Differential Regulation of TRPC4 in the vasopressin magnocellular system by water deprivation and hepatic cirrhosis in the rat.

Running Head: TRPC4 regulation by water deprivation and hepatic cirrhosis

Authors: T. Prashant Nedungadi, J. Thomas Cunningham

Department of Integrative Physiology, and Cardiovascular Research Institute
University of North Texas Health Science Centre at Fort Worth,
3500 Camp Bowie Blvd
Fort Worth, TX 76107

Corresponding author:
J. Thomas Cunningham
Department of Integrative Physiology
University of North Texas Health Science Centre at Fort Worth
3500 Camp Bowie Blvd
Fort Worth, TX 76107
Phone: 817-735-5096
Fax: 817-735-5084
E-mail: Tom.Cunningham@unthsc.edu

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Abstract

Transient receptor potential canonical subtype 4 (TRPC4) is expressed in the magnocellular paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus. In this study, the regulation of TRPC4 expression was investigated in water deprivation and hepatic cirrhosis. We utilized laser capture microdissection technique for precise dissection of pure arginine-vasopressin (AVP) cell population in the PVN and SON followed by quantitative real time RT-PCR, and immunodetection techniques by western blot analysis and immunofluorescence. Bile duct ligation elevated TRPC4 transcripts in the SON but not PVN with correlated changes in the protein expression in these regions as well as increased colocalization with AVP in the SON with no changes in the PVN. Water deprivation resulted in increased TRPC4 mRNA expression in the PVN while it decreased channel expression levels in the SON. In both these regions, protein expression measured from tissue punches were unaltered following water deprivation with no changes in the number of TRPC4 positive cells. Thus, TRPC4 expression is differentially regulated in physiological and pathophysiological models of vasopressin release.
Introduction

Transient receptor potential canonical (TRPC) channels play a role in calcium homeostasis through multiple activation mechanisms (53). They are extensively expressed in the brain (34, 42, 52, 55) and are also present in the adrenals, heart, lung, liver, spleen, kidney, testis, uterus and aorta (41, 42, 49, 51). TRPC channels are involved in the cell differentiation (72), embryonic nerve stem cell proliferation (21), BDNF signaling through TrkB activation (34), inducing dendritic spine formation (3), guidance of nerve growth cones (35), slow synaptic potentials mediated by mGluR1 synaptic signaling in cerebellar purkinje cells (32), neurite outgrowth (71) and remodeling (6), epileptiform burst firing and excitotoxicity (50) as well as in nociception (2, 16, 17, 40). Of the seven TRPC channels members identified, TRPC4 is the predominant subtype accounting for 41% of the total TRPC content expressed in the rat brain (22). In-vitro systems have demonstrated involvement of TRPC4/5, belonging to the Group 4 of TRPC channels, in triggering robust secretory response in voltage-clamped neurosecretory cells by causing Ca\(^{2+}\) influx (46). TRPC4 appears to act as a receptor operated channel in these systems since thapsigargin and IP3 did not stimulate TRPC4. Specifically, TRPC4 has been demonstrated to be involved in release of GABA from dendrites through receptor mediated Ca\(^{2+}\) influx pathways (43). In the rat cerebellum, TRPC4 expression is restricted to granule cells and their precursor, and may be essential for normal granule cell development (30). Microarray analysis identified the presence of TRPC4 gene in the supraoptic nucleus (SON) and the paraventricular nucleus of the hypothalamus (PVN) and that TRPC4 is increased in these regions following water deprivation (29). TRPC4 mRNA has been detected in the PVN using in-situ hybridization (22). The function of TRPC4 channels in these regions of the hypothalamus has yet to be determined.
Both the SON and PVN contain hypothalamic magnocellular neurosecretory cells that project to the posterior pituitary where their terminals release the hormones vasopressin (AVP) and oxytocin into systemic circulation. Circulating levels of AVP, which are primarily determined by the activity of these magnocellular cells in the hypothalamus (4, 7, 20, 38), contribute to body fluid homeostasis. Water deprivation results in increased plasma osmolality and hypovolemia which activates neurohypophyseal neurons. Plasma hyperosmolality, a characteristic feature of water deprivation, is critical to the regulation of AVP release. Non-osmotic mechanisms, such as changes in blood volume and blood pressure, also regulate AVP release and influence osmotic mechanisms (13, 25, 59, 61, 69). Dys-regulation of AVP release contributes to dilutional hyponatremia associated with liver and heart failure (60, 68). Chronic bile duct ligation, a model of liver failure, is associated with elevated plasma vasopressin levels and increased activity of the renin-angiotensin system (5, 9, 36). We and others have previously demonstrated that changes in expression of TRPV channels occur in AVP neurons during water deprivation (29, 36, 67) as well as inappropriate vasopressin release associated with bile duct ligation (10, 44).

In the present study, 48h water deprivation and the bile duct ligation model of hypoosmotic hyponatremia were used to stimulate the SON and PVN. Identifying alterations in TRPC4 in these models indicate a possible mechanism that contributes to the regulation of the magnocellular neurons of the hypothalamus.
Materials and Methods

Animal Models

Water Deprivation: Adult male Sprague-Dawley rats (250–350g b.w., Charles River Laboratories, Inc., Wilmington, MA, USA) were individually housed and maintained in a temperature-controlled (23°C) environment under a 12:12h light/dark cycle with light onset at 0700h. All experimental procedures were conducted in accordance with the guidelines of the Public Health Service and were approved by the University of North Texas Health Science Centre Institutional Animal Care and Use Committee. The control group rats were allowed \textit{ad-libitum} access to water and food throughout the experiment. Experimental group rats were water deprived for 48h (26, 31, 33) and not allowed access to water but with \textit{ad-libitum} access to food.

Bile Duct ligation: Each rat was anesthetized with isoflurane (2-3%) and its abdomen was shaved and cleaned. The common bile duct was isolated and cut between the ligatures in anesthetized rats as previously described (10, 44, 70). In sham ligated control group, the bile duct was exposed but not ligated. All rats were used for experiments 4 weeks after surgery. Liver/body weight ratio was used to verify the development of hepatic cirrhosis. Visual examination of development of fibrotic liver and jaundice also confirmed effective bile duct ligation. Rats which did not show any of these developments would be considered as unsuccessful bile duct ligations and would be removed from the study. However for this study all of the rats met the criteria for successful bile duct ligation.

Following the treatment protocols, osmolality, hematocrit, and plasma proteins were measured in rats. The rats were lightly anaesthetized (inactin 100 mg/kg ip), immediately decapitated, and trunk blood was collected into a 1.5 ml microcentrifuge tube that did not contain EDTA. Two heparin-containing hematocrirt tubes (Fisher) were filled from the microcentrifuge
tube for measuring hematocrit (Micro-Hematocrit capillary tube reader, Lancer, St. Louis, MO) and plasma protein by refractometry (National Instruments, Baltimore, MD). The remainder of the blood in the microcentrifuge tube was centrifuged for 5 min (at 10,000×g). After the blood was centrifuged, a 200 μl sample of serum was removed for measuring osmolality using a vapor pressure osmometer (Wescor Inc. Logan, UT).

**Isolation of proteins and western blot analysis from brain tissue punches**

The hypothalamic SON and PVN were micropunched from the forebrain and homogenized as previously published (10, 44) followed by Western blot analysis. Total protein concentration was determined by the Bradford method. Five or ten micrograms of total lysate were resolved by SDS-PAGE, and followed by Western blot analysis. For Western blotting as reported in our earlier studies (10, 11, 44), total lysates were loaded onto 10% acrylamide SDS gel, electrophoresed in Tris-glycine buffer under denaturing conditions and transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA) in Tris-glycine buffer with 10–20% methanol. Membranes were blocked for 1h at room temperature with 5% (wt/vol) non-fat milk in Tris-buffered saline 0.05% (vol/vol) Tween 20 followed by incubation overnight at 4°C with primary antibody raised against Transient receptor potential canonical subtype 4(Anti-TRPC4 produced in rabbit, Sigma, St. Louis, MO). This was followed by incubation in a horseradish peroxidase conjugated secondary antibody against the primary antibody host species (1:5,000; Sigma) for 1h. Enhanced chemiluminescence (ECL reagents; Amersham, Piscataway, NJ) was used for detection of immunoreactive bands and digital images for the bands were acquired from Syngene G-box (Frederick, MD). Image J (National Institute of Health) software was used for densitometry analysis of bands. A total of 24 animals were used for this study (6 Shams and 6 Bile Duct ligation (BDL); 6 Control (CON) and 6 Water Deprived (WD)). Tissue punches from
an additional 3 animals were used for experiments to validate the specificity of the TRPC4 antibody using a TRPC4 blocking peptide (New England Peptide Inc, MA) with the same amino acid sequence (-CKEKHAHEEDSSIDYDL-) used to generate the primary antibody. For these experiments, the TRPC4 antibody was incubated with 100µm of the blocking peptide prior to staining according to the manufacture’s recommendations.

**Co-localization Immunofluorescence of AVP and TRPC4**

Transcardial perfusion with 4% paraformaldehyde and brain isolation were performed as in our earlier studies (26, 33, 44, 45). AVP/TRPC4 immunofluorescence reaction was carried out with a solution containing both guinea pig anti-AVP (T-5048, 1:1000, Peninsula Laboratories, San Carlos, CA) and rabbit anti-TRPC4 (1:100, Sigma, MO). After overnight incubation, the sections were sequentially incubated in Cy3- conjugated Affinipure Donkey Anti-Guinea Pig IgG (Jackson ImmunoResearch, West Grove, PA) secondary antibody for 4 h followed by Cy2-conjugated Affinipure Donkey Anti-Rabbit IgG (Jackson ImmunoResearch, West Grove, PA) for another 4 h. At the end of the immunofluorescence reaction, the sections were mounted onto gelatin coated slides and cover slipped with Vectashield Hard Set (Vector Laboratories, Burlingame, CA). Appropriate negative controls omitting the primary antibody were performed.

In addition, a separate set of sections were processed after pre-incubation of the antibody with the blocking peptide as described above to ensure the specificity of the TRPC4 antibody. The blocking peptide, as in western blot studies, was used at a concentration of 100 µm. A total of 24 animals were used for this study (6 Shams and 6 BDL; 6 CON and 6 WD).

**Imaging**

Immunofluorescent imaging was carried out with an Olympus IX-2 DSU confocal microscope with appropriate excitation/emission filter sets. 20x and 60x magnification images
were captured using a Q-imaging Retiga-SRV camera. Image J (National Institute of Health) was used exclusively to modify brightness and contrast, to pseudocolor and merge and to count cells. Brain areas were identified using Paxinos and Watson (48). As previously published (44, 45), sections containing SON located -0.80 mm to -1.80 mm posterior to bregma were analysed while for PVN, sections located at -1.60 mm to -1.88 mm posterior to bregma were analyzed. Three coronal hypothalamic sections containing the SON that corresponded to Figures 21, 24 and 25 from Paxinos and Watson (1997) located at 0.80, 1.40 and 1.80 mm posterior to bregma were used for analysis from each rat forebrain. Immunopositive cells from these different levels of the SON was averaged for each individual animal and used for statistical analysis. AVP immunofluorescence was used to determine the rostral caudal level of the sections. For the magnocellular PVN, coronal hypothalamic sections were sampled at 2 rostral-caudal levels. Level 1 which is -1.60 mm posterior to the bregma includes the most rostral and corresponds to ventrally located magnocellular cells, while (64) level 2 which is -1.88 mm posterior to the bregma demonstrates a prominent posterior magnocellular region as previously described (65). The number of AVP and TRPC4 positive cells were then counted by individuals blind to the experimental groups and then the numbers were averaged.

**Fast Immunostaining, Laser capture microdissection (LCM) followed by transcript extraction and amplification**

To identify vasopressin positive neurons in the PVN and SON for later laser microdissection, we performed a fast immunostaining of these cells as reported earlier from our lab (12, 15, 44, 45). Briefly, snap frozen brains were cut at 10µm thickness at the level of SON and PVN using a cryostat and mounted on polyethylene naphthalate (PEN) membrane coated slides (Catalogue# LCM0522—Arcturus Bioscience). After 3 min of 100% methanol
fixation, the sections were rinsed 3 times in ice cold DEPC PBS followed by blocking in 3% horse serum containing 0.25% Triton-X for 5 min. Post–blocking, sections were incubated for 3 min in a 1:50 diluted guinea pig anti-AVP antibody (Peninsula Laboratories, San Carlos, CA) and then in Cy3 conjugated Affinipure Donkey anti-guinea pig secondary antibody (Jackson ImmunoResearch, West Grove, PA) for another 3 min.

For LCM, we utilized an Arcturus Veritas Microdissection instrument which is enabled with an IR capture laser and an UV cutting laser. Visible and completely stained neurons were selected for capture. The cutting and capture laser beams was positioned right above the brain tissue which is placed between the polyethylene naphthalate (PEN) membrane slide and a thermoplastic capture cap. The lasers were then used to cut and capture 7-10 immunostained cells onto this cap. After the capture, the cap containing the cells was immediately placed onto a 0.5ml tube containing 30 µl of ArrayPure Nano-Scale Lysis Solution with 5.0 µg of proteinase K (prod. no. MPS04050; Epicentre Biotechnol Inc. Madison, WI, USA). Total RNA was isolated from the 7-10 captured neurons using ArrayPure Nano-Scale RNA Purification Kit reagents (Epicentre Biotechnology) as previously described (12, 15, 45). 2 µl per RNA sample was amplified with TargetAmp 2-Round Aminoallyl-aRNA Amplification Kit materials (Epicentre Biotechnol Inc.), in accordance with the manufacturer’s instructions as published earlier (8). The quality and content of RNA samples were evaluated using a Nanodrop Spectrophotometer (Nanodrop 2000c Spectrophotometer, Thermoscientific). Samples of 260/280 less than 1.8 were not used further for RT reactions. A total of 26 rats (5 Shams and 6 BDL; 7 CON and 8 Water Deprived) were this study.

Reverse Transcription and Quantitative Real Time RT-PCR
Sensiscript RT Kit reagents (prod. no.205213; Qiagen Inc., Valencia, CA, USA) were used for conversion of aminoallyl a- RNA from the previous step to cDNA. For this conversion the following components and reaction volumes were used: 2µl of 10X RT buffer, 2µl of dNTP mix (final concentration: 5 mM), 2µl of oligo-dT primer solution (final concentration: 10 µm), 0.25 µl of RNase inhibitor (final concentration: 10 U/µl), 1µl of Sensiscript reverse transcriptase solution, and 4 µl of aminoallyl a-RNA dissolved in 16 µl RNase-free water to yield a total volume of 20 µl. Real time RT-PCR were performed in a Bio-Rad iQTM5 iCycler system, with the following cyclic parameters: initial denaturation at 95 ºC for 3 min, followed by 40 cycles of 1.1 min each (40 s at 94ºC; followed by 30s at 60 ºC for TRPC4 and 30s at 95 ºC followed by 1min at 65 ºC for GAPDH). The RT-PCR reaction mixture contained 1.8µl of cDNA, 4.5µl of RNase / DNase-free water, 1.2µl of primer mix, and 7.5µl of iQ SYBR Green Supermix (prod. no. 170-8880; Bio-Rad) to make a final reaction volume of 15 µl. GAPDH was chosen and used as the housekeeping gene for the PCR studies. Melt cure analysis confirmed the integrity of the RT-PCR products as confirmed by the presence of single peaks signifying the absence of primer- dimers. Controls and data analysis were performed as previously published (8, 44). The 2^−ΔΔCt method was used for quantifying gene expression. ΔCt was measured by calculating the difference between the GAPDH and the corresponding TRPC4 Ct values. For obtaining the ΔΔCt value, this value was then subtracted from the difference between the average of control GAPDH and control TRPC4 Ct values. The primer sequences are shown in Table 1.

Statistical Analysis
All results are presented as means ± SEM. For statistical analysis of data, we used unpaired t test using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA). \( p = 0.05 \) or \( p < 0.05 \) was considered to be statistically significant.

**Results**

**Effect of BDL on hemodynamic and osmotic responses**

Plasma measurements for osmolality and haematocrit for BDL and sham ligated rats are shown in Table 2. Bile duct ligation resulted in significantly lower plasma hematocrit and plasma osmolality (\( p<0.05 \)). There was a higher liver weight to body weight ratio (\( p<0.001 \)) for BDL rats compared to sham ligated rats measured at the time of sacrifice (28 days post BDL surgery). BDL induced hepatic fibrotic changes as well as jaundice in all the ligated rats evaluated by gross visual examination. These observations are consistent with our previously published reports, implying this as a valid model of reduced plasma osmolality and extracellular volume expansion (10, 15, 44, 70).

**Effect of 48h WD on hemodynamic and osmotic responses:**

As presented in Table 3, water deprivation for 48h significantly increased plasma osmolality as compared to ad-libitum euhydrated controls. (\( p<0.01 \)). Plasma protein (mg/dl) was also significantly increased by 48h water deprivation. As expected, plasma haematocrit levels were also considerably higher after 48h of water deprivation (\( p<0.001 \)). These results are in agreement with hyperosmolality and accompanying hypovolemia associated with dehydration (11, 19, 26).

**Effect of BDL on TRPC4 mRNA and protein expression in the PVN and SON using real time q-RT PCR and western blot**
The relative abundance of TRPC4 transcript was measured in laser captured vasopressin cells from the magnocellular PVN and SON. Figure 1(A&B) shows the fold change in TRPC4 mRNA expression in the magnocellular PVN and SON of BDL rats. Expression of TRPC4 was not affected by BDL in the PVN. Even though there was a 2 fold decrease in the gene expression of TRPC4, this was not significant (Fig.1A). However in the SON, there was a 4 fold significant increase in the transcriptional activation of TRPC4 in bile duct ligated rats compared to the shams (Fig.1B). TRPC4 target gene expression was normalized by coincident measurements with the expression levels of the ubiquitous GAPDH housekeeping gene. We next examined the effect of BDL on TRPC4 protein levels. The abundance of TRPC4 protein expression was significantly increased in BDL rats in the SON (Fig 1E), but not the PVN (Fig 1D). TRPC4 immunoreactive bands were seen at 100kDa for both the regions investigated (Fig 1C &1D). GAPDH was used as the loading and normalization control.

**Effect of 48h WD on mRNA and protein expression of TRPC4 in the PVN and SON using real time q-RT PCR and western blot**

After 48h water deprivation, a significant increase in TRPC4 mRNA was observed in the PVN (Fig. 2A). In the SON, 48h water deprivation decreased the transcriptional TRPC4 levels in comparison to the hydrated rats (Fig.2B). To investigate if the changes in the TRPC4 protein during 48h WD correlate with the levels of its mRNA, we performed western blot analysis for TRPC4 protein. Representative blots are shown in Figure 2D & 2F. Western blot analysis showed an immunoreactive band at 100kDa corresponding to TRPC4 protein. Figure (2C & 2E) shows quantification of western blots for TRPC4 in both the PVN and SON. Interestingly, no differences in protein levels of the canonical receptor subtype 4 were seen in both PVN and SON. Similar to the BDL study, here too we used GAPDH as the endogenous reference control.
Pre-incubation of the primary antibody with the blocking peptide for 1 hour eliminated TRPC4 immunostaining in the SON (Fig. 3C) and PVN (Fig. 3D). Similarly, TRPC4 immunoreactive bands were completely abolished after pre-incubation with blocking peptide in the SON (Fig. 3E).

Co-localization of TRPC4 channels with AVP

To test whether TRPC4 proteins are colocalized with AVP at the cellular level, we performed double immunofluorescence analysis with antisera to AVP and TRPC4. By this study we can verify if in addition to the TRPC4 transcripts seen in AVP positive cells of the magnocellular neurons, TRPC4 protein is also expressed in the magnocellular vasopressin neurons. There was an absence of TRPC4 staining in negative controls (Image not shown). Double immunofluorescence staining of AVP with TRPC4 was performed on both the experimental models of altered vasopressin release investigated. Bile duct ligation and water deprivation had differential effects on TRPC4 and AVP staining in the PVN and SON. Fig.3 and 4 shows representative confocal images of AVP, TRPC4 and AVP/TRPC4 colocalization in the SON and PVN. As previously demonstrated, an increase in AVP immunopositive cells (71 % for SON and 81 % for PVN) was observed after BDL (44). In the PVN, BDL was associated with unaltered numbers of TRPC4 positive cells as well as unchanged colocalization counts (Fig.5A). However, in the SON, BDL increased the number of TRPC4 immunoreactive cells significantly compared to naïve sham control. Here, BDL was also associated with a significant increase in the number of co-localized TRPC4/AVP cells (Fig. 5B).

The number of AVP/TRPC4 positive cells was also unchanged after water deprivation in the PVN (Fig 6A-F); however an increase in the double immunofluorescence for AVP and TRPC4 was observed in the SON (Fig.6G-L). Water deprivation did not elicit any significant
changes in the semi-quantitative immunohistochemical counts of AVP and TRPC4 in the magnocellular PVN and SON (Fig 7A & 7B) respectively.
Discussion

The present study was undertaken to elucidate the regulation of TRPC4 channels in vasopressin neurosecretory cells during a progressive homeostatic challenge and a model of inappropriate vasopressin release. Using laser capture microdissection to isolate AVP positive cells and by immunohistochemical studies we, for the first time, demonstrate the presence of TRPC4 channels in AVP positive neurons of the SON and magnocellular divisions of the PVN. Our observation of significant increases in AVP positive profiles in both the SON and PVN following bile duct ligation is consistent with our previous published reports (10, 44), suggesting that increased activity of AVP neurons in the magnocellular system leads to elevated systemic AVP during hypoosmotic hyponatremia associated with hepatic cirrhosis. Rats subjected to BDL had significantly higher TRPC4 mRNA expression in the laser capture microdissected vasopressin positive neurons and elevated protein expression from tissue punches compared with sham ligations in the SON. Even though the magnocellular PVN is a key hypothalamic center involved in the control of osmoregulation, BDL did not alter the TRPC4 levels in the PVN. Similar to our earlier report on TRPV4 (10, 44), where no difference in mRNA and protein levels were observed, we suggest an increased participation of TRPC4 in the SON to elevate circulating AVP levels via Ca\(^{2+}\) entry. Hence, hepatic cirrhosis is associated with up-regulated TRPC4 transcriptional activity and elevated translation in the SON but not PVN.

Similarly, the percentage of AVP cells that express TRPC4 was significantly increased in SON but not PVN as demonstrated by immunohistochemical experiments. These results are consistent with our western blot and mRNA findings indicating that the effect of hepatic cirrhosis on TRPC4 expression was regionally localized to the SON.
Water deprivation is a progressive physiological challenge that requires sustained increases in AVP to reduce water loss. Forty-eight hour water deprivation was characterized by down-regulation of TRPC4 transcripts in the vasopressin cells of the SON with increases in the PVN. It has been demonstrated that TRPC4 gene expression is increased approximately 3 fold in the SON after water deprivation (29). The same study also showed the presence of TRPC4 transcript in dehydrated rats in the PVN. There are several methodological considerations that may account for the differences between these studies that include the duration of the water deprivation, tissue collection and microarray analysis. The previous study employed a longer water deprivation protocol (72 h) and pooled microdissected tissue samples containing the SON or PVN from 5 rats in order to conduct a rigorous GeneChip analysis. Progressive dehydration is known to increase the functional activity of the magnocellular neurosecretory system, resulting in a further increase in plasma osmolality as well as increased secretion of vasopressin. Hence, it seems possible that 72 h of water deprivation may be required to observe increased TRPC4 expression in the SON. The differences in results between the two studies can be also be explained by the possibility of changes in TRPC4 transcripts in oxytocin and/or astrocytes cells that would have been included in the microdissected samples which are comparable to punch technique used for western blot analysis in the current study. Oxytocin, another predominant neurohypophyseal hormone synthesized in the magnocellular SON and PVN, is implicated in maintaining fluid balance and is stimulated by water deprivation (23, 28). Both the SON and PVN have an astrocytic perivascular system that mediates water fluxes, and astrocytes in these regions express vasopressin receptors (62). Our study quantitatively estimated the TRPC4 mRNA expression in only the vasopressin positive cells and compared the expression levels between the 48h water deprived and control groups.
No change in the protein levels from tissue punches were observed after water deprivation in either the PVN or the SON despite significant changes in TRPC4 transcript. These differences could be due to differences in the collection method as discussed above. Due to technical difficulties of performing western blot from low protein yield laser captured cells, we used punch samples containing the SON and PVN. TRPC4 protein expression in other cell types contained in the punch samples could be masking the actual TRPC4 translational levels in vasopressin magnocellular neurosecretory cells. Since the PVN is more heterogeneous than SON, this technical limitation could have prevented us from detecting changes in TRPC4 in Western blot analyses. In addition, there may also be TRPC4 post-transcriptional modification, increased protein degradation, or altered protein transport into the axonal terminals in vasopressin magnocellular neurosecretory cells that influence the results of the protein analysis. It still needs to be determined if the changes in the vasopressin specific transcriptional TRPC4 content associated with water deprivation are subject to post transcriptional or post translational modification.

Functional characterizations of heterologously expressed TRPC4 have revealed diverse regulatory mechanisms and permeation properties (57). Mammalian TRPC channels can form hetero-oligomeric channels in vitro (37, 73) and in vivo (24, 66). These channels have been demonstrated to be expressed in nerve terminals where the C1, C4 and C5 subunits co-associate in vivo (24). TRPC4 is known to form heteromeric cation channels with TRPC5 based on their sequence homology and functional similarities (14). Co-association of these channels could result in enhanced channel activity. The mechanism of activation of TRPC channels is still debatable, since it has been shown that depletion of intracellular Ca\textsuperscript{2+} stores activates TRPC channels (22, 27, 47, 52, 56, 74) in contrast to studies attributing the activation of TRPC
channels independent of store depletion (1, 46, 54). Also, the presence of splice variants for TRPC4 having different regulatory mechanisms even with similar mechanism of activation (58) necessitates additional efforts to be directed to understanding the function and role of TRPC channel proteins in an endogenous system. A previous study had demonstrated the involvement of G-protein coupled receptors in TRPC4 activation that contributes to a robust secretory response via Ca$^{2+}$ influx in neuroendocrine cells (46). Considering our finding that TRPC4 is differentially regulated by progressive homeostatic challenges such as water deprivation and in a model of inappropriate vasopressin release, endogenous TRPC4 may have regionally specific functions in regulating AVP release in SON and PVN.

This regional specificity appears to be at odds with the more traditional view that magnocellular neurosecretory cells in the SON and PVN are similarly regulated during physiological challenges and pathophysiological states. It could be that these results are a function of the diverse mechanisms that regulate TRPC4 and its participation in heteromeric channels and not due to differential regulation of SON and PVN. Alternatively, the differential effects on TRPC4 expression that occurred in both water deprivation and hepatic cirrhosis could be due to differences in synaptic and non-synaptic mechanisms that are specific to SON or PVN. For example, it has been demonstrated that in PVN dendritic release of peptides from magnocellular secretory cells can influence the function of pre-autonomic neurons in other subregions of this heterogeneous structure (63). This type of “interpopulation crosstalk” could potentially influence the function of PVN magnocellular neurosecretory cells but may not occur in the more homogenous SON.

Water deprivation and hepatic cirrhosis are complex physiological events and that could be the basis for their differential effects on TRPC4 expression. Vasopressin release associated with
water deprivation is driven by increased plasma osmolality along with changes in plasma volume
and activation of the renin-angiotensin system. In dilutional hyponatremia and inappropriate
vasopressin release associated with the bile duct ligation model of hepatic cirrhosis, there is an
increased level of circulating vasopressin subsequent to systemic hypotension and functional
reduction in effective plasma volume that regulates the activity of the magnocellular
neurosecretory cells of the PVN and SON. Vasopressin release in this model is traditionally
described as mediated by non-osmotic mechanisms although changes in the osmotic sensitivity
of vasopressin magnocellular secretory cells probably play at least a permissive role in this
syndrome. Although activation of the renin-angiotensin system occurs during cirrhosis, other
non-osmotic factors such hepatic-portal receptors and circulating bile have also been shown
potential to contribute to vasopressin release in this syndrome (18). The differences in the
duration of the two models may also be a contributing factor. As mentioned above, 48h of
stimulation used in the water deprivation study may not be sufficient to change TRPC4 in the
SON compared to the 4 week duration of the bile duct ligation studies.

Recently, a high throughput fluorescent screening identified a novel TRPC4/C5 antagonist
ML-204, with higher selectivity towards TRPC4 channels in cell cultures, isolated dorsal root
ganglion and smooth muscle cells (39). Pharmacological blockade using ML-204 might be
helpful in understanding the physiological role of TRPC4 channels in vasopressin release and
whether TRPC4 might be a useful target for therapeutic interventions in pathophysiological
states with altered fluid balance.
Our study has identified, for the first time, the presence of TRPC4 channels in the vasopressin cells in the magnocellular hypothalamic cells. TRPC4 expression was increased in the SON in association with hepatic cirrhosis which is characterized by dilutional hyponatremia. Similar changes were not observed in vasopressin magnocellular secretory cell of the PVN. Water deprivation was not associated with consistent changes in either region. Changes in TRPC4 may be specific to chronic activation of vasopressin neurosecretory cells in the SON. A critical challenge for understanding the functional role of TRPC4 in a physiological system is to establish whether TRPC4 channels contribute to the chronic activation of vasopressinergic neurons in the SON. Future experiments are needed to investigate this possibility. This would provide a putative therapeutic target for fluid imbalances and their complications.


34. Li HS, Xu XZ, and Montell C. Activation of a TRPC3-dependent cation current through the neurotrophin BDNF. *Neuron* 24: 261-273, 1999.


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**Table 1: Real Time q-RT PCR primer sequences for gene of interest and housekeeping gene**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tr>
<td>GAPDH Forward</td>
<td>5'-CTCATGACCACAGTCCATGC-3'</td>
</tr>
<tr>
<td>GAPDH Reverse</td>
<td>5'-TACATTTGGGGGTAGGAACAC-3'</td>
</tr>
<tr>
<td>TRPC4 Forward</td>
<td>5'-AATTACTCGTCAACAGGCGGC-3'</td>
</tr>
<tr>
<td>TRPC4 Reverse</td>
<td>5'-CACCACCACCTTCTCGACTT-3'</td>
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Table 2: Measurements of plasma osmolality, hematocrit and liver weight to body weight ratio from sham (Sham) and bile duct ligated (BDL) rats. *p<0.05 compared to sham ligation; **p<0.01 compared to sham ligation; ***p<0.001 compared to sham ligation.

<table>
<thead>
<tr>
<th>Plasma Measurements</th>
<th>Sham</th>
<th>BDL</th>
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<tbody>
<tr>
<td>Osmolality (mOsm/kg)</td>
<td>301.1± 1.7 (n=17)</td>
<td>294.4±1.1* (n=18)</td>
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<td>Hematocrit %</td>
<td>47.83 ± 0.2 (n=15)</td>
<td>43.1 ± 0.5** (n=14)</td>
</tr>
<tr>
<td>Liver weight/body weight (g/dl)</td>
<td>0.04 ± 0.003 (n=15)</td>
<td>0.083 ± 0.004*** (n=17)</td>
</tr>
</tbody>
</table>
Table 3: Measurements of plasma osmolality, hematocrit and plasma protein from Control (CON) and 48 hour water deprived (48h WD) rats. *p<0.05 compared to controls; **p<0.01 compared to controls; ***p<0.001 compared to controls.

<table>
<thead>
<tr>
<th>Plasma Measurements</th>
<th>CON</th>
<th>48h WD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osmolality (mOsm/kg)</td>
<td>299.5±2.6 (n=8)</td>
<td>316.1±2.4** (n=8)</td>
</tr>
<tr>
<td>Hematocrit %</td>
<td>42.83±0.33 (n=6)</td>
<td>49.83±0.48*** (n=6)</td>
</tr>
<tr>
<td>Plasma Protein (g/dl)</td>
<td>6.556±0.15 (n=9)</td>
<td>7.244±0.13* (n=9)</td>
</tr>
</tbody>
</table>
Figure Legends:

1. TRPC4 mRNA expression by real-time RT-PCR from 7-10 laser microdissected vasopressin cells from the PVN (A) and SON (B) in BDL versus Sham. The data depict mean ± SEM mRNA levels as calculated by the $2^{-\Delta\Delta CT}$ method. *p < 0.05 compared to sham ligation. n= 5-6 for each group. Western Blot quantification and representative immunoblots of TRPC4 protein abundance in the brain punches from PVN (C&D) and SON (E&F) of BDL versus Sham. S and B in the immunoblots represent Sham and BDL respectively. The data are presented as mean ± SEM estimated by relative intensity by normalizing with GAPDH. n= 6 for each group.

2. TRPC4 mRNA expression by real-time RT-PCR from 7-10 laser microdissected vasopressin cells from the PVN (A) and SON (B) in 48h WD versus Controls. The data depict mean ± SEM mRNA levels as calculated by the $2^{-\Delta\Delta CT}$ method. *p < 0.05 compared to sham ligation. n= 7-8 for each group. Western Blot quantification and representative immunoblots of TRPC4 protein abundance in the brain punches from PVN (C&D) and SON (E&F) of 48h WD versus Controls. C and W in the immunoblots represent Control and Water Deprivation respectively. The data are presented as mean ± SEM estimated by relative intensity by normalizing with GAPDH. n= 6 for each group.

3. Specificity of TRPC4 antibody in the magnocellular hypothalamic regions. Specific TRPC4 staining in the SON (A) and PVN (B) is completely abolished by use of TRPC4 blocking peptide in the SON (C) and also in the PVN (D). Scale bars represent 100 µm for these images. Figure E represents western blots to show complete elimination of TRPC4 immunoreactive bands using TRPC4 blocking peptide.
4. Confocal 60x pseudocolored images of AVP and TRPC4 co-localization in the SON of Sham (A-C) versus BDL (D-F) and in the PVN of Sham (G-I) versus BDL (J-L). Red immunofluorescence represents AVP staining (A, D, G and J) while green immunofluorescence represents TRPC4 staining (B, E, H and K). Merged images of AVP and TRPC4 are represented by panels C, F, I and L. Scale bar = 10 µm for all images.

5. Quantitative estimates of AVP, TRPC4 and co-localization in the PVN (A) SON (B) from Sham and BDL. *p<0.05 compared to sham ligation. n= 6 for each group.

6. Confocal 60x pseudocolored images of AVP and TRPC4 co-localization in the PVN of Control (A-C) versus 48h WD (D-F) and in the SON of Control (G-I) versus 48h WD (J-L). Red immunofluorescence represents AVP staining (A, D, G and J) while green immunofluorescence represents TRPC4 staining (B, E, H and K). Merged images of AVP and TRPC4 are represented by panels C and F. Scale bar = 10 µm for all images.

7. Quantitative estimates of AVP, TRPC4 and co-localization in the PVN (A) SON (B) from Control versus 48h WD. *p<0.05 compared to water deprivation. n= 6 for each group.
Figure A and Figure B illustrate the average number of immunopositive cells under different conditions.

**Figure A**
- **AVP**: 
  - CON: [Value] ± [Error]
  - 48h WD: [Value] ± [Error]
- **TRPC4**: 
  - CON: [Value] ± [Error]
  - 48h WD: [Value] ± [Error]
- **Colocalized**: 
  - CON: [Value] ± [Error]
  - 48h WD: [Value] ± [Error]

**Figure B**
- **AVP**: 
  - CON: [Value] ± [Error]
  - 48h WD: [Value] ± [Error]
- **TRPC4**: 
  - CON: [Value] ± [Error]
  - 48h WD: [Value] ± [Error]
- **Colocalized**: 
  - CON: [Value] ± [Error]
  - 48h WD: [Value] ± [Error]