From peppers to peppermints: Insights into Thermosensation and Pain

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by
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INTRODUCTION

In general, our work is framed by basic, curiosity-driven questions about how we experience the world through our senses. This is both a fascinating and profound goal because our senses dictate how we perceive every aspect of our physical and chemical environment, and even our internal state – all of which is determined by the biochemical, biophysical, and anatomical properties of the molecules and cells that constitute our sensory apparatus. Indeed, evolution has tuned sensory systems to suit each creature's lifestyle and thus our world is perceived quite differently from that of a bumble bee, shark, or rattlesnake.[1] Identifying and characterizing the receptors that detect relevant stimuli is therefore a critical step toward understanding how we appreciate and respond to our world and how our senses adapt to changes in our environment or ourselves.

More specifically, we are motivated by a quest to understand how we experience pain. Pain is a sub-modality of somatosensation, which we colloquially refer to as our sense of touch. Of our five senses, somatosensation is arguably the most important for our survival and wellbeing, in large part because pain serves as a main warning system that alerts us to

real or impending bodily injury and initiates appropriate protective reflexes.[2] Not surprisingly, individuals in whom this system is rendered inoperable due to genetic mutation or diseases are at great risk of injury or death.[3] The problem of course is that pain can outlive its usefulness as an acute, beneficial warning system and instead become 'maladaptive', persistent, and debilitating. Indeed, an important objective is to understand what drives this switch between acute and chronic pain, with the hope of using such knowledge to prevent or reverse this transition. Another long-term challenge in the pain field is to better define different chronic pain syndromes. Pain, like cancer, is a single word that we use to describe a myriad of distinct syndromes, but how does migraine pain differ from osteoarthritic pain, musculoskeletal pain, visceral pain, etc? What are the underlying molecular, cellular, and neural circuit mechanisms that distinguish one from the other? Resolving these questions is key to mechanistically defining and treating different pain syndromes.

Like other sensory systems, somatosensation begins with the detection of peripheral stimuli. This process occurs at the tips of primary afferent sensory nerve endings that transduce environmental or endogenous stimuli into electrochemical signals and transmit this information to the central nervous system (initially to the spinal cord and then the brain) (Figure 1).[2,4] As a group, primary afferent sensory neurons are remarkable cells because they are tasked with the job of detecting and integrating a wide range of stimuli – both physical and chemical – including temperature, pressure, environment irritants, and endogenous agents produced by tissue damage or inflammation (the so-called inflammatory soup).5 Our goal has been to understand how these diverse stimuli impinge on the primary afferent nerve terminal to modulate its excitability under normal and pathological conditions. We have focused on the subset of primary afferents that detect noxious stimuli, the so-called nociceptors.

NATURAL PRODUCTS AS PROBES OF THE PAIN PATHWAY

When we began this work some 25 years ago, identifying nociceptive mechanisms at the molecular level was somewhat challenging because genetic clues and other such tools were limited. We therefore turned to the power of natural products and folk medicine (in essence, pharmacology honed by evolution) to gain a toehold in this area. I've always been fascinated by this approach, which represents a nexus where anthropology, chemistry, and physiology come together. Some inspirational examples include Pert and Snyder's use of morphine from the opium poppy to discover opiate receptors,[6] Sir John Vane's use of salicylate from the willow bark to explore the biology of cyclooxygenases and prostaglandins,[7] and Rafael Mechoulam's identification of tetrahydrocannibinol as the psychoactive ingredient in marijuana, leading to the discovery of

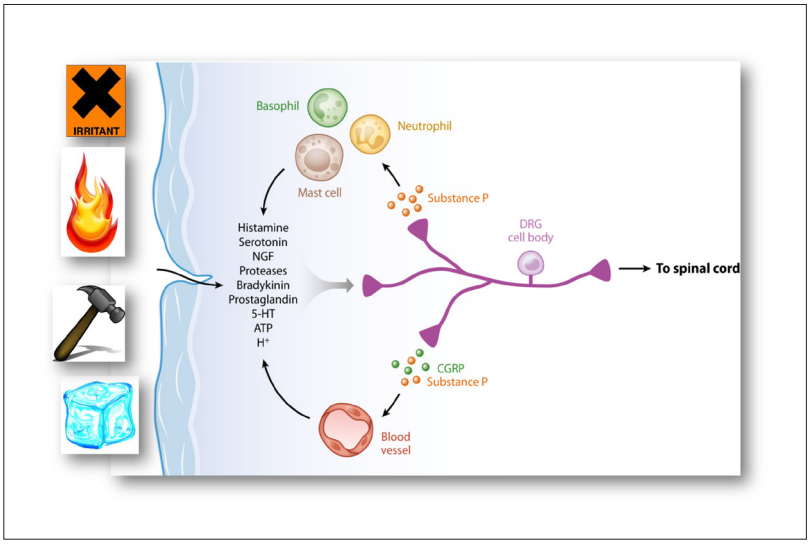

Figure 1. Primary afferent nociceptors detect and integrate noxious physical and chemical stimuli. Primary afferent nociceptors (purple neurons) detect noxious thermal, mechanical, and/or chemical stimuli and transmit this information to the spinal cord. These fibers are also sensitized by numerous 'inflammatory soup' constituents, thereby promoting pain hypersensitivity. (Adapted from reference 5).

endocannabinoids (anandamide and 2-AG) and their receptors.[8] In fact, it's striking to realize that the two main arms of analgesic pharmacotherapy used today – namely, opiate and non-steroidal anti-inflammatory drugs – were inspired by or derived from natural products. With these beautiful examples in mind, we turned the coin over on its other side to ask how natural products elicit pain, with the hope of uncovering key components of the nociceptive machinery that detect, integrate, and transduce noxious stimuli (Figure 2).

Defensive algogenic agents are produced by both plant and animal species, providing rich and chemically diverse sources of pharmacological probes for investigational and medicinal applications. But perhaps the most famous of these is capsaicin, the pungent vanilloid agent in chili peppers that has played an outsized role in the study of nociception and pain. Other notable plant-derived compounds that have featured prominently in somatosensory research (both cellular and behavioral) include isothiocyanates and thiosulfinates, which constitute the pungent irritants from mustard or allium plants such as wasabi, onion, and garlic. Another important class is represented by monoterpenes such as menthol and eucalyptol that constitute cooling agents from mint.

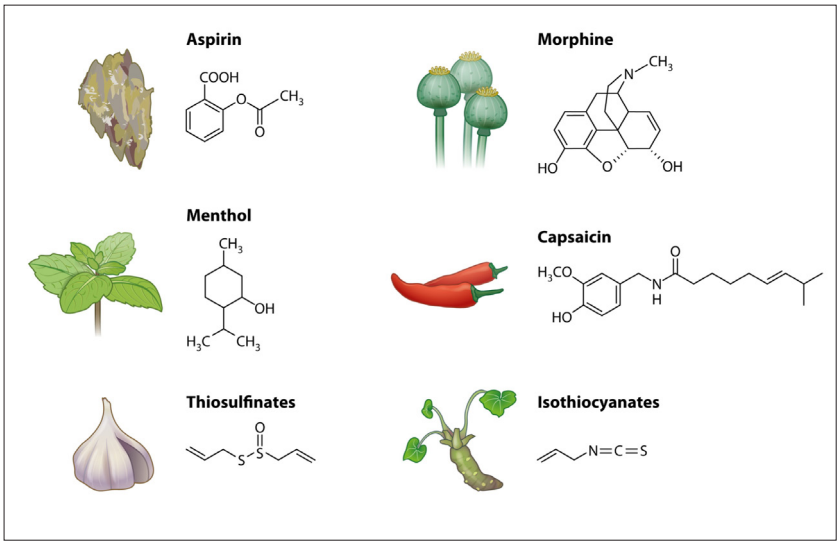

Figure 2. Natural products as probes of the pain pathway. Natural plant products have been critical pharmacological tools for identifying and studying molecules and signaling mechanisms underlying nociception and pain. These include salicylic acid (related to aspirin) from willow bark; morphine from opium poppies; menthol from mint leaves; capsaicin from chili peppers; thiosulfinates from garlic, onions, and other allium plants; and isothiocyanates from wasabi and other mustard plants. (Adapted from reference 5).

Our interest in these natural products was inspired by the work of many groups, but two stand out. One group was led by Nicholas Jancso and Aurelia Jancso-Gabor at the University of Szeged in Hungary, who showed early on (in the 1950s and 60s) that capsaicin acts as a selective excitatory agent for a subset of primary afferent nerve fibers dedicated to pain sensation (i.e., presumptive nociceptors).[9] They further showed that capsaicin produces functional desensitization of nociceptors (and at high doses can be lethal to these neurons), and that animals injected with a bolus of capsaicin experienced a profound decrease in core body temperature through peripheral and central processes that enhance vasodilation and sweating.[10–12] The other team that influenced our work was that of Yngve Zotterman and Herbert Hensel, who carried out their studies in Stockholm, Sweden. They showed that menthol activates a specific subset of primary afferent nerve fibers, and that its excitatory effect is suppressed by warming and enhanced by cooling.[13] Together, these observations suggested that the psychophysical effects of these natural products are mediated through selective, but distinct actions on somatosensory nerve fibers, providing a pharmacological window into neural mechanisms underlying pain and/or temperature sensation.

In the late 1980s and 90s, several investigators began to ask how capsaicin works from a biophysical perspective. Electrophysiological analysis of cultured sensory neurons suggested that capsaicin enhances permeability of plasma membrane to mono- and divalent cations, perhaps by acting upon a specific receptor / ion channel.[14–18] At this time there were also competing ideas about capsaicin integrating into the membrane to somehow form an ion permeation pore,[19] although this notion could not readily explain the apparent selective action of capsaicin on a subset of somatosensory neurons. Further support for the existence of a specific capsaicin 'site' came from the work of Peter Blumberg at the U.S. National Cancer Institute, who measured specific binding of radiolabeled resiniferatoxin (an ultrapotent vanilloid agonist from euphorbia plants) to somatosensory neurons.[20,21]

Thus, following on the earlier physiological and behavioral studies of Jancso, Hensel, Zotterman and others, these studies raised several intriguing questions: do natural irritants target specific sites on sensory nerve fibers? If so, what is the molecular nature of such putative receptors, and what are their true physiologic stimuli? Do they mark functionally distinct sensory neuron subtypes and, if so, what is their relationship to pain and/or temperature sensation? These exciting and profound questions were appreciated by many in the somatosensory research community, and thus finding the elusive capsaicin receptor became something of a Holy Grail in the emerging field of molecular pain research.

CAPSAICIN AND MENTHOL RECEPTORS REVEAL A MOLECULAR LOGIC OF THERMOSENSATION

Our entrée into the molecular biology of somatosensation and pain took a great leap forward when Michael Caterina (now a professor at Johns Hopkins University) joined my lab and implemented a rather beautiful and simple expression cloning approach – based solely on function – to identify a gene encoding the 'mythical' capsaicin receptor (Figure 3).[22] Starting with somatosensory neurons from rodents, Mike generated a cDNA expression library from these cells. He then introduced pools of clones (approximately 16,000 at a time) into non-excitable, non-neuronal cells (in this case HEK293 fibroblasts) with the hope of conferring capsaicin sensitivity to cells that had taken up a cDNA clone encoding a necessary receptor. If, as electrophysiological studies suggested, capsaicin promotes influx of mono and divalent cations, then we could detect capsaicin sensitivity by loading transfected HEK293 cells with calcium dyes developed by the late Roger Tsien,[23] enabling us to look for transfected cells that fluoresced because they presumably had taken up a cDNA clone rendering them responsive to capsaicin. And, indeed, in a Eureka moment, Mike called me into the microscope room to show me a small patch of

glowing cells that had been transfected with a single pool of cDNAs and exposed to capsaicin. Subsequent division of this initial positive pool soon yielded a single cDNA clone capable of rendering HEK293 or any other non-neuronal cell sensitive to capsaicin.

Together with Makoto Tominaga and others in my lab, Mike showed that this gene indeed encoded an ion channel that has permeability to both mono and divalent cations and is activated by pungent vanilloids, including capsaicin and resiniferatoxin. In fact, this channel (initially dubbed Vanilloid Receptor 1, or VR1) is a faithful reporter of spiciness; that is, if you ask how VR1 responds to extracts from peppers, ionic currents go through this receptor in direct proportion to their perceived 'hotness' using capsaicin as a standard – in essence, functioning as a molecular reporter of the famous Scoville unit as the classic behavioral measure of capsicum pungency (Figure 3). Another notable finding was that VR1-expressing HEK293 cells could be killed by prolonged exposure to capsaicin, recapitulating the lethal effects on sensory neurons described early on by the Jancso group. Despite being able to mimic this phenomenon in numerous eukaryotic cell types expressing VR1, a mechanistic basis for capsaicin-mediated cell death remains poorly understood.

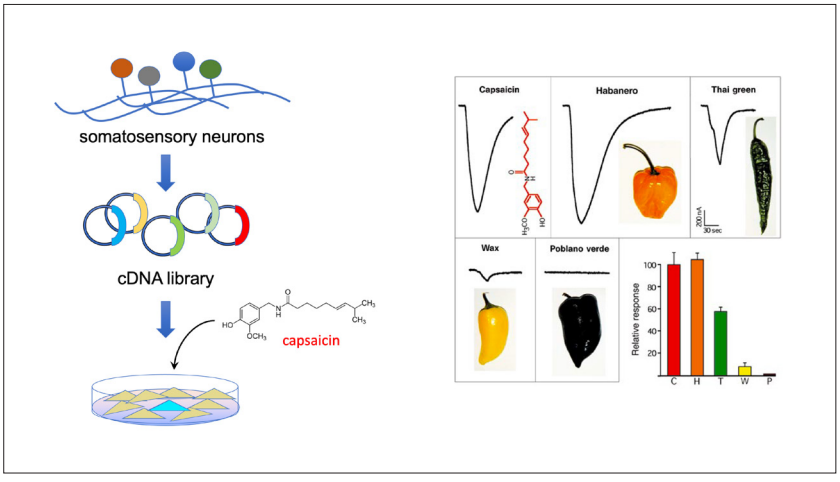

Figure 3. Molecular cloning of the capsaicin receptor. (Left) Expression cloning scheme used to isolate a functional cDNA encoding the capsaicin/vanilloid receptor, VR1 (TRPV1), as described in the text. (Right) Electrophysiological responses of TRPV1-expressing frog oocytes to capsaicin (10 mM) or to extracts derived from four varieties of peppers. Bar graph at bottom right shows relative potencies of each pepper extract normalized to responses obtained with capsaicin. Electrophysiological responses reflect relative pungencies for these pepper varieties as determined by classic Scoville psychophysical ratings. (Right panel from reference 22).

While it's fascinating (and fun) to discover mechanisms by which we appreciate spices, the 'burning' question was, of course, what role does this capsaicin receptor plays in endogenous aspects of somatosensory physiology? This critical issue was addressed when Mike, Makoto and Toby Rosen began to test several algogenic agents, such as those found in the inflammatory soup (e.g., substance P, CGRP, nucleotides, etc.), as possible VR1 activators. After several such candidates failed to activate the cloned receptor, we decided to venture beyond chemical agents to see if relevant physical stimuli might have an effect. To our amazement, we found that heterologous expression of VR1 (in mammalian fibroblasts or *Xenopus* frog oocytes) rendered cells sensitive to heat with properties that seemed both unique and relevant to thermo-nociception (Figure 4). These included steep cooperativity with a temperature coefficient (Q_{10}) of activation greater than 20 and a thermal activation threshold > 40°C, which is in keeping with heat-evoked currents characterized in nociceptive neurons,[24] as well as the psychophysical threshold at which we discriminate an innocuous warm stimulus from a noxious hot stimulus.[25] Toby was especially thrilled and enamored by what he and Mike would come to call the 'wiggle-waggle' experiment (or what I referred to as the Rorschach profile experiment), in which a frog oocyte expressing VR1 was exposed to a continually varying heat stimulus while simultaneously measuring membrane currents, thereby demonstrating the beautifully dynamic nature of the channel's response to temperature.[26] The portability of this phenomenon (namely, that it can be observed when the channel is expressed in any cell type) and its temporally dynamic nature suggested that the channel possesses intrinsic temperature sensitivity and functions as a molecular thermometer. Together, these findings suggested that VR1 (now called TRPV1) is a component of the somatosensory machinery that enables us to detect heat. This conclusion provides a parsimonious explanation for the perceived 'hotness' of chili peppers and may therefore seem obvious in hindsight, but it was not one that came immediately to mind when we first cloned TRPV1, and we were quite amazed and awestruck when we witnessed robust heat-evoked responses in cells expressing this channel.

As noted above, Hensel and Zotterman had shown that menthol, another plant-derived natural product, potentiates responses of trigeminal sensory nerve fibers to cold, and they proposed that cooling compounds mediate their psychophysical effects by interacting with a protein that is specifically involved in cold transduction[13]. Together with our findings on TRPV1, it became obvious that a menthol receptor might also be a cold receptor, but its molecular identify and any possible relationship to TRPV1 was of course unknown. David McKemy (now faculty at USC) and Werner Neuhausser in our group set out to answer these questions by following the same function-based expression cloning approach developed

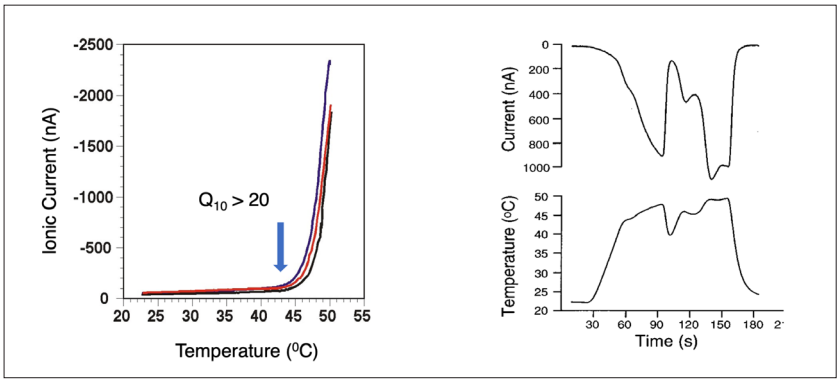

Figure 4. Heat sensitivity of TRPV1. (Left) Temperature-response profile of heat-evoked membrane currents in rat TRPV1-expressing frog oocytes (in this case a capsaicin-insensitive Y511A mutant). Steep relationship reflects high temperature coefficient of activation (Q_{10}) and marked thermal threshold (arrow). (Right) The 'wiggle-waggle' experiment showing that fluctuations in bath temperature over 40°C elicit dynamic and synchronized current responses in rat TRPV1-expressing oocytes. (Adapted from references 26 and 50).

by Mike Caterina. In this way, they were able to identify a cDNA encoding a menthol-sensitive receptor (CMR1 or TRPM8), which indeed turned out to be a cold-activated ion channel.[27] Dave and Werner showed that TRPM8 opened as the temperature dropped below ~25°C and exhibited a temperature coefficient of activation that, while not quite as steep as TRPV1, was still unusually high. Here, again, the channel's thermal activation threshold is generally consistent with a relevant psychophysical threshold, namely that at which we discriminate between warm and cool temperatures.

As one might surmise from their names, the capsaicin and menthol receptors are molecular cousins belonging to the same family of so-called TRP channels, which were initially identified in the fly phototransduction pathway and later appreciated to constitute one of the largest and most physiologically diverse group of eukaryotic ion channels.[28] This beautiful molecular convergence revealed a common biophysical mechanism for how the mammalian somatosensory system detects changes in ambient temperature. Indeed, with the precedent of TRPV1 functioning as a heat sensitive ion channel, various groups began to ask whether other TRP channel subtypes exhibit thermosensitivity, resulting in the independent identification of TRPM8,[29] as well as other thermosensitive TRP subtypes whose *in vivo* roles in temperature sensing remains a topic of ongoing investigation.[30–32]

In summary, the discoveries of TRPV1 and TRPM8 paint a picture in which capsaicin and menthol behave as allosteric modulators that lower activation thresholds for their respective receptors, enabling TRPV1 to

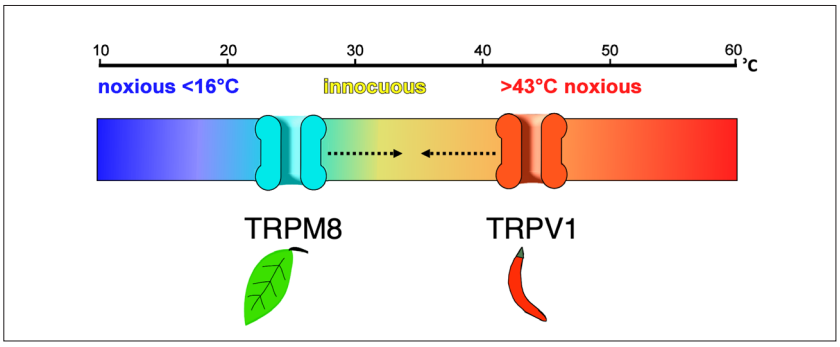

Figure 5. Natural products target molecular thermometers. Capsaicin and menthol are positive allosteric modulators of TRPV1 or TRPM8, respectively, shifting their thermal activation thresholds closer to core body temperature to mimic a hot or cold experience.

open at a lower temperature and TRPM8 to open at a higher temperature, thereby shifting their activation thresholds closer to normal body temperature. In doing so, these natural products produce a psychophysical mimic of a hot or cold experience, respectively (Figure 5). This model, and various observations noted above, lay out two key predictions: first, that TRPV1 and TRPM8 are intrinsically temperature sensitive proteins whose functional properties are consistent with known psychophysical parameters for sensing heat or cold; second, that these channels contribute to an animal's ability to discriminate ambient temperatures *in vivo*.

The first prediction was beautifully borne out by the work of Erhu Cao (now faculty at University of Utah) in my lab and Julio Cordero-Morales (now faculty at University of Tennessee, Memphis) when they purified the channel to homogeneity, reconstituted it into a totally synthetic lipid bilayer, and then together with Beiying Liu and Feng Qin at the State University of New York at Buffalo used patch clamp analysis to ask what happens when you expose this patch to a rapid burst of radiant heat from an infrared laser (Figure 6).[33] Indeed, they showed that the reconstituted channel produced characteristic outwardly rectifying ionic currents, in a temperature dependent manner. Moreover, the biophysical and thermodynamic properties of these responses – namely, temperature threshold, activation rate, temperature coefficient of activation, etc., – beautifully match the parameters that we and others (including Feng and Peter McNaughton) have measured in transfected cells expressing TRPV1 or heat-responsive sensory neurons.[24,34] So, indeed, this supports the first prediction that thermosensitive TRPs (or at least TRPV1) are intrinsically temperature sensitive and behave as molecular thermometers.

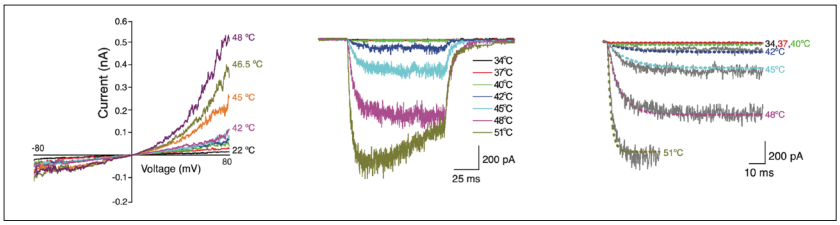

Figure 6. Intrinsic heat sensitivity of purified TRPV1 protein. (Left) Characteristic outwardly rectifying currents recorded from TRPV1-containing proteoliposome patches in response to a temperature ramp (22–48°C). (Middle) Responses elicited by rapid temperature jumps delivered to TRPV1-containing proteoliposome patches using an infrared laser (heat pulse = 100 ms). (Right) Activation time course for infrared laser-evoked responses at different stimulus temperatures fit by a single exponential. Observed parameters (e.g., activation threshold, rates, Q_{10} other biophysical properties) closely resembles those observed in TRPV1-expressing cells. (Adapted from reference 33).

Regarding the second prediction, I believe that the most elegant demonstration of this comes from an experiment that Diana Bautista (now faculty at UC Berkeley), Jan Siemens (now faculty at University of Heidelberg) and Sven Jordt (now faculty at Duke University) carried out when they were in my lab, where they generated and analyzed a menthol receptor-deficient mouse.[35] Together with Cheryl Stucky's group (Medical College of Wisconsin), we showed that cold-responsive neural activity is greatly diminished in these animals. To assess the behavioral consequence of this deficit, we designed a simple place preference chamber (currently residing in the Nobel Prize Museum) in which mice were tested for their ability discriminate between a warm (30°C) and cold (20°C) platform. Not surprisingly, normal (wild type) animals prefer to sit on the nice, warm, comfortable side, and will not venture onto the cold side. However, TRPM8-deficient mice wander freely over the apparatus and spend equal time on both plates, demonstrating an inability to discriminate between cold and warmth. Only once the temperature of the cold surface dropped to rather noxious levels (0–10°C) did TRPM8-deficient animals show a marked preference for the warm side of the chamber; moreover, their ability to avoid a noxiously hot surface remained intact. Together, such experiments from us and others[36,37] upheld the hypothesis that TRP channels are required for an animal's ability to detect and compare ambient temperatures.

Another very important insight came from examining phenotypes of TRPV1-deficient mice, especially regarding mechanisms of inflammatory pain and thermal hyperalgesia. Mice lacking TRPV1-positive afferents are largely insensitive to noxious heat38, but those lacking TRPV1 itself show only partial deficits in acute heat sensation, consistent with the idea that these heat-sensitive nociceptors express other thermosensitive TRP channels, and that acute temperature sensation involves the interplay

between TRPV1- and TRPM8-expressing fibers (see below). But where TRPV1 'knockout' mice show their most dramatic phenotype is in response to inflammation or administration of specific pro-algesic components of the inflammatory soup.[39–41] Thus, in experiments carried out with my friend and long-term collaborator Allan Basbaum and members of his lab, we found that TRPV1-deficient mice show greatly diminished thermal hyperalgesia (i.e., sensitization to heat) after local inflammation or administration of specific components of the inflammatory soup (bradykinin or nerve growth factor, NGF) (Figure 7). These findings from us and others[39–41] validate a critical role for TRPV1 in tissue injury-evoked pain hypersensitivity, providing a rationale for developing TRPV1 antagonists as a new class of analgesics (also see below).

As so beautifully demonstrated in other sensory systems (taste, vision, and olfaction), the identification of receptors for relevant stimuli provides molecular probes with which to examine coding logic of the system, at least regarding peripheral circuitry. Using antibodies against TRPV1 and TRPM8, Jan Siemens showed that these receptors are expressed in mostly distinct, non-overlapping subpopulations of somatosensory neurons35, indicative of a 'labeled line' logic in which the initial detection of hot and cold stimuli is mediated through distinct channels, upholding the so-called 'specificity theory' espoused by Sherrington, and later Ed Perl, who suggested that cellular and behavioral specificity begins at the primary afferent sensory neuron where signals are first detected, rather than at the spinal cord.[42–44] Now, of course, that doesn't mean that subsequent integration of information at the level of the spinal cord is not important for processing sensory input and shaping behavior. In fact, work from Mark Hoon and Gary Lewin suggest that the sensation of warmth

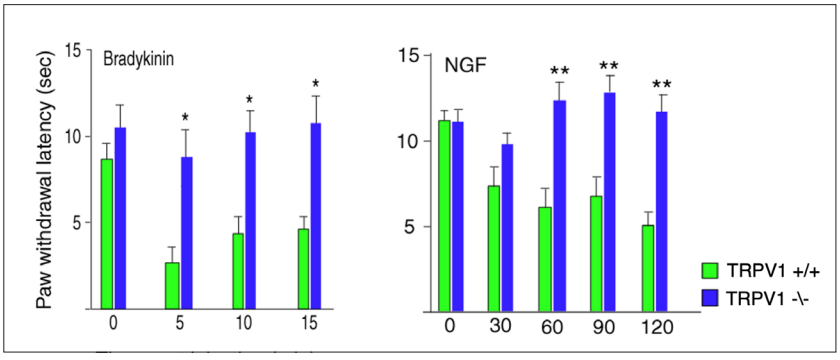

Figure 7. TRPV1 is essential for thermal hypersensitivity. Injection of pro-algesic agents, such as bradykinin or NGF, into a mouse paw enhances sensitivity to heat (i.e., decreases time to paw withdrawal from a radiant heat source). Such sensitization is not observed with TRPV1-deficient mice, illustrating the importance of this channel in the development of inflammatory hyperalgesia. (Adapted from reference 40).

requires integration of signals from hot (TRPV1-expressing) and cold (TRPM8-expressing) sensing fibers at the level of the central nervous system, thereby setting boundaries and zones for discrimination of ambient temperatures.[45,46] Moreover, while TRPV1 and TRPM8 (and their natural product agonists) have played key roles in establishing a molecular paradigm for temperature sensation, studies by Peter McNaughton, Thomas Voets, and others have implicated additional TRP channel subtypes in this process,[47,48] as noted above.

EVOLUTIONARY ADAPTATION OF THERMOSENSITIVE TRP CHANNELS

Before leaving the area of acute temperature sensation, I would like to mention work from our group concerning evolutionary adaptation of thermosensitive TRP channels – a topic which I find fascinating for its broad biological implications, as well as for the fact that it has motivated us to look beyond model genetic organisms and ask questions about animals with unusual and fascinating lifestyles and physiological attributes. Moreover, the analysis of species orthologues with distinct pharmacological or biophysical properties can leverage evolutionary pressure and time to efficiently identify key structure-function relationships. Recent advances in genome and transcriptome sequencing have greatly facilitated such projects by bringing the power of molecular genetics to the analysis of 'non-model' creatures. Here, I'll briefly describe three relevant studies from our group that have focused on TRPV1 or TRPM8.

The first of these is based on the observation that avian species are largely insensitive to capsaicin, which allows them to serve as consumers and distributors of chili pepper seeds (while also explaining why birdseed laced with capsicum extract repels foraging rodents without deterring hungry birds).[49] Sven Jordt (now faculty at Duke University) compared chicken and rat TRPV1 channels to understand this phenomenon, thereby identifying a putative vanilloid binding site and amino acid residues required for activation of the mammalian channel by capsaicin.[50] Indeed, this important study provided powerful validation of our first electron cryo-microscopy structures of TRPV1 (described below), in which vanilloid ligands were bound precisely within this region and in close apposition to these key residues.

In another wonderful, curiosity-driven study, Elena Gracheva (now faculty at Yale) and Julio Cordero-Morales (now faculty at University of Tennessee, Memphis) examined animals that possess what is colloquially referred to as a 'sixth sense' enabling detection of stimuli within the infrared range of the electromagnetic spectrum. This includes certain species of snakes (pit vipers, boas, and pythons) and one mammal – the vampire bat, whose sanguinary lifestyle necessitates that it be effective at finding

the blood supply in warm-blooded victims. This is facilitated by regions surrounding the bat's nose (so-called leaf pits) that are highly innervated by heat-sensitive nerve fibers from trigeminal somatosensory ganglia.[51] Elena and Julio asked whether there are molecular adaptations in TRP channels expressed by these fibers.[52] Indeed, they found that the vampire bat expresses two distinct isoforms of the TRPV1 channel that are generated by alternative splicing: one akin to rat or human TRPV1 having an activation threshold of ~40°C, and another with a shorter C-terminal cytoplasmic tail that is specifically expressed by trigeminal neurons and has a substantially lower activation threshold of ~30°C (Figure 8). Thus, we proposed that the short hypersensitive detector (TRPV1-S) is expressed by sensory nerve fibers innervating the anatomically specialized leaf pits, while the long form (TRPV1-L) subserves normal somatic thermosensation as carried out by other mammalian species.

Finally, to round out this brief overview of our forays into evolutionary adaptation, I want to mention a study carried out by Ben Myers (now faculty at the University of Utah), who asked if TRPM8 channels have distinct thermal activation thresholds in animals whose core body temperatures differ.[53] He looked at birds, which generally have a higher core body temperature than mammals, and cold-blooded frogs, whose body temperature is on average lower, depending upon their ecological niche. Ben found that the temperature response curve for these TRPM8 orthologues is in keeping with the average core body temperature of the cognate animal (right shifted for birds and left shifted for frogs relative to mammals) (Figure 8), again showing a beautiful example of evolutionary adaptation in which activation thresholds of thermosensitive TRP channels shift with an animal's physiology, niche, or lifestyle needs.

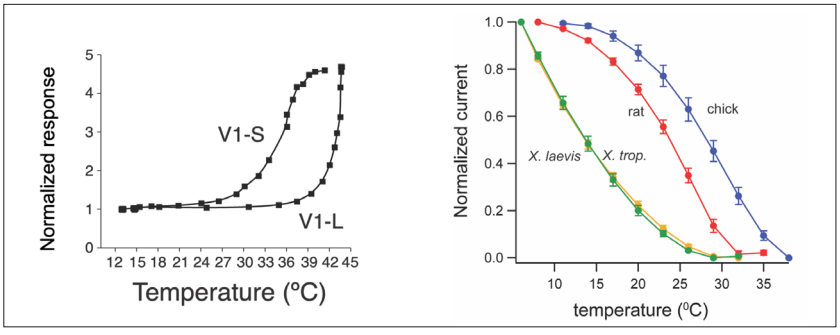

Figure 8. Evolutionary tuning of thermal thresholds. (Left) Temperature-response profiles of HEK293 cells expressing short or long isoforms of vampire bat TRPV1 (assessed by calcium imaging). (Right) Temperature-response profiles of oocytes expressing TRPM8 from amphibian, mammalian, or avian species, as indicated (assessed by electrophysiological recording). (Adapted from references 52 and 53).

TRP CHANNELS ARE POLYMODAL SIGNAL INTEGRATORS: IMPLICATIONS FOR PAIN HYPERSENSITIVITY AND THERAPEUTICS

Sensory adaptation also happens in 'real time' to modulate signaling in response to changing environmental or physiological conditions. We know from our own experience that pain thresholds change dramatically after injury. A sunburn, sprained ankle, arthritis, or other conditions associated with inflammation heighten sensitivity to pressure and temperature, and even chemical irritants – a process that is critical to enhancing protective and healing responses following injury, but which may also drive the development of persistent pain syndromes.[54] As such, an important goal in the field is to understand how changes in the physiological and chemical environment of injured or inflamed tissue alter gain of the primary afferent neuron to alter sensitivity of the pain pathway in both the short- and long-term.[4]

In this regard, TRPV1 has become an important model and locus for understanding how tissue injury enhances nociception and pain. This is because TRPV1 is modulated by various components of the inflammatory soup (see below) and as noted above, TRPV1-deficient mice do not develop thermal hyperalgesia in response to these agents (e.g., nerve growth factor or bradykinin) or following tissue inflammation.[39–41] Thus, an important goal has been to elucidate biophysical and structural mechanisms whereby pro-algesic/pro-inflammatory agents modulate TRPV1.[55] This topic pertains to other members of the TRP channel family that similarly function as polymodal signal integrators.

With respect to TRPV1 and pain, two relevant categories of physiologic modulators can be found within the inflammatory soup. These include (i) direct allosteric agents such as extracellular protons and endogenous bioactive lipids (e.g., anandamide, n-arachidonoyl-dopamine, and lysophosphatidic acid), and (ii) indirect modulators such as neurotransmitters, neurotrophins, and proteases (e.g., extracellular nucleotides, bradykinin, nerve growth factor, and thrombin) that activate their own receptors on primary afferents to sensitize TRPV1 through cytoplasmic second messenger signaling pathways, such as those downstream of phospholipase C (perhaps akin to the way in which the ancestral TRP channel in the fly eye is activated downstream of PLC-coupled rhodopsin) (Figure 9).[56] We and others have investigated numerous pathways and structure-function relationships underlying both direct and indirect modulatory mechanisms, which I can touch on only briefly in this overview by highlighting two examples from our own studies that focus on TRPV1 modulation by extracellular protons or PLC/phosphatidylinositol lipid signaling.

As Bevan, Geppetti, Reeh, and others have shown, local tissue acidosis is a major driver of inflammatory pain, and low pH potentiates cellular and behavioral responses to capsaicin[57–59] – a phenomenon that is

faithfully recapitulated in frog oocytes or transfected mammalian cells expressing the cloned TRPV1 channel.22,26 Thus, decreases in extracellular pH shift the thermal response profile of the channel leftward such that the channel opens at cooler temperatures, and responses at suprathreshold temperatures are larger than those observed at neutrality – in essence, biophysical correlates of allodynia and hyperalgesia, respectively (Figure 9). Makoto Tominaga (now faculty at the Okazaki Institute, Japan) and Sven Jordt zeroed in on two key extracellular titratable residues (glutamates at positions 600 and 648 in the rat TRPV1 channel) that account for these actions and which therefore represent important allosteric regulatory sites underlying one facet of inflammatory pain.[60]

As noted above, indirect pathways that modulate TRPV1 (or other TRP channel subtypes) likely involve multiple cellular consequences of PLC activation, including hydrolysis of plasma membrane phosphatidylinositol lipids, activation of protein kinases, and mobilization of calcium from intracellular stores.[5,55] In our studies, we've been particularly interested in the role of phosphatidylinositol lipids, in part because genetic and physiological studies have suggested that PLC-mediated hydrolysis of PIP_2 is a requisite step in activation of TRP channels in the fly eye. Indeed, when Elizabeth Prescott (now at Fred Hutchinson Cancer Institute) and Huai-hu Chuang (deceased) were in my lab, they proposed (based on pharmacological, electrophysiological, and mutagenesis experiments)

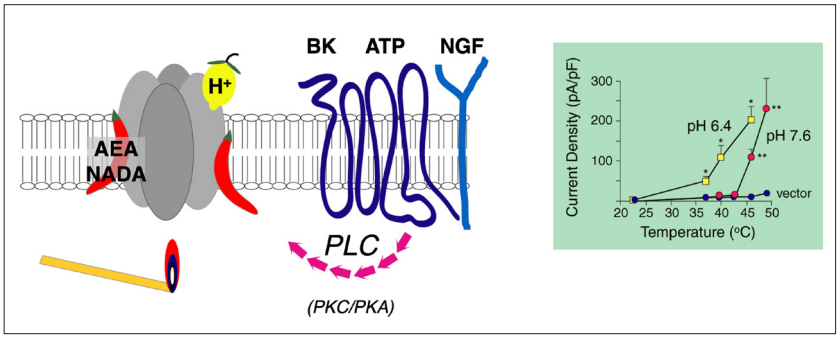

Figure 9. Direct and indirect modulation of TRPV1 by pro-algesic agents. (Left) Schematic illustrating polymodal signal integration by TRPV1. This includes direct positive allosteric modulation by extracellular protons and bioactive lipids, such as the endocannabinoids anandamide (AEA) and n-arachidonoyl-dopamine (NADA), as well as indirect potentiation downstream of metabotropic receptors for other pro-inflammatory / pro-algesic agents, such as bradykinin (BK), extracellular nucleotides (ATP), and neurotrophins (NGF). (Right) Acidosis (low extracellular pH) enhances heat sensitivity of HEK293 cells expressing the cloned TRPV1 channel. Note shift of activation threshold to lower temperature as well as enhanced response at suprathreshold temperatures. (Right panel adapted from reference 26).

that PIP_2 functions as a negative regulator of TRPV1, and that PLC-coupled receptors potentiate TRPV1 activity, at least in part, by removing this inhibitory constraint through PIP_2 hydrolysis.[40] They further proposed that a positively charged region within the channel's C-terminal tail binds to PIP_2 in the plasma membrane to mediate this regulatory interaction.[61] Broadly speaking, roles for PIP_2 and other phosphatidylinositol lipids as TRP channel modulators are well recognized and accepted, but there have been controversy and unresolved issues concerning their specific actions on TRPV1 – namely, whether they enhance or inhibit channel function and how they bind to the channel complex.[62] As discussed below, recent structural studies have begun to clarify these issues by identifying a *bona fide* binding site for phosphatidylinositol lipids and demonstrating its relevance to the mechanism of vanilloid-evoked channel activation.

Why and how might these mechanistic studies be relevant to the development of new analgesics? Over the years, TRPV1 antagonists have shown promise in clinical pain or itch trials,[63,64] but some exhibit 'on-target' side effects that have diminished enthusiasm for their use.[65–69] One such predictable effect is that individuals taking these drugs have diminished sensitivity to painful heat, and while this nicely validates a role for TRPV1 as a noxious heat sensor in people, it erodes its acute protective role. The other main side effect (which is perhaps also predictable from the early studies of Jancsó and colleagues) is a hyperthermic response that in most individuals is small and transient (increased core body temperature of ~0.5°C lasting 1 or 2 days), but which in rare cases can be more substantial. Here, again, these effects affirm a role for TRPV1 and TRPV1-expressing afferents in reporting ambient temperature to the central nervous system and eliciting thermoregulatory responses (e.g., vasodilation and sweating). While some existing antagonists may nevertheless move closer to the clinic, compounds having more nuanced modulatory effects on the channel might be of greater overall utility; they might, for example, target sites involved in allosteric modulation, such as those specifying proton and phosphatidylinositol lipid sensitivity. As discussed below, high resolution TRPV1 structures have now revealed the architecture of these and other sites, providing a foundation for rational design of such drugs.

NATURAL PRODUCTS AND PAIN: PROSPECTING BEYOND PLANTS

Plant-derived natural products have predominated in the study of pain, but animals also avert predators by deploying noxious chemical agents.[70] We have therefore mined the vast and evolutionarily honed pharmacopeia of spider, scorpion, and snake venoms as potential sources of toxins that activate nociceptors to inflict pain. In this way, we have discovered novel peptide agonists for TRPV1 and other nociceptive receptors, providing

powerful biochemical tools for identifying domains and conformational transitions associated with channel gating.[71–74]

Among these, the double-knot toxin (DkTx) from the Chinese bird spider, *Ornithoctonus huwena* (a.k.a. Earth Tiger) has been particularly impactful in our work and thus deserves special mention (Figure 10).[72] True to its name, bites from this aggressive tarantula can produce substantial pain and inflammation, making its venom a potential source of interesting new algogenic agents. Indeed, a graduate student, Christopher Bohlen (now at Genentech), purified DkTx based on its ability to activate TRPV1 in transfected cells. Together with Avi Priel (now faculty at Hebrew University, Israel), Chris showed that DkTx is a potent and persistent 'agonist' with extraordinarily high avidity for the channel (I put agonist in quotes because Avi's biophysical analysis showed that DkTx is a gating modifier that binds preferentially to the open channel state). Chris' molecular and biochemical analyses revealed the structural basis for the toxin's persistent action. Like many spider toxins, DkTx is a member of the extensive family of inhibitor cysteine knot (ICK) peptides that typically consist of 30–40 amino acids and assume a rigid, compact 'knot' scaffolded by intramolecular disulfide bonds. Owing to a presumed gene duplication event, DkTx consists of two such knots joined in tandem by a small 7 residue linker region (ergo, *double-knot* toxin). Through a bit of toxin engineering. Chris and Avi showed that DkTx bivalency accounts for its high avidity, likely reflecting interaction with its multivalent (i.e., tetrameric) channel target, as beautifully borne out by later structural studies described below.

Toxins and other natural products evolve to recognize functionally salient regions of their cellular targets, making them amazing tools for identifying structural elements that contribute to ligand binding, receptor activation, subunit assembly, and the like. For example, hanatoxin, an ICK spider toxin that inhibits voltage-gated potassium (KV) channels, binds to the voltage-sensing region of the channel, inhibiting its movement during membrane depolarization.[75] Given the overall topological similarity between KV and TRP channels, it seemed likely that DkTx might also bind to this equivalent region atop the third and fourth transmembrane helices. Instead, Chris delineated a footprint for DkTx within the outer pore region (between the 5th and 6th transmembrane domains) of TRPV1, in the general vicinity of the sites (E600 and E648) specifying proton modulation. Together, these observations bolstered accumulating evidence that regions in and around the pore of TRP channels are conformationally dynamic and contribute to channel gating and/or plasticity of the ion permeation pathway.[76–78] This stands in contrast to what has been observed with KV channels, where these region remains relatively stationary and rigid during normal gating events.[79] Indeed, these predictions

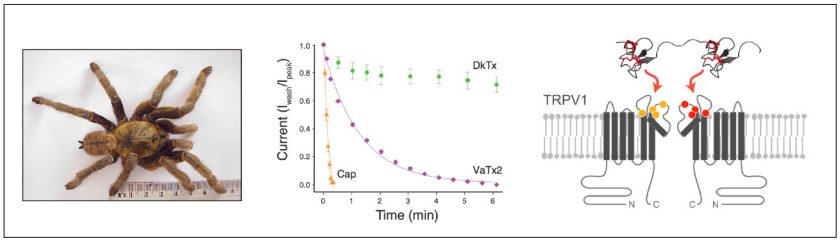

Figure 10. Double-knot toxin (DkTx) is a bivalent, high affinity TRPV1 gating modifier. (Left) The Chinese bird spider (O. *huwena*), or Earth Tiger tarantula, from Guangxi province, China. (Middle) Comparison of wash-off rates for three TRPV1 activators, suggesting very slow dissociation of DkTk from the channel when compared to capsaicin or VaTx2, another (monovalent) spider toxin. (Right) Model depicting binding of bivalent DkTx to equivalent sites on two TRPV1 subunits. Colored dots indicate residues crucial for DkTx-mediated activation. (Adapted from reference 72).

from pharmacological and mutagenesis studies were subsequently confirmed by capturing high resolution structures of TRPV1 in various states, as discussed below.

THE WASABI RECEPTOR AND CHEMO-NOCICEPTION

For the sake of brevity and organization, I focused my 'live' lecture on the capsaicin and menthol receptors and their roles in thermosensation. But in this written version, I would like to give brief mention to another very important and fascinating target of pungent natural products, namely the wasabi receptor, TRPA1.

Mammalian TRPA1 (a.k.a. ANKTM1) was initially suggested to function as a noxious cold sensor and to account for that aspect of cold sensitivity not mediated by TRPM8.[80] But subsequent findings suggest that TRPA1 plays little, if any, role in acute cold sensation and more likely contributes to injury-evoked cold hypersensitivity.[81] Interestingly, however, certain non-mammalian TRPA1 orthologues exhibit heat-sensing capabilities, as first shown by the Garrity and Patapoutian labs for the distantly related fly channel, dTRPA1.[82,83] Moreover, in yet another fascinating foray into vertebrate sensory adaptation, Elena, Julio, and our collaborators Nick Ingolia and Jonathan Weissman, pulled TRPA1 out of an unbiased transcriptome profiling screen aimed at identifying transducers of infrared stimuli in snake species (pit vipers, pythons, and boas) that possess this somatosensory modality.[84] Thus, in certain non-mammalian vertebrate and invertebrate species, TRPA1 is also a thermosensitive member of the TRP channel family.

Controversies about cold sensitivity notwithstanding, it is now generally agreed that mammalian TRPA1 plays an important role in chemo-nociception by serving as a receptor for a broad range of environmental irritants and endogenous inflammatory agents. An important step in estab-

lishing this idea was Sven Jordt's discovery that the cloned TRPA1 channel is activated by mustard oil,[85] which in behavioral studies has been used as a topical rub to elicit swelling, inflammation, and local pain hypersensitivity,[86] but without any knowledge of the underlying mechanism. Together with other members of the lab and our collaborators Peter Zygmunt and Edward Högestätt at Lund University in Sweden, Sven convincingly showed that native TRPA1 channels in mammalian sensory ganglia are, indeed, receptors for isothiocyanate compounds that constitute the pungent agents in wasabi, yellow mustard, Brussels sprouts, capers, etc. TRPA1 is also the target for volatile thiosulfinate compounds that account for the eye watering sting of chopped onion or the zing from garlic.87,88 What unites these natural product agonists is not their shape, but rather their chemical reactivity: all are reactive electrophiles that activate mammalian TRPA1 through an unusual and fascinating mechanism involving covalent modification of key cysteine residues within the channel's cytoplasmic amino terminus.[89,90] Recent structural studies by us and others have provided insights into how attachment of an electrophile at these sites promotes channel gating and thus how TRPA1 serves as a sensitive, yet broadly tuned receptor for a wide range of electrophilic irritants.[91,92] Indeed, TRPA1 is activated by caustic irritants or drug metabolites such as acrolein, formalin, or 2-pentenal, and thus serves as an important detector of these and other noxious environmental toxicants. Moreover, endogenously produced reactive electrophiles, such as 4-hydroxy-2-nonenal and J-series prostaglandins, contribute to inflammatory pain by activating TRPA1 on peptidergic nociceptors, making this ion channel an interesting target for new classes of analgesics.[93–95]

THE RESOLUTION REVOLUTION: SEEING TRP CHANNELS IN ATOMIC DETAIL

In considering the potential benefit and utility of TRP channel inhibitors that have more nuanced properties and mechanisms of action, it seemed clear that an important goal is to understand how these signal integrating machines function in atomic detail. Where are the sites that specify stimulus detection, allosteric regulation, ion selectivity, and gating? Over the years, physiological and pharmacological experiments, together with mutagenesis studies, have pinpointed resides or domains critical to these functions, but with minimal understanding of how they communicate. Thus, the next important challenge was to transform these 2-dimensional maps of TRP channel functionality into 3-dimensional ones (and preferably at the atomic level) so that structural interrelationships could be visualized and mechanistically understood.

This important goal was achieved through a wonderful and now long-term interaction with my friend Yifan Cheng and members of his lab here

at UCSF. The initial and seminal breakthrough emerged from a collaboration between Erhu Cao and Maofu Liao (now faculty at Harvard), who together took Erhu's purified (and functional) TRPV1 protein forward for analysis by single particle electron cryo-microscopy (cryo-EM).[96] At the time, we had little expectation for achieving near-atomic resolution, but after a couple of frustrating years trying to generate high quality protein crystals for X-ray diffraction, we thought that cryo-EM might at least provide some indication of protein quality and overall architecture. Fortunately for us, our UCSF colleague David Agard had just helped develop a new direct detection camera that, together with computational advances from Yifan's lab and others, greatly improved resolution,[97,98] enabling us to break the side-chain resolution barrier for membrane proteins without crystallization. Indeed, this was another Eureka moment whose impact went beyond sensory physiology and pain, ushering in what has been referred to as a 'resolution revolution' in cryo-EM and a watershed moment in structural biology – particularly for membrane proteins, whose structures could now be more readily determined using this technique.[99]

One of the remarkable and powerful advantages of cryo-EM (versus crystallography) is that single molecules or complexes can be visualized in different states, which affords one the possibility of seeing a protein in distinct conformations associated with its functional life cycle. In the case of TRPV1, we could exploit its rich pharmacology (capsaicin, resiniferatoxin, and DkTx) to bias the equilibrium between states, enabling us to trap and visualize the channel in closed, open, and intermediate conformations.[100] These initial structures (with resolutions in the 3–4Å range) confirmed proposed locations of vanilloid and toxin binding sites based on prior mutagenesis studies, thereby reciprocally validating these proof-of-principle cryo-EM structures (Figure 11). Subsequently, and with further technical advances in the field, we have visualized TRPV1 at higher resolutions and in more native environments (such as lipid nanodiscs), enabling us to better resolve structures and locations of small molecule ligands (agonists, antagonists, and modulators), permeating ions, and movements of protein domains and side chains.[101]

Among many revelations gleaned from these structures, a couple of physiologically relevant highpoints are that: (i) TRPV1 resembles KV channels in its overall subunit and quaternary architecture, including the presence of a hydrophobic restriction, or gate, at the cytoplasmic end of the ion permeation pathway. However, and as alluded to above, TRPV1 is unique in also exhibiting a dynamic restriction at the extracellular aspect of the pathway, in the vicinity of the outer pore and selectivity filter near where DkTx and extracellular protons mediate their effects; and (ii) capsaicin and other vanilloid compounds bind to a

pocket within the transmembrane core of the channel, near the lower cytoplasmic gate. In the unliganded (apo) channel, this pocket is occupied by a phosphatidylinositol lipid, suggesting that stimuli (chemical or thermal) promote channel activation by ejecting a resident phosphatidylinositol lipid(s) from this site. Together, these and other observations paint a picture in which upper and lower restrictions along the ion permeation pathway serve as key allosteric regulatory sites for exogenous and endogenous modulators (spider toxins and extracellular protons or vanilloids and bioactive lipids, respectively). In other words, these two functionally coupled sites likely account for rich physiological modulation of TRPV1 (and perhaps other TRP channels) and its ability to function as a polymodal signal integrator.

In addition to providing basic insights into structural mechanisms governing TRP channel gating and ion permeation, these studies are revealing important details about allosteric regulatory sites that could potentially be exploited to develop analgesic drugs having more nuanced modes of action (versus wholesale channel inhibition or blockade). For example, and ss noted above, proton-evoked potentiation of TRPV1 represents an important facet of inflammatory pain and thus understanding biophysical and structural underpinnings of this process could suggest strategies for diminishing hyperalgesia while sparing acute (protective) nociception. Our initial cryo-EM studies provided a mechanistic outline for how protons facilitate channel activation. In more recent studies with Yifan and postdoctoral fellow Kaihua Zhang, we captured a series of cryo-EM 'snapshots' of TRPV1 at neutral and acid pH conditions, enabling us to map stepwise conformational transitions that show how protonation of key glutamate resides alters the structure of the upper restriction and

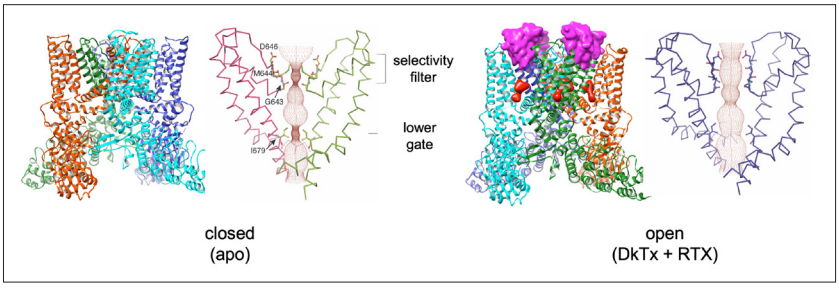

Figure 11. TRPV1 structures reveal ligand binding sites and two constrictions along the ion permeation pathway. (Left) Cryo-EM structure of rat TRPV1 channel in its unliganded (apo) closed conformation and corresponding map of the solvent-accessible pathway along the pore. Note two major constrictions at the level of the selectivity filter and lower cytoplasmic gate, corresponding to sites of regulation by spider toxins and extracellular protons or vanilloid agonists and bioactive lipids, respectively. (Right) Structure of the open channel in its liganded (DkTx, purple; resiniferatoxin, red) open state. Note substantial dilation of both upper and lower restrictions. (Adapted from references 96 and 100).

facilitates opening of the lower gate.[102] We also visualized stepwise competition between regulatory lipids and vanilloid agonists, providing further insights into the stoichiometry and geometry of this other important regulatory site. Studies such as these may suggest strategies for selectively abrogating the actions of pro-algesic agents on TRPV1 with the goal of diminishing inflammatory hyperalgesia without altering baseline thermosensation.

In the years since our initial structural studies of TRPV1, there has been tremendous progress in using cryo-EM to elucidate structures of many members of the extensive and diverse family of vertebrate TRP channels. Included among these are our other favorite nociceptive channels, TRPM8 and TRPA1. In each case, structures have been obtained in multiple states with and without agonists or antagonists, providing important new insights into mechanisms of ligand binding and channel gating. For example, and as noted above, recent TRPA1 structures elucidated by us, and others, have beautifully validated earlier structure-function studies implicating specific cytoplasmic cysteine residues as sites of electrophile modification. Furthermore, by visualizing conformational transitions associated with electrophile modification, these structures help explain how TRPA1 can function as a sensitive, yet broadly tuned receptor for a wide range of reactive environmental and endogenous chemical irritants. More generally, structures for TRPM8, TRPA1, and other subtypes have identified common elements, such as calcium binding sites that are required for channel activation, desensitization, or other forms of modulation. Moreover, several TRP channel subtypes exhibit conformational flexibility within the upper region of the ion permeation pathway, likely accounting for dynamic cation selectivity resembling that seen with TRPV1.[103]

The explosion of information gleaned from cryo-EM structures of TRP channels is by now too broad and deep to review here. But clearly that these studies have already provided unprecedented insights into the workings of these diverse and multifaceted signaling machines, including the architecture of ligand binding pockets that may serve as targets for new classes of analgesics and other relevant drugs. Still, we have only just begun to understand how conformational changes and allosteric coupling are connected to channel gating, especially for the many TRP channel subtypes that still lack specific and efficacious pharmacological tools. Another aspect of TRP channel biology that remains largely unexplored concerns their interactions with other cellular proteins. In the fly eye, genetic and biochemical studies have shown that TRP channels are part of a multiprotein complex containing relevant signal transduction proteins.[104,105] Vertebrate TRP channels have likewise been shown or proposed to interact with calmodulin, scaffolding proteins, or

kinases,103,106 but relatively little is known about whether these interactions are direct or how they are structurally manifest. Many vertebrate TRP channel subtypes possess large, structured cytoplasmic domains and thus it stands to reason that numerous protein partners and co-factors have yet to be discovered and their functional and structural interactions revealed.

CONCLUDING REMARKS

As noted above, the identification of receptors for modality-specific stimuli represents a critical step in deciphering the molecular, cellular, and circuit logic of any sensory system. In our case, the cloning of receptors for sensorial natural products has provided powerful molecular tools with which to identify and genetically manipulate primary afferent sensory neuron subtypes (at least in the mouse) to better understand their contributions to acute touch and pain sensation, as well as to maladaptive states such as inflammatory hyperalgesia. While our main and immediate motivations have been rooted in curiosity-driven basic research, we are also inspired by the hope that our findings will contribute to the development of novel analgesic therapies. Indeed, chronic or persistent pain will affect most of us at some time in our lives, at which point we will learn that existing treatments are often insufficient in providing substantial or durable relief and are limited by well-known side effects. Gaining deeper insight into the molecular and cellular mechanisms underlying specific chronic pain syndromes is key to breaking this translational barrier. Drugs targeting TRPV1, TRPA1, or other recently discovered molecules remain in various stages of testing and development, and we shall see if any emerge as safe and effective new analgesics. If so, then natural products will have once again served as inspiration for novel medicines.

Of course, I must end by thanking the many wonderful people who have made these discoveries possible. First and foremost are all the members of my lab, past and present, who have contributed to the body of work that I've described, as well as other important aspects that I haven't had the time or space to adequately include in this lecture. I thank them, as well, for their creativity, passion for discovery, collegiality, friendship, and devotion to research and mentorship. So many of my trainees remain active and successful in biomedical research and teaching and I am extremely proud of their accomplishments and potential. I must also thank several generous and devoted private foundations that have funded projects in our lab and provided fellowship support to so many students and postdoctoral scholars in our group. And, of course, the biggest shout-out goes to the National Institutes of Health, a primary and sustained supporter of work in my lab. Public support of basic research is a sign of a

healthy and vibrant culture and a society's commitment to shared values, progress, and the betterment of all. And last-but-not-least, there is the essential and warm support from my institution, mentors, and family, all of whom I have highlighted in my autobiography, to which I refer the reader for a more detailed and personal appreciation.

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