Hypernatremia-Induced Vasopressin Secretion is not Altered in TRPV1−/− Rats

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Running Head: Normal VP Secretion in TRPV1−/− Rats

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Changes in osmolality or extracellular NaCl concentrations are detected by specialized neurons in the hypothalamus to increase vasopressin (VP) and stimulate thirst. Recent in vitro evidence suggests this process is mediated by an N-terminal variant of the transient receptor potential vanilloid type 1 (TRPV1) channel expressed by osmosensitive neurons of the lamina terminalis and vasopressinergic neurons of the supraoptic nucleus. The present study tested this hypothesis in vivo by analysis of plasma VP levels during acute hypernatremia in awake control and TRPV1−/− rats. TRPV1−/− rats were produced by a Zinc-finger-nuclease 2-bp deletion in exon 13. Intravenous injection of the TRPV1 agonist capsaicin produced hypotension and bradycardia in control rats, but this response was absent in TRPV1−/− rats. Infusion of 2M NaCl (1 mL per hr, IV) increased plasma osmolality, electrolytes, and VP levels in both control and TRPV1−/− rats. However, plasma VP levels did not differ between strains at any time. Furthermore, a linear regression between plasma VP versus osmolality revealed a significant correlation in both control and TRPV1−/− rats, but the slope of the regression lines was not attenuated in TRPV1−/− versus control rats. Hypotension produced by intravenous injection of minoxidil decreased blood pressure and increased plasma VP levels similarly in both groups. Finally, both treatments stimulated thirst; however, cumulative water intakes in response to hypernatremia or hypotension were not different between control and TRPV1−/− rats. These findings suggest that TRPV1 channels are not necessary for VP secretion and thirst stimulated by hypernatremia.

Keywords (3-5 words): thirst, antidiuretic hormone, hypernatremia, osmoreceptor
INTRODUCTION

Systemic hypernatremia stimulates thirst, changes in sympathetic nerve activity, and secretion of antidiuretic hormone or vasopressin (VP). Together, these responses normalize osmotic pressure and volume (3, 25, 31). This process is initiated by specialized osmosensitive neurons located in the organum vasculosum of the lamina terminalis (OVLT) and subfornical organ (SFO) (3, 14). Indeed, the presence of osmoreponsive or osmosensitive neurons in the OVLT and SFO is supported by both in vivo and in vitro electrophysiological studies (1, 2, 5, 7, 8, 16), immunocytochemical detection of the early intermediate gene c-Fos in response to hypernatremia (11, 17), and lesion studies to suggest that thirst and VP secretion in response to acute NaCl loads depend on the integrity of these structures (4, 13, 15, 24, 29, 30). In turn, OVLT and SFO neurons project mono- or polysynaptically onto numerous hypothalamic nuclei including both parvocellular and magnocellular neurons of the hypothalamic paraventricular nucleus (PVH) and supraoptic (SON). In the past decade, evidence suggests VP magnocellular neurons may also be intrinsically osmosensitive (3, 12). A series of in vitro electrophysiological studies have reported that hyperosmotic stimuli depolarize VP magnocellular neurons through a non-selective cation conductance (3, 18, 19, 22). Together, these osmosensory processes within the OVLT, SFO, and magnocellular neurons of the SON or PVH coordinate thirst and VP secretion during hyperosmotic conditions.
Recent studies have suggested that osmosensory transduction within OVLT and SON neurons is mediated by an N-terminal variant of the transient receptor potential vanilloid type 1 (TRPV1) channel (5, 6, 22, 34). *In vitro* patch-clamp recordings of isolated OVLT or SON neurons have shown that hyperosmolality increases action potential discharge through an inward cation current that is blocked by the broad spectrum TRPV antagonist ruthenium red or a selective TRPV1 blocker SB366791, or absent in TRPV1^{−/−} mice (5, 6, 22, 34). Despite these observations, *in vivo* evidence to support a prominent role for TRPV1 in osmosensory responses has been less convincing. First, thirst responses to acute NaCl loads are either normal (10, 28) or slightly attenuated (5) in TRPV1^{−/−} versus wild-type mice. Second, acute or chronic hypernatremia in TRPV1^{−/−} versus wild-type mice produces similar numbers of Fos-positive cells in the OVLT and SFO (10, 28) as well as VPergic neurons in the SON (28). On the other hand, Sharif-Naeini et al (22) reported that the relationship between serum VP levels and osmolality during access to 2% NaCl drinking solution was significantly blunted in TRPV1^{−/−} versus wild-type mice. Unfortunately, these blood samples were collected under halothane anesthesia (22). Therefore, this prompted the current set of experiments in which plasma VP levels were assessed in conscious TRPV1^{−/−} and control rats after acute IV infusion of hypertonic NaCl. This experimental paradigm has a distinct advantage as rats can be chronically instrumented to collect blood samples before and after stimuli without the confound of anesthesia, stress, or pain associated with sc or ip injections of hypertonic solutions.
MATERIALS AND METHODS

Animals. All of the experimental procedures conform 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. Male Sprague-Dawley rats (350-450 g, Charles River Laboratories) or TRPV1<sup>−/−</sup> rats (TGRS5530, Sage Research Laboratories, Boylestown, PA) were singly-housed in dedicated metabolic cages placed in a temperature-controlled room (22±1ºC) with a 12-hour light-dark cycle. TRPV1<sup>−/−</sup> rats were developed on a Sprague-Dawley background and have a 2-bp deletion in exon 13 using a Zinc Finger Nuclease. Animals exhibit reduced expression of TRPV1 protein in the brain homogenates and increased foot licking latency to thermal heat (see www.sageresearchlabs.com or www.horizondiscovery.com). Rats were fed standard chow (Harlan Teklad Global Diet 2018), given access to deionized water, and singly housed in metabolic cages for 1 wk before any procedures. There were no differences in 24-h water intakes between groups (control: 30.3±2.3mL vs TRPV1<sup>−/−</sup>: 28.6±1.5mL; n=8 per group).

General Procedures. Animals were anesthetized with isoflurane (2-3% in 100% O₂) and instrumented with femoral arterial (microrenathane 0.012” x 0.025”) and venous (silastic 0.023” x 0.037”) catheters fused to tygon microbore tubing. Catheters were tunneled subcutaneously to exit between the scapulae and led through a tether-swivel-harness system (Instech Laboratories). Animals were treated with buprenex (0.03 mg/kg, sc), ampicillin (100 mg/kg, sc), and carprofen (5 mg/kg, sc) and allowed to
recover for at least 3 days before experiments began. Arterial catheters were flushed daily with heparinized saline (500 units/mL). Venous catheters were flushed once every 3 days with heparinized saline (40 units/mL).

Confirmation of TRPV1 Knock-Out. TRPV1\(^{-/-}\) knockouts were confirmed in two ways. First, ear biopsies were collected, digested, and analyzed through a standard PCR treated with Exo-SAP. The PCR product was isolated and subsequently sequenced by Sage Research Laboratories to confirm the 2-bp deletion from exon 13. Samples were collected at Penn State College of Medicine and sent to Sage Research Laboratories in a randomized, blinded design. Second, ABP and heart rate responses to the TRPV1 agonist capsaicin (0, 0.25, and 0.5 µg, IV bolus in 0.2mL separated by 10 min) were measured in control and TRPV1\(^{-/-}\) rats. Peak responses (1s) were compared to a 2 min baseline period.

Plasma VP and Thirst Experiments. Control and TRPV1\(^{-/-}\) rats received 2 different treatments in a randomized order separated by a minimum of 3 days. Food and water were removed ~1hr before experiments began. First, acute hypernatremia was produced by IV infusion of 2M NaCl (1.0mL/hr). Second, hypotension was produced by IV injection of the arteriolar vasodilator minoxidil (5 mg/kg, IV) and used as a non-osmotic stimulus for VP secretion. Blood samples (1.0mL) were collected at baseline, 30 and 60 min from the arterial line into microcentrifuge tubes containing heparin (10 units) and then centrifuged (10,000 x g). The plasma was stored at -80°C until VP levels
were determined. Plasma osmolality was measured in triplicate by freezing-point
depression (Advanced Instruments Inc, Model 3320). Plasma electrolytes were
analyzed using whole blood using ISTAT and 6+ cartridges (Abbott). Each blood sample
was replaced by an equal volume (1.0mL) of isotonic saline (first sample) or red blood
cells from the previous sample resuspended in heparinized saline (40units/mL) at 37°C.
After the 60 min blood sample, water bottles were returned to the cages. Cumulative
water intakes and urine outputs were measured every 15 min for the next 60 min. ABP
and heart rate were monitored throughout the experimental protocol using a BPM-832
ABP device (CWE, Inc) and Spike2 software (CED).

Analysis of Plasma VP Levels. Plasma VP levels were determined by ELISA (Enzo
Life Sciences). Briefly, samples were extracted using C$_{18}$ Sep-Pak Cartridges (1mL, 50
mg; Waters Milford MA) as described previously (21) through a 4% acetic acid wash
and eluted with a 3:1 acetonitrile:4% acetic acid solution. The extract was frozen, dried
using a Speed Vac (Savant Instruments), and then reconstituted in assay buffer (Enzo
Life Sciences). Although 100 µL samples are used in the ELISA, 200 µL samples were
extracted and reconstituted with 100µL buffer to double the sample concentration and
increase sensitivity of the assay. Each sample was assayed in duplicate. Values are
expressed as pg per mL of plasma. Recovery of VP was 87±2% (n=8) in preliminary
trials using I$_{125}$-VP. Intra- and inter-assay coefficients of variance were 8% and 4%
respectively. Sensitivity was 1.42 pg/mL. A subset of baseline samples for control and
TRPV1$^{+/−}$ rats (n=4-5 per experiment) fell below the sensitivity value but displayed
absorbance values above the 0 pg/mL standard. Values presented in the results represent extrapolated values based on the standard curve.

**Statistical Analysis.** Data are expressed as mean±SEM. All variables were analyzed by a one- or two-way ANOVA with repeated measures (Systat 10.2, Systat Software). When significant F values were obtained, independent or pair t-tests with a layered Bonferroni correction were performed. Plasma VP concentrations were measured in duplicate, averaged, and log-transformed. Linear regression analysis was performed between plasma VP and osmolality (Systat 10.2, Systat Software). A P value <0.05 was considered significant in all tests.
RESULTS

Confirmation of TRPV1⁻⁻ Rats. Figure 1 illustrates the 2bp deletion in Exon 13 of TRPV1⁻⁻ rats that was confirmed by DNA sequencing of ear biopsies. To confirm that this 2 bp deletion resulted in a disruption of TRPV1 channel function, ABP and heart rate responses to IV injection of capsaicin were tested in control and TRPV1⁻⁻ rats. IV injection of capsaicin produced a dose-dependent decrease in mean ABP and heart rate of control rats (Figure 1B). These responses were abolished in TRPV1⁻⁻ rats. There were no differences in baseline mean ABP (control: 113±4 vs TRPV1⁻⁻: 106±4 mmHg) and heart rate (control: 392±9 vs TRPV1⁻⁻: 398±19 bpm).

Effect of Acute Hypernatremia on Plasma VP Levels in Control and TRPV1⁻⁻ Rats. IV infusion of hypertonic NaCl (2M, 1 mL/h) significantly increased plasma osmolality and sodium concentration of both control and TRPV1⁻⁻ rats at 30 and 60 min after start of the infusion (Figure 2). There were no significant differences between groups at any time. The infusion of 2M NaCl also significantly increased mean ABP but did not affect heart rate of control and TRPV1⁻⁻ rats. Again, there were no differences between groups at any time. Plasma VP levels of control and TRPV1⁻⁻ rats increased significantly above baseline values at 30 and 60 min. However, there were no significant differences in plasma VP levels between control and TRPV1⁻⁻ rats at baseline or 30 and 60 min after start of the IV infusion of 2M NaCl.
As expected, a linear regression analysis revealed a significant correlation between plasma VP or log plasma VP levels versus plasma osmolality of control rats (Figure 3A). Interestingly, the same analysis revealed a significant correlation between these same variables of TRPV1<sup>-/-</sup> rats (Figure 3B). In fact, this relationship was not blunted in TRPV1<sup>-/-</sup> rats as revealed by the slope of the linear regression line.

When water was returned to the cages after the 60 min blood sample, both control and TRPV1<sup>-/-</sup> rats ingested significant amounts of water (Figure 4). However, the 60-min cumulative water intakes were not different between strains (P>0.1). Urine volume was also not different between groups before or after water access (Figure 4).

**Effect of Arterial Hypotension on Plasma VP Levels in Control and TRPV1<sup>-/-</sup> Rats.**

Both control and TRPV1<sup>-/-</sup> rats were also treated with the arteriolar vasodilator minoxidil to stimulate VP secretion and thirst independent of plasma osmolality or sodium concentrations. IV injection of minoxidil significantly decreased mean ABP and increased heart rate in both control and TRPV1<sup>-/-</sup> rats (Figure 5). There were no differences in mean ABP or heart rate between groups at any time. Plasma VP levels significantly increased from baseline values in both control and TRPV1<sup>-/-</sup> rats. However, there were no differences between groups at 0, 30, or 60 min (Figure 5). When water bottles were returned to the cages after the 60 min blood sample, control and TRPV1<sup>-/-</sup> rats ingested significant amounts of water. The amount of ingested water did not differ between groups. Urine volume did not differ between groups before (control: 0.27±0.15 vs TRPV1<sup>-/-</sup>: 0.1±0.1 mL) or during access to water (control: 0.1±0.1 vs TRPV1<sup>-/-</sup>:
0.1±0.1 mL). Minoxidil produced a small but significant increase in plasma osmolality of control and TRPV1−/− rats (Table 1). However, there were no differences between groups. Plasma sodium concentrations did not change at any time.
DISCUSSION

In vitro electrophysiological studies suggest a product of the TRPV1 gene contributes to osmosensory transduction in hypothalamic neurons to regulate VP secretion (5, 6, 22). The current study tested this notion in vivo by analysis of plasma VP levels during an acute NaCl load using unique TRPV1 \textsuperscript{-/-} rats. The present findings provide several novel observations: 1) a 2-bp deletion in exon 13 of the TRPV1 gene abolished the hypotensive and bradycardic responses to IV injection of the TRPV1 agonist capsaicin, 2) an acute NaCl load produced similar increases in plasma osmolality, electrolytes, and VP levels between control versus TRPV1 \textsuperscript{-/-} rats, 3) acute hypernatremia stimulated similar increases in water intake between strains, and 4) hypotension stimulated similar increases in plasma VP and thirst between control versus TRPV1 \textsuperscript{-/-} rats. Collectively, these observations suggest that TRPV1 channels are not necessary for VP secretion and thirst stimulated by hypernatremia in rats.

Previous in vitro electrophysiological studies suggest that TRPV1 channels contribute to the intrinsic osmosensitivity of both OVLT and VPergic SON neurons (5, 6, 22). Therefore, a plausible hypothesis is that TRPV1 channel dysfunction or deletion should disrupt osmotically-induced VP secretion and thirst. This hypothesis was tested in unique TRPV1 \textsuperscript{-/-} rats created by a 2-bp deletion in exon 13 which abolished capsaicin-evoked cardiovascular responses. Although an acute NaCl load produced similar increases in plasma osmolality and Na\textsuperscript{+} concentrations, plasma VP levels were surprisingly unaffected in TRPV1 \textsuperscript{-/-} versus control rats. This conclusion was further
validated by a linear regression analysis of plasma VP (or log plasma VP) versus plasma osmolality. It is noteworthy that a previous study reported plasma VP levels of TRPV1\(^{-/-}\) versus wild-type mice were attenuated in response to 0-48 h ingestion of 2% NaCl solution (22). Although 2% NaCl solution will provide a mixed stimulus for VP secretion including both plasma hypernatremia and volume depletion, the reasons for the apparent discrepancies are unclear aside from methodological differences. First, these studies used two different species (rats versus mice) with different targeting for the TRPV1\(^{-/-}\) channel (2bp deletion in exon 13 versus pore-loop domain). Second, plasma VP samples of mice were collected using halothane anesthesia (22). The present study has several advantages including: 1) plasma hypernatremia was produced by an acute NaCl infusion, 2) blood sampling was performed within the same animal, and 3) VP samples were collected in conscious, unstressed animals. Altogether, the findings indicate that disruption of the TRPV1 channel does not attenuate VP secretion stimulated by hypernatremia.

There are several potential explanations for the discordant observations between the \textit{in vitro} electrophysiological studies of OVLT and SON neurons (5, 6, 22) versus the current findings. First, the \textit{in vitro} electrophysiological studies have exclusively employed hypertonic mannitol solutions to investigate mechanisms underlying the intrinsic osmosensitivity of OVLT and SON neurons (5, 6, 22). Although VP secretion can be stimulated by both hypernatremia and hyperosmolality, the cellular mechanisms underlying Na\(^+\)- versus osmo-sensing in magnocellular neurons may differ (32). This raises the possibility that TRPV1 channel may contribute to VP and thirst stimulated by hyperosmolality but not hypernatremia. However, we have previously reported that
injection of hypertonic mannitol stimulates the ingestion of water in both control and TRPV1−/− mice (10). Second, other cellular mechanisms may compensate in the TRPV1−/− rat including locally release of taurine from glial cells (9), actin filaments (35), or the NaX channel (23). Clearly, future research is needed to identify cellular mechanisms regulating osmoreceptor function during both hyperosmolality and hypernatremia.

Given the potential role of TRPV1 channels within osmosensitive sites, TRPV1−/− animals may display deficits in osmotically-induced thirst. Previous studies have reported thirst responses to acute NaCl loads are either normal (10, 28) or slightly attenuated (5) in TRPV1−/− versus wild-type mice. In the present study, TRPV1−/− versus control rats ingested similar amounts of water after an acute NaCl load. Moreover, if TRPV1 channels were necessary for osmoreceptor function, TRPV1−/− animals may display deficits in body fluid homeostasis during normal conditions. Previous studies have reported either a small elevation (22) or no difference (10, 28) in plasma osmolality and electrolytes of TRPV1−/− versus wild-type mice. In the present study, plasma osmolality and Na+ concentrations at baseline conditions did not differ between TRPV1−/− and control rats. Furthermore, there were no differences in baseline plasma VP levels or 24-h water intakes. Altogether, these findings suggest TRPV1−/− animals do not display profound deficits in body fluid homeostasis.

Hypotension was used as a non-osmotic experimental treatment to stimulate VP secretion and thirst. Injection of minoxidil significantly increased VP levels and stimulated the ingestion of water in both control and TRPV1−/− rats. Under this paradigm, VP secretion is largely mediated by baroreceptor unloading (27) whereas the stimulated water intake is mediated by activation of the renin-angiotensin system and the central
dipsogenic action of angiotensin II (20, 26, 27). There was no a priori reason to hypothesize these responses would be affected in TRPV1\(^{-/-}\) rats. Instead, the paradigm was chosen as a control experiment to stimulate VP secretion and thirst independent of osmoreceptor function. Neither response was affected by deletion of TRPV1 in rats.

Experimental Limitations

The current study employed a novel KO rat in which a 2bp deletion in exon 13 was introduced to disrupt TRPV1 channel function. These animals display expected deficits such as thermal pain insensitivity (see www.horizondiscovery.com), altered brain activation to paw injection capsaicin (33), and an absence of cardiovascular responses to IV injection of capsaicin (Figure 1). Despite these deficits, the current experiments yielded negative findings in regard to hypernatremia-induced VP secretion. Since the 2bp deletion was present at development, it is possible that compensatory mechanisms such as expression of other channels preserve responses to body fluid homeostatic challenges. Furthermore, a recent report (34) provides strong in vitro evidence that a N-terminal variant of the TRPV1 channel underlies the electrophysiological responses of hypothalamic neurons to hypertonic mannitol. This TRPV1 variant lacks exons 1-4, is capsaicin-insensitive, but does contain exon 13. In the present study, it is not clear whether the 2bp deletion of exon 13 introduces a mutant mRNA or protein. Unfortunately, we are unaware of a pharmacological tool or agonist independent of mannitol to test whether the 2 bp deletion of exon 13 results in a non-functional protein. Thus, the experiments employed capsaicin to examine TRPV1
function in general. Although the results confirm functional disruption of the TRPV1 channels, it remains possible that this 2bp deletion did not disrupt the function of the N-terminal variant implicated in osmosensory transduction. Clearly, additional research is needed to identify the molecular identity of the putative TRPV1 N-terminal variant as well as other potential targets that participate in hypernatremia-induced responses.

**Perspectives and Significance**

Osmotically-induced vasopressin secretion depends on the integrity of structures in the lamina terminalis (3, 14). In turn, these regions densely innervate magnocellular neurons of the SON and PVH to subsequently regulate VP secretion through glutamatergic inputs (3, 14). Although previous studies indicate that TRPV1 channels underlie osmosensory transduction in multiple neuronal populations within this circuit, the present in vivo findings indicate that hypernatremia-induced VP secretion is unaffected by disruption of TRPV1. These findings agree with recent observations suggesting that TRPV1 deletion does not affect hypernatremia-induced thirst. These studies may highlight potential differences between osmosensory transduction versus Na\(^+\)-sensing.
ACKNOWLEDGEMENTS

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GRANTS

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DISCLOSURES

None
REFERENCES


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FIGURE CAPTIONS

Figure 1. (A) Sequence analysis of control and TRPV1^{−/−} rats. The numbers correspond to the sequence of the wild-type gene (NCBI NC_005109). Note the 2 bp deletion (grey region) present in TRPV1^{−/−} rats of exon 13. (B) IV bolus administration of capsaicin produced a dose-dependent decrease in mean ABP and heart rate of control rats. The hypotension and bradycardia were absent in TRPV1^{−/−} rats. *P<0.01 vs control rats.

Figure 2. (LEFT) Acute infusion of 2M NaCl (1mL/h, IV) produced a time-dependent increase in plasma osmolality and sodium concentration in both control and TRPV1^{−/−} rats. *P<0.05 vs 0 min. (MIDDLE) Infusion of 2M NaCl produced a significant increase in mean ABP of both control and TRPV1^{−/−} rats but did not statistically alter heart rate. (RIGHT) Infusion of 2M NaCl significantly increased plasma VP levels in control and TRPV1^{−/−} rats. However, there were no differences between groups. *P<0.05 vs 0 min.

Figure 3. Linear regression analysis between plasma VP or log plasma VP versus plasma osmolality of (A) control and (B) TRPV1^{−/−} rats during infusion of 2M NaCl. Correlational coefficients (r^2) and slopes are reported for each plot. As expected, there was a significant correlation between plasma or log plasma VP concentrations versus plasma osmolality in control rats (P<0.01). A significant correlation was found between plasma or log plasma VP levels versus plasma osmolality in TRPV1^{−/−} rats (P<0.01).
Figure 4. (TOP) 60-min cumulative water intake and (BOTTOM) urine output of control and TRPV1−/− rats during IV infusion of 2M NaCl (1mL/h). There were no differences between groups in water intake (P>0.1) or urine volume before (P>0.8) or during access (P>0.1) to water.

Figure 5. (LEFT) Mean ABP and heart rate of control and TRPV1−/− rats at baseline and after IV injection of minoxidil (5 mg/kg). Minoxidil significantly decreased mean ABP and increased heart rate in both groups. The magnitudes of these changes were not different between control and TRPV1−/− rats. (RIGHT) Minoxidil increased plasma VP and water intake in control and TRPV1−/− rats. There were no significant differences between groups. *P<0.05 vs 0 min
**TABLES**

*Table 1. Mean±SEM of plasma osmolality and sodium concentrations of control and TRPV1⁻/⁻ rats treated with minoxidil (5 mg/kg, IV). *P<0.05 vs 0 min*

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Figure 1
Functional Test of KO

A, Sequence of Exon 13

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B

- Mean ABP (mmHg)
  - IV Capsaicin (ug)
    - 0
    - 0.25
    - 0.5
    
- Heart Rate (bpm)
  - IV Capsaicin (ug)
    - 0
    - 0.25
    - 0.5

* * * *
Figure 2
Acute Hypernatremia

2M NaCl
Control (n=10)
TRPV1−/− (n=12)
Figure 3
Scatter Plot of VP

A, Control

B, TRPV1−/−
Figure 4
Water Intake - Hypernatremia

- TRPV1−/− (n=11)
- Control (n=10)
Figure 5
Hypotension