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Effect of sex chromosome complement on sodium appetite and Fos-immunoreactivity induced by sodium depletion

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Dadam FM, Caeiro XE, Cisternas CD, Macchione AF, Cambiasso MJ, Vivas L. 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, 2014. First published November 20, 2013; doi:10.1152/ajpregu.00447.2013.—Previous studies indicate a sex chromosome complement (SCC) effect on the angiotensin II-sexually dimorphic hypertensive and bradycardic baroreflex responses. We sought to evaluate whether SCC may differentially modulate sexually dimorphic-induced sodium appetite and specific brain activity due to physiological stimulation of the rennin angiotensin system. For this purpose, we used the "four core genotype" mouse model, in which the effect of gonadal sex and SCC is dissociated, allowing comparisons of sexually dimorphic traits between XX and XY females as well as in XX and XY males. Gonadectomized mice were sodium depleted by furosemide (50 mg/kg) and low-sodium diet treatment; control groups were administered with vehicle and maintained on normal sodium diet. Twenty-one hours later, the mice were divided into two groups: one group was submitted to the water-2% NaCl choice intake test, while the other group was perfused and their brains subjected to the Fos-immunoreactivity (FOS-ir) procedure. Sodium depletion, regardless of SCC (XX or XY), induced a significantly lower sodium and water intake in females than in males, confirming the existence in mice of sexual dimorphism in sodium appetite and the organizational involvement of gonadal steroids. Moreover, our results demonstrate a SCC effect on induced brain FOS-ir, showing increased brain activity in XX-SCC mice at the paraventricular nucleus, nucleus of the solitary tract, and lateral parabrachial nucleus, as well as an XX-SCC augmented effect on sodium depletion-induced brain activity at two circumventricular organs, the subfornical organ and area postrema, nuclei closely involved in fluid and blood pressure homeostasis.

sex chromosome complement; sexual dimorphism; Fos immunoreactivity; induced sodium intake; four core genotype mouse model

BIOLOGICAL DIFFERENCES between the sexes have long been recognized at biochemical, cellular, and physiological levels. Although the role of gonadal steroids in sexual dimorphism is undeniable, a growing body of evidence indicates that some sexually dimorphic traits cannot be explained solely as a result of gonadal steroid action. Males and females not only differ in their sex (males are born with testes and females with ovaries) but also carry different sex chromosome complements (SCC). Rapid advances in molecular biology have revealed the genetic and molecular bases of a number of sex-based differences, some of which are attributed to the XX-sexual genotype in the female and XY in the male. Recent evidence in gonadectomized adult mice indicates that both the sexually dimorphic ANG II-bradycardic baroreflex and hypertensive responses may be driven primarily by differences in SCC, which suggests that sex differences in genes residing in the sex chromosomes may influence these angiotensinergic sexually dimorphic traits (6, 29). Thus the genetic and/or hormone pathways could act independently or interact synergistically/antagonistically in modulating sexual dimorphic development (1, 2, 7, 10, 22).

Taking into account previous studies indicating that *1*) ANG II in the central nervous system differentially modulates cardiovascular parameters in males and females (23, 50), *2*) sex has an important influence on hydroelectrolyte and cardiovascular regulation (12, 33), *3*) SCC is involved in ANG II sexually dimorphic hypertensive and bradycardic baroreflex responses (6, 29), and *4*) furosemide-low-sodium diet (Furo/LSD) treatment leads to an increase in ANG II levels in association with sodium intake and specific brain Fos immunoreactivity (FOS-ir), we sought to evaluate whether genetic differences within the SCC may differentially modulate the known sexually dimorphic sodium appetite as well as basal or induced brain activity due to physiological stimulation of the renin-angiotensin system (RAS). To this end, we evaluated sodium-water intake and brain FOS-ir (along the forebrain and brain stem level) induced by Furo/LSD treatment in transgenic mice of the four core genotype (FCG). In this mouse model, the effect of gonadal sex (testes or ovaries) and SCC (XX or XY) is dissociated, allowing the independent assessment of sexually dimorphic traits among XX and XY females, as well as in XX and XY males.

METHODS

Animals

The FCG mouse model combines a deletion of the testis-determining gene Sry from the Y chromosome (Y^-) with the subsequent insertion of a Sry transgene onto an autosome (36, 37). Sry gene deletion in XY mice (XY^-) yields a female phenotype (ovaries). When the Sry transgene is inserted into an autosome of these mice they have testes and are fully fertile (XY^-Sry). The Y^- chromosome and the Sry transgene segregate independently, and thus four types of offspring are produced by breeding XY^-Sry males with XX females: XX and XY^- females (without Sry on the Y chromosome) and XXSry and XY^-Sry male mice (both with Sry in an autosome). All individuals possessing the Sry transgene develop testes and have a male external phenotype, regardless of their SCC, whereas individuals lacking the transgene have ovaries and external female secondary sex characteristics. Male and female are defined here according to the gonadal phenotype. Throughout the text, we will refer to XX and XY^- as XX and XY females and to XXSry and XY^-Sry as XX and XY male mice, respectively. Comparing these genotypes makes it possible to segregate the roles of *1*) SCC (comparing mice with the same gonadal type but with

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different SCC: XX vs. XY) *2*) gonadal sex (males vs. females regardless of SCC) and *3*) their interaction (1).

MF1 transgenic mice, kindly provided by Dr. Paul Burgoyne, were born and reared in the breeding facilities at the Instituto de Investigación Médica M. y M. Ferreyra (Córdoba, Argentina). They were housed in groups of 5 to 8 per cage in a temperature-controlled environment, maintained on a 12-h light/dark cycle, and fed and watered ad libitum. All experimental protocols were approved by the appropriate animal care and use committees at our institute following the National Institutes of Health guidelines for the care and use of laboratory animals.

Genotyping

Genotyping was performed on genomic DNA samples obtained from mouse tails by polymerase chain reaction (PCR). DNA extraction procedures and genotype-PCR analysis have been previously described in Caeiro et al. (6).

Gonadectomy: Surgical Procedures

To remove any activational effect of sex hormones that might mask both the modulatory action of sex chromosomes and the organizational hormonal effects, adult mice were anesthetized with ketamine-xilazine mixture, and a bilateral incision was made in the scrotum region for male mice and just below the rib cage in the female mice, to be able to perform bilateral gonadectomy. Then the vascular supply was ligated, the gonads (either testes or ovaries) were removed, and the muscle layer and incisions were sutured in place.

Urinary Osmolality and Electrolyte Analysis

Urinary concentrations of Na^+, K^+, and Cl^- were determined by an ion analyzer (Beckman), whereas osmolarity was established by a vapor pressure osmometer (VAPRO 5520).

Fos Immunohistochemistry

FOS-ir procedures have been previously described (5). Briefly, mice were transcardially perfused with isotonic saline solution followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2. The brains were then removed, fixed overnight in the fixative solution, and stored at 4°C in 30% sucrose until processing and coronal sections of 50 μm were cut using a freezing microtome. The Fos antibody used in this study was raised in rabbits against a synthetic 14-amino acid sequence corresponding to residues 4–17 of human Fos (Ab-5, batch no. 60950101; Oncogene Science, Manhasset, NY).

The brain nuclei exhibiting FOS-ir were identified and delimited according to the mouse brain atlas of Paxinos and Franklin (42). The distance from the bregma of the corresponding plates is as follows: organum vasculosum of the lamina terminalis (OVLT) 0.50 mm, median preoptic nucleus (MnPO) 0.14 mm, subfornical organ (SFO) −0.58 mm, paraventricular nucleus (PVN) −0.82 mm, supraoptic nucleus (SON) −0.82 mm, lateral parabrachial nucleus (LPB) −5.20 mm, nucleus of the solitary tract (NTS) and adjacent area postrema (AP) (NTS/AP) −7.48 mm. FOS-ir nuclei were quantified with a computerized system that included a Zeiss microscope equipped with a DC 200 Leica digital camera attached to a contrast enhancement device. Images were digitalized and analyzed using Image J PC software (version 1.42q). FOS-ir cells in each section were counted by setting a size range for cellular nuclei (in pixels) and a threshold level for staining intensity. Representative sections in each group were acquired at exactly the same level, with the aid of the Adobe Photoshop Image Analysis Program CS2 (version 9). Sections were analyzed by an experimenter blinded to the experimental groups.

Statistical Analysis

Induced water and sodium intake data were subjected to a three-way mixed ANOVA analysis with repeated measures. Treatment (depleted: DEP, control: CON), gonadal sex (male/female), and SCC (XY/XX) were considered as independent factors, and time intervals of evaluation (15, 30, 45, 60, 90, 120 min) were included as repeated measurements. Sodium balance data were subjected to a two-way mixed ANOVA with repeated measurements (gonadal sex and SCC were considered as independent factors and time intervals of evaluation were taken as repeated measurements). Urinary and FOS-ir data were subjected to a three-way ANOVA analysis with treatment, sex, and SCC as independent factors. The loci of significant interactions or significant main effects were further analyzed using the Tukey test (type I error probability was set at 0.05). Results were expressed as group means ± SE.

Experimental Design

Experiment 1: effect of SCC on sodium and water intake induced by Furo/LSD treatment. To evaluate whether SCC modulates sexually dimorphic-induced water and sodium intake, mice of the FCG mouse model aged 45–50 days were gonadectomized and, after a 15-day recovery period, were weighed, placed in individual metabolic cages, and submitted to acute sodium depletion by a combined treatment of Furo/LSD (DEP group). Diuretic-natriuretic drug furosemide (50 mg/kg) was administered subcutaneously in two consecutive doses 5 min apart. Mice were immediately placed in clean individual metabolic cages with access to a low-sodium diet (ICN, Costa Mesa, CA) and distilled water. Control groups were administered vehicle solution and maintained with ad libitum access to distilled water and normal sodium diet content (CON groups). Twenty-one hours later, mice were weighed, food was removed from the cages, and the urine voided during this period was collected for balance study, electrolytes, and osmolality analysis. The mice were immediately submitted to a two-bottle choice test (distilled water/2% NaCl solution), and intake scores were recorded every 15 min during the first hour and every 30 min during the second hour. Data are expressed as milliliters per 10 g of body weight. All experiments were carried out between 8:00 AM and 2:00 PM.

Experiment 2: effect of SCC on sodium depletion-induced brain FOS-ir. To assess whether SCC may differentially modulate basal or induced cell activity in response to acute sodium depletion, in a separate group of mice, we determined the number of FOS-ir cells in brain areas involved in hydroelectrolyte and cardiovascular homeostasis. Twenty-one hours after the administration of furosemide or vehicle solution, and immediately before the intake test took place, the animals were perfused and the brains stored until further FOS-ir processing.

RESULTS

Experiment 1: Effect of SCC on Sodium and Water Intake Induced by Furo/LSD Treatment

The statistical analysis of induced water and sodium intake showed an effect of the interaction of sex, treatment, and time [2% NaCl: $F(5,22) = 3.16$ $P < 0.01$; H_2O: $F(5,22) = 2.89$, $P < 0.01$] while no SCC factor effect was observed. As expected, DEP treatment induced an increase in sodium appetite compared with intake scores reported for CON groups. Irrespective of SCC (XX or XY), female mice showed an attenuated induced 2% NaCl intake than that reported for male mice (Fig. 1*A*, time course of cumulative NaCl intake in mice of the four genotypes, and Fig. 1*C*, representation of the significant triple interaction: sex × treatment × time in DEP and CON groups). Matching the sodium intake scores, for drinking response, DEP male mice showed an increase in water consumption, whereas DEP female mice showed the same

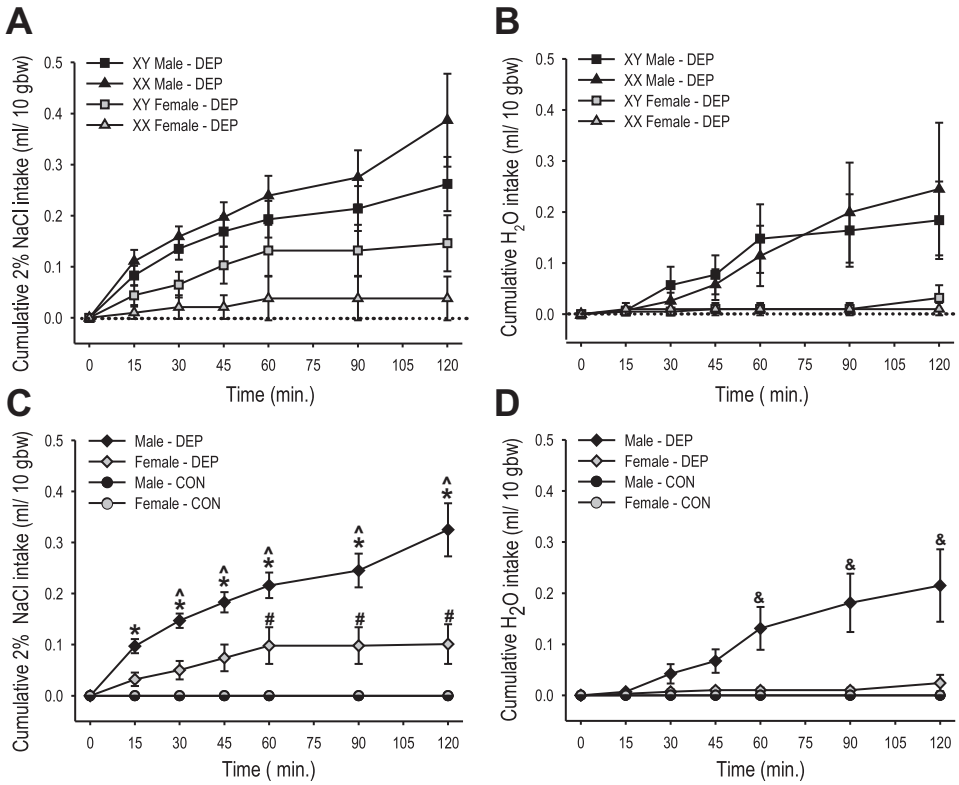


Fig. 1. Time course of cumulative 2% NaCl and H_2O intake of mice subjected to furosemide-low-sodium diet treatment. *A*: cumulative intake of 2% NaCl every 10 g body wt in XY male, XX male, XY female, and XX female mice that were subjected to acute sodium depletion (DEP). *B*: cumulative intake of H_2O every 10 g body wt in mice XY males, XX males, XY females, and XX females subjected to DEP treatment. Dotted line indicates the average intake of the control animals (CON). *C* and *D*: representation of the significant triple interaction (sex × treatment × time): cumulative 2% NaCl and H_2O intake of male and female corresponding to DEP and CON groups. *$P < 0.01$ Male-DEP vs. CON groups, #$P < 0.01$ female-DEP vs CON groups, ^$P < 0.01$ male-DEP vs. female-DEP. Values are means ± SE, n = 4–9/group.

dipsogenic response as that of the CON groups (Fig. 1*B*, time course of cumulative water intake in mice of the four genotypes, and Fig. 1*D*, representation of the significant triple interaction of water intake: sex × treatment × time in DEP and CON groups).

Urinary Data and Sodium Balance Analysis After Acute Sodium Depletion

The statistical analysis of total urinary excretion of Na^+, K^+, and Cl^- showed a significant effect of treatment factor [$F(1,41) = 28.98$, $P < 0.01$; $F(1,41) = 36.79$, $P < 0.01$ and $F(1,41) = 10.56$, $P < 0.01$, respectively], with neither the sex nor SCC factors affecting urinary electrolyte excretion. As shown in Table 1, the total urinary excretion of electrolytes increased in mice treated with Furo/LSD. Moreover, as expected, the analysis of urine output defined as urine volume per 10 g of body weight showed a significant treatment effect [$F(1,52) = 95.95$, $P < 0.01$], with mice of the DEP group showing a higher urine output than that of the CON groups. A significant increase in urinary osmolality was observed in CON versus DEP groups [$F(1,40) = 655.61$, $P < 0.01$]. All together, these data indicate that, as expected, DEP treatment induces diuresis and natriuresis with no significant treatment sex differences among groups.

Table 1. *Osmolality, diuresis, electrolyte excretion, and body weights (initial and 21 h after DEP treatment) in mice of the four core genotype model subjected to furosemide and low sodium diet treatment*

	DEP				CON			
	Male		Female		Male		Female	
	XY	XX	XY	XX	XY	XX	XY	XX
Osmolality, mosmol/kg H_2O (*t*)	531.4 ± 35.7	488.3 ± 35.0	552.2 ± 66.2	545.6 ± 63.4	2149.6 ± 58.5	2265.2 ± 186.8	2325.2 ± 174.9	2448.5 ± 184.1
Na^+, meq/10 g body wt (*t*)	0.10 ± 0.01	0.11 ± 0.00	0.10 ± 0.01	0.10 ± 0.01	0.06 ± 0.02	0.06 ± 0.02	0.06 ± 0.02	0.04 ± 0.01
K^+, meq/10 g body wt (*t*)	0.07 ± 0.01	0.07 ± 0.00	0.05 ± 0.00	0.06 ± 0.01	0.03 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	0.02 ± 0.01
Cl^-, meq/10 g body wt (*t*)	0.11 ± 0.01	0.12 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	0.08 ± 0.03	0.08 ± 0.02	0.09 ± 0.03	0.05 ± 0.02
Diuresis, ml/10 g body wt (*t*)	1.40 ± 0.20	1.30 ± 0.15	1.16 ± 0.11	1.35 ± 0.16	0.23 ± 0.10	0.40 ± 0.09	0.30 ± 0.13	0.16 ± 0.05
Initial body wt, g (*s*)	34.95 ± 0.81	35.83 ± 0.73	29.98 ± 1.15	28.29 ± 0.90	32.37 ± 1.13	35.11 ± 0.75	28.73 ± 0.89	30.15 ± 1.59
Post Furo/LSD body wt, g (*s,t*)	29.49 ± 0.77	29.97 ± 0.64	25.05 ± 1.16	23.24 ± 0.76	32.97 ± 1.05	34.43 ± 0.71	28.77 ± 0.70	30.05 ± 0.90

Values are means ± SE, n = 4–9/group. DEP, sodium-depleted groups; CON, control groups; LSD, low-sodium diet;Furo, furosemide; *t*, main effect of treatment; *s*, main effect of sex.

As shown in Table 1, the statistical analysis of initial body weight showed, as expected, a significant effect of sex factor [$F(1.79) = 56.24$ $P < 0.01$]; males were heavier than female mice irrespective of SCC (XX or XY), indicating an organizational hormonal effect. With regard to body weight after 21 h of sodium depletion, ANOVA analysis showed a significant main effect of sex [$F(1.79) = 58.16$ $P < 0.01$] and treatment [$F(1.79) = 50.88$ $P < 0.01$] factors. As described for initial body weight male mice were heavier than female and DEP mice showed (irrespectively of sex and SCC) lower body weights than those reported for CON groups. Moreover, the analysis of the percentage of mean weight loss (21 h after Furo/LSD treatment) showed, irrespective of sex and SCC, a significant main effect of treatment factor [$F(1,79) = 241.87$; $P < 0.001$]. The percentages of mean weight loss were as follows: 19.94% ± 0.84 in DEP groups and 0.28% ± 0.72 in CON groups.

With regard to sodium balance, ANOVA revealed a significant effect for time and sex factors [$F(6,15) = 25.17$, $P < 0.01$ and $F(1,25) = 4.68$, $P < 0.05$, respectively] as well as a significant interaction of sex and time [$F(6,15) = 6,98$ $P < 0.01$], whereas no SCC factor effect was seen. As shown in Fig. 2, the expected negative sodium balance was evident before the onset of the test in both male and female DEP groups. Moreover, just as with water and sodium intake profiles, irrespective of SCC (XX or XY), a differential response was observed between male and female mice, with females showing a more negative balance than males.

Experiment 2: Effect of SCC on Sodium Depletion-Induced Brain FOS-ir

Sensory circumventricular organs. FOS-ir data analysis along the sensory circumventricular organs (CVOS) revealed differential patterns of cell activation. As shown in Fig. 3, a significant effect of SCC and treatment factors was observed at the SFO [$F(1,21) = 8.33$, $P < 0.01$ and $F(1,21) = 34.08$, $P < 0.01$, respectively]. The statistical analysis also showed a significant effect of the interaction of SCC × treatment [$F(1,21) = 4.51$, $P < 0.05$] and, in the follow-up Tukey test, XX-DEP mice, irrespective of the gonadal sex, showed a significant increase in the number of FOS-ir cells compared with XY-DEP as well as to CON groups (XX and XY).

The analysis of brain activity in the AP showed an independent main effect of three factors: SCC, sex and treatment [$F(1,21) = 15.46$, $P < 0.01$; $F(1,21) = 5.06$, $P < 0.05$; and $F(1,21) = 8.73$, $P < 0.01$, respectively]. Moreover, the ANOVA analysis also showed a significant effect of the triple interaction of SCC × sex × treatment [$F(1,21) = 4.83$, $P < 0.05$] and, in a Tukey post hoc analysis, the XX male-DEP group showed a higher FOS-ir than the other DEP and CON groups (Fig. 4).

With regard to the OVLT, a significant effect of treatment factor [$F(1,22) = 52.28$, $P < 0.01$] indicated that DEP treatment, irrespective of sex and SCC, resulted in increased brain FOS-ir compared with CON groups.

Hypothalamic nuclei. The analysis of brain activity in the MnPO showed a significant main effect for the treatment factor [$F(1,21) = 8.47$, $P < 0.01$] with a similar pattern of neural activity to that reported for the OVLT nuclei, since DEP treatment was associated with increased neural activity in both brain areas (Fig. 5*A*).

The analysis of FOS-ir data at the PVN showed a significant main effect for the SCC factor [$F(1,23) = 9.50$, $P < 0.01$]. Regardless of treatment or sex, XX mice were associated with increased neural activity compared with XY mice (Fig. 5*B*). However, the brain activity analysis showed no significant differences at the SON.

Brain stem nuclei. At the LPB, the statistical analysis showed a significant main effect for the three analyzed factors. Treatment main effect analysis [$F(1,21) = 9.17$, $P < 0.01$] showed a decrease in brain activity in DEP compared with CON groups. With regard to sex, males showed greater immunoreactivity [$F(1,21) = 6.16$, $P < 0.05$] whereas post hoc analysis of the SCC main factor [$F(1,21) = 21.47$, $P < 0.01$] showed that mice with XX SCC, compared with XY, presented an increased number of FOS-ir cells (Fig. 6*A*).

The statistical analysis of FOS-ir cells at the NTS showed a significant effect due to SCC [$F(1,21) = 5.69$, $P < 0.05$], indicating that, regardless of treatment (DEP or CON) or sex (male/female), XX-SCC mice had an increased FOS-ir pattern compared with XY mice (Fig. 6*B*).

DISCUSSION

The results of this study indicate that sex differences in mice in terms of induced water and sodium intake may be due to organizational hormonal effects rather than to intrinsic differences in the SCC factor (XX and XY) or their interaction. Moreover, our results demonstrate a modulatory action of SCC on diencephalic (PVN) and brain stem (NTS and LPBN) cell activity as well as on sodium depletion-induced activity in two of the sensory CVOs (SFO and AP), nuclei closely involved in fluid and blood pressure homeostasis.

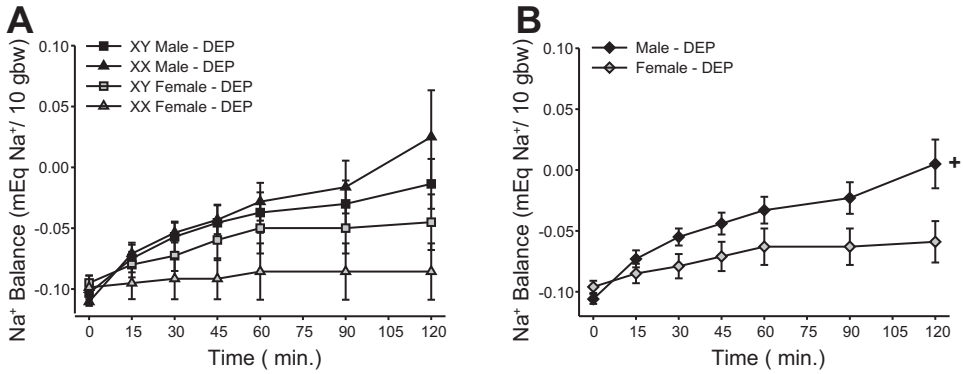
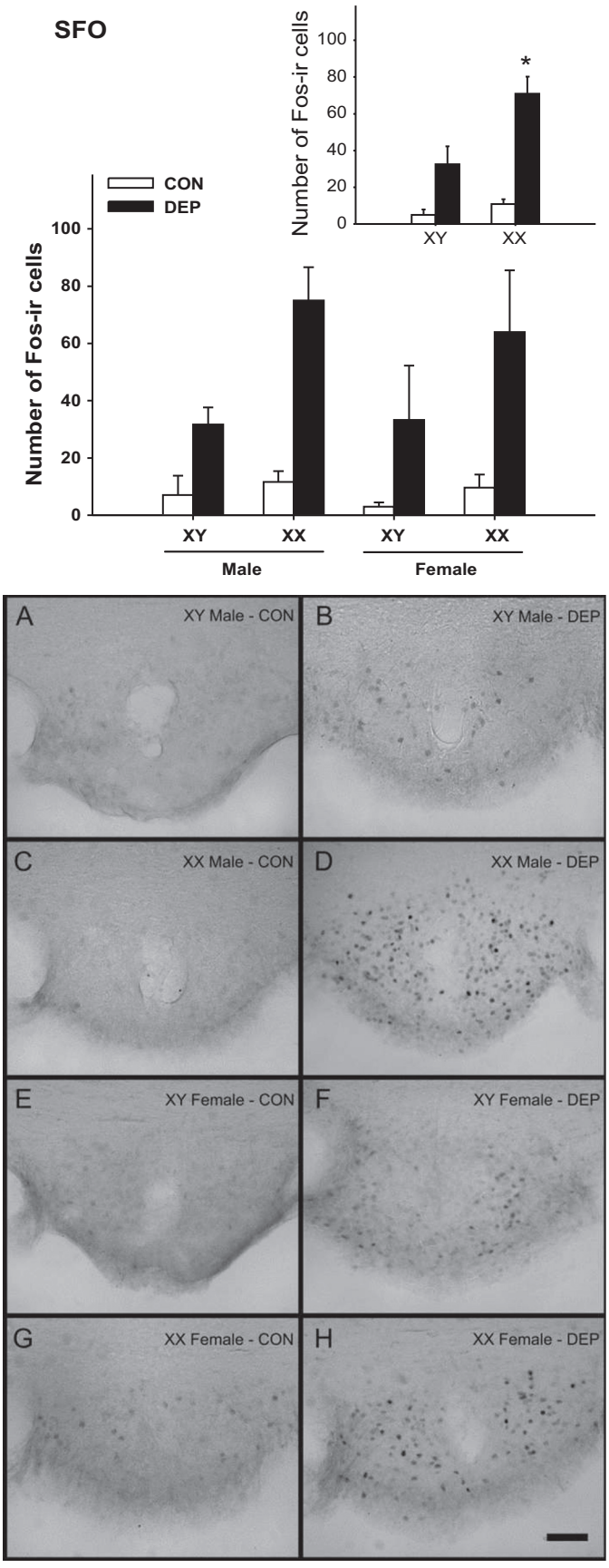


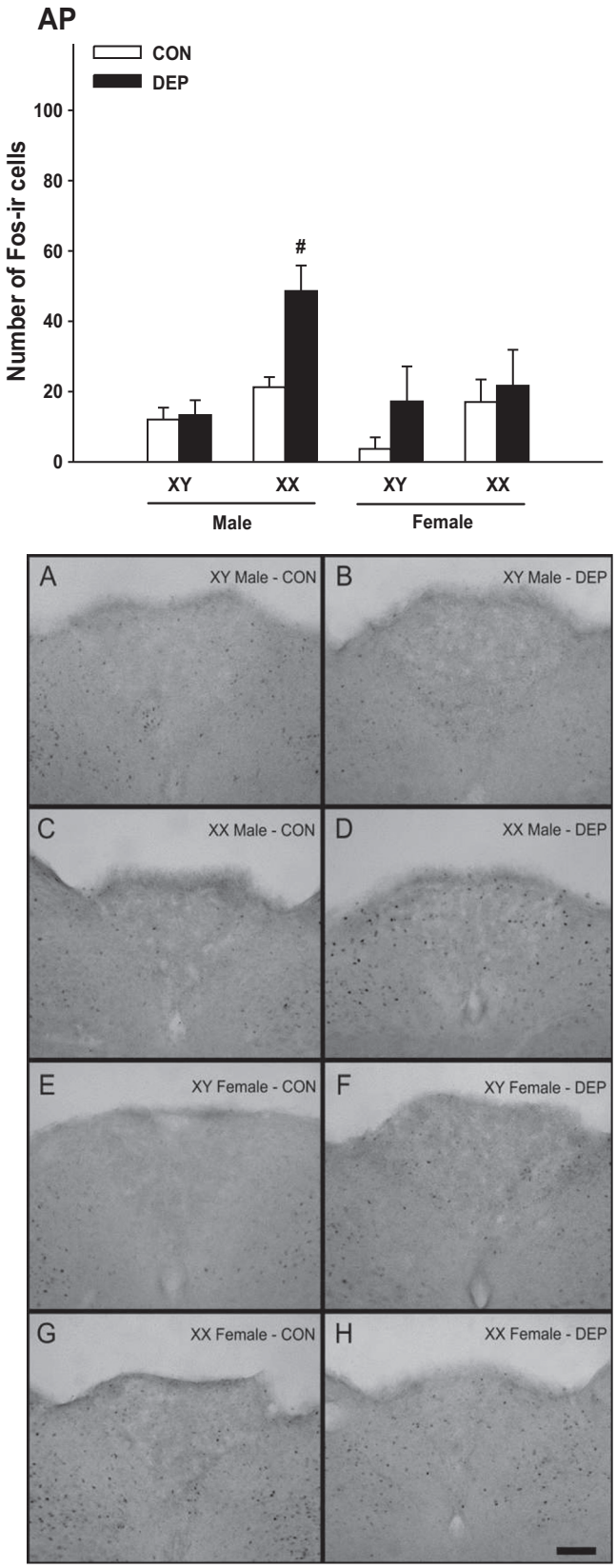
Fig. 2. Time course of cumulative sodium balance of mice subjected to furosemide-low-sodium diet treatment. *A*: cumulative sodium balance every 10 g body wt in XY male, XX male, XY female, and XX female mice subjected to acute sodium depletion (DEP). *B*: representation of the significant double interaction of sex and time factors. Significant sex main effect: $+P < 0.05$ male-DEP vs. female-DEP groups. Values are means ± SE, $n = 4$–9/group.



Although previous studies have already evaluated the impact of activational and organizational effects of gonadal steroids on sodium appetite in rats (12, 33, 46, 52), in the present study we evaluated in mice the effect of SCC on induced sodium and water intake in dissociation of the organizational and activational hormonal effects. MF1 gonadectomized male mice, regardless of the SCC, consumed a greater amount of saline than females. However, in terms of the drinking response, while DEP male mice showed the increased water consumption normally associated with enhanced sodium intake, DEP-female mice showed the same water intake as CON groups. As predicted, Furo/LSD treatment resulted in a significant increase in both electrolyte excretion and diuresis, with a consequent decrease in urinary osmolarity. As no sex differences were observed in diuretic/natriuretic effect between male and female mice, it follows that sexually dimorphic-induced water and sodium intake could not be attributed to sex differences in DEP treatment. The drinking shown by depleted females in the present study are in agreement with Rowland and Fregly (44) results where adult intact female mice subjected to Furo/LSD treatment showed an increased sodium appetite and almost no water intake during the 2-h drinking test. Moreover, the urinary output of sodium after this treatment was 0.35 ± 0.02 meq/21 h, and the mean sodium intake was about 60% of the urinary sodium loss. Thus Rowland and Fregly (44) postulated that mice may require a longer period of time to replace their sodium losses. Data from our work indicate that although this assumption may be accurate for DEP-female mice, in which sodium intake represents 42.02 ± 15.66% of the urine sodium loss, DEP-male mice consume, irrespectively of SCC, NaCl equal to or in excess of their furosemide induced-sodium deficit (111.10 ± 17.96%) in association to water intake.

Concerning this observed diminished mean sodium intake in DEP female mice related to Furo/LSD-induced urinary sodium loss, reported by both Rowland and Fregly (44) and our study, it is important to highlight that the onset of specific sodium appetite as a result of body sodium loss is a slow and complex process. In rats it has been shown that acute sodium depletion by peritoneal dialysis produces a rapid and significant decrease in both serum and CSF sodium concentration, followed by a relative slow recovery. However, sodium appetite appears only 20–24 h after sodium depletion when serum and CSF sodium concentration and blood volume have already returned to normal values, even though the rats did not have access to sodium salts, supporting the hypothesis of the sodium "reservoir" (11, 24). Likewise, studies conducted in patients to whom furosemide was intravenously administered, showed that, despite the urinary losses, no significant changes in serum osmolality or electrolytes values occurred at any sampling time (14,

Fig. 3. Brain pattern of Fos-immunoreactivity (FOS-ir) following acute sodium depletion in subfornical organ (SFO). Mean number of FOS-ir cells in XY male, XX male, XY female, and XX female mice corresponding to sodium-depleted (DEP) and control (CON) groups (black and white bars, respectively). *Inset*: significant interaction of sex chromosome complement (SCC) × treatment factors, $*P < 0.01$ XX-DEP vs. XY-DEP, XY-CON and XX-CON groups. Values are means ± SE, $n = 3$–5/group. Photomicrographs of coronal sections showing the pattern of FOS-ir cells within the SFO in CON and DEP groups (*right* and *left* panels, respectively). *A*: XY male-CON, *B*: XY male-DEP, *C*: XX male-CON, *D*: XX male-DEP, *E*: XY female-CON, *F*: XY female-DEP, *G*: XX female-CON and *H*: XX female-DEP. Scale bar 50 μm.



16). Thus the peritoneal dialysis and furosemide electrolyte data might indicate that, although female sodium intake is about half of that required in relation with urinary sodium loss, this does not indicate that female mice have decreased plasma and CSF water and sodium concentration, but instead it could be read as female mice may still have to recover their total body water and sodium content. Moreover, as we did not collect the urine volume after the intake test, we cannot assure there were not differences between males and females in the renal reabsorption of water and sodium after sodium-water ingestion. This may explain otherwise why the females are drinking presumably less than they seem to need. We may also assume that the female vasopressinergic and angiotensinergic systems, acting in the kidney are more effective, compensating in this way the lower consumption during the intake test.

There is considerable evidence of sexual dimorphism in both need-free and need-induced sodium intake (12, 33, 46, 52). Studies have addressed large variations in need-free salt and water intake in females according to the circulating levels of estrogen and sodium intake is seen to fluctuate during different stages of the estrous cycle and during pregnancy and lactation (12, 13, 25, 43). Moreover, additional data supports the idea that the sexual dimorphism of rat sodium appetite may be influenced by testosterone in particular modulating the need-free sodium intake of males and females during the organizational period and need induced intake of ovariectomized females during adulthood (activational effect) (12, 34). The present study did not explore the specific effect of testosterone on sodium need-free/induced sodium intake or either the activational effects of gonadal steroids, since we worked with gonadectomized mice; however, they confirm and extend these previous works in rats (12, 34) establishing the organizational effects of gonadal steroids on sexually dimorphic induced sodium appetite.

With regard to induced sodium intake, Rowland and Fregly (44) have demonstrated that mice are refractory to both dipsogenic and natriorexigenic effects of peripheral RAS modulation in most sodium depletion protocols; however, acute treatment with Furo/LSD is the only treatment so far tested that is capable of increasing plasma renin activity, inducing diuresis, natriuresis, and the subsequent behavioral sodium intake in this species. Moreover, Stricker et al. (48) have shown a significant increase in sodium appetite in adult male and ovariectomized female rats and a consequent decrease in sodium intake in ovariectomized females with estrogen replacement. Laboratory studies not only confirm the existence of rat sexual dimorphism in induced sodium intake but also the inhibitory effect of estrogen (activational effect) on this behavior (19, 52).

The anatomical-functional substrate, underlying the neuroendocrine and behavioral responses aimed at restoring body

Fig. 4. Brain pattern of FOS-ir after acute sodium depletion in area postrema (AP). Mean number of FOS-ir cells in XY male, XX male, XY female, and XX female mice corresponding to sodium-depleted (DEP) and control (CON) groups (black and white bars, respectively). #$P < 0.05$ XX male-DEP vs. the other DEP and CON groups. Values are means ± SE, $n = 3$–5/group. Photomicrographs of coronal sections showing the pattern of FOS-ir cells within the AP in CON and DEP groups (*right* and *left* panels, respectively). *A*: XY male-CON, *B*: XY male-DEP, *C*: XX male-CON, *D*: XX male-DEP, *E*: XY female-CON, *F*: XY female-DEP, *G*: XX female-CON and *H*: XX female-DEP. Scale bar 50 μm.

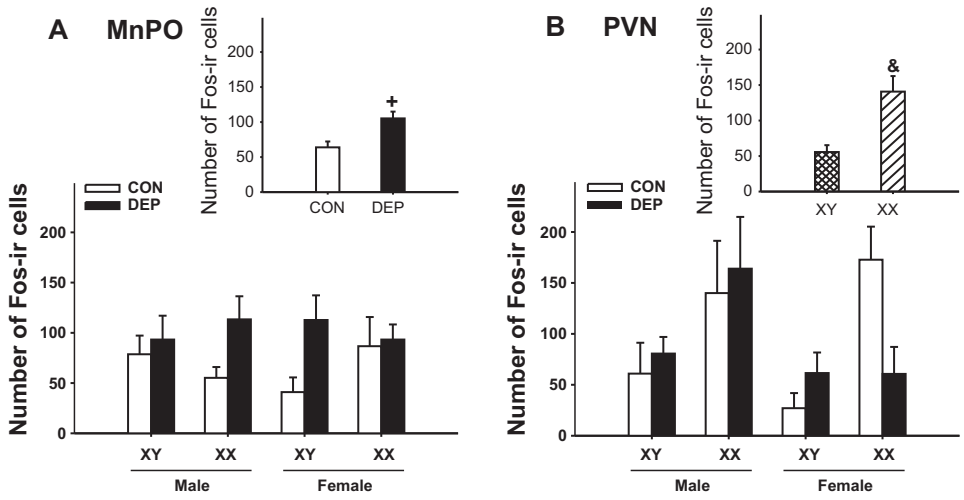


Fig. 5. Brain pattern of FOS-ir after acute sodium depletion in median preoptic (MnPO) and paraventricular (PVN) nuclei. Mean number of FOS-ir cells in XY male, XX male, XY female, and XX female mice corresponding to sodium-depleted (DEP) and control (CON) groups (black and white bars, respectively). *A*: MnPO. *Inset* shows the significant effect of treatment factor, $+P < 0.01$. *B*: PVN. *Inset* shows the significant effect of SCC main factor, $\&P < 0.05$. Values are means ± SE, n = 3–5/group.

fluid homeostasis after sodium depletion, is composed of several forebrain and brain stem neuronal groups. Changes in plasma and CSF sodium concentration, osmolarity, and ANG II concentration are sensed by the brain mainly at the level of three circumventricular organs, termed the sensory CVOs, which lack a blood-brain barrier and include the SFO and the OVLT at the third brain ventricle, and the AP at the fourth ventricle (30). The MnPO, together with the SFO and OVLT, form the lamina terminalis (LT), which has been shown to play a major role in many aspects of body fluid homeostasis (39). Furo/LSD treatment in mice induces sodium appetite and increased FOS-ir along the SFO and MnPO compared with basal or control values (18). Moreover, in rats the same sodium depletion treatment also induced an increase in *c-fos* expression along the LT (3). However, the present data not only confirm the expected sodium depletion increase in FOS-ir along the entire LT, but also show an interaction effect of treatment and sex chromosome factors on SFO cell activity. The influence of XX-SSC on SFO increased cell activity in response to sodium depletion might reflect the influence of the SSC on the angiotensinergic-dependent regulatory responses involved in fluid and cardiovascular homeostasis.

Hypotension and hyponatremia induced by sodium depletion result, among others, in the activation of the RAS. This system manages to compensate the generated hypotension restoring the extracellular volume space and inducing vasoconstriction. Increased plasma ANG II levels stimulate aldosterone secretion, which in turn increases sodium reabsorption by the kidney and also binds the SFO, OVLT, and AP (sensory CVOs). The lack of blood-brain barrier allows these CVOs to be exposed to modulatory humoral factors, giving them the potential to integrate and modulate the homeostatic response (40).

At the SFO and AP level, ANG II is involved in sodium appetite, baroreceptor reflex, and blood pressure response, modulating hydroelectrolyte and cardiovascular homeostasis. Recent studies in our laboratory, investigating the role of SCC in relation to ANG II-sexually dimorphic bradycardic baroreflex response, indicate that the ANG II differences reported

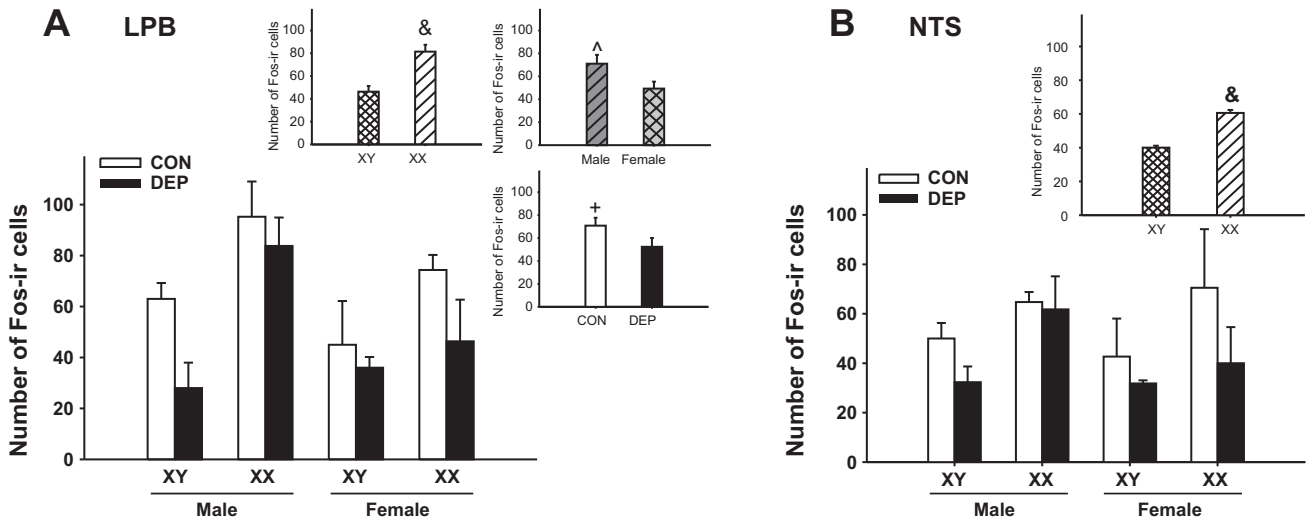


Fig. 6. Brain pattern of FOS-ir after acute sodium depletion in lateral parabrachial (LPB) and nucleus of the solitary tract (NTS) nuclei. Mean number of FOS-ir cells in XY male, XX male, XY female, and XX female mice corresponding to sodium-depleted (DEP) and control (CON) groups (black and white bars, respectively). *A*: LPB. *Inset* shows the significant main effect of treatment ($+P < 0.05$), SCC ($\&P < 0.01$), and sex ($\wedge P < 0.05$) factors. *B*: NTS. *Inset* represents significant main effect of SCC factor ($\&P < 0.05$). Values are means ± SE, n = 3–5/group.

between males and females may be due to the effect of SCC (and therefore to genes linked to sex chromosomes) and not to the organizational effects of gonadal steroids. In particular we found an XX-SCC ANG II-facilitatory bradycardic baroreflex control of heart rate (6). Although the cellular and molecular mechanisms underlying sex differences in the modulation of sodium appetite and ANG II hypertensive and bradycardic baroreflex response are still unknown, it is accepted that the efficiency of the ANG II sexually dimorphic bradycardic baroreflex response observed in females compared with males seems to be due to central actions of this peptide along the CVOs and in particular within the AP and SFO. Both the SFO and AP are subject to neural and humoral modulation and sends projections to neural centers involved in cardiovascular regulation, including NTS, dorsal vagal complex, the parabrachial nucleus, and rostral ventrolateral medulla, thereby modulating sympathetic-parasympathetic activity and baroreflex response (4, 47). Furthermore, studies conducted by Contreras and Stetson (15) have shown that, whereas AP lesion in male rats leads to an increase in spontaneous sodium intake, no differences are reported in glucose and KCl solutions consumption. In addition, injury to this brain area blocks the ANG II-hypertensive response (17, 26) and in males it prevents the decrease in baroreflex sensitivity observed after acute administration of this peptide (17, 38, 53).

Our results also show that, at brain stem level, the LPB have an increased basal neuronal activation in the control groups, with a concomitant decrease in sodium-depleted mice. These data confirm previous laboratory results indicating that this nucleus has a tonic inhibitory action on sodium intake (27). The present evidence also shows that mice bearing SCC-XX have increased neuronal activity at the PVN, LPB, and NTS. We have previously shown in male rats that the main changes in c-fos expression along these nuclei are evident after sodium consumption induced by sodium depletion, suggesting they may form a circuit subserving sodium balance regulation. These data are consistent with previous lesion, pharmacological, and physiological studies showing these nuclei are components of a inhibitory circuit modulating sodium intake (15, 27, 28, 41, 49). Although the present results confirm previous data showing a sodium-depletion induced FOS-ir along these nuclei during the appetitive phase of sodium appetite, they are also demonstrating for the first time that XX SCC mice have increased neural activity within these nuclei and above all a major basal activity in XX SCC individuals. This basal and maybe "inhibitory" activity as shown by Fos immunoreactivity along these nuclei in XX SCC mice can be the cause of the refractory sodium appetite response seen in females mice. However, in the XX SCC sodium-depleted male mice, the organizational effect of gonadal steroids may counteract the effect of XX SCC on inhibitory areas activity, resulting in an increased sodium appetite.

ANG II exerts its physiological action by binding to two G protein-coupled receptor subtypes AT1 and AT2. AT1 activation leads to elevation of blood pressure due to vasoconstriction, aldosterone, and vasopressin release, a decreased glomerular filtration rate, sodium reabsorption, water and sodium intake, and increased sympathetic activity. In contrast, binding of ANG II to AT2 receptors has the opposite effects to those of AT1 binding, inducing natriuresis, vasodilatation, and increased production of bradykinin and nitric oxide (8, 31, 35, 45).

Exciting new data indicate that X-chromosome inactivation is very far from the "all-or-none" phenomenon that was initially described, in which it was thought that one X-chromosome in somatic cells of female mammals was fully actively transcribed whereas the other was completely inactive. Although, in female mammals, most genes on one X chromosome are silenced as a result of X-chromosome inactivation, some genes escape X-inactivation and are expressed from both the active and inactive X chromosome. Microarray analysis revealed that the extent of sexual dimorphism in gene expression was much greater than previously recognized and confirmed sex-chromosome enrichment in various somatic tissues (sex-biased genes) (9, 54).

Taking into account the following: *1*) our studies indicate a modulatory action of SCC on neuronal activation at the AP and SFO of sodium-depleted mice, *2*) previous laboratory work that indicates that SCC modulates the ANG II-sexually dimorphic bradycardic response (6), *3*) that the CVOs play an important role in hydroelectrolyte and cardiovascular homeostatic regulation, *4*) that ANG II binds to both AT1 and AT2 receptors (whose actions are opposite), and *5*) that the AT2 gene (AGTR2) is located on chromosome X (21, 32), while the AT1 gene (AGTR1) is located on the autosomal chromosome 3 (51), it is tempting to speculate that sex chromosome genes showing sex-biased expression may thus serve as candidate regulators of sexually dimorphic phenotypes (20). If this is the case, the differential expression or transcription of AT1R/AT2R could be responsible for differential neuronal activation of areas closely related to angiotensinergic system activation, such as the SFO and AP. Further investigation is needed, however, to assess the contribution of SCC to AT1 and AT2 receptor expression in these specific brain areas involved in sodium depletion and cardiovascular homeostasis.

Perspectives and Significance

In summary, this study demonstrates a sexually dimorphic-induced sodium intake in mice, in which the organizational hormonal effect (but not the SCC factor) modulates the sexually dimorphic profile. Moreover we have also demonstrated that SCC modulates brain activity in nuclei closely involved in the regulatory response to RAS stimulation, suggesting a sex chromosome gene participation in the modulation of neural pathways underlying fluid and electrolyte homeostasis. Historically, most epidemiological and basic studies were performed in male subjects and, if both sexes were included, no sex differentiation was taken into account during data analysis, assuming that males and females are similar, differing only in the magnitude of the response. Nonetheless, principles learned in male models do not necessarily apply to females. Understanding in more detail sex differences on the regulatory mechanisms underlying physiological differences on water and sodium handling between males and females (both at the peripheral and brain levels) may offer important insights into designing improved oriented sex-tailored therapeutic treatments in the future.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: F.M.D., X.E.C., and A.F.M. performed experiments; F.M.D., X.E.C., A.F.M., M.J.C., and L.V. analyzed data; F.M.D., X.E.C., C.D.C., M.J.C., and L.V. interpreted results of experiments; F.M.D. and C.D.C. prepared figures; F.M.D., X.E.C., C.D.C., A.F.M., M.J.C., and L.V. approved final version of manuscript; X.E.C., M.J.C., and L.V. conception and design of research; X.E.C., F.M.D., M.J.C., and L.V. drafted manuscript; X.E.C., M.J.C., and L.V. edited and revised manuscript.

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