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The Molecular and Cellular Identity of Peripheral Osmoreceptors

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SUMMARY

In mammals, the osmolality of the extracellular fluid (ECF) is highly stable despite radical changes in salt/water intake and excretion. Afferent systems are required to detect hypo- or hyperosmotic shifts in the ECF to trigger homeostatic control of osmolality. In humans, a pressor reflex is triggered by simply drinking water which may be mediated by peripheral osmoreceptors. Here, we identified afferent neurons in the thoracic dorsal root ganglia (DRG) of mice that innervate hepatic blood vessels and detect physiological hypo-osmotic shifts in blood osmolality. Hepatic sensory neurons are equipped with an inward current that faithfully transduces graded changes in osmolality within the physiological range (~15 mOsm). In mice lacking the osmotically activated ion channel, TRPV4, hepatic sensory neurons no longer exhibit osmosensitive inward currents and activation of peripheral osmoreceptors in vivo is abolished. We have thus identified a new population of sensory neurons that transduce ongoing changes in hepatic osmolality.

INTRODUCTION

The extracellular fluid (ECF) osmolality is tightly regulated in mammals and homeostatic reflexes maintain the osmotic set-point by promoting salt/water intake or excretion. For such reflexes to function effectively, osmoreceptors are required to detect changes in ECF osmolality. Central osmoreceptive neurons located in brainstem nuclei that largely lack a blood-brain barrier play a crucial role in osmoregulation (Bourque, 2008). Electrophysiological studies have shown that osmosensitive magnocellular neurosecretory cells (MNCs), present in the supraoptic nucleus, are excited by hyperosmotic stimuli and inhibited by hypo-osmotic stimuli (Oliet and Bourque, 1993). The transient receptor potential (TRP) channel member TRPV1 is required for the transduction of hyperosmotic stimuli in MNCs (Sharif Naeini et al., 2006) and by osmosensory neurons in the organum vasculosum laminae terminalis (Ciura and Bourque, 2006). However, osmoregulation still operates in *Trpv1*$^{-/-}$ mice; thus, other osmosensitive neurons or pathways must be able to compensate for loss of central osmoreceptor function (Ciura and Bourque, 2006; Sharif Naeini et al., 2006; Taylor et al., 2008).

Osmoreceptors also exist outside the central nervous system (Adachi, 1984; Adachi et al., 1976; Baertschi and Vallet, 1981; Choi-Kwon and Baertschi, 1991; Niijima, 1969; Vallet and Baertschi, 1982) and these so-called peripheral osmoreceptors could significantly contribute to the regulation of ECF osmolality. However, it is not clear which peripheral neurons function as osmoreceptors and the molecular mechanism by which they detect changes in osmolality is unknown. Much of the older work has concentrated on vagal afferent neurons activated by hyperosmotic stimuli (Adachi, 1984; Niijima, 1969). However, a recent series of studies has provided strong evidence that an autonomic reflex can be initiated by the activation of peripheral osmoreceptors, specifically by hypo-osmotic stimuli (Boschmann et al., 2003, 2007; Jordan et al., 1999, 2000; Lipp et al., 2005; Raj et al., 2006; Scott et al., 2000, 2001; Shannon et al., 2002; Tank et al., 2003). Thus, water drinking in man (intake of 500 ml), but also in mice, can initiate an acute pressor reflex together with increased sympathetic nerve activity and thermogenesis (Boschmann et al., 2007; Jordan et al., 2000; Lipp et al., 2005; McHugh et al., 2010; Scott et al., 2000; Tank et al., 2003). It has been suggested that there is an osmosensitive sensory system in the liver that signals hypo-osmotic stimuli via the DRG and spinal cord to evoke reflex responses (Tank et al., 2003). Such a peripheral osmosensitive system has not been studied directly in animal models, although there is indirect evidence for its existence (Vallet and Baertschi, 1982; McHugh et al., 2010). In the present study, we established an animal model in which the activation of peripheral osmoreceptors could be monitored under realistic physiological conditions. We

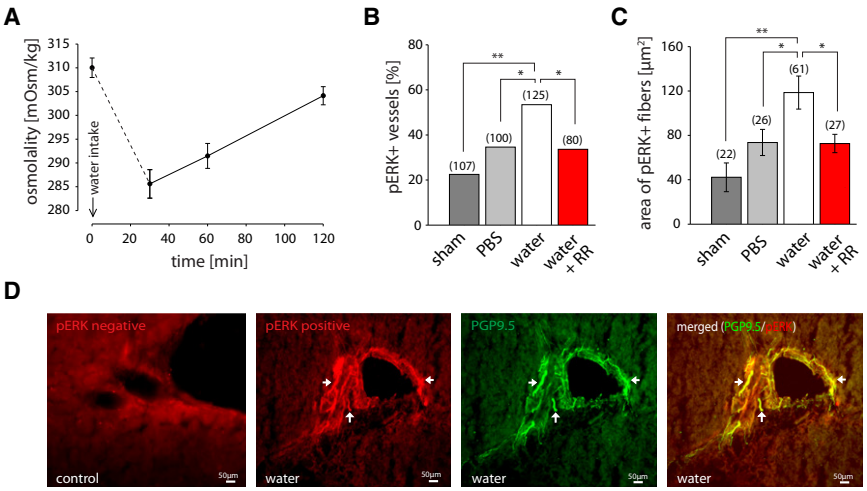


Figure 1. Water Intake Leads to Acute Activation of Hepatic Afferents In Vivo

(A) Blood osmolality in the hepatic portal vein before (time = 0) and after (t = 30, 60, and 120 min) intake of 1 ml water.

(B) Quantification of the percentage of hepatic blood vessels innervated by pERK-positive fibers after sham drinking, water intake, PBS intake and water intake laced with 100 μM RR. Numbers in parentheses = number of analyzed vessels (*$p < 0.05$; **$p < 0.01$; chi-square test).

(C) Graph shows the mean area of pERK-positive fibers in the same groups as in c (*$p < 0.05$; **$p < 0.01$; Student's t test).

(D) Fluorescence micrographs of pERK staining (red channel) around blood vessels before (control) and after water intake (water), antibodies against PGP9.5 were used to identify nerve endings (green).

identified a population of osmosensitive hepatic sensory afferents, which have cell bodies in the thoracic DRG. These neurons can detect very small hypo-osmotic shifts in the osmolality of blood flowing through the liver after water intake. Intriguingly, hepatic sensory neurons possess ionic currents activated by physiological shifts in osmolality; such osmosensitive currents have a pharmacological and biophysical profile similar to the transient receptor channel protein TRPV4. We could show that *Trpv4*$^{-/-}$ mice, lacking the TRPV4 protein, display no activation of osmoreceptors after water drinking. Furthermore, osmosensitive currents found in identified hepatic afferents are almost absent in the same neurons of *Trpv4*$^{-/-}$ mutant mice. Interestingly, patients who have undergone a liver transplant, in which the liver is devoid of osmoreceptor innervation, have a significantly higher baseline blood osmolality compared to a healthy control cohort. In summary, we have identified a peripheral neuronal population that detects physiological changes in osmolality in the liver and these neurons require TRPV4 to function as osmoreceptors.

RESULTS

Hypo-osmotic Stimulation Activates Hepatic Sensory Neurons

First, we established an animal model to study the physiological activation of peripheral osmoreceptors. We measured the magnitude of the osmotic stimulus induced in the hepatic circulation after an acute intake of 1 ml of water in the mouse (intake over < 1 min; Figure 1A). One milliliter of water corresponds to about 15% of the normal daily water intake, which is on average 6 ml per day for C57BL/6J mice (Bachmanov et al., 2002). Drinking a volume of 500 ml of water is sufficient to activate a pressor response and thermogenesis in humans (Boschmann et al., 2007; Jordan et al., 1999, 2000), and this volume also corresponds to ~15% of daily water consumption (between 2.5 and 3.5 l per day). The basal blood osmolality in the hepatic portal vein of the mouse was 310.0 ± 2.1 mOsm/kg (n = 7), and 30 min after water intake this had decreased to 285.6 ± 3.0 mOsm/kg (n = 5), an 8% change in osmolality. The hepatic portal vein blood osmolality recovered to basal levels within 2 hr after water intake (Figure 1A). The osmolality changes in the portal vein are probably determined purely by the absorption and clearance of the absorbed water bolus over time. We measured the activation of liver afferent endings using immunostaining with antibodies directed against the phosphorylated (activated) form of extracellular-signal related protein kinase (pERK). This methodology has been successfully used to visualize the activation of nociceptive neurons in the skin within minutes following stimulation (Dai et al., 2002). Increased pERK immunostaining has been observed in sensory afferents to a variety of natural stimuli, is dependent on electrical activity, and is probably a consequence of increases in intracellular calcium consequent to action potential firing (Dai et al., 2002; Fields et al., 1997). Thirty minutes after water intake (1 ml), the fixed liver was removed. We chose the 30 min time point as this time point was coincident with the maximum osmotic stimulation of hepatic afferents (Figure 1A). We observed pERK positive fibers surrounding hepatic blood vessels and double staining with the neuronal marker PGP9.5 confirmed that these structures were nerve endings (Figure 1D). We quantified the activation of liver nerve fibers by measuring the percentage of blood vessels surrounded by pERK-positive fibers (Figure 1B) and the total area of immunopositive fibers (Figure 1C). Data was collected from at least four animals per group. The number and area of pERK-stained fibers increased significantly following intake of 1 ml water compared to a sham drinking control or to ingestion of 1 ml near-isotonic PBS ($p < 0.01$ compared to sham, $p < 0.05$ compared to PBS; Figures 1B and 1C). The absence of significantly increased pERK staining after intake of 1 ml PBS suggested that liver nerve fibers detect the decreased osmolality in the hepatic circulation and not stretch or pressure changes in the vessels. Members of the TRP ion channel family have been reported to be involved in osmosensation (Clapham et al., 2005; Everaerts et al., 2010). We thus let mice drink 1 ml of water doped with 100 μM Ruthenium red (RR) a broad blocker of TRP channels and observed no increase in pERK positive fibers compared to controls (Figures 1B and 1C).

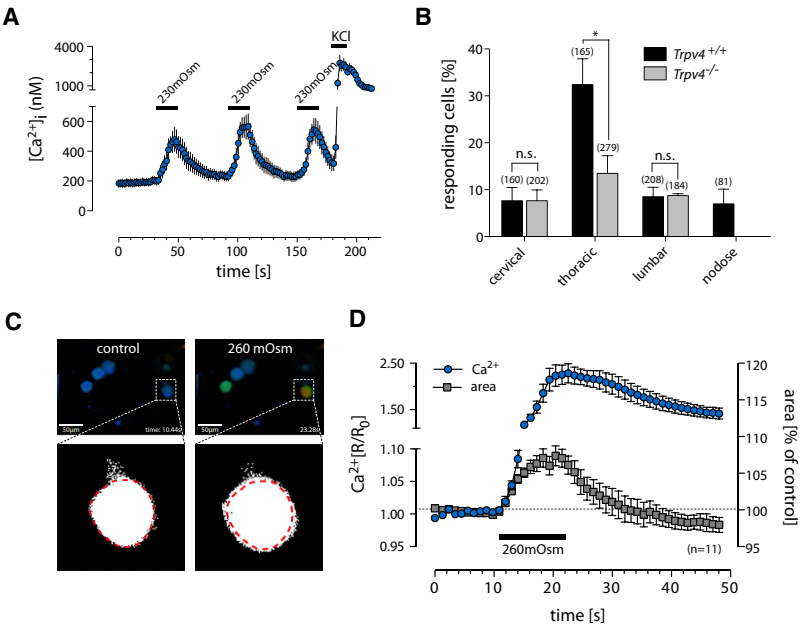


Figure 2. Thoracic DRGs Are Enriched in Osmosensitive Neurons

(A) Average changes in $[Ca^{2+}]_i$ induced by repeated hypotonic stimulation (22 s/230 mOsm) of thoracic DRG neurons measured with Ca^{2+}-imaging (n = 11); KCl was used to demonstrate cell viability. Note that repeated hypo-osmotic stimulation leads to a constant amplitude response.

(B) Proportions of osmosensitive DRG neurons in different spinal regions (cervical, thoracic and lumbar) and in nodose ganglia (average from six animals per genotype). Numbers in parentheses = total number of neurons tested from each spinal region. Osmosensitive neurons are enriched in thoracic DRGs (**$p < 0.01$, Student's t test).

(C) Images show examples for cell swelling in response to hypotonic stimulation (see also Movie S1). Top: ratio images before and during hypotonic stimulation; bottom: close up of the indicated cell after conversion into binary images (see Experimental Procedures). Red dashed line depicts the contour of the cell shape under control conditions.

(D) Time course of cell swelling (gray squares) and increases in $[Ca^{2+}]_i$ (blue circles). Note that significant increases in $[Ca^{2+}]_i$ occur concurrent with changes in cell volume.

Hypo-osmotic Stimulation of Thoracic DRG Neurons Induces $[Ca^{2+}]_i$ Increases

The liver is innervated by vagal afferents and thoracic DRG neurons (Berthoud, 2004; Carobi and Magni, 1985; Magni and Carobi, 1983). We therefore analyzed the osmosensitivity of acutely isolated vagal nodose neurons and DRG neurons from different spinal levels (cervical C4–C7, thoracic T7–T13, and lumbar L1–L6) using Fura-2-based Ca^{2+} imaging. Repeated stimulation with hypo-osmotic solutions (230 mOsm for 20 s) induced robust and reversible increases in the intracellular calcium concentration $[Ca^{2+}]_i$ in neurons from all levels with little evidence of tachyphylaxis (Figure 2A). Interestingly, the incidence of osmosensitive neurons was low in all ganglia tested (lumbar DRG 9.1%, cervical DRG 11.2%, and nodose 9.9%) with the notable exception of the thoracic ganglia, where 31% of the neurons showed responses to hypo-osmotic stimuli (Figure 2B). Most cell types increase their volume when exposed to hypo-osmotic fluids (Hoffmann et al., 2009); we thus examined the temporal correlation between cell swelling and changes in $[Ca^{2+}]_i$. Cell swelling was quantified by measuring changes in the area of the fluorescence signal after background subtraction and conversion into binary images (Figure 2C). This analysis revealed that significant increases in $[Ca^{2+}]_i$ occurred concurrent with detectable changes in cell volume (Figure 2D; see Movie S1 available online). Interestingly, not all cells that exhibited cell swelling also showed a calcium response (Movie S1). We next investigated the mechanism underlying the increase in $[Ca^{2+}]_i$. Removal of extracellular calcium almost completely abolished the Ca^{2+}-response to hypo-osmotic stimulation, whereas depletion of intracellular calcium stores by a 30 min incubation with 5 μM thapsigargin neither changed the proportion (32% of the neurons responded), nor the magnitude of the Ca^{2+}-response (Figure 3A). Blocking voltage-gated calcium channels with 100 μM Cd^{2+} did not affect Ca^{2+}-signals (Figure 3A). Consistent with our in vivo pERK findings (Figures 1B and 1C), we also observed that $[Ca^{2+}]_i$ increases were almost completely blocked with RR (100 μM) (Figure 3A). We also used Gadolinium, Gd^{3+} (10 μM) (Clapham et al., 2005; Watanabe et al., 2003), which had a reversible inhibitory effect on hypo-osmolar-induced changes in $[Ca^{2+}]_i$ (Figure 2D).

Together, these results strongly suggested that Ca^{2+} influx through a TRP-like ion channel may underlie the hypo-osmolar-induced changes in $[Ca^{2+}]_i$ that we observed in thoracic DRG neurons. One TRP-ion channel that has frequently been reported to be activated by hypotonic stimulation is TRPV4 (Everaerts et al., 2010). We therefore examined the osmosensitivity of sensory neurons from mice lacking TRPV4. Strikingly, the proportion of osmosensitive cells in thoracic ganglia was significantly decreased to 13.5% ± 3.7% ($p < 0.05$, compared with 32.4% ± 5.5% in WT mice; Student's t test), levels normally observed in lumbar and cervical ganglia. No changes were observed in ganglia from other spinal levels in *Trpv4*$^{-/-}$ mice (Figure 2B). Hence, in the absence of TRPV4 most osmosensitive neurons lose their responsiveness to hypo-osmotic stimulation, suggesting that TRPV4 is required for normal osmosensitivity in these cells.

To corroborate these findings we next tested whether osmosensitive cells are also sensitive to Phorbol 12,13-didecanoate (4α PDD), a phorbol ester that is reported to be a selective TRPV4 agonist (Vriens et al., 2004; Watanabe et al., 2002; Figure 3B). In order to verify that the agonist-induced effects were mediated by TRPV4, experiments were carried out in both *Trpv4*$^{+/+}$ and *Trpv4*$^{-/-}$ mice. Cells were first challenged with a 260 mOsm solution for 40 s to assess their osmosensitivity followed by a 100 s application of 10 μM 4α PDD. Using this protocol, we identified neurons with five different response profiles (Figure 3B, left panel). Among the osmosensitive neurons only one group responded with a reversible increase in $[Ca^{2+}]_i$ to stimulation with 4α PDD, these

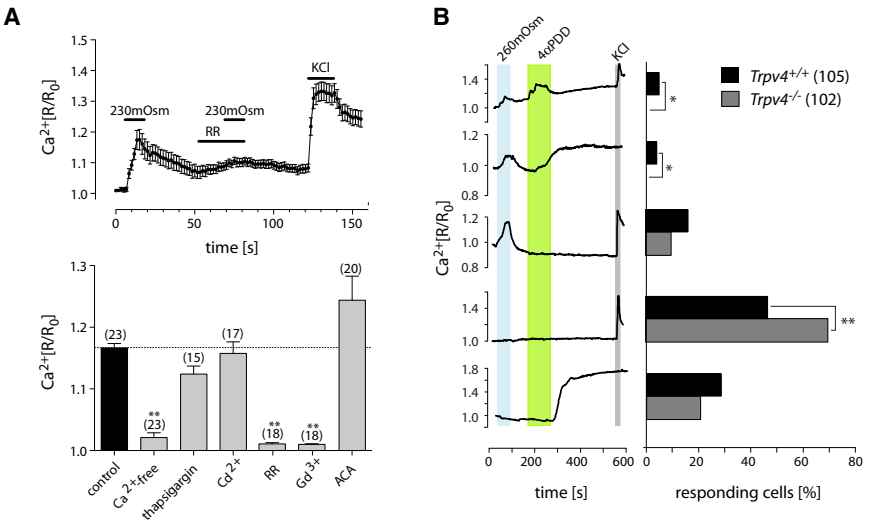


Figure 3. Ca^{2+} Responses Require Extracellular Calcium and Are Inhibited by TRP Channel Blockers

(A) Top: Example trace showing inhibition of the Ca^{2+} response by Ruthenium red (RR, 10 μM). Bottom: Summary of the pharmacological experiments; Ca^{2+} responses evoked with a 230 mOsm stimulus. Note that $[Ca^{2+}]_i$ signals are reversibly blocked by RR and Gd^{3+}, require external Ca^{2+}, but are not abolished by thapsigargin, which depletes intracellular calcium stores. Ca^{2+} responses were preserved in the presence of Cd^{2+} (100 μM) and the PLA2 inhibitor ACA (20 μM) (**$p < 0.01$; Student's paired t test).

(B) Examples of the response profiles found with a protocol where neurons were stimulated with a 260 mOsm solution followed by 4α PDD (10 μM) and 40 mM KCL (left panel). The percentage of neurons from *Trpv4*$^{+/+}$ and *Trpv4*$^{-/-}$ mice that display each type of response profile is shown on the right. Neurons responding to both 4αPDD and to osmotic stimuli are absent in *Trpv4*$^{-/-}$ mice.

neurons were relatively rare in wild-type cultures (~5%), but were never observed in cultures from *Trpv4*$^{-/-}$ mice (Figure 3, top row). In the second osmosensitive group the $[Ca^{2+}]_i$ increases induced by 4α PDD was irreversible, possibly due to toxicity (Mochizuki et al., 2009; Vriens et al., 2004), and again this population was never observed in cultures from *Trpv4*$^{-/-}$ mice (Figure 3, second row). The third group of osmosensitive neurons (10%–18% of the total) exhibited no calcium response to stimulation with 4α PDD, consistent with lack of functional TRPV4 channels in these osmosensitive neurons (Figure 3, middle row). Of the neurons lacking an osmosensitive $[Ca^{2+}]_i$ response, one group showed no response to 4α PDD (Figure 3, fourth row), and the second group only displayed a delayed and irreversible $[Ca^{2+}]_i$ response (Figure 3, bottom row). There were significantly more neurons that lack 4α PDD responsiveness and osmosensitivity in cultures made from *Trpv4*$^{-/-}$ mice compared to controls (Figure 3, fourth row). Taken together these experiments demonstrate that there are indeed osmosensitive sensory neurons with functional TRPV4 channels in the thoracic ganglia. The small numbers of neurons with clear pharmacological evidence of TRPV4 channels is probably due to toxicity of 4α PDD on up to 34% of the tested neurons. The activation of TRPV4 is thought to be mediated by phospholipase A2 (PLA2) and can thus be inhibited by the PLA2 inhibitor 3N-(p-amyl-cinnamoyl)anthranilic acid (ACA) (Vriens et al., 2004). However, hypo-osmotically induced Ca^{2+} increases in thoracic DRG neurons were not significantly altered by 20 μm ACA (Figure 3A). This lack of inhibition by ACA has not been observed for recombinantly expressed TRPV4 channels (Vriens et al., 2004) and thus it may well be that the mode of activation of TRPV4 in the physiological context of osmoreceptors is distinctive.

A Fast and Sensitive Inward Current Codes Physiological Shifts in Osmolality

These results strongly suggested that the Ca^{2+}-response was mediated by a Ca^{2+} influx through a TRP-like ion channel, most probably TRPV4. Hence osmosensitive neurons, as determined using Ca^{2+}-imaging, should also exhibit an inward current in response to hypo-osmotic stimulation. To test this hypothesis we combined simultaneous Ca^{2+}-imaging with whole-cell patch-clamp recordings. Strikingly, in 12 from 12 tested osmosensitive thoracic neurons, increases in $[Ca^{2+}]_i$ were accompanied by a fast activating inward current (Figure 4A). To test whether this osmosensitive current is carried, at least in part, by calcium ions and thus directly mediates the calcium signal we next examined its current-voltage relationship. Therefore, neurons were step depolarized from −60 mV to +20 mV for 200 ms (to inactivate voltage-gated sodium channels) followed by a 200 ms ramp depolarization from −100 mV to +100 mV (Figure 4B, inset). The osmosensitive currents reversed at membrane potentials around 0 mV (−6.7 ± 3.3 mV, n = 5), which is characteristic for nonselective cation channels. To rule out a possible contribution of swelling-activated chloride channels because such currents reverse at similar potentials under the recording conditions employed (Nilius et al., 2001), we substituted extracellular chloride with gluconate. However, changing the driving force for chloride did not affect the osmosensitive current (Figure 4D). We next applied a series of osmotic stimuli of decreasing osmolalities (260–290 mOsm) to determine the osmolality dependence of the inward current (Figure 4C). This experiment showed that the current was half-maximally activated with a stimulus of just 278.9 ± 0.6 mOsm (n = 17), which was well within the range of physiological changes in blood osmolality following water intake (Figure 1A). Hence the osmosensitive current found in thoracic DRG neurons is an excellent candidate as the primary detector of rapid and physiological changes in osmolality in hepatic blood vessels. Osmosensitive currents were substantially inhibited by 10 μM RR and Gd^{3+} (>60% reduction), and importantly the effects of both drugs were completely reversible (Figure 4D). Thus the osmosensitive current has a pharmacology very similar to that of the hypo-osmolar induced $[Ca^{2+}]_i$ increases seen in thoracic sensory neurons (Figure 2D).

Our whole-cell patch-clamp recordings from wild-type thoracic neurons indicated that many were sensitive to hypo-osmotic stimulation (67.6%; 25/37 tested neurons). Strikingly,

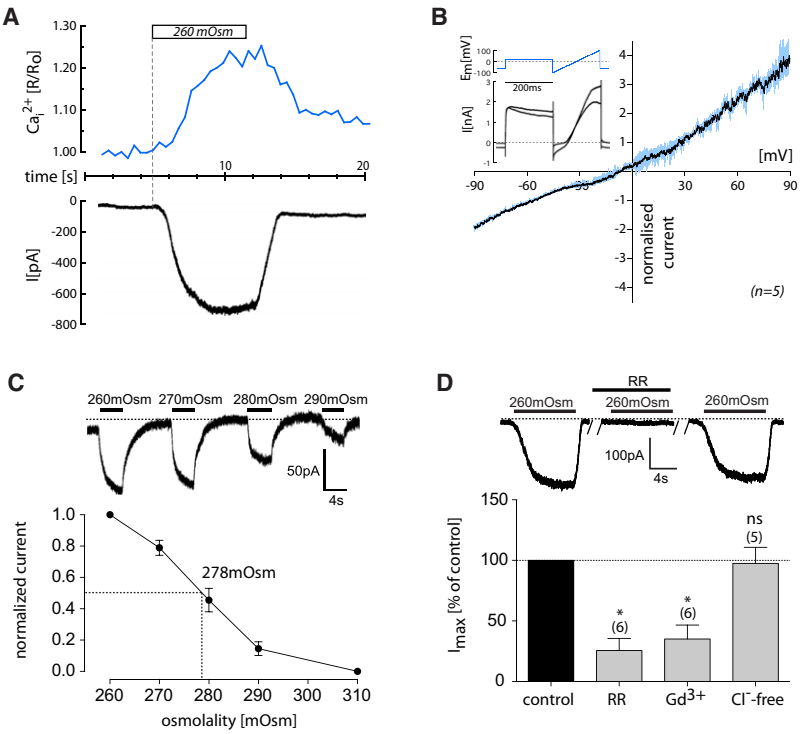


Figure 4. Thoracic Sensory Neurons Possess a Fast Osmosensitive Inward Current

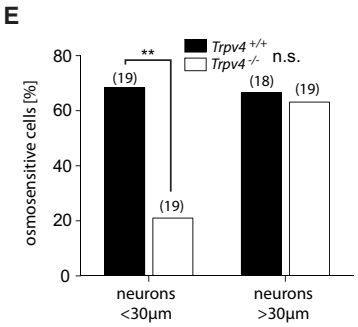
(A) Simultaneous Fura-2 calcium-imaging (top trace) and whole-cell patch-clamp recording (bottom trace) during hypo-osmotic stimulation. Ca^{2+} signals occur concurrent with a fast inward current.

(B) I-V relationship of the osmosensitive inward current (mean of five neurons); I-V curves were obtained by subtracting current responses to ramp depolarizations (−100 mV to +100 mV) under control conditions from those during osmotic stimulation (see inset).

(C) Whole-cell currents from a thoracic DRG neuron challenged successively with hypotonic stimuli (260, 270, 280, 290 mOsm; bath osmolality, 310 mOsm). Graph indicating the osmolality at which the half maximum current amplitude was reached. Current responses were normalized to the current amplitude of an intercalated 260 mOsm stimulus.

(D) Top: Typical osmosensitive current that can be reversibly inhibited with RR (10 μM). Bottom: Quantification of changes in current amplitudes produced by application of RR and Gd^{3+} or by replacing extracellular chloride with gluconate (Cl^--free).

(E) The incidence of neurons with an osmosensitive current in *Trpv4*$^{+/+}$ and *Trpv4*$^{-/-}$ mutant mice according to neuronal size.



there were significantly fewer neurons with an osmosensitive inward current in thoracic ganglia isolated from *Trpv4*$^{-/-}$ mice (42.1%; 16/38; $p < 0.05$; Chi-square test). Closer analysis of this population revealed that in wild-type ganglia both large and small neurons exhibit osmosensitive currents (66.6% > 30μm, n = 19; 68.4% < 30 μm, n = 18; Figure 4E), whereas in cultures prepared from *Trpv4*$^{-/-}$ mice only large neurons were osmosensitive (63.2%, n = 19) and only a small fraction of small neurons possessed osmosensitive currents (21.1%, n = 19). Small thoracic neurons greatly outnumber large neurons in the DRGs (~90% < 30μm) and thus the number of neurons that lose osmosensitivity in *Trpv4*$^{-/-}$ mice is larger than the uncorrected estimates shown in Figure 4E.

TRPV4 Channels Are Found at the Site of Osmosensory Transduction

Our data suggested that there is an enriched population of osmosensitive sensory neurons in thoracic ganglia requiring TRPV4 for normal function. We thus asked if these osmosensitive neurons innervate the liver and whether TRPV4 channels are present at nerve endings in the liver. We first examined TRPV4 expression in liver sections. In wild-type mice, we detected substantial TRPV4 immunoreactivity surrounding the walls of ~46% (24/52) of the PGP9.5-positive hepatic blood vessels, which was completely absent in liver sections prepared from age-matched *Trpv4*$^{-/-}$ littermate controls (compare Figures 5A and 5B). PGP9.5 is a neuron-specific marker, and thus the strong colocalization of TRPV4 and PGP9.5 immunostaining (Figure 5A, right panel) suggests that TRPV4 is indeed present at sensory nerve endings that innervate hepatic blood vessels. Consistent with these data, we also noted an enrichment of TRPV4 messenger RNA in thoracic ganglia using qPCR (Figure S1). We also made use of a BAC transgenic mouse in which EGFP is expressed under the control of the α3 nicotinic acetylcholine receptor (Gong et al., 2003) to test whether osmosensitive neurons innervate the liver. The EGFP expression pattern in the DRG of these mice was remarkable, as green fluorescent cells were highly enriched in the thoracic ganglia but were rare in cervical and lumbar ganglia (Figure 5C). Consistent with this observation, we observed that EGFP-positive fibers were rare or absent in non-visceral organs such as the skin in the BAC transgenic mice (data not shown). Interestingly, EGFP-positive fibers and cell bodies were largely negative for the lectin marker of nonpeptidergic sensory nerves isolectin-B4 (Belyantseva and Lewin, 1999; Figure 5C). Some of the EGFP-positive neurons in the thoracic ganglia also likely innervate the liver, because liver

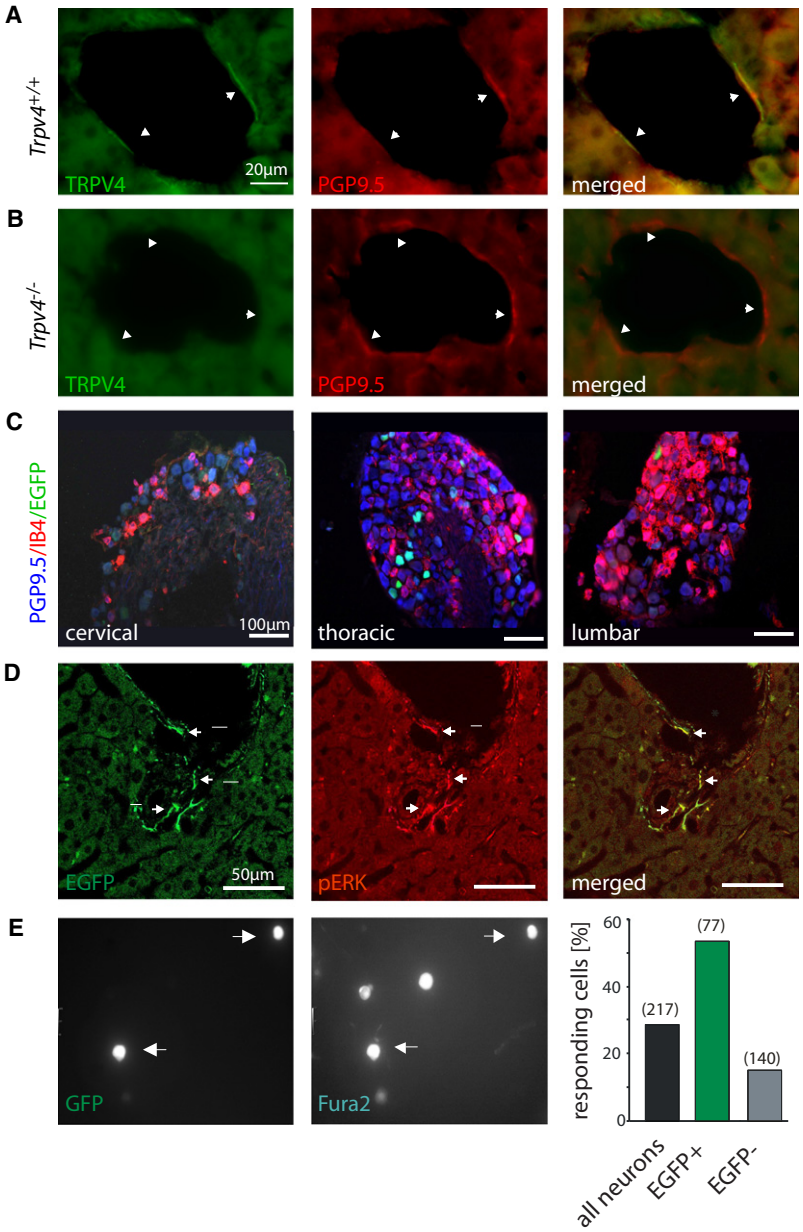


Figure 5. Hepatic Sensory Endings Are TRPV4 Positive

(A and B) Examples for TRPV4 and PGP9.5 immunoreactivity around *Trpv4*$^{+/+}$and *Trpv4*$^{-/-}$hepatic blood vessels (arrowheads).

(C) Fluorescence images of DRG sections from cervical, thoracic, and lumbar spinal regions of α3nAChR-EGFP mice. Sections were stained for PGP9.5 (blue) and IB4 (red), and green cells express EGFP. Scale bar = 100 μm.

(D) Confocal fluorescence micrograph of a hepatic blood vessel of an α3nAChR-EGFP mouse after water intake. The EGFP-positive fibers are also positive for pERK (red). Arrows indicate stained fibers. Scale bar = 50 μm.

(E) Fluorescence Fura-2 (380 nm) and GFP (488 nm) images of the same thoracic DRG neurons from α3nAChR-EGFP mice in culture (left). Arrows indicate EGFP-positive cells. Right: Quantification of the total number of thoracic neurons with an increase in $[Ca^{2+}]_i$ to a 230 mOsm stimulus taken from α3nAChR-EGFP mice. Number of analyzed cells is shown in parenthesis.

See also Figure S1.

blood vessels receive substantial innervation from EGFP-positive fibers (Figure 5D). Furthermore, the EGFP-positive fibers in the liver appear to be osmosensitive as they exhibit robust increases in pERK staining after intake of 1 ml water in transgenic mice (Figure 5D). In Ca^{2+}-imaging experiments a striking 53% of the acutely isolated thoracic EGFP-positive neurons responded to the 230 mOsm stimulus (41/77 neurons), a significantly higher proportion than found in EGFP-negative thoracic neurons (15%, 21/140 neurons) and of nonselected neurons in wild-type mice (31%, 62/217 neurons), $p < 0.01$ and $p < 0.05$ chi-square test (Figure 5E). Thus, osmosensitive neurons are enriched within the population of EGFP-positive thoracic DRGs many of which innervate the liver. However, though very supportive, these results did not clarify whether cells that give rise to pERK-positive fibers in vivo and cells that are osmosensitive in cultures constitute the same subpopulation of thoracic DRG neurons.

Osmosensitive Currents in Hepatic Sensory Neurons Are Dependent on TRPV4

To test directly whether thoracic neurons with an osmosensitive current are hepatic osmoreceptors, we retrogradely labeled hepatic sensory neurons by injecting an Alexa Fluor-488 conjugated dextran amine into the liver. Three to four days after tracer injection into the liver, the thoracic ganglia were isolated and whole-cell patch-clamp recordings made from identified hepatic afferent neurons (Figure 6A). As a control for tracer leakage, DRGs were cultured from nearby spinal segments, but no labeled neurons were found. Strikingly, almost all the hepatic afferent neurons 91.3% (21/23 cells) possessed a fast inward current in response to local perfusion of a 260 mOsm hypo-osmotic solution (Figures 6A and 6B, green bars). The proportion of osmosensitive neurons among the labeled hepatic afferent neurons (21/23 cells) was significantly higher than that found in randomly selected neurons from T7–T13 DRGs (25/37 cells, $p < 0.05$; Student's unpaired t test; Figure 6B, black bars). In contrast, identified hepatic afferent neurons from *Trpv4*$^{-/-}$ mice only infrequently exhibited an inward current to hypo-osmotic stimulation 31.6% (6/19 cells), significantly different from wild-type neurons 91.3% (21/23 cells) chi-square test $p < 0.01$. Interestingly, the remaining osmosensitive currents found in *Trpv4*$^{-/-}$ mice were kinetically indistinguishable from those found in wild-type mice (Figure S2). However, closer inspection of osmosensitive and nonosmosensitive neurons revealed that these two classes of neurons were

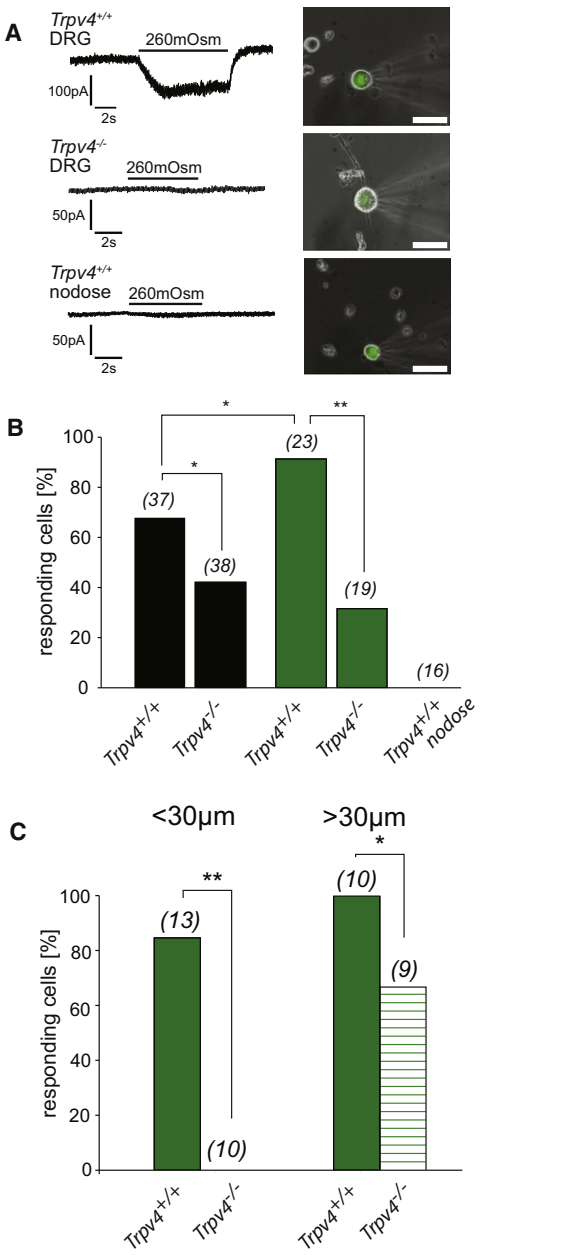


Figure 6. Hepatic Afferent Osmosensitivity Requires TRPV4

(A) Left: Example current traces of retrograde labeled *Trpv4*$^{+/+}$ and *Trpv4*$^{-/-}$ thoracic DRG neurons and of *Trpv4*$^{+/+}$ nodose ganglia. Right: Fluorescence/brightfield merge micrographs of retrogradely labeled neurons; scale bar = 50 µm.

(B) Proportions of neurons from nonlabeled thoracic DRGs (black bars), retrograde-labeled thoracic DRGs (green bars), and retrograde-labeled nodose ganglia (0%, bar not visible) with an osmosensitive current (260 mOsm stimulus). Number of tested cells in parentheses.

(C) Proportions of osmosensitive neurons shown separately for neurons with soma diameters larger and smaller than 30 µm.

See also Figure S2.

also different in other respects. Identified hepatic sensory neurons that had lost their osmosensitivity in *Trpv4*$^{-/-}$ mice were significantly smaller (23.9 ± 0.8 µm versus 35 ± 1.6 µm, $p < 0.001$, Student's paired t test) and had wider action potentials (3.35 ± 0.18 ms versus 1.60 ± 0.11 ms, $p < 0.001$, Student's paired t test) than neurons that retain their osmosensitivity. Moreover, the small TRPV4-dependent osmosensitive neurons were more excitable as they fired an average of 2.9 ± 0.2 action potentials in response to an 80 ms/320 pA current injection, whereas large TRPV4-independent neurons never fired more than one action potential. Thus, none of the hepatic sensory neurons with cell diameters <30 µm have an osmosensitive current in *Trpv4*$^{-/-}$ mice (Figure 6C). This finding might be explained by a loss of osmosensitive neurons in *Trpv4*$^{-/-}$ mice, however, we did not observe fewer retrogradely labeled sensory neurons in the mutants. Furthermore, the mean cell diameter of retrogradely labeled neurons in wild-type and *Trpv4*$^{-/-}$ mutant neurons was not different (wild-type 28.9 ± 1.2 µm; *Trpv4*$^{-/-}$ 29.5 ± 1.4 µm, $p > 0.7$ Student's t test). Osmosensitive afferent fibers innervating the liver can also originate from the nodose ganglion (Adachi, 1984; Adachi et al., 1976; Niijima, 1969). Consistent with the literature we found retrogradely labeled neurons among dissociated nodose neurons, however, none of the identified hepatic nodose neurons exhibited an inward current to hypo-osmotic stimuli (n = 16 in 5 mice) (Figure 6B). These data strongly suggest that small diameter thoracic neurons innervating the liver represent a specialized osmosensory population.

Hepatic Osmoreceptors Require TRPV4 for Normal Function

We next asked whether putative hepatic osmoreceptors present in the thoracic ganglia require the osmosensitive TRP-channel TRPV4 for function in vivo (Liedtke and Friedman, 2003; Mizuno et al., 2003). We measured the blood osmolality in the hepatic portal vein of *Trpv4*$^{-/-}$ mice and found that baseline values are elevated (316.4 ± 1.8 mOsm/kg in *Trpv4*$^{-/-}$ compared to controls 310.0 ± 2.1 mOsm/kg, $p < 0.05$ Student's t test) (Figure 7A). A similar increase in basal blood osmolality has previously been reported for mice lacking TRPV1 (Sharif Naeini et al., 2006) and could be confirmed in our measurements of hepatic portal vein blood osmolality in *Trpv1*$^{-/-}$ mice (Figure 7A). Thus *Trpv1*$^{-/-}$ mice served as controls to ensure that differences observed in *Trpv4*$^{-/-}$ mice were not solely due to increased basal blood osmolality. The osmolality change in the hepatic portal vein after intake of 1 ml of water in both *Trpv4*$^{-/-}$ and *Trpv1*$^{-/-}$ mice after 30 min was ~6%, decreasing to 298.7 ± 1.3 and 297.2 ± 1.4 mOsm/kg, respectively. We used pERK staining to ask if hepatic afferents were activated following water intake in *Trpv4*$^{-/-}$ and *Trpv1*$^{-/-}$ mice. Similar to wild-type mice, we observed both an increase in the number of pERK-positive vessels (Figure 7C) and an increase in the area of pERK-positive fibers (Figure 7D) in *Trpv1*$^{-/-}$ mice. These results demonstrated that the reduced magnitude of the osmotic stimulus in these mice (Figure 7A), is sufficient to activate hepatic sensory afferents. Strikingly, we observed no increase in the percentage of vessels with pERK positive fibers and no increase in the area of pERK staining in *Trpv4*$^{-/-}$ mice after water intake compared to mutant sham-treated mice or mutant mice following intake

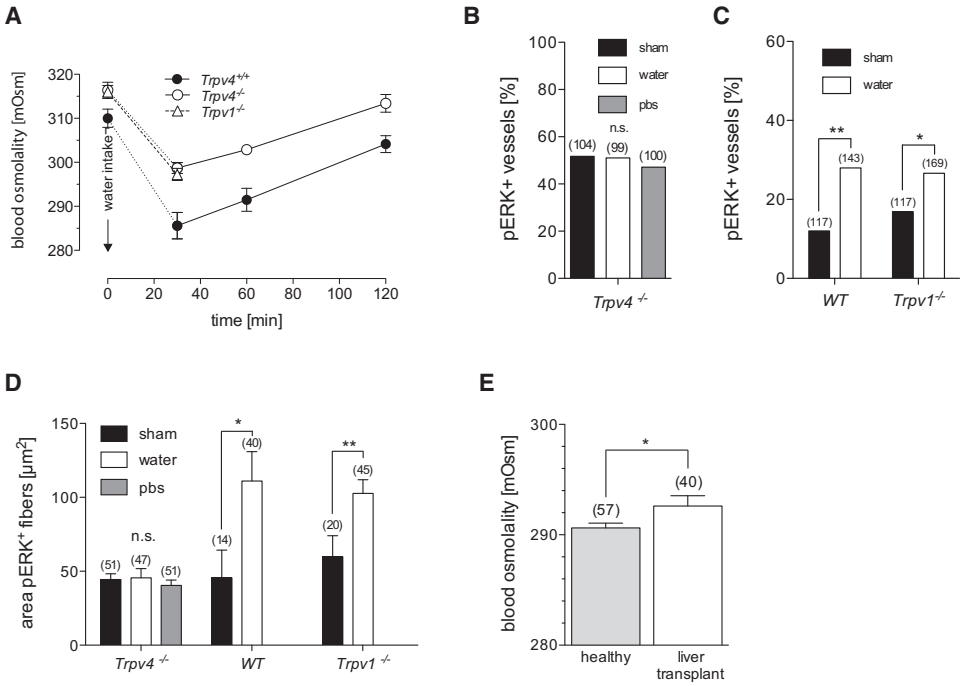


Figure 7. TRPV4 Is Required for Peripheral Osmoreceptor Function

(A) Blood osmolality in the hepatic portal vein before (time = 0) and after administration of 1 ml water in *Trpv4*$^{+/+}$, *Trpv4*$^{-/-}$, and *Trpv1*$^{-/-}$ mice.

(B and C) Quantification of the proportion pERK stained fibers around hepatic blood vessels from *Trpv4*$^{-/-}$ mice, wild-type (WT) and *Trpv1*$^{-/-}$ mice after water drinking compared to PBS and sham controls (*$p < 0.05$; **$p < 0.01$; Fisher's exact test).

(D) Quantification of the average area of pERK-positive fibers after water drinking compared to PBS and sham controls (*$p < 0.05$; **$p < 0.01$; Student's t test).

(E) Blood osmolality in human patients after liver transplantation compared to healthy controls (*$p < 0.05$; Student's t test).

of 1 ml of near isotonic PBS (Figures 7B and 7D). It should be noted, however, that the percentage of hepatic blood vessels innervated by pERK positive fibers was larger in unstimulated *Trpv4*$^{-/-}$ mice, than in controls (compare Figure 7B with Figure 7C). However, the area of pERK positive fibers per blood vessel was comparable between *Trpv4*$^{-/-}$ mice and controls (compare Figure 7B with Figure 7C). It may be that basal activation of pERK in sensory fibers is already altered in the absence of TRPV4 channels. Together these results indicate that the presence of TRPV4 is necessary for hypo-osmotic stimuli to activate hepatic osmoreceptors.

To gauge the potential influence of hepatic afferents in regulating blood osmolality in humans, we conducted an investigation of a large cohort of liver transplant recipients. We found that the blood osmolality of the liver transplant group (n = 40, age range 21–74 years) was slightly (~2 mOsm), but significantly ($p < 0.05$ Student's t test) elevated compared to a control age matched cohort (n = 57, age range 23–61 years) (Figure 7E). The levels of the C-terminal pro-arginine-vasopressin (copeptin) were also significantly elevated in samples from liver transplantees (15 ± 4.8 pmol/l) compared to healthy controls (4.16 ± 0.4 pmol/l; $p < 0.01$, Student's t test). Copeptin is a stable marker of vasopressin levels, which is upregulated in patients with experimentally induced increases in blood osmolality (Szinnai et al., 2007), the upregulation seen here suggests normal central osmoregulation in liver transplantees. These findings, which will be followed up in a more detailed longitudinal clinical study, suggest that hepatic afferents may also contribute to human osmoregulation.

DISCUSSION

Here, we have identified a specific population of TRPV4-positive hepatic sensory afferents that detect physiological changes in blood osmolality. We show that the in vivo activation of hepatic afferents by physiological changes in hepatic portal vein osmolality is absent in *Trpv4*$^{-/-}$ mutant mice. Strikingly, hepatic thoracic sensory neurons possess a fast and sensitive osmosensitive current that can dynamically signal physiologically relevant hypo-osmotic shifts in blood osmolality. We have also shown that identified small hepatic sensory neurons completely lack the osmosensitive current in *Trpv4*$^{-/-}$ mice. The pharmacological and biophysical characteristics of the osmosensitive current in hepatic afferents together with the genetic evidence, suggests that this current is, at least in part, mediated by TRPV4 channels (Figures 2 and 6). In summary, we have characterized, in detail, the cellular and molecular properties of a novel population of hepatic afferents that in humans probably forms the afferent arm of an important regulatory reflex involved in the regulation of metabolism, blood pressure, and osmolality homeostasis.

Peripheral sensory neurons that function as osmosensors have received little attention, although there is literature indicating their existence in animals (Adachi, 1984; Adachi et al., 1976; Baertschi and Vallet, 1981; Bourque, 2008; Choi-Kwon and Baertschi,

1991; Vallet and Baertschi, 1982). An increasing body of evidence indicates that there is a specific population of peripheral osmosensory neurons, which represent the afferent arm of a complex reflex response triggered by water intake (Boschmann et al., 2003, 2007; Jordan et al., 1999, 2000; McHugh et al., 2010; Scott et al., 2000, 2001; Tank et al., 2003). We postulated that osmosensory neurons detect very small hypo-osmotic shifts in blood osmolality in the hepatic circulation following water intake. Using an activity marker, we could show that hepatic afferent fibers are activated by the small osmolality changes induced by physiological water intake in the mouse. The magnitude of the stimulus was comparable to that shown in healthy humans to rapidly activate a sympathetic reflex that can elevate blood pressure and increase metabolic rate (Boschmann et al., 2007; Jordan et al., 1999, 2000; Lipp et al., 2005; Scott et al., 2000, 2001). By analogy with our animal model, the water-evoked reflexes observed in humans are also probably mediated by hepatic osmoreceptors capable of detecting a decrease of just 8% in blood osmolality. A key feature of the osmoreceptors described here, is that they can signal changes in blood osmolality well before water intake impacts systemic blood osmolality. Systemic osmolality changes following water intake will be even smaller than what we observed in the hepatic portal vein and would follow the stimulus with some delay (Adachi et al., 1976; Baertschi and Vallet, 1981; Choi-Kwon and Baertschi, 1991). We used our animal model first to identify the cellular nature of the hepatic osmoreceptor and second to characterize the physiological and molecular nature of osmosensitive transduction in these neurons.

The liver is innervated by both vagal and thoracic sensory afferents (Carobi and Magni, 1985; Choi-Kwon and Baertschi, 1991; Magni and Carobi, 1983; Vallet and Baertschi, 1982). We show that virtually all identified hepatic sensory neurons in the thoracic ganglia possess an osmosensitive current whereas nodose sensory neurons innervating the liver do not (Figure 6B). Using a transgenic animal model in which EGFP is expressed by thoracic ganglion neurons innervating the liver, we have shown that the peripheral endings of hepatic neurons are activated by physiological changes in blood osmolality. Although almost all hepatic sensory neurons could be shown to be osmosensing, it is likely that many nonhepatic thoracic sensory neurons are also osmosensitive. Thus, normally only very few thoracic ganglion neurons are labeled by injection of fluorescent tracers into the liver (<5% of the ganglia). The high proportion of osmosensitive neurons in thoracic ganglia suggests that these neurons may project through the splanchnic nerves to provide an osmoreceptor innervation to other parts of the upper viscera. In this study, we have shown that the osmosensitive ion channel TRPV4 is necessary for hepatic osmoreceptor function and other studies have indicated that this channel is present and has a functional role in a wide range of visceral sensory afferents (Brierley et al., 2008; Cenac et al., 2008). In summary, we have identified a new population of hepatic sensory afferents that are capable of detecting local decreases in blood osmolality produced by physiological water intake.

To detect physiological changes in blood osmolality, hepatic sensory neurons must possess a sensitive osmosensing mechanism. We observed specific immunostaining for the osmosensitive TRP channel, TRPV4, in fibers surrounding hepatic vessels (Figures 5A and 5B) and could show that the in vivo activation of hepatic sensory afferents by physiological water intake is absent in *Trpv4*$^{-/-}$ mutant mice (Figure 7). Furthermore, hepatic osmoreceptors possess an inward cationic current that is activated by precisely the range of hypo-osmotic stimuli found in the portal circulation in vivo (Figures 1 and 4). The half-maximal activation of this inward current, which has rectification properties similar to many TRP channels (Figure 4B), could be observed with 278 mOsm solutions, which is ~9% lower than resting osmolality. The osmosensitive current was activated with a time course essentially identical to that of increases in $[Ca^{2+}]_i$, in addition the pharmacology of current response and calcium influx were indistinguishable (Figures 3 and 4). We show that the osmosensitive inward current is the major mechanism whereby Ca^{2+} initially enters the cell after osmotic stimulation (Figures 3A and 4A).

Here, we show that the TRPV4 ion channel is essential for normal sensory responses to hypo-osmotic stimuli. However, all published studies using either native cells or cell lines heterologously expressing TRPV4, show a very slow activation of TRPV4 by hypo-osmotic stimuli (minutes) compared to the fast (seconds) activation of the osmosensitive current described here (Cenac et al., 2008; Liedtke et al., 2000; Mochizuki et al., 2009; Nilius et al., 2001; Strotmann et al., 2000; Voets et al., 2002; Vriens et al., 2004; Watanabe et al., 2002, 2003). TRPV4 and the prototypical *Drosophila melanogaster* TRP can be activated indirectly by sensory stimuli, for example through the release of lipid products or second messengers (Hardie, 2007; Vriens et al., 2004; Watanabe et al., 2003). However, here we measured the kinetics of cellular activation and changes in cell volume simultaneously and observed a striking coincidence of increased $[Ca^{2+}]_i$ with increases in cell volume in specialized osmoreceptors (Figure 2D; Movie S1). These data suggests that TRPV4 is activated in osmoreceptors more directly than in other cells types, consistent with this idea hypo-osmotic stimuli induced $[Ca^{2+}]_i$ increases were not blocked by a PLA2 blocker as shown in other cell types (Vriens et al., 2004). It is possible that small changes in membrane curvature represent a mechanical stimulus that gates the osmosensitive current, indeed there are recent reports that TRPV4 may be directly gated by membrane stretch (Loukin et al., 2010). However, the distinctive nature of the TRPV4-dependent osmosensitive current in identified osmoreceptors, rather suggests that other proteins might confer the high speed and sensitivity to hepatic sensory afferents. For example, other TRP channel proteins are activated by hypo-osmotic stimuli and they might work together with TRPV4. It has been shown, for example, that members of the TRPC subfamily of TRP channels (Birnbaumer, 2009; Clapham et al., 2005; Gomis et al., 2008; Gottlieb et al., 2008; Spassova et al., 2006), notably TRPC5 and TRPC6, are activated by hypo-osmotic stimuli (Gomis et al., 2008; Spassova et al., 2006). In addition, heterologously expressed TRPV2 and TRPM3 can also confer sensitivity to hypo-osmotic stimuli (Grimm et al., 2003; Muraki et al., 2003). Indeed all of these osmosensitive TRPs are expressed by sensory neurons (Caterina et al., 1999; Gomis et al., 2008; Lechner et al., 2009), and so it is possible that such channels could account for the residual osmosensitivity that we have found in thoracic sensory neurons

from *Trpv4*$^{-/-}$ mutant mice. Very recently, a new class of putative mechanosensitive channel proteins called Piezos were found which are blocked by RR (Coste et al., 2010), such proteins might conceivably play some role in osmosensitivity. The TRPV1 channel is involved in the detection of hyperosmotic shifts in the ECF important for central osmoreception (Bourque, 2008; Sharif-Naeini et al., 2008). We now provide genetic evidence that this sensory expressed channel is not involved in peripheral osmoreception (Figure 7); thus, TRPV4 and TRPV1 appear to play entirely complementary roles in osmoreception.

We have identified the primary afferent neurons that constitute the afferent arc of a well-characterized reflex in man and more recently also in rodents (McHugh et al., 2010). This reflex engages the sympathetic nervous system to raise blood pressure and stimulate metabolism (Boschmann et al., 2003; Jordan et al., 1999, 2000; Lipp et al., 2005; Scott et al., 2000, 2001; Tank et al., 2003). Our finding that blood osmolality is raised in *Trpv4*$^{-/-}$ mice that lack normal peripheral osmoreceptor function suggests that peripheral osmoreceptors may well contribute to the ongoing regulation of blood osmolality. Indeed, we provide some evidence here that this may also be the case in humans as in a large cohort of human liver transplantees, who presumably have denervated livers, plasma osmolality is significantly elevated compared to healthy controls (Figure 7). However, to define the role of peripheral osmoreceptors in systemic osmolality regulation, it is clear that conditional deletion of TRPV4 only in peripheral sensory neurons will be required. Activating mutations in the human *Trpv4* gene were recently shown to be present in individuals with a surprising range of genetic disorders including skeletal dysplasias, but also Charcot-Marie-Tooth disease and sensory and motor neuropathies (Auer-Grumbach et al., 2010; Deng et al., 2010; Krakow et al., 2009; Landouré et al., 2010; Nilius and Owsianik, 2010; Rock et al., 2008). It is entirely possible that the small number of individuals identified with activating mutations in the *Trpv4* gene have metabolic or cardiovascular deficits due to malfunction of the afferent pathway described here. Our study on the molecular characterization of this novel osmosensitive afferent pathway could potentially lead to the development of new procedures to target this pathway in vivo. It has already been demonstrated that orthostatic hypotension and postprandial hypotension respond to water drinking (Jordan et al., 2000; Schroeder et al., 2002; Shannon et al., 2002). Moreover, water drinking in man can prevent neutrally mediated syncope during blood donation or after prolonged standing (Claydon et al., 2006; Hanson and France, 2004; Lu et al., 2003; Schroeder et al., 2002). Finally, water drinking is also associated with weight loss in overweight individuals (Stookey et al., 2008). The characterization of the afferent arc of the reflex responsible for such effects will allow a better understanding of the physiological role of this reflex and allow the development of tools for its manipulation.

EXPERIMENTAL PROCEDURES

Oral Fluid Administration and Blood Osmolality Measurements

Water, phosphate-buffered saline (PBS), or water doped with 100 μM RR were administered orally using an application cannula with a rounded tip, fluids being dripped into the back of the mouth to evoke a swallowing reflex. Fluid intake was completed within 60 s. Small blood samples (50 μl) were taken from the hepatic portal vein and osmolality determined using a vapor-pressure osmometer (VAPRO, Wescor).

We obtained serum samples from liver transplant recipients and measured osmolality as above. We also determined C-terminal pro-arginine-vasopressin using an immunoluminometric assay (kindly provided by BRAHMS GmbH). All studies using human material were approved by the ethics committee of the Medical School Hannover.

Immunohistochemistry and Functional Labeling of Liver Afferents

Thirty minutes after drinking, animals were anaesthetized with a Ketavet (10 mg/ml)/Rompun (0.04%) mixture (Pfizer, Bayer), and perfused with chilled (4°C) 4% paraformaldehyde (PFA) in PBS. The liver was removed, postfixed in 4% PFA at 4°C for 2 hr, dehydrated with 25% sucrose in PBS for 1–3 days and 20 μm cryosections were prepared and collected on gelatine coated slides. Tissue sections were pre-incubated in 1% serum albumin (BSA) and 0.3% Triton X-100 in TBS (Tris buffered saline) for 2 hr and incubated overnight at RT with primary antibodies (pERK 1:250, Cell Signaling, PGP9.5 1:1000, UltraClone Ltd, TRPV4 1:200 ab39260 Abcam) in TBS with 0.3% Triton X-100 and 5% normal goat serum. For background controls the primary antibody was omitted. Tissue sections were washed three times in TBS for 10 min and incubated for 2 hr at RT with the secondary antibody in TBS containing 0.3% Triton X-100 and 5% normal goat serum. Sections were mounted in Aqua-Polymount and images collected using a Leica DM 5000B Upright Fluorescence Microscope and MetaVue Software.

Sensory Neuron Cultures

Mice were killed by cervical dislocation and DRGs were collected on ice in Ca^{2+} and Mg^{2+}-free PBS. DRGs cultures were prepared as previously described (Lechner and Lewin, 2009) and plated in a droplet of culture medium on a glass coverslip precoated with poly-L-lysin (20 μg/cm^2, Sigma-Aldrich) and laminin (4 μg/cm^2, Invitrogen). Cultures were used for patch clamp or calcium imaging between 18 and 48 hr after plating.

Calcium Imaging

Cultured neurons were loaded with 5 μM of Fura-2AM (Invitrogen) for 30 min at 37°C. Neurons were placed in a chamber containing extracellular buffer of isotonic osmolality (310 mOsm/kg) consisting of 110 mM NaCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 4 mM KCl, 4 mM glucose, 10 mM HEPES and 80 mM mannitol adjusted to pH 7.4. Hypotonic solutions were prepared by stepwise reducing the concentration of mannitol from 80 mM to 0 mM, osmolality was verified directly using a vapor-pressure osmometer. Cells were illuminated alternately at 340 nm and 380 nm (Polychrome IV, Visitron Systems) for 500 ms (200 ms for simultaneous patch-clamp and Ca^{2+}-imaging; Figure 4A) and ratio images were collected every 1.6 s (450 ms for Figure 4A) using MetaFluor Software and a SPOT-SE18 CCD camera. To estimate absolute changes in intracellular Ca^{2+} (Figure 2A) fluorescence ratios (R) were converted using the equation $[Ca^{2+}]i = K_{eff}^{*}(R - R_0)/(R_1 - R)$. The calibration constants K_{eff} (= 824 nM), R_0 (= 0.28) and R_1 (= 1.54) were determined as described by Vriens et al. (2004). For all other experiments ratios were normalized to the mean of the first 10 ratio images and plotted as R/R_0. Solutions were applied using a gravity-driven multi barrel perfusion system (WAS02, DITEL, Prague). Cells not responding to KCl (40 mM for 16 s) were excluded from the analysis.

Electrophysiology

Whole-cell patch-clamp recordings were made at room temperature 24–48 hr after plating of neurons as previously described (Lechner and Lewin, 2009). Patch pipettes were filled with 110 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA and 10 mM HEPES, adjusted to pH 7.3 with KOH and had tip resistances of 6–8 MΩ. The bathing solution contained 110 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 4 mM glucose, 10 mM HEPES, and 80 mM mannitol, adjusted to pH 7.4 with NaOH. All recordings were made using an EPC-10 amplifier in combination with Patchmaster and Fitmaster software (HEKA, Germany). Pipette and membrane capacitance were compensated using the auto function of Patchmaster and series resistance was

compensated by 70% to minimize voltage errors. Currents evoked by osmotic stimuli were recorded at a holding potential of −60 mV.

Retrograde Labeling

Animals were anaesthetized with a Ketavet/Rompun mixture (see above) and a small incision was made in the abdominal wall to gain access to the liver. Several injections of 3–5 μl of 2.5% Alexa-Fluor 488-coupled Dextranamin MW 3000 (total 20 μl) were made into the liver. The application needle was left inside the injection site for 30 s before retraction to avoid dye leakage. Following the injection the wound was sutured, a local anesthetic (Xylocain-Gel) administered and the animal was allowed to recover. Optimal labeling of the DRGs was found 3–4 days postinjection.

Genetically Modified Mice

Trpv4$^{-/-}$ mice were genotyped using PCR and backcrossed onto a C57Bl/6 background for at least four generations (Mizuno et al., 2003). Transgenic α3nAChR-EGFP-mice were obtained from the Gene Expression Nervous System Atlas (GENSAT) Project. Genotyping was performed by PCR using EGFP-primers according to the GENSAT-protocol.

Statistics

Statistical analyses were performed using GraphPad Prism 5.0. Means are shown ± SEM.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and one movie and can be found with this article online at doi:10.1016/j.neuron.2010.12.028.

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