
TITLE: **MECHANICAL BASIS OF OSMOSENSORY TRANSDUCTION IN MAGNOCELLULAR NEUROSECRETORY NEURONS OF THE RAT SUPRAOPTIC NUCLEUS**

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Running title: **Mechanical control of SON neurons**

Key words: Supraoptic nucleus, vasopressin, osmoregulation, microtubule, transient receptor potential vanilloid type-1 (Trpv1).

SUMMARY

Rat magnocellular neurosecretory cells (MNCs) release vasopressin and oxytocin to promote antidiuresis and natriuresis at the kidney. The osmotic control of oxytocin and vasopressin release at the neurohypophysis is required for osmoregulation in these animals, and this release is mediated by a modulation of action potential firing rate by the MNCs. Under basal (isotonic) conditions MNCs fire action potentials at a slow rate, and this activity is inhibited by hypoosmotic conditions and enhanced by hypertonicity. The effects of changes in osmolality on MNCs are mediated by a number of different factors; including the involvement of synaptic inputs, the release of taurine by local glial cells, and by a regulation of ion channels expressed within the neurosecretory neurons themselves. Here we review recent findings that have clarified our understanding of how osmotic stimuli modulate the activity of non-selective cation channels in MNCs. Previous studies have shown that osmotically-evoked changes in membrane potential and action potential firing rate in acutely isolated MNCs are provoked mainly by a modulation of non-selective cation channels. Notably, the excitation of isolated MNCs during hypertonicity is mediated by the activation of a capsaicin-insensitive cation channel which MNCs express as an N-terminal variant of the transient receptor potential vanilloid 1 (Trpv1) channel. The activation of this channel during hypertonicity is a mechanical process associated with cell shrinking. The effectiveness of this mechanical process depends on the presence of a thin layer of actin filaments beneath the plasma membrane, as well as a densely interweaved network of microtubules (MTs) that occupies the bulk of the cytoplasm of MNCs. While the mechanism by which F-actin contributes to Trpv1 activation remain unknown, recent data has shown that MTs interact with Trpv1 channels via binding sites on the C-terminus, and that force mediated through this complex is required for channel gating during osmosensory transduction. Indeed, displacement of this interaction prevents channel activation during shrinking, whereas increasing the density of these interaction sites potentiates shrinking-induced activation of Trpv1. Therefore the gain of the osmosensory transduction process can be regulated bi-directionally through changes in the organization of F-actin and MTs.

INTRODUCTION

In healthy animals, short term increases in plasma osmolality (pOsm) can be promoted by evaporative water loss during breathing or panting; by the production of sweat; or following ingestion of dry solutes (e.g. salt) or hypertonic solution (e.g. soy sauce). Conversely, acute decreases in pOsm occur readily following ingestion of hypotonic fluid. Mammals have

developed effective homeostatic mechanisms that strive to maintain pOsm near an ideal set point (~300 mosmol/kg) despite incessant challenges to the body's hydration status (1-3). Indeed, changes in pOsm that are more than 1% away from the set point (i.e. ±3 mosmol/kg) normally induce homeostatic responses such as changes in the rate of water intake (4) and secretion of vasopressin (VP, antidiuretic hormone) (5) to mediate a feedback control of pOsm **(Fig. 1)**. This process of systemic osmoregulation is of vital importance for the organism, as significant changes in cell volume caused by acute changes in extracellular fluid osmolality can cause severe organ injury. Notably changes in pOsm are mirrored by changes in cerebrospinal fluid osmolality (6, 7), and can therefore induce lethal neurological trauma (8-11).

The osmotic control of VP secretion plays a key role in systemic osmoregulation because of its ability to stimulate water reabsorption by the kidney (12, 13). Under basal conditions, VP circulates in the plasma at a concentration near 2 pg/ml, which is sufficient to promote significant water reabsorption from the kidney (14). The concentration of VP in the circulation is reduced when pOsm declines, thereby promoting diuresis and a regulatory enhancement of pOsm. Conversely, increases in pOsm enhance the concentration of VP, thereby increasing water reabsorption and promoting a homeostatic reduction in pOsm **(Fig. 1)**. Together with adaptive changes in thirst (1, 3), feedback adjustments in VP secretion mediated by changes in pOsm provide the core mechanisms responsible for systemic osmoregulation in mammals (2).

The osmotic control of VP release is an integrated process

VP is synthesized in the somata of magnocellular neurosecretory cells (MNCs) located in the paired hypothalamic supraoptic and paraventricular nuclei, where they coexist with oxytocin-

releasing MNCs (15, 16). The MNCs send axons to the posterior pituitary (17), where each axon branches into ~1800 neurosecretory terminals that abut fenestrated capillaries (18). VP secretion from these terminals is triggered by calcium-dependent exocytosis driven by the arrival of action potentials initiated at the cell soma (19-22). Therefore changes in VP release induced during changes in pOsm are mediated via the control of action potential discharge by MNC somata **(Fig. 1)**.

Previous work has shown that a variety of extrinsic and intrinsic factors are involved in the osmotic modulation of firing rate and VP secretion by MNCs (2, 23). For example, the *organum vasculosum laminae terminalis* (OVLT) is a primary osmoreceptor site within the brain that harbors a population of intrinsically osmosensitive neurons (24-26), and this area is of critical importance for osmoregulation (27-29). Neurons in the OVLT send direct axonal projections into the supraoptic nucleus (30) where they can affect the firing rate of VP neurons via excitatory synapses (24, 31-34). Other parts of the nervous system have been reported to contain osmosensitive neurons that could also contribute to the osmotic control of MNCs (23); including the subfornical organ (35) and the median preoptic nucleus (36). Moreover, osmosensitive neurons located in the periphery have been shown to regulate the firing rate of MNCs via projections ascending through vagal and spinal pathways (37-39). Furthermore, recent studies have also revealed that glia cells play a functional role in controlling the electrical activity of MNCs. Specifically, it has been shown that astrocytic processes intercalated between the MNCs release taurine as an inverse function of osmolality in the surrounding parenchyma (40). Taurine is a potent agonist at inhibitory glycine receptors (41). Thus an increase in taurine release under hypoosmotic conditions promotes a hyperpolarization of the membrane potential and a decrease of the action potential firing rate in MNCs. Conversely, hyperosmotic conditions

promote a decrease in basal taurine release and thereby depolarize and excite MNCs via de-activation of glycine receptors (40-42). Therefore both synaptic and local glial mechanisms contribute to the extrinsic regulation of MNCs during changes in fluid osmolality.

In addition to these extrinsic mechanisms, several studies have demonstrated that VP-secreting MNCs are intrinsically osmosensitive (43-47). Notably, it has been shown that the electrical activity of MNCs acutely isolated from the supraoptic nucleus of adult rats or mice is increased by hypertonicity and inhibited by hypotonicity. These responses are deemed cell-autonomous because they occur in the absence of neighbouring glial cells and without any synaptic connectivity (44). Under these conditions, hypertonic stimuli excite the cells by increasing the activity of non-selective cation channels and thus causing membrane depolarization, whereas hypoosmotic solutions inhibit MNCs through a hyperpolarization caused by a reduction in the basal activity of the non-selective cation channels **(Fig. 1)**. Interestingly, experiments on neurons acutely isolated from the mouse OVLT have revealed that the same mechanism is at play in these cells as well (24, 25). Although it is unclear if all osmosensory neurons use the same type of transduction channel, the proportional regulation of non-selective cation channel activity allows OVLT neurons and MNCs to encode physiologically-relevant increases and decreases in fluid osmolality (48, 49) and likely contributes to osmotic detection *in vivo*. In the remainder of this review we will summarize recent findings from our laboratory that have expanded our understanding of osmosensory transduction at a cellular and molecular level.

Osmosensory transduction is a mechanical process

Unlike many other types of cells which partly regulate their volume when exposed to osmotic stress (50), OVLT neurons and MNCs have been shown to display passive osmometry; that is they display changes in soma volume that vary as an inverse function of extracellular fluid osmolality and can be maintained without adaptation during perturbations lasting $\geq$60 minutes (25, 51). Although other studies have shown that non-osmometric changes in cell size can occur in MNCs exposed to chronic osmotic perturbations (52, 53), the dynamic changes in plasma osmolality that occur under physiological conditions are likely accompanied by inversely proportional changes in the volume of MNCs and OVLT neurons.

Experiments combining electrophysiological analysis and volume measurements have shown that osmotically-induced changes in non-selective cation channel activity are closely mirrored by changes in soma volume (49, 54, 55). Although this observation suggests that changes in soma size or morphology could play a role in modulating channel activity, it is important to keep in mind that osmotically-induced volume changes are accompanied by inversely proportional changes in intracellular solute concentration, and that such changes could also participate in the process of transduction.

Studies in acutely isolated MNCs and OVLT neurons have shown that the membrane potential and electrical activity of these neurons can be altered when cell volume is modified in the absence of changes in ionic strength or solute concentration (25, 49, 56, 57). For example, reducing cell volume by the application of negative pressure to the recording pipette is sufficient to depolarize and excite MNCs in the absence of osmotic stimulation **(Fig. 2A)**. Conversely, increasing cell volume by applying positive pressure to the recording pipette can effectively hyperpolarize MNCs **(Fig. 2B)**. Current-voltage analysis has confirmed that the pressure-mediated responses in MNCs and OVLT neurons reflect changes in the activity of non-selective

cation channels (49) and are quantitatively equivalent to those observed during osmotically-induced volume changes (25, 49, 55, 57). Furthermore, increases in non-selective cation channel activity induced by hypertonicity can be reversed by pressure-mediated inflation, and decreases in channel activity induced by hypotonicity can be reversed by suction-induced shrinking of MNCs and OVLT neurons. Taken together, these results provide strong evidence indicating that the intrinsic osmosensitivity of these neurons reflects a mechanical regulation of the non-selective cation channels during volume changes rather than an effect caused by changes in solute concentration or ionic strength.

Molecular identity of the osmosensory channel

Recent studies have shown that non-selective cation channels mediating osmosensitivity in MNCs and OVLT neurons can be encoded by the transient receptor potential vanilloid type-1 gene (*trpv1*) (24, 54, 58). Specifically, it has been shown that MNCs and OVLT neurons isolated from mice lacking *trpv1* display normal osmotically-induced changes in cell volume, but that these cells lack the changes in non-selective cation conductance, membrane potential, or action potential firing that are normally observed in wild type neurons. Moreover, the responses of wild type MNCs and OVLT neurons to osmotic stimulation can be inhibited by pore blockers of Trpv1, such as ruthenium red (24, 54, 58), or the selective Trpv1 inhibitor SB366791 (25). While these data support the hypothesis that *trpv1* encodes a pore-forming component of the osmosensory transduction channel, it remains unclear if the transduction channel expressed in MNCs and OVLT neurons is encoded as a full-length product of this gene (58), or as an N-terminal variant of Trpv1 (59). Although the term Trpv1 is used in the remainder of this article, it

is important to remember that the exact structure of the channel mediating osmosensory transduction in OVLT neurons and MNCs remains to be determined.

Role of actin in osmosensory transduction

Numerous studies have indicated that elements of the cytoskeleton including actin filaments (F-actin) and microtubules (MTs) can participate in the transmission and distribution of forces applied to the cell surface [see refs. (60-65) for recent reviews]. In MNCs and OVLT neurons F-actin typically forms a thin (~1.5 μm thick) but dense layer that lies beneath the plasma membrane **(Fig. 3)**. Experiments in acutely isolated MNCs have shown that this layer of subcortical F-actin plays an important role in the generation of cell-autonomous osmosensory responses (57, 66). Specifically, treating MNCs with cytochalasin D, which depolymerizes F-actin (67), has been shown to attenuate the increase in nonselective cation current, membrane depolarization and action potential firing that normally occurs upon shrinking in these cells. Conversely, responses induced by shrinking are exaggerated in MNCs treated with jasplakinolide (57, 68), a drug that promotes actin polymerization (69) and enhances the density of the subcortical F-actin layer (unpublished). These findings indicate that F-actin plays an important role in the osmotic modulation of MNCs, and that the sensitivity of the osmosensory transduction process varies in proportion with actin density. Interestingly, angiotensin II has been shown to increase the osmoresponsiveness of MNCs (70, 71), and this effect is due in part to an increase in the intrinsic osmosensitivity of these neurons (72) mediated through a receptor mediated and PLC/PKC dependent increase in subcortical F-actin density (68).

Despite these important observations, the exact mechanism by which F-actin regulates Trpv1 in MNCs and OVLT neurons remains unknown. In principle, the subcortical F-actin network could couple changes in cell size to changes in channel activity in a number of different ways. Although actin does not appear to bind directly to Trpv1 (73), F-actin could still be linked indirectly to Trpv1 channels via common binding partners and mediate a mechanical regulation of Trpv1 activity by acting as a force-transmitting tether. Alternatively F-actin could provide a structural framework that holds the channel in place while other cytoskeletal tethers mediate volume-dependent forces that actively gate the channel. Additional work is required to define the role of F-actin in osmosensation.

Osmosensory neurons feature a unique microtubule (MT) network

Our group has recently shown that MTs also play an essential role in the osmotic and mechanical regulation of Trpv1 in rat supraoptic nucleus MNCs (56). Although MTs are best known for their roles in intracellular transport and cell division, they are also recognized as the cytosketon's strongest filaments (74, 75) and for their role in the control of cell shape (75, 76). In contrast to F-actin, which forms a thin subcortical sheet (Fig. 3), we found that MTs create a highly complex 3-dimensional network that occupies the entire cytoplasm of rat MNCs and are thus ideally positioned to generate and transmit compression forces associated with cell shrinking or swelling.

As illustrated in **Fig. 4**, super-resolution imaging of immunolabeled tubulin with structured illumination microscopy (SIM) revealed that the somata of MNCs in the rat supraoptic nucleus comprise a dense and interweaved MT scaffold that extends all the way from the nucleus

to the plasma membrane (56). This complex 3-dimensional MT network contrasts sharply with the rectilinear organization of MTs that is commonly found in the soma and processes of other types of neurons [e.g. (77)], and with the centrosome-oriented MTs typically found in non-neuronal cells (78, 79). Indeed, we found that the density and complexity of the MT network is much greater in rat supraoptic nucleus MNCs than in neurons from others parts of the brain, including; cortex, hippocampus, cerebellum and suprachiasmatic nucleus. Interestingly, a high density MT network was also observed in OVLT neurons, and in MNCs of the paraventricular nucleus. Therefore, the presence of a densely interweaved cytoplasmic MT network appears to be a unique characteristic of central osmosensory neurons.

MTs are required for osmosensory transduction

Previous studies have shown that interweaved networks of elastic filaments can distribute focally-applied forces to other parts of the cell surface via tensigrity (76). The MT network observed in MNCs would therefore be ideally positioned to distribute changes in compression force to ion channels located on the plasma membrane as cells undergo dynamic volume changes associated with osmotic perturbations. Indeed, whole cell patch clamp recordings from isolated MNCs treated with the MT destabilizing drug nocodazole prevented the activation of a non-selective cation current, as well as depolarizing and excitatory responses, induced by hypertonicity or suction-induced cell shrinking (56). Conversely, these responses were enhanced in MNCs pre-treated with the MT stabilizing drug taxol. These findings indicate that MTs play a functional role in the regulation of Trpv1 channels and that the sensitivity of the transduction process can be up- or down- regulated in proportion with the stability of this unique MT scaffold.

A tubulin-TRPV1 interaction is required for transduction

As described earlier, the ion channel mediating cell-autonomous osmosensory transduction in MNCs and OVLT neurons is a variant of Trpv1 that lacks a component of the N−terminus, but retains a cytosolic C-terminus (59). Previous studies *in vitro* have shown that MTs can interact with the wild type Trpv1 channel through a pair of tubulin binding sites located on the channel's C-terminus (73, 80). We therefore examined the possibility that MTs might directly interact with Trpv1 in rat MNCs. Super resolution imaging with SIM demonstrated that MTs located at the periphery of the cytoplasmic network extend outwardly toward the cell surface, where they come into close apposition with the plasma membrane **(Fig. 5A)**. To determine if MTs that approach the cell surface can potentially interact with Trpv1, we also performed an *in situ* proximity ligation assay that uses antibodies targeting tubulin and Trpv1 to determine if these proteins are located within 40 nm of each other (56). As illustrated in **Fig. 5B**, this approach revealed numerous sites where Trpv1 channels appear to interact with tubulin at the cell surface.

To establish if an interaction between Trpv1 channels and tubulin is required for osmosensory transduction, we then examined the effect of disrupting this interaction with synthetic peptides mimicking the native tubulin binding domains (TBD1, TBD2) comprised within the C-terminus of Trpv1 (56). Immunoprecipitation experiments performed on lysates of rat supraoptic nucleus confirmed that Trpv1 interacts with tubulin *in situ*, and that TBD1 and TBD2 can significantly disrupt this interaction when included in the tissue lysates. We then made recordings from MNCs using pipettes filled with an internal solution containing TBD1

and/or TBD2 and waited several minutes before testing the cells to allow extensive diffusion of the peptides throughout the neuronal cytoplasm. As illustrated in **Fig. 6**, shrinking induced a significant increase in non-selective cation conductance in cells filled with the control peptide, whereas this response was abolished in cells filled with TBD1 and/or TBD2.

The experiments described above indicate that an interaction between tubulin and Trpv1 is necessary to mediate the mechanical activation of these channels during cell shrinking. We therefore examined if nocodazole and taxol affect osmosensory transduction through effects on the MT network, or by affecting interactions between tubulin and Trpv1 (56). Using super resolution imaging of tubulin with SIM we observed that these drugs only have minor effects on the density and complexity of the MT network in isolated MNCs. In contrast, a proximity ligation assay revealed that the density of sites at which cell surface Trpv1 channels interact with tubulin is significantly reduced by nocodazole and increased by taxol. Therefore the blocking effect of nocodazole is mediated by the elimination of tubulin-Trpv1 interaction sites, whereas taxol potentiates the osmosensory responses of MNCs by increasing the density of sites at which Trpv1 interacts with tubulin. These observations indicate that the interaction between Trpv1 and tubulin is necessary for osmosensation, and that the sensitivity of the transduction process is proportional to the density of these interactions.

Osmosensory transduction is a force-mediated process.

The observations reported above indicate that osmosensory transduction requires an interaction between Trpv1 and tubulin. However they do not establish if transduction reflects a force-mediated modulation of channel activity among a pool of preassembled complexes, or

perhaps a dynamic change in the density of sites at which tubulin and Trpv1 interact (i.e. formation of new sites during shrinking and disruption of existing sites during swelling). However an analysis of MNCs performed using the proximity ligation assay revealed that the density of tubulin-Trpv1 interaction sites does not increase in response to hypertonicity. Thus the inward current generated in response to shrinking is not caused by the formation of additional tubulin-Trpv1 complexes. This finding implies that the activation of transduction channels during cell shrinking may result from a form of "push activation" possibly mediated by the generation of shrinkage-induced compression force within MTs attached to Trpv1 channels **(Fig. 7)**. Although more work is required to confirm and extend this hypothesis, rapid indentation of the surface membrane using high speed pressure steps applied during cell-attached recordings was found to mediate channel activation with latencies on the order of a few milliseconds. This extremely fast coupling between stimulus and response is consistent with the involvement of a purely mechanical response and suggests that generation of a second messenger intermediate is not required for channel activation during cell shrinking.

Concluding remarks

Recent experiments have demonstrated that MNCs and OVLT neurons are cell autonomous osmoreceptors and that the intrinsic osmosensitivity of these cells is mediated by the volume-dependent mechanical regulation of a non-selective cation channel encoded by the *Trpv1* gene. Experiments have shown that cytoskeletal elements including F-actin and MTs are required for the osmotic and mechanical regulation of these channels. While MTs appear to mediate the Trpv1 activation by providing a pushing force to the channel during cell shrinking,

the role of F-actin, though crucial, remains to be determined. Lastly, changes in the density of F-actin, or sites at which tubulin interacts with Trpv1, have been found to cause proportional changes in the sensitivity of the osmosensory transduction process. It is therefore possible that signaling molecules such as neurotransmitters or modulators can trigger receptor-mediated changes in cytoskeletal organization that alter osmoregulatory gain under physiological and pathological conditions that affect the release of VP from MNCs. The molecular mechanisms regulating the organization of actin and MTs in these conditions should be further studied to expand our understanding of the modulation of osmoregulatory gain under physiological and pathological conditions.

Acknowledgements: Work in the authors' laboratory is supported by operating grants FRN82818 and MOP9939 from the Canadian Institutes of Health Research to CWB, and by a James McGill Chair awarded to CWB by McGill University. MPK was supported in part by a Postdoctoral Fellowship Award from the Heart and Stroke Foundation of Canada. The Research Institute of the McGill University Health Center receives generous support from Le Fonds de recherche du Québec – Santé.

FIGURE LEGENDS:

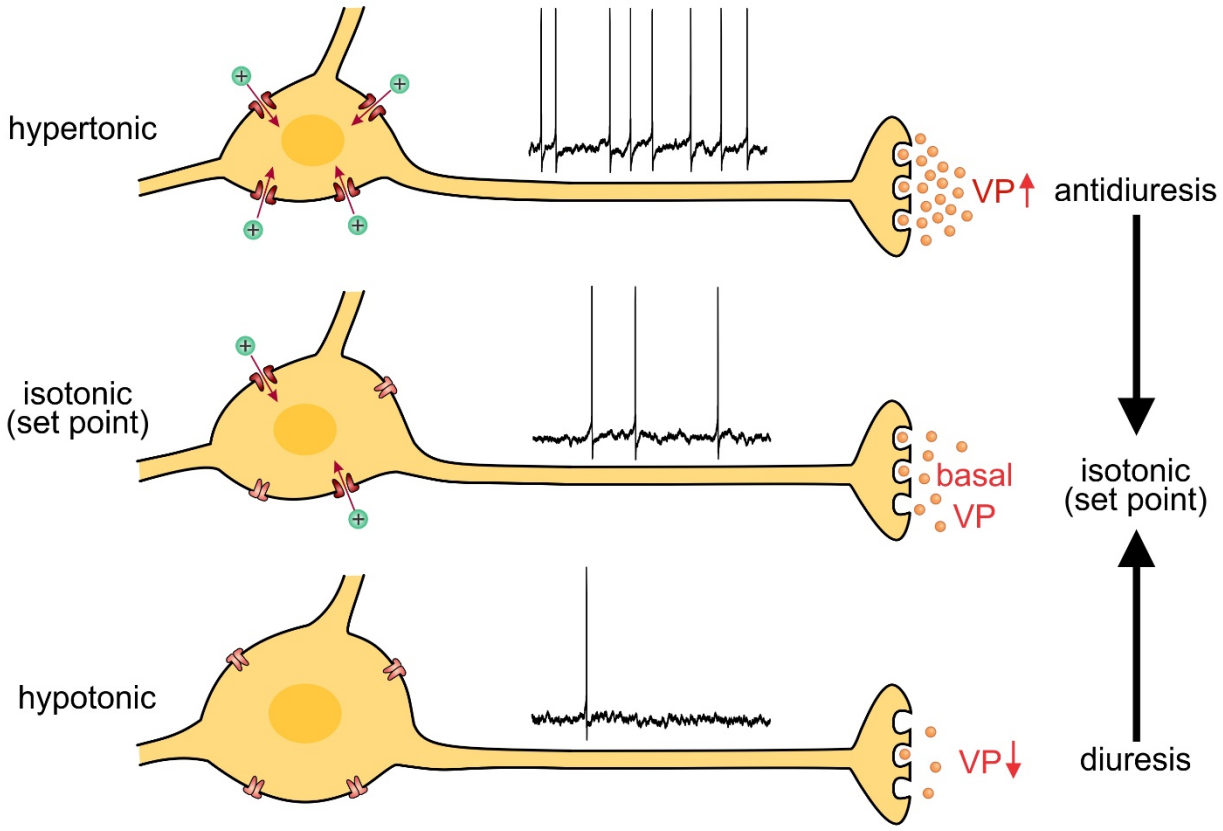

Fig. 1. MNCs are intrinsically osmosensitive. Changes in osmolality cause inversely proportional changes in cell volume. Hypertonicity-evoked shrinkage activates nonselective cation channels leading to depolarization and increase in the action-potential firing rate and vasopressin (VP) release from axon terminals in the neurohypophysis. Increased VP levels in blood enhance water reabsorption by the kidney (antidiuresis) to restore extracellular fluid osmolality toward the set point. Hypotonic stimuli cause swelling and inhibit the channels that are open under basal isotonic condition (set point), leading to hyperpolarization and decrease in the firing rate of ONs. This causes a reduction in the VP release and promotes diuresis.

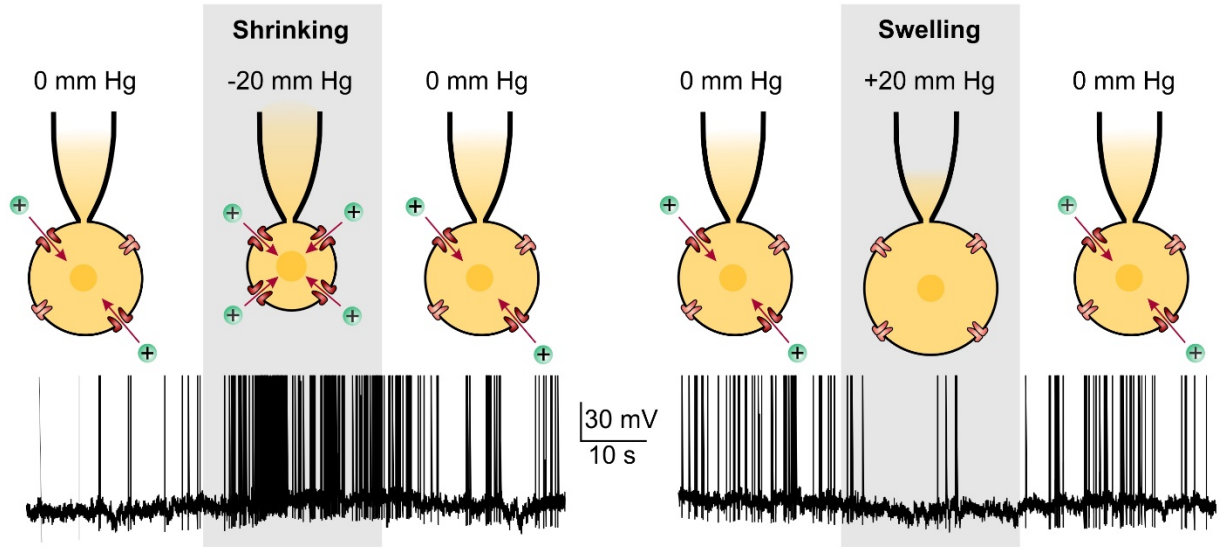

Fig. 2. Changes in cell volume modulate MNCs through a mechanical effect. Changes in cell volume provoked by the application of negative pressure (shrinking) or positive pressure (swelling) through the patch pipette have direct effects on acutely isolated MNCs. Suction-evoked shrinkage activates nonselective cation channels (upper schematic) which causes a reversible membrane depolarization and increased action-potential firing rate measured under whole cell current clamp (lower trace). Inflation-evoked swelling inhibits the cation channels that are open under basal condition (0 mm Hg), leading to hyperpolarization and decrease in the firing rate of MNCs.

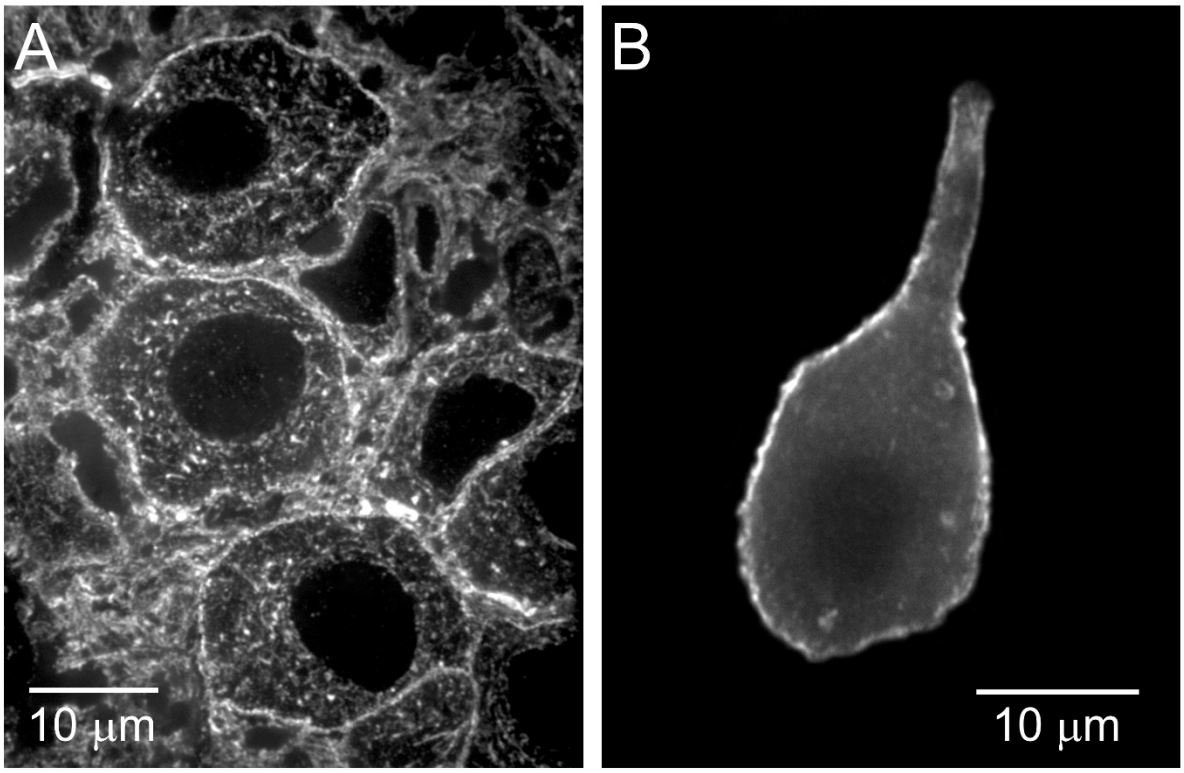

Fig. 3. MNCs feature a thin layer of subcortical actin. Confocal images show immunostaining for actin in a histological section through the adult rat supraoptic nucleus (A) and through an acutely isolated adult MNC.

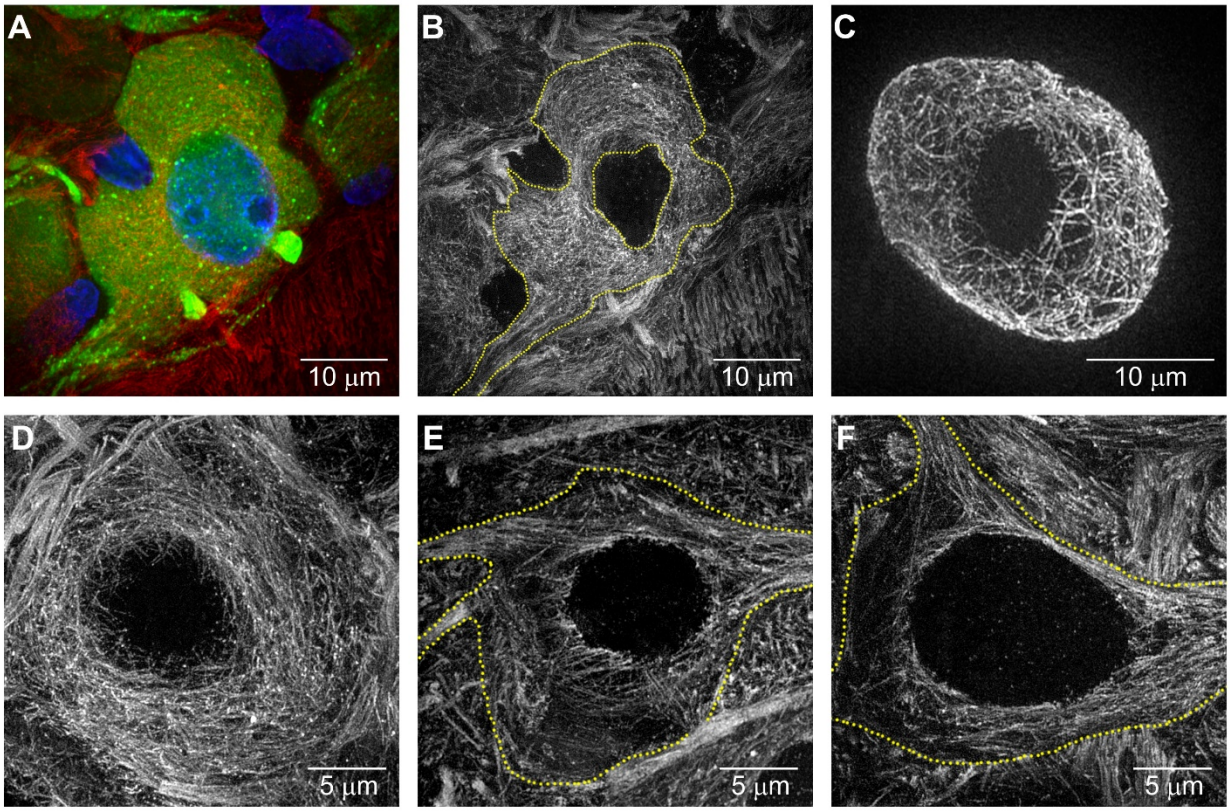

Fig. 4. MNCs feature a unique interweaved MT scaffold. Immunostaining for tubulin (red in A, white in B-F) in rat supraoptic nucleus MNCs analyzed by super-resolution structured illumination microscopy (SIM). (A,B) Triple staining of a brain section through the supraoptic nucleus of an adult rat. (A) shows a color image; tubulin (red), VP (green), and DAPI (blue). VP staining was used to define the perimeter and DAPI staining was used to define the outline of the nucleus of the cell (dotted yellow line in B). (B) tubulin staining shown in white with outline of the plasma membrane and nucleus for the cell shown in panel A. Note that a dense MT scaffold occupies the cytoplasm of this neuron. (C) High magnification SIM image showing the organization of MTs in the soma of an MNC acutely-isolated from the supraoptic nucleus of an adult rat. Note that the unique MT structure is preserved in isolated neurons. (D) High magnification SIM image of MT organization in another VP-containing MNC in a histological section. The microtubules are positioned in a wide variety of angles relative to each other, thereby creating a highly interweaved network. (E) SIM image of MT organization in a cortical neuron. (F) SIM image of MT organization in a hippocampal neuron. Adapted with permission from Ref. (56).

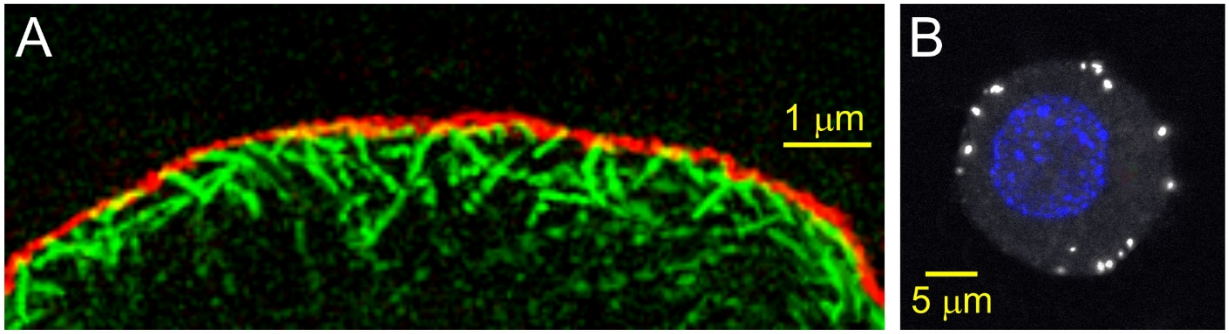

Fig. 5. MTs interact with the Trpv1 on the surface of MNCs. (A) Immunostaining for tubulin (green) and plasma membrane (red) in an acutely isolated rat MNC observed by SIM. Note that MTs extend all the way to the surface of MNCs, where they come in close contact with the plasma membrane. (B) Confocal image through an acutely isolated MNC processed using an *in-situ* proximity ligation assay (56) to visualize sites where tubulin and Trpv1 interact at the nanoscale (<40 nm; white spots; blue shows DAPI). Note that multiple sites of tubulin-Trpv1 interaction are observed at the surface of the cell, where transduction occurs. Adapted with permission from ref. (56).

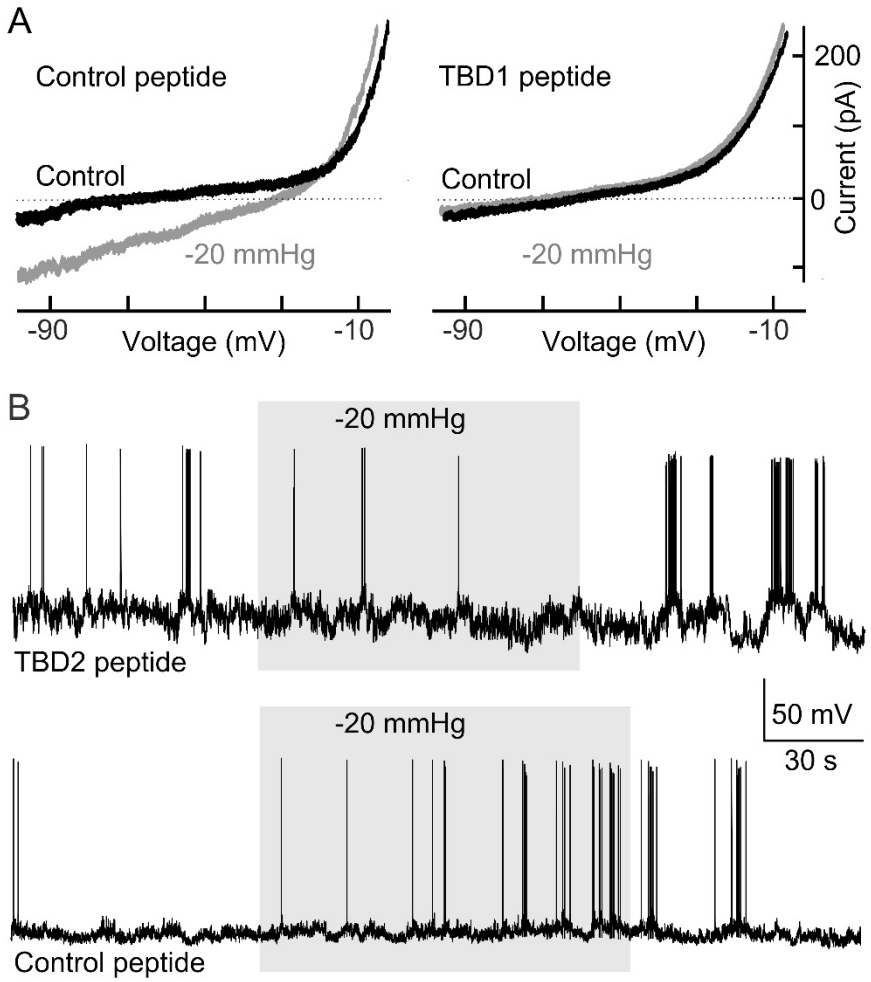

Fig. 6. Osmosensory transduction requires tubulin-Trpv1 interaction. (A) current voltage relations measured in two different MNCs acutely isolated from the supraoptic nucleus of an adult rat. The traces at left are from an MNC filled with a control peptide. In this cell, shrinking induced by negative pressure causes the activation of non-selective cation channels visible as an increase in slope conductance (gray trace). The cell on the right was filled with a synthetic peptide mimicking tubulin binding domain 1 (TBD1). Note that this cell fails to respond to cell shrinking. (B) Voltage responses of 2 MNCs measured under whole cell current clamp. Note that the cell filled with control peptide depolarizes and is excited by suction, whereas the cell filled with TBD2 peptide fails to respond. Adapted with permission from ref. (56).

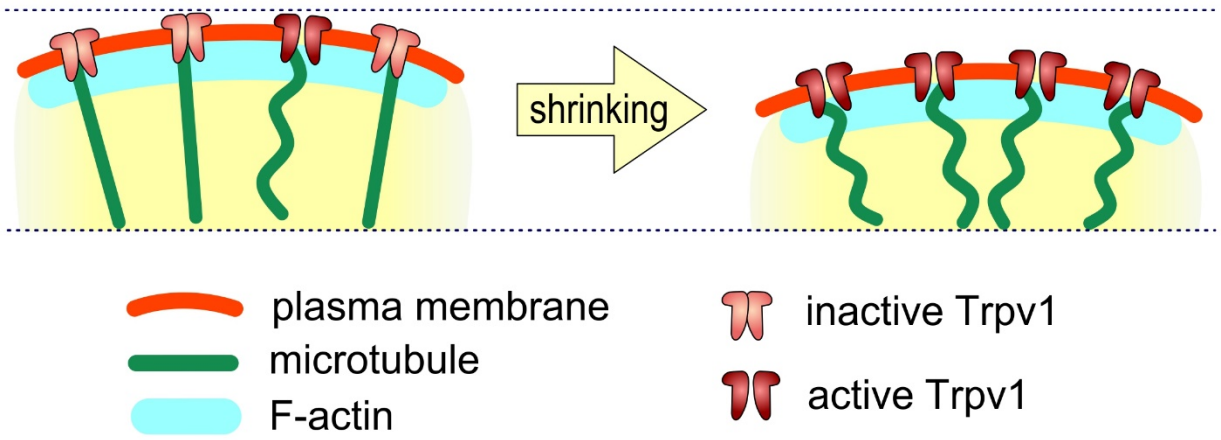

Fig 7. mechanism of mechanical regulation of Trpv1 in MNCs. *Push-activation* model for the Trpv1 activation by force through an attached MT. At rest many Trpv1 channels are bound to MTs, but few are activated due to the lack of sufficient pushing force (left). As a result of cell shrinking, the plasma membrane shifts inward (right), increasing the proportion of MTs that push onto (and activate) Trpv1 channels.

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