DIFFERENTIAL CHLORIDE COTRANSPORTER EXPRESSION IN NEUROENDOCRINE CELLS FOLLOWING OSMOTIC STRESS

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BY

John Carl Begley

APPROVED:

Dr. Jeffrey G. Tasker
Director of Thesis

Dr. Gary P. Dohanich
Second Reader

Dr. David A. Mullin
Third Reader
ABSTRACT

John Carl Begley. Differential Chloride Cotransporter Expression in Neuroendocrine Cells Following Osmotic Stress
(Dr. Jeffrey G. Tasker, Neuroscience)

The paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus control an organism’s osmotic state. Synaptic gamma-aminobutyric acid (GABA) signaling regulates vasopressin (VP) and oxytocin (OT) magnocellular neuroendocrine cells (MNCs) of the PVN and SON. The MNC response to GABAergic inputs ($E_{GABA}$) is dependent on its internal chloride concentration. This chloride concentration is established by the sodium/potassium/chloride transporters KCC2, NKCC1, and NKCC2. NKCC1 and NKCC2 are responsible for chloride import, and KCC2 for chloride export. Recent findings from our lab suggest that VP MNCs of the PVN have a depolarized GABA reversal potential, and therefore an excitatory GABAergic response at baseline. The finding of an excitatory $E_{GABA}$ was not without controversy; several labs have found that this excitatory response in VP MNCs is only possible after osmotic challenge with salt loading. Since the internal chloride concentration determines the direction of chloride movement through the membrane, my study sought to explore the expression patterns of the three chloride cotransporters using immunohistochemistry in identified MNCs in different osmotic states. Only KCC2 was found to be differentially expressed between the two populations: VP MNCs expressed significantly less KCC2 than OT neurons at baseline. Two opposing phosphorylation sites regulating KCC2 membrane stability were studied via Western blot analysis, serine-940 (S940), phosphorylation of which increases KCC2 membrane stability, and threonine-1007 (T1007), phosphorylation of which increases KCC2 internalization. KCC2 appeared to concentrate in OT MNC membranes following salt loading. Moreover, S940 phosphorylation increased in VP and OT MNCs in the PVN and SON. No significant change was seen in T1007 phosphorylation in different osmotic states. These findings suggest that VP MNCs maintain a depolarizing $E_{GABA}$ at baseline. This is the first study to report a potential decrease in KCC2 T1007 phosphorylation following osmotic challenge.
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INTRODUCTION

*Neuroendocrine Pathways for Osmoregulation*

Magnocellular neuroendocrine cells (MNCs) in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus play critical roles in functions that include fluid balance, energy homeostasis, and reproduction by secreting the neuropeptides oxytocin and vasopressin. These two neuropeptides are very similar in structure, differing by only two amino acids in their respective, nine amino acid structures. Vasopressin, also known as the antidiuretic hormone, is synthesized by magnocellular neuroendocrine cells. This hormone participates in the body’s homeostasis, principally regulating blood osmolality and water retention. Vasopressin also helps to regulate blood pressure through vasoconstriction, although this is a more restricted role of the hormone (Robertson et al., 1986).

Vasopressin is synthesized within the magnocellular neuroendocrine cells of both the PVN and the SON. These magnocellular neuroendocrine cells are part of the hypothalamic-neurohypophysial system (HNS), with the magnocellular neuronal cell bodies being located in the PVN and SON and axons projecting down to the posterior pituitary, or neurohypophysis. These neuroendocrine cells then release vasopressin or oxytocin from their axon terminals in the posterior pituitary directly into the general blood circulation (Figure 1). Once vasopressin has entered into the general circulation, the neuropeptide has the capacity to directly regulate the expression and trafficking of aquaporin receptors within the nephrons of the kidneys. An increase in vasopressin release by magnocellular neuroendocrine cells leads to an increase in the expression and trafficking of aquaporins within the kidneys. These aquaporins are permeable to water
and their expression within the kidneys directly correlates with the amount of water being reabsorbed from the renal tubules.

Figure 1: The location of the Paraventricular Nucleus (PVN) and the Supraoptic Nucleus (SON) are labeled within a cortical slice of brain tissue (shown in bottom left). The pathway for oxytocin (OT) and vasopressin (ADH) release from magnocellular neuronal cell bodies in the hypothalamus is shown on the right. Both neuropeptides begin in the PVN and SON, pass through the Pituitary Stalk (Infundibulum) and the neuropeptides’ release into the body’s general circulation in the Posterior Pituitary. Adapted from Fundamental Neuroscience, Third Edition.

The body is able to control the concentration of vasopressin within the general circulation within a range of a few picograms through an osmotic feedback inhibition mechanism (Fundamental Neuroscience, Third Edition). The brain is able to sense blood osmolarity via a circumventricular organ, the vascular organ of the lamina terminalis (OVLT). The neurons in this organ have osmoreceptors that are sensitive to changes in
sodium concentrations, and therefore blood osmolarity (Denton et al. 1996). The OVLT innervates multiple brain regions using both GABAergic and glutamatergic neurons; however, the function of these various pathways has yet to be elucidated fully. Currently, it is hypothesized that the OVLT activates the PVN and the SON promoting the release of vasopressin via glutamatergic synapses (Richard & Borque, 1996). This coincides with the release of oxytocin from the PVN and SON into the general circulation. Oxytocin can then bind to receptors in the OVLT leading to the inhibition of vasopressin MNCs in the PVN and SON. (Grafe et al. 2014).

Although the PVN and the SON share a common function in regards to water retention and osmotic response, morphologically, the two nuclei are vastly different. The SON is homogenously composed of MNCs that secrete either oxytocin or vasopressin. The PVN is much more heterogeneous. In addition to the vasopressin- and oxytocin-secreting MNCs that are of interest in the present study, there are also parvocellular neuronal populations that project to the base of the hypothalamus and to the brainstem and spinal cord (Fundamental Neuroscience, Third Edition). There are also vasopressin-secreting neurons in the suprachiasmatic nucleus (SCN) of the hypothalamus. These neurons act as a signaling molecule to relay signals within the hypothalamus about circadian rhythms and to signal the PVN and SON for the autonomic response to an osmotic stressor (Trudel & Bourque, 2012). The SCN is the primary pacemaker for mammalian circadian rhythms (Weaver, 1998). It is a secondary nucleus for osmoregulation.

Synaptic gamma-aminobutyric acid (GABA) is the primary inhibitory neurotransmitter of the central nervous system. GABA signaling is particularly crucial for
the regulation of magnocellular neuroendocrine cells of the PVN and the SON (Tasker and Dudek, 1993; Armstrong and Stern, 1997). Of the total synapses present within these two nuclei, approximately 60% have been reported to be GABAergic in nature (Decavel and Van den Pol, 1990; El Majdoubi et al., 1997). The response of a neuroendocrine cell to the release of the neurotransmitter, GABA, from a pre-synaptic interneuron is mediated mostly by post-synaptic GABA_A ionotropic receptors in the central nervous system (Cesetti et al., 2012). These ionotropic, GABA-gated ion channels are selectively permeable to chloride ($E_{Cl}$).

A Magnocellular Neuron’s response to GABA is dependent on the electrochemical driving force of this trans-membrane chloride gradient. Thus, the $E_{Cl}$ and the reversal potential for GABA_A receptor-mediated synaptic currents ($E_{GABA}$) are equivalent. The reversal potential for a given ion exists at the voltage at which the current of an ion that passively enters and exits the cell is dynamically equivalent. This value is generated by taking into account the ungated, leak channels for each of the ions, sodium (Na$^+$), potassium (K$^+$), and Chloride (Cl$^-$). For GABA_A receptors, chloride is the only ion that determines its reversal potential. (Fundamental Neuroscience, Third Edition).

On top of the existing leak channels within the membrane, there are also several counteracting cation-chloride co-transporters located in the neuronal plasma membrane that help establish the Cl$^-$ equilibrium within a given cell. These co-transporters are the potassium-chloride cotransporter 2 (KCC2) and the sodium-potassium-chloride cotransporters 1 and 2 (NKCC1 and NKCC2) which function to move chloride in opposite directions across the cell membrane. In order to maintain the endogenous chloride equilibrium potential, KCC2 is responsible for the transport of chloride out of
the cell, reducing the intracellular chloride concentration and shifting $E_{\text{GABA}}$ negative.

Conversely, NKCC1 and NKCC2 are responsible for the transport of chloride into the cell, increasing the intracellular chloride concentration and shifting the $E_{\text{GABA}}$ positive (Chamma et al., 2012). The $E_{\text{GABA}}$ of a magnocellular neuron is dependent upon the chloride gradient across the plasma membrane; a gradient maintained by the cell’s ratio of KCC2:NKCC1/2 (Chen et al, 1996). The NKCC1/2 chloride cotransporters are responsible for the internalization of chloride ions through cotransport. Until recently, NKCC2 was only thought to be expressed in the nephrons of renal cells, but recent findings suggest that NKCC2 is also found in the MNCs of the PVN and the SON and is suspected to be involved in osmoregulation. It has been proposed that NKCC2 is stimulated similarly to renal NKCC2 by vasopressin that originates via endocrine, autocrine, or paracrine signaling; all have been suggested (Konopacka et al. 2015). Previous research into NKCC2 expression had focused primarily on renal cells. In the kidneys, NKCC2, can promote water retention rather than expulsion from the excretory system by changing ion concentrations in the nephron (Giménez & Forbush, 2003).
Membrane Trafficking of Chloride Cotransporters

The expression of Cl⁻ co-transporters has been reported to be relatively fluid. During development, there is a shift in the expression of chloride cotransporters within the nervous system that induces a shift in the $E_{\text{GABA}}$. Early in development, there is a greater expression of NKCC1 and NKCC2 relative to KCC2. This increased NKCC1 and NKCC2 expression facilitates more chloride entering the cell than the chloride leaving through KCC2. When an interneuron releases GABA onto a postsynaptic vasopressin-secreting MNC, activating a GABA_A receptor, chloride exits the cell, thereby generating an excitatory postsynaptic potential. Conversely in adulthood, there is a greater

Figure 2: The function of the different chloride cotransporters is shown. Potassium-chloride cotransporter 2 (KCC2) is responsible for transporting chloride ions (Cl⁻) coupled with potassium ions (K⁺) out of the cell. Conversely, sodium-potassium-chloride cotransporters 1 and 2 (NKCC1/2) facilitate the import of chloride ions with potassium ions and sodium ion (Na⁺), increasing the intracellular concentration of chloride. Adapted from a figure constructed by Marco Fisher, adapted from Chamma, 2012
expression of KCC2 relative to NKCC1 and/or NKCC2, causing a shift in $E_{\text{GABA}}$ to the canonical, inhibitory $E_{\text{GABA}}$ (Chen et al, 1996).

The expression of the various chloride cotransporters within the membrane is controlled through a phenomena referred to as membrane trafficking. Membrane trafficking is the process through which membrane-associated proteins are transported in vesicles into endosomes and recycled back to the membrane through a combination of endocytosis and exocytosis (Fundamental Neuroscience, Third Edition). Endocytosis or internalization is the process by which a cell can down-regulate the expression of proteins imbedded in the plasma membrane by removing them and isolating them to vesicles. Exocytosis is the mechanism by which a cell can up-regulate the trafficking of a given protein to the plasma membrane.

$\text{GABA}_A$ receptors in adulthood usually generate inhibitory chloride potentials when activated by GABA (Chen et al, 2012). This is thought to be the result of a greater relative expression of KCC2 than NKCC1 and NKCC2, leading to a lower intracellular concentration of chloride compared to extracellular concentration. This leads to an influx of $\text{Cl}^-$ ion upon the opening of $\text{GABA}_A$ receptor $\text{Cl}^-$ channels, causing a hyperpolarization. However, during development, it has been found that the relative expression of these chloride cotransporters is flipped: there is a greater relative expression of NKCC1 and/or NKCC2 in comparison to KCC2. This leads to a high intracellular concentration of chloride and causes an efflux of chloride and depolarization of the membrane potential in response to $\text{GABA}_A$ receptor activation. This excitatory effect of GABA is thought to be necessary during early development as GABAergic synapses develop prior to excitatory glutamatergic synapses (Ben-Ari, 2002). It is
postulated that this change in chloride transporter expression during development is in part due to the upregulation of KCC2 through the transcription factor early growth response 4, which mediates the initiation of KCC2 transcription by brain-derived neurotrophic factor (BDNF) (Uvarov et al, 2006). However, the interaction between the expression patterns of BDNF and KCC2 is a complex one, illustrated by the finding that BDNF-knockout mice were found to exhibit a similar developmental shift in KCC2 expression (Puskarjov et al, 2015).

More evidence for the fluidity of expression of these chloride cotransporters can be found on the sub-cellular level. Differences in relative KCC2, NKCC1, and NKCC2 expression have been reported within single cells. This differential spatial regulation allows MNCs to have both an excitatory and inhibitory $E_{\text{GABA}}$ in different subcellular compartments (Wright, Raimondo, and Akerman 2011). Furthermore, changes in

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**Figure 3:** Graphical representation of the shift in the GABA$_A$ reversal potential across development. Early development is the upper left portion and the reversal potential is $\sim$-40mV. Conversely, adulthood shows that the response to GABA is between -70 and -80 mV. This figure was adapted from Chen, 1996.
chloride cotransporter expression throughout the central nervous system have also been shown to have a role in several pathological states. The dysregulation of KCC2, in particular, has been found to be associated with a robust variety of pathological states, including spasticity following spinal injury (Boulenguez et al. 2010), neuropathic pain (Coull et al. 2003), diabetes (Kim et al. 2018), epilepsy (Huberfeld et al. 2007), and dehydration through chronic salt loading (Kim et al. 2013).

**Plasticity of Chloride Cotransporter Expression**

An organism’s ability to survive coincides with its ability to rapidly adapt to its environment, including changes in osmolarity of both the blood and the extracellular milieu. Both the PVN and SON are highly labile and respond quickly to hyper- and hypo-osmotic conditions (Fundamental Neuroscience, Third Edition). Changes in the ionic concentrations of the extracellular milieu have the potential to cause significant changes in the structural morphology of the PVN and the SON. It has been observed that both nuclei respond to salt loading through the retraction of their astrocytic end feet. This response removes the insulation of synapses within the nuclei and removes any potential influence of gliotransmitters on the response of MNCs to osmotic stressors (Panatier et al, 2006).

Each chloride cotransporter has multiple amino acid residues that may be modified in a post-translational manner to alter the chloride cotransporters’ activity and expression patterns. The most readily studied of these post-translational modifications is the phosphorylation of serine/threonine phosphorylation sites. The expression of KCC2 in particular is largely influenced by an organism’s osmotic state. It was found that salt loading rats led to the post-translational modification of KCC2, which influenced this chloride cotransporter’s localization within the neuronal membrane (Chou, Trudel,
Phosphorylation of KCC2 at serine 940 is mediated by protein kinase C and has been previously shown to increase KCC2’s cell surface expression and activity in vitro (Lee et al., 2007). Here, I analyzed changes in KCC2 phosphorylation at serine 940 by salt loading using Western blot analysis.

There are also other locations for posttranslational modifications on KCC2, with several threonine residues that are conserved between KCC2 and NKCC1. These residues are phosphorylated through the WNK-SPAK/OSR1 phosphorylation cascade pathway (Kahle et al., 2005). The two chloride cotransporters respond as an activation-inactivation pair. Phosphorylation of either of the conserved threonine residues in KCC2, threonine 906 or 1007 (T906 and T1007, respectively), inhibits this cotransporter’s activity. In response to salt loading, the phosphorylation of KCC2 is increased at T906 and T1007. This increase in phosphorylation is coupled with an increase in internalization of KCC2 from the neuronal membrane (Balapattabi et al. 2019). The opposite effect is found in NKCC1, where phosphorylation of either of these residues amplifies the cotransporter’s activity (Alessi et al., 2014; Rinehart et al., 2009).

**GABA as an Excitatory Neurotransmitter**

The present study sought to build upon previous research done in our lab on the E_GABA in PVN neuroendocrine cells. Interestingly, these experiments showed that GABAergic synaptic inputs to vasopressin-secreting MNCs in the adult hypothalamus were uniformly excitatory under baseline conditions (i.e., in normally hydrated rats), whereas the GABAergic synaptic inputs to oxytocin-expressing MNCs exerted the canonical inhibitory synaptic effect (Haam et al. 2012). Native chloride concentration was maintained in these recordings by using a perforated patch-clamp method of
recording. Perforations in the membrane are created in this patch-clamp method via the use of the bacteria gramicidin. Gramicidin creates perforations in biological membranes that are permeable to only sodium ions, and not to chloride ions. This allows for an experimenter to record from a cell without altering the cell’s native intracellular chloride concentration. Using the perforated patch-clamp method in lieu of alternate methods, such as whole-cell patch-clamp recordings, showed that GABA can behave in an excitatory manner under baseline conditions in adult vasopressinergic MNCs. In the same study, whole cell patch-clamp recordings were also performed. Whole cell recordings require the cell membrane to be fully ruptured, thereby compromising a cell’s endogenous chloride concentration and equilibrium potential. In the recordings taken with whole-cell patch-clamps, the reversal potentials for chloride in both oxytocin and vasopressin cells were found to be negative, rendering the GABA-mediated synaptic currents hyperpolarizing, whereas recordings using the perforated patch-clamp method shifted the chloride reversal potential, and therefore $E_{GABA}$, positive, making the GABAergic synaptic inputs to vasopressin neurons, but not oxytocin neurons, depolarizing (Haam et al, 2012).

GABA being observed to behave in an excitatory manner in adult MNCs was in direct opposition to the canonical role of GABA in the adult hypothalamus. Previous research into $E_{GABA}$ had found that the GABA reversal potential was only excitatory in the aforementioned developmentally transitory or pathological states. These pathological states, such as with salt loading or dehydration, often cause prolonged stimulation of vasopressin neurons that is thought to lead to a depolarizing shift in the $E_{GABA}$ (Kim et al. 2013; Choe et al. 2015). Although these findings are contradictory to our own, neither is
necessarily invalid. Vasopressin concentration within the blood is tightly regulated, measuring at a total volume of less than 10 pg in circulation at a given time (Fundamental Neuroscience, Third Edition). With this in mind, our present study seeks to characterize the relationship between blood osmolality and GABAergic signaling in vasopressin-secreting neurons under different osmotic conditions, particularly through the regulation of Cl⁻ co-transporter expression.

Based on the previous data in the lab showing GABA to be excitatory in vasopressin neurons, I sought to quantify the relative expression of Cl⁻ co-transporters in vasopressin- and oxytocin-expressing MNCs. By using transgenic rats that have endogenous GFP fluorescence attached to the neurophysin II promoter, all cells that produce vasopressin fluoresce green. With this endogenous fluorescence, and a set of antibodies specific for neurophysin I, the carrier protein for the hormone oxytocin, both types of MNCs can be compared for their relative chloride cotransporter expression.

After establishing expression patterns for the three chloride cotransporters, the project then explored if any changes in expression existed in the MNCs following salt loading. KCC2 was chosen for this initial investigation. Counterarguments to GABA’s capacity to be an excitatory neurotransmitter have focused on salt loading or stressors as the reason GABA becomes excitatory in the PVN and SON. Therefore, it seems logical to also investigate if our animals show a similar response to salt loading protocols, or, if their response is entirely unique.
MATERIALS AND METHODS

Animals

A transgenic line of male Sprague Dawley rats (Charles River, Raleigh, NC), vasopressin-enhanced green fluorescent protein (VP-eGFP) transgenic rats on a Wistar background were used in the experiments in the present study. The rats were housed in house colonies originating from breeders provided by Dr. Yoichi Ueta of the University of Occupational and Environmental Health in Japan (Ueta et al., 2005; Haam et al, 2012). All rats were group housed with *ad libitum* access to food and water, with those undergoing the salt loading treatment as the lone exception. The salt-loaded group was only allowed to access saline drinking water (2% NaCl, w/v) for 5-7 days; the euhydrated control group consisted of similarly-aged male rats receiving pure tap water. Animal weight was monitored daily, with all animals being sacrificed on the 7th day of treatment, unless they lost 20% of their initial body mass, in which case they were sacrificed earlier, at 5 or 6 days. All animals were six weeks or older when sacrificed. Trunk blood was collected after decapitation and at the beginning of the perfusion process to measure plasma osmolarity. Animals in the salt-loaded group that did not show an increase in plasma osmolarity above the threshold of 310mOsm/kg were assigned to a third, new group that were all dehydration-resistant. These animals were also compared to the control and salt-loaded groups. All experimental protocols were in accordance with the US Public Health Service guidelines and were approved by the Institutional Animal Care and Use Committee of Tulane University (IACUC).
Brain slice preparation

Rats were anesthetized and sacrificed following the protocols of previous publications by Dr. Tasker’s lab: animals were subjected to isoflurane inhalation (Sigma; St. Louis, MO) and decapitated in a rodent guillotine (Tasker et al. 2019). The brains were then extracted and immersed for 1-2 minutes in chilled (0-1°C), artificial cerebral spinal fluid (aCSF) composed of (in mM): 140 NaCl, 3 KCl, 1.5 MgSO\(_4\), 1.4 NaH\(_2\)PO\(_4\), 2.4 CaCl\(_2\), 11 glucose, 5 HEPES, saturated with 100% oxygen. The osmolarity was adjusted to 290-300 mOsm and the pH was adjusted to 7.2-7.4. Measured values for pH outside of this range were adjusted using either 1M NaOH or HCl while measured values for osmolarity were adjusted by adding ddH\(_2\)O if the osmolarity is too high, and sorbitol if it is too low. The hypothalamus was blocked on ice and hypothalamic slices of 300 µm thickness were sectioned in cooled (0-2°C), oxygenated aCSF with a vibrating slicer (Vibratome Series 1000; Technical Products, Intl., St. Louis, MO). Tissue slices containing either the PVN or the SON were identified and isolated (Figure 4).

![Figure 4](image_url)

**Figure 4:** Location of the PVN and SON. *A.* Nissl-stained image of a coronal section of rat brain at the level of the hypothalamus showing the approximate locations of the SON (dashed circles). *B.* Nissel-stained image of a coronal section of rat brain at the level of the hypothalamus. The hypothalamic area has been enlarged so that the PVN can be more easily visualized. Figure adapted from Di et al. 2019
**Perfused tissue preparation**

Animals were deeply anesthetized with a mixture of ketamine and xylezine before they were transcardially perfused with phosphate buffered saline (PBS) for approximately 20-30 minutes, followed by 4% paraformaldehyde in PBS for ~15-20 minutes at a flow rate of ~10 mL/minute, at which point the brains were dissected. After dissection, brains were allowed to sit in 4% paraformaldehyde overnight, after which they were transferred to a 30% sucrose solution in 4% paraformaldehyde in PBS for cryoprotection. Cryoprotection was confirmed when the brains no longer floated in the 30% sucrose solution and sank to the bottom of their containers.

Following cryoprotection, the perfused brains were sectioned into 50 µm coronal sections using a cryostat at -20ºC. The brains were first frozen in Optimal Cutting Temperature (O.C.T.) solution, which freezes at -20ºC. The 50 µm coronal slices were taken from anterior to posterior to isolate sections containing the PVN and/or the SON. The slices containing the two, bilateral nuclei of interest were identified using the third ventricle, optic tracts, and the fornix as references (Figure 4). Isolated tissue sections were then transferred to 2 wells of PBS and stored at 4ºC. Sections that would not be stained for an extended period of time (>4 weeks) were transferred from PBS to cryoprotectant, covered with parafilm, and stored at -20ºC until needed.

**Western Blotting**

For Western blot analyses, rats were deeply anesthetized with isoflurane, 300 µm-thick brain slices were prepared as described above, and 1 mm-diameter tissue punches of the SON and/or the PVN were made bilaterally with a Miltex Biopsy Punch using the
optic chiasm/tracts, the fornix, and the third ventricle as landmarks (Figure 4). The tissue samples were added directly to lysis buffer (20 mM Tris, pH 8.0; 137 nM NaCl; 10% glycerol; 1% Nonidet P-40; protease inhibitor cocktail) and homogenized. Lysates were clarified by centrifugation and protein was determined via a modified Lowry assay (DC Protein Assay, Bio-Rad). Samples of 20 µg total protein/lysate were loaded onto kD TGX Stain-Free gels (Bio-Rad, Hercules, CA) and electrophoresed at 140 volts for 60 minutes, under reducing conditions. Proteins were then transferred from the stain-free gels to polyvinylidene fluoride membranes for immunoblotting.

Membranes containing the electrophoresed proteins were then blocked with 5% nonfat dry milk in TBS-Tween 20 at pH 7.4 for 1 hour at room temperature. The membranes were then incubated with an anti-KCC2 polyclonal antibody (1:1,000, Millipore, 07-432), anti-KCC2-Ser940 monoclonal antibody (1:1000, Santa Cruz Biotechnology, sc-293222), anti-NKCC2 monoclonal antibody (1:1000, Santa Cruz Biotechnology, sc-293222), or anti-NKCC1 monoclonal antibody (1:1000, Abcam, ab59791) with 5% normalized bovine serum albumin (BSA) overnight at 4ºC. Membranes were then cycled through five 10-minute washes in TBS-T solution, incubated in either HRP-conjugated anti-rabbit IgG or anti-mouse IgG (1:000; Cell Signaling Technologies, Danvers, MA; Upstate Cell Signaling, Lake Placid, NY), and cycled through five 10-minute washes in TBS-Tween 20 solution. To detect antibody conjugation to target proteins, membranes were incubated in chemiluminescence detecting substrate (Clarity Western ECL Substrate, Bio-Rad) and imaged on a ChemiDoc Imaging System (Bio-Rad). Image Lab software (Bio-Rad) was used to
determine optical densities of individual bands, which were normalized to total protein on the blot.

**Immunohistochemistry**

Animals were transcardially perfused and sectioned coronally as described above. Slices were rinsed six times in PBS for five minutes each, followed by an incubation in 1% NaBH₄ for 30 minutes. Slices then underwent six PBS washes for 5 minutes each. Slices were blocked in 1.5% normal donkey serum in PBS-Tx for 1 hour. Slices were then incubated in “Buffer 1” solution (chemical makeup w/v) with normalized donkey serum (1%) with an anti-KCC2 polyclonal antibody (1:1,000, Millipore, 07-432), anti-KCC2-Ser940 monoclonal antibody (1:1000, Santa Cruz Biotechnology, sc-293222), anti-KCC2-Thr1007 monoclonal antibody (1:1000, Abcam, ab49917), anti-NKCC2 monoclonal antibody (1:1000, Santa Cruz Biotechnology, sc-293222), or anti-NKCC1 monoclonal antibody (1:1000, Abcam, ab59791). These antibodies were used in combination with an anti-oxytocin polyclonal antibody (1:1000, Millipore, AB911) overnight at 4°C. The wells of tissue were then washed extensively in PBS, incubated in goat anti-mouse IgG secondary antibody, Dylight 594 conjugate (1:200, Abcam, ab96881) and donkey anti-rabbit IgG secondary antibody, Alexa Fluor 647 (1:200, Abcam, ab150075), in PBS-TX for 1 hour, and washed extensively in PBS. The tissue was then rinsed with PB to remove salt from the tissue slices and the slices were mounted on gel-coated, non-polarized, glass slides. The slides were allowed to dry and cover slipped using Fluoroshield with DAPI (~60µL, Sigma Laboratories). The slides were then imaged used a Nikon Eclipse Ti confocal microscope at 60x magnification. Imaging was focused on the hypothalamic expression of eGFP and conjugated antibodies within
the hypothalamic PVN and the SON. Staining was confirmed as cellular through a
combination of these fluorophores and the DAPI staining.

**Table 1:** Table showing the itemization of each antibody along with its specificity,
catalogue number, concentration, and antibody type is included here for convenience and
ease of replication.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Antibody Type</th>
<th>Species Specificity</th>
<th>Concentration Used</th>
<th>Catalogue ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-KCC2</td>
<td>1° polyclonal</td>
<td>Donkey anti-rabbit</td>
<td>1:1000</td>
<td>Millipore, 07-432</td>
</tr>
<tr>
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<td>1:1000</td>
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</tr>
<tr>
<td>Anti-KCC2-Thr1007</td>
<td>1° polyclonal</td>
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<td>1:1000</td>
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<td>Anti-NKCC1</td>
<td>1° polyclonal</td>
<td>Donkey anti-rabbit</td>
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<td>Abcam, ab59791</td>
</tr>
<tr>
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<td>1° monoclonal</td>
<td>Donkey anti-rabbit</td>
<td>1:1000</td>
<td>Santa Cruz Biotechnology, sc-293222</td>
</tr>
<tr>
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<td>1:1000</td>
<td>Millipore, AB911</td>
</tr>
<tr>
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<tr>
<td>Alexa Fluor 647</td>
<td>IgG 2° Antibody</td>
<td>Donkey anti-rabbit</td>
<td>1:200</td>
<td>Abcam, ab150075</td>
</tr>
</tbody>
</table>

*Quantification of Immunohistochemistry*

After subjecting perfused tissue to immunohistochemistry, the tissue slices were
mounted onto gel-coated slides, covered with the Dylight 594 “DAPI” fluorescence-
protector and visualized using a Nikon fluorescence microscope. Images were taken of
sections that contained either of the bilateral PVN’s or SON’s using 1024 pixels and
double exposure. The confocal microscope fired its lasers in an order of increasing
frequency of the light wave. The first laser fired in the sequence had the longest wave
length and therefore this laser also had the lowest energy state. Lasers were fired in a
repetitive sequence in this order because this given order minimizes any false positives
that result from the accidental excitation of fluorophores from higher intensity lasers
activating multiple recording channels at once. The relative expression of the various
chloride cotransporters was then quantified and analyzed through NIH ImageJ software.
Using the ImageJ “Freehand selections” tool, individual vasopressin or oxytocin cells
were traced. These traces were then transposed onto the same image’s chloride
cotransporter expression profile. This allowed for the relative expression of chloride
cotransporters to be measured throughout the area of individual cells.

Statistical Analyses

Statistical comparisons of immunohistochemistry data were performed using one-
tailed, unpaired t-tests for between-group analyses of the oxytocin and vasopressin-
expressing cell populations in the PVN and SON. Western blot analysis also utilized one-
tailed, unpaired t-tests for between-group analyses of total protein expression in the PVN
and SON for the various antibodies tested. The significance threshold for all analyses was
set at \( P<0.05 \). Data values are expressed as the mean ± standard error of the mean.

For all experiments conducted, oxytocin-expressing MNCs acted as controls. This
decision was made based on the morphological similarities between both the vasopressin
and oxytocin MNCs and the proximity of the two populations of cells within each of the
two nuclei.
RESULTS

The systematic analysis of the role of each of the chloride cotransporters investigated here can best be explained separately, with the NKCC’s and KCC2 being first examined individually for their opposing functions. Within each of the nuclei of the hypothalamus under study, the vasopressin MNCs had previously shown depolarizing GABAergic signals following salt loading; however, our lab is still one of the few to show depolarizing responses to GABA at baseline (Haam et al. 2012). The basal expression levels of the various chloride cotransporters had yet to be established within vasopressin MNCs in our cohort of animals. Differential chloride cotransporter expression between oxytocin and vasopressin neurons in our animals at baseline would provide molecular evidence for our previous findings.

Tissue samples were collected as described in Figure 4. The first experiment treated tissue samples using immunohistochemistry to tag oxytocin neurons and the different chloride cotransporters using the primary and secondary antibodies listed in Table 1; vasopressin MNCs were visualized through the transgenic AVP-eGFP modification. In this way, the basal levels of each chloride cotransporter was established.

NKCC Expression in the PVN and SON

The NKCC family of chloride cotransporters is primarily the group of cotransporters responsible for increasing \([\text{Cl}^-]\), (Chamma, 2012). Therefore, a higher relative expression of NKCC cotransporter relative to KCC2 in vasopressin magnocellular neuroendocrine cells would explain the depolarizing \(E_{\text{GABA}}\) observed in these cells at baseline. Immunohistochemical data on NKCC2 expression in the PVN and
SON was collected and quantified by Marco Fisher before I joined on this project (Figure 5).

**Figure 5:** Relative Expression of NKCC2 (A-E) and NKCC1 (E-I) in the SON. *A.* GFP expression in vasopressin neurons (green). *B.* oxytocin-immunoreactive neurons (red); *C.* NKCC2 expression (yellow). *D.* Merged image of all three markers together. Nuclear staining was with DAPI (blue). *E.* Graphical representation of the data presented in panels A-D for NKCC2. *F.* GFP expression in vasopressin neurons (green). *G.* oxytocin-immunoreactive neurons (red); *H.* NKCC1 expression (yellow). *I.* Merged image of all three markers together. Nuclear staining was with DAPI (blue). Data were analyzed using ImageJ software for image quantification and an unpaired, one-tailed *t*-test for all MNCs identified. There was no significant difference in expression of either NKCC2 or NKCC1 between oxytocin and vasopressin magnocellular neuroendocrine cells. Similar expression patterns were observed in the PVN.
With no significant difference having been found in NKCC2 expression between oxytocin and vasopressin magnocellular neuroendocrine cells, the project turned to the next immunohistochemical target, NKCC1. NKCC1 is also vital in transporting chloride from the extracellular space into the cytosol. I also found no significant difference between the expression of NKCC1 in the two magnocellular neuron populations of the PVN and SON (Figure 5).

**KCC2 Expression in the PVN and SON**

Having found no significant difference in the expression of either of the two chloride importers, NKCC1 and NKCC2, across the magnocellular neuronal populations of the SON and PVN, we decided to target KCC2, the cotransporter primarily responsible for the export of chloride from the cytosol to the extracellular space (Chamma, 2012).
KCC2 was found to be expressed significantly higher in oxytocin MNCs compared to vasopressin MNCs (Figure 6). Therefore, KCC2 was the only one of the three chloride cotransporters that had a significantly different expression pattern when comparing oxytocin and vasopressin MNCs. This finding guided our next experiments into the effects of salt loading on the PVN and SON.

**Figure 6:** KCC2 expression in oxytocin and vasopressin neurons. High-magnification (60x) confocal images of PVN (A-D) and SON (E-H). A. GFP expression in vasopressin neurons (green). B. oxytocin-immunoreactive neurons (red); C. KCC2 expression (yellow). D. Merged image of all three markers together. Nuclear staining was with DAPI (blue). There is a relative lack of KCC2 staining (yellow) in GFP-expressing vasopressin neurons (green) compared to immunostained oxytocin neurons (red). E. Quantification of the relative fluorescence intensity of KCC2 staining in vasopressin and oxytocin neurons of euhydrated animals. KCC2 expression was significantly lower in vasopressin compared to oxytocin neurons. These data and figure were produced by Marco Fisher.
Variations in expression of KCC2 in Salt-Loaded animals

After establishing the baseline expression patterns of the three chloride cotransporters between the oxytocin and vasopressin magnocellular neuron populations of the PVN and the SON, data could be collected on how these chloride cotransporters might change after a chronic osmotic stress, such as salt-loading. KCC2 was chosen from the three potential chloride cotransporters because of its differential expression between magnocellular neuron populations at baseline (Figure 6). Any changes in total KCC2 expression in the PVN or SON were not measured quantitatively using immunohistochemistry due to individual variations that exist between each trial run. Interestingly, the greatest change in the PVN and the SON in response to salt-loading in KCC2 was found in the oxytocin MNCs, not the vasopressin MNCs. Prior studies had found that salt loading leads to the down-regulation of KCC2. In the present study, salt loading caused KCC2 to limit its expression entirely to the membrane of the oxytocin MNCs. This was different from baseline, when KCC2 was found in the cytosol as well as at the membrane in oxytocin MNCs (Figure 7). Vasopressin MNCs did not exhibit any obvious changes in their KCC2 expression following salt loading. Any up-regulation in KCC2 expression was not immediately obvious in the immunohistochemistry performed.
Following salt loading, there still appeared to be significantly more KCC2 in oxytocin MNCs.

**Figure 7:** Changes KCC2 expression induced by salt loading in PVN (A-D) and SON (E-H). A/E. GFP expression in vasopressin neurons (green). B/F. oxytocin-immunoreactive neurons (red); C/G. KCC2 expression (yellow). D/H. Merged image of all three markers together. Nuclear staining was with DAPI (blue). There is a relative lack of KCC2 staining (yellow) in GFP-expressing vasopressin neurons (green) compared to immunostained oxytocin neurons (red).
**Figure 8**: Effect of salt loading on KCC2 expression in SON oxytocin and vasopressin MNCs. High-magnification view of sample MNCs from the SON of euhydrated (A-C) and salt-loaded animals (D-F). A-C and D-F represent identical regions within the source image, euhydrated and salt loaded, respectively. A/D: Coexpression of KCC2 (yellow) in oxytocin magnocellular neuroendocrine cells (red). B/E. Coexpression of vasopressin (green) and KCC2 (yellow). C/F: Black and white mask of KCC2 signal in SON samples from euhydrated (C) and salt loaded (F) animals.

**KCC2 Phosphorylation Patterns in the SON**

The next phase of the project went about trying to better understand how KCC2 might alter its membrane stability and up-regulate within the oxytocin magnocellular neuroendocrine cells. Using primarily western blot analysis with support from immunohistochemistry data, the phosphorylation state of KCC2 within individual neurons and across both subsets of MNCs in the PVN and the SON could be studied. Literature on the membrane dynamics of KCC2 have shown serine-940 to be an essential site at which post-translational phosphorylation might occur, thereby promoting stability...
of KCC2 within the plasma membrane. However, very little research has been done to date on how serine-940 phosphorylation might change in response to salt loading.

The first step was to confirm the presence of this serine-940 phosphorylation pattern following a salt-loading regimen through western blot analysis. Following seven-day salt-loading and confirmation of a blood plasma osmolarity >310 mOsm/ml, the phosphorylation of KCC2 at serine-940 in tissue punches generated from the PVN and the SON respectively.

![Figure 9: Change in phosphorylation of KCC2 with salt loading. A. Under euhydrated conditions (Control), there is no detectable phosphorylation of KCC2 at serine-940 in the SON. B. Following salt loading (Salt-Loaded), there is a detectable increase in phosphorylated KCC2 at serine-940 (B). C. Quantification of the optical density of KCC2-p940 bands.](image)

With this confirmation that salt-loading results in the increased phosphorylation of KCC2-p940 in both the PVN and the SON, immunohistochemistry could then be used to confirm in which cells specifically KCC2 is phosphorylated. Although oxytocin MNCs express a significantly higher total amount of KCC2 when compared to the adjacent
vasopressin MNCs, both at baseline and when salt-loaded, no significant difference was found in the KCC2 serine 940 phosphorylation (Figure 10) in the SON or PVN. Immunohistochemistry was also performed on euhydrated control animals to test for KCC2-p940; no antibody bound to the tissue in these samples.

![Image](image_url)

**Figure 10:** KCC2-p940 staining in SON MNCs. 

A. GFP expressing vasopressin neurons (green).  
B. Oxytocin-immunoreactive neurons (red).  
C. KCC2-p940 staining in the SON (yellow).  
D. Merged image of all three markers together. Nuclear staining was with DAPI (blue).  
E. Quantification of the relative fluorescence intensity of KCC2-p940 immunostaining in vasopressin and oxytocin neurons of dehydrated animals. KCC2-p940 expression was not different in vasopressin-expressing neurons compared to oxytocin-expressing neurons.

A second phosphorylation site in KCC2, threonine-1007, when phosphorylated, promotes KCC2 internalization from the plasma membrane; the opposite effect of phosphorylation at serine-940. Threonine-1007 phosphorylation has been reported to be down-regulated in response to salt-loading as part of a proposed TrKB-KCC2-NKCC1
pathway to promote an increase in KCC2 membrane localization and intracellular Cl loading in vasopressin MNCs (Balapattabi et al. 2019). Initial western blot analysis has found no significant difference between euhydrated and salt loaded SON samples (Figure 11, n=4 for each group). More research is needed on the SON and PVN to increase the number of animals tested in this experiment.

**DISCUSSION**

*Basal Expression of Chloride Cotransporters in the PVN and SON*

Vasopressin-secreting MNCs in the PVN and SON are vital in relaying information for osmotic homeostasis in mammals (*Fundamental Neuroscience*, Third Edition). It is essential that these MNCs remain stable and viable to perform their...
function of increasing an organism’s water retention through increased vasopressin release in response to osmotic stress. These experiments were designed in an attempt to try and better characterize the relationship between vasopressin MNCs, osmolarity, and GABAergic signaling.

As previously discussed, a given cell’s chloride reversal potential is established through opposing chloride cotransporters (Chen et al., 1996). By studying both oxytocin and vasopressin MNCs, comparisons in chloride transporter expression between the two cell populations could be drawn directly, potentially explaining their differential basal $E_{Cl^-}$.

Through the immunohistochemical evaluation of each of the three chloride cotransporters, KCC2, NKCC1 and NKCC2, in the PVN and the SON magnocellular neuronal populations, KCC2 was found to be the only cotransporter that was expressed at a significantly different level across the two cell populations. Vasopressin MNCs were found to express a significantly lower amount of KCC2 compared to oxytocin MNCs. We found no significant difference between the expression patterns of NKCC1 and NKCC2 in vasopressin and oxytocin MNCs at baseline.

The lower expression of KCC2 compared to NKCC1 and NKCC2 leads to an increased $[Cl^-]_i$. This finding at baseline in these vasopressin MNCs is sufficient to justify a basal depolarizing response to GABA at baseline. Less overall KCC2 expression means less overall export of $[Cl^-]$ from a cell. On the reverse, NKCC1 and NKCC2 now outperform KCC2 in vasopressin magnocellular neuroendocrine cells, leading to an increase in $[Cl^-]_i$, an intracellular-to-extracellular chloride gradient, and a positive-shifted $E_{GABA}$, which results in a depolarizing chloride membrane current. Our data further
corroborate the finding of GABA to be excitatory only in vasopressin MNCs by showing a higher expression of KCC2 in oxytocin MNCs at baseline (Figure 6). The higher KCC2 expression across oxytocin MNCs suggests these neurons maintain a lower [Cl\]-, and the expected hyperpolarized $E_{\text{GABA}}$. Oxytocin MNCs have been shown to have a hyperpolarizing GABA\(_A\) receptor-mediated chloride current at resting potential (Haam et al. 2012).

**Changes in KCC2 Expression in Response to Salt Loading**

The activity of KCC2 relative to NKCC1 and NKCC2 is vital in determining a cell’s $E_{\text{GABA}}$ (Chamma et al. 2012). KCC2 was the first chloride cotransporter to be studied under salt-loaded conditions during this project. KCC2 was chosen for analysis under salt-loaded conditions due its differential expression between the two MNC populations.

Following a standard seven-day salt-loading regimen, our animals surpassed a threshold plasma osmolarity of $>310$ mOsm/mL to qualify as sufficiently hyperosmotic. Surprisingly, there was no down-regulation of KCC2 in response to salt-loading. In fact, KCC2 appeared to remain stable in the membrane of oxytocin MNCs following salt loading. Recent studies have reported that KCC2 is down-regulated in vasopressin MNCs in response to salt loading (Balapattabi et al. 2019). Our immunohistochemical data does point to KCC2 being present in these cells at a significantly lower concentration following salt loading. The lack of marked changes in KCC2 expression in the vasopressin MNCs after salt loading is interesting. These findings led us to analyze two phosphorylation sites in the KCC2 protein, serine-940 and threonine-1007 (S906 and
T1007 respectively), which were chosen for their opposing roles in regulating the stability of KCC2 within the plasma membrane.

Following western blot analysis to confirm the presence of phosphorylation, the exact localization of these phosphorylation patterns could be quantified using immunohistochemistry. Serine-940 is discussed heavily in the literature for its capacity to increase KCC2’s membrane stability when phosphorylated, although very little, if any, research has been done on changes in phosphorylation to this site following salt loading (Lee et al. 2007). Other labs report a down-regulation in KCC2, in response to salt loading, through an increase in phosphorylation to threonine residues 906 and 1007, leading to KCC2 internalization (Chou, Trudel, Bourque, 2015). Our findings using Western blot analysis show clearly that salt loading leads to an increase in serine-940 phosphorylation in KCC2 and support an increase in KCC2 at the membrane of oxytocin magnocellular neuroendocrine cells.

Although our Western blot data suggest KCC2-p940 phosphorylation is increased in the SON and PVN following salt loading, immunohistochemistry revealed that this phosphorylation occurs equally across both vasopressin and oxytocin MNCs. With no significant difference in S940 phosphorylation, it is unlikely that this phosphorylation alone is promoting KCC2 stability within the membrane. Other factors could be responsible for the increase in KCC2 in the membrane of oxytocin MNCs in salt-loaded rats, such as KCC2 inactivation following association with lipid rafts (Hartman et al. 2009). With equal phosphorylation across oxytocin and vasopressin MNCs, it is plausible that a higher relative ratio of total KCC2 expression in vasopressin MNCs is being stabilized and activated by S940 phosphorylation in response to salt loading.
The second phosphorylation site chosen for investigation, threonine-1007, was shown previously to increase in response to salt loading in SON vasopressin MNCs. Western blot data from SON samples of control and salt-loaded animals showed no significant change in T1007 phosphorylation following salt loading in SON samples (Figure 11; n=4); however, these data trended towards significance (P=0.06). Immunohistochemistry will also have to be done to determine if this phosphorylation is robust like S940 expression, or if it is restricted to oxytocin MNCs. The exact cellular location of this phosphorylation will be important for comparison against KCC2-p940.

**Dehydration-Resistant Animals**

Individual differences amongst animals were expected; however, it is peculiar that certain animals would be able to resist changes in blood osmolarity and fail to meet the blood osmolarity threshold of >310 mOsm/ml, even after chronic salt-loading. The animals that failed to meet this criterion were assigned to a “Dehydration-resistance group.” Tissue from salt-loaded rats mimicked that of the euhydrated control that had not received 2% NaCl. Immunohistochemistry and Western blot analysis for KCC2-p940 indicated that there was not an increase in KCC2 S940 phosphorylation in these animals, similar to the euhydrated control groups. This resistance to salt-loading also did not appear to be the result of some increase in KCC2 expression in vasopressin MNCs as KCC2 immunohistochemistry appeared similar in tissue from salt-loaded and euhydrated control rats.

**Future Directions**

Further evidence pointing to vasopressin MNCs maintaining a positive response to GABAergic inputs at baseline was collected by Marco Fisher and Xin Fu using a
combination of gramicidin-perforated patch-clamp electrophysiology and stereotaxic injection in transgenic mice. They transduced a chloride channel, the SWiTChR ("switcher") channel, into vasopressin neurons that is opened and closed in response to different wavelengths of light through a viral vector.

The viral vector used in this experiment coded for a SWiTChr channel that could be opened or closed in response to a specific wavelength of light. These experiments were performed in vitro on AVP-Cre and OT-Cre transgenic mice under basal conditions. The value for the chloride reversal potential in vasopressin MNCs was -55 mV, whereas ECl- in oxytocin MNCs was ~-65 mV, representing a significant positive shift; however, this value did not reach threshold for generation of action potential generation, suggesting that the chloride currents in vasopressin MNCs are not capable of activating the cells. Given the differences measured here from the recordings in AVP-GFP rats originally, there could be differences across species in terms of how depolarizing E_{GABA} is in these vasopressin MNCs. Another possibility is that the average measured E_{Cl^−} value of -55 mV was recorded due of membrane shunting. Shunting would occur when all of the switcher channels are opened in unison, globally throughout the tissue slice, flooding any cell expressing the SWiTChr channel with chloride ions. To confirm this, both in vitro experiments and in vivo experiments in salt-loaded AVP-Cre mice must be done to determine the directionality of native chloride currents.

Other techniques have been proposed to better isolate the changes that occur in the PVN and SON in response to salt-loading. It remains to be seen how the neurons in the PVN and SON of salt-loaded animals respond to electrochemical investigations via patch-clamp. It would be interesting to determine each magnocellular neuronal
population’s respective $E_{\text{GABA}}$ and compare this to the previously recorded values at baseline.

The most promising technique explored for determining KCC2’s stability within magnocellular membranes under varying osmotic states is fractionated western blot analysis – a technique that I attempted, but that did not yield usable data. This method is used to isolate nuclear, cytosolic, and membrane fractions via a series of centrifugations, increasing the force and time of centrifugation with each fraction to be removed. By pooling tissue punches from the PVN and SON of multiple animals, a sample with a large enough protein concentration could be generated for a Western blot analysis. By comparing the relative staining of KCC2 in the cytosol and membrane of the PVN and the SON, any change in membrane expression between euhydrated and chronically salt-loaded animals could be observed. This method is being further trouble shot to be implemented again in future experiments.

Lastly, it has been shown that NKCC2 is up-regulated in response to chronic osmotic challenge (Konopacka et al. 2015). Therefore, it would be interesting to return to this chloride cotransporter to see how it changes in our animals in response to salt-loading.

**CONCLUSIONS**

This project attempts to address two areas of research in the stress response within the PVN and the SON to salt-loading. Our immunohistochemistry corroborates the findings of Haam et al., 2012. These data suggest that GABA is excitatory in vasopressin MNCs, but not in oxytocin MNCs. The significantly lower KCC2 expression in vasopressin MNCs indicates that these neurons have less of a capacity to export chloride
into the extracellular space than oxytocin MNCs. This could therefore lead to the accumulation of chloride within these neurons, causing GABAergic potentials to become depolarizing while oxytocin MNCs maintain a hyperpolarizing response.

The findings of KCC2 membrane stabilization following salt loading disagrees with the findings of other researchers in this field (Kim et al. 2013; Balapattabi et al. 2019). Beyond the initial findings of this report, there is little supporting evidence outside of our lab that corroborates the findings of the basal or salt loaded expressional changes in KCC2 in the PVN and SON. However, this oxytocin magnocellular neuron-specific increase in KCC2 membrane trafficking could provide a route for oxytocin magnocellular neuroendocrine cells to maintain their basal response to GABAergic inputs, even following salt loading.

The suggestion that depolarizing GABAergic inputs to the PVN and SON MNCs could only be generated as a direct result of salt-loading led to further questions as to whether there were variations in our animals’ stress responses to salt-loading (Kim et al. 2013). Very little, if any evidence supports the finding that KCC2 is stabilized in the membrane of oxytocin MNCs following salt loading. What remains an area of confusion in these findings is why they appear to be unique for vasopressin MNCs at baseline and presumably after salt loading. Although KCC2’s plasticity in response to stress has been widely reported, the apparent lack of this chloride cotransporter in vasopressin MNCs points to a possible genetic or environmental factor that has caused our animals to differ from those of other studies on osmoregulation in the PVN and SON. If there is some environmental factor that has caused this apparent change, are the variations in KCC2 expression between our rats and that of other labs comparable to human variance?
Overall, more research into the homeostatic osmotic-stress response, and, in particular, KCC2, is needed before a definitive direct relationship can be drawn between blood osmolarity and KCC2 expression.
REFERENCES


