Physiological and Transcriptomic Changes in the Hypothalamic-Neurohypophyseal System after 24 h of Furosemide-Induced Sodium Depletion

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Keywords

Vasopressin · Oxytocin · Osmolality · Hypothalamus · Sodium appetite

Abstract

Background/Aims: Furosemide is a loop diuretic widely used in clinical practice for the treatment of oedema and hypertension. The aim of this study was to determine physiological and molecular changes in the hypothalamic-neurohypophyseal system as a consequence of furosemide-induced sodium depletion. **Methods:** Male rats were sodium depleted by acute furosemide injection (10 and 30 mg/kg) followed by access to low sodium diet and distilled water for 24 h. The renal and behavioural consequences were evaluated, while blood and brains were collected to evaluate the neuroendocrine and gene expression responses. **Results:** Furosemide treatment acutely increases urinary sodium and

water excretion. After 24 h, water and food intake were reduced, while plasma angiotensin II and corticosterone were increased. After hypertonic saline presentation, sodium-depleted rats showed higher preference for salt. Interrogation using RNA sequencing revealed the expression of 94 genes significantly altered in the hypothalamic paraventricular nucleus (PVN) of sodium-depleted rats (31 upregulated and 63 downregulated). Out of 9 genes chosen, 5 were validated by quantitative PCR in the PVN (upregulated: *Ephx2*, *Ndnf* and *Vwf*; downregulated: *Caprin2* and *Opn3*). The same genes were also assessed in the supraoptic nucleus (SON, upregulated: *Tnnt1*, *Mis18a*, *Nr1d1* and *Dbp*; downregulated: *Caprin2* and *Opn3*). As a result of these plastic transcriptome changes, vasopressin expression was decreased in PVN and SON, whilst vasopressin and oxytocin levels were reduced in

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plasma. **Conclusions:** We thus have identified novel genes that might regulate vasopressin gene expression in the hypothalamus controlling the magnocellular neurons secretory response to body sodium depletion and consequently hypotonic stress.

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Introduction

Extracellular fluid (ECF) volume and osmolality are maintained by a complex mosaic of neuroendocrine, autonomic, hemodynamic, renal and behavioural mechanisms. The hypothalamic-neurohypophyseal system (HNS) plays a crucial role in the regulation of ECF balance [1–3]. This system is composed of magnocellular neurons that have their cell bodies located in the paraventricular nucleus (PVN) and supraoptic nuclei (SON) of the hypothalamus with axons forming tracts that end in the formation of the neurohypophysis from where the hormones arginine vasopressin (AVP) and oxytocin (OXT) are secreted into the systemic circulation [2]. A rise in ECF osmolality evokes AVP and OXT release. AVP acts to reduce urinary water excretion, whilst concomitant inhibition of sodium consumption and renal sodium excretion mediated by OXT. In contrast, a fall in ECF osmolality evokes a decrease in AVP and OXT secretion, which results in increased water excretion and sodium retention. Similarly, decreases in ECF volume stimulate AVP secretion which leads to renal water reabsorption and fluid retention. Instead, increased ECF volume stimulates a prominent secretion of OXT to increase sodium excretion and consequently reduce body fluid content [1, 3]. These processes work in concert to ensure that ECF osmolality and volume are stable. In addition to the peripheral action, AVP and OXT are also produced by PVN parvocellular neurons and released into the central nervous system as neuromodulators, exerting important autonomic and behavioural effects [4].

In clinical practice, furosemide is a diuretic that is widely used as a treatment for hypertension, oedematous disorders and is valuable in the management of hypervolemia and electrolyte disorders [5]. Furosemide acts through the blockade of the $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ co-transporter (NKCC2) in the thick ascending limb of the loop of Henle resulting in the impaired reabsorption of Na^+ , K^+ and Cl^- . NKCC2 cotransporter blockade also decreases medullary hypertonicity and the osmotic driving force for water reabsorption, resulting in an isosmotic ECF volume loss [6, 7]. As a consequence of body sodium and

ECF volume loss, the renin-angiotensin-aldosterone system (RAAS) and sodium appetite are activated [7–9]. Recently, experiments performed in transgenic rats expressing green fluorescent protein under the AVP promoter demonstrated that a single administration of furosemide acutely increased Fos-like immunoreactivity and AVP expression in the SON and in magnocellular PVN neurons [10]. These data indicate that acute ECF volume depletion is able to stimulate the HNS. However, the consequences of chronic body sodium and ECF volume depletion with furosemide on the HNS are not understood.

We and others have previously demonstrated using microarrays and RNA sequencing that the hydromineral imbalance, such as water deprivation, salt loading and 1-desamino-(8-D-arginine)-vasopressin treatment causes changes in the global expression of many genes in the HNS [11–15]. These previous results provided a quantitative assessment of whether a gene is expressed in a particular tissue or organ and whether specific transcripts are up or downregulated or remain unchanged as a consequence of hyperosmotic and hypoosmotic conditions [16]. However, there have been no such studies evaluating global changes in hypothalamic gene expression after 24 h of whole body sodium depletion associated with the neuroendocrine and behavioural adaptations.

We hypothesised that the hydromineral changes induced by 24 h of body sodium depletion is able to change global gene expression in the HNS impacting in the AVP and OXT synthesis-secretion coupling. Thus, the aim of this study was to determine the renal, endocrine, behavioural and transcriptomic adaptations related to the HNS in rats submitted to 24 h sodium depletion induced by furosemide.

Methods

Animals

Male Wistar rats obtained from the Animal Facility of the Department of Physiological Sciences-UFRRJ, and Holtzman rats obtained from School of Dentistry-UNESP were used in this study. Rats weighing 250–300 g were group housed (4 rats/cage) under controlled temperature ($23 \pm 2^\circ\text{C}$) and light conditions (12: 12 h light/dark cycle) with free access to standard food pellets and tap water. All experiments were performed in the morning (between 8.00 and 12.00 h) and approved by the Ethics Committee on animal use at the Institute of Biological and Health Sciences – UFRRJ (6492/2015-64) and School of Dentistry-UNESP (06/2015) and conducted according to the “Guide for the Care and Use of Laboratory Animals” (NIH Publication, 2011).

Experimental Design

Experiment 1: Effects of Furosemide-Induced Sodium Depletion on Hydromineral Parameters

A set of 27 Wistar rats (9 per group) was used in this experiment. Rats were placed in individual metabolic cages for 3 days of adaptation with food and filtered water *ad libitum*. After the rats were submitted to the control procedure (0.15 M NaCl vehicle *s.c.* injection) or to furosemide-induced sodium depletion (10 or 30 mg/kg *s.c.* furosemide treatment, respectively Furo10 and 30 groups) combined with free access to low sodium diet and distilled water. Water intake and urinary volume, osmolality and sodium excretion were evaluated at 2, 6 and 24 h, while food intake was evaluated 24 h after furosemide treatment. After sodium depletion for 24 h, 2 bottles containing distilled water or hypertonic saline (0.3 M NaCl solution) were offered to all animals. Water and hypertonic saline intake during the 2-bottle test were measured at 0.25, 0.5, 1, 2, 3, 4, 5, 6 and 24 h. Urinary volume, osmolality and sodium were evaluated at 2, 6 and 24 h after offering free choice between water and hypertonic saline.

Experiment 2: Plasma Hydromineral Parameters, Hormonal Changes and Quantitative PCR Transcriptome Validation after Sodium Depletion

Another set of 30 Wistar rats was used for this experiment (10 per group). These rats were submitted to the control procedure or to sodium depletion induced by furosemide (10 and 30 mg/kg *s.c.*) as described in the protocol 1. After 24 h, the rats were euthanised by decapitation and both blood and brains were collected. The blood was used to evaluate the haematocrit, plasma osmolality and sodium concentration. The levels of the hormones AVP, OXT, angiotensin-II (ANG II) and corticosterone were also quantified. The brains were used to validate transcriptomic changes in the PVN and gene expression in the SON produced by sodium depletion.

Experiment 3: RNAseq Evaluation of PVN Transcriptomic Changes Induced by 24 h Sodium Depletion

A set of 12 Holtzman rats (6 per group) was used in the present experiment. Rats were submitted to the control procedure or to 10 mg/kg furosemide-induced sodium depletion (Furo10) combined with free access to low sodium diet and distilled water. Twenty-four hours later, the rats were euthanised, brains were collected and the bilateral PVN were micropunched. Afterwards, RNA sequencing (see below) was performed to evaluate the global changes in the gene expression induced by sodium depletion.

Blood Collection, Plasma Measurements and Radioimmunoassays

After decapitation, trunk blood was collected in chilled plastic tubes containing heparin (for measurement of AVP, OXT, corticosterone, osmolality and sodium concentration) or peptidase inhibitors (for ANG II). Plasma was obtained after centrifugation (20 min, 1,600 g, 4°C) and stored at -20°C.

Haematocrit was determined by using small aliquots of trunk blood collected in capillary tubes put in microcentrifuge (200 g, 10 min) at room temperature and expressed as percentage of cells in the blood. Plasma sodium concentration was determined by B462 flame photometry (Micronal, São Paulo, Brazil), and values were expressed as mmol/L. Osmolality was analysed by Osmometer II (Precision Systems, Natick, MA, USA) based on the freezing point method, and data are expressed as mOsmol/kg H₂O.

Specific antibodies for AVP, OXT and ANG II radioimmunoassay were obtained from Peninsula (T4561, T4084 and T4007, respectively, San Carlos, CA, USA). The corticosterone antibody was purchased from Sigma (C8784), and 1,2,6,7-3H-corticosterone was obtained from GE Healthcare Life Sciences (Milwaukee, WI, USA). All hormone extractions and measurements were performed using radioimmunoassay techniques previously described [17–20] and performed in duplicate. The radioimmunoassay sensitivity and intra- and inter-assay coefficients of variation were 0.8 pg/mL, 8.1–10.1% for AVP; 0.9 pg/mL, 7.3–11.3% for OXT; 0.5 pg/mL, 12.5–11.9% for ANG II and 0.4 µg/dL, 6.7 and 9.2% for corticosterone.

Brain Micropunch and RNA Extraction

Rats were decapitated with a small animal guillotine, and the brain was rapidly removed from the cranium and placed in dry ice to allow a rapid freezing before storage at -80°C. Frozen brains were sliced into 60 µm coronal sections using a cryostat (Leica Microsystems CM1850 Cryostat). A 1 mm diameter micropunch needle was used to collect the PVN and SON from the brain slices. Thereafter, slices were stained with Toluidine Blue (0.1% v/v), and the punch location was confirmed by light microscopy, as previously described [15, 21]. Brains with incorrect dissection of the nuclei were not used in the study. The PVN and SON were then stored in QIAzol Lysis Reagent (QIAGEN, Crawley, UK). Total RNA was extracted from punched samples by combining QIAzol Lysis Reagent with RNeasy kit protocols (QIAGEN, Crawley, UK).

RNAseq and Transcriptomic Changes Analyses

Libraries from PVN RNA samples extracted from Control and Furo10 (Furosemide, 10 mg/kg) groups ($n = 3$, where each group is an independent biological pool of 2 rats each) and were constructed using the Illumina TruSeq kit. Briefly, eukaryotic messenger RNA (mRNA) was enriched using Oligo-dT beads. The products being fragmented into short fragments (between 200 and 700 bp) and then primed using random hexamer primers to create the first-strand cDNA. Following second-strand synthesis, libraries were purified prior to end polishing, and adapter ligation was conducted according to manufacturer's protocol. Fragments were enriched using PCR amplification and subject to 100 cycles of PE sequencing using the Illumina HiSeq2000 sequencer. All raw reads were preprocessed for quality assessment, adaptor removal, quality trimming and size selection using the FASTQC toolkit to generate quality plots for all libraries. RNAseq alignment and data analysis were all performed in-house using our high-performance computer; 'Hydra'. Our pipeline made use of Bash and Python scripting to accept RNAseq post trimmed data as input, before ultimately producing output tables of differentially expressed transcripts. Paired-end (2×100 bp) raw input data are initially aligned with Tophat to the sixth iteration of the Rattus norvegicus reference genome (Rn6) [22]. HTseq was used to generate read counts, using the ENSEMBL RN6 annotation for reference [23]. Our pipeline then made use of EdgeR, a R Bioconductor package to normalise and call differential gene expression (DGE) [24]. This enabled us to predict DGE and to utilise the predictions with low p values in our downstream validation. The data were ranked from lowest to highest based on EdgeR predicted p value. This highlighted the most robustly significant DGE predictions.

Table 1. List of primers used in the qPCR

Gene	Forward	Reverse
Rpl19	5'-GCGTCTGCAGCCATGAGTA-3'	5'-TGGCATTGGCGATTTTCGTTG-3'
Avp	5'-TGCCTGCTACTTCCAGAACTGC-3'	5'-AGGGGAGACACTGTCTCAGCTC-3'
hnAvp	5'-GAGGCAAGAGGGCCA CATC-3'	5'-CTCTCCTAGCCCATGACCCTT-3'
Oxt	5'-TGCCCCAGTCTTGCTTGCT-3'	5'-TCCAGGTCTAGCGCAGCCC-3'
hnOxt	5'-TGAGCAGGAGGGGGCCTAGC-3'	5'-TGCAAGAGAAAATGGGTCAGTGGC-3'
Caprin2	5'-CAGGGTTAAGTGCAAGCGAT-3'	5'-CTGGTGGTTGACTGGTTGAG-3'
Opn3	5'-CAACTCCTCTGTTTCCGCCT-3'	5'-TTCTTTGGCCTGTCCCCATC-3'
Ephx2	5'-GTTTCGACCTTGACGGAGTGC-3'	5'-TCATCTGGAAAGCGCCAAGT-3'
Ndnf	5'-GAGGAAGATAACACCCCACTGT-3'	5'-CCTGACCCATCTGCACTGGA-3'
Vwf	5'-AGTGAAGATCGGCTGCAACA-3'	5'-CCATACCAAGGGCAGAGCAA-3'
Tnnt1	5'-GGTGGCAGAGCGAGAAGAG-3'	5'-CGTCTGCAGCTCCAGTAAGT-3'
Nr1d1	5'-CTCAGCATCCTACCCTTCTGG-3'	5'-AATGGCTGAGAGAGACGAGTG-3'
Dbp	5'-CGGTTAGTGGCGGCTTGA-3'	5'-CTGGAATGCTTGACAGGGCA-3'
Mis18a	5'-GCAAATTCGTTCATGGCTGGT-3'	5'-CTCGAATCTTCCGACAGCCG-3'

qPCR, quantitative PCR.

cDNA Synthesis and Quantitative PCR Procedures

The cDNA synthesis was obtained by the reverse transcription of 200 ng of total RNA using the Quantitect reverse transcription kit (QIAGEN, Crawley, UK). Rat-specific primers were synthesised by Thermo Fisher Scientific (São Paulo, Brazil), and its sequences are shown in Table 1. The quantitative PCR (qPCR) was conducted in triplicate using SYBRgreen master mix (Roche, Jaguare, Brazil) and QuantStudio™ 3 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). The optimisation and validation of primers were performed using a qPCR standard curve with 5 concentrations and a dilution factor of 1:3. The primers efficiencies vary between 89.1 and 107.4%. The ribosomal protein L19 mRNA (Rpl19) was demonstrated to have a stable expression after osmotic challenges in both PVN and SON [14, 15]. In the present experiment, the average cycle thresholds for Rpl19 were 20.92 ± 0.12 , 20.96 ± 0.09 and 20.93 ± 0.16 in the PVN and 19.06 ± 0.17 , 18.94 ± 0.13 and 19.23 ± 0.26 in the SON for the control, Furo10 and Furo30 groups, respectively. Statistical analysis confirmed no significant difference in Rpl19 expression in the PVN ($F_{[2,15]} = 0.02$) and SON ($F_{[2,15]} = 0.53$) after body sodium depletion. Thus, the Rpl19 was used as the internal control gene for the relative quantification of gene expression by the $2^{-\Delta\Delta\text{cycle threshold}}$ method [25].

Statistical Analysis

Results are expressed as mean $\pm$ SEM. Statistical analyses were conducted by using Graphpad Prism 8.0 (Los Angeles, CA, USA). Data were submitted to Shapiro-Wilk normality test and Grubbs test to identify outliers. The data were analysed by one-way or two-way ANOVA (factors: time and treatment) followed by the Tukey's multiple comparisons test when the variances were equal or by Kruskal-Wallis test followed by Dunn's multiple comparisons test when the variances were different. The level of significance was set at $p < 0.05$.

Results

Physiological Changes Induced by Furosemide-Induced Body Sodium Depletion

Data are presented in Figure 1. Sodium excretion was significantly affected by sodium depletion ($F_{[2,24]} = 4.13$) and time ($F_{[2,48]} = 16.1$) and we observed a significant interaction between these factors ($F_{[4,48]} = 48.6$). Furo10 increased sodium excretion in relation to the control, while Furo30 increased sodium excretion in relation to both control and Furo10 group at 2 h. No differences were found in sodium excretion at 6 h, but both Furo10 and 30 groups significantly decreased sodium excretion between at 24 h in relation to the control group. Furosemide-induced sodium depletion significantly decreased urinary osmolality ($F_{[2,24]} = 46.5$) and increased urinary volume ($F_{[2,24]} = 12.9$). Urinary osmolality was lowered by both furosemide doses at all times evaluated. Urinary volume was higher at 2 and 6 h after furosemide treatment in both Furo10 and 30 groups. At 24 h, the Furo30 urinary volume was significant higher in relation to both control and Furo10 groups. Water intake was significantly affected by sodium depletion ($F_{[2,24]} = 6.21$) and time ($F_{[2,48]} = 679$), and we observed a significant interaction between these factors ($F_{[4,48]} = 14.6$). Both Furo10 and 30 groups drank less water after 24 h of furosemide-induced body sodium depletion compared to the control group. Food intake was significantly reduced ($F_{[2,24]} = 10.3$), resulting in a body weight loss ($F_{[2,24]} = 16.5$) in both Furo10 and 30 groups.

Fig. 1. Hydromineral adaptations along 24 h of furosemide-induced body sodium depletion. Changes in sodium excretion (**a**), urinary osmolality (**b**), cumulative urinary volume (**c**), water intake (**d**), food intake (**e**) and body weight (**f**). Data are analysed by

two-way (**a–d**) or one-way (**e, f**) ANOVA followed by Tukey multiple comparisons test and presented as mean $\pm$ SEM; $n = 9$ rats per group. * $p < 0.05$ compared with control; # $p < 0.05$ compared with Furo10.

Two Bottles Sodium Appetite Test after Body Sodium Depletion

After 24 h of furosemide-induced body sodium depletion, we submitted the rats to a 2-bottle test involving a

free choice between hypertonic saline and water intake (Fig. 2). Body sodium depletion induced a strong increase in hypertonic saline intake ($F_{[2,24]} = 69.7$) from 15 min up to 24 h after offering the 2-bottle free choice in

Fig. 2. Drinking behaviour and physiological responses during the 2 bottle free choice test after 24 h of furosemide-induced body sodium depletion. Changes in cumulative hypertonic saline intake (**a**), water intake (**b**), sodium intake ratio (**c**), urinary volume (**d**),

sodium excretion (**e**) and urinary osmolality (**f**). Data are analysed by two-way ANOVA followed by Tukey multiple comparisons test and presented as mean $\pm$ SEM; $n = 9$ rats per group. * $p < 0.05$ compared with control.

Fig. 3. Plasma hydromineral and hormone changes after 24 h of furosemide-induced body sodium depletion. Plasma sodium concentration (**a**), plasma osmolality (**b**), haematocrit (**c**), plasma AVP (**d**), OXT(**e**), ANG II (**f**) and corticosterone (**g**) following 24 h of sodium depletion. Data are analysed by one-way ANOVA followed by Tukey multiple comparisons test and presented as mean $\pm$ SEM; $n = 10$ rats per group. * $p < 0.05$ compared with control. AVP, arginine vasopressin; ANG II, angiotensin-II.

both Furo10 and 30 groups. In contrast, water intake was significantly reduced by body sodium depletion ($F_{[2,24]} = 11.3$) at 6 and 24 h in Furo10 group and at 24 h in Furo30 group. As a result, the hypertonic saline intake ratio was significantly increased ($F_{[2,24]} = 340$) from 15 min to 24 h of the 2 bottle free choice. During the drinking test, urinary volume ($F_{[2,24]} = 6.4$) and sodium ($F_{[2,24]} = 16$) excretion were significantly increased while urinary osmolality ($F_{[2,24]} = 27.9$) was reduced in both Furo10 and 30 groups.

Plasma Hydromineral and Hormones Changes after Body Sodium Depletion

Plasma osmolality was reduced by Furo30 ($F_{[2,27]} = 4.18$), whilst plasma sodium decreased in both the Furo10 and 30 groups ($F_{[2,27]} = 5.08$) in relation to the control rats. ECF was significantly reduced after sodium depletion, as indicated by the haematocrit increase in Furo30 group ($F_{[2,27]} = 4.87$). The hydromineral imbalance caused by furosemide-induced body sodium depletion had a marked effect on HNS hormone secretion, RAAS activation and hypothalamic-

Fig. 4. AVP and OXT gene expression in the PVN and SON nucleus after 24 h of furosemide-induced body sodium depletion. hnRNA (**a, b**) and mRNA (**c, d**) expression of AVP (**a, c**) and OXT (**b, d**), respectively, in the PVN. Expression of hnRNA (**e, f**) and mRNA (**g, h**) of AVP (**e, g**) and OXT (**f, h**), respectively, in the SON. Data are analysed by one-way ANOVA followed by Tukey multiple comparisons test and presented as mean $\pm$ SEM; $n = 6$ rats per group. * $p < 0.05$ compared with control. AVP, arginine vasopressin; OXT, oxytocin; mRNA, messenger RNA; PVN, paraventricular nucleus; SON, supraoptic nucleus; hnRNA, heteronuclear RNA.

pituitary-adrenal (HPA) axis stimulation. After 24 h of body sodium depletion, plasma AVP levels were significantly reduced in the Furo30 group ($F_{[2,27]} = 4.2$), and plasma OXT levels were reduced in both the Furo10 and 30 groups ($F_{[2,27]} = 7.34$) compared to the control group. In contrast, plasma ANG II ($F_{[2,27]} = 5.55$) and corticosterone ($F_{[2,27]} = 4.9$) were significantly higher in the Furo10 and 30 groups compared to the controls (Fig. 3).

PVN and SON AVP and OXT Gene Expression in Response to Body Sodium Depletion

We used qPCR to evaluate both the expression of the AVP and OXT precursor heteronuclear RNAs (hnRNA) and mature mRNA in the PVN and SON (Fig. 4). In the PVN, the level of the hnRNA for AVP was significantly reduced after sodium depletion ($F_{[2,15]} = 5.7$) at a similar level in both Furo10 and 30 groups. In the SON, both

hnRNA ($F_{[2,15]} = 8.8$) and mRNA ($F_{[2,15]} = 9.78$) for AVP significantly reduced in abundance in both Furo10 and 30 groups in relation to the controls. No significant differences were observed in PVN and SON hnRNA or mRNA for OXT after body sodium depletion.

Transcriptome Changes in the PVN of Sodium-Depleted Rats

We wanted to evaluate the transcriptomic changes in the PVN to better understand the integrative role of this nucleus in the integration of neuroendocrine and behavioural responses elicited by hydromineral challenge, enabling the identification of potentially important genes. As furosemide doses of 10 and 30 mg/kg have shown similar physiological and behavioural effects, we compared Furo10 sodium-depleted rats compared to controls. We found 94 differentially expressed genes in the PVN, based on an EdgeR p value < 0.05 . Of these, 63 were downregulated and 31 were upregulated. A heat map summarising the gene expression changes in the PVN is showed in Figure 5. All RNAseq data have been uploaded to the NCBI Gene Expression Omnibus under the Accession number: GSE145431. Based on our transcriptome data, we selected genes with the read count >30 and putatively downregulated (Caprin2: *Cytoplasmic Activation/Proliferation-Associated Protein-2*, Opn3: *Opsin 3/Encephalopsin*, and Miss18a: *MIS18 Kinetochore Protein A*) or putatively upregulated (Tnnt1: *Troponin T1*, Ephx2: *Epoxyde Hydrolase 2*, Ndnf: *Neuron-Derived Neurotrophic Factor*, Dbp: *D-Box Binding PAR BZIP Transcription Factor*, Vwf: *Von Willebrand Factor* and Nr1d1: *Nuclear Receptor Subfamily 1 Group D Member 1*) by sodium depletion. We also selected some genes that were already demonstrated to be changed in the PVN/SON by osmotic cues (Caprin2, Opsin3, Dbp, Vwf and Nr1d1) and some novel regulated genes (Miss18a, Tnnt1, Ndnf and Ephx2).

qPCR Validation of PVN Transcriptome Data from Sodium-Depleted Rats

Based on qPCR analysis (Fig. 6), validated genes were categorised as: (Ia) genes that showed significant downregulation; (IIa) genes that showed significant upregulation and (IIIa) genes that were not validated (i.e., false positives identified as being up or downregulated by transcriptome analysis, but revealed not to be differentially regulated by qPCR). Out of a total of 9 genes tested, 5 were significantly validated. Caprin2 demonstrated a significant reduction in expression in both Furo10 and 30 rats ($F_{[2,15]} = 6.02$), whilst Opn3 was downregulated only in Furo10 compared to the controls ($H = 9.56$, 2 df). Ephx2

($F_{[2,15]} = 8.9$) and Ndnf ($F_{[2,15]} = 6.76$) mRNA levels were upregulated in both the Furo10 and 30 groups, whilst the Vwf ($H = 8.03$, 2 df) mRNA was more abundant only in the Furo30 group compared to the controls. The RNAseq data pertaining to the Dbp, Tnnt1, Mis18a and Nr1d1 genes were not validated by qPCR.

qPCR Expression Evaluation of PVN Transcriptome Selected Genes in the SON of the Sodium-Depleted Rats

Out of the total 9 genes tested for the PVN, 6 were significantly changed in the SON as assessed by qPCR evaluation. Those genes were classified as: (Ib) genes that showed significant downregulation; (IIb) genes that showed significant upregulation and (IIIb) genes that were not differentially regulated in the SON (Fig. 7). As in the PVN, the Caprin2 ($F_{[2,15]} = 14.6$) and Opn3 ($F_{[2,15]} = 18.7$) genes were significantly downregulated in both the Furo10 and the 30 groups compared to controls. Different from PVN, the SON expression of the Dbp ($H = 9.56$, 2 df), Tnnt1 ($F_{[2,15]} = 8.4$), Mis18a ($F_{[2,15]} = 4.8$) and Nr1d1 ($H = 15.16$, 2 df) mRNAs was significantly increased by Furo30. On the other hand, Ephx2, Ndnf and Vwf mRNA expressions were not changed in the SON.

Discussion

The loop diuretic furosemide is used to induce sodium excretion and ECF volume depletion by blocking the co-transporter NKCC2 in the thick ascending limb of the loop of Henle. This approach is widely used to study the control of hydromineral balance in the hyponatremic/hypovolemic context [6–9] and for the treatment of hypertension, oedematous disorders and the management of hypervolemia and electrolyte disorders [5]. However, the physiological and molecular consequences of 24 h of body sodium and ECF volume depletion with furosemide on the HNS were not well known. Our data demonstrate that several neuroendocrine adaptations are needed after furosemide-induced sodium depletion to avoid severe hyponatremia and hypotension. Furthermore, these adaptations are required to guarantee the development of sodium appetite and inhibition of AVP and OXT synthe-

Fig. 5. Heat map summarising the gene expression changes in the PVN after 24 h of furosemide-induced body sodium depletion. We found 94 genes with the expression changed in the PVN of hypothalamus, based on an EdgeR p value < 0.05 . Of which 63 were downregulated and 31 were upregulated.

(For figure see next page.)

sis and secretion thought the HNS. The present data comprehensively describe how 24 h of sodium depletion results in hydromineral, behavioural, neuroendocrine and molecular changes related to the HNS.

Furosemide treatment increased urinary sodium excretion. These rats also show a higher urinary volume and reduced urinary osmolality compared to controls over 24 h. All these effects are a direct consequence of furosemide action through the inhibition of NKCC2 in the kidney, resulting in a dramatic reduction in renal sodium reabsorption and the abolishment of the corticopapillary osmotic gradient, leading to an isosmotic diuresis [6, 7]. As a consequence, the ECF fluid volume is reduced, as indicated by the increased haematocrit. In this protocol, rats have free access to a low sodium diet and distilled water for 24 h after acute furosemide treatment, allowing them to reestablish, at least in part, the water lost but not the sodium. This results in the development of hyponatremia and hypoosmolality and consequently inhibition of water intake as demonstrated before [7, 9, 26] and in 24 h measurements in the present study. Despite modulating thirst, changes as small as 1–2% in ECF osmolality are able to modulate neurohypophyseal hormones secretion, whereas changes of 8–15% in ECF volume are required to produce a similar effect. Thus, neurohypophyseal hormones secretion is regulated more by ECF osmolality than by ECF volume changes [27, 28]. In our protocol, decreased plasma sodium and osmolality are probably more important to modulate neurohypophyseal hormone RNA expression and secretion than the decrease of ECF volume and increase in plasma ANG II. As a result, both AVP and OXT plasma levels are reduced after 24 h of body sodium depletion.

It is known that furosemide-induced sodium depletion changes salt palatability, increases the hedonic and decreases the aversive properties of sodium taste [29]. These data are consistent with previous report that demonstrated an increased preference for salty food [30], general food avoidance [7] and reduced appetite for unsalted food [31] in sodium-depleted rats. Thus, the reduced low sodium food intake achieved in this study is not surprising. The combination of reduction in ECF volume and reduction in food intake results in a body weight loss during the furosemide-induced body sodium depletion.

After 24 h of furosemide treatment, the rats received a free choice of water or 0.3 M NaCl solution, the latter normally being aversive to sodium-depleted rats. During the 2-bottle test, the sodium-depleted rats exhibited a massive increase in hypertonic saline intake associated with a decrease in water intake, resulting in a very high salt appetite preference. Moreover, the remaining water intake

during the free choice test may be secondary to the hypertonic saline intake because it occurs after the rats have consumed a large quantity of hypertonic saline [32, 33]. Our data also show that sodium depletion induces larger hypertonic saline intake than the animals actually need to reestablish their hydromineral balance, since both urinary volume and sodium excretion were increased along the hypertonic saline intake test. This pronounced increased sodium appetite after 24 h of sodium depletion is induced by redundant neuroendocrine adaptations to ensure the animals reestablish their body sodium, such as the sodium and osmosensors [34, 35], RAAS activation [36–38], changes in sodium palatability [29], decreased dorsal raphe serotonin [9, 26, 35, 39] and hypothalamic OXT [40, 41] production and activity, amongst others. In fact, our data demonstrate increased levels of circulating ANG II, indicating that the sodium appetite induced by body sodium depletion maybe, at least in part, induced by circulating ANG II by acting on AT1 receptors located in the lamina terminalis, in particular in the organum vasculosum of the lamina terminalis and the subfornical organ, that are critical for maintaining body fluid homeostasis [32, 42]. These redundant mechanisms seem to be essential to avoid profound hyponatremia and the risk for brain swelling. We also observed increased plasma corticosterone levels after sodium depletion. In rats, corticosterone is a hormone secreted as part of the stress responses, and the depletion of body sodium is considered to be stressful, activating the HPA axis by recruiting corticotrophin-releasing hormone which is synthesised in the parvocellular part of the PVN [43, 44]. The AVP from parvocellular part of the PVN is also involved in stimulating the HPA axis [45]. It was demonstrated that acute furosemide-induced hypovolemia differentially modulates AVP expression in the magnocellular and parvocellular parts of PVN [10]. Thus, it is possible that AVP and corticotrophin-releasing hormone expression and/or release specifically from parvocellular PVN neurons are increased as a stress response to sodium depletion, resulting in the activation of HPA axis and plasma corticosterone levels. However, in the present work, we evaluated the transcriptomic changes in all PVN sub-nuclei together, which does not allowing us to look for specific transcriptomic changes in the magnocellular and parvocellular parts. It should also be taken into account that the increase of ANG II and the activation of sympathetic nervous system contribute to the activation of HPA axis. It is also important to note that furosemide caused a large release of both corticosterone and aldosterone due to stress and RAAS activation [44]. In addition, glucocorticoids

Fig. 6. qPCR validation of PVN transcriptome data from 24 h furosemide-induced sodium depletion. Validated downregulated genes classified as category Ia: Caprin2 (**a**) and Opn3 (**b**); validated upregulated genes classified as category IIa: Ephx2 (**c**), Ndnf (**d**) and Vwf (**e**); and not validated genes classified as category IIIa: Dbp (**f**), Tnnt1 (**g**), Mis18a (**h**) and Nr1d1 (**i**). Caprin2, Ephx2,

Ndnf, Dbp and Tnnt1 data are analysed by one-way ANOVA followed by Tukey multiple comparisons test, whereas Opn3, Vwf, Mis18a and Nr1d1 data are by Kruskal-Wallis test followed by Dunn's multiple comparisons. Data are presented as mean $\pm$ SEM; $n = 6$ rats per group. * $p < 0.05$ compared with control. mRNA, messenger RNA.

Fig. 7. qPCR evaluation of PVN transcriptome selected genes in the SON after 24 h furosemide-induced sodium depletion. Down-regulated genes classified as category Ib: Caprin2 (**a**) and Opn3 (**b**); upregulated genes classified as category IIb: Dbp (**c**), Tnnt1 (**d**), Mis18a (**e**) and Nr1d1 (**f**); and not changed genes classified as category IIIb: Ephx2 (**g**), Ndnf (**h**), Vwf (**i**). Caprin2, Opn3, Tnnt1,

Mis18a, Ndnf and Vwf data are analysed by one-way ANOVA followed by Tukey multiple comparisons test, while Dbp, Nr1d1 and Ephx2 data are by Kruskal-Wallis test followed by Dunn's multiple comparisons. Data are presented as mean $\pm$ SEM; $n = 6$ rats per group. * $p < 0.05$ compared with control. # $p < 0.05$ compared with Furo10. mRNA, messenger RNA.

play a role in the induction of sodium appetite along with ANG II, since both compounds potentiate the action of aldosterone on sodium appetite [37].

While the 30 mg/kg of furosemide (Furo30) was more effective in increasing renal sodium and water excretion and in reducing ECF volume and osmolality compared to the 10 mg/kg dose (Furo10), the hyponatremia and sodium appetite responses were very similar with both doses, indicating that 10 mg/kg of furosemide is enough to induce a maximal sodium appetite response, as indicated by Lundy et al. [7]. Thus, we decided to use the smaller furosemide dose to study transcriptome changes in the HNS. Since the PVN parvocellular OXT neurons have an important inhibitory role in sodium appetite control [40, 41], we decided to catalogue transcriptomic changes in this nucleus after 24 h of body sodium depletion. Our transcriptome data revealed 94 genes with expression changed in the PVN, of which 31 were upregulated and 63 were downregulated. From this list, we selected 9 genes to be validated by the qPCR in the PVN of another set of rats. Five genes out of 9 were validated in the PVN (up-regulated: *Ephx2*, *Ndnf* and *Vwf*; downregulated: *Caprin2* and *Opn3*). We have also looked at the expression of these same selected genes in the SON, where the qPCR showed a significant increase in *Tnnt1*, *Mis18a*, *Nr1d1* and *Dbp* and decreased expression of *Caprin2* and *Opn3*.

We observed that the expression of the *Opn3* (*Opsin 3*) and *Caprin2* (*Cytoplasmic Activation/Proliferation-Associated Protein-2*) genes was downregulated by 24 h furosemide-induced sodium depletion in both PVN and SON, as previously demonstrated in the SON of a sustained hypoosmolality model [11, 12]. On the other hand, *Caprin2* and *Opn3* are upregulated in the SON after induced hyperosmolality [11, 13–15, 46]. Collectively, these data indicate that *Caprin2* and *Opn3* are important for the osmotic control of magnocellular neurons function. A more recent work of Loh et al. [47] applied the unsupervised network graphical lasso (Glasso) algorithm analyses to the transcriptome of dehydrated rat SON predicting a regulatory interaction of *Caprin2* and *Opn3*. Subsequent *in vitro* experiments in differentiated PC12 cells corroborated the mathematical prediction; *Caprin2* knockdown reduced *Opn3* mRNA levels, whereas *Caprin2*-overexpression increased *Opn3* mRNA levels. These data suggest that the *Caprin2* expression changes during hyper or hypotonic conditions controlling the *Opn3* expression in the PVN and SON. It is known that light penetrates the skull and modulates the hypothalamic expression of *Opn3* [48]. Furthermore, it was recently demonstrated that SON neurons are activated by multiple classes of general anaesthet-

ic drugs and its activation induces slow-wave sleep while its inhibition disrupts natural sleep [49]. Thus, further investigations are needed to uncover the *Opn3* signalling mechanisms and their possible role integrating the neuroendocrine responses and sleep-wake rhythm according to changes in environmental light and ECF tonicity.

In addition, we have described and validated changes in the expression of a number of additional novel genes that are regulated in the PVN and SON as a consequence of this hypoosmolality, the functions of which remain to be determined. The *Vwf*, *Ephx2* and *Ndnf* mRNA are all upregulated only in the PVN, whereas *Mis18a*, *Tnnt1*, *Nr1d1* and *Dbp* were upregulated only in the SON. One previous study of the rat SON transcriptome following chronic hyper-osmotic stress with saline overload showed a reduction in *Dbp* mRNA levels [14], while we saw *Dbp* mRNA expression increased in sodium-depleted rat SON. The function of this reciprocal regulation of *Dbp* remains to be determined. A recent work using the functional enrichment analysis demonstrated changes in *Dbp* expression associated to circadian rhythm in spontaneously hypertensive rats (SHR) and both SHR and stroke-prone SHR shared changes in *Ephx2* gene expression associated to metabolic or inflammatory responses [50]. Actually, the soluble epoxide hydrolase seems to be involved in the hypothalamic regulation of blood pressure and baroreceptor reflex function in SHR [51]. Thus, changes in the expression of *Dbp* and *Ephx2* in the hypothalamus might be involved in the central nervous system control of blood pressure after whole body sodium depletion. Beside those selected genes for qPCR confirmation, our transcriptome data also show a decrease in *Creb3l1* expression in the PVN of sodium-depleted rats. We have identified *Creb3l1* (cAMP-responsive element binding protein 3 like 1) as a regulator of AVP transcription in the magnocellular neurons of the PVN and SON [13, 52]. Greenwood et al. [52] demonstrated that furosemide-induced sodium depletion coincided with a significant fall in *Creb3l1* mRNAs in the SON and PVN. In contrast, hyperosmolality induced by salt loading and water deprivation increased the *Creb3l1* expression in both PVN and SON.

The transcriptomic remodelling in the PVN and SON induced by 24 h of body sodium depletion is probably important to control the HNS hormone production and secretion, as well as the development of sodium appetite. In fact, despite the transcriptome changes we observed in the PVN, we have found a significant reduction in the expression of *hnRNA* in the PVN and in both *hnRNA* and mRNA for AVP in the SON of sodium-depleted rats. While the SON is a more homogenous collection of magnocellular

neurons, the PVN is more complex and contains both magnocellular and parvocellular neurons in different sub-nuclei dedicated to releasing numerous neuropeptides regulating diverse neuroendocrine functions [1–4, 53]. Furthermore, these brain nuclei receive projections from different brain nuclei that may be implicated in the deferential control of gene expression and neuropeptide secretion [53, 54]. This morpho-functional diversity between PVN and SON might be the reason why those nuclei responded with differences in genes expression after 24 h of body sodium depletion. Different from AVP, we did not observe significant changes in hnRNA or mRNA for OXT in the PVN and SON. In fact, we have previously shown that both *Caprin2* and *Creb3l1* are specifically involved in the control of AVP gene expression. *Caprin2*, a RNA-binding protein, binds to the AVP mRNA and mediates an increase in the poly(A) tail length, increasing its stability and abundance in response to hypertonicity [46]. Thus, it is possible that under furosemide-induced hypotonicity, the decreasing in *Caprin2* expression leads to a decrease in the AVP mRNA poly(A) tail length, reducing its stability and expression. *Creb3l1* is a transcription factor that is involved in increasing hypothalamic AVP gene expression in response to hypertonicity, cAMP and glucocorticoids [52, 55]. Thus, the reduction of *Caprin2* and *Creb3l1* expression in the PVN and SON after body sodium depletion indicates that these 2 genes are involved in the hypotonicity-induced inhibition of AVP expression after furosemide-induced sodium depletion. Moreover, both plasma AVP and OXT concentrations were decreased after 24 h of body sodium depletion. These data indicate a general decrease in the magnocellular neuronal activity, culminating on the reduction of both hormones secretion from the neurohypophysis. This is consistent with the literature, since both AVP and OXT magnocellular neurons are not only intrinsically osmosensitive but also receive osmosensory information from the lamina terminalis that reduces its activity and hormone secretion in response to hypotonicity, as induced by our sodium depletion protocol [56].

It is possible that direct furosemide action on the magnocellular neurons might contribute to decreased HNS hormone secretion. We have previously demonstrated that NKCC2 increasing expression is important to brain osmoregulation since furosemide treatment blocked AVP release in response to hyperosmolality in hypothalamic explants [21]. Balapattabi et al. [57] also show that NKCC1 is important to regulate Cl^- concentration in magnocellular AVP neurons, probably acting those neurons excitability. However, it was recently demonstrated that acute furosemide treatment in vivo activates the

magnocellular SON and PVN neurons increasing AVP expression in AVP-green fluorescent protein transgenic rats [10]. Thus, both acute stimulation of AVP expression 90 min after furosemide injection and the inhibition of AVP expression 24 h after furosemide-induced sodium depletion would appear to be more related to the ECF volume depletion and hypoosmolality/hyponatremia, respectively, than to direct furosemide action in magnocellular neurons in vivo. Nevertheless, more studies are necessary to understanding the role of NKCC1 and 2 in magnocellular neuron gene expression, activity and secretion.

The present work presents a novel and validated transcriptomic profile of genes likely to be implicated in the regulation of AVP synthesis and both AVP and OXT secretion in hypotonic stress. In summary, our results indicate that the furosemide-induced sodium depletion and the consequent hypoosmolar state induces changes in the expression of a wide variety of regulatory genes in the PVN, some of which maybe be involved in the adaptation of the HNS to hypoosmotic stress and neuroendocrine and ingestive behavioural responses.

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Disclosure Statement

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