ORIGINAL ARTICLE

Effects of bile duct ligation on the inhibitory control of supraoptic vasopressin neurons

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Abstract

Dilutional hyponatremia due to increased plasma arginine vasopressin (AVP) is associated with liver cirrhosis. However, plasma AVP remains elevated despite progressive hypoosmolality. This study investigated changes to inhibitory control of supraoptic nucleus (SON) AVP neurons during liver cirrhosis. Experiments were conducted with adult male Sprague-Dawley rats. Bile duct ligation was used as a model of chronic liver cirrhosis. An adeno-associated virus containing a construct with an AVP promoter and either green fluorescent protein (GFP) or a ratiometric chloride indicator, ClopHensorN, was bilaterally injected into the SON of rats. After 2 weeks, rats received either BDL or sham surgery, and liver cirrhosis was allowed to develop for 4 weeks. In vitro, loose patch recordings of action potentials were obtained from GFP-labeled and unlabeled SON neurons in response to a brief focal application of the GABA_A agonist muscimol (100 μ M). Changes to intracellular chloride ([CI]i) following muscimol application were determined by changes to the fluorescence ratio of ClopHensorN. The contribution of cation chloride cotransporters NKCC1 and KCC2 to changes in intracellular chloride was investigated using their respective antagonists, bumetanide (BU, $10 \,\mu\text{M}$) and VU0240551 (10 μ M). Plasma osmolality and hematocrit were measured as a marker of dilutional hyponatremia. The results showed reduced or absent GABA_A-mediated inhibition in a greater proportion of AVP neurons from BDL rats as compared to sham rats (100% inhibition in sham vs. 47% in BDL, p = .001). Muscimol application was associated with increased [CI]i in most cells from BDL as compared to cells from sham rats ($\chi^2 = 30.24$, p < .001). NKCC1 contributed to the impaired inhibition observed in BDL rats (p < .001 BDL - BU vs. BDL + BU). The results show that impaired inhibition of SON AVP neurons and increased intracellular chloride contribute to the sustained dilutional hyponatremia in liver cirrhosis.

KEYWORDS chloride, GABA, hyponatremia, vasopressin

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1 | INTRODUCTION

Arginine vasopressin (AVP) is a key component in the regulation of body fluid and electrolyte homeostasis.^{1,2} It is released from magnocellular neurosecretory cells (MNCs) in the supraoptic (SON) and paraventricular nuclei (PVH) of the hypothalamus.^{3–5} Both the SON and PVH also contain oxytocin-secreting MNCs. Classic experiments using in vivo electrophysiological recording showed that these two cell types also have electrophysiological differences.^{6,7} Putative AVP cells display phasic activity, and their spontaneous activity is interrupted by baroreceptor stimulation.^{7,8} Putative oxytocin neurons display continuous activity, and they are not inhibited by baroreceptor stimulation.^{7,8} They are instead selectively activated by peripheral injections of cholecystokinin.⁷

Circulating AVP helps regulate body fluid volume by acting on the kidneys to increase water reabsorption from the renal filtrate in the collecting duct.^{2,9} AVP also increases peripheral resistance by constricting blood vessels thus regulating blood pressure.² The release of AVP is controlled by both inhibitory and excitatory stimuli from peripheral organs and circumventricular organs located in the brain.¹⁰ A decrease in plasma osmolality or an increase in blood volume/blood pressure inhibits MNCs, decreases plasma AVP, and increases diuresis. Conversely, an increase in plasma osmolality or a decrease in blood volume or blood pressure activates MNCs to increase plasma AVP concentration.^{8,11} There is a positive linear relationship between plasma AVP concentration and plasma osmolality.⁹ This relationship is likely maintained by a well-coordinated set of excitatory, inhibitory, and neuromodulatory mechanisms.¹²

However, in some pathophysiological states such as cirrhosis and heart failure, there is a higher plasma AVP concentration which does not correlate with plasma osmolality and cannot be compensated by the body's homeostatic mechanisms.^{9,13,14} Thus, some patients with cirrhosis and heart failure develop decreased plasma osmolality, hyponatremia, fluid overload, and increased plasma AVP concentration.^{9,13,14} Previous studies have shown that similar changes occur in animal models of portal hypertension and cirrhosis.¹⁵⁻¹⁷ A recent publication from our laboratory has suggested that the increased plasma AVP concentration associated with these disorders could be caused in part by increased stimulation of SON AVP neurons by noradrenergic A1 and A2 neurons due to a decreased central blood volume.¹⁸ This is consistent with the traditional view that inappropriate AVP release in cirrhosis is due to a change in non-osmotic regulatory pathways.¹⁹ However, since patients with liver failure are hypoosmotic, a stimulus that inhibits the release of AVP, it suggests a possible failure of inhibitory mechanisms could play a role in the increased plasma AVP and dilutional hyponatremia observed.

At the cellular level, mature neurons have a low intracellular chloride concentration ([Cl]i) as compared to that in the extracellular space.²⁰⁻²² This allows an influx of chloride ions through ligand-gated GABA_A receptor channels causing hyperpolarization and cell inhibition.²⁰⁻²² GABA_A channels are bi-directional and allow the flux of chloride ions based on chloride's transmembrane ionic concentration gradient.²³ Although GABA is mainly considered an inhibitory neurotransmitter, there is some evidence for GABA-mediated excitation in normal adult mammalian neurons.²⁴⁻²⁸ In addition, GABA-mediated excitation has been implicated in certain pathophysiological conditions such as epilepsy^{29,30} and neuropathic pain.³¹ Intracellular chloride homeostasis is maintained by two cation-chloride cotransporters with opposing actions. Potassium chloride cotransporter-2 (KCC2) mediates the outward cotransport of chloride and potassium ions. This transporter is highly expressed in mature neurons helping to maintain a low [Cl]i. Sodium potassium chloride cotransporter-1 (NKCC1) mediates the inward transport of sodium, potassium and chloride into the cell and increased function of NKCC1 can increase [CI]i leading to a reversal of GABA from inhibition to excitation.^{22,32,33} For example, deoxycorticosterone acetate-salt hypertension³⁴ and chronic salt loading³⁵ are associated with a change in the reversal potential in GABA currents in MNCs that is sensitive to bumetanide, an NKCC inhibitor. Changes in KCC2 function have also been reported to contribute to similar changes in GABAA-mediated inhibition in MNCs during salt loading^{24,36} and are associated with hypertension in the median preoptic nucleus.^{37,38} However, changes in GABA₄mediated inhibition of AVP neurons during liver failure have not been fully investigated yet.

In the current study, we investigate the changes to the [CI]i and electrophysiological properties of AVP neurons in response to GABA_A receptor activation four weeks after bile-duct ligation (BDL), a rat model of cirrhosis.^{17,18,39} We used loose patch cell recordings and live-cell chloride imaging to test the hypothesis that there will be decreased GABA_A-mediated inhibition of SON AVP neurons supporting decreased plasma osmolality in BDL male rats. We further tested the contributions of NKCC1 and KCC2 in the observed changes in GABA-mediated inhibition in MNCs from BDL rats.

2 | MATERIALS AND METHODS

2.1 | Animals

These studies used adult male Sprague–Dawley rats (250–300 g, Charles River Laboratories). Rats were individually housed in a temperature-controlled (25°C) room on a 12:12 h light/dark cycle with light onset at 07:00 a.m. Food and drinking water were available ad libitum. Following bile duct ligation surgery, rats were monitored for the development of ascites and daily food and water intake. A nonsteroidal anti-inflammatory drug, carprofen (Rimadyl, 2 mg, Bio-Serv, USA), was given orally before and after surgery for pain and inflammation management. Experiments were performed according to the National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by the University of North Texas Health Science Center Institutional Animal Care and Use Committee.

2.2 | Stereotaxic surgery

Rats were anesthetized with 2%-3% isoflurane (mixed with 95% O₂/5% CO₂) and anesthesia was maintained throughout the surgery

using a commercially available vaporizer (Kent Scientific, USA) connected to a nose holder adapter on the stereotaxic frame (David Kopf Instruments, USA). The scalp of each rat was shaved and disinfected with iodine followed by alcohol. Next, the skull was secured in a stereotaxic apparatus using blunt ear bars. After the skull was exposed and leveled between bregma and lambda,⁴⁰ each rat was bilaterally injected in the SON using the following coordinates from bregma: 1.4 mm posterior, ±1.4 mm lateral, and 9.1 mm ventral as previously described.¹⁷ A hole was drilled into the skull at these coordinates and a 30-guage hypodermic tubing injector was lowered to the SON. The injector was connected to a Hamilton micro-syringe by calibrated polyethylene tubing. Each rat received a bilateral injection of 200–300 nL of the AAV2-0VP1-ClopHensorN virus^{38,39} at a titer of 1.0×10^{13} GC/mL (UNC Vector Core, USA). In another set of experiments, rats were injected with the same volume of an AAV vector that contained only the AVP promoter and the green fluorescent protein (GFP) (prepared by the University of North Carolina [UNC] Vector Core, Chapel Hill, NC, USA see³⁶). The injector was left in place for 5 min and then slowly withdrawn. Gel foam was used to fill the hole in the skull. The incision site was closed with absorbable antibiotic sutures. Animals were allowed to completely recover from anesthesia before being returned to their home cage.

2.3 Bile duct ligation (BDL) surgery

After two weeks of recovery from stereotaxic surgery, the rats were anesthetized with 2%-3% isoflurane mixed with oxygen using a commercially available vaporizer (Kent Scientific). The abdomen was shaved, cleaned, and disinfected with iodine followed by alcohol. A midline incision was performed. The common bile duct was carefully isolated from surrounding structures and cauterized between two ligatures as previously described.^{41,42} Sham rats received the same surgical procedure except their bile duct was not cauterized. Any rat showing morbidity or ascites greater than 10% of the bodyweight was euthanized with inactin (100 mg/kg ip, Sigma-Aldrich, USA) followed by decapitation. Yellow coloration of the plasma and an increase in liver weight to bodyweight ratio were used as determinants for successful BDL surgery.

2.4 Slice preparation

Four weeks after the BDL surgery, rats were anesthetized with 2% isoflurane and decapitated. From each brain, 300 µm coronal slices of the SON were cut using a Microslicer DTK Zero 1 (Ted Pella, USA) in ice-cold $(0-1^{\circ}C)$ artificial cerebrospinal fluid (aCSF) containing in mM: 3.00 KCl, 1.00 MgCl₂·6 H₂O, 2.00 CaCl₂, 2.00 MgSO₄, 1.25 NaH₂PO₄, 26.00 NaHCO₃, 10.00 D-glucose, 206.00 sucrose (300 mosM) and a pH of 7.4, oxygenated with 95% O₂ and 5% CO₂. The slices were incubated at room temperature in aCSF containing in mM: 126.00 NaCl, 3.00 KCl, 2.00 CaCl₂, 2.00 MgSO₄, 1.25 NaH₂PO₄, 26.00 NaHCO₃ and 10.00 D-glucose (300 mosM) with a pH of 7.4 for at least an hour before recording

2.5 Electrophysiology

The slices were transferred into a recording chamber on an upright microscope equipped for epifluorescence and DIC imaging (BX50WI; Olympus, USA) where they were superfused with aCSF at $31 \pm 1^{\circ}$ C. Using epifluorescence, AVP cells were identified by expression of either GFP or ClopHensorN. The electrodes (1-3 M Ω) were made from borosilicate glass capillaries (World Precision Instruments, USA) using a horizontal pipette puller (Model P-2000, Sutter Instrument Co, USA). Each recording electrode was filled with aCSF and another micropipette was used for focal drug application. Cells were identified as AVP SON neurons based on the presence of GFP (Figure 1) or ClopHensorN (Figure 2). In some slices, there was an absence of labeling from the viruses presumably due to the injections missing the SON. For these experiments, cells were considered putative AVP neurons if they showed phasic activity. In these preparations with no AAV labeling, SON cells with either phasic or continuous firing patterns were analyzed separately. Baseline action potential firing was recorded from the cells for 5 mins using loose-patch voltage clamp. Next, 30-35 nL of muscimol (100 µM in aCSF; Sigma-Aldrich), was focally applied from a glass micropipette $(2-4 \mu m)$ upstream of the recording electrode for 10s using a Pico spritzer (WPI, USA) set to 10 psi. This was followed by a 5-min post-muscimol application recording.

In some cases, focal application of muscimol (100 µM) occurred in the presence of bath applied KCC2 antagonist (VU0240551 10 µM, Tocris) or an NKCC1 antagonist (bumetanide 10 µM, Tocris). The drugs used were prepared as previously described in aCSF.³⁶ A baseline was followed by a 10 s focal application of muscimol and postmuscimol recordings. Then either bumetanide or VU0240551 added to aCSF was bath applied for 5 min. The recordings were then repeated in the presence of the antagonists. Action potential firing was grouped into 10 second bins for analysis.

Chloride imaging 2.6

In a subset of experiments, live-cell chloride imaging was performed with ClopHensorN using an upright epifluorescence microscope (BX50WI, Olympus) equipped with differential interference contrast optics. ClopHensorN is a ratiometric chloride indicator that expresses modified versions of enhanced green fluorescent protein (eGFP) and a red fluorophore, TdTomato. These constructs were combined with an AVP-specific promoter in an AAV2 vector to drive the expression of the chloride indicator in AVP-expressing SON neurons. The slice preparations were alternately excited with 445 nm light (for eGFP, "green channel") and 556 nm light (for TdTomato, "red channel") and the emission recorded between 500 and 550 nm for eGFP and between 580 and 653 nm for TdTomato. Image sets were captured every 3 s and the ratio of eGFP fluorescence, 445 nm to that of TdTomato, 556 nm (F₄₄₅/F₅₅₆ ratio) were measured at baseline and in response to 10s of focal muscimol application using the Olympus cellSens Imaging Software (Olympus Corporation, Japan).

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FIGURE 2 Digital images showing (A) supraoptic nucleus (SON) neurons using infrared differential interference contrast (IR-DIC) microscopy. (B) SON neurons expressing the chloride sensitive enhanced green fluorescent protein (eGFP) of ClopHensorN. (C) Expression of chloride-insensitive tdTomato protein of ClopHensorN in the same group of neurons. (D) Merged FITC image of the neurons showing excitation with 445 and 556 nm light during live cell chloride imaging.

2.7 | Plasma measurements

After decapitation, the trunk blood was collected from each rat into a 2 mL centrifuge tube and Vacutainer EDTA tube (containing 12 mg of EDTA). Two heparinized capillary tubes (Fisher Scientific, USA) were filled with blood from the 2 mL centrifuge tube, and the tubes were

centrifuged using a microcapillary centrifuge (Adams Micro-Hematocrit II Centrifuge, Clay Adams, USA) for measuring hematocrit with a Micro-Hematocrit capillary tube reader (Lancer, USA). The remaining blood was centrifuged at 1600 g for 5 min and the plasma was collected for measuring osmolality using a vapor pressure osmometer (Wescor, USA) and plasma protein measurement using a clinical refractometer.

2.8 | Statistical analysis

Statistical analyses were performed using GraphPad Prism 9.5.0 (GraphPad Software LLC, USA). Data are reported as mean ± SEM. Response of cells to muscimol was grouped into inhibited, no response or impaired inhibition (excited) for both sham and BDL rats. A change in firing rate or F_{445}/F_{556} ratio one standard deviation below that region of interest's individual mean was reported as inhibition or a decrease in fluorescence intensity while one standard deviation or more above the individual mean was recorded as excitation or an increase in fluorescence intensity, respectively. A change within the mean ± one standard deviation for that cell was considered as no response to muscimol. In the electrophysiology recordings, average basal firing rate was calculated from the period before muscimol application. The cells from BDL rats that were inhibited were treated as a separate group from cells that were demonstrated impaired inhibition to muscimol application. Fisher's exact test was used to analyze the proportion of cells in each group. The area under the curve postmuscimol application was analyzed using one-way ANOVA with Tukey's multiple comparisons tests. Changes in firing rate and interburst duration were analyzed using paired, two-tailed Student's t-tests and all other data were analyzed with unpaired, two-tailed Student's t-tests. A p-value less than .05 was considered statistically significant. Data were tested for normality and heterogeneity of variance in GraphPad.

3 | RESULTS

3.1 | Effects of BDL on liver weight, plasma osmolality, hematocrit, and plasma protein

BDL rats had a significant increase in liver weight to bodyweight ratio as compared to the sham rats (Table 1, t = 3.909; df = 29; p = .0005). Plasma from BDL rats had significantly lower osmolality and hematocrit values as compared to sham ligated rats (Table 1 Plasma osmolality: t = 5.191, df = 28, p < .0001; Hematocrit: t = 2.381, df = 29, p = .024). The liver is the site for the synthesis of various plasma proteins including albumin which maintains serum oncotic pressure. This prevents excessive extravasation of fluid from the blood into the extravascular space.⁴³ Since BDL caused liver failure, we determined if BDL affected this function of the liver by measuring the total plasma protein of both BDL and sham rats. Our results showed that there

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was no significant difference in the total plasma protein between the BDL and sham rats (Table 1, t = 1.740, df = 29, p = .093).

3.2 | Effects of BDL on intracellular chloride determined by chloride imaging

Changes in intracellular chloride in response to muscimol were determined by fluorescence ratio $F_{445}/_{554}$ imaging. An increase in the ratio is associated with an efflux of chloride while a decrease indicates an influx of chloride. Muscimol application resulted in a decrease in the $F_{445}/_{554}$ ratio in 38 out of 46 cells from the sham rats (Figure 3A). This is consistent with an increase in [CI]i due to GABAA receptor activation. In the remaining cells from sham controls, the $F_{445}/_{554}$ ratio increased in five cells and remained unchanged in three cells after muscimol application. A total of 21 out of 36 cells from the BDL rats showed an increase in the $F_{445}/_{554}$ ratio (Figure 3B) while the ratio decreased in eight cells and remained unchanged in seven cells. A comparison of the area under the curve (AUC) 1-min post muscimol application showed a significant increase in the $F_{445}\!/_{554}$ ratio in BDL rats versus sham rats (t = 8.087, df = 82, p < .001; Figure 3C). This observation is indicative of more cells responding to muscimol with a chloride efflux after muscimol application in slices prepared from BDL rats. Furthermore, a chi-squared analysis showed that there was a significant difference in the distribution of cell responses to muscimol application in BDL rats compared to shams ($\chi^2 = 30.24$, df = 2, p < .001).

3.3 | Effects of BDL on GABA_A-mediated inhibition determined by electrophysiology

Changes in chloride homeostasis in AVP cells affect the firing of action potentials. An influx of chloride ions after activation of $GABA_A$ receptor channels hyperpolarizes the cell and reduces action potential probability. Loose patch-clamp recordings were performed on SON cells to determine the effect of muscimol application on the cell firing rate. SON cells that showed green fluorescence or had a phasic firing pattern were considered AVP-secreting cells although some GFP-labeled SON cells showed a continuous firing pattern.^{3,4}

All labeled SON AVP neurons from sham rats were inhibited immediately following muscimol application (60s) with the average basal firing rate decreasing from 2.55 ± 0.3 Hz to 1.08 ± 0.3 Hz (Figure 4A). The firing rate returned to baseline at the end of the

TABLE 1 Bile duct ligation surgery caused a significant (A) decrease in plasma osmolality (****p < .001), (B) decrease in hematocrit (*p = .024), and (C) a significant increase in liver weight to bodyweight ratio (***p = .005) as compared to sham operated control.

	Osmolality, mOsm/kg H ₂ O	Hematocrit, %	LB ratio	Plasma protein g/dL
BDL	290.2 ± 1.14 ^{****}	40.9 ± 1.14*	0.054 ± 0.004***	7.92 ± 0.1
Sham	299.1 ± 1.25	44.1 ± 0.56	0.035 ± 0.001	8.22 ± 0.1

Note: No statistical difference in plasma protein was observed (p = .0926). Data are shown as mean ± SEM. Data were analyzed with unpaired Student's *t*-tests. Number of rats: sham, n = 14; BDL, n = 17.



5-min post-muscimol period. In slices from BDL rats, the average basal firing rate of eight out of 15 putative SON AVP neurons increased from 2.85 ± 0.5 Hz to 4.85 ± 0.9 Hz immediately after muscimol application while that of the remaining seven neurons decreased from 4.37 ± 0.9 to 1.30 ± 0.6 Hz (Figure 4B, C). Analysis of the AUC post-muscimol application by one-way ANOVA revealed a significant difference among the treatment groups (F(2, 30) = 5.7, p = .008; sham versus BDL excited p < .0095; BDL excited versus BDL inhibited, p < .0247; sham versus BDL inhibited, p = .978; Figure 5A, B).

For the unlabeled continuously active SON neurons, all 29 cells from sham rats were inhibited immediately after muscimol application with the average basal firing rate dropping from 3.54 ± 0.3 to 1.72 ± 0.5 Hz. However, in BDL rats, 28 out of 40 neurons showed impaired GABA_A mediated inhibition, with the average basal firing rate changing from 5.27 ± 0.6 to 5.22 ± 0.9 Hz immediately after muscimol application. The remaining 12 neurons were inhibited with a decrease in average basal firing rate from 4.50 ± 1.0 to 0.49 ± 0.2 Hz. Analysis of the AUC post-muscimol application by one-way ANOVA showed a significant difference across the treatment groups in the responses of the cells to muscimol application (F [2, 72] = 5.46, *p* = .006; sham versus BDL excited *p* < .013; BDL excited versus BDL inhibited, *p* < .040; sham versus BDL inhibited, *p* = .955; Figure 6A, B).

The changes in GABA_A-mediated inhibition in unlabeled phasic cells from sham and BDL rats were analyzed based on changes in cell firing rate and the interburst duration to account for their spontaneous interruptions in activity. A decrease in cell firing rate and/or an increase in the interburst duration after muscimol application was consistent with cell inhibition. In slices from sham rats, out of 13 phasic neurons, nine showed a decrease in firing rate while the frequency remained unchanged or increased in four cells. However, in BDL rats. 17/24 of phasic neurons showed an increase in firing rate following muscimol application. Fisher's exact test analysis revealed a significant difference in the response of cells from sham rats and BDL rats to the application of muscimol (p = .036). Additionally, the basal firing rate decreased from 3.14 ± 0.3 to 2.39 ± 0.3 Hz in cells from sham rats (paired t-test, t = 2.24, df = 11, p = .047; Figures 7A and 8A) while in BDL rats, there was an increase from 3.86 ± 0.4 to 5.15 ± 0.6 Hz (paired t-test, t = 2.61, df = 20, p = .017; Figure 7B and 8B).

FIGURE 3 Representative graph of chloride imaging of a neuron from (A) sham rats showing a decrease and (B) bile-duct ligation (BDL) rats showing an increase in the fluorescence ratio, $F_{445/556}$ after focal muscimol (100 µM) application. A total of 21 out of 36 cells from BDL rats showed an increase in the fluorescence ratio and 38 out of 46 cells from the sham rats showed a decrease in the ratio after muscimol application. (C) Responses of cells from sham rats and BDL rats after muscimol application expressed as area under the curve (AUC). *p*-value is from two-tailed student's *t* test (*t* = 8.087, df = 82, *p* < .0001). Number of cells = 46 from four sham rats, and 36 from five BDL rats.

FIGURE 4 Representative loose patch-clamp recordings of green fluorescent protein (GFP) labeled cells at baseline, during, and following focal muscimol (100 µM) application. (A) Cells from sham rats were completely inhibited by muscimol with a significant drop in the firing rate post muscimol similar to (B) the response of some cells from BDL rats. (C) Some cells from BDL rats had sustained cell activity during muscimol application with an increase in firing rate in the post-muscimol period.

(A)

%

Percent Baseline,

600

400

0



FIGURE 5 Effect of bile-duct ligation (BDL) and sham surgery on GABA_A-mediated inhibition of supraoptic nucleus (SON) AVP neurons. (A) All putative SON AVP neurons from sham rats were inhibited after muscimol (100 µM) application. More than half of all putative SON AVP neurons showed an increase in cell firing rate following muscimol application. (B) Analysis of the area under the curve (AUC) after muscimol application showed a significant inhibition after GABAA activation in all cells from sham rats and a mixed effect in BDL rats with some cells showing inhibition and others excitation. Number of neurons is shown in parentheses. Data were analyzed by one-way ANOVA which showed a significant main effect of treatment (F (2, 72 = 5.46, p = .006). p-values shown on the graph are from Tukey's post hoc tests.

In cells from sham rats, there was a significant increase in interburst duration after muscimol application, indicative of GABAA-mediated inhibition (paired t-test, t = 3.68, df = 11, p = .004; Figures 7A and 8C). The changes in the interburst duration of cells from BDL rats were mixed and not statistically significant (paired t-test, t = 0.63, df = 20, p = .537; Figures 7B and 8D). However, a comparison between the proportions of cells showing an increased or decreased interburst duration revealed that approximately 43% of cells from BDL rats had a decrease in interburst duration as compared to 2.3% of sham rats (p = .001, Fisher's exact test). This is consistent with a decrease in the inhibitory effects of muscimol in cells from BDL rats.

Role of chloride cotransporters in impaired 3.4 GABAA-mediated inhibition in BDL rats

The two cation chloride cotransporters KCC2 and NKCC1 are imperative in neuronal chloride homeostasis. We studied the contribution of these cotransporters in the impaired GABAA-mediated inhibition observed in some SON neurons from BDL rats by using their respective antagonists. In cells from sham rats, application of muscimol after a 5-min wash-in of bumetanide, an NKCC1 antagonist dissolved in aCSF resulted in a stronger and sustained inhibition for the entire duration of the 5-min post-muscimol period as compared to aCSF



FIGURE 6 Effect of bile-duct ligation (BDL) and sham surgery on $GABA_A$ -mediated inhibition. (A) Application of muscimol in cells from BDL rats had two effects. A total of 70% of cells studied had a blunted inhibition to muscimol application which was followed by an increase in cell activity. The remaining cells were inhibited by muscimol similar to the response of cells from sham rats. (B) Area under the curve (AUC) analysis following muscimol application in cells from sham and BDL rats. The number of cells is shown in parentheses. Data were analyzed by one-way ANOVA which showed a main effect of treatment (F [2, 72] = 5.46, p = 0.006). p-values from the Tukey's post hoc tests are shown on the graph.



FIGURE 7 Representative recordings of phasic supraoptic nucleus (SON) neurons from sham and bile-duct ligation (BDL) rats. The cells from the sham rat (A) showed an increase in interburst duration while the cells from the BDL rat (B) demonstrated a decreased interburst duration and increased firing rate.

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alone. The AUC after muscimol application showed a significant difference between the response of the cells from sham operated controls to muscimol in the presence and absence of bumetanide (main effect of treatment F(3, 32) = 37.33, p < .001; p < .001, Figures 9 and 10). Cells returned to preantagonist baseline firing rate after bumetanide washout. A similar treatment was applied to cells

FIGURE 8 Changes in cell firing rate and interburst duration in phasic cells from sham and bile-duct ligation (BDL) rats after muscimol application. (A) A significant decrease in firing rate after muscimol application was observed in cells from sham rats. (B) The frequency of action potential activity increased significantly in the phasic cells from BDL rats. (C) Cells from sham rats had an increase in interburst duration (D) The change in interburst duration after muscimol application in cells from BDL rats was mixed with no statistically significant difference between them. Data were analyzed by paired *t*-tests. *p*-values are shown on graphs. Sham, n = 12 cells, BDL. n = 21 cells.



from BDL rats with impaired GABA_A-mediated inhibition. Blocking the NKCC1 cotransporter prior to muscimol application led to the restoration of GABA_A-mediated inhibition and a decrease in cell firing rate (p < .0001, Figures 9 and 10).

VU0240551, a KCC2 antagonist was used to test the contribution of the KCC2 cotransporter to GABA_A-mediated inhibition. Following a 5-min VU0240551 wash-in, muscimol was applied to SON neurons from sham rats. There was a significant decrease in the basal firing rate in the presence of VU0240551 + aCSF as compared to aCSF (main effect of treatment (F 3, 26) = 26.05, p < .001; p < .001, Figures 9 and 11). Applying similar treatment to cells from BDL rats that showed impaired GABA_A-mediated inhibition, there were no significant differences between the response of the cells to muscimol in the presence of VU0240551 + aCSF and aCSF alone (p = .972, Figures 9 and 11).

4 | DISCUSSION

Liver cirrhosis is associated with increased plasma AVP concentrations that drive increased reabsorption of water from the collecting duct of the renal tubules resulting in dilutional hyponatremia.¹⁴⁻¹⁶ Dilutional hyponatremia is common in decompensated liver failure and leads to several complications including hepatic encephalopathy, increased susceptibility to infections, pulmonary edema and increased mortality.14,19,44,45 One of the stimuli that inhibit the release of AVP is a decrease in plasma osmolality.¹⁰ However, the increased plasma AVP levels observed in patients and rats with liver cirrhosis is maintained in the face of progressive hypoosmolality/hyponatremia.^{13–15,44} It is known that a reduction in central blood volume due to portal hypertension and splanchnic vasodilation serves as a nonosmotic stimulus for increased AVP secretion in liver cirrhosis.^{19,46} Our previous study showed that this stimulus is likely relayed to the AVP neurons via A1/A2 noradrenergic neurons because lesioning these neurons prevented the inappropriate release of AVP observed in liver cirrhosis.¹⁸ While the activation of noradrenergic neurons that project to the SON may partially explain the pathogenesis of inappropriate AVP release in this model, it does not account for why AVP neurons are not inhibited when osmolality decreases.

The inability of AVP neurons to be inhibited by hypoosmolality, such as happens during liver cirrhosis, points to a possible change in the inhibitory control of AVP release. This could contribute to the sustained elevation of plasma AVP during a worsening hypoosmolar state. We previously reported that during liver cirrhosis, there is an increased expression of BDNF in the SON that leads to increased levels of phosphorylated TrKB (pTrKB) and downregulation of



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phospho-KCC2 (pKCC2).³⁹ This was associated with the increased plasma AVP and dilutional hyponatremia observed in liver cirrhosis. Furthermore, BDNF knockdown in BDL rats prevented the downregulation of pKCC2 and decreased plasma AVP concentration, suggesting a possible involvement of chloride cotransporters in the increased plasma AVP and dilutional hyponatremia observed in BDL rats.³⁹ The cation chloride cotransporters, KCC2 and NKCC1 are vital to chloride homeostasis and normal inhibitory control in neurons. During the development of the central nervous system, there are changes in the expression of these chloride cotransporters that influence how neurons respond to GABA. In the postnatal brain, GABA is excitatory due to a high [Cl]_i resulting from a higher proportion of NKCC1 to KCC2 transporters. However, during maturation, an increase in KCC2 expression leads to a lower [CI]_i converting GABA from excitation to inhibition.^{22,47} A reduction in GABA_A-mediated inhibition due to changes in chloride cotransporter function has been reported in many disease states including epilepsy,^{29,30} neuropathic pain,³¹ saltdependent hypertension³⁴ and chronic osmotic stimulation associated with salt loading.^{24,35,36}

In the current study, we performed functional studies to investigate possible mechanisms that contribute to impaired GABA_A-mediated inhibition of SON neurons resulting in dilutional hyponatremia and the possible role of cation-chloride cotransporters NKCC1 and KCC2 during liver failure in male rats. Bile duct ligation was used to induce chronic liver failure in rats. In this model, the common bile duct, which drains bile from the liver to the small intestine, is cauterized between two ligatures.⁴² This obstructs the flow of bile causing necrosis and fibrosis of the liver leading to liver cirrhosis. The increase in liver weight to bodyweight ratio and yellow coloration of the skin and plasma were used as biomarkers of successful BDL surgery. Control rats are subjected to the same surgical procedure, but their bile duct is not cauterized. Plasma osmolality, plasma protein and hematocrit were measured for each rat as a way to monitor the development of plasma volume expansion and dilutional hyponatremia.

Our results show that liver cirrhosis was successfully induced in the BDL rats due to their relatively higher liver weight to bodyweight ratio as compared to the sham rats. This was consistent with our previous studies.^{17,18,39,41} Also, plasma osmolality and hematocrit values in the BDL rats were significantly lower than those from sham rats. This also supports the development of dilutional hyponatremia in the BDL rats. Although plasma AVP was not directly measured in this study, the presence of dilutional hyponatremia in the BDL rats is consistent with elevated plasma concentrations of AVP. This is further supported by the results of the cell electrophysiological recordings where most SON AVP neurons from BDL rats had a higher firing rate as compared to those from sham rats.

To investigate the response of SON neurons to GABA_A-mediated inhibition, we used muscimol, a GABAA receptor agonist. This was focally applied to SON neurons with basal firing activity to determine their response to GABA. It was discovered that SON AVP neurons from BDL rats were more likely to show an increase in excitability or no inhibition to muscimol application than cells from sham rats. While most putative AVP neurons from BDL rats were not inhibited by muscimol, all cells from sham rats were initially inhibited by muscimol. In addition, a greater proportion of phasic neurons from BDL rats showed an increase in firing rate and a decrease in interburst duration after muscimol application as compared to cells from sham rats. This was consistent with impaired GABAA-mediated inhibition. Similar results have been observed in studies that interrupted the baroreceptor pathway to the SON.⁴⁸

An [Cl]_i that is lower than the extracellular chloride concentration ([Cl]_e) allows an influx of chloride ions during GABA_A receptor activation leading to hyperpolarization and cell inhibition. However, GABA_A receptor channels are bidirectional, and the flux of chloride ions depends on the chloride's transmembrane concentration gradient.²³ Therefore, an increase in [Cl], will reduce the gradient causing impaired GABA_A-mediated inhibition or increased excitability of the neuron.³² We tested possible changes in [CI]_i that could support the impairment of GABA-mediated inhibition using a ratiometric chloride indicator protein called ClopHensorN.49 The ClopHensorN used in this study had an AVP-specific promoter that allowed the expression of the protein only in AVP neurons as confirmed in our previous study.³⁶ This approach ensured the experimental protocol did not alter [CI]; as could happen with whole cell patch clamp recordings. Live-chloride imaging using ClopHensorN revealed that, in the majority of cells from BDL rats, there was an efflux of chloride ions upon GABA_A receptor activation which is consistent with an increased [CI]_i. However, in cells from sham rats, a low [CI]; allowed an influx of chloride ions causing hyperpolarization and inhibition of cell firing activity. This finding supports possible changes to chloride homeostasis during cirrhosis which may contribute to the impaired inhibition observed.

In these studies, AVP SON neurons in male rats were targeted by injecting the rats with AAV vectors containing either GFP or ClopHensorN and a promotor specific for AVP neurons. We used this approach based on our earlier study that showed circulating AVP was significantly increased in male BDL rats but circulating oxytocin was not.⁵⁰ In female BDL rats, we observed the opposite effects: significantly increased plasma oxytocin but not AVP.⁵⁰ In preparations with no labeling, we separated the cells based on their spontaneous activity patterns analyzing continuously active cells separately from phasically active cells. Some of these unlabeled, continuously active cells demonstrated impaired inhibition following GABAA receptor activation

FIGURE 9 (A) Representative cell recordings of cells from sham and bile-duct ligation (BDL) rats to bumetanide. Response of cells from BDL and sham rats to muscimol application in the presence of the NKCC1 antagonist bumetanide (10 µM). Bumetanide restored GABA₄-mediated inhibition in cells from BDL rats. (B) Representative responses of supraoptic nucleus (SON) neurons from sham and BDL rats to muscimol (100 µM) application in the presence of the KCC2 antagonist VU0240551 (10 µM). VU0240551 increased the inhibition of cells from sham rats but had little effect on cells from BDL rats.



FIGURE 10 Effects of bumetanide (10 µM) on the responses of supraoptic nucleus (SON) neurons from sham-operated and bile-duct ligation (BDL) rats to muscimol expressed as a percent of baseline (A) and as the area under the curve (B). Bumetanide restored GABA_A-mediated inhibition in cells from BDL rats and increased the duration of inhibition in sham rats. There was a main effect of treatment (F(3, 32) = 37.33. p < .001). p-values from the Tukey's post hoc tests are shown.



FIGURE 11 Effect of VU0240551 (10 µM) had little effect on the response of supraoptic nucleus (SON) neurons from sham-operated and bile-duct ligation (BDL) rats to muscimol (100 μM) as a percentage of baseline (A) and expressed as an area under the curve (B). An increase in the duration of inhibition was observed in sham rats. There was a main effect of treatment (F[3, 26] = 26.05, p < .0010). p-values from the Tukey's post hoc tests are shown.

(Figure 6). It is possible that some of the unlabeled cells that demonstrated impaired inhibition to muscimol were oxytocin cells. However, we currently lack the ability to identify oxytocin neurons in vitro but such approaches are available.⁵¹⁻⁵³ Additional experiments using such approaches will be required to determine how BDL effects oxytocin neurons in male and female rats.

It is known that during cirrhosis, an increase in BDNF in the SON upregulated pTrKB and downregulated pKCC2. KCC2 is a K⁺/Cl⁻ cotransporter involved in maintaining a low [CI],³⁹ A reduction in KCC2 function has been shown in other conditions to be involved in the impairment of GABA-mediated inhibition.²⁴ NKCC1 is a Na⁺/K⁺/Cl⁻ cotransporter involved in chloride transport into the cell. NKCC1 has also been implicated in the dysregulation of chloride homeostasis of SON AVP neurons.^{34,35} To investigate the involvement of the KCC2 transporter in the increase in [Cl]_i, a KCC2 antagonist, VU0240551 was used. After a 5-min

wash-in of VU0240551, muscimol was applied to the SON neurons to test their response to GABAA-mediated inhibition. Paradoxically, KCCs antagonism increased the duration of GABAA-mediated inhibition in SON neurons from sham-ligated control rats. This was not expected since an increase in intracellular chloride is typically associated with decreased hyperpolarization. A possible explanation for this phenomenon is a depolarization shift in the Cl⁻ gradient leading to shunting inhibition or depolarization block upon GABA_A activation.^{54,55} Also, Houston et al. showed that an increase in [Cl]_i prolongs the decay of inhibitory postsynaptic currents which extends the time to counteract coincident neuronal excitation.⁵⁶ This could potentially increase the inhibitory effects of muscimol. However, their intracellular chloride concentrations were higher than could be achieved with KCC antagonism in our experiments.

The antagonism of KCC2 cotransporter in cells from BDL rats had negligible effects on the response of the cell to muscimol application.

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FIGURE 12 Illustrated summary of our results and working hypothesis. Increased activity of NKCC1 and decreased KCC2 function contribute to an increased intracellular chloride concentration that impairs GABA₄-mediated inhibition. These changes lead to increased circulating AVP that contributes to dilutional hyponatremia associated with liver cirrhosis.

This suggests that KCC2 inhibition did not influence GABA_△-mediated inhibition in BDL rats since KCC2 activity is already downregulated. This is consistent with our previous observations indicating that BDL is associated with a change in the phosphorylation status of KCC2 in the SON.⁵⁶ However, blocking the NKCC1 cotransporter with bumetanide restored the GABA_A-mediated inhibition in cells from BDL rats. NKCC1 antagonism significantly changed the response to muscimol in cells from BDL rats by restoring the decrease in activity comparable to what was observed in sham ligated controls. The responses of the BDL rats to muscimol are consistent with a reduced influx of chloride ions which would lower intracellular chloride, restore chloride's transmembrane concentration gradient, and increase GABAA-mediated inhibition. This suggests that the activity of NKCC1 may be increased in AVP neurons from BDL rats. An increase in the activity of NKCC1 could be due to either an increase in membrane expression or a change in its phosphorylation status.^{20,33} The effects of BDL on the expression of NKCC1 in the SON will be addressed in future experiments.

As mentioned above, we previously reported a contribution of A1/A2 neurons and increased BDNF expression in the SON to the increased plasma AVP concentration and dilutional hyponatremia observed in BDL rats. We also showed that BDNF plays a role in downregulating pKCC2 and contributing to an increase in [CI]_i in cells from BDL rats. Other studies have found that BDNF expression can be increased via vagus nerve stimulation and electrical stimulation.^{57–59} Likely, the increased stimulation of the SON neurons by the A1/A2 neurons could be partly responsible for the increase in BDNF and impairment of GABAA-mediated inhibition observed in BDL rats. This possibility will be explored in future experiments.

PERSPECTIVES 5

In the present study, we showed that impairment of GABAA-mediated inhibition supports dilutional hyponatremia during liver cirrhosis. The nal of Neuroendocrinology __WILEY $^{13 ext{ of } 15}$

reduced inhibition of AVP neurons associated with BDL appears to be related to a downregulation of KCC2³⁹ and increased activity of NKCC1 that increased [Cl]_i. This mechanism partly explains the impaired inhibition of AVP neurons during a state of hypoosmolality as occurs in liver cirrhosis. Additionally, the increased cell firing rate in response to GABAA receptor activation could contribute to the increased plasma AVP observed in this model from previous studies.^{18,39} Increases in hippocampal and cortical BDNF and norepinephrine are observed in male rats after vagal nerve stimulation.⁵⁷ We propose a similar mechanism via A1/A2 neurons that would increase BDNF and change the activity of cation chloride cotransporters leading to impaired GABA inhibition. These mechanisms could collectively support the increase in plasma AVP and dilutional hyponatremia (Figure 12).

AUTHOR CONTRIBUTIONS

Ato O. Aikins: Conceptualization; data curation; formal analysis; writing - original draft. George E Farmer: Conceptualization; data curation; methodology; supervision; writing - review and editing. Joel T Little: Data curation; formal analysis; methodology; writing - review and editing. J. Thomas Cunningham: Conceptualization; funding acquisition; project administration; supervision; writing - review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors have nothing to disclose.

DATA AVAILABILITY STATEMENT

Data Statement: Data available upon request from the authors.

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