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

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Nedungadi TP, Cunningham JT. Differential regulation of TRPC4 in the vasopressin magnocellular system by water deprivation and hepatic cirrhosis in the rat. Am J Physiol Regul Integr Comp Physiol 306: R304–R314, 2014. First published December 18, 2013; doi:10.1152/ajpregu.00388.2013.—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 used laser capture microdissection technique for precise dissection of pure 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 this region, as well as increased colocalization of AVP with TRPC4-positive cells. Thus, TRPC4 expression is differentially regulated following water deprivation, with no changes in the number of TRPC4-positive cells. In both of these regions, protein expression measured from tissue punches were unaltered following water deprivation, with no changes in the number of TRPC4-positive cells. Therefore, TRPC4 expression is differentially regulated in physiological and pathophysiological models of vasopressin release.

vasopressin; TRPC4; bile duct ligation; water deprivation; osmolality

TRANSIENT RECEPTOR POTENTIAL CANONICAL (TRPC) channels play a role in calcium homeostasis through multiple activation mechanisms. They are extensively expressed in the brain and are also present in the adrenal glands, heart, lung, liver, spleen, kidney, testis, uterus, and aorta. TRPC channels are involved in the cell differentiation, embryonic nerve cell proliferation, BDNF signaling through TrkB activation, inducing dendritic spine formation, guidance of nerve growth cones, slow synaptic potentials mediated by mGluR1 synaptic signaling in cerebellar Purkinje cells, neurite outgrowth and remodeling, epileptiform burst firing and excitotoxicity, as well as in nociception. Of the seven TRPC channel members identified, TRPC4 is the predominant subtype, accounting for 41% of the total TRPC content expressed in the rat brain. In vitro systems have demonstrated involvement of TRPC4/TRPC5, belonging to the group 4 of TRPC channels, in triggering robust secretory response in voltage-clamped neurosecretory cells by causing Ca$^{2+}$ influx. 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. In the rat cerebellum, TRPC4 expression is restricted to granule cells and their precursor and may be essential for normal granule cell development. 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. TRPC4 mRNA has been detected in the PVN using in situ hybridization. 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 AVP and oxytocin into systemic circulation. Circulating levels of AVP, which are primarily determined by the activity of these magnocellular cells in the hypothalamus, contribute to body fluid and electrolyte 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. Natriuresis mechanisms, such as changes in blood volume and blood pressure, also regulate AVP release and influence osmotic mechanisms. Dysregulation of AVP release contributes to dilutional hyponatremia associated with liver and heart failure. Chronic bile duct ligation, a model of liver failure, is associated with elevated plasma vasopressin levels and increased activity of the renin-angiotensin system. We and others have previously demonstrated that changes in the expression of TRPV channels occur in AVP neurons during water deprivation, as well as inappropriate vasopressin release associated with bile duct ligation.

In the present study, 48 h of 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 (250–350 g body wt; Charles River Laboratories, Wilmington, MA) were individually housed and maintained in a temperature-controlled (23°C) environment under a 12:12 h light-dark cycle with light onset at 0700. 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 rats in the control group were allowed ad libitum access to water and food throughout the experiment. Rats in the experimental group were water deprived for 48 h and not allowed access to water but with 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 the sham-ligated control group, the bile duct was exposed but not ligated. All rats were used for experiments 4 wk after surgery. Liver-to-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 that did not show any of these developments would be considered 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 anesthetized (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 hemocrit 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 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 blot analysis, 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 1 h at room temperature with 5% (wt/vol) nonfat milk in Tris-buffered saline 0.05% (vol/vol) Tween 20 followed by incubation overnight at 4°C with primary antibody raised against neurotransmitter 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 1 h. 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 (West Grove, PA) secondary antibody for 4 h followed by Cy2-conjugated Affinipure Donkey Anti-Rabbit IgG (Jackson ImmunoResearch, West Grove, PA) secondary antibody for 4 h. In addition, a separate set of sections were processed after preincubation 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 sham, six BDL; six CON, and six WD rats).

Imaging. Immunofluorescent imaging was carried out with an Olympus IX-2 DSU confocal microscope with appropriate excitation/emission filter sets. Olympus IX-2 DSU confocal microscope with appropriate excitation/emission filter sets. 

Table 2. Measurements of plasma osmolality, hematocrit and liver weight-to-body weight ratio from sham and bile duct-ligated rats

<table>
<thead>
<tr>
<th>Plasma Measurements</th>
<th>Sham</th>
<th>BDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osmolality, mOsm/kg</td>
<td>301.1 ± 1.7 (n = 17)</td>
<td>294.4 ± 1.1* (n = 18)</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>47.83 ± 0.2 (n = 15)</td>
<td>43.1 ± 0.5** (n = 14)</td>
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<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>
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BDL, bile duct ligation. *P < 0.05 compared with sham ligation; **P < 0.01 compared with sham ligation; ***P < 0.001 compared with sham ligation.

Table 3. Measurements of plasma osmolality, hematocrit, and plasma protein from control and 48-h water-deprived (48 h WD) rats

<table>
<thead>
<tr>
<th>Plasma Measurements</th>
<th>CON</th>
<th>48 h WD</th>
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<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>
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<tr>
<td>Plasma protein, g/dl</td>
<td>6.556 ± 0.15 (n = 9)</td>
<td>7.244 ± 0.13* (n = 9)</td>
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CON, control. *P < 0.05 compared with controls; **P < 0.01 compared with controls; ***P < 0.001 compared with controls.
rostrocaudal levels. Level 1, which is 1.60 mm posterior to the bregma, includes the most rostral and corresponds to ventrally located magnocellular cells, while level 2, which is 1.88 mm posterior to the bregma, demonstrates a prominent posterior magnocellular region, as previously described. 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 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 laboratory. Snap-frozen brains were cut at 10-μm thickness at the level of SON and PVN, which is –1.88 mm posterior to the bregma, demonstrates a prominent posterior magnocellular region, as previously described. The number of AVP- and TRPC4-positive cells were then counted by individuals blind to the experimental groups, and then the numbers were averaged.

Fig. 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 vs. sham. The data depict means ± SE mRNA levels as calculated by the 2^(-ΔΔCT) method. *P < 0.05 compared with sham ligation, n = 5 or 6 for each group. Western blot quantification and representative immunoblots of TRPC4 protein abundance in the brain punches from PVN (C and D) and SON (E and F) of BDL vs. sham. S and B in the immunoblots represent sham and BDL, respectively. The data are presented as means ± SE estimated by relative intensity by normalizing with GAPDH. n = 6 for each group.

Fig. 2. TRPC4 mRNA expression by real-time RT-PCR from 7–10 laser microdissected vasopressin cells from the paraventricular nucleus (PVN; A) and supraoptic nucleus (SON; B) in 48-h WD vs. controls. The data depict means ± SE mRNA levels as calculated by the 2^(-ΔΔCT) method. *P < 0.05 compared with sham ligation. n = 7 or 8 for each group. Western blot quantification and representative immunoblots of TRPC4 protein abundance in the brain punches from PVN (C and D) and SON (E and F) of 48 h WD vs. controls. C and W in the immunoblots represent control and water deprivation, respectively. The data are presented as means ± SE estimated by relative intensity by normalizing with GAPDH. n = 6 for each group.
using a cryostat and mounted on polyethylene naphthalate (PEN) membrane-coated slides (catalog no. LCM0522; Arcturus Bioscience, Mountain View, CA). After 3 min of 100% methanol fixation, the sections were rinsed three times in ice-cold DEPC PBS followed by blocking in 3% horse serum containing 0.25% Triton-X for 5 min. Postblocking, sections were incubated for 3 min in a 1:50 diluted guinea pig anti-AVP antibody (Peninsula Laboratories) and then in Cy3-conjugated Affinipure Donkey anti-guinea pig secondary antibody (Jackson ImmunoResearch) for another 3 min.

For LCM, we used an Arcturus Veritas microdissection instrument, which is enabled with an infrared capture laser and an UV cutting laser. Visible and completely stained neurons were selected for capture. The cutting and capture laser beams were positioned right above the brain tissue, which is placed between the 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 0.5-ml tube containing 30 μl of ArrayPure nanoscale lysis solution with 5.0 g of proteinase K (prod. no. MPS04050; Epicentre Biotechnology, Madison, WI). Total RNA was isolated from the 7–10 captured neurons using ArrayPure nanoscale RNA purification kit reagents (Epicentre Biotechnology), as previously described (12, 15, 45). Two microliters per RNA sample was amplified with TargetAmp 2-Round aminoallyl-aRNA amplification kit materials (Epicentre Biotechnology), 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, Thermo Fisher Scientific, Waltham, MA). Samples of 260/280 less than 1.8 were not used further for RT reactions. A total of 26 rats (five shams and six BDL; seven CON, and eight water-deprived) were used in this study.

RT and quantitative real time RT-PCR. Sensiscript RT kit reagents (prod. no. 205213; Qiagen, Valencia, CA) 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: 2 μl of 10× RT buffer, 2 μl of dNTP mix (final concentration: 5 mM), 2 μl of oligo-dT primer solution (final concentration: 10 μM), 0.25 μl of RNase inhibitor (final concentration: 10 U/μl), 1 μl 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°C for 3 min, followed by 40 cycles of 1.1 min each (40 s at 94°C, followed by 30 s at 60°C for TRPC4 and 30 s at 95°C followed by 1 min at 65°C for GAPDH). The RT-PCR reaction mixture contained 1.8 μl of cDNA, 4.5 μl of RNase/DNase-free water, 1.2 μl of primer mix, and 7.5 μl 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 curve analysis confirmed the integrity of the RT-PCR

Fig. 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. E: Western blots show complete elimination of TRPC4-immunoreactive bands using TRPC4-blocking peptide.
products as confirmed by the presence of single peaks signifying the absence of primer-dimers. Control experiments and data analysis were performed as previously published (8, 44). The $2^{-\Delta\Delta Ct}$ method was used for quantifying gene expression. $\Delta Ct$ was measured by calculating the difference between the GAPDH and the corresponding TRPC4 Ct values. For obtaining the $\Delta\Delta Ct$ 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 ± SE. For statistical analysis of data, we used unpaired t-test using GraphPad Prism (GraphPad Software, 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 hematocrit 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 with sham-ligated rats measured at the time of death (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 is a valid model of reduced plasma osmolality and extracellular volume expansion (10, 15, 44, 70).

**Effect of 48-h WD on Hemodynamic and Osmotic Responses**

As presented in Table 3, water deprivation for 48 h significantly increased plasma osmolality compared with ad libitum euhydrated controls. ($P < 0.01$). Plasma protein (mg/dl) was also significantly increased by 48 h of water deprivation. As expected, plasma hematocrit levels were also considerably higher after 48 h of water deprivation ($P < 0.001$). These

![Fig. 4. Confocal ×60 pseudocolored images of AVP and TRPC4 colocalization in the SON of sham (A–C) vs. BDL (D–F) and in the PVN of sham (G–I) vs. 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.](image-url)
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 Analysis

The relative abundance of TRPC4 transcript was measured in laser-captured vasopressin cells from the magnocellular PVN and SON. Figure 1, A and 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 twofold 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 with 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. 1C). TRPC4-immunoreactive bands were seen at 100 kDa for both the regions investigated (Fig. 1, D and F). GAPDH was used as the loading and normalization control.

Effect of 48 h WD on mRNA and Protein Expression of TRPC4 in the PVN and SON Using Real-Time q-RT PCR and Western Blot Analysis

After 48 h of water deprivation, a significant increase in TRPC4 mRNA was observed in the PVN (Fig. 2A). In the SON, the 48-h water deprivation decreased the transcriptional TRPC4 levels compared with the hydrated rats (Fig. 2B). To investigate whether the changes in the TRPC4 protein during 48 h WD correlate with the levels of its mRNA, we performed Western blot analysis for TRPC4 protein. Representative blots are shown in Fig. 2, D and F. Western blot analysis showed an immunoreactive band at 100 kDa corresponding to TRPC4 protein. Figure 2, C and E 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. Preincubation of the primary antibody with the blocking peptide for 1 h eliminated TRPC4 immunostaining in the SON (Fig. 3C) and PVN (Fig. 3D). Similarly, TRPC4-immunoreactive bands were completely abolished after preincubation with blocking peptide in the SON (Fig. 3E).

Colocalization 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 whether 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. Figure 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 with naïve sham control. Here, BDL was also associated with a significant increase in the number of colocalized TRPC4/AVP cells (Fig. 5B).

The number of AVP/TRPC4-positive cells was also unchanged after water deprivation in the PVN (Fig. 6, A–F); however, an increase in the double immunofluorescence for AVP and TRPC4 was observed in the SON (Fig. 6, G–L). Water deprivation did not elicit any significant changes in the semiquantitative immunohistochemical counts of AVP and TRPC4 in the magnocellular PVN and SON (Fig. 7, A and B), 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, for the first time, we 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 Ca2+ entry. Hence, hepatic cirrhosis is associated with upregulated TRPC4 transcriptional activity and elevated translation in the SON but not PVN.

Similarly, the percentage of AVP cells that expresses 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 three-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 five rats to conduct a rigorous GeneChip analysis. Progressive dehydra-
deprivation; influence the results of the protein analysis. It still needs to be
understood how the results reported by others in vasopressin magnocellular neurosecretory cells that
degradation, or altered protein transport into the axonal termi-
nation is known to increase the functional activity of the mag-
nocellular neurosecretory system, resulting in a further in-
crease in plasma osmolality, as well as increased secretion of
vasopressin. Hence, it seems possible that 72 h of water
depression 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 that 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 vasopres-
sin-positive cells and compared the expression levels between
the 48-h water-deprived and control groups.

No change in the protein levels from tissue punches was
observed after water deprivation in either the PVN or the SON,
despite significant changes in TRPC4 transcript. These differ-
ences could be due to differences in the collection method as
discussed above. Because of technical difficulties of perform-
ing 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.
Because 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 posttranscriptional modification, increased protein
degradation, or altered protein transport into the axonal termi-
nals in vasopressin magnocellular neurosecretory cells that
influence the results of the protein analysis. It still needs to be
determined whether the changes in the vasopressin-specific
transcriptional TRPC4 content associated with water depriva-
tion are subject to posttranscriptional or posttranslational mod-
ification.

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 coassociate in vivo (24). TRPC4 is known to form heteromeric
cation channels with TRPC5 based on their sequence homol-
gy and functional similarities (14). Coassociation of these
channels could result in enhanced channel activity. The mech-
anism 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 independ-
ent 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 toward understanding the
function and role of TRPC channel proteins in an endogenous
system. A previous study had demonstrated the involvement of
protein-coupled receptors in TRPC4 activation that contributes
to a robust secretory response via Ca\textsuperscript{2+} influx in neuroen-
docrine cells (46). Considering our finding that TRPC4 is differ-
entially regulated by progressive homeostatic challenges, such
as water deprivation and in a model of inappropriate vasopres-
sin 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 nonsynaptic mechanisms that are
specific to SON or PVN. For example, it has been demon-
strated that in PVN, dendritic release of peptides from magnocel-
lar secretory cells can influence the function of preautono-
nomic neurons in other subregions of this heterogeneous struc-
ture (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 physio-
logical 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 osmol-
ality along with changes in plasma volume and activation of the
renin-angiotensin system. In dilutional hyponatremia and in-
appropriate 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 reg-
ulates the activity of the magnocellular neurosecretory cells
of the PVN and SON. Vasopressin release in this model is

Fig. 7. Quantitative estimates of AVP, TRPC4, and colocalization in the PVN
(A) and SON (B) from control vs. 48 h WD. *P < 0.05 compared with water
depprivation; n = 6 for each group.

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tritionally described as mediated by nonosmotic 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 nonosmotic factors, such as hepatic-portal receptors and circulating bile, have also been shown to potentially 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, 48 h of stimulation used in the water deprivation study may not be sufficient to change TRPC4 in the SON compared with the 4-wk duration of the bile duct ligation studies.

Recently, a high-throughput fluorescent screening identified a novel TRPC4/C5 antagonist ML-204, with higher selectivity toward 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.

Perspectives and Significance

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.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

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