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Osmoregulatory thirst in mice lacking the transient receptor potential vanilloid type 1 (TRPV1) and/or type 4 (TRPV4) receptor

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Kinsman B, Cowles J, Lay J, Simmonds SS, Browning KN, Stocker SD. Osmoregulatory thirst in mice lacking the transient receptor potential vanilloid type 1 (TRPV1) and/or type 4 (TRPV4) receptor. Am J Physiol Regul Integr Comp Physiol 307: R1092–R1100, 2014. First published August 6, 2014; doi:10.1152/ajpregu.00102.2014.—Recent studies suggest the ability of the central nervous system to detect changes in osmolality is mediated by products of the genes encoding the transient receptor potential vanilloid-1 (TRPV1) or vanilloid-4 (TRPV4) channel. The purpose of the present study was to determine whether deletion of TRPV1 and/or TRPV4 channels altered thirst responses to cellular dehydration in mice. Injection of 0.5 or 1.0 M NaCl produced dose-dependent increases in cumulative water intakes of wild-type (WT) and TRPV1−/−, TRPV4−/−, and TRPV1−/−TRPV4−/− mice. However, there were no differences in cumulative water intakes between WT versus any other strain despite similar increases in plasma electrolytes and osmolality. Similar results were observed after injection of hypertonic mannitol. This was a consistent finding regardless of the injection route (intraperitoneal vs. subcutaneous) or timed access to water (delayed vs. immediate). There were also no differences in cumulative intakes across strains after injection of 0.15 M NaCl or during a time-controlled period (no injection). Chronic hypernatremia produced by sole access to 2% NaCl for 48 h also increased thirst. Fos immunoreactivity in the OVLT increased with the osmotic load, but the number of Fos-positive neurons in the ventral lamina terminalis largely attenuates or abolishes thirst, antidiuretic hormone secretion, and sympathetic nerve activity (3, 6, 30, 31, 41). While the relative contribution of the OVLT versus SFO to these osmoadaptation responses may vary across species, these studies highlight the importance of these structures in osmoregulatory processes.

Recent studies performed in OVLT neurons suggest the underlying osmosensory process in these neurons is mediated by members of the transient receptor potential vanilloid (TRPV) family (5, 11, 12). TRPV channels contain six membrane spanning segments and a pore-loop domain that are sensitive to temperature, chemical, and mechanical/osmotic stimuli (34, 37). These studies have demonstrated that acute or chronic hypernatremia increases Fos immunoreactivity in the OVLT and SFO (21, 36, 40, 46). Second, in vivo and in vitro electrophysiological studies indicate that acute hypernatremia or hyperosmolality increases the discharge of neurons in both structures (11, 12, 14, 17, 28, 35, 38). Third, in vitro electrophysiological studies indicate that SFO and OVLT neurons are intrinsically osmosensitive (2, 11, 12). Finally, lesion of the lamina terminalis largely attenuates or abolishes thirst, antidiuretic hormone secretion, and sympathetic nerve activity (3, 7, 20, 27, 31, 41, 51). While the relative contribution of the OVLT versus SFO to these osmoregulatory responses may vary across species, these studies highlight the importance of these structures in osmoregulatory processes.

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response to an acute sodium load (23). Ciura and Bourque (11) reported that TRPV1 knockout mice drank slightly less water than WT mice after an intraperitoneal injection of hypertonic NaCl. In marked contrast, our laboratory reported that TRPV1 knockout and WT mice ingested similar amounts of water in response to a range of acute and chronic hypertensive challenges (46). In addition, acute and chronic sodium loading produced a similar increase in the number of Fos-positive neurons in the lamina terminalis of WT versus TRPV1 knockout mice (46). Given the controversial data regarding the functional role of TRPV1 versus TRPV4 channels, the purpose of the present study was to perform a comprehensive analysis of osmoregulatory thirst in TRPV1 knockout, TRPV4 knockout, and double knockout TRPV1 knockout/V4 knockout mice. Cumulative water intake was measured in response to both acute and chronic sodium loads. We employed several different paradigms including varied injection routes (intraperitoneal vs. subcutaneous) and timed access to water (delayed vs. immediate) to permit direct comparisons to previous studies (11, 23, 46). Parallel experiments also investigated whether deletion of TRPV1 and/or TRPV4 genes disrupted Fos activation in neurons of the OVLT, SFO, and median preoptic nucleus.

MATERIALS AND METHODS

All of the experimental procedures conformed to the National Institutes of Health “Guide for the Care and Use of Laboratory Animals” and were approved by the Institutional Animal Care and Use Committee at the Pennsylvania State College of Medicine.

Animals. Double knockout mice for the TRPV1 and TRPV4 (TRPV1 knockout/V4 knockout) channels were obtained from Dr. Wolfgang Liedtke (Duke University). TRPV4 knockout mice were made previously by deletion of exon 12, which encodes the pore-loop and adjacent transmembrane domains (23). These mice were crossed with TRPV1 knockout mice (Jackson Laboratory, Bar Harbor, ME) that lack the exon encoding the fifth and sixth transmembrane and the pore-loop domains (9). Within our laboratory, the TRPV1 knockout/V4 knockout mice were crossed with C57BL/6 mice (Charles River Laboratories) to obtain TRPV1 knockout/V4 knockout strains. Animals were housed individually in a temperature-controlled room (22–24°C) and maintained on a 12:12-h light-dark cycle (lights on at 6 AM) at least 1 wk before experiments began. The room was solely dedicated for these experiments. Standard laboratory chow (Harlan Teklad Global Diet no. 7912) and deionized water were provided ad libitum except where noted. Experiments were conducted between 8 AM and 1 PM. The total number of animals used in the present study is as follows: WT (C57BL/6, n = 30), TRPV1 knockout (n = 26), TRPV4 knockout (n = 24), and TRPV1 knockout/V4 knockout (n = 23).

Thirst studies. To determine the contribution of TRPV1 and TRPV4 channels to thirst stimulated by cellular dehydration, we studied male mice (initial age: 12–15 wk) of four different strains: WT or C57BL/6 (Charles River Laboratories or Jackson Laboratories), TRPV1 knockout, TRPV4 knockout, and TRPV1 knockout/V4 knockout. At least 1 wk before experiments began, mice were housed singly and acclimated to the testing procedures daily. Briefly, food was removed from all cages at least 1 h before and throughout studies to eliminate any possibility that the recent ingestion of food impacted subsequent water intake. Each mouse was weighed and then handled identically to an injection. Finally, graduate 10-ml drinking tubes (± 0.05 ml) were placed and remained on cages throughout the entire study. Two different groups of mice were studied in two separate experimental series (see below).

Experimental series 1. The first series of experiments was a randomized, within-subjects design, and each treatment was separated by 3–5 days. WT (n = 13), TRPV1 knockout (n = 13), TRPV4 knockout (n = 12), and TRPV1 knockout/V4 knockout (n = 10) mice received an acute injection of 0.15, 0.5, or 1.0 M NaCl (10 μl/g body wt). To distinguish between thirst stimulated by hypernatremia versus hyperosmolality, mice also received an injection of 0.7 M mannitol dissolved in 0.15 M NaCl (10 μl/g body wt). Injections were performed intraperitoneally and subcutaneously using 30-gauge needles; however, the same route of injection was used only once per week. A final time-control experiment was performed in which the testing procedures were identical except mice did not receive an injection. Access to water in all tests was delayed for 30 min. Then, cumulative water intakes (± 0.05 ml) were measured at 30, 60, 90, and 120 min. In addition, water bottles were weighed (nearest 0.05 g) before and after the drinking tests to confirm the observed intakes. If there was a difference between the observed water intake and weight of the water bottles, the test was repeated on another day. However, this was rarely encountered (<5 times). Experimental series 1 lasted ~5–6 wk.

Experimental series 2. The second series of experiments was a randomized, within-subjects design, and each treatment was separated by 3–5 days. WT (n = 12), TRPV1 knockout (n = 9), TRPV4 knockout (n = 8), and TRPV1 knockout/V4 knockout (n = 9) mice received an acute injection of 0.15, 0.5, or 1.0 M NaCl (0.3 ml sc) or 0.7 M mannitol dissolved in 0.15 M NaCl (0.3 ml sc) using 30-gauge needles. In contrast to experimental series 1, these mice were given immediate access to water as previously described (46). In addition, to investigate whether TRPV1 and/or TRPV4 channels contributed to thirst stimulated by chronic hypernatremia, the same mice were given access to food and a 2% NaCl solution for 48 h. Then, food was removed and the 2% NaCl was replaced with water. To examine whether deletion of TRPV1 and/or TRPV4 genes did not result in a general disruption of thirst independent of osmotic or hypertensive stimuli, mice received an injection of the β-adrenergic agonist dl-isoproterenol hydrochloride (0.04 μg, 0.25 ml sc) or were made hypovolemic by two successive injections of the loop diuretic furosemide (0.5% Lasix, 0.25 ml sc) separated by 2 h. Again, mice had immediate access to water in all experiments except after injection of furosemide. In this experiment, water bottles were returned at 2 h after the second injection. Cumulative water intakes (± 0.05 ml) were measured at 15, 30, 60, and 120 min. Experimental series 2 lasted ~4 wk.

Blood sampling and analysis of plasma electrolytes and osmolality. A final set of experiments using a between-group design was conducted to determine whether the acute NaCl injections produced similar changes in plasma electrolytes and osmolality across mouse strains. Each treatment used both naïve mice and mice from experimental series 1 at least 2 wk after all behavioral studies were completed. The procedures were identical to those described in experimental series 1. Mice received an injection of 0.15, 0.5, or 1.0 M NaCl (10 μl/g body wt sc) but had no access to water. At 25 min, mice were anesthetized with 2–3% isoflurane (in 100% O2) and instrumented with a catheter in the left carotid artery. The entire procedure lasted <5 min. A blood sample (70 μl) was collected immediately through the carotid catheter and analyzed for electrolytes using an I-STAT1 and 6+ cartridges (Abbott; East Windsor, NJ). Additional blood (300 μl) was collected into a microcentrifuge tube containing heparin (3 units) and centrifuged (10,000 g, 1 min). Osmolality was determined in triplicate using a freezing-point depression osmometer (model 3320; Advanced Instruments). Plasma protein was measured by protein refractometry (Refractometer Veterinary ATC, VWR International).

Fos immunocytochemical studies. To determine whether lack of the TRPV1 and/or TRPV4 genes disrupted neuronal activation in the lamina terminalis, Fos immunoreactivity was quantified in the OVLT, SFO, and median preoptic nucleus.

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ml, 4°C). Brains were postfixed overnight in 4% paraformaldehyde (4°C), transferred to 30% sucrose for 1–2 days, and sectioned at 40 μm using a vibratome. Sections were collected into two serially adjacent sets and stored in 10 mM phosphate-buffered saline at 4°C for 2–3 days or cryoprotectant (52) at −20°C. Fos immunoreactivity was visualized as described previously in our laboratory (44, 46) using a rabbit Fos polyclonal anti-Fos antibody (1:10,000, PC-38; EMD Biosciences), ABC Vectastain Kit (Vector Laboratories, Burlingame, CA), and Ni-enhanced DAB reaction. The number of Fos-positive nuclei was quantified from a representative section(s) of the OVLT, dorsal MnPO, ventral MnPO, and two sections in the SFO. Counts from the two levels of SFO or dorsal and ventral MnPO were averaged or combined, respectively.

**Genotyping.** Genomic DNA was isolated from tail snips (0.5 cm) using DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). The primers used for TRPV1 genotyping were as specified by The Jackson Laboratory (oIMR297, 5'-AGGAGACTAGTGAGACGTG-3'; oIMR1561, 5'-CTGCCTCACACATGCTATTGG-3'; oIMR1562, 5'-TCCTCAGCTAGCATCAGA-3') at a concentration of 0.2 μM (oIMR297 and oIMR1561) or 0.4 μM (oIMR1562). The PCR reaction mixture contained 20 mM Tris (pH 8.0), 50 mM KCl, 1.5 mM MgCl2, 0.2 mM each dNTP, 2% DMSO, 1 unit Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA), and ~100 ng of genomic DNA. PCR cycling conditions were 94°C (3 min) followed by 35 amplification cycles (94°C, 30 s; 55°C, 1 min; 72°C, 1 min) and 72°C (10 min). The WT and null alleles generated either a 984-bp or a 600-bp product, respectively. The primers for the TRPV4 PCR (5'-CATGAAATCTGACCTCTTGTCCCC-3' and 5'-TGTGCTACCTGCACACCCAGGC-3') were used at a concentration of 0.4 μM. PCR reaction mixture contained 1× Herculase buffer (Stratagene, La Jolla, CA), 0.2 mM each dNTP, 2% DMSO, 1.25 units Herculase Enhanced DNA polymerase, 0.6 units Platinum Taq DNA Polymerase (Invitrogen), and ~100 ng of genomic DNA. PCR cycling conditions were 94°C (3 min) followed by 35 amplification cycles (94°C, 30 s; 55°C, 1.5 min; 72°C, 2 min) and 72°C for 10 min. The wild-type and null alleles generated either a 2.1- or 1.1-kb product, respectively. The PCR reaction product (15 μl) was ran on a 1.5% agarose gel containing 0.5 μg/ml ethidium bromide for 1 h at 70 V, and the PCR products were visualized with a ultraviolet transilluminator.

**Statistical analysis.** Values are expressed as means ± SE. Cumulative water intakes were analyzed in two ways: 1) two-way ANOVA (strain and time) between WT and a single strain to assess whether a strain differed from WT, and 2) a two-way ANOVA (strain and time) across all four strains. Both statistical analyses yielded the same results. The reported P values reflect the latter method. Post hoc tests were made using a layered Bonferroni with correction (Systat 10.2, Systat Software). All other data (Fos-positive nuclei, electrolytes) were analyzed by a two-way ANOVA with independent t-tests with a layered Bonferroni analysis when significant F values were obtained.

**RESULTS**

**Genotyping of WT, TRPV1−/−, TRPV4−/−, and TRPV1−/−TRPV4−/− mice.** The PCR product generated from tail snips of WT contained the expected 984-bp and 2100-bp fragments corresponding to the TRPV1 and TRPV4 alleles, respectively (Fig. 1). TRPV1−/− mice displayed the shorter fragment or null allele at 600 bp for TRPV1 gene. TRPV4−/− mice had the predicted null allele at 1100 bp for the TRPV4 gene. Double-knockout or TRPV1−/−TRPV4−/− displayed both null alleles for the TRPV1 and TRPV4 gene.

**Effect of TRPV1 and/or TRPV4 gene deletion on osmotically stimulated thirst.** A major goal of the present study was to determine whether deletion of the TRPV1 and/or TRPV4 gene disrupted thirst stimulated by cellular dehydration and produced by injection of hypertonic NaCl. Acute injection of 0.5 and 1.0 M NaCl produced dose-dependent increases in water intake across all strains including WT, TRPV1−/−, TRPV4−/−, and TRPV1−/−TRPV4−/− mice (Fig. 2). However, there were no differences in water intake across strains at any time. This observation was consistent regardless of the route of injection (intraperitoneal versus subcutaneous, Fig. 2A, experimental series 1) or whether mice had delayed versus immediate access to water (Fig. 2, A vs. B, experimental series 1 vs. 2). In addition, there were no differences in water intake across strains after a control injection of 0.15 M NaCl. Time-control studies (experimental series 2), in which water intake was measured but no injection was performed, indicate there were no differences in water intake across strains at 120 min (WT: 0.03 ± 0.02 ml, TRPV1−/−: 0.01 ± 0.01 ml, TRPV4−/−: 0.02 ± 0.02 ml, TRPV1−/−TRPV4−/−: 0.04 ± 0.03 ml, P > 0.9 from overall ANOVA). In addition, there were no differences in body weight across groups or cumulative water intakes when normalized to 20 g body weight (data not shown).

Analysis of plasma electrolytes and plasma osmolality after injection of NaCl indicate there were no differences across WT, TRPV1−/−, TRPV4−/−, and TRPV1−/−TRPV4−/− mice (Fig. 3). Injection of 0.5 or 1.0 M NaCl produced dose-dependent increases in plasma [Na⁺], [Cl⁻], and osmolality in all strains. Again, there were no differences across strains. As expected, plasma protein concentration decreased significantly after injection of 0.5 or 1.0 M NaCl (data not shown).

Since the majority of studies investigating the intrinsic osmosensitivity of hypothalamic neurons have increased bath osmolality using mannitol rather than NaCl (11, 12), an additional set of acute experiments was conducted using hyperosmolality via addition of mannitol (0.7 M dissolved in 0.15 M NaCl) as a stimulus for thirst. Injection of 0.7 M mannitol (or 1.0 osmol/l) significantly increased water intake across all strains. However, there were no differences in cumulative water intakes across strains regardless of the route of injection (intraperitoneal or subcutaneously, Fig. 4A, experimental series...
ries I) or whether animals had delayed or immediate access to water (Fig. 4, A vs. B, experimental series I vs. 2).

When mice were given access to 2% NaCl, all strains ingested similar amounts over 48 h (WT: 6.5 ± 0.6 ml/day, TRPV1⁻/⁻: 7.2 ± 0.3 ml/day, TRPV4⁻/⁻: 5.9 ± 0.4 ml/day, TRPV1⁻/⁻ V4⁻/⁻: 5.8 ± 0.5 ml/day). When water bottles were returned to the cages, all groups drank similar amounts of water (Fig. 5).

It is noteworthy that similar statistical results were obtained for all experiments when cumulative water intakes were normalized to 20 g body weight (data not shown).

**Effect of TRPV1 and/or TRPV4 gene deletion on thirst stimulated by extracellular fluid volume deficits.** To determine whether deletion of the TRPV1 and/or TRPV4 resulted in a general disruption of thirst, cumulative water intakes were measured after injection of two agents commonly used to induce extracellular fluid volume deficits without changes in plasma electrolytes or osmolality. Injection of the β-adrenergic agonist isoproterenol increased water intake in all strains of mice, but cumulative water intakes were not different across strains (120 min: WT, 0.18 ± 0.03 ml; TRPV1⁻/⁻: 0.24 ± 0.03 ml; TRPV4⁻/⁻: 0.20 ± 0.04 ml; TRPV1⁻/⁻ V4⁻/⁻: 0.16 ± 0.02 ml; P > 0.3 from overall ANOVA). Injection of furosemide also increased water intake in all mice (120 min: WT, 0.78 ± 0.07 ml; TRPV1⁻/⁻: 0.66 ± 0.05 ml; TRPV4⁻/⁻: 0.79 ± 0.07 ml; TRPV1⁻/⁻ V4⁻/⁻: 0.57 ± 0.09 ml), but there were no statistical differences between groups (P > 0.1 from overall ANOVA). Similar statistical results were obtained for both isoproterenol and furosemide tests when cumulative water intakes were normalized to 20 g body weight (data not shown).

**Effect of acute hypernatremia on Fos activation in the forebrain lamina terminalis.** A final set of experiments was performed to determine whether deletion of the TRPV1 and/or TRPV4 gene attenuated Fos activation in the forebrain lamina terminalis in response to an acute sodium load. Few Fos-positive cells were observed in the OVLT, MnPO, or SFO of any strain after injection of 0.15 M NaCl (Fig. 6A). Injection of 0.5 M NaCl significantly increased the number of Fos-positive cells in all areas of all strains (Fig. 6B). However, there were no differences in the number of Fos-positive nuclei in the OVLT between WT versus TRPV1⁻/⁻ (P > 0.8), TRPV4⁻/⁻ (P > 0.4), or TRPV1⁻/⁻ V4⁻/⁻ (P > 0.3) mice treated with 0.5 M NaCl. Similarly, there were no differences in the MnPO between WT versus TRPV1⁻/⁻ (P > 0.8), TRPV4⁻/⁻ (P > 0.8), or TRPV1⁻/⁻ V4⁻/⁻ (P > 0.5) mice treated with 0.5 M NaCl. For SFO, there were no differences in the number of Fos-positive nuclei between WT and TRPV1⁻/⁻ (P > 0.2) or TRPV4⁻/⁻ (P > 0.02) mice treated with 0.5 M NaCl. However, the number of Fos-positive nuclei in the SFO was significantly less in TRPV1⁻/⁻ V4⁻/⁻ versus WT mice after treatment with 0.5 M NaCl compared to untreated controls (P < 0.05).
electrophysiology studies performed in vitro have suggested that the osmosensitivity of isolated OVLT neurons is a mechanical process mediated by the TRPV1, but not TRPV4, gene products (11, 12). However, the present study demonstrates that cellular dehydration produced by acute injection of hyperosmotic NaCl or mannitol produced similar increases in water intake in WT versus TRPV1−/− mice. This was a consistent observation regardless of the route of injection (intraperitoneal vs. subcutaneous) or whether mice had immediate versus delayed access to water. These findings confirm a previous report from our laboratory (46). The lack of a difference in cumulative water intakes cannot be explained by the ability of TRPV1−/− mice to excrete the NaCl load more efficiently as we (46) and others (11) have reported that acute NaCl injections produce similar increases in plasma [Na+] or osmolality between WT and TRPV1−/− mice. Indeed, the present study confirms this observation as plasma electrolytes and osmolality did not differ between WT and TRPV1−/− mice (or TRPV4−/− and TRPV1−/−V4−/−) after injection of 0.5 or 1.0 M NaCl. If osmoregulatory function was compromised in TRPV1−/− mice, it may be expected that an acute NaCl load should produce a greater increase in plasma electrolytes or osmolality due to a reduced capacity to concentrate urine and effectively excrete the NaCl load. Furthermore, compared with WT mice, TRPV1−/− mice did not have an elevated basal intake over the testing period, which could have masked a response to acute NaCl loads as reported previously (46). In addition, these mice drank normally in response to nonosmotic challenges such as isoproterenol or furosemide thereby suggesting that deletion of TRPV1 and TRPV4 channels did not alter thirst responses in general. Altogether, these findings suggest that TRPV1 channels do not play a pivotal role in thirst stimulated by cellular dehydration.

What explains the discrepancy between the previous study of Ciura and colleagues (11) versus the findings of the present study and Taylor and colleagues (46)? It is not clear but it is unlikely to be due to differences in experimental methods since the present study incorporated a variety of experimental protocols including those of previous studies (11, 46). The only apparent methodological difference is that Ciura and colleagues removed food at the time of injection, whereas the present study removed food 1 h before injections to limit the impact of recent food ingestion on subsequent water intake. While Ciura and colleagues (11) reported an attenuated water intake of TRPV1−/− versus WT mice after an acute NaCl load,
the reported difference was small (~20–25%). In other words, in the study by Ciura and colleagues (11), deletion of the TRPV1 gene slightly attenuated the water intake stimulated by acute hypernatremia. Yet, lesions of the lamina terminalis or anteroventral region of the third ventricle in mice have been reported to significantly reduce water intake to acute hypernatremia by >75% (20, 27). Collectively, these behavioral data would suggest that deletion of the TRPV1 gene produces slight or no differences in thirst stimulated by acute increases in plasma sodium concentration or osmolality.

TRPV4 channels have also been implicated in central and peripheral osmoregulatory processes (22, 23, 29). TRPV4 mRNA and immunoreactivity has been reported in the ventral lamina terminalis (23). Furthermore, Liedtke and colleagues (23) reported a smaller number of Fos-positive nuclei within the OVLT and a smaller water intake after an acute NaCl load (23) reported a smaller number of Fos-positive nuclei within the lamina terminalis (23). Furthermore, Liedtke and colleagues (23) reported a smaller number of Fos-positive nuclei within the lamina terminalis (23). Furthermore, Liedtke and colleagues (23) reported a smaller number of Fos-positive nuclei within the lamina terminalis (23).

In the current study, TRPV4 mRNA and immunoreactivity has been reported in the ventral lamina terminalis (23). Furthermore, Liedtke and colleagues (23) reported a smaller number of Fos-positive nuclei within the OVLT and a smaller water intake after an acute NaCl load in TRPV4−/− mice versus WT mice (15, 23). In the current study, TRPV4−/− mice ingested similar amounts of water as WT mice over a range of hypernatremic and hyperosmotic stimuli. TRPV4−/− and WT mice also displayed similar number of Fos-positive neurons after acute NaCl load. While these findings contradict the original report of Liedtke and colleagues (23), it does corroborate the in vitro electrophysiological data (12). Again, the reason for the discrepancy remains unclear. The prior study of Liedtke and colleagues (23) administered a single injection of 0.5 M NaCl (0.4 ml/10 g body wt ip) that stimulated a robust increase in water intake, osmolality, and Fos immunoreactivity. The magnitude of these changes exceeded those of the present study after injection of 0.5 or 1.0 M NaCl and likely attributed to the much larger NaCl load. Although TRPV4−/− mice drank significantly less than WT, the difference was again <15% (23). In the current study, thirst responses were tested over a range of hypernatremic and hyperosmotic stimuli using a number of different experimental protocols. Collectively, these findings suggest TRPV4 channels do not play a prominent role in thirst stimulated by hypernatremia or hyperosmolality.

Since previous reports indicated a role for both TRPV1 and TRPV4 channels in osmoregulatory thirst (11, 23), we measured cumulative water intakes in TRPV1−/−V4−/− mice (double knockout) after a number of different stimuli. Surprisingly, these mice ingested similar amounts of water versus WT mice in response to acute hypernatremia or hyperosmolality despite similar increases in plasma electrolytes and osmolality in response to 0.5 or 1.0 M NaCl. The number of Fos-positive nuclei was also not different between WT and TRPV1−/−V4−/− in the OVLT or MnPO after injection of 0.5 M NaCl; however, there was a statistically smaller number of Fos-positive cells within the SFO of TRPV1−/−V4−/− versus WT mice. This is consistent with the presence of osmosensitive neurons in the SFO (1, 2). Collectively, the combined deletion of TRPV1 and

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**Fig. 6.** A: means ± SE of Fos-positive nuclei in the lamina terminalis (OVLT), subfornical organ (SFO), and median preoptic nucleus (MnPO) of WT, TRPV1−/−, TRPV4−/−, and TRPV1−/−V4−/− mice after injection of 0.15 or 0.5 M NaCl (0.3 ml sc). Injection of 0.5 M NaCl increase the number of Fos-positive in all three brain regions of all strains (*P < 0.05). The only difference across strains was a smaller number of Fos-positive nuclei in the SFO of TRPV1−/−V4−/− versus WT mice (**P < 0.05). B: examples of Fos-positive nuclei in the OVLT, SFO, dorsal MnPO (dMnPO), and ventral MnPO (vMnPO) in WT, TRPV1−/−, TRPV4−/−, and TRPV1−/−V4−/− mice after injection of 0.5 M NaCl (0.3 ml sc). Black bar represents 100 μm in each area.
TRPV4 channels did not further disrupt thirst stimulated by hypernatremia or hyperosmolality.

In the present study, we used Fos immunocytochemistry as an index of neuronal activation within the lamina terminalis. The approach has been used by a number of investigators to identify neurons responsive to hypernatremia and other stimuli (21, 36, 40, 46). Although a common interpretation of Fos immunoreactivity is a synaptic and/or membrane depolarization, it is possible that neurons in the OVLT, SFO, and MnPO were responsive to the acute hypernatremia but might have displayed a significantly blunted discharge response in TRPV1−/−, TRPV4−/−, and TRPV1−/−V4−/− versus WT mice. Hypernatremia also stimulates the secretion of antidiuretic hormone from the posterior pituitary (5, 42), and TRPV channels on magnocellular neurons within the supraoptic nucleus have been postulated to participate in this response (5, 39). We did not quantify the number of Fos-positive neurons within the supraoptic (or hypothalamic paraventricular nuclei), but Fos activation was present in these regions across all strains (unpublished observations). Future experiments will be necessary to provide a comprehensive analysis of antidiuretic hormone (and oxytocin) secretion across a number of paradigms in these mice.

There are several explanations for the lack of apparent differences in thirst or Fos immunoreactivity between WT versus TRPV1−/−, TRPV4−/−, and TRPV1−/−V4−/− mice. First, TRPV1 and/or TRPV4 channels do not play a pivotal role osmosensation and thirst responses stimulated by cellular dehydration. Alternatively, another channel either plays a primary role or contributes only after deletion of the TRPV1 and TRPV4 gene at development to cause an adaptive mechanism within osmoregulatory circuits. For example, TRPV2 channels respond to changes in osmolality (24) and have been reported in the OVLT and SFO (33). In addition, extracellular hyperosmolality produces cellular shrinking and regulatory volume increase accomplished by the influx of inorganic and organic osmolytes (4, 8) and secondary movement of water. In magnocellular neurons, one such organic osmolyte postulated to participate in hypertonicity-evoked changes in electrical activity is taurine (5, 13). Whether such mechanisms contribute to osmoregulatory transduction in OVLT neurons has not been tested, but a recent study by Ciura and colleagues (12) suggests the osmosensitivity of OVLT neurons is strictly a mechanical process. As recently reviewed (50), a second possibility is extracellular acidification. In vascular smooth muscle cells or renal mesangial cells, hyperosmolality can increase extracellular pH (32, 43) and increase activity or expression of the Na+/H+ exchanger. This raises the possibility that Na+/H+ exchanger or acid-sensing ion channels could participate in osmoregulatory transduction. Presently, none of these possibilities has been directly tested. Finally, a series of recent reports suggest the central nervous system detects changes in [Na+] through the voltage-dependent Na+ channel (15, 16). While these studies report NaX knockout versus WT mice have a lower Na+ intake or preference during dehydration or lateral ventricle infusion of hypertonic NaCl, cumulative water intakes were not different between strains during these same experimental manipulations. Moreover, the threshold for activation of the NaX channel was reported 157 mM Na+. So far, the available data would not support a role for NaX channel as a central mediator of thirst responses to changes in osmolality or [Na+], but its function warrants further investigation.

A potential confounding variable in the present study is that the majority of experimental manipulations such as the injection of hypertonic NaCl or mannitol may produce visceral pain to subsequently alter thirst responses or central Fos immunoreactivity. Therefore, it is theoretically possible that TRPV1−/− and/or TRPV4−/− mice have an attenuated thirst response but this is masked by removal of the painful stimulus. While this might explain the lack of apparent differences between strains, it does not explain the discordance between the findings of Ciura and Bourque (11) versus the present study or Taylor and colleagues (46). In fact, such experimental manipulations have been consistently used in both rats and mice to investigate the central control of thirst and vasopressin secretion (6, 18, 20, 23, 46). It is also possible that nociceptive activity or the lack thereof may impact Fos immunoreactivity in circumventricular organs. However, we are unaware of a role for such structures in pain responses or central processing of nociceptive inputs. Finally, it is noteworthy that the electrophysiological studies performed to date have focused on the underlying osmosensitivity of OVLT neurons (11, 12). The exact contribution of OVLT neurons to osmoregulatory thirst may be species dependent. For example, classical studies performed in dogs reported that selective lesion of the OVLT greatly diminished thirst and vasopressin secretion stimulated by an intravenous infusion of hypertonic NaCl but not in response to hemorrhage (47, 48). Lesion of the SFO in dogs does not affect these thirst or vasopressin responses to hypertonic NaCl (49). Studies performed in sheep indicate that lesion of the OVLT only does not greatly disrupt osmoregulatory thirst (31). Instead, osmoregulatory thirst was attenuated in animals with combined lesion of the OVLT and SFO, OVLT, or MnPO, or the entire lamina terminalis (31). In rodents, lesions of the AV3V region largely attenuate or eliminate osmotically induced drinking (7, 20, 27), whereas lesion of the SFO produce small deficits in rodents (18, 25). To date, there are no data regarding the contribution of OVLT neurons as selective lesions have not been produced and evaluated. Collectively, these data highlight the potential species differences and multiple forebrain hypothalamic sites that may mediate osmoregulatory responses. Whether the osmosensory processes differs across these structures or between neurons participating in different osmoregulatory responses (thirst, vasopressin secretion, sympathetic nerve activity, natriuresis) remains to be determined.

**Perspectives and Significance**

In summary, deletion of the TRPV1 and/or TRPV4 gene did not disrupt thirst stimulated by acute hypernatremia or hyperosmolality. In addition, acute hypernatremia produced similar increases in the number of Fos-positive neurons within the lamina terminalis of WT, TRPV1−/−, TRPV4−/−, and TRPV1−/−V4−/− mice. Collectively, these findings suggest that products encoded by the TRPV1 and/or TRPV4 genes do not greatly contribute to osmoregulatory thirst. Moreover, these findings highlight that the molecular identity of putative signaling mechanisms by which neurons sense changes in osmotic pressure or cell volume to underlie osmosensory processes remains unknown.
THIRST IN TRPV1 AND/OR TRPV4 KNOCKOUT MICE

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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