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Effects of salt-loading on supraoptic vasopressin neurones assessed by ClopHensorN chloride imaging

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Abstract

Salt-loading (SL) impairs GABA_A inhibition of arginine vasopressin (AVP) neurones in the supraoptic nucleus (SON) of the hypothalamus. Based on previous studies, we hypothesised that SL activates tyrosine receptor kinase B (TrkB), down-regulating the activity of K⁺/Cl⁻ co-transporter2 (KCC2) and up-regulating Na⁺/K⁺/Cl⁻ co-transporter1 (NKCC1). These changes in chloride transport would result in increased [Cl⁻]_i in SON AVP neurones. The study combined virally-mediated chloride imaging with ClopHensorN with a single-cell western blot analysis. An adeno-associated virus with ClopHensorN and a vasopressin promoter (AAV2-0VP1-ClopHensorN) was bilaterally injected in the SON of adult male Sprague-Dawley rats that were either euhydrated (Eu) or salt-loaded (SL) for 7 days. Acutely dissociated SON neurones expressing ClopHensorN were tested for decreases or increases in [Cl⁻]_i in response to focal application of the GABA_A agonist muscimol (100 μmol L⁻¹). SON AVP neurones from Eu rats showed muscimol-induced chloride influx ($P < 0.05$; 23/35). SON AVP neurones from SL rats either significantly increased chloride efflux ($P < 0.05$; 27/39) or did not change chloride flux (12/39). The SON AVP neurones that responded to muscimol appeared to be viable and expressed KCC2 and β-actin. Neurones that did not respond during chloride imaging did not show KCC2 and β-actin protein expression. The KCC2 antagonist (VU0240551, 10 μmol L⁻¹) significantly blocked the chloride influx in cells from Eu rats but did not affect cells from SL rats. A NKCC1 antagonist (bumetanide, 10 μmol L⁻¹) significantly blocked the chloride efflux in cells from SL rats but had no effect on cells from Eu rats. Blocking NKCC1 using bumetanide had less of an effect on the muscimol-induced Cl⁻ influx in Eu rat neurones compared to the KCC2 antagonist. The TrkB antagonist (AnA-12) (50 μmol L⁻¹) and protein kinase inhibitor (K252a) (100 nmol L⁻¹) each significantly blocked chloride efflux in SON AVP neurones from SL rats. Salt-loading increases [Cl⁻]_i in SON AVP neurones via a TrkB-KCC2-NKCC1-dependent mechanism in rats.

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CONFLICT OF INTERESTS

The authors declare that they have no conflicts of interest.

DATA AVAILABILITY

The data that support the findings of the present study are available from the corresponding author upon reasonable request.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Keywords

chloride imaging; SON and salt-loading; vasopressin

1 | INTRODUCTION

Magnocellular neurosecretory cells (MNCs) located in the supraoptic nuclei (SON) and the paraventricular nuclei of the hypothalamus produce and release the hormone arginine vasopressin (AVP) into the circulation.^{1–3} An important systemic effect of AVP is water reabsorption via V2 receptors in the kidney. Excess water retention associated with heart and liver failure negatively influences the morbidity and mortality associated with these disorders.^{4–6} Although AVP contributes to water retention in these disorders, the mechanisms responsible for the inappropriate secretion of AVP are not known.^{7,8}

Previous studies have shown that salt-loading (SL; drinking water replaced by 2% NaCl solution for 7 days) can cause a sustained increase in AVP secretion as a result of changes in the excitability of AVP neurones.^{1,2,9} One mechanism that has been shown to contribute to sustained AVP release in SL rats is a change in the polarity of the response of neurones to GABA. Although controversial, GABA-mediated neurotransmission is generally described as a primary inhibitory mechanism in SON AVP neurones.^{9–11} The inhibitory effect of GABA depends on a low intracellular chloride concentration ($[Cl^-]_i$). Increases in $[Cl^-]_i$ can change GABA-mediated inhibition to excitation by reversing the direction of chloride movement across the cell membrane. The balance between neural excitation and inhibition is crucial for maintaining the concentration of circulating AVP within normal limits.^{9,12} In most neurones, $[Cl^-]_i$ is regulated by cation chloride cotransporters such as KVCF co-transporter 2 (KCC2), which causes the efflux of Cl^- ions, and $Na^+/K^+/Cl^-$ cotransporter 1 (NKCC1), which causes the influx of Cl^- ions.^{13–15} Changes in the expression or activity of these transporters have been shown to influence the strength and polarity of the neuronal response to GABA in several systems.^{16–18}

Previous studies demonstrate that SL, probably via the actions of brain-derived neurotrophic factor (BDNF), decreases the activity of KCC2 in SON causing increased AVP secretion.^{1,2,19} However, the mechanism leading to increases in $[Cl^-]_i$ is not completely understood. The present study aimed to determine the role of tyrosine receptor kinase B (TrkB) receptor, NKCC1 and KCC2 co-transporter with respect to the increase in intracellular chloride concentration and impairment of GABA_A-mediated inhibition in salt-loaded (SL) rats. Based on previous studies,^{1–3,19} we hypothesised that SL activates the BDNF receptor, TrkB and down-regulates the activity of KCC2 co-transporter at the same time as up-regulating NKCC1. Down-regulation of KCC2 decreases the efflux of chloride ions causing increases in $[Cl^-]_i$ in SON AVP neurones. Also, the expression of KCC2 in the SON AVP neurones is controversial because some studies suggest that AVP neurones lack KCC2,²⁰ whereas others have shown the immunohistochemical expression of KCC2 in these neurones.² The up-regulation of NKCC1 was previously shown to disrupt $[Cl^-]_i$ in paraventricular neurones of spontaneously hypertensive rats,²¹ although the role of NKCC1 in chloride regulation in SON during SL is not completely understood.

In the present study, we used virally-mediated ClopHensorN, a genetically encoded ratiometric chloride fluorescence imaging technique,²² to measure changes in $[Cl^-]_i$ in dissociated AVP neurones by combining it with an AVP-specific promoter.²³ ClopHensorN is composed of a Cl^- sensitive variant of green fluorescent protein (E2GFP) and a Cl^- insensitive red-fluorescent protein (TdTomato) acting as an invariant reference for ratiometric imaging.^{24,25} Ratiometric analysis (E2GFP/TdTomato) of the emitted fluorescence provides the Cl^- measurement. Increased $[Cl^-]_i$ decreases E2GFP fluorescence relative to TdTomato and vice versa. ClopHensorN allows for measurement of changes of neuronal $[Cl^-]_i$ at the single-cell level without disrupting the native cellular milieu that would be associated with whole-cell patch-clamp recording and possibly gramicidin-based perforated patch recording. Furthermore, we combined single-cell protein expression measurements with chloride imaging aiming to determine KCC2 expression in SON AVP neurones and the modulation of neuronal $[Cl^-]_i$ in response to various antagonists.

1.1 | Significance

The present study uses virally-mediated live cell chloride imaging to test the roles of TrkB, KCC2 and NKCC1 with respect to how SL alters the GABA responses of AVP neurones of the SON. This is the first study to use chloride imaging for the measurement of chloride flux in SON AVP neurones and to correlate the chloride imaging data with single-cell western blot analysis of KCC2 protein expression.

2 | MATERIALS AND METHODS

2.1 | Animals

The experiments were conducted in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. All of the animal protocols used in the present study were approved by the UNT Health Science Center Institutional Animal Care and Use Committee. The outbred adult male (weighing 250-300 g) Sprague-Dawley rats (Charles River, Wilmington, MA, USA) were individually housed as a result of the use of survival surgery and the individual fluid intake measurements in the protocol. All animals were maintained under a 12:12 hour light/dark cycle at 24-26°C and 40%-60% relative humidity with access to food (Teklad LM-485 RatSterilisable Diet with 0.3% Sodium, Envigo) and water available ad lib. unless otherwise indicated. Aseptic techniques were used to conduct survival surgeries. All rats were s.c. administered with procaine penicillin G (30 000 U) to prevent postoperative infection. Carprofen (2 mg) (Rimadyl; Zoetis Inc., Kalamazoo, MI, USA), a non-steroidal anti-inflammatory drug, was given orally before and after surgery for pain management.

2.2 | Stereotaxic surgery

The AAV2-0VP1-ClopHensorN was bilaterally injected into the SON of each rat. Separate group of rats were injected in the SON with an AAV vector that contained only the AVP promoter and green fluorescent protein (GFP) (prepared by the University of North Carolina [UNC] Vector Core, Chapel Hill, NC, USA). For each injection, the rats were anaesthetised with isoflurane (2%-3%) and placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). Their skulls were exposed and levelled between two cranial suture

landmarks: lambda and bregma.²⁶ A micromanipulator was used to position the injector at the SONs (1.4 mm posterior, 9.1 mm ventral and ± 1.4 mm lateral from bregma). The AAV2-0VP1-ClopHensorN or the AAV2-0VP1-eGFP vectors were bilaterally injected in the SON (300 nL each side) at a titre of 1.0×10^{13} GC mL⁻¹ (UNC Vector Core). Each injection was made over a 10-minute period. After the injection, the injector was left in place for 5 minutes then slowly removed. The incision was closed with absorbable antibiotic sutures.

2.3 | Immunohistochemistry

Four weeks after the injections with AAV2-0VP1-eGFP, rats were deeply anaesthetised with inactin (100 mg kg⁻¹, i.p.), perfused transcardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in PBS. After the perfusion, brains were extracted from the skull and placed in 30% sucrose until dehydrated. The brains were serial sectioned at 40- μ m thickness. Separate sets of serial sections from each rat were processed for either AVP using guinea-pig polyclonal Anti-(Arg⁸)-VP (dilution 1:1000; catalogue no. T-5048; Peninsula Labs, San Carlos, CA, USA) or oxytocin (OXT) using anti-OXT mouse monoclonal immunoglobulin (Ig)G (dilution 1:10 000; catalogue no. MAB5296; Millipore, Billerica, MA, USA) immunofluorescence. Sections were incubated in primary antibodies for 2 days at 4°C. After primary antibody incubation, sections were then rinsed with PBS, followed by sequential incubation in respective secondary antibodies against host species conjugated to Cy3 fluorophores (Cy3-conjugated anti-guinea pig IgG (dilution 1:10 000; catalogue no. 706-165-148; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) or Cy3-conjugated Anti-Mouse IgG (dilution 1:10 000; catalogue no. 715-165-151; Jackson ImmunoResearch Laboratories, Inc.). Sections were rinsed again in PBS, mounted on gelatin-coated slides and cover slipped with Vectashield (Vector Laboratories, Inc., Burlingame, CA, USA) mounting media. Sections were examined for co-localisation of the GFP reporter with AVP or OXT immunofluorescence to confirm vector transfection specificity.

2.3.1 | Histological analysis—Tissue sections were inspected using a microscope BX41 microscope (Olympus Corp., Tokyo, Japan) equipped for epifluorescence and images were recorded using a DP70 digital camera with DP MANAGER, version 2.2.1 (Olympus Corp.). Images were uniformly adjusted for brightness and contrast. Regions of the brain were identified using the rat brain stereotaxic atlas of Paxinos and Watson.²⁷

The GFP positive cells in SON were counted using IMAGEJ (NIH, Bethesda, MD, USA). The total number of GFP positive colocalised neurones with either AVP or OXT neurones in the SON as indicated by fluorescence staining was counted blind to the experimental conditions of the subjects for all experiments. Six to eight sections were analysed from each rat. Counts from each section were averaged for each individual animal for statistical analysis.^{28,29}

2.4 | Salt-loading

After 2 weeks of recovery from surgery, daily fluid intake was measured by filling the water bottles up to a predetermined weight in grams and subtracting the remaining weight 24 hours later. The rats were provided with ad lib. access to water for first 7 days to record baseline fluid intake and body weight. After the baseline measurements, a subset of rats had

their water replaced with 2% salt (NaCl) to drink for 7 days as previously described.² The euhydrated (Eu) rats were used as controls and the SL rats were used as treatment groups. At the end of the 7-day SL protocol, the rats were anaesthetised with inactin (100 mg kg⁻¹ i.p.; MilliporeSigma, Burlington, MA, USA) and decapitated to isolate neurones from the SON.

2.5 | Plasma measurements

Trunk blood was collected from each rat after decapitation and was centrifuged as described previously.¹ Plasma was collected from each sample for measuring plasma osmolality, haematocrit, circulating AVP and copeptin (CPP). CPP is widely used as biomarker for AVP secretion³⁰ and was measured in the present study in addition to AVP.

Separate blood samples (5-6 mL) were collected in Vacutainer tubes containing the anticoagulant ethylenediaminetetraacetic acid (12 mg). The proteinase inhibitor aprotinin (0.6 TIU mL⁻¹ of blood; Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA) was added to the sample and centrifuged at 1600 *g* for 15 minutes at 4°C. Next, 2-3 mL of plasma was removed from each sample and an aliquot of plasma was used to extract peptides by solid phase extraction using C-18 SEP-Column (Phenomenex, Torrance, CA, USA). After the extraction, each sample was subjected to vacuum centrifugal concentration. The extracted peptide was used to measure circulating AVP with a specific enzyme-linked immunosorbent assay (ELISA) (EK-065-07; Phoenix Pharmaceuticals, Inc). CPP concentration was measured in plasma samples using a specific ELISA in accordance with the manufacturer's instructions (MBS724037; MyBioSource, Inc., San Diego, CA, USA). Four parametric logistic analysis (4-PL) was performed to quantify the concentration of AVP and CPP.

Another 1-2 mL of blood was collected in a 2-mL microcentrifuge tube and prepared for measuring plasma osmolality and haematocrit as described previously.³¹ Two heparinised capillary tubes (Fisher Scientific, Waltham, MA, USA) were filled with blood for measuring haematocrit using Micro-Hematocrit capillary tube reader (Lancer, Cambridge, UK). The remaining blood sample was centrifuged at 1600 *g* for 5-10 minutes and plasma was collected to measure plasma osmolality using vapor pressure osmometer (Wescor, Logan, UT, USA).

2.6 | SON dissociation

The brains were rapidly removed and placed in ice-cold (0 to +4°C) artificial cerebrospinal fluid (aCSF), bubbled with carbogen gas (95% O₂, 5% CO₂). The composition of the standard aCSF was 120 mmol L⁻¹ NaCl, 3 mmol L⁻¹ KCl, 2 mmol L⁻¹ MgCl₂, 2 mmol L⁻¹ CaCl₂, 1.2 mmol L⁻¹ NaH₂PO₄, 23 mmol L⁻¹ NaHCO₃ and 11 mmol L⁻¹ D-glucose. The carbogen gas maintained the pH between 7.35 and 7.40. The osmolality was between 290 and 300 mosmol kg⁻¹. The SONs were dissected from surrounding tissue using a microscope and iris scissors. The extracted tissue containing the SONs was placed in calcium-free Hibernate media that contained papain (0.2% w/v; Worthington Biochemical Corporation, Lakewood, NJ, USA). The tissue was incubated at 32°C water bath for 30 minutes for enzymatic dissociation. The cells were dissociated mechanically by gentle trituration in Hibernate A media (Gibco Life Technologies, Grand Island, NY, USA) with B27 supplement (2% v/v; Gibco Life Technologies). The dissociated cells were then

centrifuged and suspended in Neurobasal A medium (Gibco Life Technologies) with B27 supplement (2% v/v), L-glutamine (200 mmol L⁻¹) and penicillin/streptomycin (100 U mL⁻¹). Dissociated SON neurones were plated on coverslips coated with 0.1% poly-D-lysine (Sigma-Aldrich, St Louis, MO, USA) and incubated at 37°C in 5% CO₂ humidified incubator for 2 hours before recording.^{32–36} Images were collected after a 2-hour incubation period and recordings continued for up to 6 hours after the end of the incubation period.

2.7 | ClopHensorN chloride imaging

The coverslips with SON neurones were transferred to a recording chamber on the stage of an inverted microscope (Olympus Corp.), which was equipped for epifluorescence imaging. The cells were continuously perfused with 95% O₂ and 5% CO₂ oxygenated aCSF. The dissociated neurones were visualised using fluorescence as an estimate of neuronal viability. To determine whether successful expression of ClopHensorN resulted in the corresponding expression of both green fluorophore (E2GFP) and the fused red fluorophore (TdTomato), cells were excited at 445 nm (for E2GFP, “green channel”) and at 556 nm (for the TdTomato, “red channel”). Emission was collected by switching filters (Prior Lumen 200 Pro; Prior Scientific, Inc., Cambridge, UK) between 500 and 550 nm for the green channel and between 580 and 653 nm for the red channel.^{24,37} The reliability of cellular viability was confirmed by each cell’s ability to respond to antagonists during imaging and for some experiments by single-cell measurements of β -actin protein by single-cell western blot (Simple Wes) analysis.

Individual SON neurones were visualised using a 40 $\times$ water-immersion objective and selected for imaging. Putative healthy AVP neurones with uniform bright appearance indicative of an intact plasma membrane and expressing both E2GFP and TdTomato were chosen for imaging. Imaging for ClopHensorN was performed by sequentially exciting the individual AVP neurones alternately at 445 and 556 nm. The emission was collected between 500 and 550 nm for the 445 nm excitation and between 580 and 653 nm for the 556 nm excitation. Images were captured with camera (Camera Controller C10600; Hamamatsu Photonics, Hamamatsu, Japan) using Meta Fluor software (Molecular Devices, Sunnyvale, CA, USA). Each image was processed for background (measured from a non-fluorescent area) subtraction and fluorescence was measured every 3 seconds at a constant rate of 0.33 Hz within regions of interest selected from individual neurones using a F_{445}/F_{556} ratio.

In a subset of experiments, whole-cell micropipettes were used to induce changes in intracellular chloride concentrations that were measured using ClopHensorN fluorescence. The electrodes were prepared from borosilicate glass capillaries (World Precision Instruments, Sarasota, FL, USA) using a horizontal pipette puller (Model P-2000; Sutter Instruments, Novato, CA, USA). The diameter of the tip was approximately 1–2 μ m for each micropipette. For these experiments, the micropipette solution consisted of either 130 mmol L⁻¹ CsCl, 10 mmol L⁻¹ KCl, 10 mmol L⁻¹ Hepes, 1.1 mmol L⁻¹ EGTA, 2 mmol L⁻¹ Mg₂ATP and 0.3 mmol L⁻¹ Na₃GTP to create a high [Cl⁻]_i solution or 140 mmol L⁻¹ K-gluconate, 10 mmol L⁻¹ Hepes, 1.1 mmol L⁻¹ EGTA, 2 mmol L⁻¹ Mg₂ATP and 0.3 mmol L⁻¹ Na₃GTP for a low [Cl⁻]_i solution. Baseline fluorescence F_{445}/F_{556} ratio was measured for

90-120 seconds prior to whole-cell access. After whole-cell access, the fluorescence was recorded for 10 minutes.

In other experiments, micropipettes were used for focal drug application by adding the GABA_A agonist muscimol to the aCSF solution. After recording the baseline fluorescence for 1 minute, muscimol (100 $\mu\text{mol L}^{-1}$) was focally applied for 10 seconds (10 psi) using a pneumatic Picospritzer (World Precision Instruments) with a patch pipette placed 100-150 μm upstream of the AVP neurone. Following the initial 4-minute recording, bath solution (aCSF) was switched to aCSF containing antagonists and imaging resumed 5 minutes later. After 5 minutes of perfusion with the antagonist, the muscimol injection was repeated to determine its effects on the muscimol response.^{37,38} The duration and experimental conditions utilised were first verified using control imaging to muscimol response with no bath application of antagonists.

At the end of some of the experiments, the cell was harvested with a patch pipette filled with aCSF. The cell was aspirated into the patch pipette by applying negative pressure while observing the process using the microscope.

2.8 | Drugs and reagents

Stock solutions of muscimol (100 mmol L^{-1} ; Sigma-Aldrich) were created by first dissolving the muscimol in 0.05 mol L^{-1} HCl and diluting it to the final concentration in aCSF (100 $\mu\text{mol L}^{-1}$). The other antagonists used in these studies (all obtained from Tocris Bioscience, St Louis, MO, USA) were first dissolved in dimethyl-sulphoxide (DMSO) (Sigma-Aldrich; Stock Concentrations: K252a, 100 $\mu\text{mol L}^{-1}$; AnA-12, 10 mmol L^{-1} ; VU0240511, 10 mmol L^{-1} and bumetanide, 50 mmol L^{-1}) and then diluted to their final concentrations in a CSF for bath application. The TrkB receptor was blocked using either broad-spectrum protein kinase inhibitor K252a (100 nmol L^{-1}) or the TrkB receptor antagonist AnA-12 (50 $\mu\text{mol L}^{-1}$). KCC2 antagonist VU0240511 (10 $\mu\text{mol L}^{-1}$) and NKCC1 cotransporter antagonist bumetanide (10 $\mu\text{mol L}^{-1}$) were also used. The final concentration of DMSO used to solubilise the antagonists ranged from 0.5% to 0.02% when added to the bath solution.

AAV2-0VP1-ClopHensorN: An pAAV-bGH(+) shuttle vector containing poly(A) tail downstream of the multicloning site (Vector BioLabs, Malvern, PA, USA) was used to prepare the plasmid for packaging as an AAV. The ClopHensorN plasmid (#25940 pcDNA3-PalmPalm-ClopHensorN; Addgene, Watertown, MA, USA) was cloned into the pAAV-bGH(+) vector along with an AVP-specific promoter (p2.0VPI.EGFP; Addgene Plasmid #40868 obtained from Harold Gainer²³) using *EcoRI/NotI* restriction sites. The promoter and reading frame were sequenced to confirm the direction of ClopHensorN cDNA insertion and verify the entire sequence. The resulting plasmid-0VP1-ClopHensorN was sent to the UNC Vector Core packaging in an AAV2 vector.

2.9 | Single-cell Wes analysis

Individual AVP neurones were used for Simple Wes analysis to measure KCC2 and β -actin expression. Each cell that was collected as described above was released from the micropipette into a standard (volume) polymerase chain reaction (PCR) tube using a syringe.

Each PCR tube contained 5.0 μ L of RIPA lysis buffer containing dithiothreitol, chelators and protease phosphatase inhibitor cocktail. The samples were frozen on dry ice immediately for later protein analysis. As a control for contamination, an aCSF sample from the same preparation was collected in separate tube and this was analysed in parallel with the single-cell samples. The cells were lysed by vortexing the tube for 1-2 minutes followed by indirect sonication for 5 minutes in ice bath.^{39,40}

The Simple Wes system (Protein Simple, San Jose, CA, USA) was used to quantify total KCC2 and β -actin protein expression in the individual neurones. In brief, the standard matrix (12-230 kDa) and Anti-Rabbit Detection Module were used. The undiluted protein samples were mixed with Fluorescent Master Mix and heat denatured at 95°C for 5 minutes. The samples, blocking reagent, primary antibodies: total KCC2 (rabbit; dilution 1:50; 07-432; Millipore) and β -actin (rabbit; dilution 1:500; NB600-532; Novus Biologicals, Littleton, CO, USA), horseradish peroxidase-conjugated secondary antibodies and chemiluminescent substrate were pipetted into the separation module plate. The assay plate and the capillary cartridge were loaded into the Simple Wes instrument. The samples were processed using default settings and in accordance with the manufacturer's instructions: stacking and separation at 375 V for 30 minutes; blocking reagent for 5 minutes, primary and secondary antibody both for 30 minutes; luminol/peroxide chemiluminescence detection for approximately 15 minutes (exposures of 1-2-4-8-16-32-64-128-512 seconds). The specific peaks for total KCC2 and β -actin were obtained from resulting electropherograms using COMPASS, version 2.7 (Protein Simple).

2.10 | Statistical analysis

Statistical analyses were performed using SIGMAPLOT, version 12.0 (Systat Software Inc., Chicago, IL, USA). Data are reported as the mean $\pm$ SEM. For immunohistochemistry, the numbers of cells labelled with eGFP were manually counted using IMAGEJ and double-labelling of AVP-GFP and OXT-GFP was calculated and expressed as a percentage of neurones containing double-labelling. Chloride imaging data were analysed by a chi-squared test and two-way repeated measures ANOVA with treatment (SL vs euhydrated) as first factor and bath applied antagonists as second factor along with Tukey comparisons. Fluid intake data were analysed by separate two-way repeated measures ANOVA with treatment (SL vs euhydrated) as first factor and time as second factor followed by Tukey post-hoc tests. All other data were analysed using Student's *t* test. Composite figures were assembled using the 'magick' package in RSTUDIO (<https://www.rstudio.com>). $P < 0.05$ was considered statistically significant for all tests. The group sizes were determined by power analysis and the effect size calculated from our previously reported work^{2,31,41} and preliminary data using SIGMAPLOT. The minimal number per group for each experiment was decided to enable an appropriately powered study with an effect size of approximately 0.7 (70%). Numbers (n) in the chloride imaging data indicate the number of cells that were obtained from 4-6 rats in each group.

3 | RESULTS

3.1 | Immunohistochemistry

To verify the specificity of the AVP promoter, we injected an AAV2 vector (p3.0VP1.EGFP) with an AVP promoter and an eGFP reporter targeted to the SON. Previous studies have shown the specific expression of eGFP in AVP neurones in the SON using this promoter.²³ Co-localisation of GFP with either AVP or OXT neurone was determined by calculating the percentage double-labelling using a fluorescence microscope. Our results indicate the co-localisation of GFP and AVP MNCs in the SON (89% GFP-AVP double-labelling, $n = 3$) (Figure 1A–C) and GFP and OXT (0.08% GFP-OXT double-labelling, $n = 3$) (Figure 1D–F). Given this demonstration of successful vector transduction, we can conclude that vectors using this promoter are primarily selective to AVP expressing MNCs.

3.2 | Changes in fluid intake and body weight

Fluid intake was measured daily for 7 days before and during the 7-day 2% SL protocol. The fluid intake increased steadily during the SL and these rats consumed larger volumes of fluid compared to the control Eu rats (oncrease in fluid volume in mean $\pm$ SEM in Eu: -0.8 ± 0.98 ; SL: 27.4 ± 1.58). In addition, the SL rats had a lower increase in body weight compared to the Eu rats. Two-way repeated measures ANOVA revealed a significant interaction indicating that fluid intake was affected by time and the treatment protocol (Time $\times$ Treatment: $F_{13,65} = 26.040$, $P < 0.001$). Post-hoc multiple comparisons between the factors revealed that, although there were no differences between the groups during baseline, SL significantly increased fluid intake. Also, the average body weight increase during SL was significantly lower in SL group compared to the Eu group (increase in body weight in mean $\pm$ SEM in Eu: 48.21 ± 13.73 ; SL: 7.25 ± 1.81 ; $t = 2.956$; $df = 10$; $P < 0.05$).

3.3 | Changes in plasma AVP, CPP, osmolality and volume

At end of the study, plasma samples were collected to measure AVP and CPP concentrations along with osmolality and haematocrit measurements. Plasma samples from SL rats had higher AVP and CPP concentrations compared to Eu rats. Seven days of SL significantly increased circulating AVP ($t = -4.682$; $df = 10$; $P < 0.001$) and CPP ($t = -4.831$; $df = 10$; $P < 0.001$) compared to Eu rats (Table 1).

Plasma osmolality and haematocrit values were significantly different between the groups (plasma osmolality $t = -6.799$; $df = 16$; $P < 0.001$; haematocrit $t = -6.123$; $df = 16$; $P < 0.001$). Our data show that SL significantly increased plasma osmolality and haematocrit values compared to the Eu group in accordance with previous studies.^{1,42}

3.4 | Chloride imaging

3.4.1 | ClopHensorN calibration for chloride measurement—The whole-cell access was verified by the presence of capacitance transients and an access resistance < 20 M Ω in response to a 5-mV depolarising step. Images of AVP neurones expressing the ClopHensorN plasmid are shown in Figure 2. Note that an increase in intracellular chloride decreases the fluorescence F_{445}/F_{556} ratio (Figure 2Ai,ii), whereas a decrease in intracellular chloride increases the F_{445}/F_{556} ratio (Figure 2Bi,ii). When caesium chloride (CsCl, 130

mmol L⁻¹) with high [Cl⁻]_i was used as patch pipette solution, ClopHensorN fluorescence decreased following whole-cell access, indicating Cl⁻ influx as a result of a change in the concentration gradient (Figure 2Aiii). Dialysis with potassium gluconate (KGlu, 140 mmol L⁻¹), which should have decreased [Cl⁻]_i, produced an increase in ClopHensorN fluorescence consistent with Cl⁻ efflux (Figure 2Biii). Calibration curves with CsCl and KGlu were obtained by averaging the responses of five to seven cells.

3.4.2 | Protein expression using Simple Wes—We use an optimised Simple Wes protocol to measure KCC2 protein from single SON AVP neurones following imaging experiments. The β-actin protein was used as control. The specificity of the KCC2 and β-actin antibodies used was verified by including no lysate control in the experiment. The total KCC2 antibody did not generate any background, whereas the β-actin antibody had background at 200 kDa in no lysate blot (see Supporting information, Figure S1). The KCC2 and β-actin antibodies used in the present study could detect up to 4 ng μL⁻¹ of protein in Simple Wes. A sharply defined chemiluminescent signal was obtained for total KCC2 (Figures 3B,F) from both the SL and Eu neurones that responded to muscimol during chloride imaging (Figure 3A,E). The data are also displayed using KCC2 and β-actin protein bands in Figure 3D,H. Cells that did not respond to muscimol typically displayed no KCC2 or β-actin signals at the appropriate molecular weights (see Supporting information, Figure S2). These results indicate that most AVP neurones from the SON express KCC2.

3.4.3 | Muscimol-mediated changes in intracellular chloride—In our experiments, GABA_A receptors were activated by exogenous application of muscimol (100 μmol L⁻¹; 10 seconds; three cycles), a GABA_A agonist, in acutely dispersed SON AVP neurones from Eu or SL rats. The duration and experimental conditions utilised were first verified by recording the changes in chloride concentration to muscimol in the absence of antagonists. The response of the neurone to muscimol applications was recorded at a time interval similar to that used for the subsequent experimental conditions but with no bath application of antagonists (Figure 4). Two-way repeated measures ANOVA revealed no significant difference between the repeated muscimol applications ($F_{18} = 0.0312$, $P = 0.864$). These data show that cells were viable and responded uniformly for the entire duration of recording.

The majority of cells from Eu rats (23 out of 35) showed a decrease in the F_{445}/F_{556} ratio consistent with chloride influx (Figure 4A). The decrease in fluorescence from baseline was significant in the responsive cells ($t = 3.195$; $df = 3$; $P < 0.05$) and a similar response could be evoked 10 minutes later (Figure 4A). In SON AVP neurones from SL rats, 27 out of 39 cells showed significant increases in the F_{445}/F_{556} ratio indicative of chloride efflux ($t = -3.755$; $df = 7$; $P < 0.05$) (Figure 4B) associated with muscimol application, whereas 12 out of 39 had no change in chloride flux ($\chi^2 = 49.92$; $df = 2, 74$; $P < 0.001$).

3.4.4 | Role of KCC2 in chloride regulation—Because our data indicate Cl⁻ efflux in SON AVP neurones from SL rats, we tested the hypothesis that the reduced activity of KCC2 and the decreased chloride efflux contribute to this change in the polarity of the muscimol response.^{13,14} To test for KCC2 in setting [Cl⁻]_i, we used VU0240551, an antagonist that selectively targets the KCCs.¹² The muscimol-induced increase in [Cl⁻]_i was

inhibited by VU0240551 in neurones from Eu rats (Figure 5A,C). By contrast, VU 0240551 did not have any effect on the SL rat neurones because KCC2 membrane expression is known to be down-regulated in SL rats^{1,2} (Figure 5B,C). Two-way repeated measures ANOVA revealed a significant interaction, indicating that the chloride concentration was affected by treatment and bath antagonist protocol (Treatment $\times$ Antagonist: $F_{1,11} = 6.549$, $P < 0.05$). Post-hoc multiple comparisons between the factors revealed that KCC2 antagonist (VU 0240551, $10 \mu\text{mol L}^{-1}$) significantly blocked the chloride influxes in SON AVP neurones from EU rats (Tukey $t = 4.906$; $P < 0.05$) (Figure 5A,C).

3.4.5 | Role of NKCC1 in chloride regulation—Previous studies have implicated NKCC1 in $[\text{Cl}^-]_i$ regulation in various neurones.^{12,43} To test for a contribution of NKCC1 to $[\text{Cl}^-]_i$ on SON AVP neurones during SL, we used the NKCC1 antagonist bumetanide ($10 \mu\text{mol L}^{-1}$), which targets NKCC1 cotransporter without influencing KCC2 activity.⁴³ Bumetanide inhibited the muscimol-induced decrease in $[\text{Cl}^-]_i$ in AVP neurones from SL rats. By contrast, bumetanide did not alter the response of Eu rat neurones to muscimol. Two-way repeated measures ANOVA followed by post-hoc multiple comparisons revealed that the bumetanide did not have any effect on the Cl^- influx of Eu rat neurones with muscimol application (Figure 5D,F). Bumetanide significantly blocked the chloride efflux in SON AVP neurones from SL rats (Tukey $t = 3.629$; $P < 0.05$) as shown in Figure 5E,F.

3.4.6 | TrkB signalling in chloride regulation—Previous studies have demonstrated the role of the BDNF-TrkB-KCC2 signalling mechanism in the SON neurones with respect to modulating AVP secretion during high SL in rats.^{2,3,19,32} However, the role of this signalling mechanism with respect to mediating changes in intracellular chloride concentration is not known. In the present study, we tested the role of TrkB receptor activation on GABA_A-induced Cl^- flux associated with SL. Experiments were conducted using either a broad-spectrum protein kinase inhibitor, K252a, or a specific TrkB antagonist, AnA-12. Neurones from SL rats exhibited marked inhibition in muscimol-induced decrease in $[\text{Cl}^-]_i$ with both K252a and AnA-12 application. However, the antagonists did not alter the response of Eu rat neurones to muscimol. Two-way repeated measures ANOVA revealed a significant interaction, indicating that the chloride concentration was affected by treatment and K252a (Treatment $\times$ Antagonist: $F_{1,11} = 20.647$, $P < 0.05$). Post-hoc multiple comparisons between the factors revealed that protein kinase inhibitor (K252a, 100 nmol L^{-1}) significantly blocked the blocked muscimol-induced chloride efflux in SL rats (Tukey $t = 8.358$; $P < 0.05$) (Figure 6B,C). Similarly, specific TrkB antagonist (AnA-12, $50 \mu\text{mol L}^{-1}$) significantly blocked the blocked muscimol-induced chloride efflux in SL rats (Tukey $t = 4.611$; $P < 0.05$) (Figure 6E,F). The significant increase in $[\text{Cl}^-]_i$ in AVP neurones from Eu rats in response to muscimol application was unaffected by both the kinase inhibitor and TrkB antagonist (Figure 6A,D).

4 | DISCUSSION

In the present study, ClopHensorN was expressed in AVP neurones using an AAV vector with a vasopressin-specific promoter.²³ Our results confirmed the specificity of the promoter in a separate group of rats using immunohistochemistry for vasopressin and oxytocin as described previously.^{23,41} This approach allowed us to measure changes in $[\text{Cl}^-]_i$ associated

with the focal application of muscimol in acutely dissociated SON AVP neurones. This could offer several advantages compared to gramicidin perforated patch recording as a result of leaving membrane voltage and intracellular chloride unperturbed.^{12,25} Our methodology using dissociated neurones did not allow us to investigate subcellular differences in $[Cl^-]$. Our future studies with higher resolution imaging techniques will address the somatodendritic Cl^- gradients in SON AVP neurones.

Previous studies have shown that SL with 2% NaCl increases thirst and gradually causes hypernatraemia and hyperosmolality.^{1,44,45} Also, SL was associated with a decrease in body weight gain, possibly as a result of the anorexia that accompanies the drinking of hypertonic saline.⁴⁶

We measured plasma CPP in addition to AVP in our experiments. CPP is a peptide that is derived from the C-terminus of pre-prohormone of AVP and is often used as a surrogate marker for AVP. Previous studies have shown that plasma AVP and CP correlate strongly over a wide range of osmolalities.³⁰ The reported AVP and CPP plasma concentrations were in accordance with previous studies and both were significantly increased by SL.^{1,42}

In the adult nervous system, GABA primarily produces membrane hyperpolarisation by opening GABA_A receptors leading to Cl^- influx. However, there have been several reports showing that, in some systems, the valence of the GABA response can be altered so that it causes membrane depolarisation as a result of Cl^- efflux.^{2,15–17,20} Similar changes have been reported in the AVP neurones.^{2,15} To assess how SL influences the valence of GABA transmission in SON AVP neurones, we performed focal applications of the GABA_A agonist muscimol in the presence of various antagonists applied individually aiming to understand the specific role of various cotransporters and receptors. The time course and the concentrations for the drug treatments were chosen based on previous studies showing that this time course can cause transient shifts in $[Cl^-]_i$ affecting GABA polarity.^{37,38}

The relative activity of NKCC and the KCC cotransporters can determine resting $[Cl^-]_i$ in most neurones.^{2,13} Normally, $[Cl^-]_i$ is kept low in neurones by the tonically active KCC2.^{13,15,47} Membrane KCC2 expression is stabilised by post-translational modification, comprising phosphorylation at Ser940, which is shown to correlate with its cellular activity.⁴⁸ The results of the present study demonstrate that individual SON AVP neurones express KCC2 and the chloride imaging results support previous studies suggesting that SL effects this cotransporter to alter the inhibitory control of AVP release.^{1,2}

In addition to decreased KCC2 activity, increased activity of NKCC1 could also mediate the increase in $[Cl^-]_i$.¹⁵ To understand the role of NKCC1 in mediating the change in GABA-mediated chloride flux followed by SL, we examined the functional contribution of this transporter in SON AVP neurones from EU and SL rats using NKCC1 inhibitor bumetanide.

In SON AVP neurones from Eu rats, KCC2 inhibition significantly decreased the chloride influx associated with muscimol application, which is consistent with increased $[Cl^-]_i$ caused by reduced chloride excretion. By contrast, KCC2 inhibition had no effect on the muscimol responses of SON AVP neurones from SL rats. This suggests that SL had already reduced KCC2 activity. However, NKCC1 inhibition with bumetanide significantly

decreased in the amplitude of GABA-induced Cl^- efflux in SON AVP neurones from SL rats but did not affect the responses of neurones from Eu rats. In the cells from Eu rats that presumably have low $[\text{Cl}^-]_i$, blocking chloride excretion had a greater impact on the muscimol response compared to inhibiting NKCC1-mediated influx. This is consistent with GABA having an inhibitory effect in SON AVP neurones from Eu rats as a result of a low $[\text{Cl}^-]_i$ that is dependent on KCC2. Inhibiting KCC2 had no effect on the muscimol responses of SON AVP neurones from SL rats, presumably because its activity had already been reduced.² In SON AVP neurones from SL rats, blocking NKCC1 did significantly reduced muscimol-mediated chloride efflux. The significant reduction in chloride efflux associated with NKCC1 inhibition might only be partially explained by reduced chloride transport into the cells from SL rats. Chloride extrusion mediated by KCC2 would be reduced in cells from SL rats. Additional experiments will be required to obtain a complete understanding of the role of NKCC1 in the regulation of $[\text{Cl}^-]_i$ in SON AVP neurones during SL.

Capillary electrophoresis-based Protein Simple Wes was used in the present study because of its ability to analyse very low concentrations of protein and also because of its automation.⁴⁹ This technology allowed us to show that KCC2 and β -actin expression is found in the neurones that responded to muscimol during chloride imaging. The subset of neurones not responding during chloride imaging did not have KCC2 or β -actin protein expression. The absence of β -actin expression and its unresponsiveness to muscimol indicates that these neurones might not have been viable. Although previous immunohistochemical studies have demonstrated colocalisation of KCC2 with AVP in SON neurones,² the present study is the first to correlate KCC2 protein expression in individual AVP neurones with chloride imaging. KCC2 cotransporter expression in the AVP neurone has been controversial.^{2,13,15,20} Differences in methodology are likely to underlie some of these inconsistencies. Although the response of the AVP neurones to bumetanide indicates the expression of NKCC, both the expression of NKCC and the phosphorylation status of KCC2 in individual neurones will be addressed in future studies.

Previous studies have shown that TrkB-mediated signalling contributes to the down-regulation of KCC2 co-transporter in the SON neurones and increases AVP secretion in rats during SL.^{1,2,9} The effects of the TrkB inhibitor, AnA-12, and broad-spectrum protein kinase inhibitor, K252a, on muscimol evoked responses in SON AVP neurones were also analysed in the present study. Although they did not significantly influence the responses to muscimol in neurones from Eu rats, both inhibitors significantly blocked the effects of SL in SON AVP neurones responses to muscimol. This suggests that BDNF-TrkB signalling is a mechanism that contributes to the increase in $[\text{Cl}^-]_i$ associated with SL. However, the synaptic mechanisms leading to BDNF-mediated activation of TrkB receptor are not known and should be the focus of future studies.

5 | CONCLUSIONS

In the present study, we used virally-mediated expression of a chloride-sensitive indicator and a Simple Wes approach to examine the effects of SL on GABA signalling in SON neurones. Our data show that a TrkB-KCC2-NKCC1-dependent mechanism contributes to the increases in $[\text{Cl}^-]_i$ of SON AVP neurones from SL rats.^{1,2} In addition, the results show

that SON AVP neurones express KCC2 and they also suggest the expression of a bumetanide-sensitive NKCC co-transporter. The relative contribution of these transporters to the chloride homeostasis of vasopressin neurones is dependent on the physiological state of the animal. Future studies will aim to test whether other physiological challenges can influence the valence of the GABA response in SON AVP neurones.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

A, Arginine vasopressin (AVP) staining within the supraoptic nucleus (SON). B, Immunofluorescence of AAV2-0VP1-GFP virus. C, Merged digital image. Cells labeled with GFP and AVP are indicated with arrows. D, Oxytocin (OXT) staining within the SON. E, Immunofluorescence of AAV2-0VP1-GFP virus. F, Merged digital image. Cells labelled with GFP and OXT are indicated by arrows. Scale bar = 50 μm

FIGURE 2.

Representative digital image of supraoptic nucleus arginine vasopressin neurone expressing ClopHensorN plasmid showing the F_{445}/F_{556} ratio. Ai, Fluorescence of the cell during baseline. Aii, Representative image showing the decrease in fluorescence with an increase in $[Cl^-]_i$ cell during the peak response. The inset table is a colour scale for changes in the F_{445}/F_{556} ratio (0.2-0.5). Aiii, Ratiometric chloride imaging with ClopHensorN using whole-cell patch with a CsCl filled electrode. An arrow indicates the start of whole-cell access. Bi, Fluorescence of the cell during baseline. Bii, Representative image showing the increase in fluorescence with a decrease in $[Cl^-]_i$ cell during the peak response. Biii, Ratiometric chloride imaging with ClopHensorN using whole-cell patch with a low chloride K gluconate electrode. Note that increasing intracellular chloride with CsCl decreases the F_{445}/F_{556} ratio, whereas as decreasing intracellular chloride with K gluconate increases the F_{445}/F_{556} ratio.

FIGURE 3 :

(A-D) Representative protein expression of a single supraoptic nucleus (SON) arginine vasopressin (AVP) neuron from an euhydrated rat. A, Chloride imaging of the neurone from which protein expression was analysed. This neurone responded to GABA_A agonist muscimol during imaging. B, Chemiluminescent signal showing a sharp peak for total K⁺/Cl⁻ co-transporter2 (KCC2) protein expression at 174 kDa (electropherogram view). C, Electropherogram plot showing a chemiluminescent signal for β-actin protein expression at 24-26 kDa. D, Total KCC2 and β-actin expression of the same neurone in traditional densitometry view using single-cell western blot analysis. (E-H), Representative protein expression of a single SON AVP neuron from a salt-loaded (SL) rat. E, Chloride imaging of the neurone from which protein expression was analysed. This neurone responded to muscimol during imaging. F, Chemiluminescent signal showing a sharp peak for total KCC2 protein expression at 174 kDa (electropherogram view). G, Electropherogram plot showing a chemiluminescent signal for β-actin protein expression at 24-26 kDa. H, Total KCC2 and β-actin expression of the same neurone in traditional lane view

FIGURE 4.

Control experiment to verify changes in $[Cl^-]_i$ in acutely dissociated supraoptic nucleus (SON) arginine vasopressin (AVP) neurones with no bath application of antagonists. A, response of a cell from an euhydrated rat to focal application of muscimol ($100 \mu\text{mol L}^{-1}$; 10 s; three cycles). Artificial cerebrospinal fluid (aCSF) was bath applied without any antagonists for the second response of the same cell to muscimol focal application. B, response of a cell from a salt-loaded rat to focal application of muscimol ($100 \mu\text{mol L}^{-1}$; 10 s; three cycles) twice without bath application of antagonists. The duration and experimental conditions utilised in this control imaging were similar to the drug treatment recordings

FIGURE 5.

(A-C) K^+/Cl^- co-transporter2 (KCC2)-mediated changes in $[Cl^-]_i$ measured by ClopHensorN in acutely dissociated supraoptic nucleus (SON) arginine vasopressin (AVP) neurones. A, response of a cell from an euhydrated (Eu) rat to focal application of muscimol (100 $\mu\text{mol L}^{-1}$; 10 s; three cycles) and KCC2 antagonist VU0240511, 10 $\mu\text{mol L}^{-1}$ (incubated for 5 min and continuously bath applied for second half recording). Cells from control rats showed muscimol-induced Cl^- influx (7/9; $P < 0.05$). VU0240511 significantly blocked the Cl^- influx. B, 2% salt-loaded (SL) rat AVP neurone responses to muscimol and VU0240511 application. Muscimol application to SON AVPs from SL rats caused either a significant increase in chloride efflux (7/10; $P < 0.05$) or no change in chloride flux (3/10). C, Quantification graph showing average changes in the peak to muscimol from baseline before and after VU0240511 application in both Eu and SL rats (D-F), NKCC1-mediated changes in $[Cl^-]_i$ measured by ClopHensorN in acutely dissociated SON AVP neurones. D, response of a cell from an Eu rat to focal application of muscimol (100 $\mu\text{mol L}^{-1}$; 10 s; three cycles) and NKCC1 antagonist bumetanide, 10 $\mu\text{mol L}^{-1}$ (incubated for 5 min and continuously bath applied for second half recording) (5/8). Bumetanide did not have any effect on Cl^- influx in Eu rats. E, 2% SL rat AVP neurone responses to muscimol and bumetanide application (6/10). Bumetanide significantly blocked the Cl^- efflux. F, Quantification graph showing average changes in the peak to muscimol from baseline before and after bumetanide application in both Eu and SL rats. The statistical difference is calculated using a two-way repeated measures ANOVA, a chi-squared test and Bonferroni post-hoc analysis. Data are the mean $\pm$ SEM. * $P < 0.05$ vs artificial cerebrospinal fluid (aCSF) with muscimol.

FIGURE 6.

Tyrosine receptor kinase B (TrkB)-mediated changes in $[Cl^-]_i$ measured by ClopHensorN in acutely dissociated supraoptic nucleus (SON) arginine vasopressin (AVP) neurones. A, response of an euhydrated (Eu) rat to focal application of muscimol ($100 \mu\text{mol L}^{-1}$; 10 s; three cycles) and protein kinase inhibitor K252a, 100 nmol L^{-1} (7/10). B, 2% SL rat AVP neurone responses to muscimol and K252a application. K252a significantly blocked the Cl^- efflux (6/8). C, Quantification graph showing average changes in the peak to muscimol from baseline before and after K252a application in both Eu and SL rats. D, response of a cell from an Eu rat to focal application of muscimol ($100 \mu\text{mol L}^{-1}$; 10 s; three cycles) and TrkB antagonist AnA-12, $50 \mu\text{mol L}^{-1}$ (incubated for 5 min and continuously bath applied for second half recording) (5/9). E, 2% SL rat AVP neurone responses to muscimol and AnA-12 application (6/10). AnA-12 significantly blocked the Cl^- efflux. F, Quantification graph showing average changes in the peak to muscimol from baseline before and after AnA-12 application in both Eu and SL rats. The statistical difference is calculated using a two-way repeated measures ANOVA, a chi-squared test and Bonferroni post-hoc analysis. Data are the mean $\pm$ SEM. * $P < 0.05$ vs artificial cerebrospinal fluid (aCSF) with muscimol

TABLE 1

Plasma osmolality, haematocrit, arginine vasopressin (AVP) and copeptin (CPP) concentration in euhydrated (Eu) and salt-loaded (SL) rats

	Euhydrated (Eu)	Salt-loaded (SL)
Osmolality (mosmol kg ⁻¹)	298.8 ± 1.12 (11)	308.6 ± 0.55 (7) ^a
Haematocrit (%)	42.5 ± 0.5 (11)	47 ± 0.43 (7) ^a
Plasma AVP (pg mL ⁻¹)	14.8 ± 5.31 (6)	57.6 ± 7.91 (6) ^a
Plasma CPP (pg mL ⁻¹)	49.4 ± 1.98 (6)	77.8 ± 5.53 (6) ^a

Values are the mean ± SEM; n, number of rats in each group (given in parenthesis).

^a*P* < 0.05 vs Eu.