Characterization of a Novel Pre-Pore Loop Antibody Against Rat TRPV1

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Abstract

Splice variants of the transient receptor potential vanilloid type-1 (TRPV1) channel appear to be involved in the physiological detection of extracellular fluid (ECF) osmolality in the supraoptic nucleus (SON) and organum vasculosum lamina terminalis (OVLT). It remains to be determined whether these splice variants are directly involved as pore-forming proteins in the osmosensory transduction complex. Since these TRPV1 splice variants are not sensitive to capsaicin antagonists, such as capsazepine (Sharif Naeini et al., 2007), novel tools that specifically interfere with ion permeation through TRPV1 are required for functional studies on the involvement of this channel. In this study, we developed rabbit polyclonal antibodies targeting specifically the extracellular pre-pore loop region of rat TRPV1 (PH-4281). Histological results showed that PH-4281 is specific to rat TRPV1 and TRPV1 expression is found in regions that are known to be osmosensitive. PH-4281 could be used as a specific tool to study the osmosensory transduction complex.

Résumé

Des variantes d'épissage du récepteur vanilloide à potentiel transitoire de type 1 (TRPV1) semblent être impliquées dans la détection physiologique de l'osmolalité du liquide extracellulaire (ECF) dans le noyau supraoptique (SON) et dans l'organe vascularisé de la lame terminale (OVLT). À savoir si ces variantes d'épissage sont directement impliquées comme protéines formant le pore du canal osmosensible demeure inconnu. Puisque ces variantes d'épissage du TRPV1 ne sont pas sensibles aux antagonistes de la capsaicine, comme la capsazepine (Sharif Naeini et al., 2007), de nouveaux outils originaux qui interfèrent avec la perméation d'ions par le TRPV1 sont requis pour les études fonctionnelles sur le rôle de ce canal. Dans cette étude, nous avons développé des anticorps polyclonaux de lapin ciblant en particulier la région pré-pore extracellulaire du TRPV1 de rat (PH-4281). Les résultats histologiques ont montré que le PH-4281 est spécifique au TRPV1 et que ce canal s'exprime dans les régions reconnues pour leur osmosensibilité. Le PH-4281 pourrait donc être utilisé comme outil spécifique pour étudier le complexe de transduction d'osmosensibilité.

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List of Abbreviations and Symbols

ATP:	adenosine triphosphate
BLAST:	basic local alignment search tool
Ca^{2+}:	calcium
CHO:	chinese hamster ovary
CTX:	charybdotoxins
DAG:	diacylglycerol
DRG:	dorsal root ganglion
ECF:	extracellular fluid
GFP:	green fluorescent protein
HEK293:	human Embryonic Kidney
HEPES:	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IgG:	immunoglobulin G
IP3:	inositol-1,4,5 triphosphate
I-V:	current-Voltage
K^{+}:	potassium
kDal:	kilodalton
M:	molar
MNC:	magnocellular neuroscretory cells
MnPO:	median preoptic nucleus
mV:	millivolt
Na^{+}:	sodium
OT:	oxytocin
OVLT:	organum vasculosum lamina terminalis
pA:	picoampere
PBS:	phosphate buffered saline
PCR:	polymerase chain reaction
pF:	picoFarad
PFA:	paraformaldehyde
PIP2:	phophatidylinositol-4,5-biphosphate
PIPIES:	piperazine-N,N-bis(2-ethanesulfonic acid)
PLCβ:	phospholipase Cβ
SDS-PAGE:	sodium dodecyl Sulfate polyacrylamide gel electrophoresis
s.e.m:	standard error of mean
SFO:	subfornical organ
SICC:	stretch-inactivated cationic channels
SON:	supraoptic nucleus
TBST:	tris-Buffered Saline Tween-20
TRPV:	transient receptor potential vanilloid
VP:	vasopressin
WT:	wild-type
µg:	microgram
µM:	micromolar

Chapter 1: Introduction

1.1 - Osmoregulation

Osmoregulation is one of the body's most essential homeostatic mechanisms. It is a process by which water and electrolyte balance are maintained. The extracellular fluid (ECF) osmolality of perfused tissues can fluctuate which can lead to disruption of normal volume, metabolism, and function of individual cells [1, 2]. It has been reported in the literature that large acute osmotic perturbations can cause confusion, paralysis, coma, and convulsive seizures [3-11]. Under extreme conditions, osmotic changes cause brain swelling or shrinkage of such magnitude that it can lead to traumatic or lethal consequences [12-14]. Therefore, osmoregulation uses behavioural and physiological mechanisms to counteract osmotic perturbation to restore homeostasis and avoid these effects. In mammals, when the ECF osmolality deviates from its homeostatic set-point, the behavioural response, mediated by the hypothalamus, consists of regulating the intake of salt and water through changes in sodium appetite and thirst (Figure 1.1) [15, 16]. Simultaneously, the hypothalamus mediates the physiological mechanisms by adjusting renal excretion of water and sodium through changes in the release of anti-diuretic and natriuretic hormones (Figure 1.1) [1, 2, 17]. The anti-diuretic and natriuretic hormones, vasopressin and oxytocin respectively, are synthesized by magnocellular neurosecretory cells (MNCs) in the hypothalamus [18, 19].

Oxytocin is known for its role as a humoral trigger for uterine contractions at birth and for milk ejection during lactation [20]. However in the rat, studies have shown that oxytocin contributes to sodium balance and osmoregulation because of its natriuretic effects [21, 22] and because its release is stimulated by increases in osmolality [23, 24].

1.2 - Discovery of Osmoreceptors

It was observed by Gilman in 1937 that drinking in dogs can be induced by systemic infusion of hypertonic sodium chloride, a membrane-impermeant solute, but not by an equal osmotic amount of membrane-permeant urea [25]. Gilman concluded that the sensation of thirst arises as a consequence of cellular dehydration (i.e reduced cell volume), a process where the efflux of cytoplasmic water was due to the increase ECF osmolality [25]. Although this suggested that changes in cell volume play a role in cellular detection of ECF osmolality, there was no evidence available to determine whether sensation of thirst was generated due to a decrease in volume of all cells or from the activation of specialized receptors. Similar to Gilman's findings on thirst, Verney discovered that vasopressin release in dogs can be evoked by systemic infusion of hypertonic solutions [26]. Again, this was only observed with membrane-impermeant solutes, such as sodium chloride, sodium sulphate, or sucrose and not with membrane-permeant urea [26]. As will be described below, evidence

now exists to support the involvement of specific osmoreceptors, sensors specialized for detecting changes in ECF osmolality, in the control of osmoregulatory responses [1, 17, 27].

1.3 - Osmoreceptors in the Central Nervous System

It has been well demonstrated from lesion studies that structures near the anterior part of the hypothalamus are important for vasopressin secretion [28-37], osmotic regulation of thirst [28, 30, 31, 35-37], sodium appetite [38], and natriuresis [39-43]. These observations and other studies [1] suggested that osmotic control of most osmoregulatory responses is mediated by neurons in three structures: the subfornical organ (SFO), the median preoptic nucleus (MnPO), and the organum vasculosum lamina terminalis (OVLT). Electrophysiological experiments *in vivo* have demonstrated the presence of osmoresponsive neurons in each of the loci mentioned above [1]. In addition, recordings *in vitro* under conditions where synaptic transmission is blocked demonstrated that at least some of the neurons located in the SFO [1, 44] , MnPO [17] , and OVLT [45] are intrinsically sensitive to changes in fluid osmolality. This suggested that neurons within these areas may act as cellular osmoreceptors. However, cellular osmoreceptors are defined not only by their intrinsic sensitivity to changes in ECF osmolality, but also by their ability to regulate neuronal systems responsible for the production of specific

osmoregulatory responses [17]. Because the neural circuits mediating thirst and sodium appetite are largely unknown, the exact role of cellular osmoreceptors regulating these responses are still unclear [17]. On the other hand, pathways involved in the osmotic control of anti-diuretic and natriuretic hormones, are well characterized and can be used to investigate the cellular basis for osmoreception [17].

As mentioned in section 1.1, MNCs synthesize anti-diuretic and natriuretic hormones, vasopressin and oxytocin respectively, which are essential for proper osmoregulatory responses [18, 19]. Furthermore, Mason reported that MNCs of the supraoptic nucleus (SON) are themselves intrinsically osmosensitive under conditions where synaptic transmission is blocked [46]; a similar behaviour seen in neurons from SFO, MnPO, and OVLT. Because MNCs are easily identifiable in various *in vitro* preparations from rat brain [17] and because they release hormones crucial to generating normal osmoregulatory response [18, 19], most of what is currently known about the ionic basis for osmoreception was produced from experiments in rat MNCs.

1.4 - Cellular Basis for Osmoreception

The mechanisms by which changes in ECF osmolality are transduced into an electrical signal were unknown until a study was done by Oliet and Bourque where patch-clamp recordings were obtained from MNCs acutely isolated from

the SON of adult rats [47]. A variation in ECF osmolality changes the cell volume and it has been shown that bath application of hypertonic solution (i.e cell shrinking) induces a sustained and reversible membrane depolarization accompanied by an increase in firing in MNCs (Figure 1.2). On the other hand, reducing the osmolality of the perfusate (i.e cell swelling) hyperpolarizes and inhibits firing in MNCs (Figure 1.2). To determine whether changing cell volume mechanically is enough to activate the transduction mechanism, Oliet and Bourque applied negative pressure to the inside of whole cell patch pipettes to produce changes in cell volume comparable to those elicited by hypertonic stimulation. The pressure response seen is similar to the osmotically-evoked stimulus; MNCs respond with a reversible depolarization and increase in firing rate. Voltage-clamp analysis revealed a similar reversal potential for both osmotically- and mechanically-evoked currents. Moreover, increasing extracellular potassium concentration shifts both the reversal potential of osmotically- and mechanically-evoked currents to similar values in the positive direction. These finding suggest that the response of MNCs to changes in pipette pressure or ECF osmolality results from the modulation of the same population of non-selective cationic channels [47]. Oliet and Bourque concluded that changes in membrane tension associated with osmotically induced volume changes lead to the modulation of stretch-inactivated cationic channels (SICC) that underlie osmoreception in hypothalamic MNCs (Figure 1.2) [47]. The molecular identity of the SICC channels involved in the osmosensory

transduction complex has yet to be reported. For the remainder of this chapter, I will be discussing studies that provide important clues that may help us identify the composition of these osmoreceptors.

1.5 - OSM-9 Role in Osmoreception in *C.Elegans*

In 1997, a study was done on roundworms, *Caenorhabditis elegans (C. elegans),* where Colbert et al. described the molecular characterization of the *osm-9* gene [48]. *Osm-9* encodes an ion channel expressed in sensory neurons that mediates chemosensory, osmosensory, and mechanosensory transduction. It was shown that upon mutation of the *osm-9* gene, the behavioural avoidance response to high osmotic strength was disturbed in *C. elegans* in a manner that was consistent with impaired osmoreception. It was observed that by placing *C. elegans* on an agar plate and encircling the plate with a high osmotic strength ring, wild-types avoided the ring whereas *osm-9* mutants did not. A basic local alignment search tool (BLAST) search of the GenBank database showed similarity between *osm-9* and the *Drosophila* transient receptor potential (TRP) channel family in the area of the sixth putative transmembrane domain, a region strongly conserved between TRP family members. Moreover, both *osm-9* and *Drosophila* TRP channels contain ankyrin motifs in their amino terminus, six predicted membrane-spanning regions, and a hydrophilic C terminal domain. The discovery

of *osm-9* carried with it the suggestion that members of the TRP channel family might have critical roles in transduction of osmotic stimuli [49].

1.6 - TRP Channels

The founding member of the TRP superfamily was identified as a gene product involved in *Drosophila* phototransduction [50, 51]. In flies, the processing of light is initiated by rhodopsin activating G-αq, which in turn stimulates a phospholipase Cβ (PLCβ) [52, 53]. The PLCβ cleaves phophatidylinositol-4,5-biphosphate (PIP2) to produce inositol-1,4,5 triphosphate (IP3) and diacylglycerol (DAG) leading to influx of Ca^{2+} and Na^{+} [54]. Thus, photoreceptor cells are depolarized in response to light and Ca^{2+} is involved not only in depolarization, but is also critical for adaptation and termination of the photo-response [52, 53]. In voltage-clamp recordings, photoreceptor cells of wild-type flies exposed to constant light produces an initial large transient inward current that decays significantly within 200ms and is followed by a smaller sustained inward current [55]. In *trp* mutant flies which lack a functional TRP channel, only the transient response is seen during sustained exposure to light [55]. Therefore, while *trp* mutants initially display a large response to a bright light stimulus, they behave as if they were blind within a few milliseconds of sustained exposure to light [54, 56]. However, if TRP is a light-sensitive ion channel, it cannot be the only one since there is still an initial transient light response in *trp* mutants [54]. In fact,

there are two other TRP related proteins expressed in photoreceptor cells, TRPL and TRPγ [57, 58]. Moreover, a *trpl/trp* double mutant is completely unresponsive to light [59, 60]. The TRP channel therefore underlies the sustained current in these photoreceptor cells.

1.7 - The TRP Superfamily of Cation Channels

Since the identification of the founding member of the TRP channels in fruit flies [50, 51], some 70 TRP channels have been uncovered in other organisms including worms, zebrafish, mice, rats and humans. This superfamily of channels is divided into group 1 and 2, which are themselves divided into seven subfamilies. This separation of the group 1 and 2 TRPs is based on sequence and topological differences. The group 1 TRPs consist of five subfamilies, which have the strongest sequence homology to *Drosophila* TRP: TRPC (canonical or classical), TRPV (vanilloid), TRPM (melastatin), TRPA (ANKTM1), and TRPN (NO mechanotransducer potential C). Group 2 TRPs consist of TRPP (polycystin) and TRPML (mucolipin) which are distantly related to group 1 TRPs. The names of the subfamilies are based on the original designation of the first recognized members of each subfamily. This superfamily of cation channels are now known to mediate several sensory responses, including responses to light [50, 55, 56], pheromones [61], olfaction [48], taste [62], mechanical changes [63], temperature [64], pH [64], osmolality [65-67], vasorelaxation of blood vessels

[68], and metabolic stress [69]. Of this superfamily of channels, TRPV channels were the first subfamily to be implicated with the osmotic transduction process in mammals.

1.8 - TRPV Family of Ion Channels

The mammalian TRPV family consists of six members, TRPV1 to TRPV6. The first member of this family to be cloned, TRPV1, is also known as the capsaicin receptor [64]. TRPV1 was identified from a rat dorsal root ganglion (DRG) cDNA library using an expression cloning strategy based on Ca^{2+} influx after application of capsaicin, a vanilloid compound present in hot chilli peppers [64]. TRPV2 to 4 were later identified by searching the GenBank database for sequences related to TRPV1 [70-74]. TRPV5 and TRPV6 were isolated by expression cloning of genes coding for proteins that mediated Ca^{2+} transport in kidney and intestine, respectively [75, 76]. However, TRPV5 and TRPV6 are more distantly related to TRPV1 (20-30% amino acid homology) [77] whereas TRPV2-4 are more closely related to TRPV1 (40-50% amino acid homology) [78]. Functionally, there is a Ca^{2+} permeability difference between TRPV5/TRPV6 and the other TRPVs. TRPV1 to 4 are low Ca^{2+} selective channels ($P_{Ca}/P_{Na} \sim 3\text{-}10{:}1$) [77] whereas, TRPV5 and TRPV6 are the most highly Ca^{2+} selective TRP channels ($P_{Ca}/P_{Na} > 100{:}1$) [78]. In addition, when each channel is individually expressed in a heterologous system, TRPV1 to 4 are temperature sensitive [64, 70, 73, 79, 80] whereas TRPV5 and

TRPV6 are not [78]. Interestingly, some of the temperature sensitive TRPVs also appeared to be osmosensitive or at least be modulated by osmotic stimuli [63, 65, 74, 81, 82]. Therefore in the following section, focus will be placed only to TRPV1 to TRPV4.

1.8.1 - TRPV1

As mentioned, TRPV1 channels are activated by vanilloid compounds such as capsaicin [64]. In addition, TRPV1 channels can be activated at temperatures above 43^{0}C and by extracellular protons [64]. Extracellular protons regulate TRPV1 via two distinct mechanisms. At pH 6 to 7, protons sensitize the channel to other stimuli such as capsaicin and heat but at higher concentrations (pH < 6), protons directly activate the receptor [64, 83]. There are relatively few studies reporting the potential osmosensitivity of TRPV1. It has been reported that TRPV1-transfected HEK293 cells did not display any change in $[Ca^{2+}]_i$ in response to hypotonic stimulation [65]. Moreover, expression of TRPV1 into *osm-9* mutant *C. elegans* did not rescue their high osmotic avoidance behaviour [84].

However, a study by Birder et al. measured distension-evoked ATP release from cultured urothelial cells isolated from TRPV1 knockout (*trpv1-/-*) or wild-type mice [85]. The authors observed that urothelila cells from wild-type mice responded to a hypotonic swelling stimulus with a release of ATP, whereas

trpv1-/- urothelila cells showed significantly less hypotonic-evoked ATP release. In contrast, *trpv1-/-* had no significant effect on phenylephrine-evoked ATP release. Thus, *trpv1* gene disruption appeared to impair the ability of urothelial cells to release ATP specifically in response to stretch stimuli [85]. To directly illustrate TRPV1's involvement to ATP release, the authors added capsazepine, a competitive antagonist of TRPV1, to culture rat urothelial cells and showed that there was significantly less ATP release in response to a hypotonic stimulus. Together, these results suggested that TRPV1 was necessary for hypotonic-evoked ATP release [85].

Moreover, a study by Liu et al. investigated how capsaicin-activated TRPV1 receptors expressed on dissociated trigeminal neurons, are affected by changes in osmotic pressure [86]. Under control conditions, the authors showed that when a trigeminal neuron was exposed to an isotonic solution, the application of capsaicin evoked a small inward current. When the same neuron was exposed to either hypotonic or hypertonic solutions, re-application of capsaicin evoked a significantly larger inward current. The current-voltage (I-V) responses of these neurons under different osmotic conditions (isotonic, hypotonic, or hypertonic) showed outward rectification and similar reversal potential. Application of capsazepine to these neurons reduced the capsaicin evoked currents during changes in osmotic pressure. These results suggested that increase sensitivity to capsaicin evoked currents by changes in tonicity is through TRPV1 receptors [86].

Recent studies examined the potential role of *trpv1* gene products to the intrinsic osmosensitivity of osmoreceptors [81, 82]. From reverse transcriptase-PCR and immunohistochemistry results, Sharif Naeini et al. found that MNCs in the SON expressed a capsaicin-insensitive splice variant of TRPV1, in which a portion of the N-terminal domain is truncated (Figure 1.3) [82]. Furthermore, electrophysiological analysis of SON and OVLT neurons from *trpv1-/-* mice revealed *trpv1* gene products may contribute to the intrinsic osmosensitivity of these neurons [81, 82]. It was shown that a hyperosmotic stimulus to isolated SON and OVLT neurons still caused a reduction in cell volume in *trpv1-/-* mice but it was no longer linked with an increase in membrane conductance, membrane depolarization, or increase in firing frequency that was normally seen in neurons obtained from wild-type mice (Figure 1.4) [81, 82]. In agreement with these results, hyperosmotic stimulus evoked thirst and vasopressin release were both found to be significantly reduced in *trpv1-/-* mice when compared to wild-type [81, 82].

1.8.2 - TRPV2

Unlike TRPV1, vanilloids and acidification do not produce any response in heterologous system expressing TRPV2 channels [70]. However, TRPV2 can function as a heat-gated channel, similar to TRPV1 channels, but at a threshold of activation at temperatures above 53^{o}C [70]. TRPV2 channels can also be

modulated by osmolality and therefore might be involved in osmoreception [63]. It has been demonstrated by Muraki et al. that TRPV2 channels expressed in murine vascular smooth muscle cells are activated by cell swelling; whether they are mechanically- or osmotically-evoked [63]. When isolated mouse aortic myocytes are exposed to a hypoosmotic solution, it causes cells to swell and leads to activation of non-selective cation current and elevated $[Ca^{2+}]_i$ [63]. Treatment of myocytes with an antisense oligodeoxynucleotide specific against TRPV2 reduces the hypoosmotic-induced responses. This suggests that TRPV2 channels are associated with the non-selective cation currents and contributes to elevation of $[Ca^{2+}]_i$ [63]. Lastly, the authors expressed TRPV2 channels into Chinese hamster ovary (CHO) cells and applied membrane stretch through the recording pipette or hypotonic stimulation which resulted in activation of non-selective cation currents [63]. As suggested by Muraki et al., homo-multimeric TRPV2 channels may be mechanosensitive in that they function as stretch-activated channels. However, as previously mentioned (see section 1.4), MNCs in the SON express SICC channels, therefore homo-multimeric TRPV2 channels are unlikely to be involved in the osmotransduction process of these neurons.

1.8.3 - TRPV3

TRPV3 channels are similar to TRPV2 channels in that they have no response to vanilloids or to extracellular acidification but are temperature sensitive [73]. It

was shown by Peier et al. that when temperatures rose above 33°C, an increase in current is seen in TRPV3-expressing CHO cells [73]. Because of its recent identification, very few studies have examined the osmosensitvity of TRPV3 channels. Peier et al. showed that when TRPV3-expressing cells were exposed to hypo-osmotic solution containing 70mM NaCl, no current response is observed [73]. Thus, homo-multimeric TRPV3 channels are unlikely involved in the osmosensory transduction process because the SICC channels in MNCs responded to hypo-osmotic solution with a decrease in channel activation.

1.8.4 - TRPV4

In heterologous expression systems, TRPV4 channels have been reported to be activated by heating to temperatures above 25°C [80] or 30°C [79]. This discrepancy in temperature threshold between these two studies could be related to the experimental methodology or preparation. For instance, Watanabe et al. used Cs-aspartate in their internal solutions whereas Guler et al. used standard KCl electrodes. TRPV4 channels do not respond to vanilloids [65] but are activated by extracelluar protons [87]. There was one report that stated TRPV4 heterologously expressed in CHO cells is activated by lowering the pH to values less than 6, and activation reached a maximum at around pH 4 [87].

Although TRPV4 channels are activated by various stimuli, TRPV4 channels are the first type of TRPV channels described to be modulated by osmolality [65, 74,

88]. Moreover, *in situ* hybridization analysis also showed that TRPV4 channels are expressed in regions that are osmosensory, including the OVLT, MnPO and SFO [65, 74, 88].

When expressed as homo-multimers in heterologous systems, TRPV4 channels are activated by cell swelling [65, 74, 88] but are unresponsive to cell shrinkage [65]. However, this channel is unlikely to be directly mechanosensitive, since no response to changes in pipette pressure is seen in single-channel cell-attached recordings [74]. It was later shown that TRPV4 activation during cell swelling was due to intracellular signalling via second messengers [89]. This intracellular signalling is believed to be dependent on phospholipase A2-mediated arachidonic acid release and its subsequent P450-epoxygenase-dependent metabolism to 5'-6'-epoxyeicosatrienoic acid [89]. To establish the properties of TRPV4 in a sensory system *in vivo*, TRPV4 was expressed into *C.elegans* with *osm-9* mutations, animals that were almost completely defective in their response to high osmotic strength (see section 1.5) [84]. From osmotic avoidance assays, it was shown that expressing TRPV4 channels in *osm-9* mutants restored the worms' ability to behaviourally avoid high osmotic strength [84].

To further support TRPV4 channels involvement in osmotic regulation, Liedtke et al. generated TRPV4 knockout (*trpv4-/-*) mice and demonstrated that the *trpv4* gene contributed to fluid balance and to the osmotic control of vasopressin release [90]. Liedtke et al. noticed that under basal conditions, *trpv4-/-* mice

drank less water and became more hyperosmolar than did wild-type littermates [90]. In addition, when hypertonic saline was injected into *trpv4-/-* mice, there was significantly lower plasma level of antidiuretic hormone than the wild-types [90]. However, Mizuno et al. observed that disrupting the *trpv4* gene did not influence drinking behaviour or serum osmolality [91]. In addition, they reported that hypertonic stimulation of hypothalamic slices obtained from *trpv4-/-* mice resulted in an increase in vasopressin release compared to WT mice [91].

Therefore, Liedtke and Friedman suggested that TRPV4 is involved in stimulating vasopressin release in response to hypertonicity, where as Mizuno et al. suggested that this channel may have an inhibitory role in vasopressin release [90, 91]. One possibility for this discrepancy was in the method used to generate the knockouts. Liedtke et al. deleted the entire exon 12 of TRPV4, which encodes the pore-loop domain and the adjacent transmembrane domains 5 and 6 [90]. On the other hand, Mizuno et al. inserted a neo cassette in exon 4, coding for the second ankyrin repeat in the intracellular amino-terminal domain [91]. The former method presumable produced a non-functional TRPV4 channel. Liedtke and Friedman confirmed the absence of a *trpv4* gene product in SFO and OVLT of *trpv4-/-* mice by immunohistochemistry. In contrast, the latter method may have still produced a functional TRPV4 channel. For example, it is possible that an alternative splice site was present downstream of the neo cassette and that a truncated TRPV4 channel might still be expressed. This hypothesis has been supported by recent evidence reporting the existence of five N-terminal splice

variants of TRPV4, all of which lack different portions of their cystolic N-terminal domain [92]. While Mizuno et al. confirmed the absence of TRPV gene products in kidneys of *trpv4-/-* mice, they did not examine the OVLT and SFO [91]. Thus, the possibility remains that an N-terminal variant of TRPV4 expressed in these areas can be implicated in the osmotic control of vasopressin release. Although the reason underlying the discrepancies between the two studies remains to be determined, it still suggests that the TRPV4 channels are involved in the osmotic control of vasopressin release.

1.9 – Osmotic Responses from Homomulteric TRPV Channels Differ from SICC Channels in MNCs

Of the TRPV channels mentioned above, TRPV1, TRPV2, and TRPV4 appeared to be modulated by osmolality. Birder et al. suggested that TRPV1 channels expressed in urinary bladder are responsible for hypoosmotic-evoked ATP release [85]. Also, Liu et al. demonstrated that capsaicin-evoked TRPV1 currents are modulated by changes in osmotic pressure (hypotonic or hypertonic) [86]. Homo-multimeric TRPV2 or TRPV4 channels expressed in heterologous systems are activated by hypotonicity; cell swelling [63, 65]. However, these results differ from what is seen in MNCs of the SON where hypotonicity (i.e cell swelling) hyperpolarize and inhibit firing in MNCs. On the other hand, hypertonicity (i.e cell shrinkage) activated SICC channels leading to depolarization and increase in firing rate in MNCs [47]. These opposite observations suggested that although

TRPV1, TRPV2, and TRPV4 channels on their own (homomultieric complexes) are modulated by osmolality, they do not appear to be the SICC channels involved in the osmosensory transduction process in MNCs of SON [47]. To our knowledge, only one molecularly defined cation channel has been shown to display properties consistent with that of MNCs during osmotic stimulation [93].

1.10 - Cloning of a Stretch-Inactivated Non-Selective Cation Channel

A homologue of the TRPV1 channel was isolated from a rat kidney cDNA library and found to be inactivated by membrane stretch (SIC_{art}) [93]. An alignment of the SIC_{art} amino acid sequence showed that it starts at the 308^{th} amino acid of the full-length TRPV1 and has a different C-terminal region [93]. It was later confirmed that the C-terminal of SIC_{art} (beginning at the 460^{th} amino acid) was homologous to the corresponding C-terminal domain of TRPV4 [94]. The SIC_{art} cDNA encodes a 563-amino acid protein in which the six transmembrane segments and pore regions is similar to TRPV1. However, the N-terminal of SIC_{art} contains one ankyrin repeat domain, whereas TRPV1 has three. When SIC_{art} is expressed in CHO cells, hypertonicity caused an increase in current amplitude whereas hypotonicity led to a decrease in current amplitude [93]. Thus, SIC_{art} is an alternative splice variant, lacking a part of the N-terminal region of TRPV1 with a TRPV4 C-terminal region and it behaves like a stretch-inactivated channel. Unfortunately, genomic analysis by Xue et al. suggested that SIC_{art} may be a

cloning artifact because it was not encoded by the mouse genome [94]. Nonetheless, these findings raised the possibility that TRPV1 variants alone, or in conjugation with other TRPV subunits (such as TRPV2 or TRPV4), may contribute to the osmoreceptor in MNCs.

1.11 - Potential Role of TRPV1 Gene Products in Osmoreceptors

As previously stated (see section 1.8.1), MNCs of SON expressed a splice variant of TRPV1 in which the N-terminal is truncated. Unlike the TRPV1 expressed in dorsal root ganglia, electrophysiology results showed that these splice variants are insensitive to capsaicin (Figure 1.3) [82]. Furthermore, SON neurons from *trpv1-/-* mice showed no increase in membrane conductance, membrane depolarization, or increase in firing rate upon hypertonic stimulation as compared to wild-type (Figure 1.4) [81, 82]. However, lack of osmosensitivity in *trpv1-/-* does not directly prove that TRPV1 is part of the osmoreceptor. Hypothetically, lack of *trpv1* gene products could have resulted in a developmental problem; trpv1 gene products could have been necessary during development for neurons to acquire an osmosensory phenotype dependent on another gene. However, Ciura and Bourque reported that *trpv1-/-* mice displayed no gross neuroanatomical abnormalities and the number of cells expressing neuronal-specific nuclear protein appeared similar in the OVLT of wild-types and *trpv1-/-* animals [81]. Another possibility is that *trpv1* gene products may be

needed for the assembly or targeting of the osmoreceptor to the surface of the membrane. Finally, it is possible that a product of the *trpv1* gene plays a functional role as a pore-forming channel protein in the osmosensory transduction complex. Evidence supporting the latter hypothesis remains to be obtained.

1.12 - Specific Pharmacological Tools Against TRPV1 Channels

There are a few approaches that can be used to determine whether *trpv1* gene products are part of the pore-forming channel in the osmosensory transduction complex. One method is to isolate the osmoreceptor (i.e SICC channels in MNCs of SON) and observe if it is homologous to TRPV1 channels. Unfortunately, attempts at cloning SICC channels have been unsuccessful. Alternatively, one could conditionally knockout the *trpv1* gene in brain regions of full grown rodents that are known to be osmosensitive (e.g SON, OVLT, SFO, MnPO) and observe whether neurons from these areas are still responsive to osmotic stimuli.

A third approach consists of utilizing specific pharmacological tools, which will acutely antagonize TRPV1-mediated ion influx and observe whether osmotically evoked currents are impaired. This would confirm whether trpv1 gene products are part of the pore-forming channels responsible for the osmosensory transduction process. Currently, there are tools such as capsazepine, which acts

as a specific competitive antagonist of capsaicin-mediated activation of TRPV1 [95]. However, the osmosensory responses of wild-type MNCs are not sensitive to capsazepine. This came as no surprise since the splice variant of TRPV1 expressed in MNCs was not activated by capsaicin (see section 1.11) [82]. Recently, small molecules such as SB-366791 have been identified which appear to be specific TRPV1 antagonists [96]. Although SB-366791 is found to be ineffective in blocking TRPV4 channels, whether it is also ineffective in blocking other members of the TRPV family (i.e TRPV2 and TRPV3) remains to be determined [96]. The use of SB-366791 is also limited only to functional studies of TRPV1 channels; the drug cannot be used for histological (e.g. western blots or immunohistochemistry) or biochemical (i.e co-immunoprecipation) studies. Thus a novel tool, capable of multiple applications, specifically targeting TRPV1 channels is required to help study whether trpv1 gene products are involved in the osmosensory transduction complex.

1.12.1 - Immunological Strategy: Extracellular Pre-Pore Loop Antibodies

One approach that can be used to develop a specific ion-channel blocking tool (that can be used both for a functional and histological studies) is the generation of extracellular pre-pore loop antibodies [97, 98]. Studies have shown that antibodies binding to the pre-pore region can act as ion-channel blockers [97, 98]. Though the underlying mechanism is unclear, there are two possible mechanisms on how these extracellular pore loop antibodies can act as channel inhibitors as suggested by Zhou et al. [97]. First, the target peptide sequence is in the ion flux pathway, thus when the antibody binds to this region, it may act like a plug and physically block permeation of ions [97]. A similar blocking mechanism was previously described with charybdotoxin (CTX), a scorpion toxin [99]. MacKinnon and Miller reported that CTX binds to the pore region of Ca^{2+}-activated K^+ channels, acting like a plug, and blocks ion flux [99]. Thus, because the antibody is also targeting the pore region, its blocking mechanism on ion-channels may be similar to how CTX blocks Ca^{2+}-activated K^+ channels. Alternatively, it is possible that the binding of the antibody causes a conformation change in the ion-channel and causes closing of the ion flux pathway [97]. Thus, instead of acting like a plug as described above, the antibody could be an allosteric effector causing the channel to stay in a closed position hence impairing ion flux.

Xu et al. were the first to develop an extracellular pre-pore loop antibody against a member of the TRP family; they specifically focused on TRPC5 channels [98]. To establish whether the antibody can act as an ion channel inhibitor, cells expressing TRPC5 channels were activated by the lanthanide gadolinium and then they bath applied the TRPC5 specific pore loop antibody [98]. It was observed that the TRPC5 specific antibody blocked the gadolinium–induced current by about 50% after 5-10 minutes (Figure 1.5) [98]. On the other hand, bath application of a non-specific immunoglobulin-G (IgG) ,or phosphate buffered saline (PBS), did not block the gadolinium-induced current [98]. In addition to blocking the gadolinium-induced currents in TRPC5 channels, the authors demonstrated that the pre-pore loop antibody is specific to TRPC5 channels [98]. From western blot analysis, they showed that the antibody only detected a band in the lane containing protein lysates of HEK293 cells transfected with TRPC5 channels and not in other lanes containing lysates of HEK293 cells transfected with TRPC1 or TRPC4 (TRPC5's closest relatives) (Figure 1.5) [98]. These studies indicate that extracellular pre-pore loop antibodies could represent a useful approach to investigate whether *trpv1* gene products form part of the ion-channel involved in osmoreception.

1.13 - Hypothesis

The exact molecular composition of the osmoreceptor has yet to be determined but *trpv1* gene products seem to be potentially involved. With the use of specific pharmacological tools such as an extracellular pre-pore loop antibody specific to rat TRPV1, one could investigate whether trpv1 gene products are responsible for forming parts of the ion-channel involved in the osmosensory transduction complex. In this study, therefore, attempted to develop an antibody specific to rat TRPV1. As such, the following hypotheses were formulated and tested:

1. Injecting rabbits with synthetic peptide comprising an amino acid sequence specific only to TRPV1 subunits, we will produce antibodies that will specifically bind to TRPV1 channels.
2. An extracellular pre-pore loop anti-TRPV1 specific antibody will act as a functional blocker of TRPV1 channels
3. Anti-TRPV1 antibody will bind within regions of the hypothalamus that are known to be osmosensors; SON, OVLT, SFO, and MnPO.

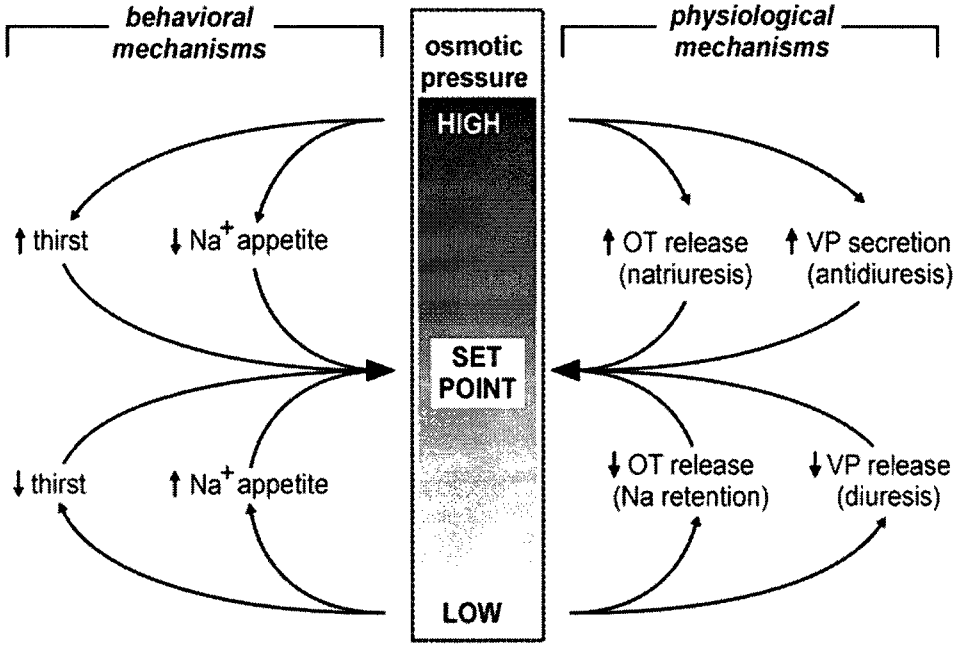


Figure 1.1 - Osmoregulatory Responses to Osmotic Perturbations
Changes in extracellular fluid (ECF) osmolality modulate behavioral (left) and physiological (right) mechanisms that affect sodium and water balance to restore homeostasis. In mammals, when the ECF osmolality deviates from its homeostatic set-point, the behavioral response consists of regulating intake of salt and water through changes in sodium appetite and thirst. Simultaneously, the physiological response consists of releasing anti-diuretic (vasopressin, VP) and natriuretic (oxytocin, OT) hormones. Together, both mechanisms respond in order to restore homeostasis upon osmotic perturbations. Modified, with permission (License # 2007781024501) , from Frontiers in Neuroendocrinology, Vol 15, Issue 3, Bourque CW, Oliet SH, Richard D. Osmoreceptors, osmoreception, and osmoregulation. Pages 231-74, 1994.

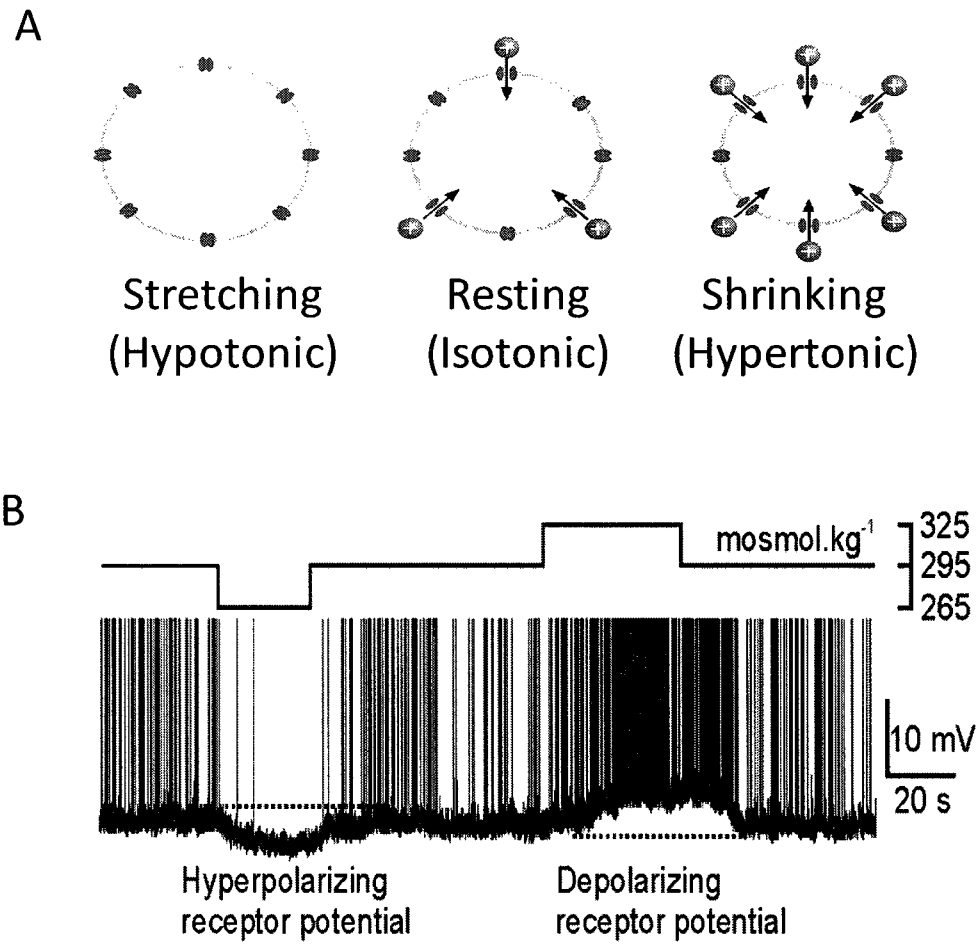


Figure 1.2 – Osmosensory Transduction is Mediated by Stretch-Inactivated Cation Channels (SICC)

In panel A, schematic drawings illustrate the phenotypes of magnocellular neurosecretory cells (MNCs) when faced with different osmotic stimuli. Under resting conditions (A, middle) a proportion of SICC channels are active and allows the influx of cations. When MNCs are exposed to a hypotonic solution, the cell stretches and inactivates SICC channels thus decreasing the influx of cations (A, left). On the other hand, when MNCs are exposed to a hypertonic solution, the cell shrinks and activates more SICC channels thus increasing the influx of cations (A, right). In Panel B, tonic action potential firing is seen during resting conditions (i.e isotonic solution). When exposed to a hypertonic solution, an increase in cation influx depolarizes the membrane and causes an increase in action potential firing frequency (B, right end of trace). Under hypotonic solution, the SICC channels are inactivated and the loss of cation influx causes hyperpolarization and inhibits firing (B, left end of trace). Modified, with permission (License # 2012690019351), from J Physiol. 1992 Sep;455: Oliet SH, Bourque CW, P.291-306.

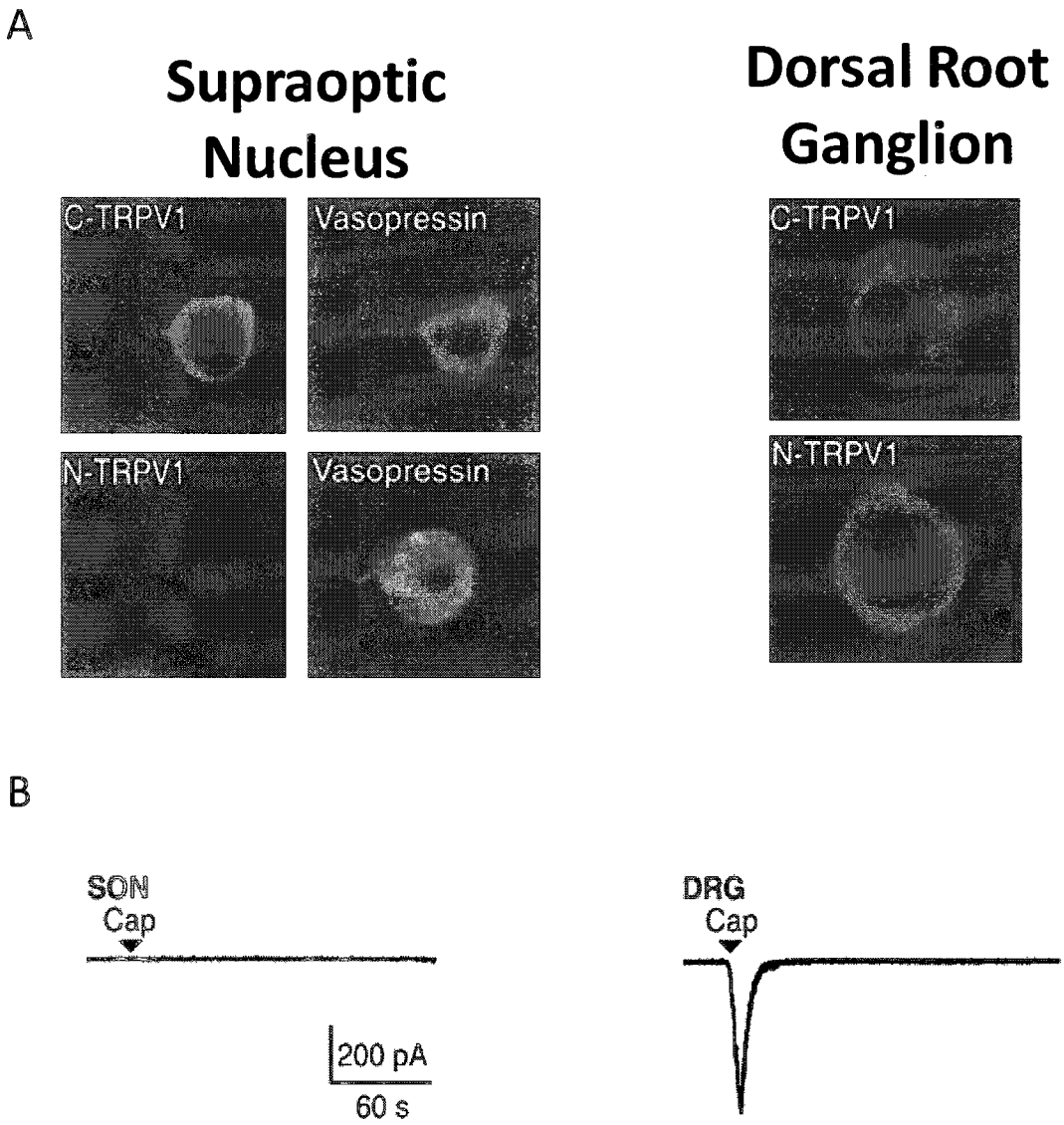


Figure 1.3 – MNCs of SON Express a Capsaicin-Insensitive N-Truncated Splice Variant of TRPV1

In panel A, immunohistochemical staining confirms the presence of the full-length TRPV1 in dorsal root ganglion neurons (left). On the other hand, only the C-terminal of TRPV1 is detected in vasopressin-positive SON neurons. In panel B, whole-cell voltage-clamp recordings show that dorsal root ganglion neurons respond to capsaicin (right) whereas SON neurons do not (left). Figure, reproduced with permission (License # 2012681396883), from Nat Neurosci. 2006 Jan;9(1), Sharif Naeini R, Witty MF, Séguéla P, Bourque CW, P.93-8.

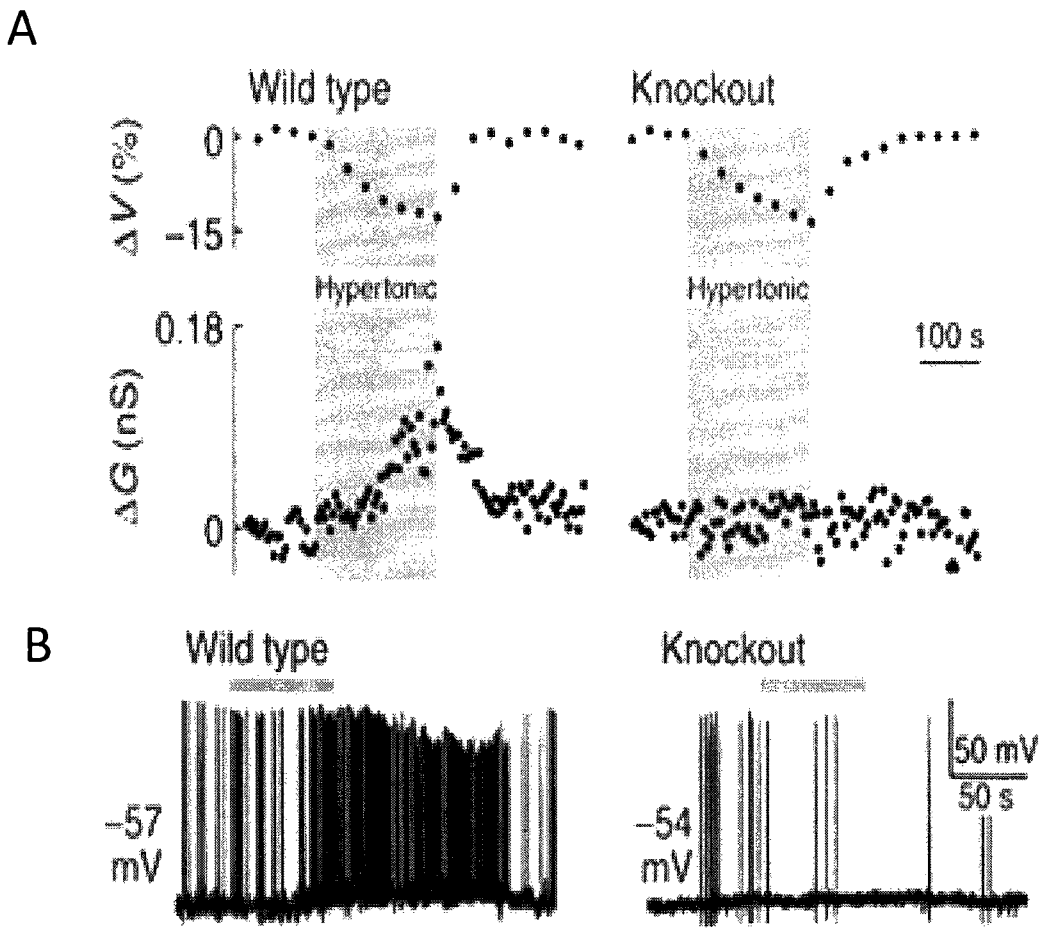


Figure 1.4 – Lack of Osmosensory Transduction in MNCs from TRPV1 Knockout Mice

In panel A, when MNCs from wild-types and trpv1 knockout mice are exposed to a hypertonic stimulus, a decrease in cell volume is observed in both conditions. However, only MNCs from wild-type mice showed an increase in membrane conductance. In panel B, whole-cell current clamp recordings show that when exposed to a hypertonic stimulus, membrane depolarization and an increase in firing frequency is only seen in MNCs of wild-type mice. Figure, reproduced with permission (License # 2012681396883), from Nat Neurosci. 2006 Jan;9(1), Sharif Naeini R, Witty MF, Séguéla P, Bourque CW, P. 93-8.

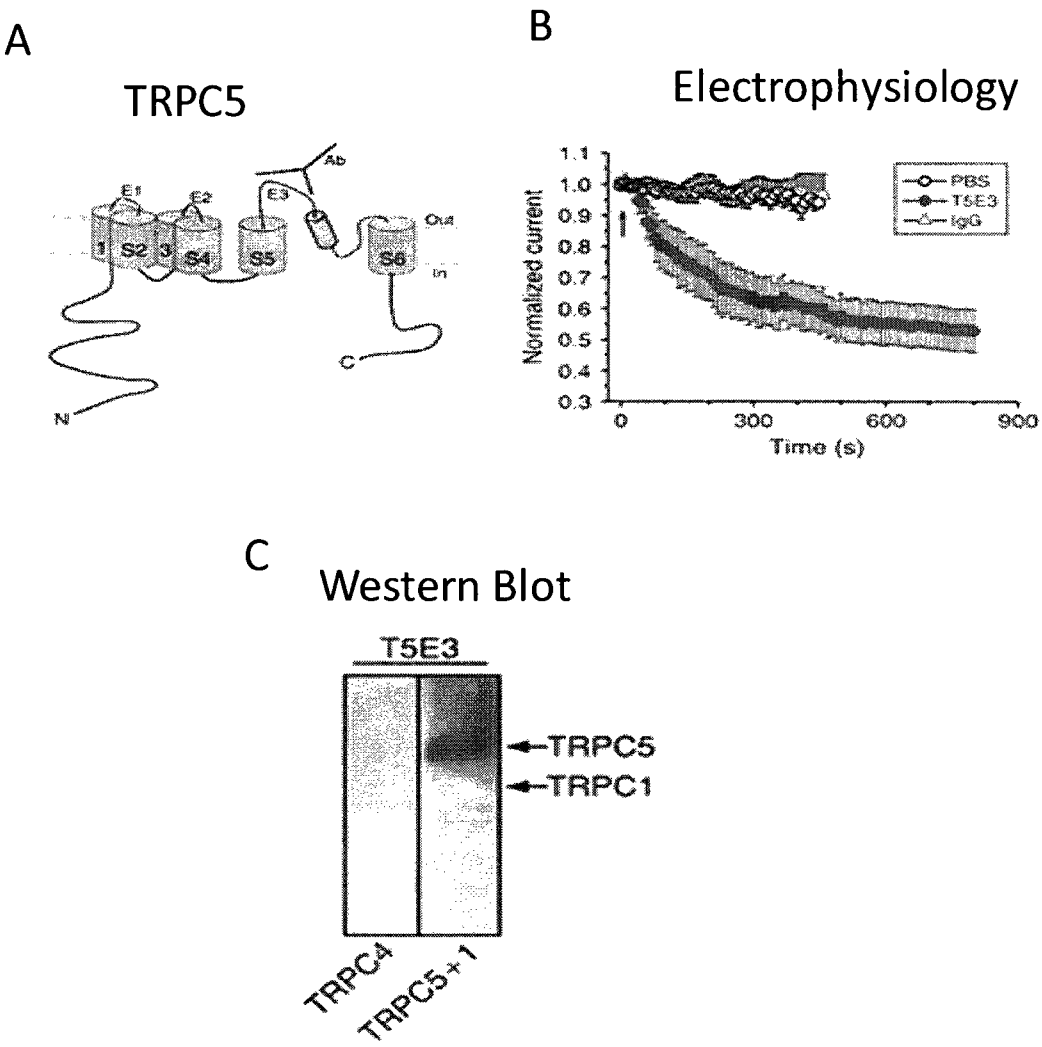


Figure 1.5 – An Extracellular Pre-Pore Loop Antibody Can Act as a Specific Ion-Channel Blocker

Panel A illustrates a schematic drawing of a TRPC5 subunit with six transmembrane segments (S1-S6), three extracellular loops (E1-E3), with intracellular N- and C-termini. As shown, the pre-pore loop antibody (T5E3) specifically targets the region between S5-S6 at the extracellular loop (E3). In panel B, cells transfected with TRPC5 channels are activated by the lanthanide gadolinium. Bath application of the T5E3 antibody inhibit the gadolinium-induced current, but not phosphate buffered saline (PBS) or a non-specific IgG. In panel C, western blot results show that TRPC5-E3 specifically detects TRPC5 and not TRPC1 or TRPC4. Figure, reproduced with permission (Licence # 2012680902011), from Nat Biotechnol. 2005 Oct;23(10), Xu SZ et al, P.1289-93.

Chapter 2: Methods and Materials

2.1 - Peptide Synthesis

The 18 amino acid sequence, CGKNNSLPMESTPHKCRG, highly specific to the rat TRPV1, is located between the fifth and sixth transmembrane domains on the extracellular pre-pore loop region of the rat TRPV1 channel. We obtained 10mg of synthetic peptide comprising the sequence mentioned above with purity >= 75% from McGill University: Sheldon Biotechnology Centre located at Montreal, Quebec (Canada).

2.2 - Generation of Rabbit Polyclonal Antibodies to Pre-Pore Loop of Rat TRPV1

For this protocol, all materials and detailed instructions are provided by Pierce Biotechnology: EZ Sulfhydryl Reactive Antibody Production and Purification Kit with mcKLH (Product # 77614).

2.2.1 - Synthetic Peptide Conjugation with Maleimide-Activated mcKLH

First, a bottle containing *Imject® Maleimide-Activated mcKLH* was dissolved with 200µL of ultrapure water. Then, we weighted 2mg of the synthetic peptide (see section 2.1) and added it to 300µL of of *Maleimide Conjugation Buffer*. Both

solutions (total volume of 500µL) were mixed together and incubated for 2 hours at room temperature.

2.2.2 - Conjugate Purification by Desalting

One bottle of *Imject® Purification Buffer Salts* was dissolved by adding 60mL of ultrapure water. Then, we prepared the *D-Salt™ Dextran Desalting Column* by sequentially removing the top and bottom caps, allowed the storage solution to completely drain out, and then washed the column by adding 25mL of the dissolved *Imject® Purification Buffer Salts.* Next, we added 500µL of the synthetic peptide conjugated with mcKLH solution (see section 2.2.1) to the *D-Salt™ Dextran Desalting Column.* Once the mixture was settled, we added 500µL of the dissolved *Imject® Purification Buffer Salts* and collected the solution that drained out of the column into clean tubes (approximately 500µL). This last step was repeated 14 times and in the end, we had 15 tubes with aliquots of 500µL.

2.2.3 - Collecting Synthetic Peptide Conjugated with mcKLH

In order to locate which tubes contained the conjugated peptide, we used a spectrophotometer to measure the absorbance of each sample from section 2.2.2. First, we adjusted the wavelength to 280nm (a value where proteins in

solution maximally absorb ultraviolet light) and changed the setting on the spectrophotometer so that it displayed the absorbance value. Next, we calibrated the spectrophotometer with 500µL of dissolved *Imject® Purification Buffer Salts* (see section 2.2.2). We recorded the absorbance from each sample (i.e 15 tubes) and after every reading, we re-collected the sample and washed the glass cuvette with distilled water. Samples containing little or no protein display low absorbance values (approximately zero) were discarded where as samples containing protein display an absorbance peak were kept.

2.2.4 - Antibody Production

The conjugated peptide samples were given to the McGill University Animal Resource Centre where they mixed it with Freund's Complete Adjuvant and then injected into a rabbit (250µg/300µL) of the conjugated peptide per rabbit). We also provided three additional conjugated samples (100µg/300µL) where they mixed it with Freund's Incomplete Adjuvant and will be used for three subsequent booster shots. At the facility, the rabbit was bleed after each injection and the serum was collected for us.

2.3 - Affinity Column Preparation

For this section, all materials and detailed instructions are provided by Pierce Biotechnology: EZ Sulfhydryl Reactive Antibody Production and Purification Kit with mcKLH (Product # 77614).

2.3.1 - Reducing Synthetic Peptide for SulfoLink® Column

One mg of synthetic peptide (see section 2.1) was dissolved in 1mL of *SulfoLink® Sample Preparation Buffer* and then added to the *SulfoLink® Reductant* bottle. We incubated the mixture for 1.5 hours at 37°C and then allowed it to cool to room temperature. During the incubation, we prepared the *D-Salt™ Polyacrylamide Desalting Column* by washing it with 20mL of *SulfoLink® Coupling Buffer*. Once the incubation time was over, we added this peptide mixture to the *D-Salt™ Polyacrylamide Desalting Column* and allowed the mixture to settle in the column gel bed. We then added 3mL of *SulfoLink® Coupling Buffer* to the column and collected the 3mL that drained out.

2.3.2 - Immobilizing Peptide to SulfoLink® Column

First, we removed the caps on the the *SulfoLink® Column* and allowed the storage solution to drain out. We then prepared the *SulfoLink® Column* with 8mL

of *SulfoLink® Coupling Buffer*. Next, we replaced the bottom cap back on the *SulfoLink® Column* and added 2.5mL of the reduced synthetic peptide sample (see section 2.3.1). The top cap was placed back on the *SulfoLink® Column* and then we mixed entire column (end-over-end) at room temperature for 15 minutes followed by 30 minutes incubation without mixing. Once time was over, we sequentially removed the top and bottom caps, allowed the solution to drained, and washed the column with 6mL of *SulfoLink® Coupling Buffer*.

2.3.3 - Blocking Non-Specific Binding Sites in SulfoLink® Column

We weighted 15.8mg of L-Cysteine•HCl and added it to 2mL of *SulfoLink® Coupling Buffer*. Then we replaced the *SulfoLink® Column*'s bottom cap, added this mixture to the column and replaced the top cap. We then mixed the entire column (end-over-end) for 15 minutes at room temperature and then incubated the reaction without mixing for another 30 minutes at room temperature. After incubation, we sequentially removed the top and bottom caps and allowed the solution to drain from the column. We then washed it with 12mL of *SulfoLink® Wash Solution* and then washed the again with 4mL of phosphate buffered saline. Next, we replaced the bottom cap and added an additional 3mL of phosphate buffered saline. Lastly, we inserted a porus disc by sliding it to within 1mm of the gel bed using the open tube end of a serum separator. The top cap

was replaced and the entire column was placed upright at 4°C until it was ready to be used.

2.4 - Affinity Purification of Anti-TRPV1 Antibodies

For this protocol, all materials and detailed instructions are provided by Pierce Biotechnology: EZ Sulfhydryl Reactive Antibody Production and Purification Kit with mcKLH (Product # 77614).

2.4.1 - Sample Preparation

We first diluted 500µL of rabbit serum (see section 2.2.4) with 500µL of *Sample Buffer* (0.025M Tris, 0.15M NaCl, pH 7.2 – BupH Tris Buffered Saline Pack – Product # 28379). We then removed both caps on the *SulfoLink® Column*, allowed the excess storage solution to drain out and then washed it with 6mL of *Sample Buffer.* Next, we applied 1mL of the sample prepared above to the column and allowed it to completely enter the gel bed. We then applied 0.2mL of *Sample Buffer* and once it entered the gel bed, we replaced the bottom cap. We added an additional 0.5mL of *Sample Buffer* to the column, replaced the top cap, and then incubated the column for 1 hour at room temperature. After incubation, we removed both caps and washed the column with 12mL with *Sample Buffer.* In order to collect the antibody, we applied 0.5mL of elution

buffer (ImmunoPure® IgG Elution Buffer – Product # 21004) to the column and collected the 0.5mL sample into a fresh tube. We added another 0.5mL of elution buffer (ImmunoPure® IgG Elution Buffer – Product # 21004) to the column and this was repeated another 15 times; in the end, we had 16 tubes with aliquots of 0.5mL.

Similar to the protocol described in section 2.2.3, we used a spectrophotometer to locate which tubes contained the purified antibody. First, we adjusted the wavelength to 280nm (a value where proteins in solution maximally absorb ultraviolet light) and changed the setting on the spectrophotometer so that it was displaying the absorbance value. Next, we calibrated the spectrophotometer with 500µL of elution buffer (ImmunoPure® IgG Elution Buffer – Product # 21004). We recorded the absorbance from each sample (i.e 16 tubes) and after every reading, we re-collected the sample and washed the glass cuvette with distilled water. Samples containing little or no protein display low absorbance values (approximately zero) were discarded where as samples containing protein display an absorbance peak were kept.

2.4.2 - Regenerating and Storing Affinity Column

In order to reuse the *SulfoLink® Column*, we regenerated the column by washing it with 16mL of *Sample Buffer* to remove any residual protein and reactivate the gel. Then, we added 8mL of *Sample Buffer* containing 0.05% sodium azide to the

column. Lastly, we replaced the bottom cap and added 2mL of *Sample Buffer* to column. We then replaced the top can stored the column upright at 4°C until we used it again.

2.5 - HEK293 Cell Culture

We chose to culture HEK293 cells because they lack TRPV1 channels. In 100mm cell culture dishes, we incubated HEK293 cells at 37°C and 5% CO_2 and cultured them with 10mL of Dulbecco's Modified Eagle Medium (DMEM - Gibco # 10313-021), supplemented with 10% Fetal Bovine Serum (Gibco # 16000-044), 1% Penicillin-Streptomycin (Gibco # 15140-122), 1% MEM Non-Essential Amino Acids (Gibco # 11140-050), and 1% L-Glutamine (Gibco # 25030-081). When the HEK293 cells were approximately 60% to 80% confluent, we removed the culture medium and washed the HEK293 cells with 5mL of Dulbecco's Phosphate Buffered Saline (D-PBS - Gibco # 14190-144). We then removed the D-PBS and added 1mL of Trypsin-EDTA (Gibco # 25300-054). The cells were then incubated at 37°C and 5% CO_2 for 2-3 minutes and then we gently tap/swirl the dish until it was clearly seen that all the HEK293 cells were lifted from the dish. Next, we transferred all the dissociated cells into a fresh tube and added 9mL of culture medium. Then, we re-plated 1mL of the dissociated HEK293 cells onto a fresh 100mm cell culture dish and added an additional 9mL of culture medium. The plate was placed back in the incubator at 37°C and 5% CO_2. This entire protocol

was repeated once the HEK293 cells became approximately 60% to 80% confluent.

2.6 - Transfection of HEK293 Cells

Prior to transfecting HEK293 cells, we took 1mL of the dissociated HEK293 cells (see section 2.5), plated it on 60mm culture dish with an additional 4mL of culture medium and incubated the cells at 37°C and 5% CO_2. When the dish was approximately 40% to 80% confluent, it was ready for transfection.

First, we diluted approximately 4µg of cDNA (a combination of rat TRPV1 and 1:10 GFP) dissolved in TE buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA) with only DMEM (no supplements) to a total volume of 150µL. We then added 40µL of PolyFect Transfection Reagent (Qiagen # 301105) to the cDNA solution, mixed it by pipetting up and down, and then incubated the solution at room temperature for 10 minutes. In the meanwhile, we removed the culture medium from the 60mm dish and add 3mL of fresh culture medium. When incubation was over, we added 1mL of culture medium to the sample described above, mixed it by pipetting up and down, and then quickly transferred the total volume to the 60mm dish. We gently swirled the dish and then placed it back into the incubator at 37°C and 5% CO_2 for 48 hours. After 2 days, we used a florescence microscope to determine whether HEK293 cells were GFP positive and if they were, they would be furthered processed. This protocol was repeated for other transfection

(i.e GFP/TRPV2, GFP/TRPV3, and GFP/TRPV4). For more details instructions, please refer to the Qiagen: PolyFect® Transfection Reagent Handbook (Product # 301105).

2.7 – Transfected HEK293 Cells Lysate Preparations

2.7.1 - Protein Extraction

Dishes containing transfected HEK293 cells (see section 2.6) were removed from the incubator and placed on ice. We gently removed the culture medium and added 3mL of cold 1X PBS (see Table 1). We repeated this step 3 times and every time we used a new tip to aspirate the 1X PBS. After the last removal, we added 0.2mL of RIPA/PI (see Table 2) to the dish, gently swirled it, and then left it on ice for 15 minutes. Next, we scraped the cells with a rubber policeman, a hand-held flexible natural-rubber used to scrap cells from a plate to a suspension, and collected approximately 0.2mL of the lysate into a clean tube. The tube was placed on ice for another 15 minutes and it was vortexed occasionally for the lysis process. Lastly, the tube was centrifuged at maximum speed (Eppendorf Centrifuge Model # 5412) for 30 minutes at 4^{o}C and the supernatant was transferred into a clean tube and the pellet was discarded.

2.7.2 - Protein Dosage

We prepared a protein standard ladder by diluting BSA (10mg/mL) with RIPA/PI to certain concentrations (see Table 3). We also diluted the transfected HEK293 cell lysate to 1:5 and 1:10 in RIPA/PI buffer. Next, with the reagents provided in the Bio-Rad DC Protein Assay Kit (Product # 500-0112), we mixed 1mL of *Reagent A* and 20µL of *Reagent S*.

With the microplate, we added 5µL of protein solution to each well; each sample was duplicated (i.e two wells of 0.2mg/mL protein standard, two wells of 0.5mg/mL protein standard, two wells of transfected HEK293 cell lysates at 1:5, etc.). Next, we added 25µL of *Reagent A + S* mixture to each well. Lastly, we added 200µL of *Reagent B* in each well. We waited 15 minutes to allow the reaction to fully take place before measuring the absorbance of each sample with a microplate reader. From the absorbance values, we applied the Beer-Lambert's Law to calculate the concentration of the sample containing the transfected HEK293 cells.

2.7.3 - Sample Preparation for Gel Electrophoresis

Prior to making the sample, we added 12% β-mercaptoethanol to 4X Sample Buffer (see Table 4) (i.e 12µL of β-mercaptoethanol to 88µL of 4X Sample Buffer). Next, we diluted the 4X Sample Buffer with the protein lysates of transfected

HEK293 cells making it 1X Sample Buffer. After, we placed safe caps on the tube, boiled it (at approximately 100°C) for 7 minutes and then froze the aliquot at -20°C. We would thaw the aliquot when we were ready to use them for gel electrophoresis.

2.8 - Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

2.8.1 - Electrophoresis Set Up

We used a Vertical Electrophoresis System (Bio-Rad) to set up the gel cast. After preparing the separating gel solution (see Table 5), we quickly poured it into the cast until it reached the 5cm mark as indicated on the apparatus and then added some water to level off the gel. When the separating gel was polymerized, we just poured the water out. Next, we inserted the combs that made the wells and quickly poured the prepared stacking gel solution (see Table 6). We waited until the gel completely polymerized before removing the combs. We then inserted the gel cast into the electrophoresis chamber and filled it up with 1L of running buffer (see Table 7). Depending on the experiment, we did either section 2.8.2A or section 2.8.2B.

2.8.2A - Protein Loading and Gel Running – Dilution Series

We loaded into each lane 20µg of protein lysates of HEK293 cells transfected with rat TRPV1 (see section 2.7.3), and at least two wells with 5µL of a pre-stained SDS-PAGE Standards, Low Range Weight Ladder (Bio-Rad Product # 161-0305). We then turned on the power supply (200V) and watched the progression of migration of each sample. We stopped running the gel after 90 minutes.

2.8.2B– Protein Loading and Gel Running – Specificity

We loaded each lane with 20µg of protein lysates of HEK293 cells transfected with either rat TRPV1, TRPV2, TRPV3, or TRPV4 (see section 2.7.3) and one lane was loaded with 5µL of a pre-stained SDS-PAGE Standards, Low Range Weight Ladder (Bio-Rad Product # 161-0305). We then turned on the power supply (200V) and watched the progression of migration. We stopped running the gel after 90 minutes.

2.8.3 - Protein Transfer

Following section 2.8.2A or section 2.8.2B, we transferred the protein on the gel to nitrocellulose membrane via a transfer cassette in a tray with 500mL of transfer buffer. First, we made sure the black side of the transfer cassette was the bottom layer. Then we soaked the sponge with transfer buffer (see table 8) and placed in on top followed by Watman paper (10x8 cm). Next, we carefully

placed the gel on top and then placed the nitrocellulose membrane. Finally, we added another piece of Watman paper followed by a sponge and then we closed the cassette. The cassette was placed into the transfer apparatus filled with transfer buffer along with a stirring bar and was set for transfer at 20V overnight at 4°C.

2.9 - Western Blot

Following section 2.8.3, we disassembled the cassette and placed the membrane in a small tray with Ponceau (1X) and shook it for 3 minutes. We then rinsed it with distilled water until proteins were visible, and then placed the membrane in a Ziploc bag and made a photocopy of it. Next, we added 5% blocking solution (see Table 9) to the Ziploc bag and then completely sealed it. The bag was placed on a shaker for 60 minutes at room temperature.

To determine the optimal range to use the anti-TRPV1 antibody, we used the nitrocellulose membrane prepared from section 2.8.2A. With this experiment, we prepared a range of dilutions of anti-TRPV1 antibodies (1:100, 1:500, 1:1000, and 1:5000) in 5% blocking solution in order to determine the minimum dilution needed to detect a signal. We then compared the photocopied paper of our membrane to see where exactly where the proteins were and then used a razor to cut out different lanes on the actual membrane. Each lane was placed into a

different Ziploc bag with a different dilution of anti-TRPV1 antibody (at least 0.5mL per lane) and was sealed.

To determine the specificity of anti-TRPV1 antibody, we used the nitrocellulose membrane prepared from section 2.8.2B. In this case, we placed the entire membrane into the Ziploc bag and added 10mL of anti-TRPV1 antibody at 1:100 and sealed the bag.

In both situations mentioned above, the Ziploc bags are incubated overnight at 4°C. The following day, membranes are removed and washed three times for 10 minutes with TBST (see Table 10) in small trays. We replaced the membranes back into sealed Ziploc bags with diluted secondary antibody: goat-anti-rabbit-IgG-HRP in blocking solution (1:10 000) and incubated them for 2 hours at room temperature on a shaker. After incubation, we washed the membranes three times for 10 minutes with TBST. After the final wash, we placed the membranes back into their original orientation on a Ziploc bag, went to the dark room. In the room, we mixed the two Amersham ECL™ Western Blotting Detection Reagents (Product # RPN2109) and immediately poured it evenly on the membrane, waited 30 seconds and removed excess reagent. We then taped the Ziploc bagged membrane into a developing cassette and turned off the lights. We took a Kodak BioMax Light Film (Product # 868 9358) and placed it over the membrane. We closed the cassette and exposed the film for 30 seconds before

we took it out and placed it in the developer. This last step was repeated at different exposure times (1, 3, 5, 7 minutes).

2.10 - Immunohistochemistry - Transfected HEK293 Cells – Dilution Series

We used five 60mm dishes with HEK293 cells transfected with rat TRPV1 (see section 2.6), we removed 4mL of culture medium and then we dissociated them with 1 mL of Trypsin-EDTA (Gibco # 25300-054). We placed the dishes in the incubator at 37^0C and 5% CO_2 for 2-3 minutes and then added 4mL of fresh culture medium. We then drew wax circles in five 35mm culture dishes, added 200µL of the re-suspended transfected HEK293 cells within this marked circle and incubated them at room temperature for 1 hour. Following incubation, we gently removed 200µL of culture medium and added 200µL of 4% PFA within the waxed circle and waited 10 minutes at room temperature. We then flooded the entire dish with 0.1M PBS and washed it 3 times for 5 minutes with 0.1M PBS. After the final wash, we flooded the dish with blocking solution (10% normal goat serum, 0.05% Triton-X in 0.1M PBS) and incubated the dish at room temperature for 1 hour.

To determine the optimal range, we prepared different anti-TRVP1 antibody dilutions in blocking solutions (1:100, 1:500, 1:1000, and 1:5000). Following incubation with blocking solution, we washed each dish containing HEK293 cells transfected with rat TRPV1 with 0.1M PBS three times for 5 minutes. We then

added a different dilution of anti-TRPV1 antibody to each plate and each plate was placed overnight at 4°C.

The next day, we washed each dish three times for 5 minutes with 0.1M PBS. We then added secondary antibodies, Alexa Fluor® 568 goat anti-rabbit IgG at 1:500 (Invitrogen) and incubated the dish at room temperature for 90 minutes. Lastly, we washed each dish three times for 5 minutes with 0.1M PBS and after the last wash, we flooded the dish with 0.1M PBS and stored it at 4°C until we were ready for imaging at the confocal fluorescence microscope. The confocal microscope used is a Leica DM LFSA and images were captured simultaneous with either a 10x or 40x objective lenses. GFP-positive cells were excited at 488nm and emission was collected at 525nm. TRPV1-positive cells were excited at 568nm and emission was collected at 607nm.

2.11 - Immunohistochemistry – Transfected HEK293 Cells – Specificity

With the transfected HEK293 cells (see section 2.6), we removed 4mL of culture medium and then we dissociated them with 1 mL of Trypsin-EDTA (Gibco # 25300-054). We placed the dish in the incubator at 37°C and 5% CO_2 for 2-3 minutes and then added 4mL of fresh culture medium. With 35mm culture dishes, we used a wax pencil to mark a circle, added 200µL of the re-suspended cells within this marked circle and incubated them at room temperature for 1 hour. Following incubation, we gently removed the 200µL of culture medium and

added 200µL of 4% PFA within the waxed circle and waited 10 minutes at room temperature. We then flooded the entire dish with 0.1M PBS and washed it 3 times for 5 minutes with 0.1M PBS. After the final wash, we flooded the dish with blocking solution (10% normal goat serum, 0.05% Triton-X in 0.1M PBS) and incubated the dish at room temperature for 1 hour.

To determine the specificity, we used dishes that contained HEK293 cells transfected with either TRPV1, TRPV2, TRPV3, or TRPV4. Within these dishes, we added anti-TRPV1 antibody (1:100) diluted with blocking solution. The dishes were incubated overnight at $4^{o}C$.

The next day, we washed each dish three times for 5 minutes with 0.1 PBS. We then added secondary antibodies, Alexa Fluor® 568 goat anti-rabbit IgG at 1:500 (Invitrogen) and incubated the dishes at room temperature for 90 minutes. Lastly, we washed each dish three times for 5 minutes with 0.1M PBS and after the last wash, we flooded the dish with 0.1M PBS and we stored it at $4^{o}C$ until we were ready for imaging at the confocal fluorescence microscope. The confocal microscope used is a Leica DM LFSA and images were captured simultaneous with either a 10x or 40x objective lenses. GFP-positive cells were excited at 488nm and emission was collected at 525nm. TRPV1-positive cells were excited at 568nm and emission was collected at 607nm.

2.12 - Immunohistochemistry – Isolated Neurons

For this part of the study, we used male Long Evans rats (Charles River, Quebec, Canada). These experiments were performed in accordance to the guidelines of the Canadian Council on Animal Care and were approved by the Animal Care Committee of McGill University under the protocol number 1190. Long Evans rats were anaesthetized with halothane (Sigma) in a chamber and decapitated using a small animal guillotine (Stoelting), and brains were rapidly removed from the cranial vault. Coronal slices about 1 mm thick were cut through the hypothalamus using razor blades and tissue blocks of either OVLT, MnPO, SFO or SON were dissected under a dissection microscope using forceps and fine scissors. Tissue blocks were incubated at room temperature for 90 minutes in oxygenated PIPES (130mM NaCl, 5mM KCl, 1mM $MgCl_2$, 20mM PIPES, 1mM CaCl, 10mM Glucose) with 0.5mg/mL Protease-X and 0.5mg/mL Protease-XIV. Tissue blocks were then washed in protease-free PIPES, triturated (i.e gently mixing up and down with a glass pipette) and plated on 35mm culture dishes within a small circled traced using a wax pencil. We then incubated the dish at room temperature for 30 minutes and then added 4% PFA for 10 minutes. Next, we flooded the entire dish with 0.1M PBS and washed it 3 times for 5 minutes with 0.1M PBS. After the last wash, we flooded dish with blocking solution (10% normal goat serum, 0.05% Triton-X in 0.1M PBS) and incubated the dish at room temperature for 1 hour.

The dishes containing rat SON neurons were doubled stained with either rabbit polyclonal anti-TRPV1 antibodies (PH-4281, 1:100) and mouse monoclonal anti-Vasopressin neurophysin (PS41, 1:50, gift from H.Gainer) antibodies or rabbit polyclonal anti-TRPV1 antibodies (PH-4281, 1:100) and mouse monoclonal anti-Oxytocin neurophysin (PS38, 1:50, gift from H.Gainer) antibodies in blocking solution. The other dishes containing either rat OVLT, MnPO, or SFO neurons were stained with rabbit polyclonal anti-TRPV1 antibodies (PH-4281, 1:100) and mouse monoclonal NeuN antibodies (1:50), a neuronal specific marker (Chemicon) in blocking solution. These dishes were then incubated overnight at 4^{o}C.

Twenty-four hours later, we washed each dish 3 times for 5 minutes with 0.1M of PBS. We then added the secondary antibody, Alexa Fluor® 568 goat anti-rabbit IgG at 1:500 and Alexa Fluor® 488 goat anti-mouse IgG at 1:500 (Invitrogen) with blocking solution and incubated the dishes at room temperature for 90 minutes in complete darkness. Lastly, we washed 3 times for 5 minutes with 0.1M PBS and after the last wash, we flooded the dish with 0.1M PBS and stored it at 4^{o}C until it was ready for detection by confocal fluorescence microscopy. The confocal microscope used is a Leica DM LFSA and images were captured simultaneous with either a 10x or 40x objective lenses. NeuN-positive cells were excited at 488nm and emission was collected at 525nm. TRPV1-positive cells were excited at 568nm and emission was collected at 607nm.

2.13 - Live Staining – Transfected HEK293 Cells

With the HEK293 cells transfected with rat TRPV1 (see section 2.6), we removed 4mL of culture medium and then we dissociated them with 1 mL of Trypsin-EDTA (Gibco # 25300-054). We placed the dish in the incubator set at 37°C and 5% CO_2 for 2-3 minutes and then added 4mL of fresh culture medium. With 35mm culture dishes, we used a wax pencil to mark a circle, added 200µL of the re-suspended cells within this marked circle and incubated them at room temperature for 20 minutes. After incubation, we removed the culture medium and added 200µL of anti-TRPV1 antibody (1:100) within the waxed circle. Immediately after, we fixed the cells with 4% PFA at different time points: 0, 30, 60, and 120 minutes. Once all the cells were fixed, we removed the 4% PFA and washed the dishes 3 times for 5 minutes with HEPES (140mM NaCl, 3mM KCl, 1mM MgCl2, 10mM HEPES, 1mM CaCl, 10mM Glucose). We then added the secondary antibody, Alexa Fluor® 568 goat anti-rabbit IgG at 1:500 (Invitrogen) in blocking solution and incubated them for 90 minutes at room temperature in complete darkness. Following incubation, we removed the secondary antibody and washed with HEPES (140mM NaCl, 3mM KCl, 1mM MgCl2, 10mM HEPES, 1mM CaCl, 10mM Glucose) 3 times for 5 minutes . After the last wash, we flooded the dish with additional HEPES and stored the dish at 4°C until it was ready for detection by confocal fluorescence microscope. The confocal microscope used is a Leica DM LFSA and images were captured simultaneous

with either a 10x or 40x objective lenses. TRPV1-positive cells were excited at 568nm and emission was collected at 607nm.

2.14 - Electrophysiology

2.14.1 - Electrophysiology – Set Up

Two days after transfection, we removed 4mL of culture medium from culture dishes containing HEK293 cells transfected with rat TRPV1 (see section 2.6) and then we dissociated them with 1mL of Trypsin-EDTA (Gibco # 25300-054).
We placed the dish in the incubator at 37°C and 5% CO_2 for 2-3 minutes and then added 4mL of fresh culture medium. With 35mm culture dishes, we used a wax pencil to mark a circle, added 200µL of the re-suspended cells within this marked circle and incubate them at 37°C and 5% CO_2 for 20 minutes. We then removed the 200µL of culture medium and then added 200µL of HEPES (140mM NaCl, 3mM KCl, 1mM MgCl2, 10mM HEPES, 1mM CaCl, 10mM Glucose) or 200µL of HEPES with anti-TRPV1 antibodies (1:100) and incubated for 1 hour before recording and then we gently flooded the dish with HEPES.

Patch pipettes (1.2 mm o.d. glass, A-M Systems Inc., WA, USA) were pulled using a P-87 Flaming-Brown puller (Sutter Instruments Co., CA, USA). The recording tip was polished with a micro-forge (Narishige; Scientific Instrument Lab – Tokyo, Japan) and the other end was fire polished using an ethanol lamp containing 100% ethanol. The patch pipettes were then filled with internal solution

containing (in mM): K-gluconate, 1mM $MgCl_2$, 1mM EGTA, 10mM HEPES, pH 7.2, Osm: 265mOsm/kg.

The solution filled pipette was placed into a sealed plexiglass holder connected to a headstage attached to a micromanipulator (Siskiyou - Grants Pass, Oregon). Approximately 50 mmHg of positive pressure (as detected by a pressure monitor) was applied to clear any debris in its path once in solution (World Precision Instruments Inc. – Sarasota, Florida). The pipette was brought down until it was almost touching one of the HEK293 cells and then we released the positive pressure. Once we obtained a 1 GIGA-seal, we used the "ZAP" function on the Axoclamp 200B amplifier (Molecular Devices, CA, USA) to enter whole-cell configuration. Capacitance and series resistance were neutralized electronically, and values of whole cell capacitance and series resistance were noted.

2.14.2 - Electrophysiology – Without Antibody Incubation

Whole-cell voltage clamp recordings (holding potential at -60mV) of transfected HEK293 cells were performed using an Axoclamp 200B amplifier (Molecular Devices, CA, USA). We used a fast stepper device (Fast-Step Perfusion System, SF-77B, Warner Instrument Co., CT, USA) for drug application. Perfusion at 0.2mL per minute was achieved via a three-barrel assembly controlled by the fast stepper device, allowing rapid (20ms) switching between solutions. We first had a barrel perfusing the patched cell with HEPES-buffered saline solution (140mM

NaCl, 3mM KCl, 1mM MgCl2, 10mM HEPES, 1mM CaCl, 10mM Glucose, 312mOsm/kg with mannitol).

For transfected HEK293 cells without anti-TRVP1 antibody incubation, we recorded the current amplitude when the stepper was switched to a different barrel containing, capsaicin (10mM in ethanol) diluted in HEPES to 10µM. When we saw a saturated current, we then switched back to the HEPES-buffered saline solution barrel. We allowed the cell's holding current to recover back to baseline. In some recordings, we then switched to a different barrel containing both capsaicin (10µM) and capsazepine (10µM) and then back to the HEPES-buffered saline solution barrel. In these cases, we subsequently went back to the barrel containing, capsaicin (10mM in ethanol) diluted in HEPES to 10µM and then back to the HEPES-buffered saline solution barrel.

For steady-state current-voltage (I-V) analysis, voltage was ramped up from –100mV to 100mV for 1 second at intervals of 20 seconds.

2.14.3 - Electrophysiology – With Antibody Incubation

Similar to section 2.14.2, whole-cell voltage clamp recordings (holding potential at -60mV) of transfected HEK293 cells were performed using an Axoclamp 200B amplifier (Molecular Devices, CA, USA). We used a fast stepper device (Fast-Step Perfusion System, SF-77B, Warner Instrument Co., CT, USA) for drug application. In this experiment transfected HEK293 cells were incubated with anti-TRPV1

antibody for 1 hour and for controlled conditions, transfected HEK293 cells were incubated with HEPES for 1 hour. We recorded the current amplitude when the barrel switched from HEPES-buffered saline solution to capsaicin (10mM in ethanol) diluted in HEPES to 10μM.

2.15 - Statistical Analysis

All values in this thesis are reported as +/- standard error of the mean (s.e.m). For the live staining experiments, the intensity level (i.e brightness of staining) was measured with the MetaMorph Imaging software (PerkinElmer Biosignal Inc. Montreal, QC). The comparisons of means observed in different groups (0, 30, 60, 120 minutes) were performed using a Student's *t*-test (SigmaStat). Similar for the electrophysiology, the current density (i.e peak current / whole-cell capacitance) between transfected HEK293 in the presence of anti-TRPV1 antibodies and in the absence of antibodies were compared using an unpaired Student's *t*-test (SigmaStat).

Compound	Concentration (mM)	MW (g/mol)	1L (g)
NaCl	1380	58.44	80.65
KCl	25	74.56	1.86
Na_2HPO_4-H_2O	100	142.00	14.20
KH_2PO_4	18	136.10	2.45

Table 1 –1X PBS (pH 8) was used for Protein Extraction
10X phosphate buffered saline (PBS) (recipe above) was diluted to 1X PBS with distilled water. Ice cold 1X PBS was used to wash HEK293 three times cells prior to protein extraction (see section 2.7.1 for details).

Compound	Concentration (mM)	MW (g/mol)	300mL (g)
NaCl	150	58.44	2.63
Tris-HCl	50	121.10	1.82
EDTA	1	380.40	200µL at 0.5M
TritonX-100	1%	628.00	3mL

Table 2 –RIPA Buffer (pH 7.4) Mixed with Protease Inhibitor (RIPA/PI) was used to Lyse Cells
100µL of protease inhibitor cocktail (Sigma) mixed with 10mL of RIPA buffer (recipe above) was added to HEK293 cells in order to lyse cells (see section 2.7.1 for details).

Standard (mg/mL)	0	0.2	0.5	0.7	1.0	1.4
10 mg/mL BSA (µL)	0	2	5	7	10	14
RIPA/PI (µL)	100	98	95	93	90	86

Table 3 – Bovine serum albumin (BSA) used as Standard Protein Ladder
Standard protein ladder was prepared by diluting BSA (10mg/mL) with RIPA/PI, as indicated above. The mixture will provide a known amount of protein. This standard protein ladder was used to determine the concentration of unknown protein samples (see section 2.7.2 for details).

Compound	Concentration	Unit	100mL
SDS	8%	g	8.0
Glycerol	40%	mL	40.0
Tris-HCl pH 8 1.5M	0.5M	mL	33.0

Table 4- Preparing 4X Sample Buffer with Protein Extract for Gel Electrophoresis
Twelve percent β-mercaptoethanol was added to 4X sample buffer (recipe above). This mixture was diluted to 1X by adding the protein extract sample. The sample was then boiled at 100°C for 7 minutes and then frozen at -20°C until ready to be loaded into gel (see section 2.7.3 for details).

Compound	10% (mL)
Milli-Q H_2O	9.7
1.5 M Tris-HCl pH 8.8	5.0
10% SDS	0.2
40% Acrylamide/bis	5.0
10% APS	100 µL
TEMED	10 µL

Table 5 – Preparing Separating Gel for SDS-PAGE
Separating gel was prepared by adding compounds mentioned above. Ammonium persulfate (APS) was freshly prepared and TEMED was only added at the end when everything was ready to be poured into the gel cast (see section 2.8.1 for details).

Compound	4% (mL)
Milli-Q H_2O	6.4
0.5 M Tris-HCl pH 6.8	2.5
10% SDS	0.1
40% Acrylamide/bis	1.0
10% APS	50 µL
TEMED	10 µL

Table 6 – Preparing Stacking Gel for SDS-PAGE
Stacking gel was prepared by adding compounds mentioned above. Ammonium persulfate (APS) was freshly prepared and TEMED was only added at the end when everything was ready to be poured into the gel cast (see section 2.8.1 for details).

5X Tris Glycine-SDS

Compound	Concentration	MW (g/mol)	4L (g)
Glycine	1 M	75.07	287.5
Tris	0.1 M	121.1	60.5
SDS	0.5%	288.38	20.0

Running Buffer

Compound	1L
5X Tris Glycine-SDS	200 mL
Water	800 mL

Table 7 – Solutions Required to Run SDS-PAGE
Running buffer was used while loading protein extract samples as well as when running SDS-PAGE. Running buffer was made from 5X Tris Glycine-SDS (recipe above) mixed with distilled water (recipe above). See section 2.8.1 for details.

5X Tris Glycine

Compound	Concentration	MW (g/mol)	4L (g)
Glycine	1 M	75.07	287.5
Tris	0.1 M	121.1	60.5

Transfer Buffer

Compound	4L
Methanol	800 mL
5X Tris Glycine	800 mL
Water	2400 mL

Table 8 - Solutions Required for Protein Transfer from Gel to Membrane
Transfer buffer was used while assembling the transfer cassette as well as when transferring protein from the gel to the membrane. Transfer buffer was made from 5X Tri Glycine (recipe above) mixed with methanol and distilled water (see section 2.8.3 for details).

Tris-Buffered Saline Tween-20 (TBST)

Compound	Concentration	2L
Tris-HCl pH 7.5	0.05M	12.11g
NaCl	0.9%	18g
Tween 20	0.1%	2mL

Blocking Solution

Compound	50mL
Skim Milk	2.5g
TBST	50mL

Table 9 - 5% Blocking Solution Used to Block Non-Specific Binding Sites
In western blot experiments, we used 5% skim milk powder mixed with Tris-Buffered Saline Tween-20 (TBST) as blocking solution. Blocking solution was incubated with the membrane for one hour at room temperature in order to block non-specific binding sites (see section 2.9 for details).

Chapter 3: Characterization of a Novel Pre-Pore Loop Antibody Against Rat TRPV1

3.1 - Introduction

The first evidence indicating a role for TRPV channels in osmoreception came from experiments performed on the worm *C. elegans* by Colbert et al [48]. The authors demonstrated that worms lacking a functional OSM-9 protein (which shares homology with the *Drosophilia* TRP channel) failed to display normal avoidance behaviour when encountering a high osmolality solution [48].

The characterization of *osm-9* suggested that TRP family members might have critical roles in transduction of osmotic stimuli. TRPV4, a homolog of OSM-9, was the first mammalian TRPV channel shown to be osmosensitive [65, 74]. When expressed as a homo-multimeric channel in a heterologous system, TRPV4 is activated by cell swelling, but is unresponsive to cell shrinkage [65]. Strotmann et al. showed that this channel is not directly mechanosensitive because no response was observed upon a change in pipette pressure during cell-attached single-channel recordings. Because of its activation by cell swelling, its unresponsiveness to cell shrinkage, and its lack of direct mechanosensitivity, TRPV4 channels are unlikely to function on their own (as homo-multimeric complexes) as the transduction of central osmoreceptor neurons in rodents as described in section 1.8.4.

TRPV2 was also identified as another member of the TRPV family that can be modulated by osmolality and might therefore play a role in osmoreception [63]. Muraki et al. demonstrated that TRPV2 expressed in murine vascular smooth muscle cells are directly activated by membrane stretch [63]. When isolated cells from mouse aorta were exposed to hypotonic solutions, the cell swelling lead to the activation of a non-selective cation current and an increase in $[Ca^{2+}]_i$ [63]. Treatment of these isolated cells with TRPV2 antisense oligonucleotides resulted in suppression of these hypotonicity-induced responses [63]. Finally, stimulation of TRPV2-transfected Chinese hamster ovary cells by application of membrane stretch through the recording pipette or hypotonic stimulation consistently activated single non-selective cation channels [63]. Although these experiments suggested that homo-multimeric TRPV2 channels may be directly mechanosensitive, the channels functionally operate as stretch-activated channels, rather than SICC channels described in section 1.8.2. Therefore, it would appear that homo-multimeric TRPV2 channels are unlikely to function as the mechanosensitive transducers in rodent osmosensory neurons.

As described above, homo-multimeric TRPV channels (i.e TRPV2 and TRPV4) expressed in heterologous systems display a phenotype opposite to what is seen in MNCs of the SONs where cell swelling caused channel inhibition and cell shrinking induced channel activation [47]. The possibility remains, however, that a hetero-multimeric complex comprised of various TRPV channel subunits (including TRPV2 and/or TRPV4, or splice variants of these channels) might

display stretch-inactivated gating and thus play an integral role in the osmosensory transducer complex.

In two recent studies, the role of *trpv1* gene products was examined in the intrinsic osmosensitivity of osmoreceptors [81, 82]. Using reverse transcriptase-PCR and immunohistochemistry, Sharif Naeini et al. found a splice variant of TRPV1 in SON neurons where a portion of the N-terminal domain is truncated [82]. The authors also showed from voltage-clamp recordings that this TRPV1 splice variant is capsaicin-insensitive [82]. Moreover, electrophysiological analysis of SON and OVLT neurons of TRPV1-knockout (*trpv1-/-*) mice revealed that products of the *trpv1* gene may contribute to the intrinsic osmosensitivity of these neurons [81, 82]. The authors demonstrated that hyperosmolality caused a decrease in cell volume in SON and OVLT neurons isolated from *trpv1-/-* mice but this effect is no longer linked to an increase in membrane conductance, membrane depolarization or increase in firing frequency, as normally observed in cells obtained from wild-type mice (Figure 1.4) [81, 82].

The osmosensory response of wild-type MNCs is not sensitive to antagonists, such as capsazepine, that are competitive antagonists of capsaicin-mediated activation of TRPV1 [82]. This is not surprising since, as mentioned above, the form of TRPV1 expressed in osmosensory neurons is not activated by capsaicin. Thus, a novel tool that specifically interferes with ion flux through TRPV1 channels is required for functional studies on the involvement of this channel in

osmoreception. The goal of this project is to generate a rabbit polyclonal antibody specifically targeting the extra-cellular pre-pore region of rat TRPV1 and to investigate whether this antibody can be used as a specific tool to study the osmosensory transduction complex.

3.2 - Results

Serum was obtained from a rabbit immunized against a peptide sequence (CGKNNSLPMESTPHKCRG) contained in the rat TRPV1 subunit as described in section 2.2. The 18-amino acid sequence is located between transmembrane regions 5 and 6 at extra-cellular pore-forming region of the protein as described in section 2.1. The serum was affinity purified as outlined in section 2.4. The purified antibody is henceforth termed PH-4281.

3.2.1 - Western Blot Analysis of PH-4281

We first wanted to determine the optimal dilution of PH-4281 for binding to TRPV1 using Western blots. PH-4281 was diluted to different ratios (1:100, 1:500, 1:1000, and 1:5000) and probed against lanes containing protein lysates (20µg) of HEK293 cells transfected with rat TRPV1 (see section 2.6). Our results showed a clear single band, at the expected molecular weight of 95 kDal, between the ranges of 1:100 to 1:1000 (Figure 3.1). As a positive control, we also probed the same lysate with a commercial anti-N-Terminal-TRPV1 antibody

(Neuromics) at 1:100. The expected TRPV1 band was visible; however other bands were also clearly visible at other molecular weights (Figure 3.1). To determine the specificity of PH-4281, we used it to probe other members of the TRPV family; TRPV2, TRPV3 and TRPV4 at a dilution of 1:100. Each lane was loaded with 20μg of protein lysates of HEK293 cells transfected with TRPV1, TRPV2, TRPV3, or TRPV4. A positive signal was only seen in the lane containing TRPV1 and not in the lanes containing TRPV2, TRPV3 or TRPV4 (Figure 3.1). These results suggested that PH-4281 specifically targets rat TRPV1 when used at dilutions between 1:100 and 1:1000.

3.2.2 - Immunohistochemistry Analysis of PH-4281

We investigated the optimal dilution range of PH-4281 that could be used for immunohistochemistry. To this end, PH-4281 was diluted to different ratios (1:100, 1:500, and 1:1000) and probed against HEK293 cells transfected with rat TRPV1 (see sections 2.6). Staining was observed along the perimeter of cells expressing TRPV1 with PH-4281 dilutions ranging between 1:100 to 1:1000 (Figure 3.2). We next investigated the specificity of PH-4281 by staining cells expressing other members of the TRPV family. PH-4281 was used at 1:100 in HEK293 cells co-transfected with either TRPV1, TRPV2, TRPV3 or TRPV4 and GFP. The GFP cDNA concentration used was ten times less than the TRPV cDNA (TRPV1, TRPV2, TRPV3 or TRPV4). As described in a different study involving M-

type K+ channels (KCNQ2 and KCNQ3), when KCNQ2/3 and GFP cDNAs are co-transfected, > 95% of GFP-positive cells expressed KCNQ2/3 currents in control experiments [100]. Thus, we assumed that because the desired TRPV cDNA was more concentrated than GFP cDNA, GFP-positive HEK293 cells should express the TRPV channel. As shown in Figure 3.2, HEK293 cells were GFP-positive but TRPV1 staining with PH-4281 was only seen in cells that were also transfected with TRPV1 (Figure 3.2). These data suggested that PH-4281 can specifically stain the cells transfected with rat TRPV1.

3.2.3 - Immunohistochemistry Analysis of Acutely Isolated Neurons with PH-4281

As stated in section 1.3, regions such as SON, OVLT, SFO, and MnPO are believed to be comprised of osmosensitive neurons. Recently, studies have shown that SON and OVLT neurons from *trpv1-/-* mice lack the osmosensory transduction process [81, 82]. This suggests that TRPV1 might have a role as an osmosensor but whether *trpv1* gene products are involved in forming the ion-channel responsible for osmorecepretion has yet to be determined. In this study, we investigate whether PH-4281 can detect TRPV1 in neurons from osmosensor regions. Isolated SON neurons were incubated with a solution containing PH-4281 and antibodies specific to vasopressin-neurophysin and oxytocin-neurophysin. Results show TRPV1 staining with PH-4281 in both vasopressin- and oxytocin-positive neurons (Figure 3.3). As shown in Figure 3.4, TRPV1 staining is

observed in acutely isolated OVLT cells that are positive for NeuN, a specific neuronal marker. Moreover, we observed that 31% (13/42) of NeuN positive OVLT neurons did not stain for TRPV1. Lastly, NeuN positive cells isolated from MnPO (Figure 3.5) and SFO (Figure 3.6) also stained for TRPV1. These results suggest that trpv1 gene products are expressed in all osmosensory regions. Whether TRPV1 mediates osmoreception in all of these regions is still under investigation.

3.2.4 – Staining of Live Transfected HEK293 Cells with PH-4281

We next investigated the length of time it took PH-4281 to bind to the extra-cellular pre-pore region of TRPV1 in live transfected HEK293 cells. The results obtained would help us determine the incubation time with PH-4281 required before we can test whether it can act as an ion-channel blocker on transfected HEK293 cells (see section 3.2.5). In this experiment, we used a live staining protocol with PH-4281 to determine when TRPV1 staining was observed on the cell surface. HEK293 cells transfected with rat TRPV1 were incubated with PH-4281 for a various intervals (30, 60, and 120 minutes) and then fixed with 4% PFA. The time lengths chosen ensured that cells would have attached itself to the bottom on the dish and to be sure that the cells were still viable. As illustrated in figure 3.7, staining intensity gradually increased over time. MetaMorph Imaging software was used to determine the intensity value (i.e brightness of staining) of

TRPV1 staining around the cells' perimeter for all time intervals. The intensity value was averaged over six different points around the cell. This was done for multiple cells and the overall average of the intensity values measured for each time interval were then expressed as the percent intensity difference compared with the control condition (i.e zero minute incubation) as shown in figure 3.7. Performing a student's t-test showed a significant difference ($P < 0.05$) in percent of staining intensity at 60 and 120 minutes. These results suggest that prior to testing the antibody's functional effects, a minimum of 60 minutes is required for PH-4281 to bind to the transfected HEK293 cell.

3.2.5 – Characterization of Rat TRPV1 by Whole-Cell Recordings

Prior to investigating whether PH-4281 can functionally impair TRPV1-mediated ion influx, we wanted to confirm that our construct of rat TRPV1 had the same electrophysiological profile as reported by others [64].

Voltage-clamp recordings (holding potential at -60mV) obtained from TRPV1 transfected HEK293 cells demonstrated an inward current upon application of 10μM capsaicin (-89.63 +/- 16.47pA; n = 5) (Figure 3.8). As shown in figure 3.8, the current was reversibly blocked by capsazepine (0.63 +/- 3.09 pA; n = 5; $P<0.05$) (10μM). Steady-state current-voltage analysis showed that the capsaicin-induced current displayed outward rectification and a reversal potential of ~0mV

(n = 5) (Figure 3.9). These data are consistent with previous studies characterizing the electrophysiological profile of TRPV1 channels [64] .

3.2.6 – Effects of PH-4281 on Capsaicin-Induced Currents in Transfected HEK293 Cells

To investigate whether PH-4281 can functionally impair TRPV1-mediated ion influx, HEK293 cells transfected with rat TRPV1 were incubated with PH-4281 for 60 minutes (see section 3.2.4). We compared voltage-clamp recordings (holding potential at -60mV) from non-incubated transfected HEK293 cells (n = 19) (Figure 3.10) and transfected HEK293 cells incubated with the PH-4281 (n = 19) (Figure 3.10). In both conditions, an inward current was seen upon application of capsaicin. The average peak current for the non-incubated and incubated cells was calculated and was not significantly difference (-973.54 +/- 153.85 pA and -1301.46 +/- 214.74 pA; $P>0.05$; respectively) (Figure 3.10).

The current amplitude reflects the number of activated channels which is related to the size of the cell (that is, one can see larger current amplitudes simply because the cell is larger and contains more channels). Because we recorded from transfected cells of different sizes, we also determined the current density (i.e current/ whole-cell capacitance) which measures the current per unit surface area and this allowed us to compare the current from any sized cells. We found that there was no significant difference in current density between the non-incubated and incubated groups (-85.48 +/- 13.07 pA pF^{-1} and -113.38 +/- 18.03

pA pF^{-1} ; P>0.05; respectively) (Figure 3.10). These results suggested that pre-incubating PH-4281 did not reduce capsaicin-evoked currents.

3.3 - Discussion

3.3.1 - PH-4281 is Specific to TRPV1

Our results indicated that PH-4281 specifically interacts with rat TRPV1 and not TRPV2, TRPV3, or TRPV4. Although anti-TRPV1 antibodies are commercially available, (e.g. anti-TRPV1-C Terminus or anti-TRPV1-N-Terminus) the specificity of these antibodies against other members of the TRPV family is rarely reported. Our analysis using western blots indicated that the anti-TRPV1-N-Terminus antibody (1:100) produced by Neuromics detected a band at the expected molecular weight of TRPV1 (~95kDal) as well as additional bands; two of which were greater than 95kDal and one that was less than 95kDal. These additional bands may be due to non-specific binding of the anti-TRPV1-N-Terminus antibody. The anti-TRPV1-N-Terminus antibody could be tested in HEK293 cells not transfected with TRPV1 to see if the bands are still present. In contrast, PH-4281 (1:100) detected only a single band at the expected molecular weight of TRPV1.

We also reported from immunohistochemistry experiments that TRPV1 staining was predominately localized along the perimeter of the transfected HEK293

cells. Even though we used a permeabilizing agent, which breaks down the cell membrane allowing PH-4281 to enter the cell, TRPV1 staining was mainly observed along the perimeter of the cell. This suggested that a greater density of TRPV1 channels are along the cell's surface. This observation came as no surprise because a high concentration of TRPV1 cDNA was used during the transfection process. Thus, a greater amount of TRPV1 channels was expected to be produced and brought towards the cell's surface.

Furthermore, PH-4281 was tested against other members of the TRPV family that are closely related to TRPV1. As mentioned in section 1.8, only TRPV2, TRPV3 and TRPV4 are of interest because of their close sequence homology to TRPV1. Each of these channels were over-expressed into a heterologous system and it was confirmed using both western blots and immunohistochemical methods that PH-4281 is specific only to rat TRPV1.

3.3.2 - TRPV1 Expression in Acutely Isolated Neurons of Osmosensory Regions

It has been well established in the literature that regions such as the SON, OVLT, MnPO and the SFO are osmosensitive (see section 1.3). Recently, knockout studies have suggested that trpv1 gene products may be involved in the osmosensory complex [81, 82]. In this study, we used an immunohistochemical approach to investigate whether TRPV1 is expressed in these regions with PH-4281.

It has been previously shown that acutely isolated vasopressin-releasing neurons in the SON expressed an N-terminal variant of TRPV1 [82]. However, the SON contains two populations of intrinsically osmosensitive neurons, those that release vasopressin and those that release oxytocin [46]. These two hormones are crucial to the generation of normal osmoregulatory responses (see section 1.1). In this study, we confirmed using PH-4281 that TRPV1 is expressed in vasopressin neurons and we further revealed that this channel is expressed also in oxytocin-producing neurons of the SON.

A recent study done by Ciura and Bourque showed that wild-type OVLT neurons are intrinsically sensitive to an increase in the osmolality of the extracellular fluid and that the cation channel dependent osmosensory signal transduction cascade observed in wild-type neurons was absent in isolated OVLT neurons from TRPV1 knock-out mice [81].

Although Hollis et al. showed from coronal sections of rat brains that TRPV1 is expressed in OVLT neurons, the staining pattern of TRPV1 on the cell is not clear [101]. In this study, we confirmed the expression of TRPV1 in OVLT neurons isolated from rat, a preparation that allowed us to better observe the staining pattern of TRPV1 on the cell. If we compare our results to Hollis et al., we can clearly see that in our study TRPV1 staining was much more intense along the cell's surface. Furthermore, it was reported by Ciura and Bourque that 63% of OVLT neurons were osmoresponsive [81]. Therefore if TRPV1 is part of the

osmoreceptor, the non-osmoresponsive population of OVLT neurons should not express TRPV1. As reported here, 31% OVLT neurons stained with NeuN, a specific neuronal marker, but TRPV1 staining with PH-4281 was not observed. Whether TRPV1 containing neurons correspond to the osmosensitive ones remain to be demonstrated.

Studies have demonstrated that in an *in vitro* slice preparation from rats containing the MnPO, neurons are sensitive to osmotic pressure [102, 103]. These authors showed that application of a hyperosmotic stimulus to the slice caused an increase in neuronal activity but to our knowledge, intrinsic osmosensitivity of acutely isolated MnPO neurons has yet to be shown. In the current study, we reported that acutely isolated MnPO neurons from rats do express TRPV1. Although expressed, the function of *trpv1* gene products in MnPO neurons in respect to osmoreception remains unexplored.

Finally, Anderson et al. showed that isolated SFO neurons are intrinsically osmosensitive [104]. Their results demonstrated that 66% of SFO neurons responded to changes in osmolality in a predictable manner, exhibiting hyperpolarization and decrease in action potential frequency in hypoosmotic solutions and depolarization and increase in action potential frequency during hyperosmotic exposure [104]. However, contrary to what was described by Oliet and Bourque in MNCs where osmoreception is mediated by non-selective stretch-inactivated channels, the mechanism involved in the osmosensitivity in

SFO neurons is independent of the non-selective stretch inhibited cation channels [47]. Here, we report the expression of TRPV1 in SFO neurons but whether or not they play a role in the osmosensitivity of SFO neurons remain unknown.

3.3.3 – PH-4281 Effects on Capsaicin Evoked Reponses

As frequently mentioned above (see 1.11), electrophysiological data from trpv1 knockout mice suggested that *trpv1* gene products may be involved with osmosensitivity in SON and OVLT neurons [81, 82]. However as stated in section 1.11, the lack of osmosensitivity in trpv1 knockouts does not prove that TRPV1 is part of the osmosensory transduction complex. Although it is possible that a product of the *trpv1* gene plays a functional role as a pore-forming channel in the osmosensory transduction complex, direct evidence supporting this hypothesis has yet to be reported. There are a few approaches to test this hypothesis as described in section 1.12. Here, we developed an extracellular pre-pore loop antibody, PH-4281, that is specific to rat TRPV1. As stated in section 1.12.1, antibodies directed at the extracellular pre-pore loop region may act as ion channel blockers. Although it was shown in section 3.2.4 that PH-4281 can bind to live HEK293 cells, electrophysiological analyses indicated that PH-4281 was unable to inhibit responses evoked by the perfusion of capsaicin containing solution (see figure 3.10). A possible reason for this is that by using a fast

perfusion system, the fast flowing perfusate is washing PH-4281 off its antigen site on the cell hence removing, if any, the antagonist effect. As described by Harlow and Lane, an antibody binding to its antigen is non-covalent and is reversible [105]. It is possible that PH-4281 has a low affinity and this forms a weak antibody-antigen complex [105]. Furthermore, the half-time for dissociation of a low affinity antibody may only be a few minutes or less. Thus, if PH-4281 is a low affinity antibody, it would have dissociated from its antigen (i.e the pore loop of the TRPV1 channel) and with the fast perfusion system, PH-4281 would have been washed away from the cell`s vicinity thus not allowing it to re-bind.

To verify if PH-4281 was being washed away, future studies can be done where PH-4281 could be used to stain for TRPV1 in live cells and then on a confocal fluorescence microscope, measure the difference between the staining intensity of cells before and after fast perfusing them. If staining intensity is decreased after perfusing the live cells, it is possible that using a fast perfusion system washed off PH-4281 and that is why we did not observe any antagonistic effect.

If this is the case, another study should be done where TRPV1 channels are activated in a less invasive manner so that we would not disturb the interaction between PH-4281 and its antigen. As stated in 1.8.1, TRPV1 channels are also temperature-sensitive, thus one possible method is to heat up the entire bath above 43^{0}C to activate TRPV1 channels [64] without perfusing the dish. This

method of TRPV1 channel activation should not disturb the PH-4281 and antigen interaction as much as the fast perfusion system and not wash away PH-4281 away from the cell. This will allow us to observe whether PH-4281 is able to impair ion influx. If so, PH-4281 could be used for future studies to investigate *trpv1* gene products involvement in osmoreception and thermosensation in native cells.

3.4 - Closing Statement

In closing, we have successfully developed a novel extra-cellular pre-pore loop antibody against rat TRPV1. Histological results showed that it is specific to rat TRPV1 and TRPV1 expression is found in regions that are known to be osmosensitive. Future studies will be required to determine whether the pre-pore loop antibody can act as an ion channel blocker and be used to help identify whether *trpv1* gene products are part of the osmosensory transduction complex. Regardless of whether PH-4281 may act as an ion channel blocker, the specificity of PH-4281 will be useful to others for studying the cellular localization of the TRPV1 channels. PH-4281 could also be used for biochemical experiments, such as co-immunoprecipitation assays, to help identify hetero-multimeric complexes involving TRPV1 that might serve as molecular transducers. Furthermore, because PH-4281 can be used to stain live cells, translocation studies may be

done where it can be used to investigate the sensitization and desensitization of TRPV1 channels.

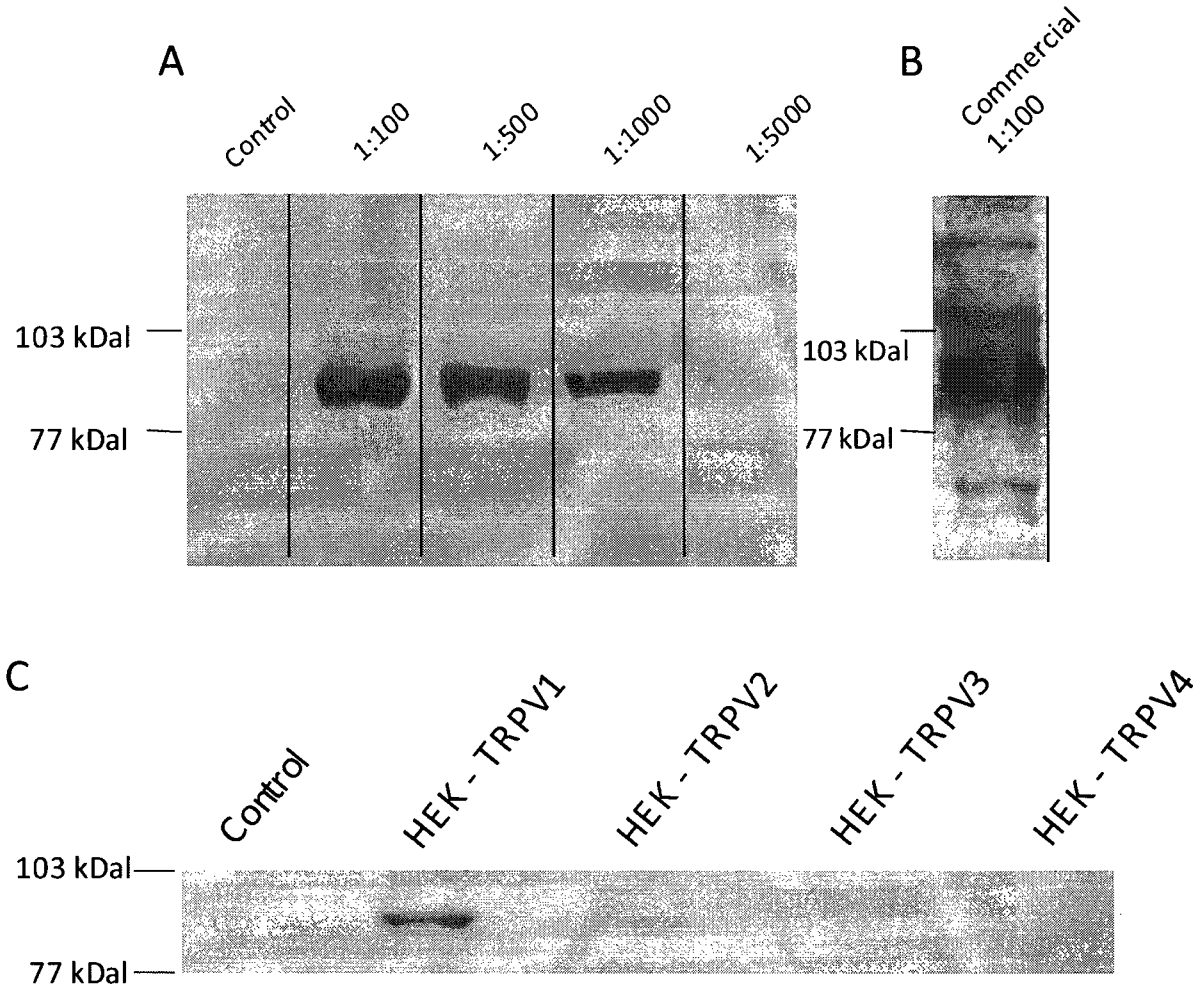


Figure 3.1 – Western blot analysis of PH-4281's optimal range and specificity to rat TRPV1.

In panel A, protein lysates of HEK293 cells transfected with rat TRPV1 were probed with different dilutions of PH-4281 (1:100, 1:500, 1:1000, and 1:5000). A single band was clearly detected at the expected molecular weight of TRPV1 (~95kDal) between 1:100 and 1:1000. In panel B, PH-4281 diluted at 1:100 was compared with a commercially available TRPV1 antibody diluted at 1:100 with the same protein lysate. Multiple bands were seen with the commercial antibody. In panel C, protein lysates of HEK293 cells transfected either with rat TRPV1, TRPV2, TRPV3 or TRPV4 were probed with PH-4281 at 1:100. The only detected band was in the lane containing rat TRPV1.

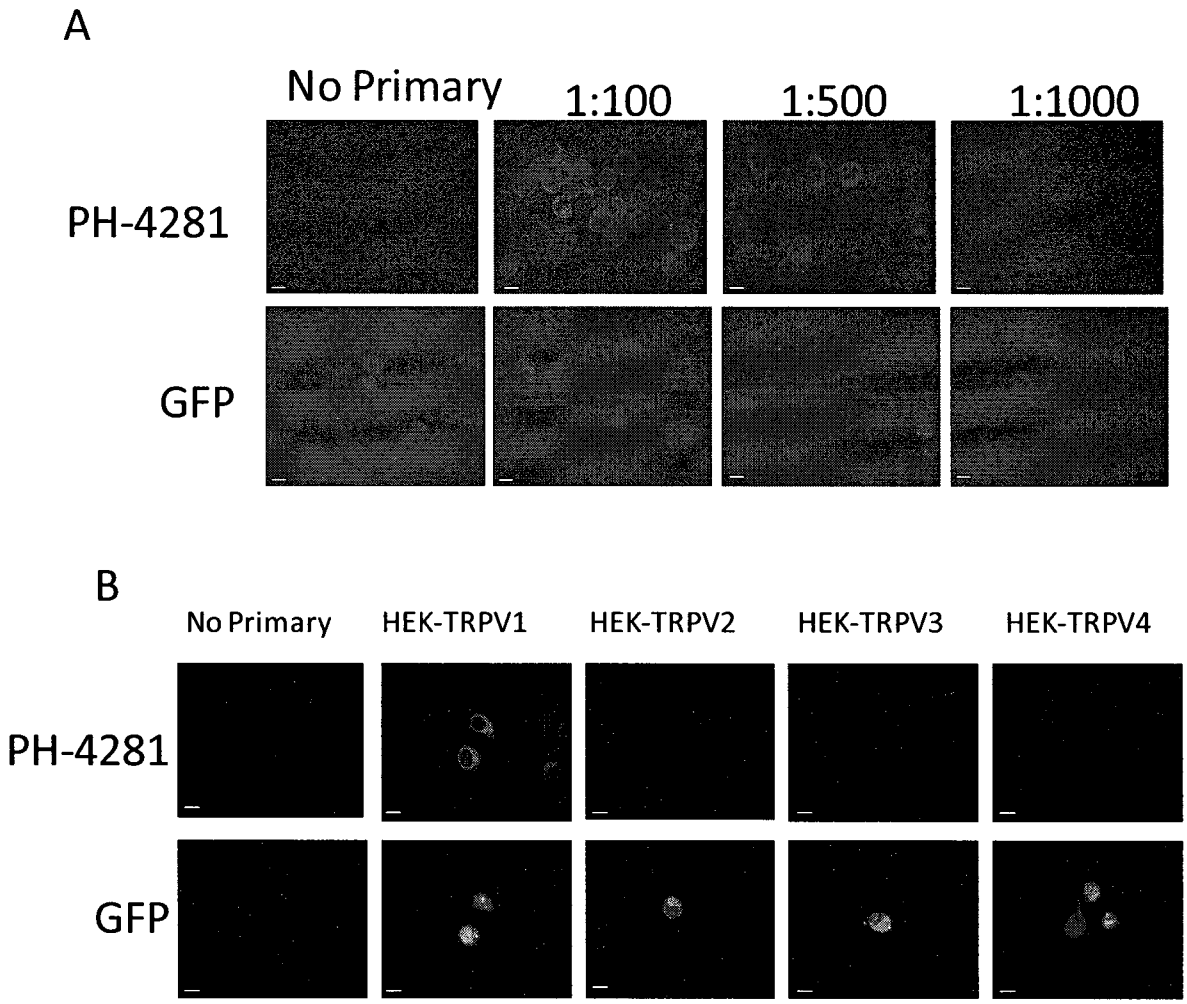


Figure 3.2 – Immunohistochemistry analysis of PH-4281's optimal range and specificity to rat TRPV1

In panel A, HEK293 cells were co-transfected with GFP (green, lower panels) and TRPV1 and probed with PH-4281 (red, upper panels) at different dilutions (1:100, 1:500, and 1:1000). No staining was observed in control conditions (no PH-4281) but TRPV1 staining was clearly seen between 1:100 and 1:1000. In panel B, PH-4281 diluted at 1:100 was probed against HEK293 cells that were transfected either with rat TRPV1, TRPV2, TRPV3 or TRPV4. Staining was only seen in cells transfected with TRPV1. All scale bars are 30μm.

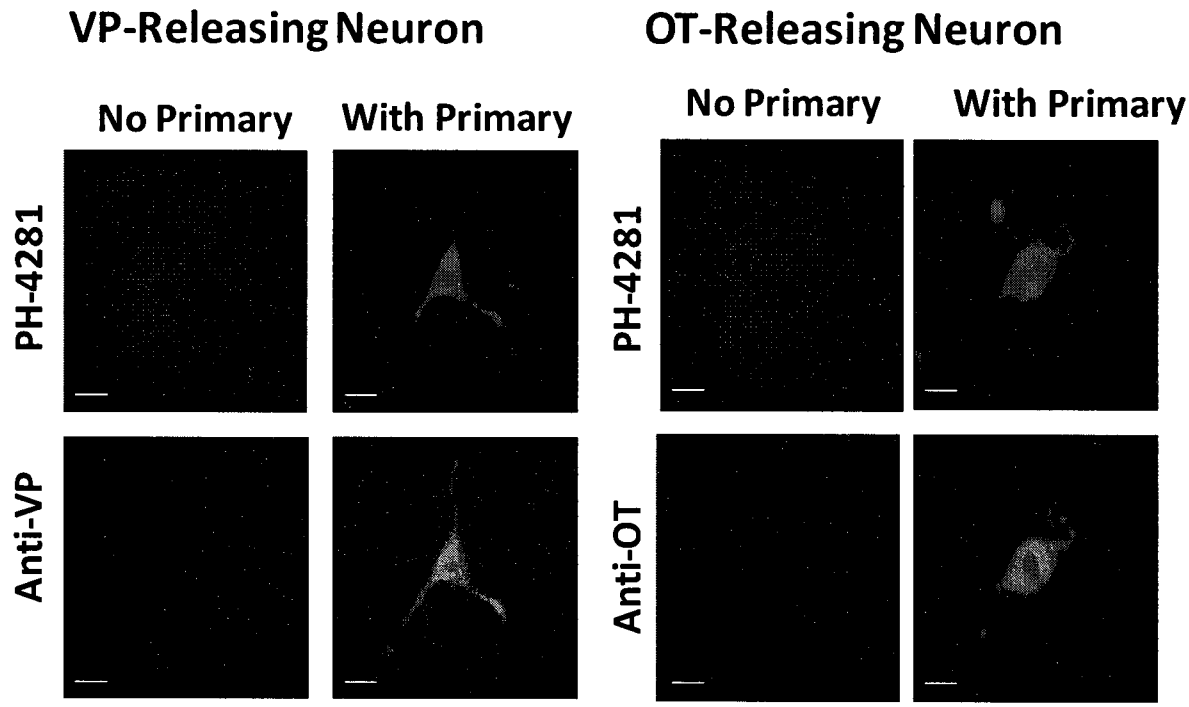


Figure 3.3 – TRPV1 is Expressed in Vasopressin- and Oxytocin-Releasing Neurons in SON

Acutely isolated rat SON neurons were doubled stained with PH-4281 (1:100) and with with anti-vasopressin antibodies (1:50, Anti-VP) or anti-oxytocin antibodies (1:50, Anti-OT). TRPV1 staining was observed in vasopressin- and oxytocin-positive neurons. All scale bars are 30μm.

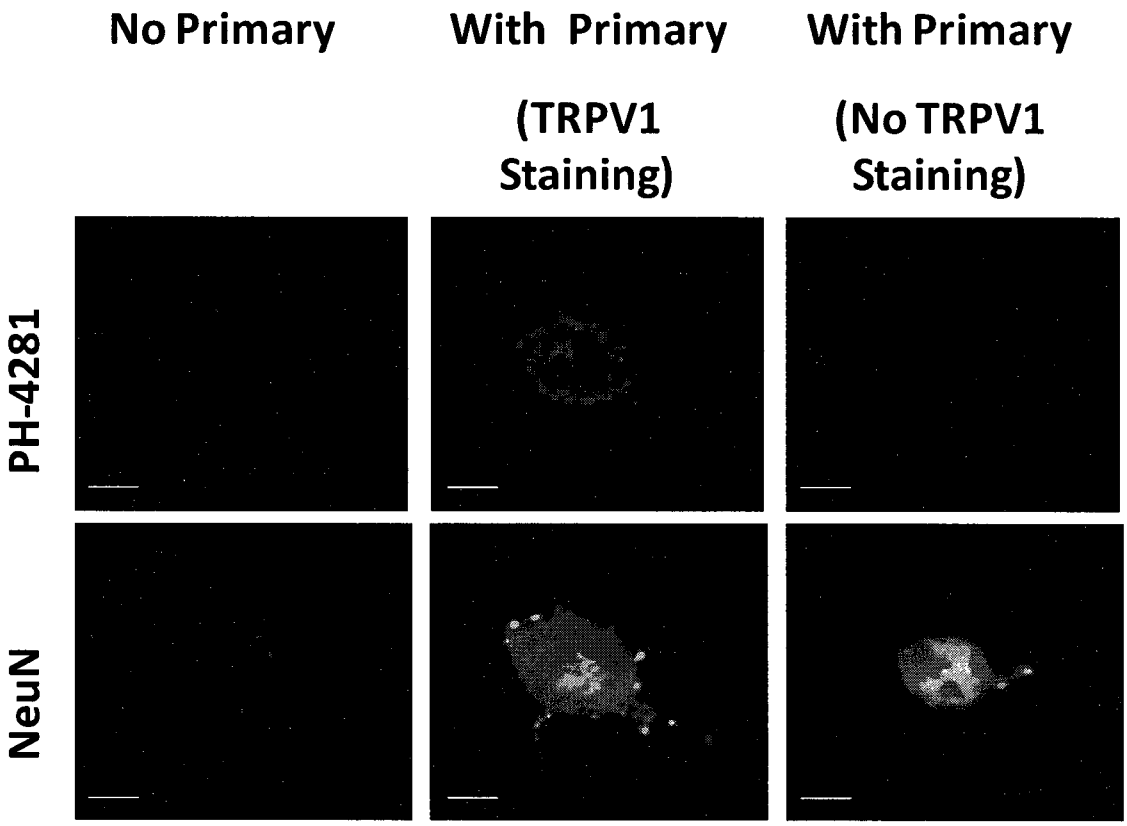


Figure 3.4 – TRPV1 is Expressed in OVLT Neurons
Acutely isolated rat OVLT neurons were double stained with PH-4281 (1:100) and NeuN antibodies (1:50), a specific neuronal marker. NeuN-positive cells showed TRPV1 staining. 31% (13/42) of NeuN-positive neurons did not stain for TRPV1. All scale bars are 15µm.

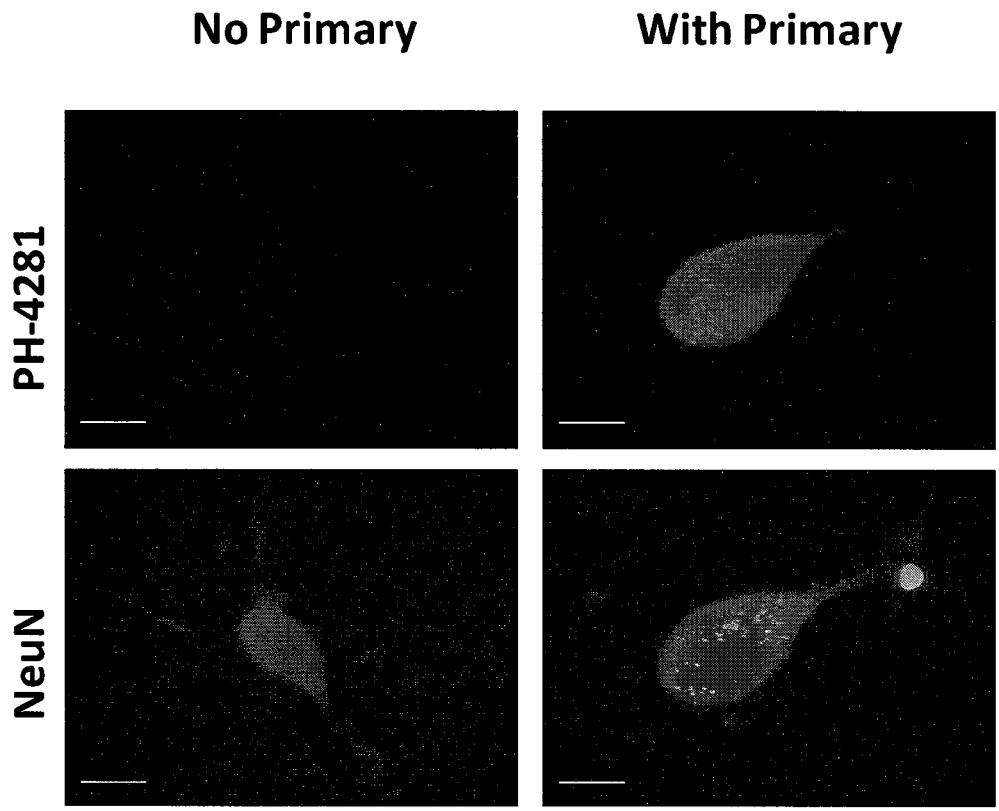


Figure 3.5 – TRPV1 is Expressed in MnPO Neurons
Acutely isolated rat MnPO neurons were double stained with PH-4281 (1:100) and NeuN antibodies (1:50), a specific neuronal marker. NeuN-positive cells showed TRPV1 staining. Some NeuN-positive cells lack TRPV1 staining (not shown). All scale bars are 15µm.

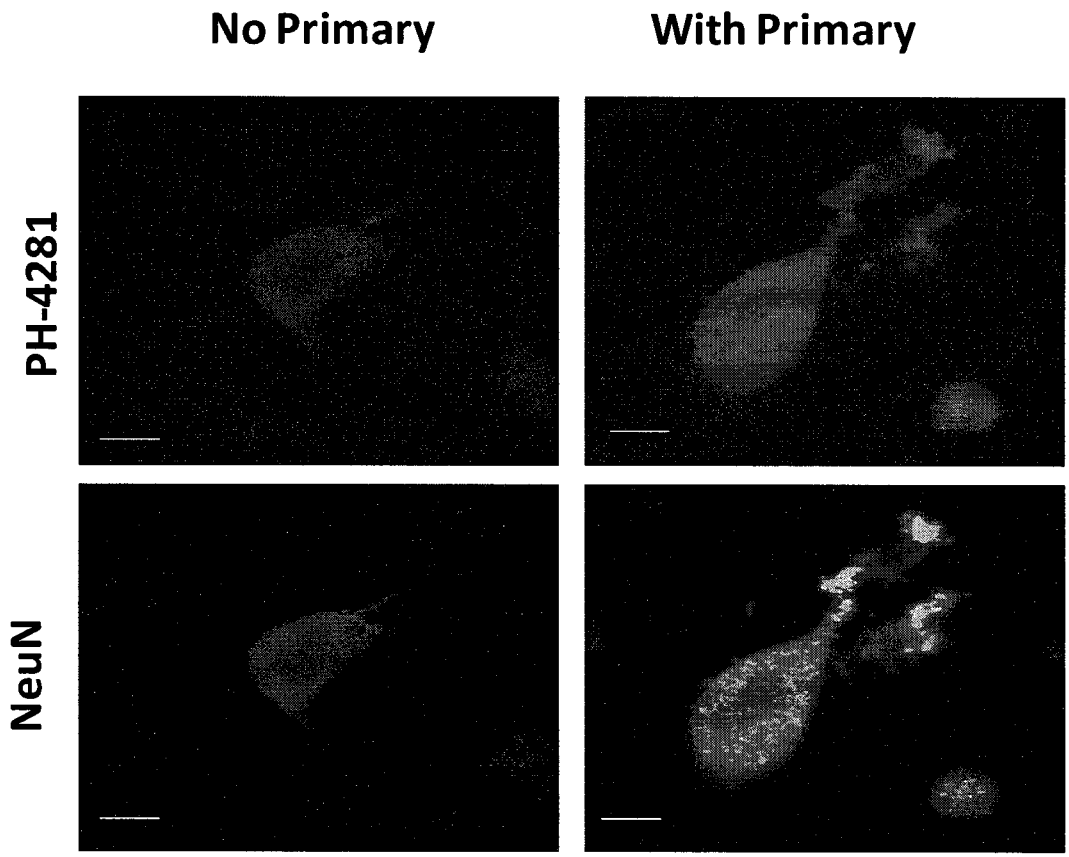
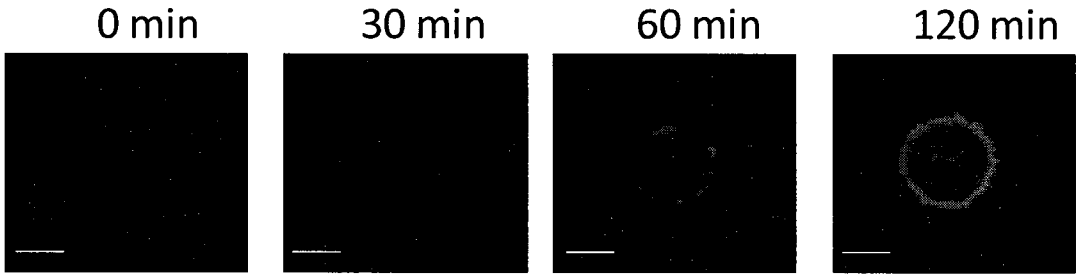


Figure 3.6 – TRPV1 is Expressed in SFO Neurons
Acutely isolated rat SFO neurons were double stained with PH-4281 (1:100) and NeuN antibodies (1:50), a specific neuronal marker. NeuN-positive cells showed TRPV1 staining. Lack of TRPV1 staining was also seen in some NeuN positive cells (not shown). All scale bars are 15μm.

A



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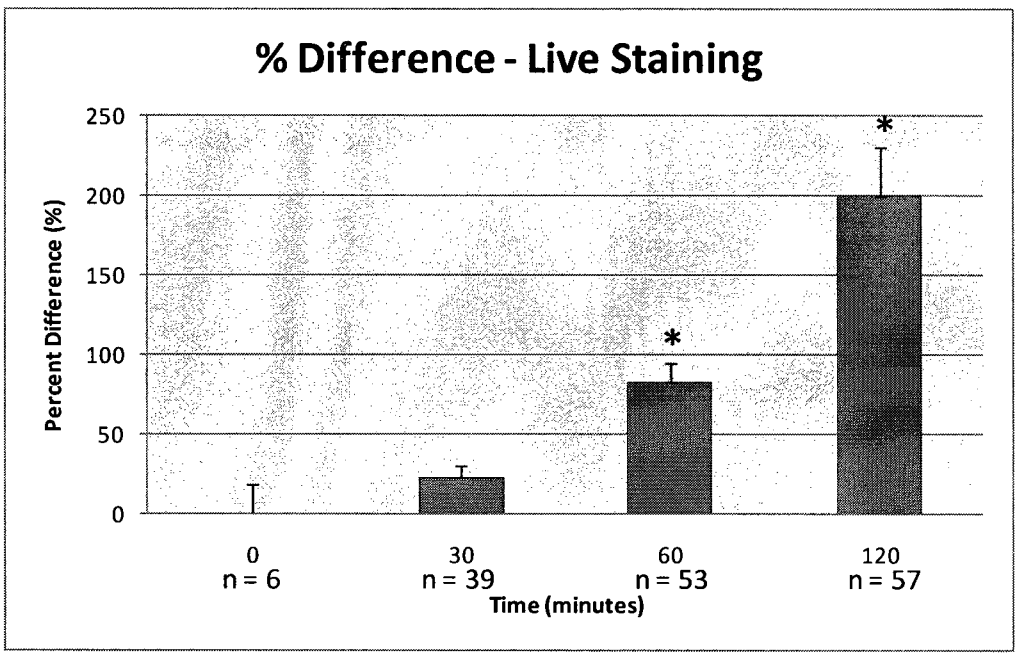

Figure 3.7 – PH-4821 Can Stain Live Transfected HEK293 Cells
Using a live staining protocol (see section 2.13) HEK293 cells transfected with rat TRPV1 were incubated with PH-4281 at different time intervals (0, 30, 60, 120 minutes) and then fixed with 4% paraformaldehyde (PFA). TRPV1 staining around the perimeter of the cell became brighter as time interval increased (Panel A). All scale bars are 15µm. In panel B, the percent intensity difference was compared with the control condition (i.e zero minute incubation) showed a significant difference ($P < 0.05$) in percent difference of staining intensity at 60 and 120 minutes.

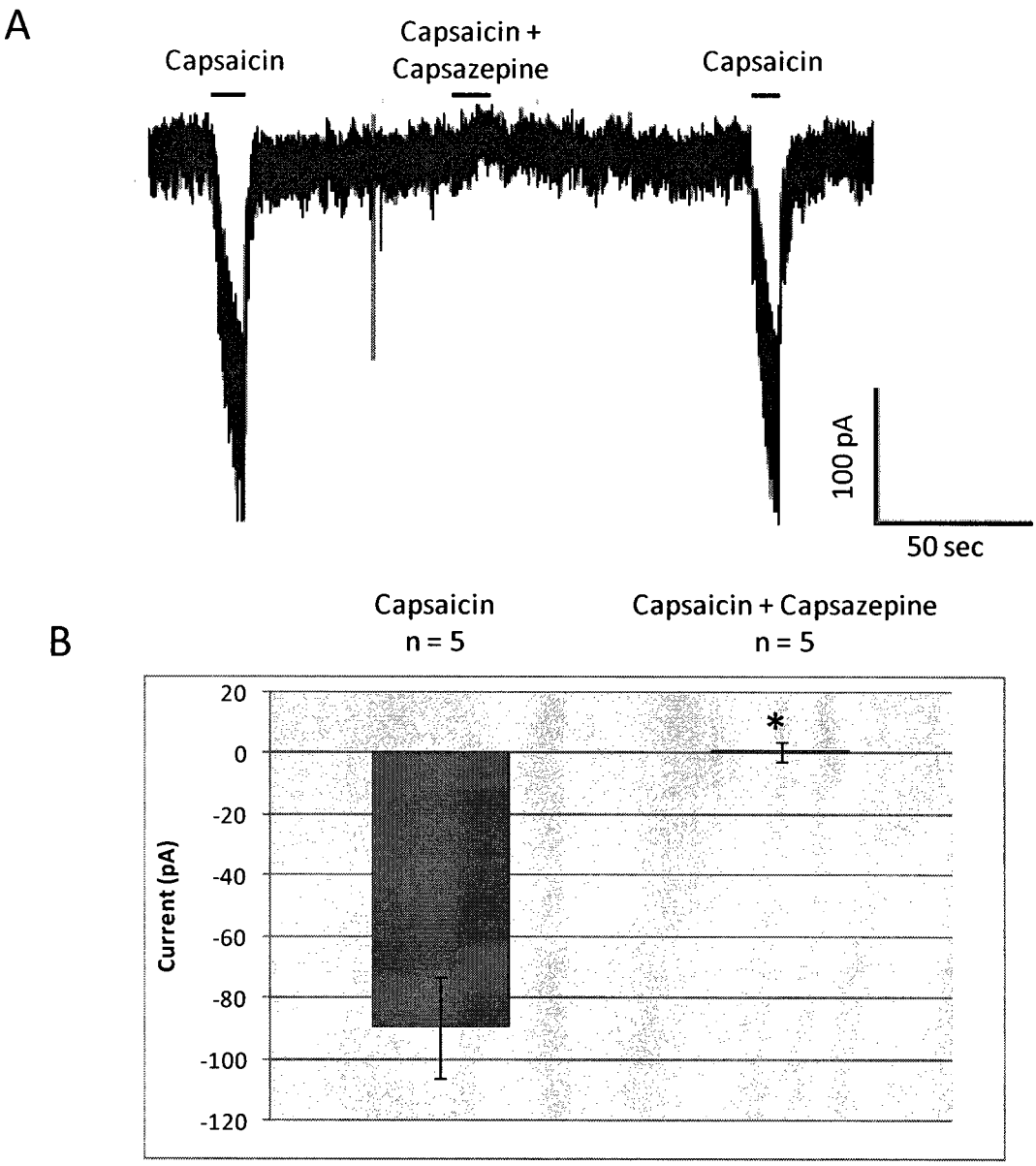

Figure 3.8 – Capsazepine Blocked Capsaicin-Evoked Responses in TRPV1 Transfected HEK293 Cells

Panel A: Whole-cell voltage-clamp recording of a HEK293 cell transfected with rat TRPV1 showed an inward current at the on-set of capsaicin (10μM). Switching to a solution containing capsaicin (10μM) and capsazepine (10μM), the capsaicin-evoked response was suppressed. Re-application of capsaicin (10μM) induced an inward current. Panel B: The current amplitude (pA) was compared between the first application of capsaicin (10μM) and the on-set of capsaicin (10μM) + capsazepine (10μM). The average peak current amplitude during capsaicin application (10μM) was -89.63 pA +/- 16.47 (n=5). When capsaicin (10μM) + capsazepine (10μM) was applied, the current was significantly reduced to an average current of 0.63 pA +/- 3.087 (n=5, $P<0.05$).

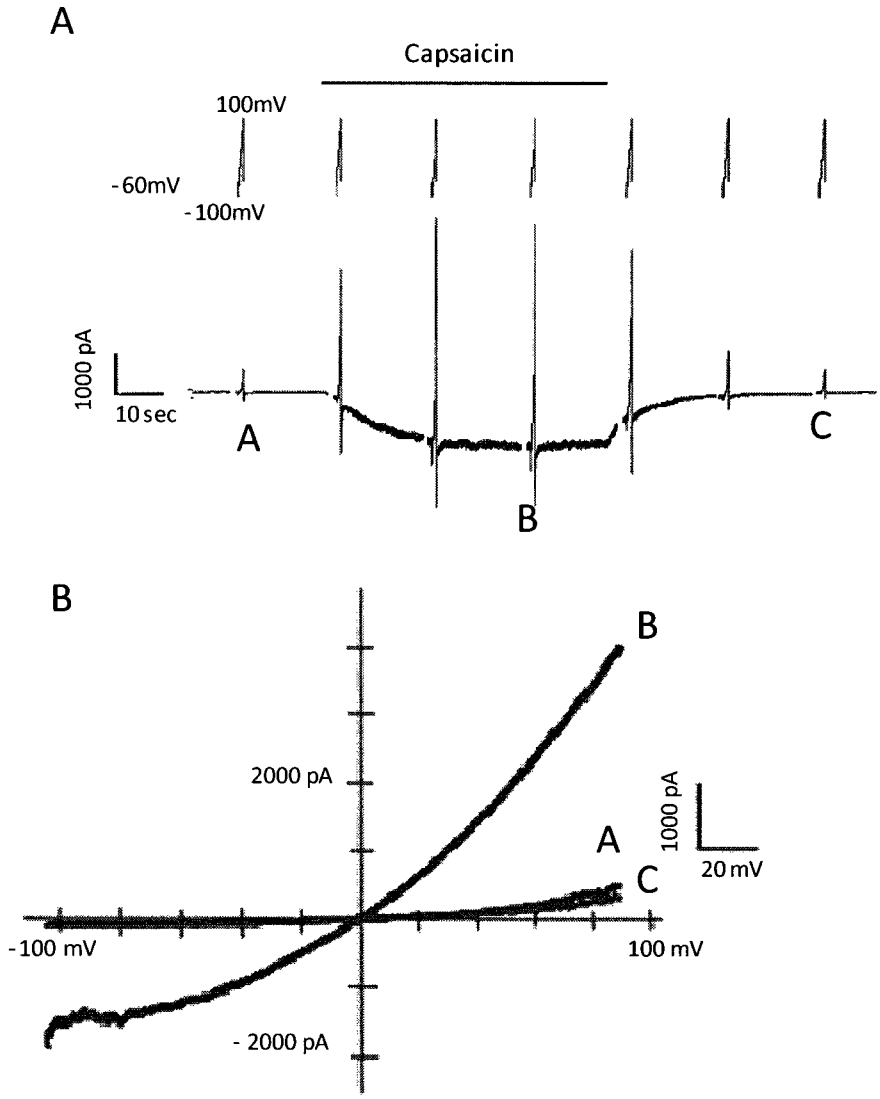

Figure 3.9 – Steady-State Current-Voltage Analysis of Capsaicin-Induced Responses in TRPV1 Transfected HEK293 Cells

Panel A: From whole-cell recordings (holding potential at -60mV), voltage was ramped up from -100mV to 100mV for 1 second at intervals of 20 seconds. Application of capsaicin (10μM) resulted in an inward current and the current magnitude increased as the voltage was ramped up. Panel B: Steady-state current-voltage (I-V) analysis was done at three time points (A, B, and C). Trace A illustrated that prior to capsaicin, and little or no current was observed. Trace B was during capsaicin application and outward rectification was observed. After a brief wash out period, trace C was similar to trace A in that little or no current was observed. The reversal potential was approximately ~0mV (n = 5).

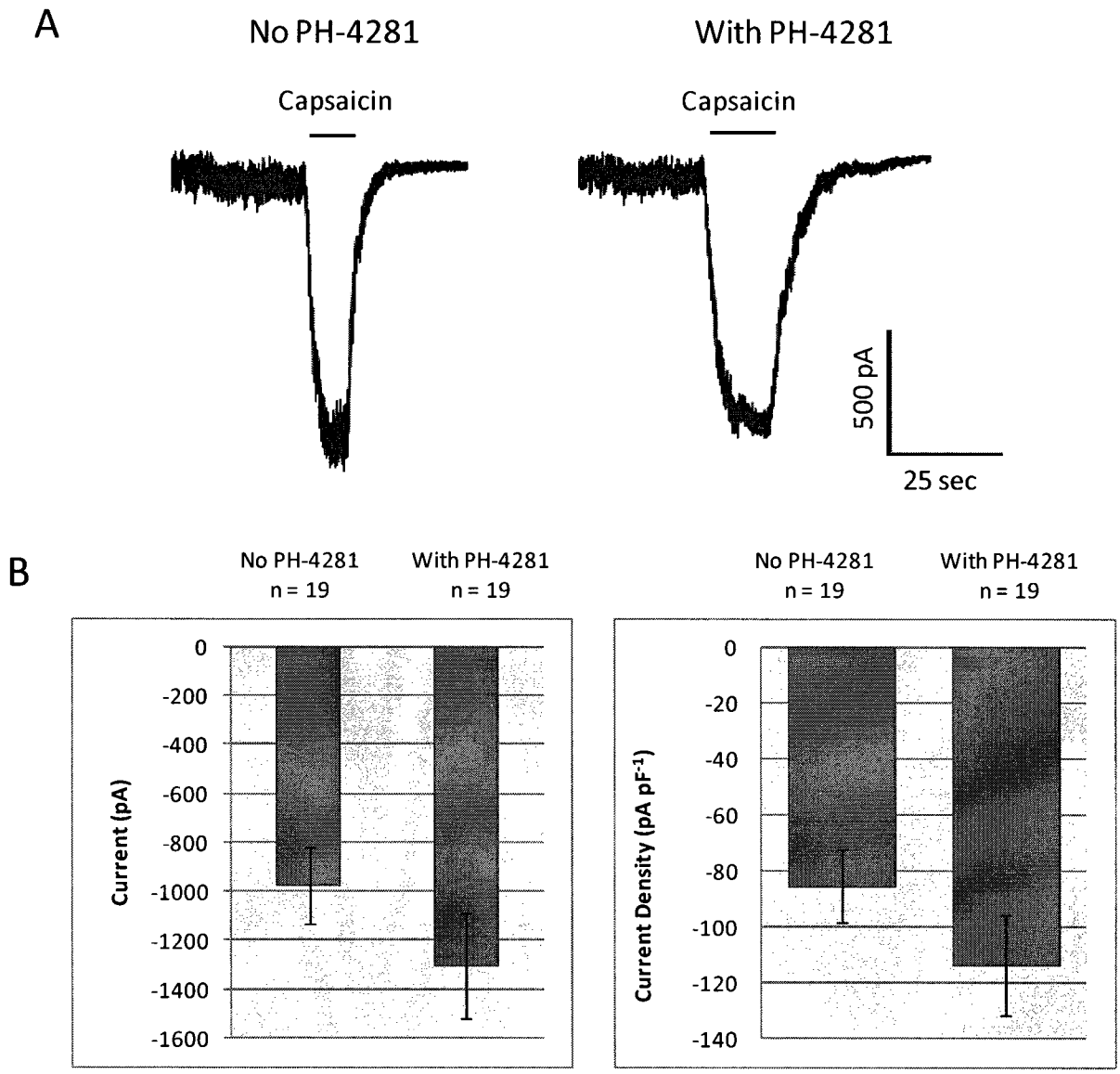

Figure 3.10 – The Effects of PH-4281 on Capsaicin-Evoked Responses in Transfected HEK293 Cells

Panel A: Whole-cell voltage-clamp recordings were done on HEK293 cells transfected with rat TRPV1. Under conditions where there was no PH-4281 incubation (left trace, n=19), capsaicin-evoked an inward current. When PH-4281 was incubated for 1 hour (right trace, n=19), capsaicin also evoked an inward current. Panel B: The average peak current was not significantly different between cells with no PH-4281 and with PH-4281 incubation (-973.54 +/- 153.85 pA and -1301.46 +/- 214.74 pA; $P>0.05$; respectively). The average current density was also not significantly different ($P>0.05$) different between cells that had no PH-4281 incubation and with PH-4281 incubation (-85.48 +/- 13.07 pA pF^{-1} and -113.38 +/- 18.03 pA pF^{-1} respectively).

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Only minor changes have been made.

Please note that (as per their respective training) S. Ciura works only with mice, E. Trudel works only with rats, and that Y. Choe, P. Hua and J Sudbury work with both rats and mice.

Breeding procedures:
Breeding (transgenics/knockouts): B6.129S4-Trpv1m1jul mice obtained from Jackson Labs will be bred in the animal facility according to UACC-SOP#4 to produce the animals required for the procedures described below. The transgene is a commercial product with no phenotypic abnormalities and is not being produced as part of the protocol.

Breeding (transgenics/knockouts): TRPV4 knockout mice obtained from Duke University Medical Center will be bred in the animal facility according to UACC-SOP#4 to produce the animals required for the procedures described below. The transgene bears no phenotypic abnormalities and is not being produced as part of the protocol.

THE FOLLOWING TEXT HAS BEEN DELETED -Breeding (transgenics/knockouts): FVB/N-tg(GFAP GFP) 14m es/j mice obtained from Jackson Labs will be bred in the animal facility according to UACC-SOP#4 to produce the animals required for the procedures described below. The transgene is a commercial product with no phenotypic abnormalities and is not being produced as part of the protocol. THE TEXT ABOVE HAS BEEN DELETED BECAUSE THESE MICE WILL NOT BE USED.

BREEDING (TRANSGENICS/KNOCKOUTS): TG-VP-EGFP RATS (FOUNDERS WERE OBTAINED FROM QUEENS UNIVERSITY IN 2007) WILL BE BRED IN THE ANIMAL FACILITY ACCORDING TO UACC-SOP#4 TO PRODUCE THE ANIMALS REQUIRED FOR THE PROCEDURES DESCRIBED BELOW. THE TRANSGENE BEARS NO PHENOTYPIC ABNORMALITIES AND IS NOT BEING PRODUCED AS PART OF THE PROTOCOL.

Acute non-survival preparations made from adult mice and rats:
Fresh brain tissue will be harvested from single adult rats and mice. Animals are brought in a single cage from their housing location in the Conventional Facility of the Montreal General Hospital Research Institute (Livingston wing, L13-419 for mice and L13-307 for rats) to room R2-117 where they are kept in a quiet environment prior to the procedure (usually less than 15 minutes later).

Procedure for rats (this procedure will be done in isolation from other animals), E. Trudel only: when ready for the procedure, the animal is picked up by the tail and calmly guided into a polyethylene restraint cone. The rats spontaneously enter these cones when guided in their direction. Although the animal is immobilized when the investigator holds the rear side of the flexible cone, it can still breathe through a small opening cut into the front end of the cone. The animal is usually restrained for less than 5 seconds prior to euthanasia by decapitation with a small animal guillotine (#51330; Stoelting Co. Wood Dale, IL, USA). The use of the restraint cone ensures that the rat does not change its position and thus permits a very rapid (less than a tenth of a second) and precise (C1-C2 vertebrae) procedure. Tissue is then obtained by dissection and the remains of the animal are disposed of at the appropriate location in the animal housing facility. The viability of the supraoptic neurons in hypothalamic slices or explants obtained from anaesthetized rats is significantly reduced compared to preparations obtained from unanaesthetised animals (Bourque, 1990). It is for this reason that Mr. Trudel makes his experimental preparations from rats euthanized by decapitation. The use of videopatch microscopy (introduced in my laboratory during the past 2 years) has greatly improved our ability to visually identify viable neurons that can be used for

experiments. Other rat users (J. Sudbury and T. Stachniak) which can bebefit from this approach are now making their preparations following decapitation of anaesthetised rats (as described below). We expect to phase out the use of rodent restraint cones and euthanasia of rats by decapitation before the end of the 2008-2009 protocol year.

Procedure for collecting viable/fresh brain tissue from anaesthetised rats: when ready for the procedure, Halothane (0.5 ml) is injected into the lower compartment of an induction chamber. The rat is then picked up by the tail and placed in the upper compartment of the anaesthetizing chamber, where it cannot come in direct contact with halothane. Anaesthetic induction is performed in a fume hood with external exhaust. The rat is then removed when fully anaesthetized (20-40 seconds) and is euthanized by decapitation for the purpose of harvesting brain tissue by dissection. Although isoflurane is recommended instead of halothane as an anaesthetic because of the reduced cardiopulmonary depression and hepatocellular necrosis, the procedure being used is a non-survival one and its outcome will not be affected by the type of inhalant* anaesthetic.

Procedure for mice: when ready for the procedure, the mouse is picked up by the tail and placed in a small anaesthetizing chamber. Halothane (0.3 ml) is then injected into the chamber. Animals placed in the induction chamber will not come in direct contact with halothane. Anaesthetic induction is performed in a fume hood with external exhaust. The mouse is then removed when fully anaesthetized (10-20 seconds) and decapitated for the purpose of harvesting brain tissue. Although isoflurane is recommended instead of halothane as an anaesthetic because of the reduced cardiopulmonary depression and hepatocellular necrosis, the current procedure is a non-survival one and its outcome will not be affected by the type of inhalant anaesthetic.

Animal perfusion fixation for histology: for the purpose of performing immunocytochemistry, animals will be anaesthetized with a mixture of ketamine-xylazine-acepromazine or urethane (1000 mg/kg i.p.) as per SOP, and perfused intracardiacally with paraformaldehyde. Perfusion procedures are performed in a bio-safety cabinet with external exhaust. The use of urethane will be preferred on occasions where a single animal is to be used over a period of several weeks or months because urethane stored as dry powder has a long shelf-life and is therefore more convenient and cost-effective when isolated experiments require perfusion.Urethane provides rapid and profound anaesthesia for the very short time that is required to initiate transcardiac perfusion of fixative and is acceptable and approved (as per SOP) for acute terminal procedures on adult rodents. We will use ketamine-xylazine-acepromazine solution whenever a group of animals is used for histological experiments.

Procedures for tissue culture: neonatal (P3-P5) mice (mouse c57/bl6 and b6.129s4-trpv1m1jul) OR P3-P6 RATS (TG-VP-EGFP WISTARS) will be anesthetized by indirect hypothermia for 4-5 minutes according to UACC SOP-11, section 4.1, then euthanized by decapitation according to UACC SOP-13 section 2.1. tissue will then be obtained by dissection and animal remains will be disposed of at the appropriate location in the animal housing facility.

Animal identification and genotyping: All procedures to be performed in the L13 facility to avoid contamination. For animals less than 3 weeks of age individuals will be marked by ear notching and a 0.5 cm tail segment will be removed as per UACC SOP-4 section 4.1. For older mice, animals will be anaesthetized with isoflurane as per UACC SOP-2 and a 0.5 cm segment of the distal part of the tail will be cut using a clean razor blade according to UACC SOP#4. hemorrhage will be stopped using hemostop powder (Professional Veterinary Laboratories) to the tip of the tail. This procedure will be done only once in the animal's lifetime.

Procedure for chronic dehydration or salt-loading. As stated in section 5b, objective #5, we aim to define plastic changes in sensory transduction, synaptic transmission and neuro-glial signaling that normally serve to enhance osmoregulatory homeostasis during chronic dehydration and salt-loading. These experiments will be performed on tissue obtained as indicated under "Acute non-survival preparations made from adult rats and mice" (above). The only differences are that:

For dehydration: the water bottle will be removed from the cage housing the animal 12-48 hours (mice) or 12-72 hours (rats) prior to the tissue isolation procedure. Reference for rat: Hayashi M, Arima H, Goto M, Banno R, Watanabe M, Sato I, Nagasaki H, Oiso Y. (2006) Vasopressin gene transcription increases in response to decreases in plasma volume, but not to increases in plasma osmolality, in chronically dehydrated rats. Am J Physiol Endocrinol Metab. 290, E213-E217. Reference for mice: Morris M, Means S, Oliverio MI, Coffman TM. (2001) Enhanced central response to dehydration in mice lacking angiotensin AT(1a) receptors. Am J Physiol Regul Integr Comp Physiol. 280, R1177-R1184.

For salt-loading: the water bottle will be replaced with a bottle containing 2% NaCl 1-7 days prior to the tissue isolation procedure. Reference for rats: Summy-Long JY, Hu S, Pruss A, Chen X, Phillips TM (2006) Response of interleukin-1beta in the magnocellular system to salt-loading. J Neuroendocrinol. 18, 926-937. Reference for mice: Theodosis DT, Schachner M, Neumann ID. (2004) Oxytocin neuron activation in NCAM-deficient mice: anatomical and functional consequences. Eur J Neurosci. 20, 3270-3280.

These protocols are well-documented in the literature (where dehydration is fequently prolonged to 4-5 days rather than the maximum of 3 days (for rats) or 2 days (for mice) being performed here; and where salt loading is frequently performed for 8-10 days rather than the maximum of 7 days being proposed here). The protocols cause no observable distress or behavioral changes in the animals, and are easily and completely reversible without adverse effects. The dehydration protocol causes a progressive increase in plasma osmolality and accompanying reduction in blood volume, whereas the salt-loading protocol causes a progessive increase in plasma osmolality with little change in blood volume. The use of these two protocols allows a determination of the effective variable (osmolality or blood volume) on the parameters being measured.

b) For D level of invasiveness,

Include here ALL procedures described in the original protocol. New and changed procedures in CAPS *(was section 10a in main protocol); Please only attach SOPs related to new and changed procedures to this renewal form.*

7. Endpoints

a) For B and C level of invasiveness,

The procedures are the same as the original protocol: YES☒ NO☐

IF NO, supply new endpoints that are different from the original protocol:

Experimental endpoints:

Clinical endpoints:

b) For **D level** of invasiveness,

Include here ALL endpoints, including the ones described in the original protocol as well as new and changed endpoints in CAPS:

Experimental endpoints:

Clinical endpoints:

8. Hazards (check here if none are used: ☐)

a) Are the hazards different from original protocol? (infectious, radioactive, toxic, carcinogen, tumours)

YES ☐ NO ☒ **if yes, supply details (material, risks, precautions):**

b) Have the cell lines been tested for human and animal pathogens? YES:☐ NO:☐ None used:☒

9. Description of Animals to be used in the coming year (only):

Quality Control Assurance: To prevent introduction of infectious diseases into animal facilities, a health status report or veterinary inspection certificate may be required prior to receiving animals from all non-commercial sources or from commercial sources whose animal health status is unknown or questionable. Quarantine and further testing may be required for these animals. *If more than 6 columns are needed, please attach another page*

	Sp/strain 1	Sp/strain 2	Sp/strain 3	Sp/strain 4	Sp/strain 5	Sp/strain 6
Species	rat	rat	mouse	mouse	mouse	
Supplier/Source	Charles River	Breeding	Charles River	Jackson Labs	Duke University Medical Center	
Strain	Long Evans	Tg-VP-EGFP-Wistar	C57/BL	B6.129S4-Trpv1m1Jul	Trpv4 k/o	
Sex	male	males and females	male	male/female	male/female	
Age/Wt	40-160 g	40-160 g	6-24 weeks	6-24 weeks	male/female	
# To be purchased	450	0	250	0	0	
# Produced by in-house breeding	0			504	450	
# Other (e.g.field studies)						
TOTAL# /YEAR	450	300	250	504	450	

10. Explanation of Animal Numbers:

BASED ON THE EXPERIMENTAL OBJECTIVES OF THE PROJECT, describe the number of animals required for one year. Include information on experimental and control groups, # per group, and failure rates.

For breeding, specify how many adults are used, number of offspring produced, and how many offspring are used in experimental procedures. The arithmetic explaining how the total of animals for each column in the table above is calculated should be made clear.

The total number of Long Evans rats needed for this year (450) is based on the fact that experiments related to objectives 1-4 involve two electrophysiology recording rigs used 4 days per week for 50 weeks. Since each rig needs a separate rat on each experimental day, we need 8 rats per week for a total of 400 per year. Another 50 rats are needed to perform basic histological, immunocytochemical and tissue RT-PCR detection studies related to each project, bringing the average to 9 rats per week and a total of 450 for the year.

The total number of Tg-VP-EGFP Wistar rats needed for this year (300) is based on the fact that experiments related to objectives 1-4 will require preparations for two electrophysiology recording rigs on 3 days per week for 50 weeks. Since each rig needs a separate rat on each experimental day, we need 6 per week, or 300 rats per year (2x3x50=300). These rats will all be produced by breeding.We aim to generate these rats by generating approximately 60 litters of 5 pups each (average).

The number of adult male C57/BL mice from Charles River needed for this year (250) reflects the fact that one adult C57 mouse is used for electrophysiological analysis on two rigs two days per week for 50 weeks (2x4x50= 200), and a need for 50 additional adult C57 mice for immunocytochemical and molecular biological analysis.

protocols which must be designed once initial observations are made. Thus we cannot predict all of the protocols or reagents that will be used throughout the year. As individual observations must be reproduced 5-10 times to reach statistical significance, it is imperative that each experiment be repeated at a frequency which allows us to reach this minimum number of observations. Lastly, our research program goes well beyond the present year (present funding is approved until 2010) and regardless of how quickly individual objectives can be reached, new objectives will always be defined to optimize productivity. Research in this field is extremely competitive and requires optimal use of the six rigs available in my lab (each valued at >$100,000).

Submit to your local Facility Animal Care Committee. Please note that after two renewals, a full protocol needs to be submitted.

This approval does not imply that space will be made available. If a major increase of space needs is anticipated, please contact the appropriate animal facility manager.