Brain-Derived Neurotrophic Factor and Supraoptic Vasopressin Neurons in Hyponatremia

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K⁺/Cl[−] cotransporter 2 · Tyrosine receptor kinase B · Bile duct ligation and liver failure

Abstract

Hyponatremia due to elevated arginine vasopressin (AVP) secretion increases mortality in liver failure patients. The mechanisms causing dysregulation of AVP secretion are unknown. Our hypothesis is that inappropriate AVP release associated with liver failure is due to increased brain-derived neurotrophic factor (BDNF) in the supraoptic nucleus (SON). BDNF diminishes GABA_A inhibition in SON AVP neurons by increasing intracellular chloride through tyrosine receptor kinase B (TrkB) activation and downregulation of K⁺/Cl[−] cotransporter 2 (KCC2). This loss of inhibition could increase AVP secretion. This hypothesis was tested using shRNA against BDNF (shBDNF) in the SON in bile duct ligated (BDL) male rats. All BDL rats had significantly increased liver weight ($p < 0.05$; 6–9) compared to shams. BDL rats with control shRNA injections (BDL scrambled [SCR]) developed hyponatremia with increased plasma AVP and copeptin (CPP; all $p < 0.05$; 6–9) compared to sham groups. This is the first study to show that phosphorylation of TrkB is significantly increased

along with significant decrease in phosphorylation of KCC2 in BDL SCR rats compared to the sham rats ($p < 0.05$; 6–8). Knockdown of BDNF in the SON of BDL rats (BDL shBDNF) significantly increased plasma osmolality and hematocrit compared to BDL SCR rats ($p < 0.05$; 6–9). The BDL shBDNF rats had significant ($p < 0.05$; 6–9) decreases in plasma AVP and CPP concentration compared to BDL SCR rats. The BDNF knockdown also significantly blocked the increase in TrkB phosphorylation and decrease in KCC2 phosphorylation ($p < 0.05$; 6–8). The results indicate that BDNF produced in the SON contributes to increased AVP secretion and hyponatremia during liver failure.

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Introduction

Hyponatremia is characterized by serum sodium < 135 mEq/L in humans and is the most frequent electrolyte abnormality. The cost of its treatment in the United States has been estimated to be USD 1.6–3.6 billion per year [1]. Hyponatremia is often associated with either congestive heart failure or liver cirrhosis. Hyponatremia related to liver failure is caused by inappropriate arginine vasopres-

sin (AVP) release that contributes to ascites, seizures, pulmonary, and cerebral edema [2–5]. A relative decrease in plasma volume and vasodilation in response to portal hypertension during liver failure are reported to increase AVP secretion as a compensatory mechanism [2, 5–8]. However, sustained AVP secretion is maintained despite decreased plasma osmolality. This inappropriate AVP release eventually leads to hyponatremia, which increases morbidity and mortality of patients with liver failure [2–5]. Once hyponatremia is established in the setting of liver failure, conventional therapies are frequently ineffective [6, 9]. Although treatments with AVP antagonists can improve hyponatremia, the cause of inappropriate AVP release is unknown [1, 5, 10, 11]. The development of effective therapeutic approaches for dilutional hyponatremia may require understanding molecular mechanisms behind the abnormality.

The secretion of AVP is regulated by magnocellular neurosecretory cells (MNCs) located in the supraoptic nucleus (SON) and paraventricular nucleus of the hypothalamus [12–19]. The dysregulation of these MNCs located in the hypothalamic neurons can contribute to elevated AVP secretion during liver failure. Sustained AVP release is also reported in high salt loaded rats and in DOCA-salt hypertension [20, 21]. In these models, MNCs continue to release AVP into the systemic circulation despite elevated blood pressure forgoing baroreflex-mediated inhibition of AVP MNCs [20, 22, 23]. Previous studies have shown that during high salt loading and dehydration, MNCs upregulate brain-derived neurotrophic factor (BDNF) [20, 22–24]. BDNF can have profound effects on GABA neurotransmission through several mechanisms, including the regulation of chloride transporters through tyrosine receptor kinase B (TrkB) activation. Increase in intracellular chloride $[Cl]_i$ can diminish or reverse the inhibitory effects of GABA on AVP neurons creating a feed-forward loop that drives AVP release [23, 25]. The aim of this study is to explore the role of BDNF-TrkB mechanism in increased AVP secretion associated with an animal model of liver [26, 27]. In rats, chronic bile duct ligation (BDL) is a model of liver failure with dilutional hyponatremia caused by elevated circulating AVP. Similar to the human condition, this rat model has significantly decreased plasma osmolality, hypervolemic ascites formation, and increased drinking behavior in addition to elevated circulating AVP [26, 28].

We hypothesize that BDNF in the SON contributes to the increased AVP release associated with liver failure. BDNF diminishes GABA_A inhibition in SON AVP neurons by increasing $[Cl]_i$ through TrkB receptor activation

and downregulation of K⁺/Cl[–] cotransporter 2 (KCC2). This loss of inhibition could increase AVP secretion. In this study, we used adeno-associated viral vectors with shRNA against BDNF (shBDNF) to test our hypothesis by knocking down BDNF in the SON. This study reveals a novel mechanism that contributes to increase in AVP secretion during liver failure that could contribute to other diseases such as heart failure and neurogenic hypertension.

Materials and Methods

Animals

All the experimental protocols involving animals were conducted in accordance to the National Institute of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the UNT Health Science Center Institutional Animal Care and Use Committee. Six-week-old outbred adult male Sprague-Dawley rats with body weights of 200–250 g (Charles River Laboratories, Inc., Wilmington, MA, USA) were used to conduct all the experiments. The animals were maintained in a temperature-controlled (23 °C) environment with a 12-h light dark cycle with the light phase lasting from 7 a.m. to 7 p.m. Food and water were available *ad libitum* to the animals unless otherwise indicated. Rats were individually housed due to the use of survival surgery and individual fluid intake measurements in the protocol. Survival surgeries were conducted using aseptic techniques, and procaine penicillin G (30,000 U) was subcutaneously administered to prevent postoperative infection. Nonsteroidal anti-inflammatory drug, carprofen (Rimadyl, 2 mg), was given orally before and after surgery for pain management.

Virally Mediated BDNF Knockdown in the SON

An adeno-associated virus (AAV) serotype 2 conjugated with a U6 promoter, a mCherry reporter, and a shBDNF and an AAV2 with a scrambled (SCR) sequence of shRNA were used in this experiment. Both the viruses were obtained from Vector Biolabs (Malvern, PA, USA). Rats were anaesthetized with isoflurane (2–3%), placed in a Kopf (Tujunga, CA, USA) stereotaxic frame, and their scalps were disinfected with alcohol and iodine. Their skulls were exposed and leveled between lambda and bregma [29]. A micromanipulator was oriented to lower the probe to the targeted coordinates of SONs (1.4 mm posterior, 9.1 mm ventral, and ± 1.4 mm lateral from bregma). A burr hole was drilled at the injection site, and a 30-gauge stainless steel injector was lowered to the SON.

Rats were bilaterally injected in the SON (300 nL/side) with either AAV2-U6-mCherry-shBDNF or with a control virus AAV2-U6-mCherry-SCR. The vectors were injected at a titer of 1.0×10^{13} genomic particles/mL (Vector Biolabs). The injector was connected to a Hamilton 5 μ L syringe (#84851, Hamilton) by calibrated polyethylene tubing that was used to determine the injection volume. Each construct was injected in both the SONs over a 10-min period. The injector remained inserted for 5 min to allow for absorption of injected particle and then slowly withdrawn. Gel foam was used to fill the openings in the cranium, and the incision site was closed with absorbable antibiotic sutures.

BDL Surgery

After 2 weeks of recovery from stereotaxic injections, the rats were anaesthetized with isoflurane (2–3%). The abdomen was shaved, cleaned, and disinfected. A midline abdominal incision of approximately 2 cm was performed. The bile duct was carefully isolated from the flanking portal vein and hepatic artery using micro-serrations forceps. The bile duct was secured in position, double ligated with 6–0 silk sutures, and cauterized between 2 ligatures as previously described [26, 27]. Sham rats received the same surgical procedure except their bile duct was not cauterized. The abdominal layers were closed with absorbable antibiotic sutures. Visual inspection of ascetic fluid in the peritoneal cavity was performed daily after surgery. Any rat showing morbidity or ascites of >10% of the body weight was euthanized with inactin (100 mg/kg, ip, St. Louis, MO, USA). The survival rates of the animals used in this study were over 98%. Successful BDL was verified at the end of the study by yellow coloration of plasma and an increase in liver weight to body weight ratio.

Metabolic Cage Study

After 2 weeks of recovery from BDL or sham ligation surgery, the rats were moved into metabolic cages (Lab Products, Seaford, DE, USA) to measure food intake, fluid intake, and urine excretion every day. The rats were provided with *ad libitum* access to food and water for 14 days to record various parameters as previously described [22]. Food intake was measured by filling the food containers up to a predetermined weight of ground chow (Teklad Diets, Madison, WI, USA) in grams. After 24 h, the food was reweighed, and the amount consumed was calculated by the difference. Fluid intake was measured by filling the bottles with a known amount of water in grams and subtracting the remaining weight 24 h later. Mineral oil was added to the urine collection vials to prevent evaporation of water from urine. Urine excretion volume was recorded, and urine samples were collected for measuring daily sodium excretion.

Measurement of Electrolyte Concentration

The daily urine samples were collected from each rat in 50-mL centrifuge tubes, and a 1–2 mL aliquot was taken from each daily sample. Debris was removed by centrifuging (20 min; 10,000 g) the urine samples. The sodium concentration of each of the urine samples was determined using flame photometer after a 1:500 dilution (Jenway PFP7, VWR International, Radnor, PA, USA). Varying concentrations of sodium chloride were used as standards, and the instrument was calibrated with standards for each analysis. Final sodium concentration in mEq/L was calculated from linear calibration curve derived from sodium standards using Sigma plot (version 12.0, Systat Software Inc., San Jose, CA, USA).

Laser Capture Microdissection and Quantitative Real-Time PCR

Four weeks after BDL, the rats were anesthetized with inactin (100 mg/kg ip, Millipore Sigma, Burlington, MA, USA) and decapitated. The brains were removed from the skulls, flash frozen using precooled 2-methyl butane, and stored at –80°C. Each brain was prepared for laser capture microdissection (LCM) [30, 31] by sectioning the frozen brain at a thickness of 10 µm through the hypothalamus at the levels containing the SON. The coronal sections were directly mounted onto poly(p-phenylene) sulfide membrane-coated slides (Leica Microsystems, Buffalo Grove, IL, USA).

Table 1. Primer sequences for qRT-PCR

Gene	Primer sequences
BDNF forward	5'-ATGACCATCCTTTTCCTTACTATG-GT-3'
BDNF reverse	5'-TCTTCCCCTTTTAATGGTCAGTG-TAC-3'
hnAVP forward	5'-GCCCTCACCTCTGCCTGCTA-3'
hnAVP reverse	5'-CCTGAACGGACCACAGTGGT-3'
S18 forward	5'-CAGAAGGACGTGAAGGATGG-3'
S18 reverse	5'-CAGTGGTCTTGGTGTGCTGA-3'

qRT-PCR, quantitative real-time PCR; BDNF, brain-derived neurotrophic factor.

Separate sets of 40 µm sections were mounted on gelatin-coated slides and were used to independently verify the injection sites. The Leica Microsystems LCM instrument utilized in this experiment uses a UV cutting laser to dissect the region of interest into collection tubes containing the lysis buffer. The accuracy of the injection sites was verified by visualizing the mCherry reporter in the viral construct, and the SON regions were specifically collected. Minimum of 7–9 SON regions were laser captured and collected from each rat for RNA extraction and amplification. The SONs collected from LCM were used to measure changes in BDNF mRNA and AVP hnRNA using quantitative real-time PCR. The RNA was extracted and purified from each sample using Array-Pure Nano-Scale RNA Purification Kit reagents (Epicentre Biotechnologies, Madison, WI, USA). The concentration and quality of each RNA sample was evaluated using a Nanodrop Spectrophotometer (Thermo Scientific, Waltham, MA, USA) and reverse transcribed to cDNA with Sensiscript RT Kit reagents (Qiagen, Valencia, CA, USA) [30]. Real-time PCR was performed on a CFX96 C1000 Cyclor (Bio-Rad, Hercules, CA, USA) with SYBR green fluorescent label. Samples (15 µL final volume) contained: SYBR green master mix (Bio-Rad), 3–5 pmol of each primer, and equal concentration of cDNA. Forward and reverse primers for target genes (Table 1) were obtained from Integrated DNA Technologies (Coralville, IA, USA). The housekeeping gene S18 was used to normalize RNA expression. Cycling parameters were as follows: 95 °C:3 min, then 40 cycles of the following 95 °C:10 s, 60 °C:1 min. A melting temperature-determining dissociation step was performed from 65 to 95 °C at increments of 0.5 °C every 5 s at the end of the amplification phase. Melt curves generated were analyzed to identify nonspecific products and primer-dimers. The data were analyzed by the $2^{-\Delta\Delta C_t}$ method [32, 33]. ΔC_t was measured by calculating the difference between the S18 and the corresponding gene of interest C_t values. For obtaining the $\Delta\Delta C_t$ value, this value was then subtracted from the difference between the average of control S18 and control gene of interest C_t values [22, 28, 31, 34].

Western Blot Analysis

Four weeks after BDL, the rats were anesthetized with inactin (100 mg/kg ip) and decapitated. Punches containing the SON were collected from 1 mm coronal section from each brain as pre-

Table 2. Liver to body weight ratio, plasma osmolality, and hematocrit in sham-ligated rats injected with the control vector (Sham SCR), sham rats injected with shRNA against BDNF (Sham shBDNF), bile duct ligated rats injected with control vector (BDL SCR), and bile duct ligated rats injected with shRNA against BDNF (BDL shBDNF) groups

	Sham SCR	Sham shBDNF	BDL SCR	BDL shBDNF
Liver Wt/body Wt	0.052±0.001 (6)	0.048±0.003 (9)	0.086±0.002 (6)*	0.086±0.002 (8)*
Osmolality, mOsm/kg	299.9±0.94 (6)	301.4±1.11 (9)	289.4±0.72 (6)*	295.5±0.53 (6)*, +
Hematocrit, %	45.4±0.53 (6)	45.5±0.44 (8)	39.1±1.09 (6)*	42.9±0.45 (6) ⁺

Values are mean ± SEM; *n*, number of rats in each group, in parenthesis. SCR, scrambled; shBDNF, shRNA against BDNF; BDL, bile duct ligated; BDNF, brain-derived neurotrophic factor.

* *p* < 0.05 versus control groups (liver to body wt: *F*[3,25] = 59.304; osmolality: *F*[3,23] = 32.115; hematocrit: *F*[3,22] = 20.771).

⁺ *p* < 0.05 versus BDL SCR.

viously described [30, 31]. The tissue was lysed using RIPA lysis buffer containing DTT, EGTA, EDTA, and protease phosphatase inhibitor cocktail. The tissue extracts were sonicated, and insoluble material was spun down at 14,000 g for 20 min. Protein concentration was determined by Bio-Rad DC assay (detergent compatible) with varying concentrations of BSA as reference standards. Total lysate (20–25 µg) was mixed with SDS-loading buffer dye and heat denatured at 95 °C for 5 min. The lysates were loaded onto a 4–15% SDS PAGE gel and separated by electrophoresis using Tris-glycine buffer with denaturing conditions. The protein was transferred to polyvinylidene difluoride membrane (Immobilon-P; EMD-Millipore, Burlington, MA, USA) in Tris-glycine buffer (25 mM Tris, 192 mM glycine, 0.1% SDS; pH 8.3) with 20% (v/v) methanol. Membranes were blocked with 5% BSA in Tris-buffered saline-Tween 20 (25 mM Tris base, 125 mM NaCl, 0.1% Tween 20) for 30 min at room temperature. The primary and secondary antibodies and their respective concentrations were chosen based on previous studies [20, 22, 35]. The membranes were incubated with primary antibodies made in 5% BSA overnight at 4 °C. The primary antibodies used were: phosphorylated TrkB (Y515; rabbit polyclonal; 1:1,000; ab109684, Abcam, Cambridge, MA, USA), total TrkB (goat; 1:1,000; GT15080, Neuromics), phosphorylated KCC2 (Ser940; rabbit polyclonal; 1:500; 612–401-E15, Rockland, ME, USA), mCherry (rabbit polyclonal; 1:500; ab167453, Abcam), and GAPDH (mouse monoclonal; 1:2,000; MAB374, Millipore).

Membranes were washed 3 times at 10 min intervals with TBS-Tween followed by a 2-h incubation at room temperature with a horseradish peroxidase-conjugated secondary antibody against the primary antibody host species (anti-rabbit, anti-goat, or anti-mouse; 1:1,000; Sigma, St. Louis, MO, USA). The membranes were washed 3 times at 5 min intervals with TBS-Tween. Proteins were visualized using an enhanced chemiluminescence substrate kit (Supersignal West Femto Maximum Sensitivity kit; Thermo Scientific, Waltham, MA, USA). Blots were developed, and the digital image was obtained by using Gbox (Genesnap program), and densitometry analysis of the bands was performed using ImageJ. Densitometry measurements of the immunoreactive bands were normalized using GAPDH as the loading control.

Plasma Measurements

Plasma osmolality, hematocrit, circulating AVP, and copeptin (CPP) were measured in plasma collected from trunk blood of each rat after decapitation. CPP is widely used as a biomarker for AVP secretion [36] and was measured in this study in addition to AVP. An aliquot of 1–2 mL blood was collected and prepared for measuring plasma osmolality and hematocrit as previously described [31, 37]. Blood was filled in duplicate heparinized capillary tubes (Fisher Scientific, Hampton, NH, USA) and centrifuged for 10 min. A micro-hematocrit capillary tube reader (Lancer, St. Louis, MO, USA) was used for measuring hematocrit. The remaining blood sample was centrifuged at 1,600 g for 5–10 min, and plasma was collected and maintained at 4 °C to measure osmolality using vapor pressure osmometer (Wescor, Logan, UT, USA).

Separate 5–6 mL of blood was collected in vacutainer tubes containing the K₂ EDTA (12 mg) to prevent coagulation of the blood. The proteinase inhibitor, aprotinin (0.6 TIU/mL of blood; Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA), was added to each tube before blood collection. The blood samples were centrifuged at 1,600 g for 15 min at 4 °C. Two to 3 mL of plasma was collected 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 specific ELISA (EK-065-07, Phoenix Pharmaceuticals, Inc.). The CPP concentration was measured in plasma samples by using specific ELISA according to the manufacturer's instructions (MBS724037, MyBioSource, Inc., San Diego, CA, USA). Four parametric logistic analyses were performed to quantify the concentration of AVP and CPP using Sigma plot (version 12.0).

Experimental Groups and Statistical Analysis

In all the experiments in this study, the rats were divided into 4 groups as follows: (1) Sham rats injected with SCR virus (Sham SCR), (2) Sham rats injected with shBDNF virus (Sham shBDNF), (3) BDL rats injected with SCR virus (BDL SCR), and (4) BDL rats injected with shBDNF virus (BDL shBDNF).

Data from the metabolic cage studies and urine sodium concentration were analyzed by separate two-way repeated measures

Fig. 1. **a** Representative digital image of mCherry fluorescence in the SON illustrating a successful injection. **b** Quantitative real-time PCR data showing BDNF mRNA expression from the SON of rats with successful SON injections. **c** AVP hnRNA expression from the SONs of rats with successful SON injections. Groups: Sham-ligated rats injected with SCR virus (Sham SCR, $n = 5$); Sham rats injected with shBDNF virus (Sham shBDNF, $n = 6$); BDL rats injected with SCR virus (BDL SCR, $n = 6$), and BDL rats injected with shBDNF virus (BDL shBDNF, $n = 6$). Data are mean $\pm$ SEM. * $p < 0.05$ versus all other groups (BDNF: $F[3,19] = 5.68$; AVP: $F[3,19] = 5.552$). + $p < 0.05$ versus BDL SCR. SON, supraoptic nucleus; OT, optic tract; BDNF, brain-derived neurotrophic factor; SCR, scrambled; shBDNF, shRNA against BDNF; BDL, bile duct ligation.

ANOVA with time as first factor and treatment (BDL/sham with stereotaxic injection) as second factor followed by Bonferroni post hoc tests. All other data were analyzed using one-way ANOVA with Bonferroni post hoc tests using Sigma Plot (version 12.0). Figures were assembled using the “magick” package in RStudio. The group sizes were determined by power analysis and effect size calculated from our previously published work [20, 27, 31] and preliminary data using Sigma plot (version 12.0). The considerations for calculating power analysis were $p < 0.05$, α of 0.8, largest difference between means, and the largest standard deviation we had observed from our studies. The effect sizes were calculated by η^2 (η^2) method using sum of squares of effect and total from our previous studies [20, 26, 27, 31]. The sample sizes were independent of each other in all the groups, and the minimal n 's per group for each experiment were chosen to have an appropriately powered study with the effect size (η^2) of approximately 0.7 (70%).

Results

Liver to Body Weight Ratio

The liver weight was measured 4 weeks after BDL/sham ligation surgery. The increase in liver weight is

widely used as a biomarker to verify the liver failure animal model [27, 28]. The liver weight to body weight ratio was very high in both BDL SCR and BDL shBDNF rats compared to the sham rats. One-way ANOVA followed by Bonferroni multiple comparisons show that both SCR and shBDNF rats with BDL had significantly increased ratios compared to the sham groups ($F[3,25] = 59.304$, $p < 0.05$; Table 2), and the BDNF knockdown did not affect the liver to body weight ratio.

BDNF and AVP Gene Expression in SON

LCM was used to visualize the mCherry reporter to verify the accuracy of the stereotaxic injection (Fig. 1a) and to collect the SONs for RNA extraction. For this study, BDNF mRNA and AVP hnRNA expression were measured using $2^{-\Delta\Delta C_t}$ method. The BDNF and AVP gene expression were increased in the SON of BDL rats compared to the sham-ligated rats. The shBDNF injections in the BDL rats lowered BDNF mRNA and AVP hnRNA expression in the SON compared to the rats with SCR injections. Less than 5% of total rats had injections that did

Fig. 2. The effects of BDL and BDNF knockdown in the SON on protein expression. **a** Sample Western blot images showing changes in protein expression of SON punches. mCherry protein expression images shows successful injections at SON. **b** Quantification of phosphorylated TrkB (normalized to total TrkB). **c** Phosphorylated KCC2 normalized to GAPDH. Data are mean $\pm$ SEM. * $p < 0.05$ versus all other groups (TrkB: $F[3,20] = 14.931$; KCC2: $F[3,20] = 5.973$). + $p < 0.05$ versus BDL SCR. Groups: Sham SCR ($n = 6$); Sham shBDNF ($n = 6$); BDL SCR ($n = 6$); BDL shBDNF ($n = 6$). SCR, scrambled; shBDNF, shRNA against brain-derived neurotrophic factor; BDL, bile duct ligation; TrkB, tyrosine receptor kinase B.

not hit the SON; these rats were not included in any subsequent analyses. One-way ANOVA revealed significant difference between the groups for both BDNF ($F[3,19] = 5.68$, $p < 0.05$) and AVP ($F[3,19] = 5.552$, $p < 0.05$) gene expression. Post hoc multiple comparison of mRNA levels between BDL SCR and BDL shBDNF groups showed that SON injections of shBDNF significantly blocked the increases in BDNF mRNA (Bonferroni $t = 3.111$, $p < 0.05$; Fig. 1b) and AVP hnRNA (Bonferroni $t = 3.437$, $p < 0.05$; Fig. 1c) in the SON of BDL rats.

Phosphorylation of TrkB and KCC2 in SON

The SONs from the BDL rats with SCR injections had increased phosphorylation of TrkB receptor and decreased phosphorylation of KCC2. The phosphorylation or activation of TrkB receptor was decreased in the BDL rats with BDNF knockdown in the SON. In addition, the downregulation of phosphorylated KCC2 was prevented by BDNF knockdown in the BDL rats. The mCherry expression was used to verify the specificity of stereotaxic injections (Fig. 2a). One-way ANOVA analysis revealed significant difference between the groups in TrkB phosphorylation ($F[3,20] = 14.931$, $p < 0.05$; Fig. 2b) and KCC2 phosphorylation ($F[3,20] = 5.973$, $p < 0.05$; Fig. 2c). BDL for 4 weeks significantly increased TrkB phosphorylation without affecting total TrkB expression and decreased

phosphorylation of KCC2 (Bonferroni t tests, all $p < 0.05$, Fig. 2) in the SON of rats injected with the control vector compared to the sham-ligated rats. Virally mediated BDNF knockdown in the SON of BDL rats significantly prevented the increase in TrkB phosphorylation and decrease in KCC2 phosphorylation compared to sham rats (Bonferroni t tests, all $p < 0.05$, Fig. 2). One to 2 rats in each group did not have successful virus injections in the SON, which were verified at the end of the experiment and were excluded from the data analysis in all the experiments.

Plasma AVP, CPP, Osmolality, and Volume

The plasma osmolality, hematocrit, AVP, and CPP were measured 4 weeks after BDL/sham ligation surgery. The development of hyponatremia in BDL rats was associated with decreased plasma osmolality and hematocrit values in BDL rats with SCR injections compared to the sham rats. Hyponatremia associated with BDL was not observed in rats with BDNF knockdown of the SON. The concentration of AVP and CPP in BDL rats injected with the control vector was higher compared to the sham rats. This increase in plasma AVP and CPP values was not seen in the BDL rats with SON injections of shBDNF.

One-way ANOVA followed by post hoc multiple comparisons show that plasma osmolality and hematocrit values were significantly different between the groups (plas-

Fig. 3. The effects of BDL and BDNF knockdown in the SON on (a) plasma AVP concentration and (b) plasma CPP concentration. Data are mean $\pm$ SEM. * $p < 0.05$ versus sham groups (AVP: $F[3,18] = 157.817$; CPP: $F[3,23] = 40.581$). + $p < 0.05$ BDL shBDNF versus BDL SCR. Groups: Sham SCR ($n = 6$); Sham shBDNF ($n = 6$); BDL SCR ($n = 6$); BDL shBDNF ($n = 6$). AVP, arginine vasopressin; SCR, scrambled; shBDNF, shRNA against brain-derived neurotrophic factor; BDL, bile duct ligation; CPP, copeptin.

ma osmolality $F[3,23] = 32.115$, $p < 0.05$; hematocrit $F[3,22] = 20.771$, $p < 0.05$). The BDL SCR rats also had plasma osmolality and hematocrit values that were significantly lower than all other groups (Bonferroni t tests, all $p < 0.05$). The BDL shBDNF group had significantly higher plasma osmolality (Bonferroni $t = 4.269$, $p < 0.05$; Table 2) and hematocrit (Bonferroni $t = 5.945$, $p < 0.05$; Table 2) as compared to the BDL SCR group. One-way ANOVA revealed significant difference between the groups in plasma AVP and CPP concentration (AVP: $[F(3,18) = 157.817]$, $p < 0.05$; CPP: $[F(3,23) = 40.581]$, $p < 0.05$). Bonferroni multiple comparisons show that BDL rats with either SCR or shBDNF injections had significantly increased circulating AVP and CPP compared to all the other groups (Bonferroni t tests, all $p < 0.05$; Fig. 3). Plasma AVP and CPP were significantly lower in BDL rats with knockdown of BDNF in the SON as compared to the BDL SCR group (AVP: Bonferroni $t = 8.999$, $p < 0.05$, Fig. 3a; CPP: Bonferroni $t = 5.763$, $p < 0.05$, Fig. 3b).

Fluid Intake, Urine Excretion, Food Intake, and Body Weight

Fluid intake, urine excretion, and food intake were measured daily for 14 days beginning 2 weeks after BDL or sham ligation surgery. Both the fluid intake and urine excretion were increased in BDL rats with SCR injections compared to the sham rats. But this increase in both fluid intake and urine excretion was not observed in the BDL rats injected with shBDNF. Two-way repeated measures ANOVA revealed significant differences indicating that fluid intake was affected by time and the treatment protocol (Time: $F[13,156] = 5.734$, $p < 0.05$; Treatment: $F[3,156] = 11.818$, $p < 0.05$; Fig. 4a). Post hoc comparisons between the factors revealed that BDL significantly increased fluid intake (vs. Sham SCR: Bonferroni $t = 4.942$;

$p < 0.05$; vs. Sham shBDNF: Bonferroni $t = 5.271$; $p < 0.05$). In contrast, fluid intake was significantly lower in BDL rats with BDNF knockdown as compared to the BDL rats with SCR injections (Bonferroni $t = 4.027$; $p < 0.05$). A significant interaction between the factors are observed in the volume of urine excreted (time $\times$ treatment $F[39,351] = 24.419$, $p < 0.05$; Fig. 4b). The average daily urine volume significantly increased in BDL SCR compared to the sham rats (Bonferroni t tests, $p < 0.05$). Post hoc comparisons show that the urine excretion was not significantly increased in BDL shBDNF rats compared to the sham rats. The food intake was not significantly different among the 4 groups ($F[3,19] = 0.595$, $p = 0.626$). Concomitantly, the average body weight increase was not significantly different among the 4 groups.

Urine Sodium Excretion

Urine sodium excretion was measured every day for 14 days starting 2 weeks after BDL or sham ligation surgery. One-way ANOVA analysis revealed significant difference between the groups in daily average sodium excretion ($F[3,19] = 119.215$, $p < 0.05$; Fig. 5a). The BDL SCR rats had significantly decreased average sodium excretion compared to both the sham-ligated control groups (Bonferroni t tests, all $p < 0.05$; Fig. 5a). These data were in accordance with the previous studies [28]. Two-way RM ANOVA indicates that average daily changes in sodium excretion were significantly affected by time and the treatment protocol ($F[39,156] = 2.175$, $p < 0.001$; Fig. 5b). The BDL shBDNF rats had significant decrease in daily average sodium excretion compared to the sham rats (Bonferroni t tests, all $p < 0.05$; Fig. 5b). This decrease was similar to that of BDL SCR rats. The average daily sodium excretion in BDL rats with BDNF knockdown in the SON was not significantly different compared to the BDL SCR rats.

Fig. 4. The effects of BDL and BDNF knockdown in the SON on (a) daily average fluid intake and (b) daily average urine excretion. Data are mean $\pm$ SEM. * $p < 0.05$ versus sham groups (Fluid intake: Time: $F[13,156] = 5.734$, Treatment: $F[3,156] = 11.818$; Urine excretion: Time $\times$ Treatment $F[39,351] = 24.419$). + $p < 0.05$ BDL shBDNF versus BDL SCR. Groups: Sham SCR ($n = 6$); Sham shBDNF ($n = 6$); BDL SCR ($n = 6$); BDL shBDNF ($n = 5$). SCR, scrambled; shBDNF, shRNA against brain-derived neurotrophic factor; BDL, bile duct ligation.

Fig. 5. Urine sodium excretion. a Average urine sodium concentration. * $p < 0.05$ versus sham groups ($F[3,19] = 119.215$). b Daily average urine sodium excretion after 2 weeks of BDL. Data are mean $\pm$ SEM. * $p < 0.05$ versus sham groups ($F[39,156] = 2.175$). Groups: Sham SCR ($n = 6$); Sham shBDNF ($n = 6$); BDL SCR ($n = 6$); BDL shBDNF ($n = 5$). SCR, scrambled; shBDNF, shRNA against brain-derived neurotrophic factor; BDL, bile duct ligation.

Discussion

It is well-established that the liver failure causes portal hypertension due to obstruction of portal blood flow because of massive structural changes associated with fibrosis and intrahepatic vasoconstriction [38]. The increase in hepatic vascular resistance to blood flow during liver failure is coupled with hypovolemia and vasodilation. These systemic responses to portal hypertension negatively in-

fluence normal circulatory physiology [3, 5, 6, 39, 40]. As part of a complex neurohumoral response, AVP secretion is increased as a compensatory mechanism to maintain blood volume. However, AVP secretion is not suppressed as plasma osmolality decreased, leading to hyponatremia in liver failure patients. This dilutional hyponatremia is common in end-stage liver disease, and it leads to life-threatening complications such as cerebral and pulmonary edema [9, 41].

Hyponatremia is characterized by relative excess of body water relative to body sodium content. The management of hyponatremia in liver failure is challenging as conventional treatments, including fluid restriction and loop diuretics to enhance the renal solute-free water excretion, are frequently inefficacious. Diuretic therapy often leads to a worsening of the plasma sodium status and has been shown to have very little effect on improving free water clearance [5, 39]. The majority of patients find it difficult to adhere to fluid restriction, and the discontinuation of diuretics may further worsen ascites [9]. However, the rapid correction of serum sodium may lead to serious neurological complications such as central pontine myelinolysis, or seizures [9, 41]. The logical step in the treatment of hyponatremia represents AVP antagonists, vaptans that selectively antagonize the effects of AVP on the V2 receptors in the kidney tubules. However, most currently available vaptans increase risk for hepatic failure and mortality [1, 5, 41].

It is known that the decrease in blood volume during liver failure acts as a nonosmotic stimulus for initial increase in AVP, but the mechanism leading to the sustained increase in AVP secretion during liver failure is not known. In this study, we show a mechanism that contributes to the increased AVP secretion in an experimental model of liver failure. This study used chronic BDL male rats as an animal model for liver failure. This model is characterized by hyponatremia due to increased AVP secretion induced by liver failure. This model is widely used as it is reproducible and the survival rate associated with this model is higher compared to other liver failure animal models [42, 43]. The cauterization of bile duct in BDL rats causes obstruction of the bile duct resulting in cholestasis. Cholestasis is a condition where bile cannot flow from the liver to the duodenum and it leads to jaundice. The accumulation of bile in the liver causes necrosis of hepatocytes leading to liver failure. The yellowish pigmentation of skin, plasma and increase in liver to body weight ratio can be used like biomarkers to verify the disease model in this study [26, 28, 31, 44].

We measured CPP in addition to plasma AVP in our experiments. CPP is secreted at equal molar concentration (1:1) to AVP but has longer half-life compared to AVP. Previous studies from our lab and several others have shown the correlation of plasma CPP with AVP measurements at various plasma osmolalities [36, 37, 45]. Plasma AVP and CPP observed in this study were in accordance with the literature. Although the circulating AVP and CPP were significantly decreased in BDL rats

with BDNF knockdown compared to the BDL rats with SCR injections, these values were still higher compared to the sham control rats. While we specifically targeted the SON in this study, paraventricular nucleus, which contain AVP neurons could contribute to the increase in AVP and hyponatremia in this model. This indicates BDNF-TrkB mechanism in the SON contributes partially to the elevated AVP secretion and includes the possible contribution of other signaling and physiological mechanisms.

The fluid intake and urine excretion in BDL rats are very high compared to the sham rats despite their lower plasma osmolalities, which suggests the role of nonosmotic mechanisms in fluid intake of BDL rats. The fluid intake and urine excretion were decreased with BDNF knockdown in BDL rats. Previous studies from our laboratory reported the association between BDL, increased drinking behavior, increased AT1R expression in the SFO, and elevated peripheral renin angiotensin system [27, 28, 44]. The renin angiotensin system is activated in response to systemic hypotension in BDL rats. The current study shows the similar decrease in drinking behavior and urine excretion in BDL rats. The BDNF knockdown in the BDL rats might have influenced the decrease in mean arterial pressure thereby preventing angiotensin-mediated thirst response, although this was not evaluated in the current study. Also, osmoreceptors in the SON are known to regulate plasma osmolality [13, 40, 46–48]. It is possible the BDNF plays a role in the transduction pathways of the local osmoreceptors.

The body fluid and electrolyte homeostasis are maintained by the integration of physiological and behavioral systems [5, 46, 49]. In the context of this disease model, decreased AVP secretion and fluid intake could contribute to the moderate increase in plasma osmolality associated with BDNF knockdown in BDL rats. But the role of BDNF from the SON in thirst is not well understood and will be the focus of future studies.

The inhibitory effect of GABA depends on a low $[Cl^-]_i$ concentration ($[Cl^-]_i$) [50]. The low $[Cl^-]_i$ is determined by the relative activity of KCC2 and $Na^+/K^+/2Cl$ cotransporter 1 (NKCC1) [50]. Increases in $[Cl^-]_i$ can reverse the direction of chloride movement across the cell membrane and prevent GABA_A-mediated inhibition [51–53]. The balance between neural excitation and inhibition is crucial to maintain the concentration of circulating AVP within normal limits [52, 53]. KCC2 is the major cotransporter for the efflux of chloride ions, as KCC2 is down-regulated, Cl concentration within the neuron is increased which reverses the flux of ions through the GA-

BA_A receptor. This impairs GABA-mediated inhibition of SON neurons [20, 23, 54]. The loss of synaptic inhibition in SON neurons could result in a feed-forward loop that drives inappropriate secretion of AVP [20, 22, 55]. Previous salt loading studies have suggested that in addition to KCC2, NKCC1 contributes to the increase in [Cl⁻]_i, modulating the valence of GABA_A in the SON AVP neurons [20, 37, 50]. The role of NKCC1 in increase in AVP secretion during liver failure is yet to be understood.

This is the first study to demonstrate the increased activation of TrkB receptor (pTrkB^{Y515}) and decreased phosphorylation of KCC2 cotransporter (pKCC2^{S940}) in BDL rats compared to sham-ligated rats. The knockdown of BDNF, the primary ligand of TrkB receptor in the SON of BDL rats, prevented both the activation of TrkB receptor and downregulation of KCC2 cotransporter. The BDNF knockdown decreased the hnRNA expression of AVP along with a decrease in circulating AVP and CPP in BDL rats.

The AAV-U6 – shBDNF used in this study to knockdown BDNF specifically in the SON is based on previous studies, which show a stable knockdown of BDNF and high transduction efficiency with this viral construct [20, 22]. Studies from our lab previously demonstrated increased FosB staining and AVP hnRNA expression in the SON neurons of BDL rats [27, 28]. In addition, the increase in AVP secretion in SL rats was prevented by inhibiting the increase in BDNF in the SON [22]. Based on these previous results, the bilateral SON was chosen in the current study to inject shBDNF.

While changes in the posttranslational modification of KCC2 in the SON could imply that there are changes in [Cl⁻]_i [52, 56], the effect of BDL and BDNF knockdown on the chloride homeostasis and GABA_A-mediated inhibition is not directly tested in this study. The synaptic mechanisms contributing to the increase in BDNF in SON are yet to be determined.

Conclusions

Our results are the first to demonstrate that BDNF from SON contributes to the increase in AVP secretion observed in an animal model of liver failure that results in hyponatremia. The changes in the activity of AVP neurons caused by BDNF may represent an adaptation to excess activity that contributes to inappropriate AVP release in a variety of experimental models. While the KCC2 data suggest that this mechanism is related to

changes in GABA, several recent experiments suggest that excitatory mechanisms are also important [57, 58]. In addition, the knockdown of BDNF in the SON reduced the thirst and urine excretion associated with liver failure.

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Disclosure Statement

The authors have nothing to disclose.

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Author Contributions

J.T.C. and K.B. designed experiments; K.B., J.T.L. and M.B. performed experiments; K.B., M.B., and J.T.C. analyzed data; K.B. and J.T.C. wrote the manuscript.

Data Availability Statement

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

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