Physiology & Behavior 136 (2014) 91–96

Contents lists available at ScienceDirect

Physiology & Behavior

journal homepage: www.elsevier.com/locate/phb

Acute hypernatremia promotes anxiolysis and attenuates stress-induced activation of the hypothalamic–pituitary–adrenal axis in male mice ☆

Justin A. Smith [a], Lei Wang [a], Helmut Hiller [a], Christopher T. Taylor [a], Annette D. de Kloet [b], Eric G. Krause [a,*]

[a] Department of Pharmacodynamics, College of Pharmacy, University of Florida, PO Box 100487, Gainesville, FL 32611, United States
[b] Department of Physiology and Functional Genomics, College of Medicine University of Florida, PO Box 100274, Gainesville, FL 32610, United States

HIGHLIGHTS

- Hypernatremia decreased activation of the HPA axis and anxiety-like behavior.
- Hypernatremia activated OT neurons but inhibited CRH neurons.
- Excess dietary sodium intake may alleviate the impact of psychological stress.

ARTICLE INFO

Article history:
Received 23 January 2014
Received in revised form 24 March 2014
Accepted 25 March 2014
Available online 2 April 2014

Keywords:
Oxytocin
Corticotropin-releasing-hormone
Anxiety
Depression
Hypertension
Hypernatremia

ABSTRACT

Previous investigation by our laboratory found that acute hypernatremia potentiates an oxytocinergic tone that inhibits parvocellular neurosecretory neurons in the paraventricular nucleus of the hypothalamus (PVN), attenuates restraint-induced surges in corticosterone (CORT), and reduces anxiety-like behavior in male rats. To investigate the neural mechanisms mediating these effects and extend our findings to a more versatile species, we repeated our studies using laboratory mice. In response to 2.0 M NaCl injections, mice had increased plasma sodium concentrations which were associated with a blunted rise in CORT subsequent to restraint challenge relative to 0.15 M NaCl injected controls. Immunofluorescent identification of the immediate early gene product Fos found that 2.0 M NaCl treatment increased the number of activated neurons producing oxytocin in the PVN. To evaluate the effect of acute hypernatremia on PVN neurons producing corticotropin-releasing hormone (CRH), we used the Cre-lox system to generate mice that produced the red fluorescent protein, tdTomato, in cells that had Cre-recombinase activity driven by CRH gene expression. Analysis of brain tissue from these CRH-reporter mice revealed that 2.0 M NaCl treatment caused a dramatic reduction in Fos-positive nuclei specifically in CRH-producing PVN neurons. This altered pattern of activity was predictive of alleviated anxiety-like behavior as mice administered 2.0 M NaCl spent more time exploring the open arms of an elevated-plus maze than 0.15 M NaCl treated controls. Taken together, these results further implicate an oxytocin-dependent inhibition of CRH neurons in the PVN and demonstrate the impact that slight elevations in plasma sodium have on hypothalamic–pituitary–adrenocortical axis output and anxiety-like behavior.

1. Introduction

In mammals the plasma sodium concentration (pNa^+) is regulated by neural, humoral and behavioral mechanisms that maintain blood tonicity at levels that allow normal physiological function. Sodium deficiency causes hyponatremia and increases circulating levels of angiotensin II (Ang-II) and aldosterone (ALDO) which activate receptors in the kidney and brain to restore the pNa^+ to homeostatic levels by promoting the retention and consumption of sodium. Conversely, excess sodium causes hypernatremia and suppresses Ang-II and ALDO but causes the secretion of vasopressin (AVP) and oxytocin (OT) into the systemic circulation which alleviates the elevated pNa^+ by promoting renal water retention and sodium excretion. Thus, the pNa^+ is tightly regulated by neurohumoral compensatory responses that act in the brain and periphery to maintain blood tonicity at homeostatic levels when challenged with sodium deficiency or excess.

The neuropeptides and hormones that maintain the pNa^+ are also known to influence mood, affect, and stress responsiveness. For example, studies conducted in humans and animals have found that elevated circulating levels of Ang-II and ALDO are predictive of affective disorder [1–4], and that OT and AVP are mediators of stress responsiveness and anxiety [5,6]. As mentioned, the secretion of Ang-II, ALDO, AVP, and

☆ Support: NIH HL096830 (EGK), HL116074 (ADdK).
* Corresponding author. Tel.: +1 352 273 6977.
E-mail addresses: smith6jt@ufl.edu (J.A. Smith), stone0338@ufl.edu (L. Wang), hhiller@cop.ufl.edu (H. Hiller), gator.ctaylor@gmail.com (C.T. Taylor), adekloet@ufl.edu (A.D. de Kloet), ekrause@cop.ufl.edu (E.G. Krause).

http://dx.doi.org/10.1016/j.physbeh.2014.03.027
0031-9384/

OT are heavily influenced by the pNa^+, and therefore, it is possible that alterations in body sodium levels affect stress responding and anxiety-like behavior through manipulation of these neuroendocrine signals. In this regard, previous work from our group and others have found that sodium depletion is anxiogenic [7] but acute salt loading is anxiolytic and dampens stress responsiveness in laboratory rats [8,9]. While the changes in the pNa^+ are found to influence mood and stress responding in rats, whether these effects generalize to mice, and therefore, allow the use of the genetic manipulations that mouse models afford to investigate central mechanism(s) underlying the stress limiting effects of acute hypernatremia has not been evaluated.

The goal of the present study was to determine whether acute modest increases in the pNa^+ affect anxiety-like behavior and hypothalamic–pituitary–adrenal (HPA) axis activation in laboratory mice. Mice were rendered mildly hypernatremic via systemic administration of 2.0 M NaCl, and subsequently, were subjected to psychogenic stress or tests of anxiety-like behavior. Administration of 2.0 M NaCl produced a modest but significant increase in the pNa^+ relative to control injection of 0.15 M NaCl. This modest rise in the pNa^+ was associated with attenuated anxiety-like behavior and decreased stress-induced HPA activation. Both quantitative and qualitative neuroanatomical studies were conducted to provide insight towards neural mechanisms contributing to the anxiolytic effects of acute mild hypernatremia. Collectively, the results demonstrate that the stress limiting and anxiolytic effects of slight elevations in the pNa^+ also occur in mice. The implication is that acutely increasing the pNa^+ may trigger interactions between neurons expressing OT and corticotropin-releasing-hormone (CRH) to limit responding to psychological stress.

2. Materials and methods

2.1. Animals

Studies examining the effects of acute hypernatremia on anxiety-like behavior and HPA activation used adult male C57BL/6 mice obtained from Harlan. Neuroanatomical studies utilized the Cre-LoxP system to generate male mice that express red fluorescent protein (tdTomato) in cells that produce CRH. Briefly, these CRH-reporter mice were generated by breeding mice that have Cre recombinase expression directed to CRH-producing cells (Jackson Laboratory Stock # 012704) to mice with a mutation of the Gt(ROSA)26Sor locus with a *loxP*-flanked STOP cassette preventing transcription of a CAG promoter-driven sequence coding for tdTomato (Jackson Laboratory Stock # 007914). All mice were 9–10 weeks old at the initiation of the study and were individually housed on a 12:12 h light/dark cycle in clear plastic ventilated cages with plumbed water supply. Standard mouse chow (Harlan) was suspended in a wire rack that also supported an accessory water bottle allowing *ad libitum* access to both food and water except where otherwise noted. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Florida.

2.2. Restraint stress and blood sampling

Mice were injected subcutaneously with 0.1 mL of either 2.0 M ($n = 10$) or 0.15 M NaCl ($n = 10$) and returned to their home cages where water was made unavailable. Saline injections were preceded by 2% lidocaine (~0.01 mL) to minimize discomfort. Sixty-minutes after saline injections, mice were placed in clear plastic ventilated tubes to initiate a stress response in the context of normal or elevated pNa^+. Tail blood samples (~20 μL) were collected in chilled EDTA-coated plastic collection tubes immediately at the onset of restraint and again after 30 min of immobilization in plastic restrainers. Mice were then released and allowed to recover in their home cages where two more blood samples were taken at 60 min and 120 min relative to the initiation of restraint. Blood samples were kept on ice until centrifuging at 4 °C at 6500 rpm for 15 min. Microcapillary samples were measured for hematocrit, and plasma was extracted and stored at −80 °C until pNa^+, plasma proteins, and CORT analyses took place. Plasma sodium levels were determined for the blood sample taken at the onset of restraint using an auto flame photometer as previously described [9] (Instrumentation Laboratory, Lexington, Massachusetts). Plasma CORT was determined for each time point a blood sample was taken using an ^{125}I RIA kit (MP Biomedicals, Santa Ana, CA) as previously described [9]. Plasma proteins and hematocrit were determined for the blood sample taken at the onset of restraint using a handheld refractometer (VET 360, Reichert) and a microcapillary reader, respectively.

2.3. In situ hybridization

RNAscope *in situ* hybridization (ISH) was performed on brain tissue collected from CRH-reporter mice to determine the extent to which CRH mRNA co-localizes with tdTomato in the PVN. Mice were overdosed with sodium pentobarbital, and then transcardially perfused with 0.9% saline followed by 4% paraformaldehyde (PFA). Subsequently, brains were extracted, coronally sectioned at 20 μm into 6 series and then immediately rinsed and mounted onto Superfrost Plus Gold slides. Tissue collection, sectioning and mounting of sections were performed in RNase-free conditions. Slides were allowed to air dry for 20–30 min and then were stored at −80 °C until processing for *in situ* hybridization. Three slides containing separate series of sections through the PVN were allowed to reach room temperature for 30 min prior to performing the manufacturer's protocol (Advanced Cell Diagnostics; Hayward, CA). RNAscope ISH was performed using the following probes: (1) Negative Control, DapB, (2) Positive control, Ubc, (3) CRH. All images were captured at 40× magnification and the exposure time was adjusted for each image using the best-fit feature in Axiovision. Subsequently, the min–max feature was utilized to minimize background fluorescence and provide optimal visualization of RNA signal. All images were processed using the same automated parameters.

2.4. Immunohistochemistry

2.4.1 Two separate histological studies were performed: CRH-reporter mice ($n = 6$) and ($n = 8$) were each further divided into groups given either 2.0 M NaCl or 0.15 M NaCl and then restrained 60 min later as described above. Mice were sacrificed 120 min after the onset of restraint (180 min after injections) and stimulated Fos induction, a marker of neuronal activity, is known to peak during this time [10]. Mice were overdosed with sodium pentobarbital inducing a level of anesthesia that rendered them unresponsive to toe-pinch before transcardial perfusion with 0.9% saline. Following the clearing of blood, mice were perfused with 4% PFA and brains were carefully extracted then post-fixed for 2 h in 4% PFA before cryoprotection in 30% sucrose. Four series of coronal 30 μm brain sections were cut on a Leica CM3050 S cryostat (Leica, Buffalo Grove, Illinois) and then stored in cryoprotective solution at −20 °C.

2.4.2 Rinses and solutions were made using 50 mM potassium phosphate buffered saline (KPBS) and took place at room temperature on an orbital shaker unless otherwise noted. Immunofluorescent labeling of Fos in brain sections from CRH-reporter mice began by rinsing free-floating sections 5 × 5 min to remove cryoprotectant. Blocking consisted of 2% normal donkey serum (Jackson ImmunoResearch, West Grove, Pennsylvania) with 0.2% Triton-X (Sigma) for 1 h followed by primary antibody incubation with rabbit anti-Fos (sc-52 1:1000; Santa Cruz) in a blocking solution overnight at 4 °C. The second day consisted of rinses 5 × 5 min and incubation for 2 h in a blocking solution with donkey anti-rabbit Alexa-Fluor 647 (1:500; Jackson ImmunoResearch, West Grove, Pennsylvania). After a final series of rinses, sections were mounted on Superfrost Plus slides (Fisher) in KPB, allowed to air dry, and then coverslipped using polyvinyl alcohol with DABCO (Sigma).