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Chronic salt-loading reduces basal excitatory input to CRH neurons in the paraventricular nucleus and accelerates recovery from restraint stress in male mice

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Abstract

Neurons synthesizing corticotrophin-releasing hormone (CRH) in the paraventricular nucleus of the hypothalamus (PVN) are activated during acute stress and act via the hypothalamic-pituitary-adrenal (HPA) axis to increase systemic levels of corticosterone (CORT). Recent data indicates that CRH neurons in the PVN are inhibited by acute salt-loading, and that this inhibition blunts the response to restraint stress as measured by increases in plasma CORT. The current study evaluates the effects of chronic rather than acute salt-loading on stress-induced activation of the HPA axis. Relative to euhydrated controls, chronic salt-loading over a 5-day period elevated plasma sodium and fluid intake without eliciting hypovolemia or substantial alterations in food intake or body weight. Chronic salt-loading also decreased expression of CRH mRNA in the anterior but not posterior portion of the PVN. Similarly, whole cell patch clamp recordings revealed that salt-loading effectively decreases spontaneous excitatory input to CRH neurons in the PVN without altering spontaneous inhibitory input. Generally consistent with these observations, chronic salt attenuated HPA axis activation as indicated by a significant reduction of plasma CORT during recovery from restraint stress.

Introduction

The paraventricular nucleus of the hypothalamus (PVN) contains peptidergic neurons that respond to dehydration through endocrine and neural compensatory mechanisms that maintain and restore hydromineral balance. Specifically, the intracellular dehydration that occurs with acute elevations in the plasma sodium concentration (pNa^+) influences the activation of PVN neurons to prevent diuresis and promote natriuresis and the maintenance of blood pressure by controlling the systemic and central release of arginine vasopressin (AVP), oxytocin (OT) and corticotrophin-releasing hormone (CRH). Acute elevations in the

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pNa⁺ or hypernatremia activates magnocellular AVP and OT neurons in the PVN to elicit neurohypophyseal secretion of these neuropeptides which act peripherally on the kidney to promote water retention and the excretion of sodium in urine, respectively (Ludwig et al., 1994; Pirnik et al., 2004; Verbalis et al., 1991). Centrally, acute hypernatremia also inhibits parvocellular neurosecretory CRH neurons in the PVN resulting in blunted stress-induced activation of the hypothalamic-pituitary-adrenal (HPA) axis (Frazier et al., 2013; Krause et al., 2011; Smith et al., 2014). Indeed, recent work suggests that this effect is likely mediated by local paracrine effects of OT released from PVN magnocellular neurons (Frazier et al., 2013; Smith et al., 2015; Smith et al., 2014). In contrast, dendritic release of vasopressin from PVN magnocellular neurons has an excitatory effect on nearby parvocellular preautonomic neurons (Son et al., 2013).

Prior studies have associated chronic salt-loading, as induced by drinking hypertonic saline instead of water, with increased osmoregulatory responses, and attenuated HPA axis activity (Amaya et al., 2001; Lightman and Young, 1987; Sapirstein et al., 1950; Watts, 1992; Watts, 1996). These changes are likely to occur concomitantly with altered expression of CRH in the PVN. For example, studies conducted in rats found that magnocellular neurons of the PVN and supraoptic nucleus (SON) adapt to chronic salt-loading by upregulating CRH (Kovacs and Sawchenko, 1993; Lightman and Young, 1987), which may facilitate the systemic release of OT and thereby natriuresis (Verbalis et al., 1991); however, parvocellular neurons in the PVN down-regulate CRH expression (Amaya et al., 2001).

In the current study we use a CRH-reporter mouse line that has been found to reliably colocalize CRH mRNA with the red fluorescent protein, tdTomato (Smith et al., 2014; Wamsteeker Cusulin et al., 2013) to evaluate the effects of chronic salt-loading over a five day period on plasma sodium, body weight, fluid intake, CRH mRNA expression in PVN, excitatory and inhibitory neurotransmission to known CRH neurons, and the HPA response to acute restraint stress. Our results indicate that chronic salt-loading increases pNa⁺ and fluid intake, reduces CRH mRNA expression in the neurosecretory regions of the PVN, decreases excitatory input to CRH neurons, and reduces the HPA response to restraint stress. Collectively, these results extend our understanding of chronic salt-loading in a mouse model and highlight interesting differences in the centrally mediated effects of acute vs. chronic salt-loading.

Materials and Methods

Animals and Procedure

Adult male CRH reporter mice were generated as previously described (Smith et al., 2014; Taniguchi et al., 2011). Briefly, induction of tdTomato red fluorescent protein to indicate CRH transcription in neurons was accomplished by the generation of B6(Cg)-Crh^{tm1(cre)Zjh/J} knockin mice (Jackson Laboratory Stock # 012704) expressing a Cre recombinase coding region immediately after the STOP codon terminating CRH transcription. These mice were then crossed with Gt(ROSA)26Sor^{tm14(CAG-tdTomato)Hze} congenic mice (Jackson Laboratory Stock # 007914) expressing a *loxP*-flanked STOP cassette preventing tdTomato transcription. CRH reporter mice were 8–12 weeks old at the beginning of the experiments and were maintained on a 12:12 h light/dark cycle in clear

plastic ventilated cages. Prior to the experimental period all animals were maintained with *ad libitum* access to pelleted chow (Harlan Teklad) and water. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Florida.

Salt-loading and HPA axis assessment

During the 5-d experimental period mice either continued *ad libitum* access to water (control group; Water) or had water replaced with a 2% NaCl solution for five days (experimental group; 2% NaCl). Assessment of body weight, food intake, and fluid intake was performed at the same time each day for the duration of the 5-day study. Food intake and fluid intake were expressed relative to body weight. Before initiating the 5-day salt-loading protocol tail blood samples (~20 µL) were collected from mice in both groups using chilled EDTA-coated plastic collection tubes to assess basal levels of hematocrit, plasma sodium and plasma proteins. After the fifth consecutive day of salt-loading, tail blood samples were again taken prior to the onset of restraint stress. These samples were used to determine the effect of salt-loading on hematocrit, plasma sodium, plasma proteins and non-stressed levels of CORT. After the onset of restraint tail blood samples were taken at 30 min, mice were released from the restrainers and tail blood samples were collected at 60 and 120 min during recovery. Restraint was accomplished by placing mice in clear plastic ventilated restraint devices constructed of 50 mL conical tubes for 30 min. Mice were then overdosed with sodium pentobarbital and transcardially perfused with 0.9% saline (30–50 mL) followed by ice-cold 4% paraformaldehyde (PFA; 30–50 mL). Brains were carefully extracted and post-fixed for 4 h in 4% PFA before cryoprotection in 30% sucrose. Four series of free-floating coronal 30 µm sections were cut on a Leica CM3050 S cryostat (Leica, Buffalo Grove, Illinois) and then stored in cryoprotective solution at –20° C. Prior to processing for *in situ* hybridization (below), sections were rinsed and mounted onto microscope slides. Blood samples were kept on ice until centrifuged at 4° C at 6500 rpm for 15 min. Plasma was extracted and stored at –80° C until pNa⁺ levels were determined using an auto flame photometer (Instrumentation Laboratory, Lexington, Massachusetts) and plasma protein concentrations were determined using a hand-held refractometer (Reichert, Depew, New York). Plasma CORT was determined for each time point a blood sample was taken using an ¹²⁵I RIA kit (MP Biomedicals, Santa Ana, California) as previously described (Frazier et al., 2013).

In situ hybridization for CRH mRNA

Localization of mouse CRH mRNA was performed using a digoxigenin (DIG)-conjugated RNA probe that is based on the rat CRH mRNA probe (Choi et al., 2008; Herman et al., 1994; Ostrander et al., 2006). Mouse hypothalamic RNA was extracted using RNeasy mini columns (Qiagen) and cDNA was synthesized using iScript (BioRad). PCR amplification of the 747 bp CRH cDNA fragment for probe generation was performed on mouse hypothalamic cDNA using the following primers: Forward: 5' GAT CCG CAT GGG TGA AGA ATA; Reverse: 5' GCG CGT AAT ACG ACT CAC TAT AGG GGC G-TCC ACT GCA GCT CCA AAT AAA. The reverse primer contains the T7 RNA polymerase site at the 5' end for subsequent synthesis of the antisense mRNA probe. The purified PCR product (150 ng) was mixed with DIG-11-UTP (0.35 mM) and unadulterated UTP (0.65 mM). The mix incubated at 37° C for 2 h and unincorporated NTPs were removed using G50 columns (Roche cat#1 274015). Finally, 100% Ultrapure formamide was added at a 1:1 ratio and the

mixture was stored at -80°C . Hybridization was accomplished by diluting the probe mix 1:1000 in a hybridization buffer (50% Formamide, 0.1 mg/mL yeast tRNA, 10% dextran sulphate, 1x Denhardt's, 0.2 M NaCl, 5 mM EDTA, 10 mM Tris-HCl, 5 mM $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 5 mM Na_2HPO_4) warmed to 65°C . The probe solution was then pipetted onto slide-mounted sections ($\sim 500\text{ }\mu\text{L/slide}$) before coverslipping. Slides were then placed in a humidified tray overnight at 65°C .

DIG Immunohistochemistry—Localization of mRNA probe for CRH was accomplished with an alkaline phosphatase reaction. Following the overnight hybridization, coverslips were removed and slides were washed 3 X 5 min in MABT at room temperature and 2 X 30 min in 50% formamide, 1x SSC, 0.1% Tween-20. Sections were then blocked for 1 h at room temperature with 1x Blocking Reagent (Roche) in MABT with 10% heat-inactivated sheep serum before coverslipping and incubation with primary antibody (Sheep anti-DIG AP Fab Fragment, 1:1500; Roche) overnight at 4°C . The following day, coverslips were removed and slides were rinsed in MABT 3 X 10 min and pre-developing buffer (100 mM Tris pH 9.8, 100 mM NaCl, 50 mM MgCl_2) for 2 X 2 min at room temperature. Slides were then transferred to developing solution (100 mM Tris pH 9.8, 100 mM NaCl, 50 mM MgCl_2 , 5% polyvinyl alcohol, 0.11 mM nitroblue tetrazolium salt in 70% dimethylformamide, 5-bromo-4-chloro-3-indolyl-phosphate in 100% dimethylformamide) in a foil-wrapped coplin jar at 37°C for 36 h. Slides were then briefly rinsed in tap water to stop the color reaction. Sections were then dehydrated in 2 min rinses of increasing concentrations of ethanol, defatted 2 X 2 min in xylene, and then coverslipped with DPX mounting media (VWR).

Image capture and analysis

Brightfield images of CRH mRNA-DIG *in situ* hybridization labeled sections were taken with a Plan-Apochromat 10x/0.45 M27 objective using light filters to obtain maximum contrast. Densitometry of CRH mRNA labeling was performed using Image J (NIH) by first outlining a region of interest based on boundaries established in The Mouse Brain in Stereotaxic Coordinates 3rd Ed (Franklin and Paxinos, 2008) and Biag et al. (2012) for separate anterior-posterior levels of the PVN, and then using the “measure” function to determine the raw integrated density of the outlined area. Bi-lateral densities for each PVN atlas-matched level were averaged for brain sections taken from control and salt-loaded mice. Signal density data are then reported as percentages, relative to the mean water control data for each atlas section.

In vitro electrophysiology

Male CRH reporter mice (2–5 months old) were administered ketamine (80–100 mg/kg, ip) and were rapidly decapitated using a rodent guillotine. The brain was quickly removed, and coronal sections (300 μm thick) through the PVN were made using a Leica VT 1000s vibratome. Slices were incubated for 30 minutes in a dissecting solution maintained at $30\text{--}35^{\circ}\text{C}$, and then allowed to equilibrate at room temperature for at least 30 minutes prior to experimental use. The external solution for dissection and incubation contained in mM: 124 NaCl, 2.5 KCl, 1.23 NaH_2PO_4 , 2.5 MgSO_4 , 10 D-glucose, 1 CaCl_2 , and 25.9 NaHCO_3 , saturated with 95% O_2 –5% CO_2 . During whole cell patch clamp recording, slices were continuously perfused at a rate of 1.2–1.5 mL/min with artificial cerebral spinal fluid (aCSF)

that contained in mM: 126 NaCl, 3 KCl, 1.2 NaH₂PO₄, 1.5 MgSO₄, 11 D-glucose, 2.4 CaCl₂, and 25.9 NaHCO₃. This solution was saturated with 95% O₂ and 5% CO₂, and bath temperature was maintained at 28 ± 2°C.

CRH neurons expressing tdTomato were initially identified in PVN slices using epifluorescence microscopy. Whole-cell voltage-clamp recordings from CRH neurons were then initiated under differential interference contrast microscopy using micropipettes pulled from a borosilicate glass with a Flaming/Brown electrode puller (Sutter P-97; Sutter Instruments, Novato, California). Voltage-clamp experiments were performed using an Axon Multiclamp 700A or 700B amplifier (Molecular Devices, Sunnyvale, CA). Data were sampled at 20 kHz, lowpass filtered at 2 kHz, and recorded on a computer via a Digidata 1400 A/D converter using Clampex version 10 (Molecular Devices, Sunnyvale, CA). To isolate spontaneous excitatory postsynaptic currents (sEPSCs) neurons were voltage clamped at -70 mV in the presence of the GABA_A receptor antagonist picrotoxin (100 μM), using an internal solution that contained (in mM): 130 K-gluconate, 10 KCl, 10 NaCl, 2 MgCl₂, 1 EGTA, 2 Na₂ATP, 0.3 NaGTP, and 10 HEPES, pH adjusted to 7.3 using KOH and volume adjusted to 285–300 mOsm. To isolate spontaneous inhibitory postsynaptic currents (sIPSCs) neurons were voltage clamped at -70 mV in the presence of the ionotropic glutamate receptor antagonists DNQX (20 μM) and DL-2-amino-5-phosphonopenatanonic acid (APV, 40 μM), using an internal solution that contained (in mM): 100 K-gluconate, 40 KCl, 10 NaCl, 2 MgCl₂, 1 EGTA, 2 Na₂ATP, 0.3 NaGTP, and 10 HEPES, also pH adjusted to 7.3 using KOH and volume adjusted to 285–300 mOsm. The higher internal chloride in this solution allowed sIPSCs to be detected as inward currents in neurons voltage clamped at -70 mV. Spontaneous synaptic currents were recorded continuously for at least four minutes approximately 10–15 minutes after establishment of whole cell recording. A 50 msec voltage step from -70 mV to -80 mV was delivered every 5 seconds during and used to monitor access resistance, whole cell capacitance, and input resistance, and holding current. Cells which displayed unstable access resistance or holding current during the four-minute recording period were excluded from further analysis. Event detection was performed in all other cells using parameter based event detection software written by in OriginC (Originlab, Northampton, MA) by CJF. All chemicals used in the electrophysiological experiments were obtained from either Tocris Cookson or Sigma-Aldrich.

Statistics

All group data are presented as mean ± SEM. Fluid intake, food intake, body weight and plasma CORT were assessed with a two-factor analysis of variance. Integrated plasma CORT responses were calculated as the area under the curve (AUC) of the time course data. Main effects or interactions ($P < 0.05$) were assessed post-hoc with the Bonferroni method. Within group (pre vs post salt-loading) and between group CRH mRNA, pNa⁺, hematocrit, and plasma protein percentage were each assessed with a paired and unpaired 1-tailed t-test, respectively. Primary parameters of spontaneous synaptic events (frequency, amplitude) from control and salt loaded animals were compared using a two-tailed unpaired Student's T-test (with Welch correction in all cases where datasets had unequal variance). Significance for all analyses was set at $P < 0.05$. Statistical analyses were performed and graphs were

created using GraphPad Prism 5 (Graphpad Software, La Jolla, CA) and/or OriginPro 2017 (OriginLab, Northampton, MA).

Results

Salt-loaded mice exhibit increased fluid intakes and plasma sodium concentrations, but maintain normal indices of blood volume

Daily monitoring of fluid intake revealed that mice in the control condition drank an average of 5.27 ± 0.10 mL/30 g of body mass of water each day and intake did not significantly change over the course of the study. In contrast, mice with 2% NaCl as a sole source of fluid drank significantly more after 48 h of salt exposure (time x treatment interaction; $[F_{(4, 115)} = 3.14, (P < 0.05)]$) and intake remained significantly higher ($P < 0.0001$ on days 2, 3, and 4; $P < 0.001$ on day 5) than controls for each day thereafter (Fig. 1, A). Consumption of 2% NaCl for five days significantly elevated the pNa^+ relative to Day 1 and water drinking controls ($P < 0.05$) (Fig. 1, B). Despite the challenge to hydromineral homeostasis, hematocrit and plasma protein concentrations were similar between groups both at the beginning and the end of the experiment (Fig. 1, C & D).

Salt-loaded mice maintain food intake and body weight

There was a main effect of treatment [$F_{(1, 115)} = 9.53, (P < 0.05)$] with a tendency for salt-loading to reduce food intake on Day 1 (Fig. 2A, Day 1: Water 6.92 ± 1.12 ; 2% NaCl 4.33 ± 0.72); however, there were no effects of time or time x treatment interactions. Figure 2B shows the daily average body weights for each group. Despite variation in fluid and food intake, both groups maintained stable body weights throughout the five-day period.

Salt-loading decreases expression of CRH mRNA in neurosecretory regions of the PVN without significantly altering it in preautonomic regions

Figure 3 shows representative images of sequential (A and C, B and D) coronal brain sections taken from a series through the PVN and processed for *in situ* hybridization and digoxigen immunohistochemistry. At Bregma -0.70 mm, the CRH mRNA labeling represents a dense triangular region (Fig. 3A) that is markedly reduced in the ventral portion with salt-loading (Fig. 3B). At Bregma -0.82 mm, CRH expression following salt-loading appears localized to a cluster in the dorsal PVN (Fig. 3C) that has reduced area, but not density (Fig. 3D) with salt-loading (Fig. 3D). Quantification of the average density for each PVN region revealed a significant ($P < 0.05$) decrease in CRH mRNA density with salt-loading at Bregma -0.70 mm, but no overall difference at Bregma -0.82 mm (Fig. 3E).

Salt-loading reduces frequency of spontaneous excitatory postsynaptic currents in PVN CRH neurons, without altering inhibitory postsynaptic currents

CRH neurons from control or salt-loaded mice were identified in acute slices through the PVN using epifluorescence microscopy and voltage clamped at -70 mV (See Methods). Spontaneous excitatory or inhibitory synaptic currents were recorded for a minimum of 4 minutes approximately 10–15 minutes after establishing a whole cell recording. Salt-loading significantly reduced the frequency of sEPSCs (5.2 ± 1.5 Hz in control vs. 0.9 ± 0.2 Hz in neurons from salt loaded mice, $p = 0.03$) without altering their amplitude (Figs. 4A and B).

By contrast, salt-loading had no significant effect on either frequency or amplitude of sIPSCs (Figs. 4C and D).

Salt-loading blunts restraint-induced elevations in plasma CORT during recovery

Assessment of plasma CORT revealed a significant time X treatment interaction [Figure 5, $F_{(3,54)} = 3.32$, ($P < 0.05$)]. Restraint increased CORT at 30 min compared to baseline concentrations in both groups; however, salt-loaded mice recovered faster and plasma CORT at 120 min was significantly reduced compared to water controls ($P < 0.05$). This attenuation was specific to this time points as neither baseline nor 60 min CORT concentrations were significantly different between groups. The area under the curve was not different relative to controls (Figure 5, inset).

Discussion

The current study uses mice to evaluate the effects of chronic salt-loading on ingestive behavior and indices of HPA axis activity. These results provide new information about the physiological, behavioral, and anatomical impact of chronic salt-loading, and insights into potential underlying mechanisms.

From a physiological and behavioral standpoint, chronic salt-loading in mice produced robust increases in fluid intake and clear osmotic stress as indicated by increased pNa^+ . However, interestingly, food intake and body weight were not altered, and there was no change in hematocrit or plasma protein. While hematocrit and plasma protein concentrations are not direct measures of blood volume, it is interesting to note that rats presented with saline instead of water exhibit highly variable fluid intake and decreased food consumption resulting in significantly reduced blood volume and body weight (Boyle et al., 2012; Watts, 1992; Watts et al., 1995; Yue et al., 2008). The renal handling of sodium and water seems to be more efficient in mice relative to rats (Christensen et al., 2014; Zhai et al., 2006). Whereas rats respond to forced saline consumption by limiting food intake, mice quickly adapt to maintain intake levels by drinking and excreting large volumes of fluid. Consistent with this, the higher ratio of short to long loop nephrons in the mouse kidney (4.5:1, (Zhai et al., 2006)) compared with rats (2.3:1, (Kriz, 1967)) is predictive of the lower rise in plasma osmolality observed in mice (3.3%, (Polito et al., 2006)) compared with rats (6.5%, (Watts et al., 1995)) following five days of drinking 2% NaCl. Greater resiliency to osmotic stress in mice likely explains the lack of dehydration induced anorexia in the current study, despite the fact that this phenomenon has been thoroughly characterized in rats (e.g. see Salter-Venzon and Watts, 2008; Watts, 1999; Watts, 2000; Watts and Sanchez-Watts, 2007). A higher concentration of sodium or a longer duration of salt-loading may elicit similar effects in mice; however, this possibility was not evaluated in the present study.

From a neuroanatomical perspective, the current study indicates that total CRH expression was moderately reduced in neurosecretory regions of the PVN, but unaltered in preautonomic regions. Specifically, we noted an apparent reduction in both density and area of CRH mRNA in more anterior regions of the PVN with a likely change in area but not density in dorsal and posterior regions of the PVN. Collectively, prior work in rats suggests that chronic salt-loading alters the relative expression of CRH in parvocellular and

magnocellular neurons (Amaya et al., 2001; Kovacs and Sawchenko, 1993; Lightman and Young, 1987), and augments Fos induction in neurons expressing CRH in the lateral hypothalamic area but not in the parvocellular neurosecretory region of the PVN (Watts and Sanchez-Watts, 2007). The current study in mice is generally consistent with these results; however additional studies are necessary to better understand the change in CRH expression within specific subdivisions of the PVN.

From a cellular and synaptic perspective, we also report that chronic salt-loading produced a selective reduction in excitatory but not inhibitory synaptic inputs to PVN CRH neurons. The current study is one of relatively few to use *in vitro* electrophysiological approaches to directly evaluate function of CRH neurons in PVN (e.g. see Senst et al., 2016; Wamsteeker Cusulin et al., 2013), and the first to do so after chronic salt-loading. It is interesting to note that prior work in rats has indicated that chronic variable stress (CVS) produces an increase in VGluT2 appositions to PVN CRH neurons without altering the number of GABAergic-immunoreactive boutons (Flak et al., 2009), suggesting the possibility that CVS and chronic salt have opposing physiological impact on excitation of CRH neurons. It might be expected that decreased excitatory input to PVN CRH neurons as indicated by the *in vitro* studies would predict lower basal plasma CORT, similar to what occurs in salt-loaded rats (Amaya et al., 2001). It is possible that in the current study, basal plasma CORT was simply too low in the control condition to reliably observe this effect. Neurons within the PVN that synthesize CRH receive dense glutamatergic inputs from diverse brain regions and further work is needed to determine the specificity of the excitatory inputs that are downregulated following chronic salt-loading.

Finally, we report that chronic salt-loading attenuated the acute HPA axis response during recovery from stress. This result stands in interesting contrast to prior studies in both rats and mice which indicate that acute hypernatremia produces robust inhibition of the HPA axis response to restraint as observed at the initiation of the stress but not during recovery (Frazier et al., 2013; Krause et al., 2011; Smith et al., 2014). Accumulating evidence suggests that acute hypernatremia is likely to inhibit PVN CRH neurons via a mechanism that depends on an oxytocin mediated paracrine signaling mechanism (Frazier et al., 2013; Smith et al., 2015; Smith et al., 2014). Indeed, PVN magnocellular neurons are osmosensitive, and acute hypernatremia produces a much larger, albeit much shorter, increase in plasma osmolarity than observed here. Thus it seems likely that the effects of chronic salt on stress responsiveness are less dependent on acute activation of magnocellular neurons, and more likely to involve structural and/or physiological changes that downregulate excitatory input to PVN CRH neurons and/or their ability to activate the HPA axis. Indeed, a general reduction in CRH expression in neurosecretory neurons and decreased basal excitatory input could plausibly work synergistically to hasten recovery from acute restraint stress, as observed here. It will be interesting for future studies to evaluate CRH expression and HPA function in these animals in response to rehydration.

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Highlights

- The effects of chronic salt loading in mice are evaluated.
- Food intake, body weight, plasma protein, and hematocrit are unaltered.
- Expression of CRH mRNA in neurosecretory regions of the PVN is decreased.
- Frequency of spontaneous excitatory inputs to PVN CRH neurons is reduced.
- Restraint induced elevation of plasma corticosterone is blunted during recovery.

Figure 1.

Fluid consumption and the effects on indices of hydration. Mice with access to 2% NaCl (shaded circles) instead of water (open squares) drank significantly more (time x treatment interaction; [$F(4, 115) = 3.14$, ($P < 0.05$))] each day after the first 48 h (A). Mice maintained on 2% NaCl had higher pNa^+ on Day 5 compared with Day 1 (B; $P < 0.05$). Hematocrit (C) and plasma proteins (D) were similar between groups. Water $n=12$, 2% NaCl $n=13$.

Fig. 2.

Daily food intake and body mass measurements. The consumption of chow was similar between salt-loaded mice and water-drinking controls as there were no significant differences in daily intakes (A), however, there was a main effect of treatment [$F(1, 115) = 9.53$, ($P < 0.05$)]. Body weights did not differ significantly (B). Water $n=12$, 2% NaCl $n=13$.

Fig. 3.

Representative images of PVN sections processed for *in situ* hybridization of digoxigenin-conjugated probe and CRH mRNA. Comparable sections from mice in the Water (A, C) and 2% NaCl (B, D) groups. Salt-loading was associated with a decrease in density at Bregma -0.70 mm, but did not differ at Bregma (E, $P < 0.05$). Water $n = 4-6$, 2% NaCl $n = 4$. Scale bars = 100 μm . 3v, 3rd ventricle.

Fig. 4.

Chronic salt-loading reduces spontaneous excitatory input to CRH positive neurons in PVN without altering spontaneous inhibitory input. A) Representative raw data traces illustrating isolated sEPSCs in CRH positive neurons voltage clamped at -70 mV (See Methods). B) Summary data for sEPSC experiments. Mean sEPSC frequency was 5.2 ± 1.5 Hz in neurons from control animals vs. 0.9 ± 0.2 Hz in neurons from salt loaded animals (asterisk indicates $p=0.03$, $n=6, 7$ respectively). Mean sEPSC amplitude was 9.1 ± 1.1 pA in neurons from control animals vs. 10.3 ± 0.6 pA in neurons from salt loaded animals ($p=0.3$, $n=6, 7$ respectively). C) Representative raw data traces illustrating isolated sIPSCs in CRH positive neurons voltage clamped at -70 mV (See Methods). D) Summary data for sIPSC experiments. Mean sIPSC frequency was 4.6 ± 0.8 Hz in neurons from control animals vs. 3.4 ± 1.1 Hz in neurons from salt loaded animals ($p=0.4$, $n=6$ in both groups). Mean sEPSC amplitude was 27.0 ± 5.74 pA in neurons from control animals vs. 30.0 ± 4.30 pA in neurons from salt loaded animals ($p=0.7$, $n=6$ in both groups).

Fig. 5. Chronic salt-loading reduces the CORT response to restraint during the recovery period. The integrated CORT response was not significantly reduced. * $p < 0.05$. AUC=Area Under the Curve. Error bars indicate SEM.