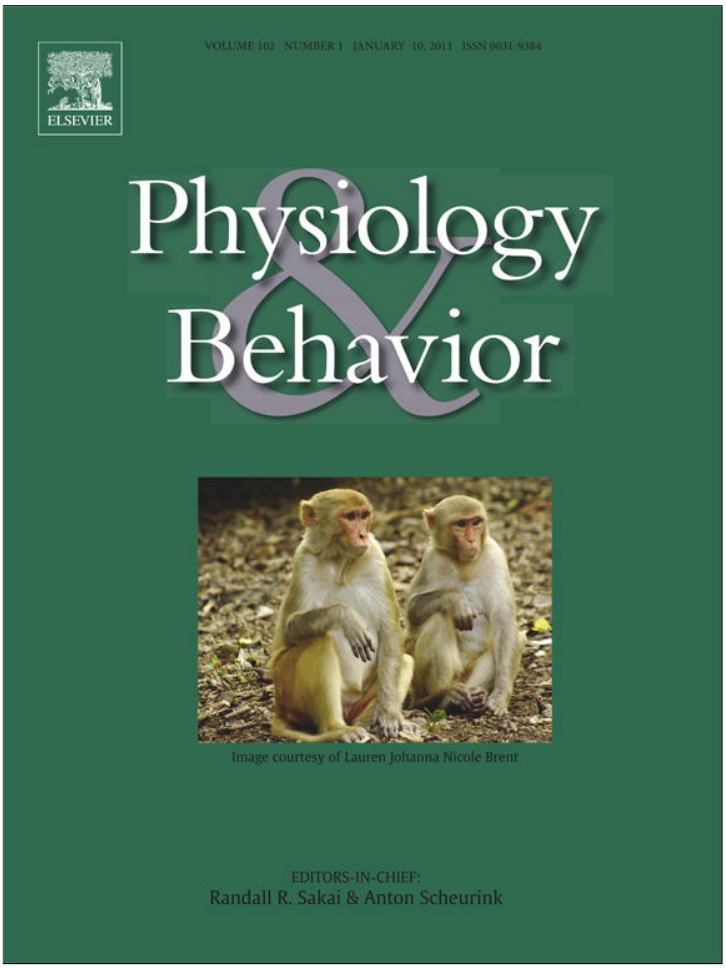

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Availability of a rich source of sodium during the perinatal period programs the fluid balance restoration pattern in adult offspring

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ABSTRACT

Osmoregulatory mechanisms can be vulnerable to electrolyte and/or endocrine environmental changes during the perinatal period, differentially programming the developing offspring and affecting them even in adulthood. The aim of this study was to evaluate whether availability of hypertonic sodium solution during the perinatal period may induce a differential programming in adult offspring osmoregulatory mechanisms. With this aim, we studied water and sodium intake after Furosemide–sodium depletion in adult offspring exposed to hypertonic sodium solution from 1 week before mating until postnatal day 28 of the offspring, used as a perinatal manipulation model [PM-Na group]. In these animals, we also identified the cell population groups in brain nuclei activated by Furosemide–sodium depletion treatment, analyzing the spatial patterns of Fos and Fos–vasopressin immunoreactivity.

In sodium depleted rats, sodium and water intake were significantly lower in the PM-Na group vs. animals without access to hypertonic sodium solution [PM-Ctrol group]. Interestingly, when comparing the volumes consumed of both solutions in each PM group, our data show the expected significant differences between both solutions ingested in the PM-Ctrol group, which makes an isotonic cocktail; however, in the PM-Na group there were no significant differences in the volumes of both solutions consumed after Furosemide–sodium depletion, and therefore the sodium concentration of total fluid ingested by this group was significantly higher than that in the PM-Ctrol group.

With regard to brain Fos immunoreactivity, we observed that Furosemide–sodium depletion in the PM-Na group induced a higher number of activated cells in the subfornical organ, ventral subdivision of the paraventricular nucleus and vasopressinergic neurons of the supraoptic nucleus than in the PM-Ctrol animals. Moreover, along the brainstem, we found a decreased number of sodium depletion-activated cells within the nucleus of the solitary tract of the PM-Na group.

Our data indicate that early sodium availability induces a long-term effect on fluid drinking and on the cell activity of brain nuclei involved in the control of hydromineral balance. These results also suggest that availability of a rich source of sodium during the perinatal period may provoke a larger anticipatory response in the offspring, activating the vasopressinergic system and reducing thirst after water and sodium depletion, as a result of central osmosensitive mechanism alterations.

1. Introduction

Environmental changes during critical periods of intrauterine life and/or the early postnatal stage (perinatal period) have a strong impact, even in the long term, differentially programming the systems in developing individuals. The process by which early insults at critical stages of development have irreversible, permanent and long-term effects in tissue structure and function is known as intrauterine programming and may result in adult disease originated *in utero*, and this is often called the 'developmental origins of adult disease' hypothesis [1–3]. At present, a great amount of research is being carried out to evaluate how the adult phenotype is a consequence of environmental signals operating on genes during perinatal development. Permanent changes in the homeostatic regulation of these systems could lead to increased risk factors for certain increasingly prevalent diseases in our society, such as hypertension, non-insulin dependent diabetes, impaired glucose tolerance and obesity [2,4–9].

Hydroelectrolytic homeostatic systems are not exempt from the effects of perinatal programming and many studies indicate that, during sensitive periods of ontogeny, different perinatal stimuli such as dehydration, hypernatremia, sodium overload, sodium depletion or

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angiotensin converting enzyme (ACE) inhibitors, induce an imprinting on osmoregulatory mechanisms, altering their endocrine and behavioral regulatory responses [10–16]. Perinatal experiences of the stimuli of sodium overload (water deprivation, dietary sodium overload) or sodium depletion (diuretics administration, repeated vomiting during the first trimester of pregnancy, etc.) alter salt preference, sodium appetite and/or fluid intake of the offspring in adult life [11,13,17–22]. Despite the varying results, all these studies provide increasing evidence that exposure to altered osmotic environments during ontogeny can program subsequent adult systems governing thirst and sodium appetite and, if persistent through adulthood, these alterations may have adverse clinical effects.

On the other hand, it is well known that an increased sodium intake, like that of many other mineral and nutrient requirements, occurs during pregnancy, [23–26]. Barelare and Ritcher (1938) [27] for example, demonstrated for the first time that, when pregnant dams have access to a rich sodium source, the total sodium intake increases between 60–98% compared with pregnant rats who have access to water only. Taking this into account, the present study sought to determine if voluntary access to hypertonic sodium chloride solution during the perinatal period may be sufficient to produce lasting changes in osmoregulatory mechanisms in adult offspring.

The study used two groups of pregnant rats, both fed *ad libitum*, one with access to water, and the other with access to water and also a hypertonic sodium chloride solution (0.45 M NaCl), in order to evaluate whether availability of hypertonic sodium solution during the perinatal period may induce a differential programming of central osmoregulatory mechanisms involved in offspring fluid intake control. For this purpose, we investigated the water and sodium intake induced by Furosemide–sodium depletion treatment in adult offspring. We also analyzed the pattern of cell activity, as shown by Fos-immunoreactivity, within vasopressinergic hypothalamic nuclei and along other brain neuron groups involved in fluid balance regulation.

2. Materials and methods

2.1. Animals

Twenty Wistar-derived female rats, born and reared in the vivarium of the Instituto Ferreyra (INIMEC-CONICET, Córdoba, Argentina), were used in the experiments. Animals 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. Animal handling and experimental procedures were approved by the appropriate animal care and use committee of our institute, and the National Institutes of Health (NIH) Guidelines were followed.

As shown in Fig. 1, seven days before mating, female rats were randomly divided in two groups to receive the corresponding perinatal manipulation (PM): one group had free access to tap water and standard commercial diet (Cargill Inc. Argentina, containing approx. 0.18% NaCl) [PM-Ctrol group] and the other group, in addition to tap water and commercial diet, had voluntary access to a hypertonic sodium chloride solution (0.45 M NaCl) [PM-Na group]. After a week of adaptation, one couple per cage was placed for mating in the same standard holding chamber until a sperm-positive test was obtained, maintaining the hypertonic sodium chloride solution access in the PM-Na group. When the pregnancy was confirmed (1 to 5 days), males were withdrawn and pregnant rats were maintained in the same holding chamber. These experimental conditions were maintained throughout pregnancy. 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 perinatal conditions until pups were weaned at postnatal days 21–22 (PD21–22). At the weaning, dams and their female pups were sacrificed for subsequent plasma parameter analysis. Only male pups continued the experiments, and these received the same conditions as their dam until reaching a month of life (PD28). From then on, males of both experimental conditions were kept in standard conditions of water and food until 2 months of age (PD60–70). No more than 3 males/dam were used for the same condition in each experiment. As we were aware of the sexual dimorphism of sodium appetite [28,29] and also how estrogen level changes influence female fluid intake [30], we decided to analyze only the males' intake in the present study.

2.1.1. Experiment #1.a: Maternal water and hypertonic sodium chloride solution (0.45 M) intake

From the week of adaptation and throughout pregnancy and lactation, the maternal water intake of both PM groups was recorded daily and averaged weekly. The same procedure was also performed with the hypertonic sodium chloride solution intake of PM-Na dams. Data are shown as average of fluid consumed per dam (water, sodium and total fluid ingestion) during the following periods: adaptation week; first week of pregnancy and total pregnancy period; and first, second and third lactation weeks. Dams' intake during the days needed for pregnancy confirmation was not taken into account to calculate the volume drunk in any period. The number of pregnancy days, litter size born and percentage of male offspring were also recorded.

2.1.2. Experiment #1.b: Plasma sodium concentration, osmolality and protein assays in dams and female pups

On the weaning day, plasma of dams and female pups was collected by decapitation. Trunk blood was collected in plastic tubes containing EDTA (final concentration 2 mg/ml blood) and immediately centrifuged at 4 °C for 20 min at 3.000 *g*. Then plasma was removed and kept at −20 °C until determination (note that pooled blood was not necessary for the pup measurements). Plasma sodium concentration [Na^+],was determined using an Ion Selective Electrode (Hitachi Modular P + ISE. Roche 8 Diagnostic). Plasma osmolality was analyzed by vapor pressure osmometry (VAPRO 5520) and plasma volume was indirectly inferred by the plasma protein concentration, measured in an

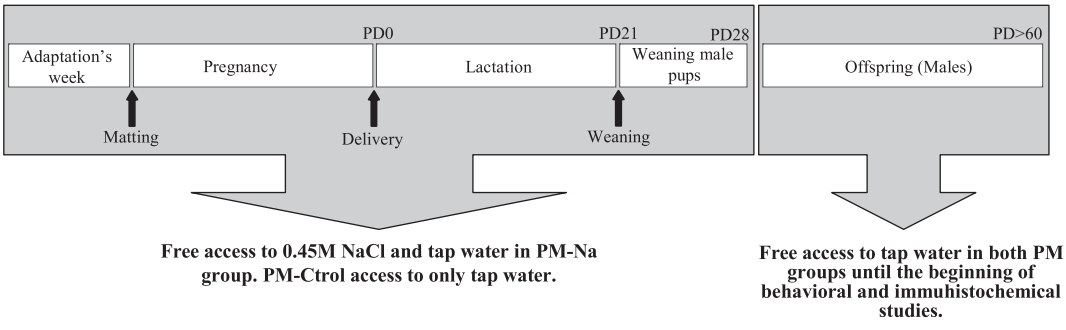


Fig. 1. Schematic diagram showing the conditions to which female rats and offspring were subjected from adaptation week until the beginning of behavioral and brain immunohistochemical studies. Note that from PD28 until PD60, males of both PM groups were kept in standard conditions.

absorbance microplate reader (BioTek EL800), according to the protocol proposed by Lowry et al. (1951); [31].

2.1.3. Experiment #2.a: Need-free water and sodium intake in adult offspring

To assess adult offspring sodium and water intake, males (≥PD60) were placed in individual metabolic cages with access to commercial diet, deionized water and hypertonic sodium solution (0.45 M NaCl) in graduated tubes. Twenty-four hours' spontaneous intake of both solutions was recorded in the morning before sodium-depletion treatment. It is important to note that the first 24 h of the spontaneous intake of both solutions were discarded. Body weight (BW) of animals of both PM conditions was analyzed at the beginning of the experimental protocol (BW-day 1) and before sodium-depletion treatment (BW-day 3).

2.1.4. Experiment #2.b: Water and sodium intake after Furosemide–sodium depletion treatment in adult offspring

Males from Exp.#2.a were randomly assigned to sodium-replete [Veh] or Furosemide–sodium depleted [Furo] groups. Sodium depletion was carried out by a combined treatment of Furosemide and sodium-free diet (SFD), according to the Sakai et al. protocol (1986); [32]. The animals were injected with Furosemide (Lasix, Sanofi Aventis lab, sc; 40 mg/kg.) in 2 injections separated by 2 h (at noon). At the time of the first injection, commercial diet was replaced by SFD and hypertonic sodium chloride solution was withdrawn. The sodium-replete group was injected with the corresponding volume of vehicle solution (0.15 M NaCl) and had access to a commercial diet. Twenty hours after, the animals were subjected to the sodium and water intake test for 2 h. The cumulative volume ingested was measured at 5, 10, 15, 30, 60, 90 and 120 min.

To quantify the effect of Furosemide–sodium depletion treatment, overnight water intake, body weight at the time of the need-induced intake test (BW-day 4) and percentage of body weight lost (% BW Lost) were recorded. Finally, sodium concentration (mM NaCl) of the ingested fluid was calculated as [(NaCl intake (ml/100gbw) × molality of the hypertonic sodium solution (450 mM NaCl))/total fluid intake (ml/100gbw)] at 15, 30, 60, 90 and 120 min during water and sodium intake test.

2.1.5. Experiment #2.c: Brain pattern of Fos-ir and double-immunolabeled (Fos-AVP) cells after Furosemide–sodium depletion in adult offspring

For the immunohistochemical studies, we used separate groups of males, different from those used in behavioral studies. All animals from both PM conditions were sodium depleted using two different doses of Furosemide: 8 mg/kg [Low Furo] and 40 mg/kg [High Furo]. The groups were randomly assigned to one of two different Furosemide doses. Males were injected with Furosemide (Lasix, Sanofi Aventis lab, sc) in 2 injections separated by 2 h (at noon). At the time of the first injection, commercial diet was replaced by sodium-free diet (SFD) and hypertonic sodium chloride solution was withdrawn. Twenty hours after sodium depletion, animals were subjected to immunohistochemical detection of Fos and Fos-AVP. It is important to note that despite *c-fos* being an immediate early gene, normally activated transiently and rapidly in response to a variety of cellular challenges, our previous works [33–36] demonstrated that a persistent stimulation, such as hyponatremia and/or hypovolemia induced by sodium depletion, produced an increased Fos immunoreactivity that persisted over the time until homeostatic balance was achieved.

2.1.6. Immunohistochemistry

Animals were anesthetized with chloral hydrate, 6% (0.6 ml/100 gbw, ip) and perfused transcardially with ~100 ml of normal saline solution followed by ~400 ml of 4% paraformaldehyde 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. They were then incubated in 10% normal horse serum (NHS- Gibco, Auckland, NZ) in PB for 1 h to block non-specific binding sites. Fos-ir was detected using a standard avidin–biotin peroxidase protocol. The staining procedures followed the double-labeling procedures described in Franchini and Vivas, 1999 and Godino et al., 2005; [33,37]. The free-floating sections were incubated overnight at room temperature in an antibody raised in rabbits against a synthetic 14-amino acid sequence corresponding to residues 4–17 of human Fos (Ab-5, Oncogene Science, Manhasset, NY), diluted 1:10,000 in a solution of PB containing 2% NHS and 0.3% Triton X-100 (Sigma Chemical Co., St. Louis, MO, USA). After being washed in PB, the sections were incubated in biotin-labeled anti-rabbit immunoglobulin (Jackson Immunoresearch Laboratories, diluted 1:500 in 1% NHS-PB) and avidin–biotin peroxidase complex (Vector Laboratories Inc., Burlingame, CA, USA) diluted 1:200 in 1% NHS-PB for 1 h 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.

Subsequently, hypothalamic Fos-labeled sections were processed for immunocytochemical localization of vasopressin (AVP). Sections were incubated for 72 h at 4 °C in polyclonal rabbit anti-AVP antibody (Chemicon International Inc.) diluted 1:10,000 in a solution of PB containing 2% NHS and 0.3% Triton X-100 (Sigma Chemical Co., St. Louis, MO, USA). After incubation, sections were rinsed and incubated in biotin-labeled anti-rabbit immunoglobulin (Jackson Immunoresearch Laboratories, diluted 1:500 in 1% NHS-PB) and avidin–biotin peroxidase complex (Vector Laboratories Inc., Burlingame, CA, USA, diluted 1:200 in 1% NHS-PB). Cytoplasmic AVP-ir was detected with unintensified DAB to produce a brown reaction product. Finally, the free-floating sections were mounted on gelatinized slides (with Albrecht's gelatine), air-dried overnight, dehydrated, cleared in xylene, and placed under a coverslip with DPX mounting medium (Fluka, Buchs, Switzerland).

2.1.7. 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 of Paxinos and Watson (1997); [38].

The numbers of Fos-ir nuclear profiles in the sections were counted at three levels for different PVN subnuclei, i.e., medial magnocellular (PaMM), lateral magnocellular (PaLM), posterior (PaPo), ventral (PaV) and medial (PaPM) parvocellular subdivisions (distance from the bregma of the corresponding plates: from −0.92 to −2.12 mm). The Fos-ir cells of all the other nuclei were counted at one level. The distance from the bregma of corresponding plates is indicated between brackets: *organum vasculosum of the lamina terminalis* (OVLT, −0.20 mm), median preoptic nucleus (MnPO, −0.40 mm), subfornical organ (SFO, −0.92 mm), supraoptic nucleus (SON, −1.3 mm), dorsal raphé nucleus (DRN, −8.00 mm), lateral parabrachial nucleus (LPBN) and locus ceruleus (LC) in plate with a distance from bregma of −9.3 mm. Finally, area postrema (AP) and nucleus of the solitary tract (NTS) in plate with a distance from bregma of −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 digitized 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 done in four to six 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 investigator who conducted the counting of Fos-ir cells was blinded to the experimental groups. Because one section of each nucleus was quantified, no corrections were necessary to avoid double counting. Two series of sections were used in the quantitative analysis; in one series of sections, Fos-ir cells were counted using the computerized image analysis previously described, and in the other, Fos and AVP-labeled cells were hand counted.

2.1.8. Statistical analysis

All data are expressed as mean ± SE. All variables were analyzed by appropriate analyses of variance (ANOVA) with repeated measures (time or solution) where appropriate. Both main effects and interactions were considered to be statistically significant at $p<0.05$. Analyses were performed utilizing Statistica Stat-Soft Inc. version 5.0 Tulsa, OK, USA. Experimental design for each ANOVA was:

- 1-way ANOVA for maternal sodium intake (Exp.#1.a); plasma sodium concentration, osmolality and protein concentration assays in dams and female pups at PD21 (Exp.#1.b) and for body weight analysis (days 1 and 3) in adult offspring;
- 2-way ANOVA for percentage of body weight loss and body weight to day 4, overnight water intake after Furosemide–sodium depletion (Exp.#2.b); and cerebral nuclei analyses (Exp.#2.c);
- 2-way ANOVA with repeated measures for maternal water and total intake (Exp.#1.a); need-free water and sodium intake in adult offspring (Exp.#2.a) and for sodium chloride concentration of total fluid ingested (mM NaCl) after Furosemide–sodium depletion in adult offspring (Exp.#2.b);
- 4-way ANOVA with repeated measures for water and sodium intake after Furosemide–sodium depletion in adult offspring (Exp.#2.b). It is important to note that, although Fig. 5 was constructed with cumulative data, the 4-way ANOVA was done using non-cumulative data.

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

3. Results

3.1. Experiment #1.a: Maternal hypertonic sodium chloride solution and water intake and pregnancy parameters

As expected, average maternal water intake progressively increased throughout the different periods [$F(5,80)=53,36$; $p<0.001$, Fig. 2]. With respect to PM conditions, no significant differences were observed in average water intake in dams of both PM groups. There was no significant interaction between PM groups and periods (see Fig. 2A). Maternal hypertonic sodium chloride solution intake data showed a significant main effect of Period factor [$F(5,36)=2,56$; $p<0.05$] (see Fig. 2B). Pairwise comparisons revealed that maternal hypertonic sodium intake increased significantly during the first pregnancy week and total pregnancy period compared to the adaptation week and the lactation period (p values <0.05 and 0.01, respectively). Moreover, total maternal fluid intake, predictably, increased throughout the different periods [$F(5,70)=42,48$; $p<0.001$]; however, no significant differences were observed between PM groups, nor significant interaction between PM groups (see Fig. 2C).

Pregnancy parameters were also recorded from PM-Ctrol Dams ($n=6$) and PM-Na Dams ($n=6$). No significant differences were observed between dams of either PM group in number of pregnancy days (22.33 ± 0.23 vs. 22.83 ± 0.18), litter size (10.33 ± 1.12 vs. 9.83 ± 0.82 pups per dam) or male percentage of offspring (46.13 ± 7.42 vs. 35.88 ± 4.55), suggesting that perinatal manipulation would not alter these pregnancy parameters.

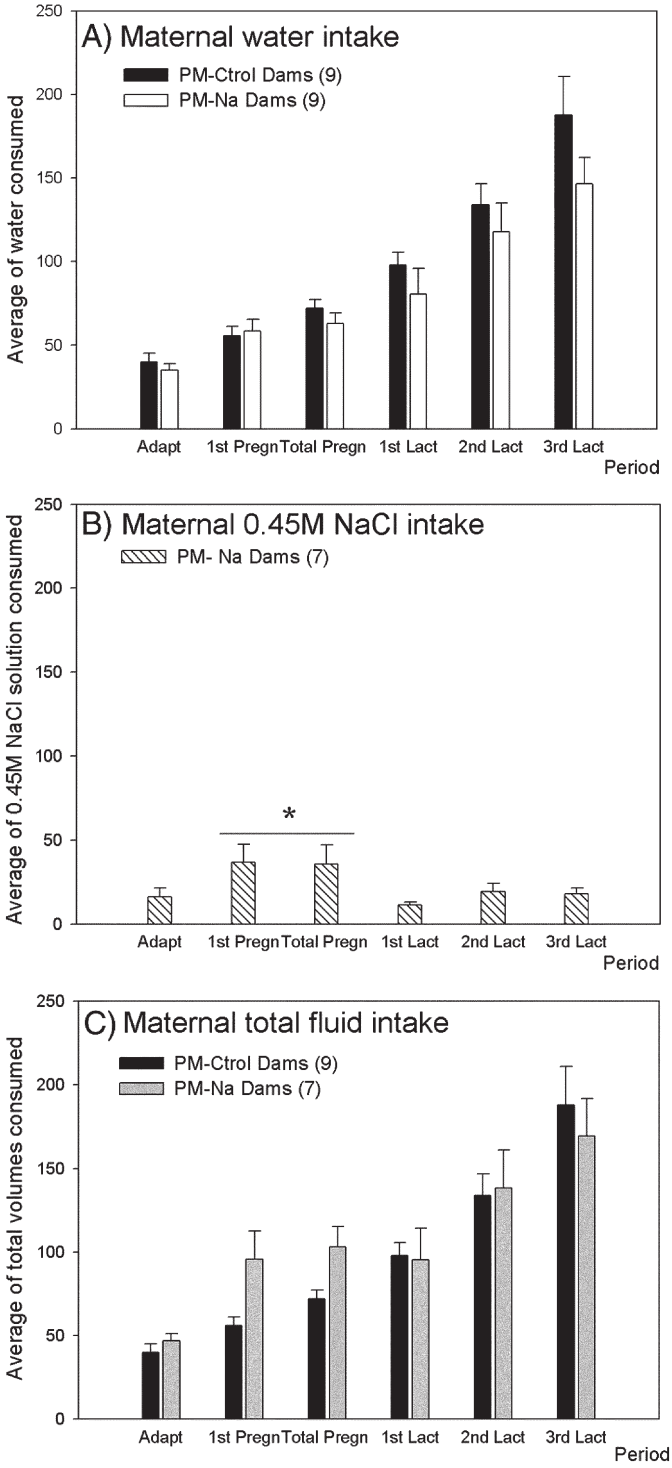


Fig. 2. Maternal water (A), 0.45 M NaCl (B) and total fluid (C) intake in dams of both PM conditions. Values are means ± SE, (n). Adapt: adaptation week, 1st Pregn: First pregnancy week, Total Pregn: Total pregnancy period, 1st Lact: First lactation week, 2nd Lact: 2nd lactation week, 3rd Lact: 3rd lactation week. (*) $p<0.05$ 1st pregnancy week and Total pregnancy significantly different vs. Adaptation week and lactation periods in PM-Na Dams.

3.2. Experiment #1.b: Plasma sodium concentration, osmolality and protein assays in dams and female pups

3.2.1. Rat dams at weaning

As shown in Table 1, access to hypertonic sodium chloride solution during the perinatal period did not affect plasma sodium concentration, osmolality or protein concentration in dams. The absence of differences in those parameters might be explained by the efficiency of maternal fluid homeostatic systems compensating for variations caused by the voluntary ingestion of hypertonic sodium chloride solution. It is equally possible that the plasma variables between the groups were similar because intake was not elevated at that time, and for the three periods before the dams were killed (see Fig. 2).

3.2.2. PD21 female pups

Results show that availability to hypertonic sodium chloride solution during the perinatal period specifically affects plasma sodium concentration and osmolality [$F(1,14)=5{,}76$; and $F(1,14)=4{,}71$, respectively; $p<0.05$] of female pups at PD21 (Table 1). Furthermore, no significant difference was observed in plasma protein concentration.

The increase observed in plasma sodium concentration and osmolality of PM-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 sodium chloride solution.

3.3. Experiment #2.a: Need-free water and sodium intake in adult offspring

As expected, need-free sodium intake was significantly lower than need-free water intake [$F(1,57)=193{,}33$; $p<0.001$]. However, as shown in Fig. 3, whatever the solution ingested, need-free total intake of PM-Na animals was significantly lower than need-free total intake of PM-Ctrol animals [$F(1,57)=5{,}13$; $p<0.05$]. There was no significant interaction between PM groups and solutions.

It is important to note that no significant differences were observed in body weight measurements between animals of both PM groups at the beginning of the experimental protocol (BW-day 1) or before Furosemide–sodium depletion treatment (BW-day 3), showing that PM has no effects on body mass of animals (see Table 2).

3.4. Experiment #2.b: Water and sodium intake after Furosemide–sodium depletion treatment in adult offspring

As shown in Fig. 4, overnight water intake was significantly diminished by Furosemide treatment as expected, [$F(1,57)=23{,}51$; $p<0.001$]; however, and continuing with a similar pattern observed in need-free ingestion (Exp.#2.a), where PM-Na animals drink less water than PM-Ctrol animals, our results show that overnight water intake was significantly reduced in the PM-Na group compared with the PM-Ctrol group [$F(1,57)=4{,}31$; $p<0.05$]; besides there was a

Table 1
Plasma sodium concentration, osmolalities and protein concentrations measured in dams and female pups of both PM conditions at weaning (PD21).

	PM conditions	Plasma sodium concentration (mM)	Plasma osmolality (mosmol/kgH_2O)	Plasma protein concentration (g/dl)
Dams	PM-Ctrol	147.86 ± 1.51 (5)	303.17 ± 2.45 (5)	48.4 ± 2.66 (5)
	PM-Na	146.20 ± 1.26 (6)	303.03 ± 1.64 (6)	54.99 ± 3.57 (7)
Female pups	PM-Ctrol	161.79 ± 2.05 (8)	296.88 ± 1.82 (8)	42.24 ± 1.46 (8)
	PM-Na	170.60 ± 3.35 (8)*	304.06 ± 3.04 (8)*	43.04 ± 1.34 (8)

Values are means ± SE, (n).
* $P<0.05$ significantly different from PM-Ctrol group in female pups.

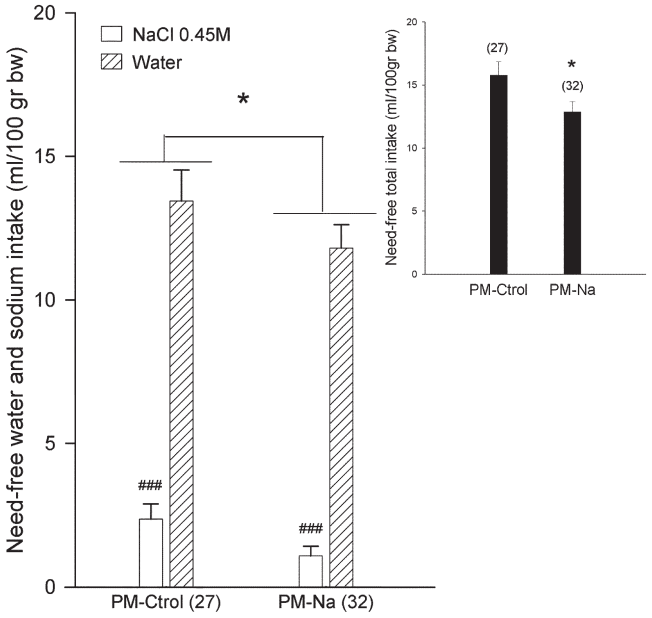


Fig. 3. Need-free water and sodium intake during 24 h before Furosemide–sodium depletion. Inset in panel represents the need-free total intake before Furosemide–sodium depletion, irrespective of solutions. Values are means ± SE, (n). (*) $p<0.05$ significantly different from PM-Ctrol group. (###) $p<0.001$ significant differences between solutions.

significant interaction between PM conditions and Furosemide treatment [$F(1,57)=4{,}05$; $p<0.05$]. Pairwise comparisons revealed that overnight water intake did not differ between PM groups in Furosemide–sodium depleted animals [Furo-group], whereas in sodium-replete conditions [Veh-group] the PM-Na animals consume significantly lower volumes of water vs. the PM-Ctrol group (p values <0.01).

Together with the overnight water intake analysis, we studied the body weight (BW-day 4) and percentage of body weight lost (% BW lost) in response to Furosemide sodium-depletion treatment, in both PM groups before the need-induced intake test. These analyses made it possible to rule out differences not related to sodium-depletion. Note that, as expected, BW-day 4 and% BW lost were affected for Furosemide–sodium depletion [$F(1,58)=10{,}07$; $p<0.01$ and $F(1,58)=255{,}24$; $p<0.001$, respectively] but these parameters were not affected for PM conditions, suggesting that Furosemide–sodium depletion had an equivalent effect among animals of both PM groups (see Table 2).

Predictably, need-induced sodium and water intake were significantly increased by Furosemide–sodium depletion [$F(1,50)=67{,}37$; $p<0.001$] and water volumes consumed were higher than those of sodium [$F(1,50)=9{,}16$; $p<0.01$]. In PM groups, data showed a significant PM main effect [$F(1,50)=5{,}79$; $p<0.01$]. There were significant interactions between PM conditions and sodium-depletion treatment [$F(1,50)=6{,}68$; $p<0.01$] and between PM conditions and solutions [$F(1,50)=7{,}86$; $p<0.001$]. There were also significant 3-way interactions between PM conditions, sodium-depletion treatment and solutions [$F(1,50)=5{,}97$; $p<0.01$].

Pairwise comparisons revealed that, in basal conditions [Veh group], there were no differences between PM groups in the ingestion of both solutions. However, when the animals were Furosemide–sodium depleted [Furo group], the PM-Na group showed decreased water intake at minute 15 and decreased sodium intake at minute 60 of the intake test (p values <0.01; Fig. 5A and B). Interestingly, comparing the volumes consumed of both solutions (water/sodium) in each PM group subjected to Furosemide–sodium depletion, data show significant differences between solutions in the PM-Ctrol group (p value <0.001) but no significant differences were observed

Table 2
Body weights (BW) at the beginning of the experimental protocol (BW-day 1), before sodium-depletion (BW-day 3), at time of the need-induced intake test (BW-day 4) and percentage of body weight lost (% BW Lost) in animals of both PM conditions.

PM condition	BW-day 1	BW-day 3	BW-day 4		% BW lost	
			Veh	FURO	Veh	FURO
PM-Ctrol	289.26 ± 8.17 (23)	280.93 ± 7.38 (27)	287 ± 14.42 (9)	252.16 ± 8.64 (18) ##	2.80 ± 1.30 (9)	−10.54 ± 0.49 (18) ###
PM-Na	299.33 ± 5.15 (33)	293.57 ± 5.30 (35)	289.66 ± 9.56 (12)	268.83 ± 5.54 (23) ##	1.61 ± 1.00 (12)	−9.66 ± 0.59 (23) ###

Values are means ± SE, (n). (##) P<0.01 and (###) P<0.001 significantly different from Veh group.

when comparing the volumes consumed of both solutions in the PM-Na group (p = 0.49).

As would be expected following the above observations, given the differences in sodium and water intake during the 2-h test in the PM groups, the sodium chloride concentration of the total fluid ingested (mM NaCl) was significantly affected by PM conditions [F(1,31) = 11,18; p<0.01] and by the times analyzed [F(4,124) = 27,23; p<0.0001] (see Fig. 5C). In Furosemide–sodium depleted animals, sodium chloride concentration of total fluid ingested by the PM-Na group was significantly higher than that ingested by the PM-Ctrol group (p value<0.001). These data suggest that the PM-Na animals, when stimulated to drink water and sodium, make a hypertonic cocktail instead of the isotonic one usually made by the control animals.

3.5. Experiment #2.c: Brain pattern of Fos-ir and double-immunolabeled (Fos-AVP) cells after Furosemide–sodium depletion in adult offspring

3.5.1. Lamina terminalis

Along the OVLT and MnPO nuclei, there were no significant differences in the number of Fos-ir cells in relation to the PM conditions (PM-Ctrol vs. PM-Na groups) or in the levels of Furosemide–sodium depletion (Low-Furo and High-Furo doses), (see Fig. 6). With respect to the SFO, there was a significant increase in the number of Fos-ir cells in the PM-Na group in comparison to the PM-Ctrol group [F(1,15) = 4,86; p<0.05] and also between the two levels of Furosemide–sodium depletion (Low-Furo and High-Furo doses), [F(1,15) = 8,99; p<0.01]. Nevertheless, there was no significant interaction between the factors (PM conditions and Furosemide doses). The pattern of labeling in SFO activated cells is shown in Fig. 9.

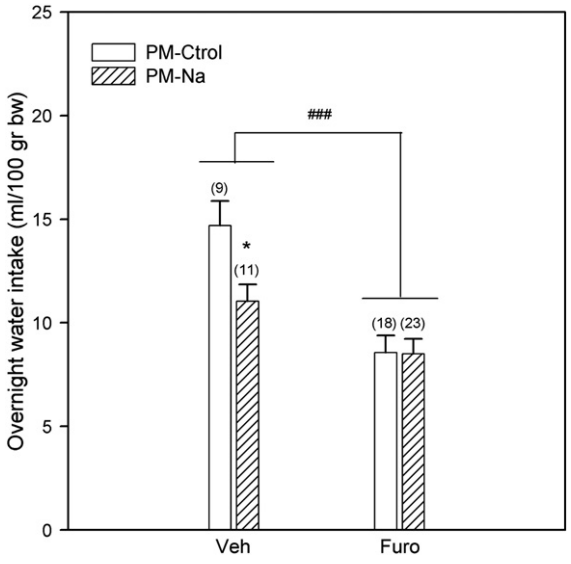


Fig. 4. Overnight water intake after Furosemide–sodium depletion. Values are means ± SE, (n). (*) p<0.05 significantly different from PM-Ctrol group in sodium-replete animals. (###) p<0.001 Veh vs. Furo group.

3.5.2. SON and PVN hypothalamic nuclei

The entire supraoptic nucleus (SON) showed an increased level of *c-fos* expression in the PM-Na group compared to the PM-Ctrol group [F(1,15) = 7,88; p<0.01], see Fig. 7A. Analyzing the SON vasopressinergic cells activated by Furosemide–sodium depletion, we found significant differences between both PM groups [F(1,15) = 8,62; p<0.01], Fig. 7B. The pattern of double-labeling cells in the SON is shown in Fig. 9.

No significant differences were found in Fos activation in relation to Furosemide doses nor a significant interaction between the factors (PM conditions and Furosemide doses). As regards the percentage of Fos-AVP cells activated after Furosemide–sodium depletion in both PM groups, approximately 22% of the activated cells were vasopressinergic in the PM-Ctrol group with a Low-Furo dose, while in the High-Furo dose, no double-labeled cells were observed. In addition, 40% and 35% of the activated cells in the PM-Na group with Low and High Furo, respectively, were vasopressinergic. Thus, the data show a greater number of vasopressinergic cells activated by both Furosemide doses in PM-Na animals, suggesting a comparatively major activation of the vasopressinergic system.

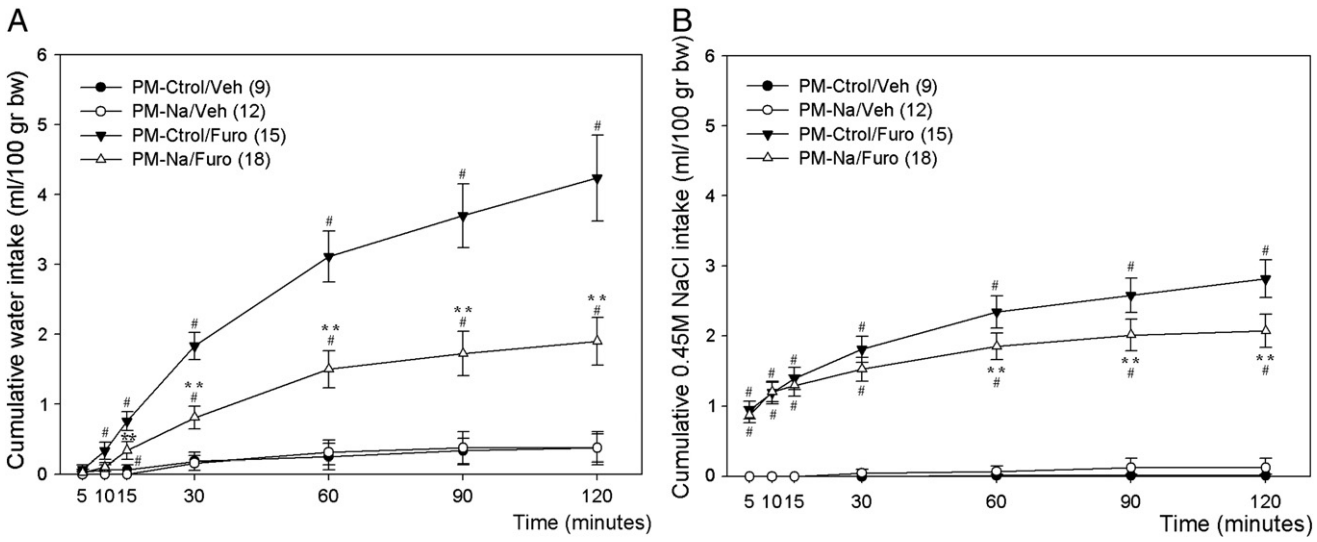
The following PVN subdivisions analyzed, PaLM, PaPM, PaMM and PaPo, showed no significant differences in the number of Fos-ir cells between both factors (PM conditions and Furosemide doses). However, within the ventral subdivision of the paraventricular nucleus (PaV), the data showed a significant main effect of the PM conditions with a high *c-fos* expression in the PM-Na group compared with PM-Ctrol group [F(1,15) = 27,54; p<0.001]. In addition, a significant interaction between the PM conditions and sodium-depletion treatment was observed [F(1,15) = 6,28; p<0.05], see Fig. 7A. Pairwise comparisons showed an increased number of Fos-ir cells of the PM-Na group compared with the PM-Ctrol group when animals were exposed to High-Furo doses, (p value<0.001).

3.5.3. Brainstem nuclei

No significant differences were found in the number of Fos-ir cells between the PM conditions and levels of Furosemide-doses along the LC, NDR and AP nuclei (see Fig. 8). Regarding the cell activity found within the NTS in response to PM, we observed a significantly reduced number of Fos-ir cells in the PM-Na group [F(1,13) = 4,71; p<0.05, Fig. 8]. The pattern of labeling cells in NTS is shown in Fig. 9. On the other hand, with respect to the *c-fos* expression observed along the LPBN, there was a significant increase induced by the higher dose of Furosemide, regardless of the PM conditions [F(1,16) = 10,93; p<0.01]. There was no significant interaction between the factors (PM conditions and Furo doses) in any of the brainstem nuclei analyzed.

4. Discussion

The present study is the first to demonstrate how the availability of a rich source of sodium, by voluntary access to a hypertonic sodium chloride solution during the perinatal period, can modify the induced-fluid intake of adult offspring and also their brain pattern of cell activity as shown by Fos immunoreactivity. Our results show that PM-Na descendants drink reduced amounts of water, not only



C

Minutes	NaCl concentration of total fluid ingested (mM NaCl)	
	PM-Ctrol/Furo (15)	PM-Na/Furo (18)
15	301.67±28.55	388.68±23.70 **
30	229.84±11.78	319.59±26.06 **
60	203.21±10.25	276.65±23.34 **
90	197.26±9.91	271.52±22.69 **
120	194.15±8.60	262.79±22.50 **

Fig. 5. Cumulative water (A), and 0.45 M NaCl intake (B) of sodium-depleted adult offspring. Furo group (triangles); Veh group (circles). PM-Ctrol group (filled symbols); PM-Na group (open symbols). Panel C shows sodium chloride concentration of total fluid ingested (mM NaCl) in sodium-depleted animals of both PM conditions at different times after Furosemide-induced fluid intake. Values are means ± SE, (n), bw: body wt. (**) $p<0.01$ significantly different from PM-Ctrol group when animals were Furosemide–sodium depleted. (#) $p<0.001$ significantly different from Veh group.

during Furosemide–sodium depletion but also during need-free conditions. These data are consistent with the findings of Contreras' laboratory made in adult offspring subjected to dietary sodium overload (3% NaCl) during the perinatal period. They observed a marked decrease of water intake associated with sodium intake after 10 days of dietary sodium deprivation [11]. Previous work from Contreras and Kosten (1983) [21] also showed that both male and female offspring raised on a high salt diet showed a greater preference for sodium solution as adults, with differences in water intake being responsible for the significant effects on preference. It is important to note that, in both these works, the animals are subjected to obligatory sodium overload, while the present work analyzes the effect in adult offspring of perinatal hypertonic sodium consumption as a result of a free choice. Although the aim of the present study was to analyze a similar protocol but giving the animals the opportunity to drink hypertonic sodium chloride, this protocol does not enable us to rule out whether the changes observed later in life are due to maternal intake during pregnancy, or during lactation, or by offspring voluntary consumption until PD28, and at which stage of those periods. Our previous unpublished data shows, for example, that an animal's access to hypertonic sodium chloride after weaning and until adulthood did not induce any significant difference in water and sodium ingestion stimulated by sodium depletion, demonstrating that this period at least may be not enough to induce any programming. Further studies are necessary to discriminate the precise stage responsible for the changes observed later in life.

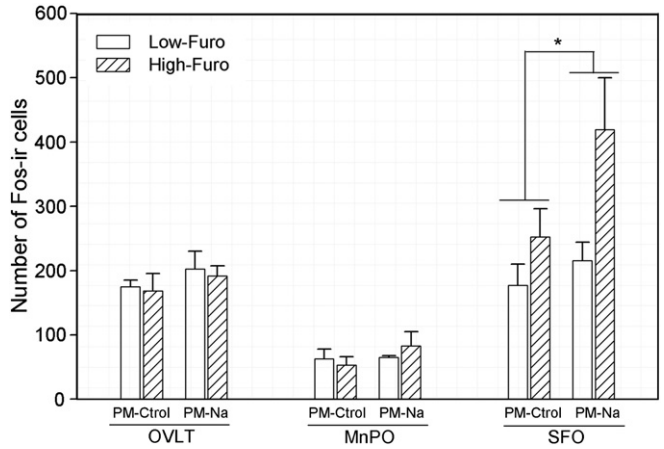


Fig. 6. Average number of Fos-immunoreactive (Fos-ir) cells in the *lamina terminalis* nuclei (OVLT, MnPO and SFO) of both PM groups, after injection of two different doses of Furosemide (Low-Furo and High-Furo). Values are means ± SE. (*) $p<0.05$ significantly different from PM-Ctrol group. SFO = subfornical organ; OVLT = *organum vasculosum of the lamina terminalis*; MnPO = median preoptic nucleus.

Previous studies [10–13,16,21,22,39] have shown that early challenges to osmoregulatory mechanisms involving electrolyte and/or endocrine environmental changes, lead to persistent changes in need-free and stimulated fluid intake and, while most of the work has shown perinatal osmotic alterations, they have contradictory results with regard to how they affect adult offspring fluid intake, and specifically, whether the perinatal manipulation increases or decreases water or sodium ingestion of the adult offspring. Whatever the final PM effect, sometimes the physiological result in terms of body sodium status may be similar. For example, if reduced sodium ingestion in offspring is associated with relatively

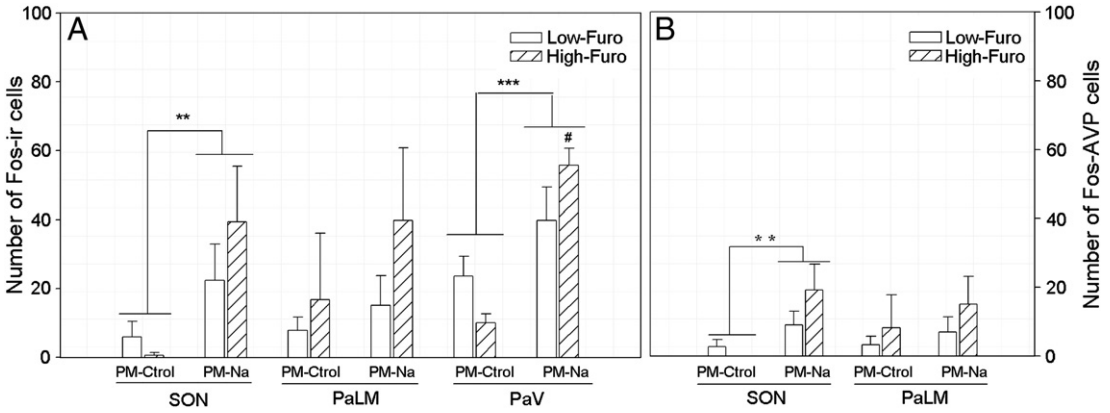


Fig. 7. A) Average number of Fos-immunoreactive (Fos-ir) cells in the supraoptic nucleus (SON), and paraventricular hypothalamic nucleus along the lateral magnocellular and ventral subdivisions (PaLM and PaV, respectively) of both PM groups after injection of two different doses of Furosemide (Low-Furo and High-Furo). B) Average number of Fos/vasopressin-immunoreactive (Fos/AVP-ir) double-labeled cells in the SON and PaLM subdivision of the paraventricular nucleus of both PM groups after injection of two different doses of Furosemide (Low-Furo and High-Furo). Values are means ± SE. (#) $p<0.05$ significant differences between PM-Na/High-Furo vs. PM-Ctrol/High-Furo. (**) $p<0.01$ and (***) $p<0.001$ significantly different from PM-Ctrol group.

lower water ingestion, the sodium concentration of the total fluid ingested (mM of NaCl) is high, and the body sodium overload may be similar to when sodium appetite is stimulated without changes in the associated water intake. Our results in sodium-exposed animals show small differences in sodium chloride intake but larger differences in water intake, i.e., reduced water consumption when adult offspring are stimulated by Furosemide–sodium depletion. Thus, the sodium chloride concentration of the total fluid ingested was proportionally higher in the PM-Na group during induced intake, suggesting that PM-Na descendants compose a more hypertonic cocktail to restore their hydromineral balance, drinking less water for each unit of NaCl consumed. Consistent with our results, Mouw et al. (1978) [39], in an opposite treatment of prenatal and early postnatal sodium diet deprivation, found that this perinatal manipulation had no effect on salt preference; however, they observed a persistent increase in the fluid intake of the adult animals due to increases in water intake (a 20% enhancement approximately).

Our results also show that early sodium availability induces comparatively higher brain cell activity after Furosemide–sodium depletion in key areas involved in osmoreception, such as the subfornical organ and the hypothalamic supraoptic nucleus. The fact that sodium-exposed animals have increased activity of these areas may reflect a sensitization of osmosensitive circuits in these animals as a result of plasticity changes induced by perinatal manipulation. Moreover, in the case of the SON of PM-Na animals, we found a greater number of vasopressinergic cells activated by both Furosemide doses (20% and 35% increase in response to Low and High dose of Furosemide, respectively) as compared with PM-Ctrol animals, suggesting a comparatively major activation of the vasopressinergic system. Since this system is mainly involved in renal water recapture, minimizing the fall in blood pressure as a result of hypovolemia caused by sodium depletion, our results show increased vasopressinergic activity and decreased thirst during the test. These observations may be explained by a SFO-SON pathway sensitization induced by perinatal sodium availability that allows PM-Na animals to have a larger anticipatory response, reabsorbing more water after Furosemide treatment and thus drinking less water, making a more hypertonic cocktail during the intake test.

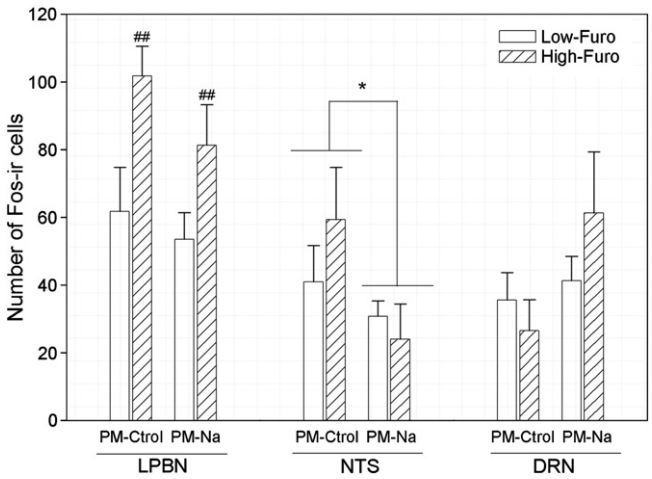


Fig. 8. Average number of Fos-immunoreactive (Fos-ir) cells in lateral parabrachial nucleus (LPBN), nucleus of the solitary tract (NTS), and dorsal raphé nucleus (DRN) of both PM groups after injection of two different doses of Furosemide (Low-Furo and High-Furo). Values are means ± SE. (*) $p<0.05$ significantly different from PM-Ctrol group. (##) $p<0.01$ significant differences between Furosemide doses (Low vs. High).

The subfornical organ plays a major role in many aspects of body fluid homeostasis; it is a circumventricular organ, and thus lacks a blood–brain barrier and contains cells which are sensitive to humoral signals, such as changes in plasma and cerebrospinal fluid sodium concentration, osmolality and ANG II levels. The SFO cells project, directly or indirectly via the MnPO, to the magnocellular portion of the hypothalamic supraoptic and the paraventricular nuclei. Vasopressin synthesis and release, and subsequent hydroelectrolytic balance regulation, are modulated by means of these projections [40,41].

Several studies have clearly identified mechanisms through which increases in CSF sodium concentration and circulating ANGII may exert effects on SFO excitability, and have also addressed the central pathways through which such activation controls neuroendocrine and autonomic output, such as vasopressin, oxytocin, and ACTH secretion as well as sympathetic modulation. One of the main pathways involved in these functions comprises the SFO efferent projections to the supraoptic and the paraventricular nuclei [42–47]. It seems likely, therefore, that, during the present imprinting, SFO excitability may trigger SFO-SON pathway sensitization and, when the offspring is challenged by a hypovolemic thirst model, the vasopressinergic cells are more active and in this way modulate fluid drinking secondarily.

Another explanation of our results may involve plasticity changes in the osmosensitive mechanisms that may alter the osmotic threshold for AVP release and subsequent water drinking. Previous studies of Ross' laboratory have demonstrated that the plasma osmolality threshold for AVP release was increased in offspring exposed in utero to an 8–10 mEq/l increase in maternal hypernatremia in response to maternal water restriction. In the same model, the authors demonstrated alterations in pituitary AVP content and hypothalamic AVP synthesis.

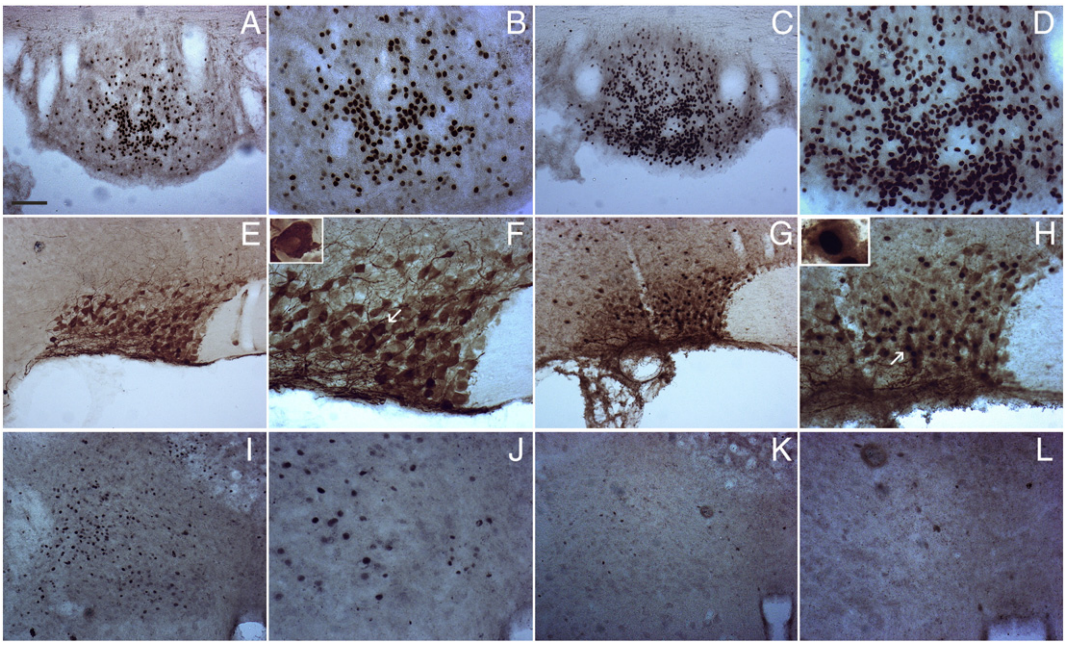


Fig. 9. Photomicrographs showing the pattern of Fos-immunoreactivity in the subfornical organ (SFO, A–D) and in the nucleus of the solitary tract (NTS, I–L) and double immunoreactivity cells (Fos-AVP) in the supraoptic nucleus (E–H) of PM-Ctrol (A,B,E,F,I,J) and PM-Na (C,D,G,H,K,L) animals after the injection of a high dose of Furosemide. Plates B, D, F, H, J, L are higher magnifications (20×) of the plates shown in A, C, E, G, I and K (10×). Small squares in plates F and H show higher magnifications (40×) of cells indicated by arrows. Scale bar = 100 μm.

However, in this imprinting model, together with maternal hypernatremia, the nutritional stress provoked by dehydration-induced anorexia may induce chronic alterations of renal AVP responsiveness [14,15].

An additional possibility is that the present imprinting may alter the complex mechanisms that underlie the regulation of vasopressin release from magnocellular neurosecretory cells. Previous studies [48–50] have shown that these cells have endogenous osmosensitivity and, through the modulation of stretch-inactivated cation channels, depolarize in response to hypertonic stimuli to release vasopressin, and hyperpolarize in response to hypotonic stimuli, decreasing AVP release. Besides the mechanosensitivity of magnocellular neurosecretory cells, they are also under the modulation of several peptides such as angiotensin II. The excitatory effect of hypertonic stimuli is greater when these cells are exposed to Ang II, and conversely, depolarizing responses to Ang II are inhibited under hypotonic conditions [51,52]. In our model, PM-Ctrol animals show the usual response to Ang II in a hypotonic condition, just as after Furosemide–sodium depletion, where the excitatory response to Ang II is canceled by the hyponatremia and thus AVP release is inhibited. On the other hand, in the PM-Na group, persistent perinatal access to a hypertonic stimulus may alter the interaction between osmotic (hyponatremia) and peptidergic stimuli (Ang II), inducing a major activation of vasopressinergic cells.

Two other nuclei were differentially activated by the present imprinting: the parvocellular subdivision of the paraventricular nucleus PaV and the nucleus of the solitary tract (NTS). This result is consistent with the fact that vasopressinergic neurons in the PaV innervate the NTS, while most of NTS neurons receiving arterial baroreceptor afferent inputs are AVP sensitive, giving an important role to AVP and the PVN-NTS pathway in modulating volume and cardiovascular responses [53,54]. Cardiac vagal afferents terminating in the NTS are believed to participate in stimulating neurohypophysial secretion of vasopressin as well as increased ingestion of water and NaCl solution in response to decreased blood volume. This area is known to be important for the integration of cardiovascular reflexes and is the primary termination site within the brain for sensory afferents innervating the heart and circulation. Several studies have demonstrated that AVP may influence the cardiovascular-related functions of the NTS, increasing arterial pressure, heart rate and circulating catecholamines, suggesting activation of the sympathetic nervous system. There is evidence that the increased release of AVP may enhance the sensitivity of some neurons to the same level of baroreceptor stimulation, increasing baroreflex sensitivity [55–57].

In summary, the present study provides new information about the programming of offspring fluid reestablishment patterns in the perinatal availability of a rich source of sodium as a manipulation model. This imprinting has a significant impact that alters the development of thirst associated with sodium ingestion, and sensitizes brain circuits involved in hydroelectrolyte and volume regulation, both of which may rely on time-dependent neuroplastic changes in brain functioning.

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