

STRUCTURAL, BIOCHEMICAL AND COMPUTATIONAL STUDIES OF
TRP CHANNEL TRANSMEMBRANE DOMAIN MODULARITY



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A thesis submitted in partial fulfilment of the
requirements for the degree of Doctor of Philosophy at
the University of Oxford

Christ Church
Department of Biochemistry

November 2013

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Doctor of Philosophy at the University of Oxford, Trinity 2013

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Abstract

Transient receptor potential (TRP) channels are expressed throughout the central nervous system and have a unique ability to detect a wide range of stimuli including changes in voltage, temperature, pH, lipid environment, small molecule agonists, and mechanical stress. While it is known that TRP channels contain the same six transmembrane helix (S1-S6), tetrameric architecture as voltage-gated channels, the degree to which functional and structural analogies are relevant remains poorly understood. This thesis describes a multidisciplinary approach toward understanding the structure and function of TRP channel transmembrane domains by focusing on the S1-S4 transmembrane helices of the TRPV1. This focus is inspired by the voltage-sensor domain (VSD) of the S1-S4 helices of voltage-gated channels, for which a range of studies show functional and structural independence. While some TRP channels are voltage-sensitive, their S4 helix does not contain the positive string of amino acids of canonical VSDs. However, the S1-S4 helices are functionally significant as the binding site of small molecule ligands in both TRPV1 and TRPM8 (for capsaicin and menthol, respectively).

The question of TRP channel transmembrane domain modularity is addressed in this thesis by expression and purification trials as well as radioligand-binding assays. It is demonstrated that the S1-S4 and S1-S6 helices of TRPV1 can be properly inserted, overexpressed, and show signs of stability upon detergent-extraction from *Saccharomyces cerevisiae* membranes. However the TRPV1 S1-S4 and S1-S6 helices do not show wildtype (WT)-like binding in [³H]-RTX binding assays. These results indicate that the TRPV1 transmembrane domains are likely structural but not functional domains. The *S. cerevisiae* expression system remained promising for the overexpression of TRP transmembrane domains as well as the production of functional, though not stable upon detergent-extraction, WT TRPV1. This WT TRPV1 was subsequently found to functionally bind both RTX, used in ligand binding assays, as well as the double-knot toxin (DkTx), targeted to the pore domain (the S5-S6 helices). An effect of DkTx on RTX binding affinity demonstrates an allosteric interaction indicative of a possible tighter packing between the two transmembrane domains than is seen in voltage-gated channels containing the canonical VSD.

Computational approaches additionally allowed for the investigation of the intramembrane capsaicin binding site in the TRPV1 S1-S4 helices, crucial to the initial motivations of this study. While the literature locates the capsaicin binding site to the TRPV1 S1-S4 helices, a 'binding pocket' has yet to be defined, with regards to the orientation of bound capsaicin and its access route to the site via the bilayer. Using molecular dynamics (MD) simulations the preferred location of capsaicin within the bilayer is defined, as well as the elucidation of capsaicin flip-flop between bilayer leaflets as a key event prior to TRPV1 binding. A transient binding was also observed between a homology model of the TRPV1 S1-S4 helices and capsaicin, possibly encouraging the idea that the S1-S4 helices still contain a partial binding site, though of too low affinity to be observed in the binding experiments performed here.

Acknowledgements

First and foremost I must thank my supervisors: Prof. Mark. S. P. Sansom, Dr. Simon Newstead, and Dr. Kenton J. Swartz. There have been surprisingly few difficulties in conducting this project across two continents in four labs, with three *other* collaborators, in what feels like two separate fields, and for this I owe my gratitude and thanks in tolerating this arrangement and sticking by this project even when nothing was going right.

After that I would like to thank my supervisors from undergrad: Prof. Lin Chen and Dr. Cosma Dellisanti at the University of Southern California and Prof. Santiago Schnell at Indiana University. These people have had nothing to do with the work in this thesis, but I have no doubt that without their support I would not have attempted it.

More to the point, I have enormous amounts of gratitude for the swaths of people, straddling the Atlantic, who have helped me achieve the work in this thesis. The NIH-Oxford-Cambridge Scholars program, including specifically Dr. Michael Lenardo and Dr. Matt Vogt, has been inspirational from the beginning and helpful til the end. My NIH supervisor Kenton J. Swartz at the NINDS has provided funding and mentorship all four years despite my physical location in Oxford so much of that time. The Swartz lab, and particularly Dr. Jeet Kalia, Dr. Toshi Kawate, Dr. Mufeng Li, Dr. Shai Silberberg, Dr. Dmitriy Krepkiy, Dr. Andres Jose-Oseguera, Dr. Jaime Smith, Dr. Frank Bosmans, Tsg-Hui (Helena) Chang, Dr. Gil Toombes, Kelsey Ladit, Dr. Mirela Milesescu, and Dr. Feng Zhang for their scientific support and laughter over the years. I'd also like to thank Dr. Mark L. Mayer for his support and unexpected thoughtfulness. Dr. Peter M. Blumberg, though he was never officially a fourth supervisor, is someone to whom I probably owe the most gratitude of all, as he and his lab at NCI taught, helped, and collaborated with me on the radio-ligand binding assays presented in Chapter 3 and 4. Without their help, kindness, and rigor this thesis would be much weaker. Specifically Larry Pearce, Dr. Noemi Kedei, and Nancy Lewin of this lab were so welcoming when I dropped in from England proposing to do ligand binding assays for a month (and then kept coming back).

Then there is everyone at Oxford, in the Newstead lab, SBCB and beyond, who have been nothing short of life-changing friends and colleagues during these last few years. Jane Kwok, Nicolae Solcan, Johnny Beale, Dr. Sabrina Schulze, Daphne Amevenu, Marko Sus-tarsic, Dr. Joanne Parker, and Simon himself of the Newstead lab have provided the sound

foundation and a small, stable family to count on. Simon's unwavering support of myself and a project that has little to do with the peptide transporters the rest of his lab focuses on, has no doubt kept this project moving forward. And of course the men and women of SBCB: Dr. Craig Lumb, Dr. Sarah Rouse, Dr. Ben Hall, Dr. Joseph Goose, Dr. Jussi Aittoniemi, Dr. Phill Stansfeld, Dr. Phil Fowler, Dr. Matthieu Chavent, Dr. Mickael Lelimosin, Dr. Heidi Koldsø, Jean H  lie, Jerome Ma, Firdaus Samsudin, Dr. Prafulla Aryal, Matthias Schmidt, Pragya Chohan, Amanda Buyan, Caroline Dahl, David Shorthouse, Dr. Shabana Vohra, Dr. Tyler Reddy, Jemma Trick, Dr. Danny Parton, Dr. Chen Song, Dr. Maria Musgaard, etc., and especially Mark himself are more than thanked for the countless discussions, scripts, conference travel help, movie competitions, etc! And of course Team Stat Mech (LLAS for short): Greg Ross and Lukas Stelzl, revived a raw appreciation for science when it was most needed. May the dream live on! Crucially are also the others at Oxford who have been involved in this project, such as those at the MPL at Harwell, and collaborators Dr. Stuart Conway and Dr. Esther Becker.

There are also about a million other people who have made these last few years fun and educational like Kate Wright, Jacob Brady, Narin Hengrung, Sarah Dixon, Michael Tee and the other squirrels whose class I abducted, Hilary Johnson, Chris Lander and 12 Abbey, the 181 Woodstock crew, all the NIHers (Austin Swafford, Prantik Kundu, the 'Sticky Fingers' ultimate folks, Abhi Kohl, Adjoa Smalls-Mantey, Eugene Oteng, Alex Ritter*, Aaron Alexander-Block, Jenny Dworzak, Jeff Cloutier*, Stefano Lantorno*, Bennet Waxse*), and 9206 Cypress (including Katy Graef, Lydia Durant, Ambika Bumb, and Joel Meyerson). Many thanks especially to Molly Perkins*, Nollanne Chang*, and Cynthia Chang* who all at one point or another, in addition to providing unending emotional support, lent me sheets or a floor or a bed (indicated by *) so I could actually have a place to sleep while doing some of these experiments. Also a shoutout to all the Christ Church, OBR, TMB/USC alums who helped me remember every once in a while that there is more to life than a PhD.

Finally, I'd like to thank my parents (including Pat) and brother, the rest of my family (Aunt Ardie and Uncle Royce, Betsy and Katie, Aunt Phyllis and Uncle Lance, Laramee, Uncle Don, Craig and Janice, Grandma Betty and Grandpa Al, etc.), and Nina Byers for all of their love and support.

November 10, 2013

S.M.H.

If nature does not answer first what we want,
it is better to take what answer we get.

– Josiah Willard Gibbs, Lecture XXX, Monday, February 5, 1900

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List of Abbreviations

Å	Angstrom(s)
aa	amino acid
Amp	ampicillin
ARD	ankyrin repeat domains
ATP	adenosine triphosphate
BSA	bovine serum albumin
B _{max}	maximum number of ligand binding receptors
°C	degrees Celsius
C12E8	octaethylene glycol monododecyl ether
CaM	calmodulin
CG	coarse-grained
CHO	Chinese Hamster Ovary
CMC	critical micelle concentration
CNG	cyclic nucleotide-gated
D7PC	1,2-Diheptanoyl-sn-glycero-3-phosphocholine
Da	Dalton
DAG	diacylglycerol
DkTx	double-knot toxin
DM	n-decyl- β -D-maltoside
DDM	n-dodecyl- β -D-maltoside
DNA	desoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DRG	dorsal root ganglion
EDTA	ethylenediaminetetraacetic acid
EGFR	epithelial growth factor receptor
EM	electron microscopy

FC12	n-Dodecylphosphocholine
FC16	n-Hexadecylphosphocholine
FSEC	fluorescence-coupled size exclusion chromatography
g	acceleration of gravity
GFP	green fluorescent protein
GPCR	G protein-coupled receptor
gromacs	Groningen machine for chemical simulations
ICK	inhibitory cysteine knot
K	degrees Kelvin
Kir	inward rectifying K^+
K_d	dissociation constant
K_v	voltage-gated potassium
L	litre
LDAO	N,N-Dimethyldodecylamine N-oxide
M	moles per liter
MBP	maltose-binding protein
MD	molecular dynamics
MW	molecular weight
MWCO	molecular weight cut-off
Nav	voltage-gated sodium
NMR	nuclear magnetic resonance
OG	n-octyl- β -D-glucoside
OPLS-AA	Optimised Potential for Liquid Simulations-All Atom
PAGE	polyacrylamide gel electrophoresis
PBS	probable batch system or phosphate-buffered saline
PCR	polymerase chain reaction
PDB	Protein Data Bank
pI	isoelectric point
PIP ₂	phosphatidylinositol 4,5-bisphosphate
PEG	polyethylene glycol
PKC	protein kinase C
PLC	phospholipase C
PME	particle mesh Ewald
PMF	potential of mean force
POPC	1-palmitoyl-1,2-oleoyl-sn-glycero-3-phosphocholine

POPE	1,2-dioleoyl-sn-glycero-3-phosphoethanolamine
POPS	1,2-dioleoyl-sn-glycero-3-phospho-L-serine
POPG	1,2-distearoyl-sn-glycero-3-phospho-(1'-rac-glycerol)
RFU	relative fluorescence units
RMSD	root-mean-square deviation
RNA	ribonucleic acid
RTX	resiniferatoxin
SDS	sodiumdodecylsulfate
SEC	size exclusion chromatography
SPC	simple point charge
Taq	<i>Thermus aquaticus</i>
TEV	tobacco etch virus (protease)
TM	transmembrane (helix)
TRP	transient receptor potential
TTX	tetrodotoxin
Tris	2-amino-2-hydroxymethyl-propane-1,3-diol
VGL	voltage-gated-like
VSD	voltage-sensor domain
WHAM	Weighted Histogram Analysis Method
w/v	weight per volume
WT	wildtype

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Introduction

If we were to name the most powerful assumption of all, which leads one on and on in an attempt to understand life, it is that *all things are made of atoms*, and that everything that living things do can be understood in terms of the jiggings and wiggings of atoms.

—Richard P. Feynman, *The Feynman Lectures on Physics*, 1964

MORE than three billion years ago an amalgamation of molecules enveloped in a lipid film created the first rudimentary cell. Safely encompassed inside this ion-impermeable membrane the components could develop as an entity and follow the course of evolution to become the cells of life as we know it today. However mechanisms for remaining in contact with the environment outside the cell, either by allowing the passage of molecules through the membrane or by detecting the state of the environment outside the membrane, were necessary. While cells have developed more organism-wide mechanisms of responding to the outside environment since these early days, the basics remain the same: membrane-embedded proteins are necessary to actively and passively transport molecules through the membrane, as well as to detect the range of physical and chemical stimuli from the outside environment.

Though the scientific understanding of many of these processes has had some phenomenal successes, the understanding of many aspects of how cells detect the pressure, temperature, light, and small molecules in the outside environment remains an area of research with gaps to fill. This thesis focuses on members of a family of proteins called the transient receptor potential (TRP) channels which are important for conferring heat and cold sensitivity, among other things, in animals. In order to put the studies of this thesis into context, this chapter covers (1) the historical and contemporary studies of ion gradients and ion channels as a universal mechanism of cell communication, (2) the recent developments in membrane protein structural biology, (3) the background of the physiological and biophysical understanding of membrane protein interactions with lipids and lipophilic molecules, and finally, (4) the utility of breaking proteins into structural and functional domains. The chapter then concludes with an overview of the main aims of this thesis.

1.1 Ion channels

While TRP channels are one of the more recently discovered and less well-understood ion channels, the understanding of their possible mechanisms has been able to develop relatively rapidly due to the firm foundations of over a century of ion channel research.

1.1.1 Ion channels as essential eukaryotic proteins

Ion channels are proteins that facilitate passive diffusion of ions through the cell membrane, with different types of ion channels often selective for different types of ions. An asymmetric distribution of ions across the cell membrane creates an electrochemical gradient, which makes this diffusion energetically favorable (Figure 1.1). This definition of ion channels, however, has not always been known. Since the late 1800s it has been conjectured that ions were integral to cell excitability, demonstrated, for example, by the experiments

of Sidney Ringer in the 1880s in which to keep a frog heart beating a solution of specific concentrations of the ions K^+ , Na^+ , and Ca^{2+} was required [231, 179]. Then in the 1900s Julius Bernstein hypothesised that cell excitability could be a direct result of the selective permeability of membranes [25].

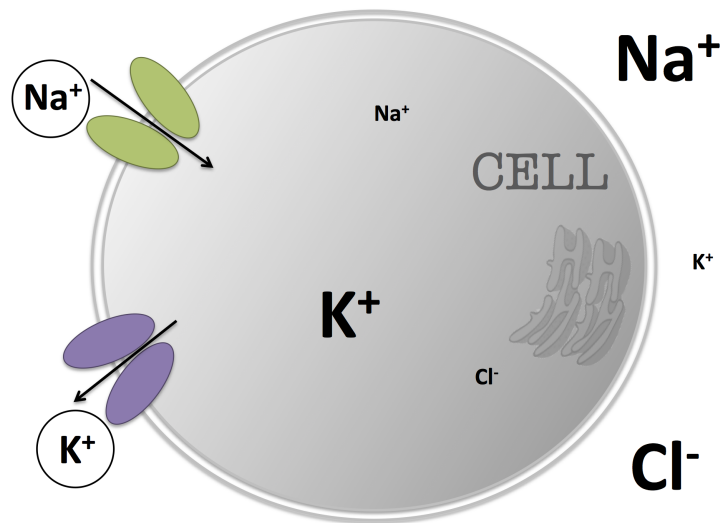


Figure 1.1: **Ion channels allow for the passive diffusion of ions through the cell membrane.** All cells have an electrochemical gradient that allows ions to passively diffuse through ion channels.

In the 1950s, during a period sometimes referred to as the heroic era of classical biophysics, a small group of biophysicists including Alan L. Hodgkin and Andrew F. Huxley performed a series of experiments mostly on the squid giant axon that resulted in the definition of sodium- and potassium- selective ion channel proteins by the 1970s. Initial experiments measured voltage changes across the membrane of the squid giant axon simply by recording the electrical current that flows through a wire from the inside of the axon to the outside sea water bath. This allowed for the first recordings of action potentials, which are the change in the potential across the membrane that travels down the length of the axon as the result of an initial electrical stimulus (Figure 1.2a) [42]. However, even with the ability to measure this action potential, exactly what it was remained unclear until new

techniques were developed. Soon, scientists were able both to control the voltage across the membrane, via voltage clamp, and to directly measure the ionic currents through the membrane [104]. Eventually the currents due to Na^+ and K^+ ions could be decoupled using ion substitution experiments that recorded clear differences in currents when Na^+ ions were removed from the bath (Figure 1.2b) [104].

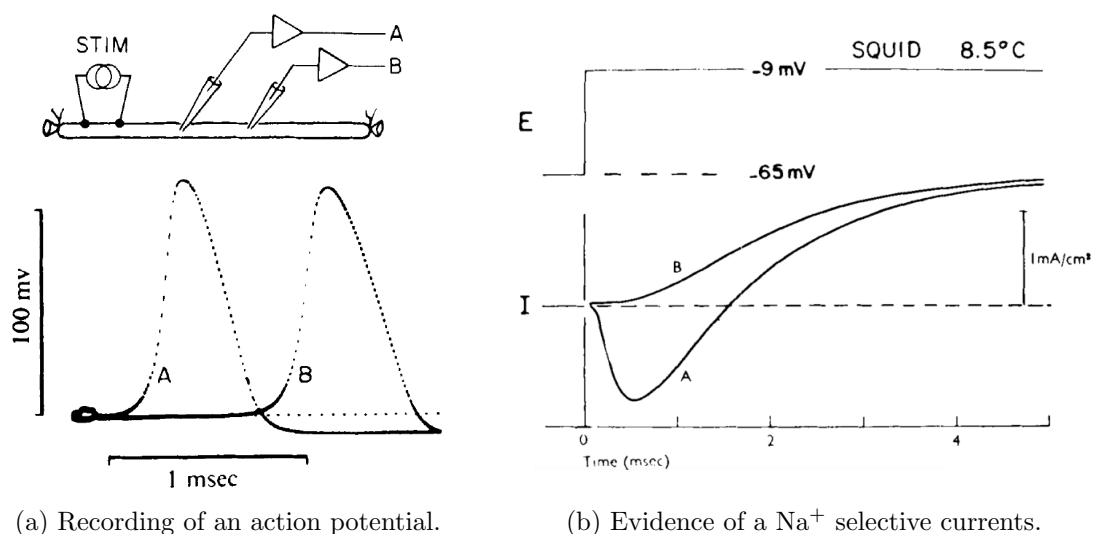


Figure 1.2: **Hodgkin and Huxley prove ion selectivity in squid axon in 1950s.** (a) Measuring the electrical pulse at two places (A and B) along a squid giant axon shows the propagation of the action potential. Figure from [42] modified in [103]. (b) When applying a voltage step (E) known to open the channels of a squid axon, this figure shows clear current differences in (A) normal seawater and (B) low-sodium seawater with 90% of the NaCl replaced. Figure from [104] modified in [103].

Even with these experimental advances, it was not clear what was responsible for the ion selectivity: possibly proteins, the lipid membrane, or even nucleotides. Then a series of toxins were discovered, including tetrodotoxin (TTX) from the poisonous Japanese puffer fish, that could block the Na^+ currents selectively, showing very similar traces to the ion substitution experiments [191]. Similarly, an organic molecule was discovered that conversely blocked the K^+ currents selectively [93, 11]. These molecules provided invaluable tools to ion channel research, and allowed for a remarkably accurate understanding of the structure of these Na^+ and K^+ ion channels as early as 1977 (Figure 1.3) [102].

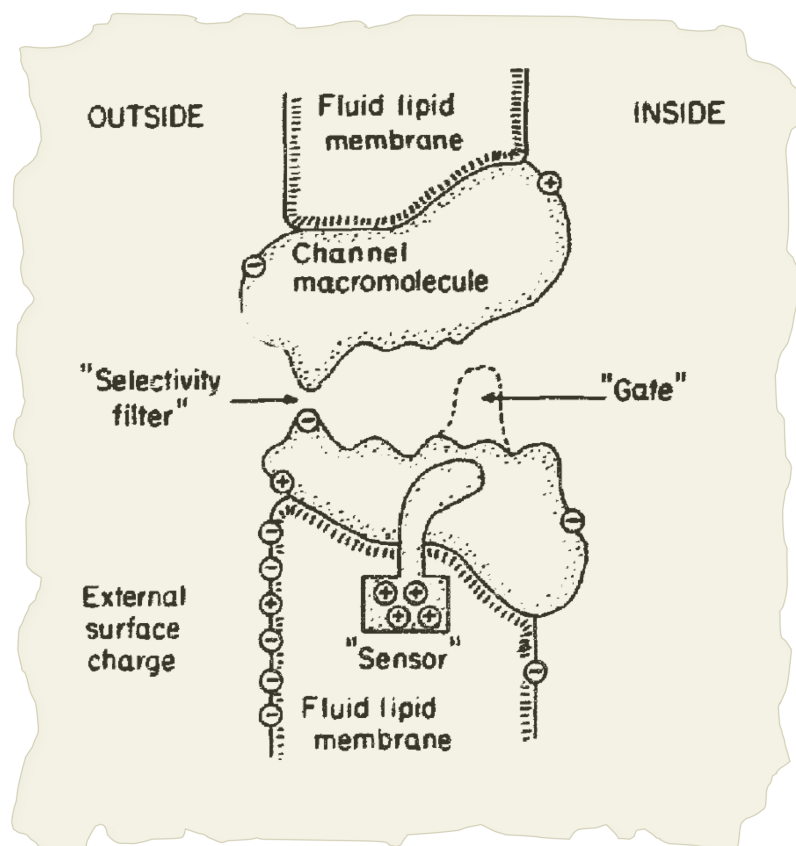


Figure 1.3: **Bertil Hille's schematic of ion channel understanding circa 1977.** This schematic drawing of the contemporary understanding of the main structural elements of voltage-gated Na^+ and K^+ channels drawn by Bertil Hille in the late 1970s [102] strongly resembles the crystal structures solved in the early 2000s. At that time, the understanding of the selectivity filter, the voltage sensor, the inner vestibule, and the intracellular gate were already well-developed.

Even with the improved characterisation of what were termed the voltage-gated channels (discussed further in section 1.1.2), first facilitated by the patch clamp technique and then by the development of the pharmaceutical tools of toxins, the first cloned channels began a new era of understanding the biochemical and biophysical mechanisms of channel proteins. Soon numerous channels were found to share similar topology to voltage-gated Na^+ and K^+ channels (Figure 1.4), now ion channels of this topology form the superfamily of the voltage-gated-like (VGL) channels and include the TRP channels studied most

specifically in this thesis. By the 1980s, it became apparent that the human genome contained several hundred genes for ion channels that we now know can mostly be categorised into the following families or superfamilies: the VGL channels, a group of tetrameric, 6 transmembrane (TM) helix channels architecturally similar to voltage-gated channels (Figure 1.4) defined by William Catterall in a 2004 review [307]; glutamate receptors, a group of tetrameric channels significant in neuronal signalling [274]; cys-loop receptors, a group of pentameric channels that includes both cation and anion channels [148]; Orai Ca^{2+} channels (not shown) [108]; voltage-sensitive Cl^- channels (ClC) (not shown) [67]; and the P2X receptor/ASIC/ENAC channels [13] (Figure 1.5).

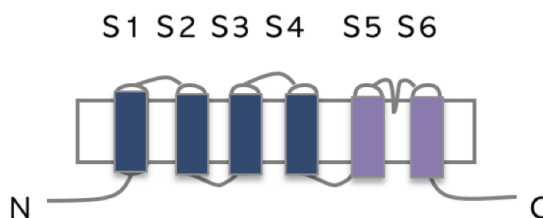


Figure 1.4: **VGL channels have 6 TM helices.** With the era of cloning and advancing biochemical techniques, came a more structural understanding of voltage-gated channels and their homologues. The six transmembrane α -helices were dubbed S1-S6, with Bertil Hille's 'channel macromolecule' being formed of the tetramerisation of the S5 and S6 helices.

In addition to being integral to the propagation of neuronal action potentials (Figure 1.2a) ion channels have been associated to a wide range of diseases and physiological processes: the muscarinic nicotinic acetylcholine receptors of the cys-loop receptor family are involved in muscle contraction [8], the inward rectifying potassium channels have been found in to be mutated in types of diabetes [87], a rare disease that results in the inability to perceive pain is produced by a mutant in a voltage-gated sodium channel [57], and taste and smell are mediated by a range of ion channels [150], to name a few. While our knowledge of ion channels and their importance in human physiology is making rapid progress, including the relatively recent availability of the molecular structures solved from x-ray

diffraction patterns of almost all relevant channel families (Figure 1.5), there remain wide gaps in the knowledge of both molecular mechanisms of ion channels and their downstream physiological effects.

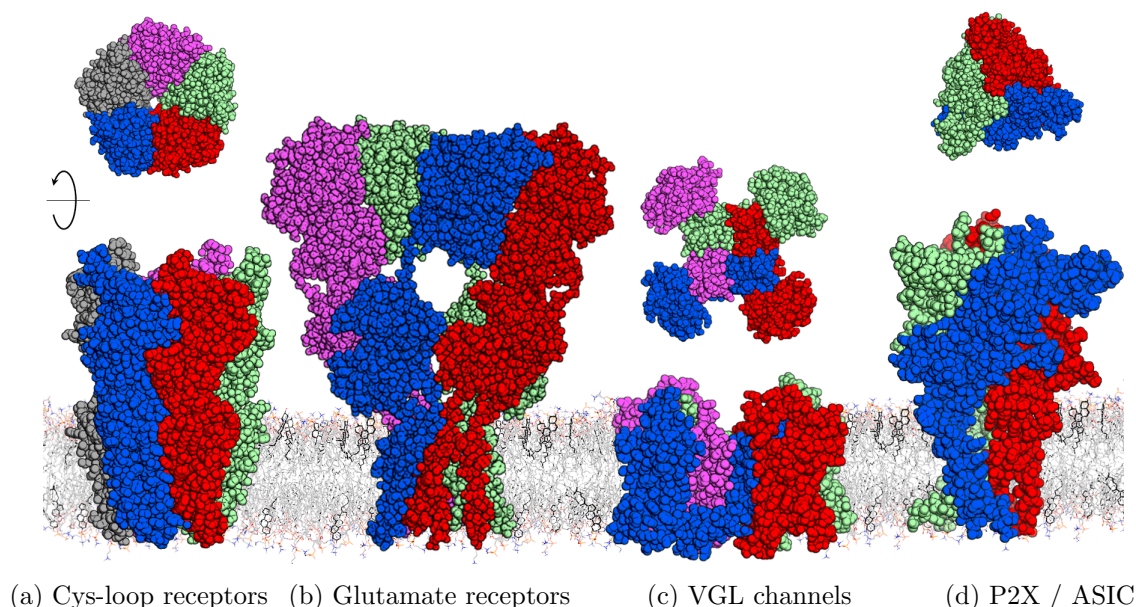


Figure 1.5: Representative structures of known ion channel types. (a) The crystal structure of the bacterial *Gloeobacter* ligand-gated ion channel (GLIC) [209] (PDB ID: 4F8H) is representative of the pentameric Cys-loop receptor family of ion channels of which the acetylcholine receptor cation channels and glycine receptor anion channels are a member. (b) The crystal structure of the rat α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)-sensitive glutamate receptor (GluA2) [253] (PDB ID: 3KG2) is representative of the tetrameric cation-selective glutamate receptor family. (c) The crystal structure of a rat voltage-gated K^+ channel (Kv1.2) [156] (PDB ID: 2R9R) is representative of the cation-selective tetrameric voltage-gated-like channels (d) The crystal structure of the zebrafish P2X receptor [132] (PDB ID: 3H9V) is representative of the cation-selective P2X receptors, ENAC, and ASIC channels which have been seen to have surprisingly similar trimeric architectures. Figure was made using PyMol [244].

1.1.2 A brief overview of voltage-gated ion channel biophysics

While ion channels take advantage of the electrochemical gradient for the passive diffusion of ions, this asymmetric distribution of ions across the cell membrane has another effect

on ion channels, alluded to briefly in the previous section: the electric potential across the membrane actually holds voltage-gated Na^+ and K^+ channels closed at the cell's resting potential (around -70 mV) (Figure 1.6). While all cells have and use a similar membrane potential a special type of cell in more complex organisms called excitable cells, which include neurons, secretory cells, and muscle fibers, take advantage of this membrane potential to produce and respond to electrical current. In the case of neurons, the electric current is the propagation of the action potential shown in Figure 1.2a that Hodgkin and Huxley showed to be a cascade of channel openings down the giant squid axon. As positive ions flow into the cell through Na^+ channels, the local negative potential across the membrane increases, depolarising the cell and releasing the voltage sensors of adjacent channels [104]. Subsequently, voltage-gated K^+ channels follow this opening of Na^+ channels to return the membrane potential back to the $\sim -70 \text{ mV}$ resting potential.

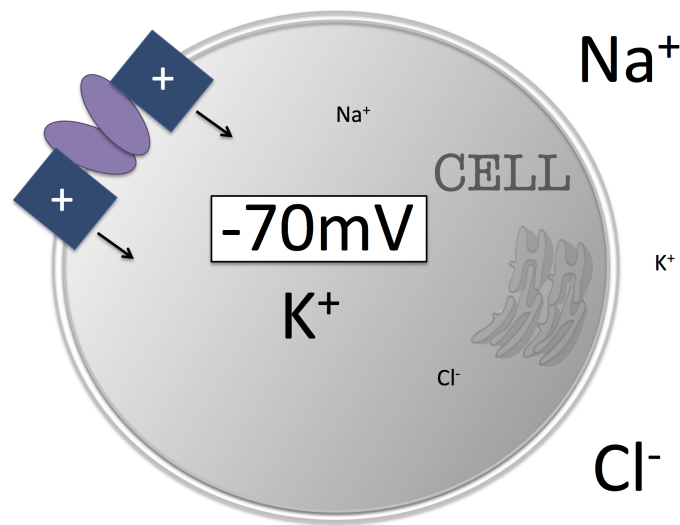


Figure 1.6: **Voltage-gated channels are kept closed at the cell's resting potential.** All cells have a membrane potential that allow ions to passively diffuse through ion channels.

This voltage sensitivity was a clear characteristic of the currents Hodgkin and Huxley measured and a series of charged residues within the membrane was imagined to be the

gating charge responsible for this voltage sensitivity (labeled ‘Sensor’ in Figure 1.3). The gating charge, z , was initially used by Hodgkin and Huxley to mathematically model how a voltage-sensitive channel would work [105] (Equation 1.3). This equation simply uses the Boltzmann equation (Equation 1.1), which gives the relative probabilities at equilibrium of finding a particle in state 1 or state 2 if the energy difference between those states is $u_2 - u_1$, with k_B as Boltzmann’s constant and T as temperature. To use this equation to describe a simplistic model of channel opening (Equation 1.2), where channel open probability is dependent on a gating charge z that moves within a membrane potential, all one needs is to know is ω , the energy of opening without a membrane potential, and q , the elementary charge. This model allowed for an estimate of the gating charge, simply by measuring the change in open probability as a function of membrane voltage (V).

$$\frac{p_2}{p_1} = \exp\left(-\frac{u_2 - u_1}{k_B T}\right) \quad (1.1)$$



$$\frac{O}{C} = \exp\left(-\frac{\omega - zqV}{k_B T}\right) \quad (1.3)$$

While this model was described by Hodgkin and Huxley in the 1950s, it wasn’t until the 1970s that the movement of the gating charges was measured, and the molecular definition of these gating charges did not start to become clear until the first voltage-sensitive channels were cloned in the 1980s [45, 199]. With the primary sequence of the first sodium channels available, a conserved string of positively charged amino acids along the length of the putative fourth transmembrane helix became a striking candidate for the molecular basis of voltage sensitivity [45, 199] (Figure 1.7a). Mutagenesis experiments [247] and the cloning

of a wide range of voltage-sensitive channels and proteins [228, 112, 265] confirmed and elaborated on this hypothesis again and again, until in the early 2000s came the first crystal structures of this ‘voltage-sensor domain’ (Figure 1.7b). Today, debates remain about the exact mechanisms of this voltage sensor-mediated gating [78, 115, 270], but the basic model described above remains the standard background for these variations.

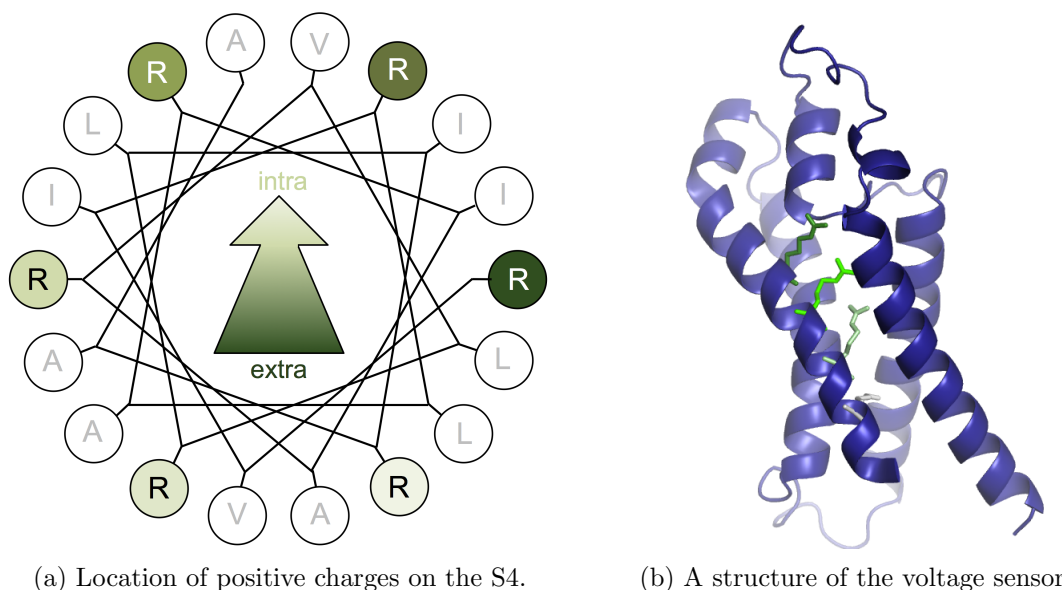


Figure 1.7: The voltage sensor of voltage-gated channels. A voltage sensor of a string of charges was suspected before any voltage-sensitive channels were cloned. (a) On cloning the first voltage-sensitive channel, an Na^+ channel from eel [199], the organisation of positive charges on one side if one of the transmembrane α -helices is striking (here it is the S4 from the subunit IV of the Na^+ channel). (b) Decades later, the transmembrane positioning of these positively charged amino acids have been revealed in a few crystal structures. Here is the voltage sensor of the rat Kv 1.2 / 2.1 chimera (PDB ID: 2R9R) with the arginines represented as green sticks [156]. Image created using PyMol.

1.1.3 Transient Receptor Potential (TRP) channels as stimulus detectors and integrators

The members of the transient receptor potential (TRP) channel family are a diverse group of ion channels that have been identified gradually. Even today, with all mammalian

subtypes identified, TRP channels are grouped into subfamilies due to sequence homology rather than functional characteristics. The first member of the TRP channel family was identified in 1969 with the identification of a gene in *Drosophila melanogaster* that when knocked out, removed responsiveness to light beyond an initial pulse [55]. This channel was cloned in the 1980s and determined to be a Ca^{2+} channel similar in topology to voltage-gated channels [186, 98, 113]. There are now three known *Drosophila* TRP genes: TRP, TRPL, and TRP γ . It was first suspected that there were vertebrate TRP homologues when sequences deduced via PCR cloning revealed sequences similar to those of *Drosophila* TRP channels in frog and mouse [220], and in the mid 1990s the first mammalian TRP channels were cloned [310, 311]. While initially called the Htrp1-3, they were soon associated with the TRPC, for 'canonical' TRP, subfamily of which there are now seven known members. With this broad range of TRP channels, there are still no prokaryotic TRP homologues known, though the single-celled eukaryotic yeast has a single TRP channel, also homologous to the TRPC subfamily [208].

Between the years of 1996 and 2003, a range of channels with homology to the *Drosophila* TRP with increasingly interesting properties were discovered: in 1997 the VR1 (now the TRPV1), a capsaicin and heat sensitive channel [44]; in 1998 the melastatin gene (now the TRPM1), a TRP-like gene down-regulated in melanoma [66]; in 1999 VRL1 (now the TRPV2), another heat sensitive channel [43]; in 2002 the CMR1 (now the TRPM8), a menthol and cold sensitive channel similar in sequence to the melastatin gene [174, 217]; and in 2003 the ANKTM1 (now the TRPA1), another cold sensitive channel [259]. In 2003, it was determined that instead of referring to the separately named VR1 or hTRP1 or ANKTM1 or CMR1 as TRP channel homologues, all TRP channels should be referred to under a unified naming convention, however, it was noted that [51]:

At present, no one feature other than overall 6TM architecture and homology define the TRP family. Thus, we expect that the definition of TRP channels will

evolve as functions and structures are clarified.

Now, a decade later with the full library of the 28 mammalian TRP channels grouped into 7 subfamilies (N, M, C, A, ML, P, and V), not much more has been clarified concerning a unified TRP channel identity.

One unifying characteristic, however, is the significant involvement of TRP channels in sensory mechanisms. TRP channels are mostly non-selective cation channels that when opened, depolarise the cell and initiate action potential propagation by voltage-gated Na^+ channels. Even the earliest studies of the TRPV1, TRPM8, and TRPA1 showed these channels are highly expressed in subsets of peripheral sensory neurons that innervate the skin [44, 174, 259].

While TRP channel topologies remain diverse, one element that ties them together and strengthens their relationship to voltage-gated channels is evidence for a selectivity filter between the S5 and S6 transmembrane α -helices in every subfamily [206]. For example, in TRPV5 an aspartate-to-alanine mutant in this region abolishes Ca^{2+} permeation [198], in TRPC5 neutralisation of three glutamates in this region resulted in a loss of La^{3+} potentiation [125], and in TRPM3 splice variants with variations within this region were found to have an effect on selectivity [202]. Worth noting, however, is that while the selectivity filter lies in a consistent location, the sequences and effects of mutants have a wide diversity. Other elements of the channel family are even less conserved across subfamilies, and in some cases, are not even conserved inside subfamilies. The most conserved region across all TRP channels is a short series of amino acids at the end of the S6 called the ‘TRP Box’ domain, which has been implicated in channel regulation by lipid second-messengers (Figure 1.8) [32]. Outside of these two domains, which define TRP channel definition are several other recurring domains, such as: the Ankyrin Repeat Domains (ARD) present in TRPCs, TRPVs and TRPA1; the calmodulin binding domain of TRPCs, TRPMs and TRPVs; and the coiled-coil domains of TRPCs, TRPMs, and TRPPs. While some of these

cytosolic domains have been crystallised, the heterogeneity of the N- and C-termini of both the lengths and predicted secondary structures of TRP channels leaves open the question of conserved architectures and tail-mediated mechanisms (Figure 1.9).

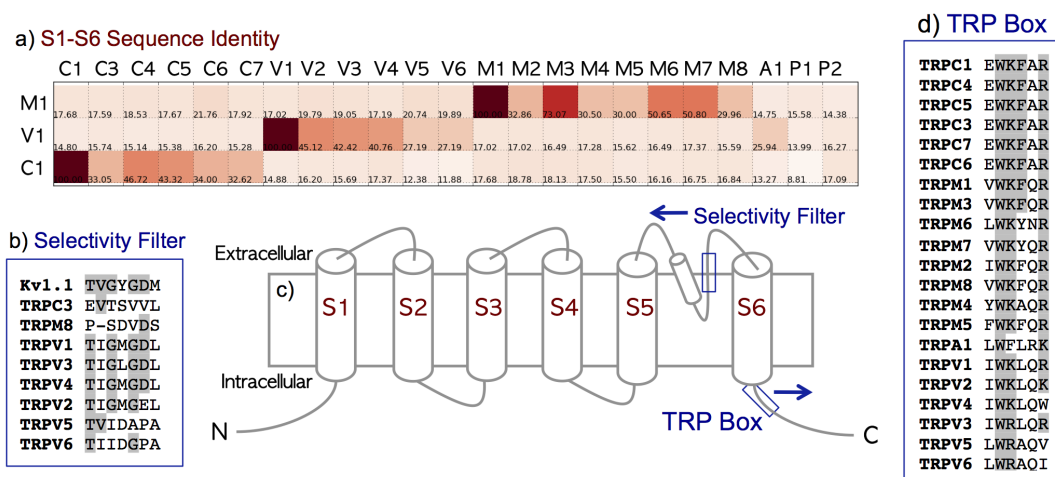


Figure 1.8: **An overview of human TRP channels: transmembrane domains.** Note that not included are TRPC2, which is a pseudogene in humans, and TRPN, which is not found in mammals. (a) Heat map of the sequence identity of the S1-S6 helices of 23 human TRP channels to TRPM1, TRPV1 and TRPC1, with 100% identity represented as this dark red. Alignments performed with Clustal Omega. (b) Sequence alignment of putative selectivity filters of selected TRP channels. (c) Topology diagram of TRP channels. (d) Sequence alignment of the TRP Box motif at the end of the S6 of all TRP channels. Figures and alignments were created for the purpose of this thesis from UniProt sequences.

1.1.4 Progress toward understanding the heat sensitivity of thermoTRP channels

The temperature sensitivity of certain TRP channels has become a common theme of the family and is a property that has been suggested for almost a third of known TRP channels, though not all TRP channels have been systematically tested (Table 1.1). Temperature gradients are ubiquitous throughout cellular environments and have wide ranging effects on cellular processes. Indeed all proteins are temperature sensitive through the general thermodynamic effects of temperature changes on, for example, rates of enzymatic reactions,

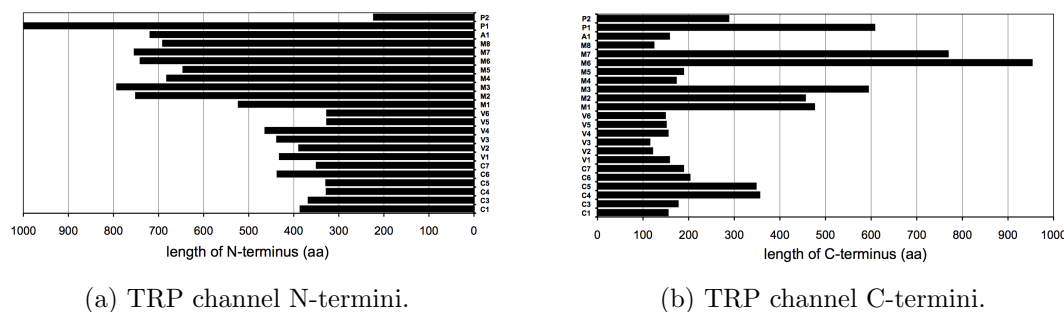


Figure 1.9: **An overview of human TRP channels: cytosolic domains.** Note that not included are TRPC2, which is a pseudogene in humans, and TRPN, which is not found in mammals. (a) A visualisation of the heterogeneity of the lengths of the N-Termini of TRP channels. Note that the TRPP1, has been cut-off. (b) A visualisation of the heterogeneity of the lengths of the C-Termini of TRP channels. Figures were created directly from UniProt sequences.

protein denaturing, membrane fluidity, and nucleotide super-coiling. One can imagine that the effect of temperature on the rates of enzymatic reactions could have a larger collective effect, allowing the cell to respond to changes in temperature, but in reality these effects are too small to result in the needed fast, direct response to temperature. Additionally, while temperature increases membrane fluidity, the effect is likely not sharp, as membranes are heterogeneous mixtures of different kinds of lipids with different phase transition temperatures. Though there could be a case for sharper transitions in such formations as lipid rafts, little evidence has been shown that this is related to a temperature-sensing mechanism in cells.

Considering the importance of sensing temperature gradients to all phyla of life, it is interesting that there are no direct prokaryotic TRP channel homologues, indicating that the family is a relatively new mechanism for temperature sensitivity. Perhaps this is related to the need for fast, organism-wide reactions to temperature in eukaryotes, requiring the development of a temperature-sensitive ion channel. This touches on one of the most intriguing aspects of the TRP channel family: the family seems to appear only in eukaryotes to allow for the ‘sensing’ of a wide variety stimuli, since only at this point in evolution does

Table 1.1: The ThermoTRPs

Name	Temperature Sensitivity	References
TRPV1	$> 42^{\circ} \text{ C}$	[44]
TRPV2	$> 52^{\circ} \text{ C}$	[43]
TRPV3	$> 33^{\circ} \text{ C}$	[252, 303]
TRPV4	$\sim 27\text{-}42^{\circ} \text{ C}$	[294]
TRPM8	$< 25^{\circ} \text{ C}$	[174, 217]
TRPA1	$< 18^{\circ} \text{ C}$	[259, 128]
TRPM3	$> 45^{\circ} \text{ C}$	[290]
TRPC5	$< 16^{\circ} \text{ C}$	[312]

the notion of ‘sensing’ begin to have meaning. To have a similar protein in prokaryotes would not be necessary, as the scale over which they need to integrate a range of signals from disparate locations into a single organism-wide behaviour is considerably smaller.

Indeed, proteins with distinct temperature-dependent behaviours *have* been found in bacteria [61], though it is not clear if there are any parallels between their mechanisms and those of TRP channels. For example, the cyanobacterium *Synechocystis* offsets the effect of cold temperatures on bilayer fluidity by expressing acyl-lipid desaturases that increase the cisunsaturation of the lipid membrane [264, 2, 1]. Also, it is known that the flagellar motor of *Escherichia coli* switches between a clockwise and counterclockwise state based on a temperature-controlled switch [276], and though the mechanism is not well-known it is expected to be related to a set of small membrane proteins [190]. Finally, heat shock proteins exist that help regulate the folding and unfolding of proteins in the presence of stressors. Heat shock proteins are found in all organisms and have been widely studied since their discovery in *Drosophila* in the early 1960s [232]. One mechanism that has been proposed for their heat sensing mechanism is the RNA thermometer [292], though it has also recently been shown that TRPV1 activation also induces the heat shock protein mechanism in animal cells [36].

While interesting and relevant to keep in mind, none of these mechanisms are thought

to be involved in TRP channel heat sensitivity. Attempts have been made to biologically and structurally address TRP temperature sensitivity. When ThermoTRPs were first discovered, it was unclear if the temperature sensitivity was direct or indirect, however it has now been shown that both TRPV1 and TRPM8 are intrinsically temperature-sensitive when reconstituted into pure lipid bilayers [40, 309]. While this narrows the search for a mechanism to just the ThermoTRP itself, a variety of experiments have been performed to try and isolate a possible ‘heat sensor domain’ in TRP channels with limited success. Experiments on TRPV1 have seen effects on temperature sensitivity upon mutating amino acids in both the pore ‘turret’ [305] and the C-Terminal region [33, 285], with no consensus on which are the most significant or even most reproducible results. Other evidence hints that the S6 is significant to the temperature-sensitivity of TRPV3 [89]. To complicate matters more, the only studies of the temperature-sensitivity of TRPA1 point toward the N-terminal ARD [54]. The strongest experiment that makes a case for the role of the C-terminus in temperature sensitivity is the characterisation of chimeras created by swapping the C-terminal domains of TRPV1 and TRPM8, in which a corresponding swap in temperature sensitivity was observed [33]. Overall, two things remain unclear: (1) whether there is in fact a modular temperature sensing domain in any TRP channel and (2) if there is a unified mechanism of temperature sensitivity throughout the family.

Though no mechanism has been agreed on for the heat sensitivity of TRP channels, the tools developed for mathematical modelling of electrophysiological recordings has provided insights into the thermodynamic parameters significant to channel opening. One parameter that has helped to quantitatively define the temperature sensitivity of TRP channels in comparison to, for example, voltage-gated Na^+ and K^+ channels is the Q_{10} value (Equation 1.4). This value simply gives the change in current with a ten degree change in temperature, but provides a useful quantitative basis for temperature sensitivity with TRPV1 and TRPM8 currents giving values of ~ 26 and ~ 24 respectively when other channels have Q_{10} values closer to ~ 3 [153, 31]. The Q_{10} value has however, come under some scrutiny with

regards to its reproducibility due to the sensitivity of its measurement to, among other factors, background current and the temperature-dependence of ion permeation [286].

$$Q_{10} = \left(\frac{I_2}{I_1} \right)^{10/(T_2 - T_1)} \quad (1.4)$$

Even with no exact mechanism postulated for the TRP heat-sensing mechanism, fitting electrophysiological recordings with Boltzmann's equation has predicted a high energetic difference between open and closed states. This can be done simply by taking equation 1.3 from the previous section and substitute in the definition of Gibbs free energy, ΔG (Equation 1.5), for the ω term, the energy of opening without a membrane potential. With the resulting equation (Equation 1.6) one can fit values for ΔH and ΔS , to understand their respective contributions to channel opening. In the case of TRPV1, a very large change in enthalpy (ΔH) was found upon channel opening (~ 150 kcal/mol), suggesting large conformational changes upon opening[153]. In the case of TRPM8, a relatively large free energy change was also seen (~ -60 kcal/mol), but was negative instead of positive, as expected [31]. In both cases, the change in entropy (ΔS) was also very large, compensating for the large enthalpy change, resulting in a free energy (ΔG) that was maintained at a low level. This avenue of investigation has allowed for at least the beginnings of a mechanistic understanding for temperature sensitivity, but clues to what to attribute this large enthalpy change are scarce. One suggestion has been that ΔH is, in fact, itself temperature dependent as a result of large changes in molar heat capacity, however this theory has yet to see any experimental evidence, and its mechanistic explanation for temperature-sensitivity remains that a large conformational change upon channel opening must be occurring [50].

$$\omega = \Delta G = \Delta H - T\Delta S \quad (1.5)$$

$$\frac{O}{C} = \exp \left(- \frac{\Delta H - T\Delta S - zqV}{k_B T} \right) \quad (1.6)$$

While TRP temperature sensitivity remains enigmatic, one hope is that perhaps the mild voltage-sensitivity seen in some TRP channels will provide some insight, as cooperativity has been observed between the temperature-sensitivity, voltage-sensitivity and ligand-sensitivity in certain TRP channels [197]. Though TRP channels lack the string of positive residues in the S4 that is so well-conserved in voltage-gated Na^+ and K^+ channels, some TRP channels have shown voltage-sensitive currents (Figure 1.10). For example, as early as 2001 it was seen that TRPM4 had a linear current-voltage relationship [218, 196]. In fact, according to reference [197], voltage-dependent currents have been seen in several TRP channels, including in the original studies of TRPV1 and TRPM8, subsequent studies of TRPV5, TRPV6, and TRPV3, and in TRPM3, TRPM4, and TRPM5. While TRP channel mechanisms may not end up being universal, it is clear that the wide variety of activating stimuli resulting in the ability of a single channel to serve as a molecular integrator of chemical and physical stimuli is common across all TRP subfamilies. TRPM8 and TRPV1, for example, are both activated by ligands (menthol and capsaicin, respectively), temperature and voltage, thus addressing the question of how these activating stimuli interact with each other's kinetics is appealing, and indeed in reference [197] they do just this, and show that temperature and ligands shift the voltage sensitivity of these channels toward more physiological voltage ranges, suggesting a shared gating mechanism within a single TRP, if not within thermoTRPs (Figure 1.11).

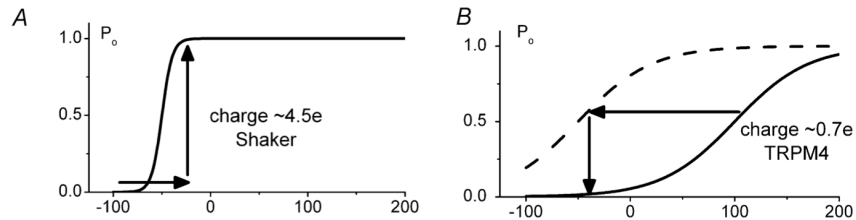


Figure 1.10: **The first voltage-sensitive TRP.** (a) Voltage sensitivity as seen in a voltage-gated K^+ channel with a large z value. (b) Voltage sensitivity as seen in TRPM4 with a small z value. Image adapted from reference [197].

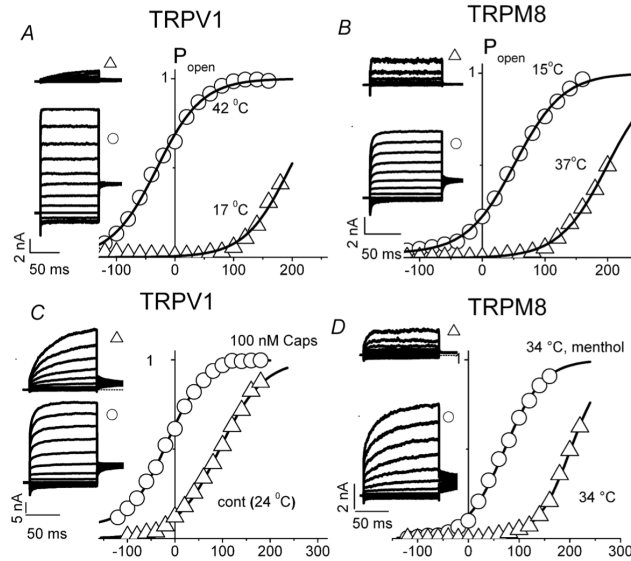


Figure 1.11: **Cooperativity between voltage sensitivity and other activating stimuli in TRPs.** (a) Heat shifts the voltage-sensitivity of TRPV1 to physiological ranges. (b) Cold shifts the voltage-sensitivity of TRPM8 to physiological ranges. (c) Capsaicin shifts the voltage-sensitivity of TRPV1 to physiological ranges. (d) Menthol shifts the voltage-sensitivity of TRPM8 to physiological ranges. Image adapted from reference [197].

1.2 Difficulty and success in membrane protein structural biology

While many structural and functional characteristics of ion channels, even TRP channels, have been determined surprisingly accurately via pharmacological and biophysical methods, with the first crystal structures of membrane proteins came a transformation of what were previously ideas and sketches into defined, Angstrom-resolution models with which understanding and predictions could be refined accordingly. While the understanding of the transmembrane (pore and voltage-sensor-like) domains of TRP channels lack such high-resolution models, the progress made with structures of other VGL channels and of TRP channel soluble domains have already been invaluable.

1.2.1 A short history of membrane protein structural biology

In 1960, John Kendrew and Max Perutz solved the first crystal structures of proteins: the soluble proteins myoglobin and hemoglobin [134, 219]. After that, it was 25 years until the first crystal structure of an integral membrane protein was solved. In fact this was believed impossible, until by chance Hartmut Michel noticed his bacteriorhodopsin samples might be showing evidence of crystallisation propensity. Then after almost a decade of work and optimisation, he and his colleagues were able to solve the first structure of an integral membrane protein to 3 Å resolution [60]. After that it was another thirteen years until 1998, when the first membrane protein crystal structure was solved from a recombinant system, and even then it was a bacterial KcsA gene from *Streptomyces lividans* expressed in *E. coli*, also a bacterial host system [62]. Since this, however, techniques in membrane protein crystallography have advanced rapidly, for example, since the first structure of a non-rhodopsin G-protein-coupled receptor (GPCR) in 2007 [229] there have now been 16 distinct structures of these receptors [130].

Major advances in x-ray crystallographic methods occurred in the 1940s and 1950s, facilitating the historical determination of the structures of haemoglobin and myoglobin, mentioned above, insulin, lysozyme, etc. Now the problems that took heroic efforts to surmount during early crystallography, like the phase problem and the sheer amount of data to process, have been standardized and automated. Now, however, the most difficult areas of crystallography are the fields of large multi-protein complexes and membrane proteins. With these projects, a completely different end of the workflow, the production of diffracting protein crystals, becomes the limiting factor (Figure 1.12). The production of protein crystals is often hampered by the early stages of overexpression and purification. In this light, it is not surprising that the earliest membrane proteins to be crystallised are those naturally abundant in the native membrane, like bacteriorhodopsin and aquaporin. Overexpressing enough of a functional, stable membrane protein that is not naturally abundant becomes

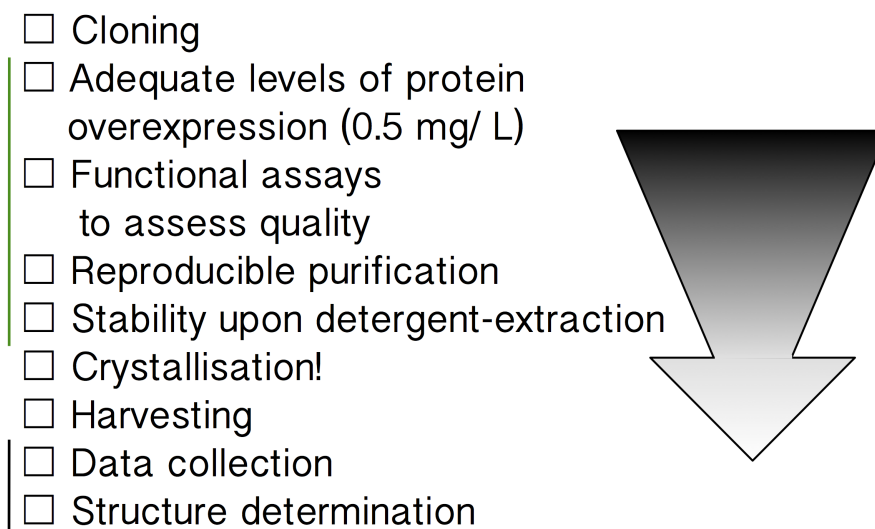


Figure 1.12: **Crystallography pipeline.** This pipeline shows the steps of crystallography in chronological order from cloning to structure determination. Highlighted with a black line are the areas optimised in the early, heroic years of crystallography. Highlighted with a green line are the areas that remain a struggle for more complex crystallographic targets, such as membrane proteins.

a challenge on its own. One factor that contributes to this challenge is that membrane proteins are often pores or transporters, which can be toxic if expressed to high density in the cell's membrane. In addition, the possibility of secreting soluble proteins means that there is essentially no limit to their overproduction, unlike membrane proteins which can only occupy the limited area of the cell's membranes (Figure 1.13).

Even once mg quantities of a membrane protein can be achieved, one of the notoriously difficult aspects of membrane protein crystallography is extracting a stable protein from the membrane by solubilising broken cells with detergent. This step is crucial because often membrane proteins will aggregate or become otherwise unstable after being removed from their native membrane. This potential instability upon detergent extraction is even more of a problem for eukaryotic membrane proteins than for prokaryotic membrane proteins. This can be attributed to the fact that prokaryotic membranes are less rigid to begin with, lacking some of the stabilising elements, like cholesterol, of eukaryotic membranes (Figure 1.14).

While eukaryotic and prokaryotic membranes are mostly composed of the same broad components, mostly phospholipids, a representative member of which (1-palmitoyl-1,2-oleoyl-sn-glycero-3-phosphocholine or POPC) is presented in Figure 1.14, the small differences can add up. Bacterial membranes mainly consist of POPE and POPG which have either ethanolamine or glycerol, respectively, in the place of the choline of POPC, as opposed to eukaryotic membranes with POPC, POPS (serine instead of choline), and POPE as the main constituents of a lipid bilayer with an asymmetric distribution of these phospholipids as well as sphingomyelin, cholesterol, and phosphatidylinositol 4,5-bisphosphate (PIP₂) in the two bilayer leaflets.

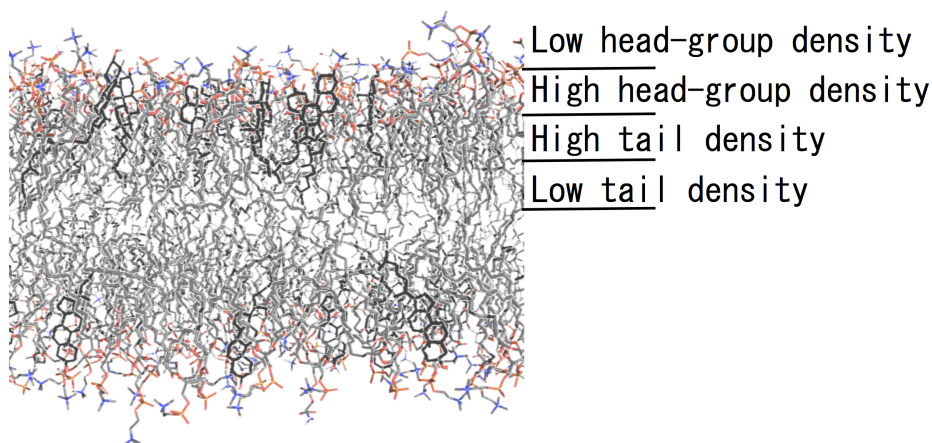


Figure 1.13: **The lipid membrane.** Integral membrane proteins are hard to work with because in physiological conditions they must be stable within the lipid bilayer, which has a highly hydrophobic center composed of the lipid tails (acyl chain carbons in grey) while the charged lipid head-groups line the water-bilayer interface (phosphates in red and choline groups in blue) able to hydrogen bond with the polar water molecules. This bilayer is composed of POPC and cholesterol (black). Figure made using PyMol.

To facilitate the earliest stages of membrane protein crystallography, a broad range and combination of detergents can be tested. Additionally new detergents such as amphiphiles and lipid-like detergents, which have seen much success in membrane protein NMR have been developed to increase protein stability upon extraction by mimicking the behaviour of the native membrane. Aside from testing a variety of detergents, various other methods

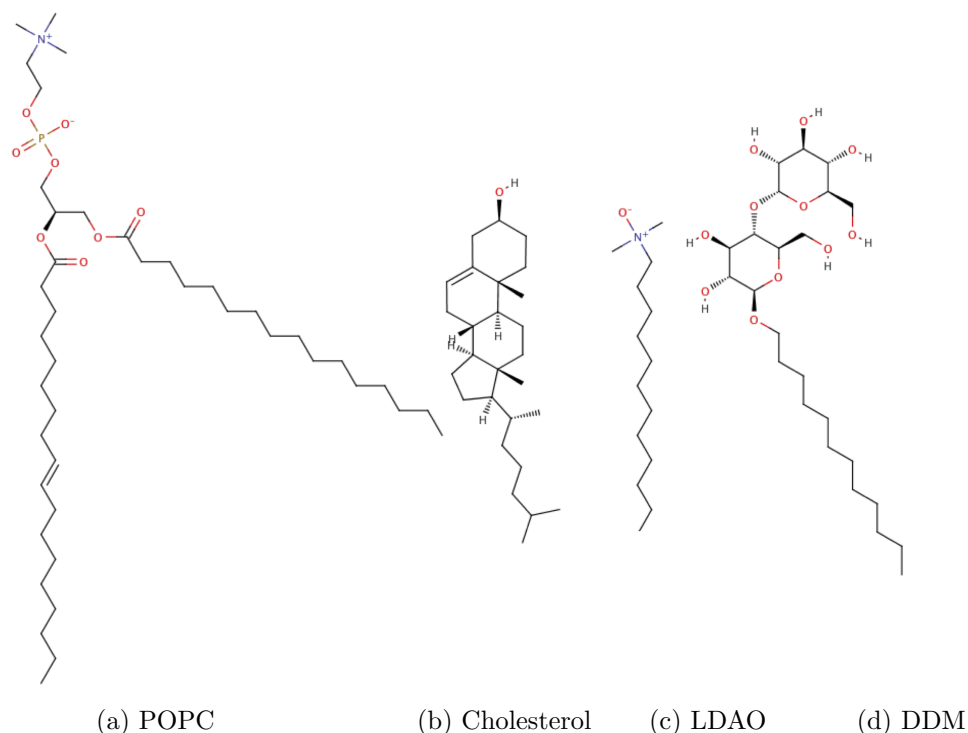


Figure 1.14: **Common lipids and detergents.** There are many kinds of lipids and detergents, but here is a representative sample of important molecules to consider when working with membrane proteins. (a) POPC is a lipid molecule often used to model the eukaryotic membrane. The two branched tails (note that one is saturated and the other unsaturated) and the phosphate head-group are common in phospholipids. (b) Cholesterol is ubiquitous in eukaryotic membranes. Though its percentage in the membrane varies from organelle to organelle, though on average it is expected to make up 25%. LDAO (c) and DDM (d) are two common detergents used to extract and solubilise membrane proteins from their native bilayer. Figure made using the MarvinSketch software from ChemAxon.

have been attempted, especially for GPCRs. For example the use of maltose-binding protein (MBP) tags, or in the case of one structure a protein was engineered in the middle of the GPCR to increase its stability. Additionally for GPCRs Chris Tate's lab developed a pipeline for determining thermostable mutants that would be amenable to crystallography [299, 271]. Additionally a method adding a C-terminal GFP tag to membrane proteins to track overexpression and stability was recently developed [131, 194, 63], and has been used in the pipeline for many successful structural studies so far. And lastly, adding binding

partners such as toxins or antibodies to stabilise proteins, has seen its own range of successes within membrane crystallography [229].

The rate at which membrane protein crystal structures are solved by x-ray diffraction is now on par with the rate at which soluble structures were being solved in the 1970s [254]. This means that though techniques are rapidly developing, only a handful of labs are able to consistently master the specialised techniques and regularly solve structures of eukaryotic membrane proteins [26, 132, 229, 156]. In the not too distant future, perhaps the techniques of membrane protein crystallography will reach the current state of soluble protein structural biology, but until then, trends indicate that a great investment in time, people and resources are still required to approach each project undertaken in eukaryotic membrane protein crystallography.

1.2.2 Chronology of successes of structural biology of the VGL-channome

The first crystal structure of a membrane protein from recombinant sources in 1998 was also the first crystal structure of an ion channel [62]. This structure is of a potassium channel from *Streptomyces lividans*, showing the first pore at high resolution with two α -helices (equivalent to the S5 and S6) forming a tetrameric K^+ -selective pore. In the fifteen years since, over a hundred structures have been solved of channels with this characteristic pore architecture of the voltage-gated-like channel (VGL) family (Table 1.2). While this resource is invaluable, relatively few of these structures have the full six transmembrane helices characteristic of voltage-gated and voltage-gated-like channels. In many cases this is due to the channel, like with inward rectifying K^+ (Kir) channels and KcsA, not having the four-helix voltage sensor domain in the first place. In another case, McCusker et al took advantage of the modularity of voltage-gated channels and removed the voltage sensor to be able to isolate the open form of the pore, otherwise held closed by the voltage sensors in the absence of a voltage gradient [173].

Table 1.2: **Structures of the VGL channel superfamily.** Data curated from the membrane protein structure database at <http://blanco.biomol.uci.edu/mpstruc/>.

Channel Name	Reference	PDB ID(s)	Species
Voltage-gated K⁺ Channels			
KcsA	Doyle et al. (1998)	1BL8	<i>S. lividans</i>
	Zhou et al. (2001)	1K4C; 1K4D	<i>S. lividans</i>
	Lockless et al. (2007)	2ITC; 2ITD; 2NLJ	<i>S. lividans</i>
	Uysal et al. (2009)	3EFF; (3EFD)	<i>S. lividans</i>
	Thompson et al. (2009)	(3IGA); 3GB7	<i>S. lividans</i>
	Cuello et al. (2010)	3F5W; 3F7V; 3F7Y; 3FB6; 3FB5	<i>S. lividans</i>
	Cuello et al. (2010)	3HPL; 3FB7	<i>S. lividans</i>
	Uysal et al. (2011)	3PJS	<i>S. lividans</i>
	Chakrapani et al. (2011)	3OR7; 3OR6	<i>S. lividans</i>
	Raghuraman et al. (2012)	3STL; 3STZ	<i>S. lividans</i>
KvAP	Jiang et al. (2003)	<u>1ORQ</u> ; <u>1ORS</u>	<i>A. pernix</i>
	Lee et al. (2005)	<u>2A0L</u>	<i>A. pernix</i>
	Butterwick & MacKinnon (2010)	<u>2KYH</u> (NMR)	<i>A. pernix</i>
Kv1.2	Long et al. (2005); Chen et al. (2010)	<u>2A79</u> ; <u>3LUT</u>	<i>R. norvegicus</i>
Kv1.2/2.1 Chimera	Long et al. (2007)	<u>2R9R</u>	<i>R. norvegicus</i>
Kv1.2/2.1 Chimera	Tao et al. (2010)	<u>3LNM</u>	<i>R. norvegicus</i>
KvLm	Santos et al. (2012)	4H33; 4H37	<i>L. monocytogenes</i>
Two Pore K⁺ Channels			
TWIK-1	Miller & Long (2012)	3UKM	<i>H. sapiens</i>
TRAAK	Brohawn et al. (2012)	3UM7	<i>H. sapiens</i>
TRAAK	Brohawn et al. (2013)	4I9W	<i>H. sapiens</i>
Inward Rectifying K⁺ Channels			
KirBac1.1	Kuo et al. (2003)	1P7B; 2WLL	<i>B. pseudomallei</i>
Kir3.1/pro Chim.	Nishida et al. (2007)	2QKS	<i>M. musculus</i> & <i>B. xenovornas</i>
Kir2.2	Tao et al. (2009)	3JYC	<i>G. gallus</i>
	Hansen et al. (2011)	3SPI; 3SPC; 3SPH; 3SPJ; 3SPG	<i>G. gallus</i>

Continued on next page

Table 1.2 – continued from previous page

Channel Name	Reference	PDB ID(s)	Species
KirBac3.1	Clarke et al. (2010)	2WLJ; 1XL4; 2WLK; 1XL6; 2WLI; 2WLO; 2WLM; 2WLN; 2WLH; 2X6A; 2X6B; 2X6C	<i>M. magnetotacticum</i>
Kir3.2	Bavro et al. (2012)	3ZRS	<i>M. magnetotacticum</i>
	Whorton & MacKinnon (2011)	3SYO; 3SYA; 3SYC; 3SYP; 3SYQ	<i>M. musculus</i>
Ca²⁺ -gated K⁺ Channels			
	Ye et al. (2010)	3LDC; 3LDD; 3LDE	<i>M. thermautotrophicus</i>
	Posson et al. (2013)	4HYO; 4HZ3	<i>M. thermautotrophicus</i>
Ligand-gated K⁺ Channels			
GsuK	Kong et al. (2012)	4GX0; 4GX1; 4GX2; 4GX5; 4GVL	<i>G. sulfurreducens</i>
Cyclic nucleotide-gated K⁺ Channels			
MlotiK	Clayton et al. (2008)	<u>3BEH</u>	<i>M. loti</i>
NaK Channels			
NaK	Shi et al. (2006)	2AHY; 2AHZ	<i>B. cereus</i>
	Alam et al. (2007)	2Q67; 2Q68; 2Q69; 2Q6A	<i>B. cereus</i>
	Alam & Jiang (2009)	3E86	<i>B. cereus</i>
	Derebe et al. (2011)	3K0D; 3K06; 3K0G; 3K04; 3K08	<i>B. cereus</i>
	Derebe et al. (2011)	3OUF; 3K03; 3OUS	<i>B. cereus</i>
	Sauer et al. (2011)	3T1C; 3T2M; 3TET; 3T4Z; 3T4D; 3TCU	<i>B. cereus</i>
NaV / NaK Chimera	Irie et al. (2012)	3VOU	<i>B. weihenstephanensis</i> & <i>S. pontiacus</i>
Voltage-gated Na⁺ Channels			
NavAb	Payandeh et al. (2011)	<u>3RVY</u> ; <u>3RVZ</u> ; <u>3RW0</u>	<i>A. butzieri</i>
	Payandeh et al. (2012)	<u>4EKW</u>	<i>A. butzieri</i>
NavRh	Zhang et al. (2012)	<u>4DXW</u>	<i>Alpha proteobacterium</i> HIMB114
NavMs	McCusker et al. (2012)	4F4L	<i>M. marinus</i>
TOTALS	48 papers since 1998	103 (<u>14 S1-S4</u>) structures	17 species (4 euk.)

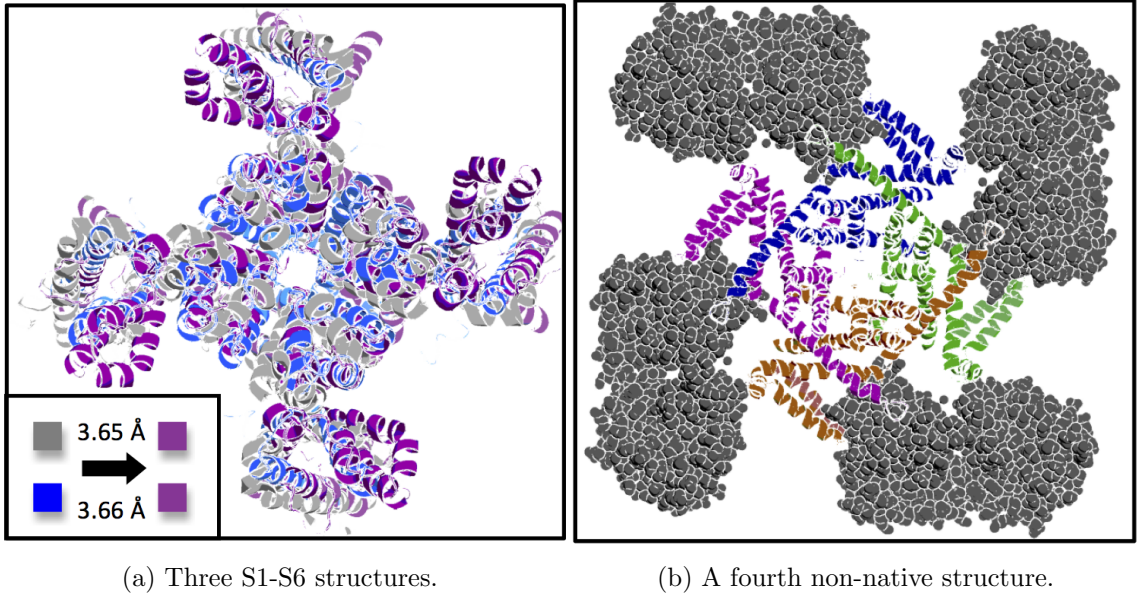


Figure 1.15: **Structural templates for VGL channel S1-S6 helices.** (a) Overlay of the three representative structures. Here NavAb is shown in grey, the Kv 1.2 /2.1 Chimera is shown in purple, and the MlotiK is shown in blue. This superposition was performed using Coot [70], which calculated the RMSD between the MlotiK and Chimera structures (3.66 Å), between the NavAb and Chimera structure (3.65 Å) and between MlotiK and NavAb (5.37 Å). In order to perform the superposition, Coot performs a sequence alignment. Despite the similar RMSDs, the sequence identity between the structures was poor at 17.97% between the MlotiK and Chimera sequences, 12.79% between the NavAb and Chimera sequences, and 10.18% between the MlotiK and NavAb sequences. (b) The structure of KvAP S1-S6 with antibodies bound (PDB ID: 1ORQ). Each chain of the channel is coloured differently and shown with cartoon representation, while the antibody fragment is coloured grey and shown as spheres.

The first crystal structure of a channel that included the four helices of the S1-S4 voltage-sensing domain was achieved in 2003 [116]. Two structures were published simultaneously in this paper, both with an antibody bound to the same epitope, one with just the S1-S4 helices and the other with the full S1-S6 tetramer. The S1-S4 of these two structures, however, differed markedly, with some of the helices of the tetramer structure practically parallel to the bilayer. Since this first result, several other structures of the full S1-S6 transmembrane domain have been solved (underlined in Table 1.2), which are likely in

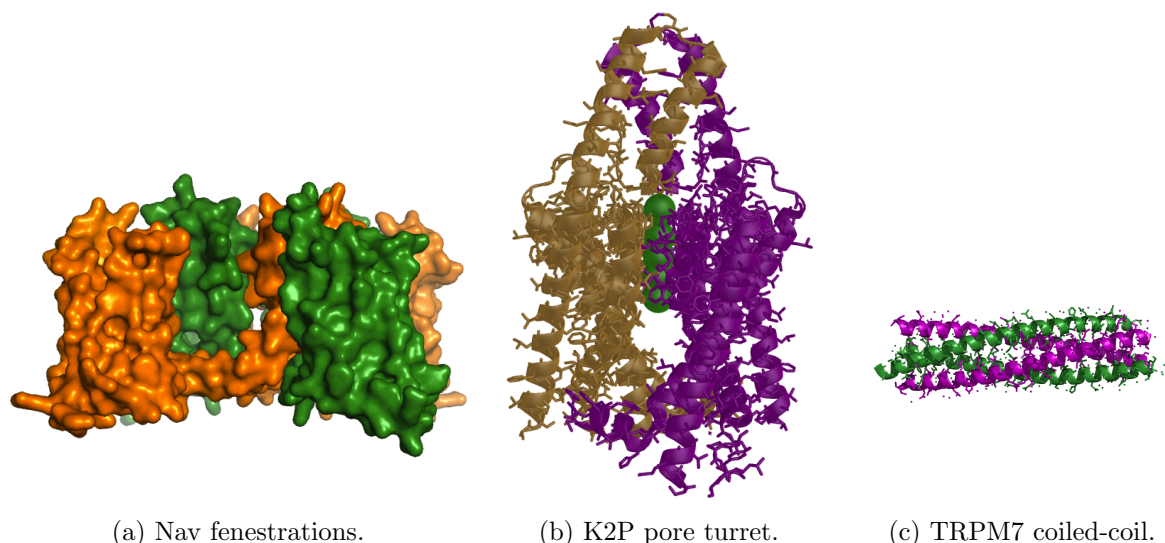


Figure 1.16: **Recent unexpected findings within VGL structures.** Peculiar elements of VGL channels have recently been elucidated by crystallography that may also be relevant to understanding TRP channel mechanisms. (a) Fenestrations visible in the crystal structure of a bacterial Nav channel [215]. (b) The pore turret of K2P channels [224]. (c) Evidence of asymmetry in the two-fold symmetry of the crystal structure of the coiled-coil C-terminal domain of TRPM7 [82]. Figures made with PyMol [244].

more physiological conformations than this original structure (Figure 1.15). The handful of unique structures solved of S1-S6 domains include a bacterial voltage-gated K^+ channel, the rat Kv 1.2 channel, a bacterial homologue to the cyclic nucleotide-gated (CNG) K^+ channels, and a bacterial Nav channel. Except for the initial structure of the bacterial voltage-gated K^+ channel, the remaining structures align remarkably well considering their low sequence identity (Figure 1.15). Variations of the S1-S6 tetramer structure of the rat Kv 1.2 channel are so far the only eukaryotic S1-S6 structure available, and the highest resolution structure of these is the known as the Kv ‘Paddle-Chimera’ structure, as it was obtained by cloning the Kv 2.1 voltage sensor ‘paddle motif’ (the end of the S3 and beginning of S4) into the Kv 1.2 channel [156].

While the voltage sensor and selectivity filter have been a topic of functional and structure studies for decades, a few recent insights into the architecture of VGL channels help

paint a complete picture of what TRP channel transmembrane domains likely look like. One example is the observation of fenestrations that could allow lipids or ligands to snake into the pore region, which have been uncovered in the structures of both a two pore potassium (K2P) channel and the bacterial voltage-gated Na^+ channel (Figure 1.16) [215, 35]. Also from the recent structures of human K2P channels was the revelation of a pore turret above the selectivity filter made of a continuation of one of the pore helices and a second smaller α -helix (Figure 1.16) [35, 178]. This novel structural element is suggestive of both a structural and functional role for the long supposedly unstructured loops between the two pore helices in many TRP channels, though secondary structure predictions do not provide much support for this hypothesis. Furthermore, a crystal structure of the homotetrameric bacterial voltage-gated Na^+ channel revealed a two-fold rather than four-fold axial symmetry [214]. Combining this peculiarity with the crystal structure of the coiled-coil C-terminal region of TRPM7 that forms a dimer of dimers with similarly two-fold axial symmetry, opens up a discussion of whether TRP channels could be oligomerising asymmetrically (Figure 1.16) [82].

1.2.3 Insights from molecular dynamics (MD) simulations of VGL channel structures

Within a year of the publication of the KcsA crystal structure, the first molecular dynamics (MD) simulations were conducted of the 5 amino acids that constitute the K^+ selectivity filter [6]. While this simulation did not model in the membrane, it would have been an option as almost a decade earlier the first membrane protein simulations were conducted, including the 100 ps simulations of the major coat protein of bacteriophage M13 to compare the stabilities of proposed α and β secondary structures; these simulations used a hydrophobic potential to represent the lipid bilayer [238]. This was fourteen years after the first simulation of any protein in 1977 (in vacuo) [171], and the techniques of molecular

simulation had been progressing since then, including encouraging the concept of proteins as 'jiggling and wiggling' rather than as static structures. These molecular simulations take the coordinates of crystal structures and solve Newton's equations iteratively to describe the motion of the atoms with time, providing insights into the dynamics of the mechanisms crucial to protein function (more details can be found in Chapter 2) [91].

After the first simulation of KcsA, subsequent simulations continued to provide insights into voltage-gated channels and their homologues. For example, the insight that the K^+ selectivity filter functioned via a single-file passage of ions, with alternating water molecules and K^+ ions in the filter was refined [250, 241] (Figure 1.17a). Additionally, an increase in computational power over the years has allowed for membranes to be simulated explicitly as individual lipid molecules, crucial to addressing the growing evidence that the membrane has significant effects on K^+ channel function [242]. Most relevantly, simulating the original voltage sensor structure of KvAP in explicit membrane showed that the lipid bilayer could accommodate the highly charged S4 helix. The protein had significant effects on bilayer structure, including an extensive penetration of water molecules into the voltage sensor domain, effecting the apparent electrostatic potential around the voltage sensor [240, 137] (Figure 1.17b). Finally, homology models of the Hv1 proton channel based on the KvAP and Chimera S1-S4 helices were able to provide insights into the mechanism of proton transport through the membrane, even without a high resolution crystal structure [227].

1.2.4 TRP channel structural biology and homology modelling so far

While TRP channel structural biology has not so far seen the success of other members of the VGL channel superfamily, the field has nonetheless seen some progress in structural determination of various domains. Eight separate crystal structures have been determined of cytosolic domains of TRP channels. The N-terminal Ankyrin Repeat Domains (ARD) of the TRPV1, TRPV2, TRPV4, and TRPV6 have all been crystallised [152, 111, 172,

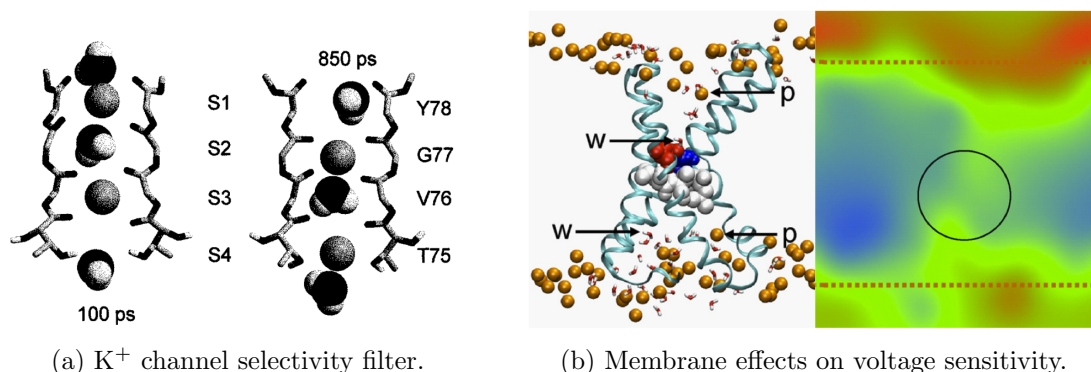


Figure 1.17: **Insights from simulation toward VGL channel function.** (a) Passage of alternating water and ions through a K⁺ channel selectivity filter from [250]. (b) Water penetration (red and white lines) and bilayer deformation (phosphate headgroups as orange spheres) mediated by the S1-S4 helices effects the electrostatic potential across the bilayer. Image adapted from [240].

117, 221]. As mentioned above, the structure of C-terminal coiled-coil domain of TRPM7 has been determined [82]. Additionally, the kinase domain unique to the C-terminus of TRPM7 has been crystallised [304]. Finally, a complex between calmodulin (CaM) and a 35-residue peptide of the TRPV1 C-terminus has been recently crystallised, which though small, has profound functional implications for the role of the C-terminus of TRPV1 [142]. EM structures of several full length TRP channels have been determined at low resolution, discussed in more detail in Chapter 3.

Despite these successes, homology modelling remains the only structural resource available for the transmembrane domains of TRP channels. One of the earliest attempts at TRP channel modelling was a homology model used to address phosphatidylinositol 4,5-bisphosphate (PIP₂) binding to and regulation of TRPV1 in 2007 [32]. Another approach was the modelling of the full-length TRPV1, including all cytosolic domains, to attempt to draw an initial picture of what the field could expect, however at the time, the information available was far from sufficient to create a reliable picture [74]. Then, most recently a model was built to address capsaicin and RTX binding to the transmembrane domains of

TRPV1 [144]. Also included in these modelling efforts are a variety of models of the selectivity filter to attempt explanations of the various experiments mentioned above to probe the understanding and variety of TRP channel selectivity [206]. Though still of marginal utility due to low sequence identity of every TRP subfamily except TRPV's to existing crystal structures in this region, these may be the best use of modelling so far, as the selectivity filter is the most conserved region in TRP channels. More detailed discussion of TRP channel models is discussed in Chapter 5.

1.3 Lipophilic ligands as important tools for understanding TRP channel mechanisms

Though TRP channels themselves were not identified until within the lifetime of the author of this thesis, their manipulation by spices and herbs has been a part of human culture for millennia. While it was the use of these small molecules that facilitated the discovery of the canonical thermoTRPs in the first place, many questions still remain regarding the action of these molecules, and much is left to learn from their use in the structural and functional characterisation of TRP channels.

1.3.1 A developing understanding of membrane protein interactions with lipids and lipid-like drugs

When ion channel were just beginning to be characterised, the defining models of drug binding interaction kinetics had already been developed and tested [34]. But today, membrane proteins constitute more than 40% of therapeutic targets [205] and models of drug interactions through membranes are not nearly as well developed (Figure 1.18). In the modern pharmaceutical industry, drug discovery abandons many drug candidates due to poor adsorption, distribution, metabolism, excretion, and toxicology (ADMET). A variety

of recent clinical failures have been due to poor bioavailability and pharmacokinetics, both of which relate to drug interactions with the membrane bilayer [267]. In addition, experiments point to the non-specific interaction of drugs with the lipid bilayer as a previously underestimated possible cause of drug side effects [159]. While these general effects of drug interaction with a membrane are important, these small molecules also specifically target membrane proteins within their transmembrane domains. For example, the recently crystallised GPCR S1P1 has a catalytic domain in the middle of the lipid bilayer [96]. While surprisingly little research has been done to compare ligand binding within a membrane to ligand binding in solution, this type of system is exactly the system expected for agonist and antagonist binding to TRP channels, e.g. menthol binding to TRPM8 and capsaicin binding to TRPV1.

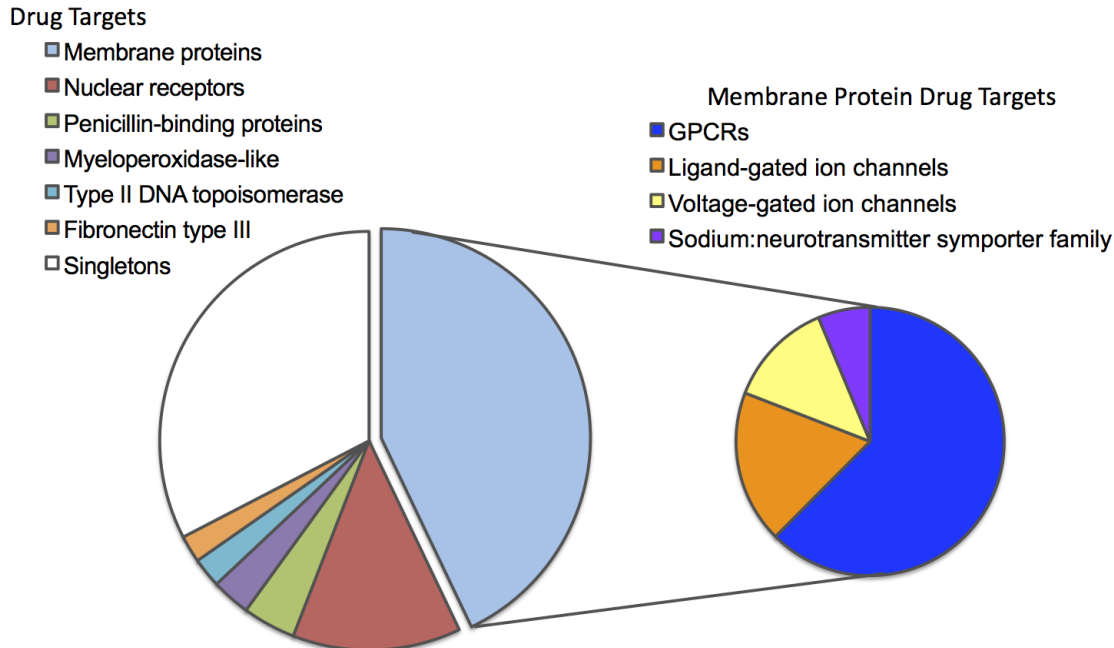


Figure 1.18: **Membrane proteins as drug targets.** Membrane proteins make up at least 43% of drug targets. GPCRs are the largest group of membrane proteins targeted, making up 27% of all drug targets. Voltage-gated ion channels are 6% of drug targets. Data acquired from [205].

With the first crystal structures of KcsA and KvAP, the importance of the lipid environment to ion channel structure and function started to become clearer [146, 277]. Soon after that, the subtleties of these interactions became more defined with studies on the importance of charged lipids and membrane deformation to voltage sensor function [137, 242]. Though even before this, experiments had demonstrated the regulation of Kir channels by the anionic lipid PIP₂ [100, 71]. It is now widely recognised that PIP₂ regulation can be extended to all classes of ion channel [275], and most importantly for this thesis plays an important and role in TRP channel physiology [32, 154].

While the effects of the lipid environment on K⁺ channels were becoming clearer, so too was the general complexity of the eukaryotic lipid bilayer. While bacterial lipid bilayers have relatively simple symmetric compositions of about 75% POPE, 20% POPG, and 5% Cardiolipin, eukaryotic bilayers are much more complex, and perhaps more variable, with an asymmetry between the upper and lower leaflets with mostly POPC and sphingomyelin with a little bit of POPE, POPS and phosphatidylinositols like PIP₂ in the outer leaflet, and mostly POPS and POPE with a smaller amount of POPC, sphingomyelin and phosphatidylinositols in the inner leaflet [280, 279]. In addition to PIP₂, several of these components have been found to have a variety of effects on membrane proteins, for example cholesterol has been seen to have specific effects on both TRPV1 and the β 2-adrenergic receptor GPCR [222, 313] and glycolipids have been seen to regulate the EGF receptor [56]. This increased understanding of the complexity of lipid bilayers has also allowed for the development of larger-scale theories of bilayer-mediated behaviour, such as the formation of lipid domains, for example, lipid rafts, for which a range of physiological implications have been suggested [151].

1.3.2 MD simulations of lipids and ligands in the membrane

Molecular simulation has provided significant insight to the understanding of the synergistic relationship between membrane-embedded proteins and the lipid bilayer. While the initial evidence for the importance of these interactions has come from experiment, for example in the case of PIP₂, understanding the mechanistic implications cannot simply be done by looking at a crystal structure, even if one is lucky enough to have one for the system in question. One advantage of the ‘Paddle-Chimera’ structure of the rat Kv 1.2/2.1 is that it explicitly shows lipids bound to the channel [156]. However even this is far from a complete bilayer, and the role of these lipids remains unclear. Some techniques such as solid state NMR, neutron scattering or a range of fluorescence methods, can provide data regarding how the average lipid within the bilayer may be interacting with the protein [245, 137]. However, before approaching these often highly specialised techniques made even more difficult by the presence of a lipid bilayer, embedding a crystal structure or homology model of a membrane protein into a simulation-derived lipid bilayer and observing the time-evolved interactions can be invaluable. For example, in the case of PIP₂ binding to Kir channels, the binding site for the molecule was predicted surprisingly accurately via computational methods two years before a crystal structure of the same complex was solved [255, 95] (Figure 1.19). In this case, a homology model and a coarse-grained (CG) PIP₂ molecule were used to probe the general nature of the interaction with the larger sampling space available in CG simulations before converting the prediction to atomistic representation. Thus, the predicted contacts are not exact, but show the same trend as those seen in the crystal structure. Similar interactions between lipids and proteins have been seen in simulations of aquaporin and cytochrome *bc₁* with cardiolipin [256, 12]. Other insights regarding the clustering and diffusion rates of lipids and membrane proteins within large bilayers have also been made available through molecular simulation [88, 211, 135].

So far, most simulation-based studies of the concerted interactions of lipophilic molecules

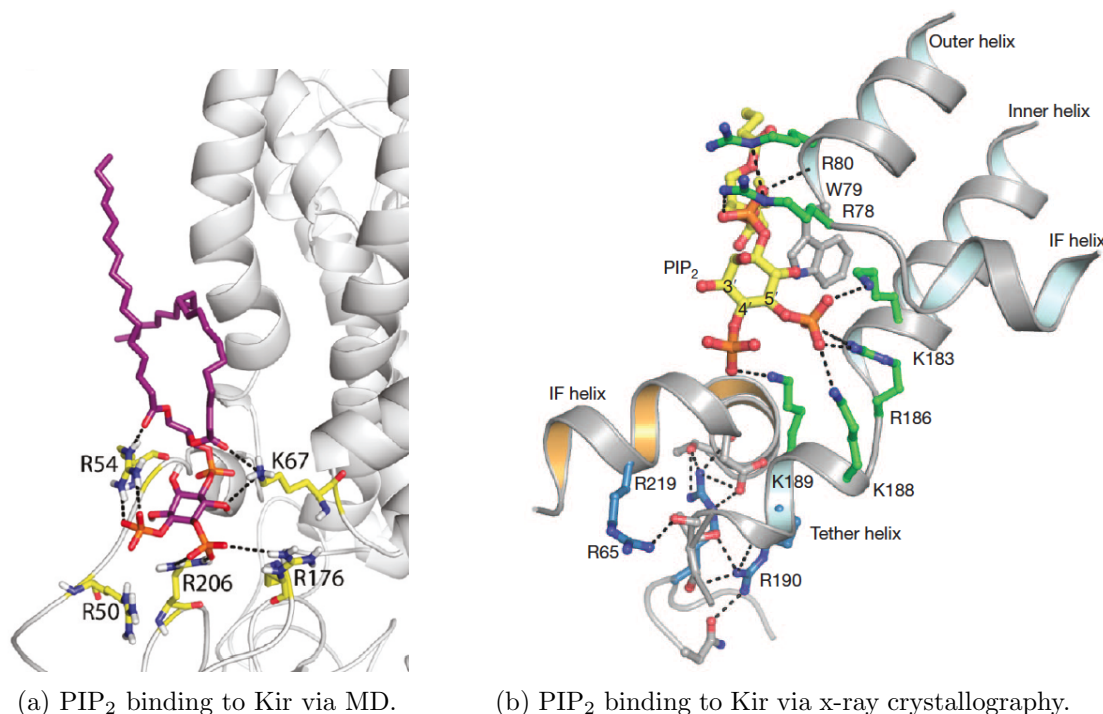


Figure 1.19: **MD predicts PIP₂ binding site.** (a) CG simulations converted to atomistic representation predicts a strong interaction between PIP₂ and charged residues near the water/bilayer interface of Kir6.2. Image adapted from [255]. (b) A crystal structure of PIP₂ bound to Kir2.2 shows the same trend [95].

with membrane proteins have focused on lipid molecules and not drugs, but simulations with a range of small molecules and their interactions with protein-less bilayers have been conducted and begun to show some concerted patterns. The earliest MD simulations to look at solute diffusion through lipid bilayers were in the early 1990s looking at the diffusion of benzene within a DMPC bilayer [15], but the first systematic studies were in the mid-nineties by Marrink and Berendsen, who calculated the force required to maintain a Lennard-Jones particle at various distances from the bilayer center [164]. This method was later taken a step further when [20] compared this energetic distribution for some of the most common chemical functional groups. Since then, a variety of simulations have studied the interactions of molecules with lipid bilayers, ranging from steroids [284] to nucleosides [296] to fatty acids [46] to pharmaceutical drugs [267], though the studies of larger molecules often focus on

empirical observations rather than precise free energy calculations, as larger molecules are less likely to see convergence of the calculations.

One example of this kind of empirical observation is a recurring focus on lipid or lipid-like molecule flip-flop from one bilayer leaflet to the other. One of the first examples of observing this phenomenon in simulations was a simulation of DMPC lipids in which a pronounced imbalance of ions created an electric field across the bilayer, increasing the likelihood of the spontaneous formation of water and ion pores within the bilayer, which facilitated spontaneous pore-mediated lipid flip-flop [92]. Most of the other examples of flip-flop observed in simulation address this event in cholesterol and other steroids, for example how the addition of chemical groups effects the rate of flip-flop [234, 21]. In the case of cholesterol flip-flop the event is spontaneous and does not need to be facilitated by the creation of pores by an electric field, however both types of events suggest that the understanding of this event is physiologically significant, and could be significant not only to lipids and steroids, but also to lipophilic drug mechanisms.

1.3.3 Lipids and lipophilic ligands in TRP channel physiology

In addition to these specific interactions of lipids with TRP channels, there are several instances of TRP channels interacting with and/or sensing the larger scale dynamics of the lipid membrane. For example, there has been some discussion of whether TRPM8 channels associate with lipid rafts [187]. In addition, numerous TRP channels have shown evidence of mechanosensitive properties. The main physiological role of TRPV4 is to detect cellular hypotonicity indicates a possible osmosensitive or mechanosensitive channel, though data indicates this is sensed indirectly [261]. The role of the TRPP's in detecting fluid flow in the kidney also suggested they may have mechanosensitive properties [192]. While TRPCs, TRPVs, and TRPAs have also been suggested to have mechanosensitive properties, the most powerful evidence for TRP mechanosensitivity comes from the yeast and drosophila

TRP channels [262].

While non-endogenous compounds, such as menthol and capsaicin, evolved in plants to target the TRP channels of animals have been useful tools for the characterisation and discovery of the family, they may be targeting already existing mechanisms of endogenous TRP channel activity. Practically all TRP channel subfamilies have been shown to be involved GPCR/PKC/PLC mediated signaling pathways that are predicted to have their ultimate effect on TRP channels via second messenger lipids like PIP₂ and diacylglycerol (DAG) [97]. While these lipids clearly regulate TRP channels, for example TRPM8, TRPV1, and the canonical TRPC's, it is clear that the mechanisms and effects are not consistent across all TRP channels. For example, it is agreed that PIP₂ positively regulates TRPM8 [154], but whether PIP₂ positively or negatively regulates TRPV1 is debated [152, 40]. A range of other endogenous compounds have now been identified as regulators of TRP channels, such as the products of lipoxygenase that regulate TRPV1 including the first 'endovanilloid' anadamide [110]. So far most endogenous compounds seen to specifically interact with TRP channels are also endogenous lipids, like PIP₂ and anadamide, but extend to fatty acids and their metabolites [207].

With the topological similarities of TRP channels to voltage-gated channels, the significance of both specific and non-specific interactions with the membrane comes as no surprise. While many details have still to be discovered and/or clarified it is clear that a similar range of lipid signalling molecules, such as PIP₂, and hydrophobic toxins, the inhibitory cysteine knot (ICK) toxins, target both TRP channels and other members of the VGL family. In addition to these, a surprising number of herbal compounds have been found to activate TRP channels: TRPC6 has been found to be activated by hyperforin, a key ingredient of the anti-depressant St. John's wort; TRPV1 is activated by capsaicin from chilli peppers, gingerol from ginger, piperine from black pepper, resiniferatoxin (RTX) from a cactus-like plant native to Morocco, and a wide range of other compounds discussed further in the

next section; TRPV2 has been found to be activated by Δ 9-tetrahydrocannabinol (THC) the psychoactive substance found in cannabis; TRPV3 has been found to be activated by caracrol from oregano, thymol from thyme, vanillin from vanilla and eugenol from cloves; TRPV4 has been found to be activated by bisandrographolide, an anti-inflammatory agent used in parts of Asia; TRPM8 is activated by menthol from mint, the synthetic agonist icilin, eucalyptol from eucalyptus and hydroxyl-citronellal from lemon and lemongrass; TRPA1 is activated by allyl isothiocyanate from mustard oil, allicin from garlic and cinnamaldehyde from cinnamon, as well as eugenol and gingerol which also activate some TRPV's [289]. This clustering of herbal compounds that activate TRP channels, familiar to most for their culinary applications, has yet to be explained but there is no doubt they provide a diverse toolbox for studying the mechanistic effect of how amphipathic small molecule compounds target TRP channels, most likely through the membrane.

1.3.4 Capsaicin as the canonical TRPV1 ligand

While all of these molecules have been recognised as TRP ligands, none of them have been as thoroughly studied as capsaicin, the ligand to the canonical thermoTRP TRPV1. The 'chili' is the fruit of a plant originally from South America, used by the Aztec for millenia. It is reported that the Incas burned dried chilis to blind the Spaniards as they were invading. The first record of the chili in Western history is from a doctor in the fleet of Christopher Columbus, and eventually the name 'chili pepper' was adopted in reference to the already known similarly hot-tasting black pepper. In the middle ages, the plant was given the scientific name 'Capsicum' by a French botanist, perhaps from the Latin word 'capsa' for box, referring to the hollowness of the pepper [269].

The pungent chemical of these Capsicum plants was first isolated and characterised in the late 19th century, and given the name capsaicin. At this time it was predicted that capsaicin had a vanillin element, as the vanilla odour was apparent with certain preparation,

though the complete structure was not determined until 1919 [193]. Still used today to measure the potency of peppers, Wilbur Scoville developed the Scoville Unit in 1912, which essentially quantified the 'hotness' of a pepper by the amount of capsaicin it contained [246]. As early as 1925, the importance of the various functional groups on capsaicin to its pungency were being measured via a method that required scientists to dilute and taste the compound [120]. While TRPV1 was not discovered until 1997, the importance of the functional groups of capsaicin had been well characterised by 1994 [302] (Figure 1.20 and Table 1.3), leading to the discovery of the TRPV1 antagonist capsazepine, and the earliest strong evidence that the effect of capsaicin was due primarily to a specific effect on a protein and not a general action on the lipid membrane [293]. Because while it had been observed that applying capsaicin to sensory neurons increased membrane conductance of cations, it was still several years before the revelation that capsaicin was the agonist for an ion channel [167].

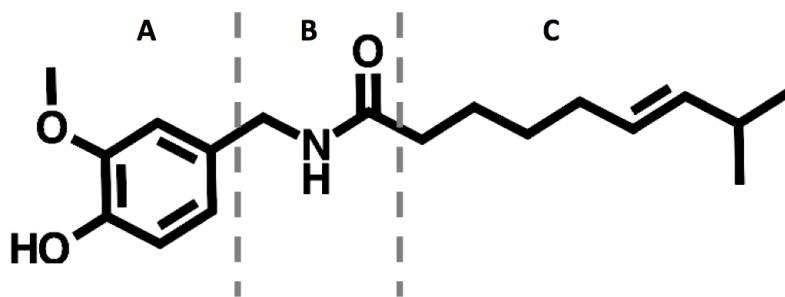


Figure 1.20: **The functional groups of capsaicin.** The functional groups of capsaicin: (A) the substituted aromatic region or 'vanillyl' group, (B) the dipolar amide-bond region, and (C) the hydrophobic tail region.

While capsaicin had proved a valuable tool for identifying and characterising sensory neurons, its hydrophobicity masked the occurrence of saturable binding. Thus with the discovery that the compound resiniferatoxin (RTX) (Figure 1.21), which had been identified as a potent irritant with both a phorbol ester and a vanilloid group, bound the capsaicin

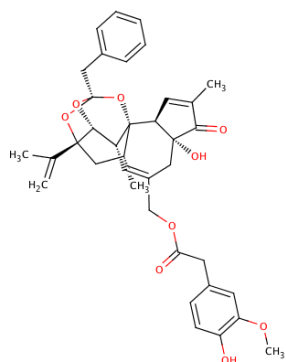
Table 1.3: **Known effects of varying the functional groups of capsaicin**, where A,B, and C are as in Figure 1.20. Data curated from [302].

A	B	C
Alkylation or removal of 4-OH removes activity	Important to have both an H-bonding donor and acceptor	Hydrophobicity is most important characteristic
Changing 3-OCH ₃ to 3-OH has only minimal effects	Amide or reverse amide are equally potent	Octyl is ideal: either (CH ₃) ₆ CH(CH ₃) ₂ or (CH ₂) ₇ CH ₃
	N-methylation reduces activity	Length can be varied but too short [(CH ₂) ₃ CH ₃] or too long [(CH ₂) ₁₅ CH ₃] have reduced activity

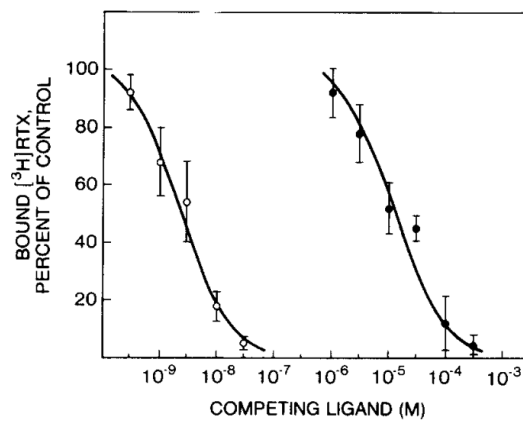
receptor with high specificity, binding constants became measurable through saturable binding curves (Figure 1.21) [268]. It's worth noting, however, the same non-specific binding to the cell membrane that had prevented these measurements for the hydrophobic capsaicin molecule was also an initial hurdle only overcome by the addition of the soluble drug binding protein α -glycoprotein to decrease the background in the RTX binding assays [269].

In addition to the range of TRPV1 mutants that effect capsaicin activity, mentioned briefly in section 1.1 and addressed in more detail in the next section, the hydrophobicity of capsaicin is hypothesised to be a key player in preventing a confident definition of the binding site of the molecule in TRPV1. While it is now clear that the dominant effects of capsaicin are through its specific interaction with TRPV1, its non-specific effects on cell membranes at physiological concentrations remain debated. Early experiments indicate that capsaicin could form pores within a bilayer without any protein interaction [72], but more likely are reports that claim that capsaicin increases bilayer fluidity [10], explaining possible non-specific effects on other membrane proteins beside TRPV1 [160].

Another debate effected by the hydrophobicity of capsaicin is whether it preferentially activates TRPV1 from the extracellular or intracellular side of the membrane. While some



(a) The structure of resiniferatoxin.



(b) The discovery of ultrapotent RTX.

Figure 1.21: **The discovery of RTX.** (a) The structure of resiniferatoxin has both a phorbol ester group and a vanilloid group. (b) Binding curves of (○)RTX and (●)capsaicin inhibition of [³H]-RTX binding. Figure adapted from [268].

reports suggest capsaicin can only activate TRPV1 from the intracellular side[123], others claim the opposite, that capsaicin preferentially activates TRPV1 from the extracellular side [47, 291]. A range of caged capsaicin analogues have even been developed to address this question of asymmetric capsaicin activity, but the results of these experiments continue to produce conflicting results [53]. Despite these discrepancies, the mutational data that points to residues in the intracellular S2-S3 loop as crucial to capsaicin binding, seems to sway the mindset of the field toward a dominant interpretation: an intracellular capsaicin binding site is accessible via the spontaneous diffusion of the hydrophobic capsaicin molecule across the cell membrane.

1.4 Benefits of dividing TRP channels into domains for structural and functional studies

The ability of members of the TRP channel family to integrate multiple stimuli into channel opening is one of the only unifying characteristics attributed to the family. But it is not clear how or if the structure of these channels reflects this functional polymodality. With the regulation and channel activation, for example, of TRPV1 divisible into temperature, pH, Ca^{2+} , ligands, possibly membrane stretch, ATP, lipid second messengers, etc. the protein must be organised in one of two ways: (1) the channel has many separate modular domains that communicate to the pore separately for each of these sensitivities or (2) has only a few modular domains that interact with one another before the signal reaches the pore. Note that neither of these models incorporates the proposal that the temperature sensitivity of TRP channels has a protein-wide, rather than modular effect. Whichever the answer, and it will probably not be black and white. Complicating matters more, exactly how to break TRP channels into modular domains remains another elusive riddle, the answer to which would be a valuable tool toward understanding this peculiar channel family.

1.4.1 Protein domains as structural and functional entities

Before the structures of proteins started to be determined through x-ray crystallography in the 1960s, Linus Pauling predicted that proteins would likely adopt some form of secondary structure beyond the primary structure of their amino acid sequence that he called α -helices and β -strands [213]. When this was proved to be true, the question remained if there was even more organisation within protein structure. Gradually it became clear that certain folds were common and that individual proteins tended to segregate into distinct structural regions, or domains [298]. Today, that many different folds of conserved

and recognisable domains build up functional proteins is practically a law of biochemistry. In the Prologue to ‘Modular Protein Domains’ Sir Tom Blundell makes the analogy that domains are the words to the sentence of the protein [27].

Domains are now defined as independently folding structures usually defined by a specific function. They have proven to be invaluable tools both for the functional and structural characterisation of proteins. Since many larger proteins are constructed of smaller domains connected by flexible linkers, it is common practice in crystallography to divide proteins into these more rigid structural domains instead of tackling a full length protein. A classical example of domain structure in proteins is the epithelial growth factor receptor (EGFR), where the separate cytosolic and transmembrane domains have been both structurally and functionally characterised separate from the other [73, 99].

Thus, to find that TRP channels adopt the 6-TM VGL channel architecture alongside a range of well-known soluble domains in the N- and C-terminal tails comes as no surprise. These cytosolic domains, whose crystal structures are covered in the previous section, include, for example, the TRPM7 kinase domain that has more than 500 similarly folded and functioning proteins in the human genome [127]. One of the most common TRP domains is the Ankyrin Repeat Domain (ARD), defined more by structure than function and commonly found to mediate protein-protein interactions [84]. Additionally there is the calmodulin binding domain, which regulates Ca^{2+} responses via calmodulin (CaM) and is found in a variety of membrane signalling proteins, including small-conductance calcium-activated potassium (SK) channels and the sarcoplasmic reticulum Ca^{2+} pump. Finally, there are the coiled-coil domains expected to mediate TRP channel oligomerisation, initially found in α -keratin, but now exist ubiquitously, such as in the muscle protein tropomyosin [169]. Even with this range of structural and functional knowledge to draw from, it remains difficult to put all the pieces of any individual TRP channel together into a sensible picture, especially since there remain large regions of the N- and C- termini of TRP channels with

no straightforward prediction of their structure or even clues to if they form more than one domain.

1.4.2 Addressing modularity in TRP channel transmembrane domains

Just as some cytosolic domains of TRP channels are well-defined while others remain mysterious, the transmembrane region of TRP channels seems to clearly define itself as a member of the VGL channel superfamily while maintaining some clear ambiguities. Concerning the S5-S6 helices, pore helix, inner vestibule and selectivity filter the architecture of the TRP channel pore domain seems relatively clear, but whether the S1-S4 helices of the transmembrane region can be defined as a structural and functional domain has remained largely unaddressed.

1.4.2.1 The S1-S4 domain in voltage-gated proteins

While the idea of a voltage sensor in voltage-gated cation channels, has been crucial to the understanding of these channels since they were first characterised (Figure 1.3), the fact that the voltage sensor formed a clearly defined structural and functional domain has only recently become clear. In the late nineties it was determined that a spider toxin, hanatoxin, was targeted to the S1-S4 helices of the voltage-sensor domain (VSD) of the *Shaker* Kv channel [266]. Then with the first crystal structure containing this VSD from the *Aeropyrum pernix* Kv channel, it became clear that this was an independent structural domain that could be expressed separately from the rest of the channel in a physiological fold, shown by the ability of this separated VSD to bind similar spider toxins [116]. Around this same time (early 2000s) the functional modularity of the VSD was illustrated by numerous functional chimeras of voltage-gated channels in which voltage-sensitive properties were transferred with the VSD [158, 157, 3]. Finally, completing the picture of the VSD as a functionally and structurally modular domain, came the discovery of the VSD in voltage-sensitive proteins

that are not cation channels, for example in the voltage-sensing phosphatase from *Ciona intestinalis*, Ci-VSP [112] and the voltage-sensitive proton channel, Hv1 [228] (Figure 1.22).

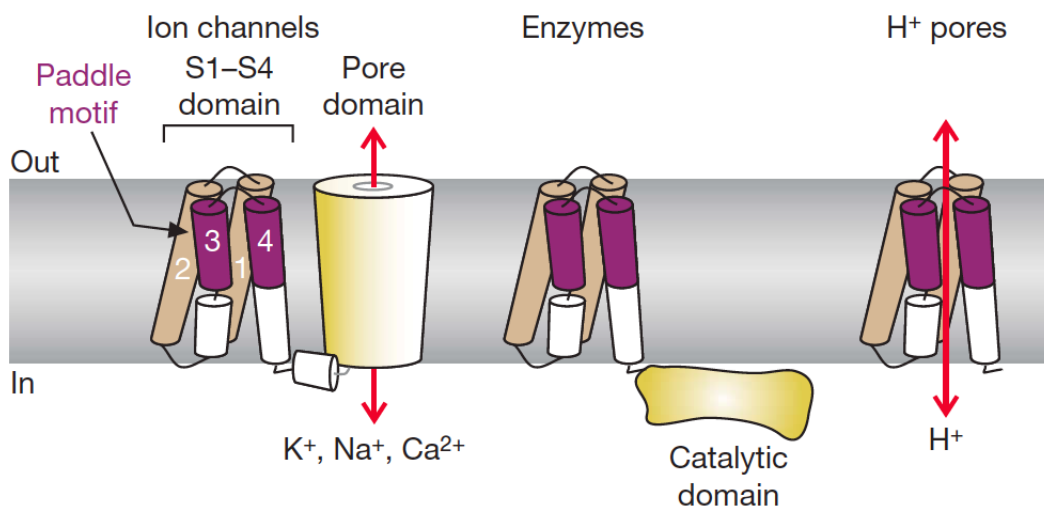


Figure 1.22: **The S1-S4 voltage-sensing domain.** The S1-S4 voltage-sensing domain is not only found in members of the voltage-gated-like ion channel superfamily, but also functions as a voltage sensor in the Ci-VSP voltage-sensitive enzyme and the voltage-sensitive proton channel. Figure adapted from [265].

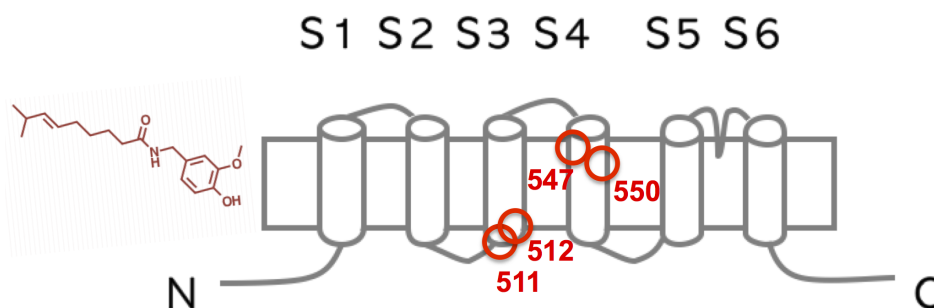
1.4.2.2 The S1-S4 helices of TRP channels: a voltage-sensor-like domain?

Despite this clear evidence for the S1-S4 helices constituting a domain in the formal sense in members of the VGL channel family as well as other voltage-sensitive proteins it remains unclear whether the S1-S4 helices of TRP channels are similarly functionally and structurally significant. While some TRP channels exhibit voltage sensitivity, as discussed in section 1.1.4, the absence of the conserved string of positive residues of a classical VSD in the S4 helix of TRP channels is worrying. It remains unclear if this region is a functional domain in TRP channels: Is this transmembrane region involved in voltage-sensitivity? does it facilitate the ligand- and temperature-sensitivity through a shift in voltage-sensitive properties as the experiments discussed in Section 1.1 suggest? Or is it just an evolutionary

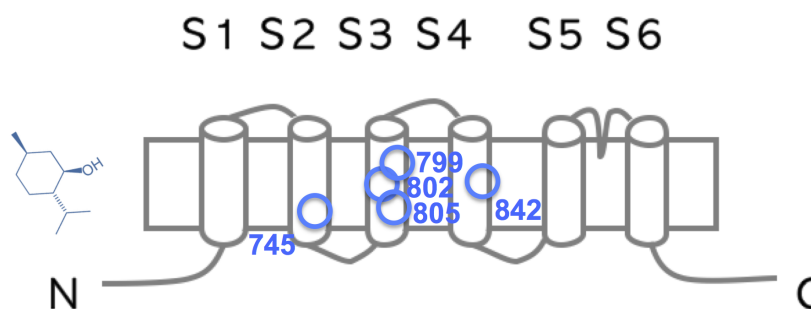
artifact adopted with the pore domain with no practical relation to voltage-sensitivity?

So far voltage-sensitive properties of TRP channels have been attributed to a mutant at the end of the TRPM8 S4 [287], the C-terminus of TRPM4 [206] and the S4-S5 linker in TRPVs [30]. This heterogeneity does not help isolate either the voltage sensor or the function of the S1-S4 helices of TRP channels. However, a range of other parallels between TRP channels and voltage-gated channels allow us to speculate that the S1-S4 helices would have a similarly modular functional and structural role in TRP channels. First, the S1-S6 tetrameric architecture in itself is a strong clue. Second, the similarities in the selectivity filter of TRP channels to VGL channels. Third, evidence toward an S6 gate as well as sensitivity to quaternary ammonium ions, indicates a general mechanistic similarity to VGL channels [114]. Fourth, three different spider toxins in the same family, inhibitory cysteine knot (ICK) toxins, as those that bind to the Kv channel VSD have been found to activate TRP channels: vanillotoxins activate TRPV1, the double-knot toxin (DkTx) activates TRPV1, and the GsMtx-4 activates TRPA1 [101, 28, 251]. A fifth indication is that despite not being the case in all TRP channels, neutralizing a positively charged residue in the TRPM8 S4 helix, mentioned above, did indeed negatively effect voltage sensitivity [287]. And the last strong piece of evidence for a functionally significant S1-S4 domain in TRP channels, is the identification of residues essential to ligand activation of TRPV1 and TRPM8 in the S1-S4 helices.

The first experiment highlighting the S1-S4 as a ligand binding domain in TRP channels was in 2002, and focused on the fact that bird TRPV1 is insensitive to capsaicin but maintains the same pH- and heat-sensitive properties [162, 121]. Their hypothesis was that chicken TRPV1 might contain a partial binding site that they could identify by comparing with species TRPV1 to TRPV2. Assessing the capsaicin-induced currents and the [³H]-RTX binding of first chimeras centered around the S2-S4 helices and then point mutants, they identified crucial adjacent tyrosine and serine residues in the S2-S3 intracellular loop whose



(a) Capsaicin and the TRPV1 S1-S4.



(b) Menthol and the TRPM8 S1-S4.

Figure 1.23: Functional significance of the S1-S4 in TRP channels. (a) Diagram showing the residues of the TRPV1 S1-S4 suggested to be significant to capsaicin sensitivity. (b) Diagram showing the residues of the TRPM8 S1-S4 suggested to be significant to menthol sensitivity.

mutations selectively removed capsaicin activation (Y511A and S512Y, respectively). Notably, both of these residues are identical in rat and chicken TRPV1, supporting the claim of birds having a partial ‘capsaicin binding pocket’. A second paper in 2004 supported this region as crucial for capsaicin binding by also investigating species differences in capsaicin activation and RTX-binding affinities of TRPV1[86]. Here experiments focused on differences between rat and rabbit TRPV1, to identify M547 and T550 in the S4 helix as responsible for the higher affinity of rat TRPV1 to these two ligands (Figure 1.23). Similarly, mutagenesis experiments on menthol and icilin binding to TRPM8 have identified important residues in the S1-S4 (Figure 1.23) [48, 287, 14].

1.5 Thesis Aims

This thesis will address the hypothesis that in TRP channels, like in voltage-gated potassium channels, the S1-S4 helices form a significant sensory domain. In the process issues in eukaryotic membrane protein structural biology (Section 1.2) and lipophilic ligand interactions with membrane proteins (Section 1.3) come to the forefront.

While it is clear that the mechanisms of voltage sensing of TRP channels are not identical to those of the canonical voltage-gated Na^+ and K^+ , the conserved six helix architecture, ligand binding and toxin binding propensity of the helices suggests that gating mechanisms and the function of the S1-S4 helices may be related to those of other members of the VGL channel family (1.1,1.4). This thesis aims to address the structural and functional modularity of the TRP channel S1-S4 transmembrane helices by:

1. Biochemically and biophysically addressing the structural modularity of the S1-S4 helices of TRPV1 and other channels.
2. Determining the functional modularity by ligand binding assays of the S1-S4 helices of TRPV1, after first assessing the functionality of the WT overexpressed protein.
3. Investigating the modularity of and ligand binding to TRPV1 using computational modelling and simulation methods.

In the subsequent chapters, after an explanation of the materials and methods (Chapter 2), these aims are addressed by first validating expression systems and functional assays for wild-type TRPV1 (Chapter 3), addressing both the structural and functional modularity of the transmembrane helices using these methods (Chapter 4), building TRP channel homology models and using molecular simulation to address TRP transmembrane modularity (Chapter 5), and finally, investigating the significance of the interaction of TRPV1 ligands with the lipid bilayer to mechanisms of binding and channel activation (Chapter 6).

Materials and methods

The materials and methods used in this thesis are composed of two distinct parts: the first are the experimental methods, which focus on the biochemical and biophysical characterisation of TRP channels expressed in *Saccharomyces cerevisiae*, and the second are the computational methods, which focus on molecular dynamics simulations and homology modelling.

2.1 Experimental materials and methods

The experiments in this thesis were conducted either in the lab of Dr. Simon Newstead at the Department of Biochemistry of the University of Oxford, or in the lab of Dr. Peter M. Blumberg at the National Cancer Institute of the U.S. National Institutes of Health. Expression and purification trials in *S. cerevisiae* were performed in the former and radio-labeled resiniferatoxin binding assays were performed in the latter. The instrumentation and chemicals available in each lab are slightly different, therefore some sections have been divided by location.

2.1.1 Instruments

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- AllegraTMX-22R centrifuge [Beckman Coulter]
- Microfuge[®] 16 [Beckman Coulter]
- AvantiTMJ-20 centrifuge [Beckman Coulter]
- OptimaTML-100K Ultracentrifuge [Beckman Coulter]
- Microfuge[®]22-R [Beckman Coulter]
- Innova[®]44 shaker incubators [New Brunswick Scientific]
- iCyclerTM PCR thermal cycler [Bio-Rad Laboratories]
- SpectraMax M3 Microplate Reader [Molecular Devices]
 - Nunc 96-well black optical bottom plate [Nunc]
- TS Series Benchtop E1061 Cell Disruptor [Constant Systems] with Neslab ThermoFlex1400 Recirculating Chiller [Thermo Fisher Scientific]
- Peristaltic pump P-1 [GE Healthcare]
- 5-mL His-trap columns [GE Healthcare]
- PowerEase[®] 500 Power Supply with Novex Mini-Cell [Invitrogen]
- FLA-3000 fluorescent gel imager [FujiFilm]
- ÄKTApurifier system with Box-900, UPC-900, P-900 [GE Healthcare] with FP-2020 Intelligent Fluorescence Detector [JASCO]
 - Superose 6 10/300 GL Tricorn gel filtration column [GE Healthcare]
 - Superdex 200 10/300 GL Tricorn gel filtration column [GE Healthcare]
- Spinning disk confocal Olympus IX8 microscope system (Perkin Elmer UltraVIEW) with an Olympus 100X 1.35 N.A. objective and an EMCCD camera (Hamamatsu) controlled by the VolocityTM program

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- Beckman 367570 Allegra 21 refrigerated centrifuge with Beckman 363000 rotor assembly, bowl H6002 [Beckman Coulter]
- Wallac 1409 DSA liquid scintillation counter

2.1.2 Yeast strain, media, chemicals and kits

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- 0.5 mm glass beads [BioSpec Products]
- PageRuler Prestained Protein Ladder [Thermo Scientific]
- Fluorescent BenchMark Ladder [Invitrogen]
- Amersham ECL PlusTM Western Blotting Detection Reagents [GE Healthcare]
- Ni-NTA superflow resin [Qiagen]
- Complete protease inhibitor cocktail tablets [Roche]
- N,N-Dimethyldodecylamine N-oxide (LDAO), n-Decyl- β -D-maltopyranoside (DM), n-Dodecyl- β -D-maltopyranoside (DDM), n-Dodecylphosphocholine (FC12), n-Hexadecylphosphocholine (FC16), Polyethylene(8)dodecyl ether (C12E8), n-Octyl- β -D-Glucoside (OG), 1,2-Diheptanoyl-sn-Glycero-3-Phosphocholine (D7PC) [Anatrace]
- All other reagents were purchased from Sigma-Aldrich, VWR, Invitrogen and Fisher, unless otherwise noted: PEG 3350, Lithium Acetate, Single-stranded carrier DNA (salmon sperm), Imidazole, Tris, NaCl, Glycerol, Lactate, Glucose, Galactose, etc.

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- Opti-MEM[®] Reduced Serum Media [Gibco]
- Lipofectamine[®] LTX and PLUSTM Reagents [Invitrogen]

- Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) [Gibco]
- 0.25% Trypsin-EDTA [Gibco]
- Resiniferatoxin (2 mM in DMSO)
- ^3H -labeled Resiniferatoxin (5.22 μM in EtOH)
- DPBS with Mg^{++} and Ca^{++}
- Bovine Serum Albumin
- α -glycoprotein
- Ultima GoldTM scintillation liquid (PerkinElmer)
- Fura-2, AM

2.1.3 Plasmids and primers

Cloning TRP channels into the *S. cerevisiae* overexpression system by homologous recombination was performed with a series of plasmids and primers, listed below (Tables 2.1 and 2.2). Primers were designed by the author and ordered from Eurofins MWG Operon. DNA sequencing to confirm clones was performed by SourceBioscience.

Table 2.1: Plasmids used for expression and cloning.

TRP gene (if applicable)	Vector	Source
-	pDDGFP2 GAL Promoter	Newstead Lab
-	pDDGFP2 TEF Promoter	Newstead Lab
-	Leu2d pDDGFP2	Newstead Lab
<i>R. norvegicus</i> TRPV1	pGEMHE	Swartz Lab
<i>R. norvegicus</i> TRPM8	pGEMHE	Swartz Lab
<i>G. gallus</i> TRPV1	pcDNA3	Dr. David Julius, UCSF
<i>G. gallus</i> TRPM8	pFROG3	Dr. David Julius, UCSF
<i>X. laevis</i> TRPV1	pcDNA3	Dr. David Julius, UCSF
<i>X. laevis</i> TRPM8	pUCHE	Dr. David Julius, UCSF
<i>R. norvegicus</i> TRPV1	pEGFP	Blumberg Lab

Table 2.2: **Primers used in cloning.** Capital letters correspond to the sequence required for homologous recombination, except in the case of the Y511A mutant where they correspond to the residue that confers the mutation.

Construct	Sense Primer / Antisense Primer
<i>R. norvegicus</i> TRPV1 WT	ACCCCGGATTCTAGAACTAGTGGATCCCCCatggaacaacgggctagctta AAATTGACCTTGAAAATATAAATTTTCCCCtttctcccctgggaccatgga
<i>R. norvegicus</i> TRPV1 Y511A	tgtggacagcgc C agtgagatac gtatctcact G gcgctgtccaca
<i>R. norvegicus</i> TRPV1 S1-S4 (LL)	ACCCCGGATTCTAGAACTAGTGGATCCCCCatggtacaggacaagtgggac AAATTGACCTTGAAAATATAAATTTTCCCCtctgagatcatcttctcaat
<i>R. norvegicus</i> TRPV1 S1-S4 (SS)	ACCCCGGATTCTAGAACTAGTGGATCCCCCatgaagcgcattcttctacttc AAATTGACCTTGAAAATATAAATTTTCCCCaatcatgacagcatagatgcc
<i>R. norvegicus</i> TRPV1 S1-S6	- AAATTGACCTTGAAAATATAAATTTTCCCCgaaggccttctcatgca
<i>R. norvegicus</i> TRPM8 WT	ACCCCGGATTCTAGAACTAGTGGATCCCCCatgtccttcgagggagccagg AAATTGACCTTGAAAATATAAATTTTCCCCtttgattttattagcaatctc
<i>R. norvegicus</i> TRPM8 S1-S4 (LL)	ACCCCGGATTCTAGAACTAGTGGATCCCCCatggagcaatggtatggagag AAATTGACCTTGAAAATATAAATTTTCCCCgatgagcatccgctgcagcat
<i>R. norvegicus</i> TRPM8 S1-S4 (SS)	ACCCCGGATTCTAGAACTAGTGGATCCCCCatggacacgaagaactggaag AAATTGACCTTGAAAATATAAATTTTCCCCcaggttcctgctcacggt
<i>R. norvegicus</i> TRPM8 S1-S6	- AAATTGACCTTGAAAATATAAATTTTCCCCgaaggggaaggggatgtt
<i>G. gallus</i> TRPV1 WT	ACCCCGGATTCTAGAACTAGTGGATCCCCCatgtcttccattcttgag AAATTGACCTTGAAAATATAAATTTTCCCCgactgacttgcagcgattc
<i>G. gallus</i> TRPV1 S1-S4 (LL)	ACCCCGGATTCTAGAACTAGTGGATCCCCCatgcaagacaagtgggac AAATTGACCTTGAAAATATAAATTTTCCCCtcttaggatcatctttgc
<i>X. laevis</i> TRPV1 WT	ACCCCGGATTCTAGAACTAGTGGATCCCCCatgaagaaaatgggcagtcc AAATTGACCTTGAAAATATAAATTTTCCCCctcagccttggatgtcac
<i>G. gallus</i> TRPM8 WT	ACCCCGGATTCTAGAACTAGTGGATCCCCCatgaggcaccaagaatggc AAATTGACCTTGAAAATATAAATTTTCCCCtttgattttgctggaatctc
<i>X. laevis</i> TRPM8 WT	ACCCCGGATTCTAGAACTAGTGGATCCCCCatgactgtcagaccatcc AAATTGACCTTGAAAATATAAATTTTCCCCctttatcttgcttctgatttc

2.1.4 Expression and purification of eukaryotic membrane proteins from *S. cerevisiae*

The protocols in this section are mostly adopted from Screening membrane protein overexpression using whole-cell and in-gel fluorescence of Drew et al, Nature Protocols (2008) [64].

2.1.4.1 Cloning

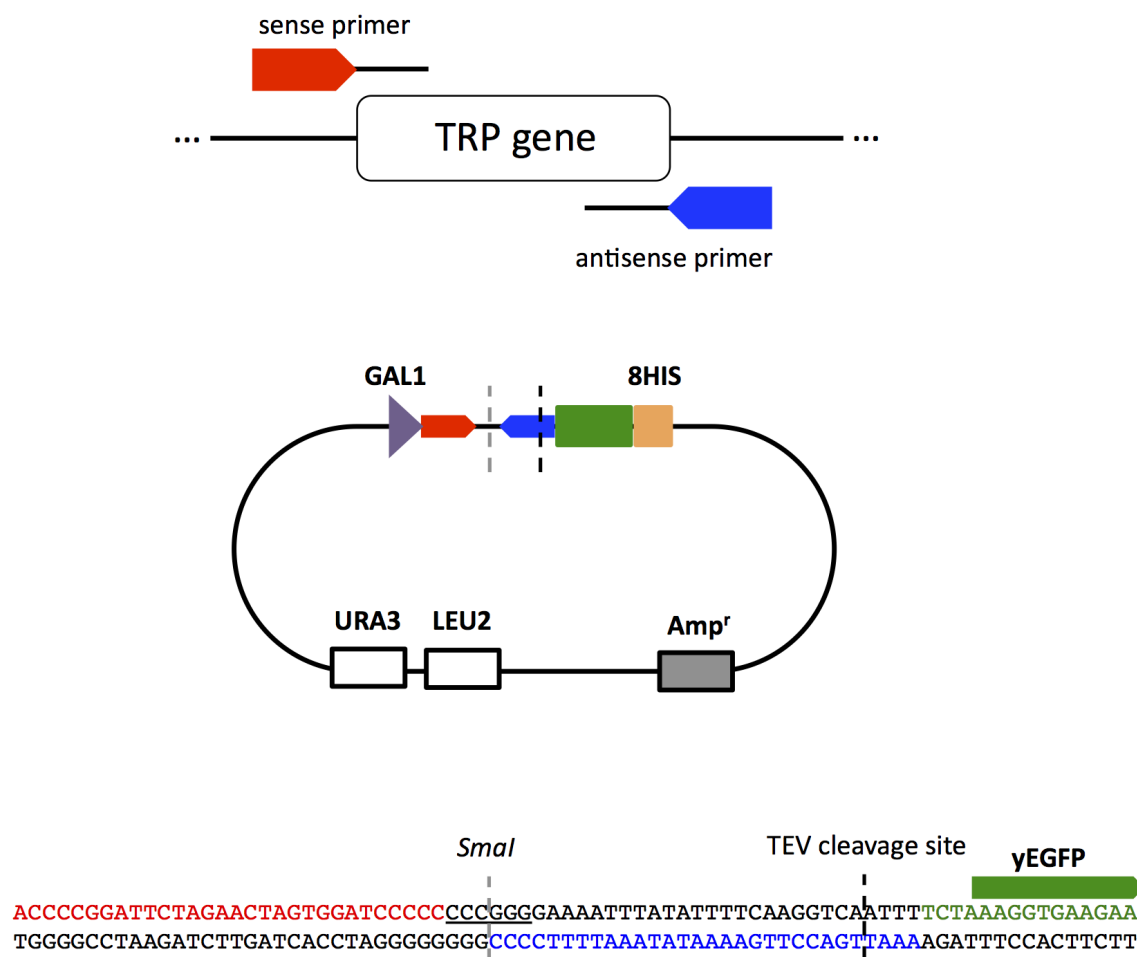
Using the primers and TRP channel plasmids listed above, a recombination sequence was added to both ends of the desired TRP channel sequence, which allowed the gene to be incorporated by homologous recombination into an *Saccharomyces cerevisiae* 2 μ GFP-fusion vector. To create adequate amounts of the DNA fragment, polymerase chain reaction (PCR) was conducted using the PCR thermocycler in the instruments list above and following the protocol below.

SOLUBILISATION SOLUTION

- 30-50 mL membranes
- 1x PBS
- 10% glycerol
- 150 mM NaCl
- 1% detergent
- 1 pellet EDTA-free protease inhibitor
- dH₂O to add up to desired volume

2.1.4.2 Expression trials

Small scale expression trials were then conducted to assess the membrane overexpression of each TRP channel construct. First the construct must be transformed into yeast to allow for the incorporation of the gene. Using a sterile loop, 5 mL of yeast peptone dextrose (YPD) medium in a 50-mL capped tube was inoculated with yeast strain (BJ 3460). This was then

Figure 2.1: **Homologous recombination.**

incubated in an orbital shaker at 280 RPM overnight at 30°C. This overnight culture was then diluted into 50 mL of YPD in a 250-mL shaker flask to an OD₆₀₀ of 0.1. These cells were then grown in an orbital shaker at 280 RPM at 30°C until an OD₆₀₀ of 0.50.6 (~4 hrs). Cells were then centrifuged at 3,000g for 5 minutes at 4°C, and the supernatant discarded. The cell pellet was then resuspended in 25 mL of sterile dH₂O and centrifuged at 3,000g for 5 minutes at 4°C. The supernatant was then discarded and the pellet resuspended in 1 mL of 100 mM lithium acetate (LiOAc). The suspension was then transferred into a 1.5-mL tube and centrifuged at 8,000g for 15 seconds. The supernatant was then discarded and the

pellet resuspended in 400 μ L of 100 mM LiOAc.

One 1.5-mL Eppendorf tube was then prepared on ice containing 240 μ L of 50% (wt/vol) PEG 3350 for each transformation, in which 50 μ L of cell suspension was added before vortexing. Then 25 μ L of 2mg/mL single-stranded carrier DNA (ssDNA) was added to each tube before vortexing. A 50 μ L DNA mix must be prepared in advance that contains 3 μ L of 25ng/mL SmaI-digested pDDGFP2-Leu2D vector, 5 μ L of 150 ng/ μ L PCR product and 42 μ L of sterile dH₂O. This was then added to the 1.5-mL Eppendorf tube and vortexed. The PEG, ssDNA, cells and DNA mixture was then incubated for 30 minutes at 30°C and then heat shocked for 25 minutes at 42°C.

After this the mixture was centrifuged at 8,000g for 15 seconds at room temperature and the supernatant discarded. Excess PEG was then removed with a pipette, and the pellet resuspended with 100 μ L of sterile dH₂O. The cell suspension is then pipetted onto -URA selective plates and incubated for 23 days at 30°C, at which point any creamy colony was restreaked onto a LEU selective plate and incubated for 23 more days at 30°C.

A creamy colony is then picked from the LEU restreak using a sterile loop and is then used to inoculate 10 mL of LEU selective medium with 2% glucose in a 50-mL aerated capped tube, which is then incubated at 30°C in an orbital shaker at 280 RPM for ~22 hrs. This overnight culture can be used for a yeast miniprep to confirm cloning and be transformed into *E. coli* for the creation of a stable plasmid (see yeast miniprep and confirmation via PCR). The overnight culture was then diluted into 10 mL of LEU with 2% lactate in a 50-mL aerated capped tube to an OD₆₀₀ of 0.3. Cells were then grown at 30°C in an orbital shaker at 280 RPM to an OD₆₀₀ of 1.0 (12-20 hours). Protein expression was then induced by adding 2% galactose (1 mL 20% (wt/vol) galactose). Cells were then harvested after 24 hours by centrifugation at 3,000 g for 5 minutes. The supernatant was then removed and the pellet resuspended in a total volume of 200 μ L, adding 1x PBS if necessary. The 200 μ L cell suspension was then transferred to a black Nunc 96-well optical

bottom plate to measure GFP fluorescence by excitation at 488 nm and emission at 512 nm in the SpectraMax M3 fluorescence plate reader, using the bottom read option. At this point, the cell suspension can be transferred to a 1.5-mL Eppendorf and stored at -20°C for future analysis.

Yeast miniprep and confirmation via PCR. From the overnight culture 200 μ L of cell suspension was transferred to 1.5-mL Eppendorf tube and 250 μ L P1 buffer (or equivalent to this Qiagen miniprep buffer) was added along with 100 μ L glass beads (0.5 mm glass beads from BioSpec Products). This mixture was then vortexed at 4°C for 10 minutes. Cells and beads were then centrifuged at 8,000 g for 15 seconds and the supernatant transferred to a clean 1.5-mL tube. The standard miniprep procedure (e.g. Qiagen manufacturers protocol, etc.) was then followed and the DNA concentration was then estimated by measuring the OD at 260nm using e.g. a NanoDrop system. This plasmid can then be transformed into *E. coli* and sequenced per standard procedure. A PCR with (lower efficiency, cheaper) GoTaq polymerase can be performed on fresh yeast minipreps to confirm the presence of the target gene, using homologous recombination site primers. By running 5 μ L samples from each PCR on a 10% Agarose gel (incl. SyberSafe and 1kb ladder) cloning could be quickly confirmed, the same as protein expression was induced.

	Recipe	Protocol (25x)
4 μ L	5x Green GoTaq Buffer	95°C for 2 min
2 μ L	2mM dNTPs	95°C for 30 sec
10 μ L	dH ₂ O	55°C for 30 sec
1 μ L	Forward Primer	72°C for 3 min
1 μ L	Reverse Primer	72°C for 5 min
1 μ L	dsDNA	4°C for ∞
0.1 μ L	GoTaq Polymerase	

2.1.4.3 Fluorescence spectroscopy

Confocal microscopy of *S. cerevisiae* overexpressing TRPV1 constructs was conducted with help from and in close collaboration with Sarah Dixon and Kok-Lung 'Chris' Chan in the lab of Kim Nasmyth in the Department of Biochemistry at the University of Oxford.

A small-scale expression trial was conducted, and after determining by whole cell fluorescence that the protein had expressed, 1 mL of cell culture was centrifuged at 13000 RPM for 30 seconds. The supernatant is discarded and the cells resuspended in the pellet resuspended in the remaining supernatant. A 2% agarose stamp was made by microwaving 17.8 mL -MED medium with 2 mL 20% glucose, 0.2 mL complete synthetic media, and 0.4 g agarose. Of this, 40 μ L was pipetted onto a slide using a cut 200 μ L tip. This slide was then covered with another slide immediately such that the agarose dries as a very thin circular patch. Then 0.8 μ L of the cell resuspension was then pipetted onto the patch and covered with a cover slip. The cells were then imaged using an EMCCD camera (Hamamatsu) mounted on an Olympus IX8 microscope with 100X 1.35 N.A. objective.

2.1.4.4 In-gel fluorescence and Western blots

While whole cell fluorescence can give a good first indication of protein overexpression, in-gel fluorescence and Western blots allow for a better indication of the quality of the protein, and also serve as a triple check of proper cloning. This is achieved by performing a crude membrane prep of the small scale culture. The 200 μ L cell suspension was transferred to a 2.0-mL tube with clasped lid and 500 μ L 1x PBS was added along with 100 μ L glass beads (0.5 mm glass beads from BioSpec Products). The cells were then vortexed with these beads for 10 minutes 4°C for 10 minutes. The beads and unbroken cells were then spun down by centrifuging at 8,000 g for 15 seconds. A 500 μ L aliquot of the supernatant was then removed to a clean 1.5-mL tube and another 500 μ L 1x PBS was added before

vortexing at 4°C for another 10 minutes. The beads and unbroken cells were then spun down at 8,000 g for 15 seconds and 500 μ L supernatant removed to the 1.5-mL tube.

To pellet crude membranes, this 1 mL of 'broken' cells was then centrifuged at 20,000g in a desktop centrifuge at 4°C for 1 hour. Membranes can also be pelleted using a desktop ultracentrifuge at 120,000g for 1 hour. The supernatant was then carefully removed from the pellet and resuspended in 50 μ L 1x PBS. These membranes can be frozen and stored at -80°C for future analysis.

This membrane fraction was then be combined loaded (2x) Loading Buffer onto a Tris-Glycine (10%, 12%, 15% acrylamide depending on desired protein resolution) gel along with 2 μ L of Invitrogens Fluorescent BenchMark Ladder. The gel was then typically run for for 90 minutes at 125 V. The gel was then rinsed in dH₂O and imaged using a FLA-3000 fluorescent gel imager from FujiFilm with excitation wavelength set to 473 nm and the read-out cut-off filter is set to 520 nm.

For proteins without a GFP tag, a Western Blot using a HIS-directed primary antibody is necessary. Gels of the small-scale membrane fraction were run as above and then soaked in 1x NuPAGE Transfer Buffer before being transferred to a PVDF membrane that has been activated in methanol. With cardboard and sponges on either side inside the transfer cassette, filled with 1x NuPAGE Transfer Buffer, the transfer was conducted for 60 minutes at 170 mA and 30V. The membrane was then blocked in 5mL PBS-T (200mL PBS+ 200 μ L Tween 20 = 0.1% Tween) + 5% milk (1g per 20 mL) for 30 min at room temperature in a 50 mL conical tube with protein side of membrane facing inward. New 5mL PBS-T + 5% milk + His-Tag Primary at 1:3000 dilution (2 μ L Ab in 6 mL PBS-T + milk) was then added and the membrane was incubated at 4°C overnight (or for 2 hrs at room temperature). The membrane was then rinsed with 3x 10 mL PBS-T for 10-15 minutes at room temperature before being incubated with PBS-T + 5% milk + 1:10 000 rabbit HRP-conjugated mouse secondary antibody for 1 hour at room temperature. The membrane was then rinsed with

3x 10 mL PBS-T for 10-15 minutes at room temperature before the membrane was placed protein side up on clingfilm. The membrane was then covered with Amersham ECL PlusTM Western Blotting Detection Reagents (2mL A + 50 μ L B) and allowed to incubate for 10 minutes. The membrane, wrapped in clingfilm, is then exposed to film in a dark room for 10 seconds to 15 minutes, depending on the exposure necessary, and developed.

2.1.4.5 Fluorescence-coupled size exclusion chromatography

For constructs that show high levels of overexpression in whole cell fluorescence and low levels of degradation in either in-gel fluorescence or Western blots, medium scale preparations of 2L final volume can be used to assess the detergent solubility and predicted quality of protein upon solubilisation. A sterile loop with cells from the -LEU restreaks from the small scale trials is used to inoculate 10 mL of LEU selective medium containing 2% glucose in a 50-mL aerated capped tube. This culture was then incubated at 30°C in an orbital shaker at 280 RPM for ~22 hrs. After it was ensured cells were growing the entire 10mL overnight culture was poured into a second 100 mL LEU 2% glucose overnight which was then incubated at 30°C in an orbital shaker at 280 RPM for ~22 hrs.

This second overnight culture was then diluted into 2 L of LEU with 2% lactate to an OD₆₀₀ of 0.3, and the cells were then incubated at 30°C in an orbital shaker at 280 RPM to an OD₆₀₀ of 1.0 (12-20 hours). Protein expression was then induced by adding 2% galactose. After checking expression levels by whole cell fluorescence of 10 mL of culture, cells were harvested 24 hours after induction by centrifugation at 4,000g for 10 minutes. The supernatant was then removed and the pellet weighed before being resuspend in 20 mL cold 1x PBS per Liter of culture. At this point cells can be stored and frozen in 50-mL conical tubes at -80°C.

After this medium scale culture of cells have been harvested, a proper membrane preparation can be performed using the Constant Systems cell disruptor. Cells were broken in

four passes at 25kpsi, 30kpsi, 35kpsi, and 40kpsi. Debris was then removed by centrifugation at 15,000g for 10 minutes. Fluorescence can be used to check cell lysis efficiency by measuring the fluorescence before and after spinning down 'debris.' Membranes were then centrifuged from the supernatant of this by centrifugation at 41,000 RPM at 4°C for 2 hours in OptimaTML-100K Ultracentrifuge using the Ti45 rotor. Membranes were then resuspended in 10 mL cold 1x PBS per membrane pellet using a 10mL syringe. The fluorescence of the resuspended membranes can give an estimate of the protein yield. At this point membranes can be flash-frozen and stored in -80°C in 1 mL aliquots in 1.5 mL Eppendorf tubes.

Then 900 μ L of membranes were transferred to a clean 1.5mL Eppendorf tube, and 100 μ L of 10% detergent (DDM, LDAO, DM, OG, etc.) was added to make 1% final detergent concentration. This membrane detergent mixture was then solubilised for 1 hour at 4°C with gentle agitation. Unsolubilised membranes were then centrifuged out at 100,000g for 45 minutes at 4°C in a desktop ultracentrifuge (a normal desktop centrifuge at 22,000xg may be sufficient). An estimate of solubilisation efficiency can be measured by comparing fluorescence before and after centrifugation. The solubilised membranes were then transferred to a clean 1.5 mL tube before 500 μ L were loaded onto the Superose 6 10/300 column coupled to a fluorometer.

2.1.4.6 Purification

Once it was determined that a construct showed both promising overexpression levels and indications of monodispersity upon detergent solubilisation, large-scale cell cultures were prepared for purification. Cultures were grown as for the medium-scale cultures in the previous section, with the exception of a second overnight culture 1 L LEU 2% glucose instead of 100 mL and this then being diluted into 10x 1L cultures. Membranes from this amount of culture can be stored in a 50-mL conical tube at -80°C after flash freezing.

After membranes have been prepared they are detergent solubilised in a beaker with stirring for 1 hour at 4°C with the recipe below.

Solubilisation Solution

30-50 mL membranes
1x PBS
10% glycerol
150 mM NaCl
1% detergent
1 pellet EDTA-free protease inhibitor
dH₂O to add up to desired volume

Unsolubilised membranes were removed by centrifugation for 1 hour at 4°C at 41,000 RPM in a Beckman Ultracentrifuge with the Ti45 rotor. Solubilisation efficiency can be calculated by measuring fluorescence before and after centrifugation. Solubilised membranes were then incubated with Ni-NTA resin for 2 hours with stirring at 4°C. Before this the Ni-NTA has been washed and resuspended in wash buffer made according to the recipe below. After this two hour incubation the solution was poured through a column. Binding

Wash Buffer

1x PBS
10% glycerol
150 mM NaCl
20 mM Imidazole
0.1% detergent (DDM/LDAO)
dH₂O to add up to desired volume

efficiency can be calculated by measuring the fluorescence of this solution with resin (R) and after flowing through the column (FT). The column was then washed twice: first with 50 mL 20mM Imidazole wash buffer (W1) and then with 150 mL 30mM Imidazole wash buffer (W2). The protein is then eluted with 20 mL 250mM Imidazole wash buffer (E). An estimate of protein yield can be obtained from the fluorescence of the Elution. The column and resin were then cleaned with 0.5 M Imidazole, water, and 20% EtOH for storage at 4°C and reuse.

A Tris-Glycine gel was then run of all purification fractions and stained with Coomassie. If it was determined that pure protein had been obtained from the first step of purification, the elution (E) was then cleaved during overnight dialysis at 4°C with tobacco etch virus (TEV) protease (2mg TEV/1 mg protein as estimated by fluorescence) in 3,500 MWCO dialysis tubing with stirring in 1.5 L dialysis buffer according to the recipe below.

Dialysis & Gel Filtration Buffer
150 mM NaCL
20 mM Tris
3x CMC of detergent

After dialysis the presumably cleaved protein solution (D) was then transferred into clean beaker and filtered through a 22 μ M filter using a syringe (D_{FIL}). Using a peristaltic pump this sample was then passed through a Ni Sepharose High Performance HisTrap column. The flow through (F_{HT}) should contain purified protein from which the GFP has been cleaved, as the GFP and contaminants for the first step of purifications should stick to the higher affinity HisTrap column. The flow through was then concentrated (Conc.) from ~20 mL to 500 μ L using a 50,000 MWCO Vivaspin 20 concentrator, while the HisTrap column was washed (W_{HT}) with 20 mL 20 mM Imidazole Gel Filtration Buffer and then the GFP, uncleaved protein, TEV, and contaminants were eluted (E_{HT}) in 20 mL 250 mM Imidazole Gel Filtration Buffer. The column and resin were then cleaned with 0.5 M Imidazole, water, and 20% EtOH for storage at 4°C and reuse.

Once the flow through from the HisTrap had been concentrated to 500 μ L, and a gel was run to determine that cleavage and the second step of purification were likely to have retained worked, it was run on a Superdex 200 10/300 column in Gel Filtration Buffer, collecting 500 μ L fractions. The fractions corresponding to the peaks on the Size Exclusion Chromatography trace were then run on a Tris-Glycine gel and coomassie stained, to determine the yield and purity of the protein of interest.

2.1.5 Radioligand binding assays using [³H]-labeled resiniferatoxin

This work was performed in the Blumberg lab at NCI, NIH. While the radioligand binding assays are the sole work of the author, the other work done with stable and transient CHO cells was done very much in collaboration with Noemi Kedei, though the author often helped perform elements of the experimental procedures.

2.1.5.1 CHO cell expression

Control RTX-binding experiments were conducted on cell pellets of Chinese Hamster Ovary (CHO) cells stably expressing TRPV1 from *R. norvegicus*.

Subsequent experiments to test the effects of TRPV1 over-expression on RTX binding affinity were conducted on cell pellets of CHO cells transiently expressing different levels of TRPV1 from *R. norvegicus*. This variability in expression levels was achieved by varying the amount of DNA transfected, though it is acknowledged that this does not always provide a predictable correlate to protein expression levels, expression levels determined by GFP fluorescence and the $B_{\max}$ achieved in the binding assays showed the variability did tend to correspond to the amount of DNA transfected.

Radioligand binding assays were ultimately conducted on pellets composed of cells compiled from two identical plates of transfected cells. Three concentrations of the TRPV1 vector were transfected into CHO cells: 4.7, 1.2, and 0.8 μg / plate. Each plate of cells first requires incubating two solutions for 15 minutes in a fume hood at room temperature: (1) the appropriate amount of DNA with 20 μL PLUSTM reagent and 750 μL Opti-MEM[®], and (2) 750 μL Opti-MEM[®] with 30 μL Lipofectamine[®]. These two solutions were then combined and incubated for another 15 minutes. The media was then aspirated from the plates and the 1.5 mL mixture above was added to 5 mL Opti-MEM[®] and then added to the plate of cells. The plates were then incubated for 4 hours at 37°C. At this point the

transfection mix was removed and 10 mL CHO cell media was added (F12 with 10% FBS) before the cells were left to incubate for one day at 37°C. The media was then removed and the cells washed with 3.5 mL DPBS (with Ca^{++} and Mg^{++}). The cells were then removed from the plate by incubating with 1.2 mL Trypsin for 3 minutes at 37°C. The cells were then collected with 9mL DPBS per two plates and added to a 15 mL conical tube. Cells were then pelleted at 1000g for 5 minutes, the supernatant aspirated, and the pellet in the 15 mL conical stored at - 80°C.

2.1.5.2 Radioligand binding assays

A published description of a similar protocol and justifications for aspects of the method and data analysis of radioligand binding to a membrane-bound protein can be found in Lewin & Blumberg, Methods Mol Biol, (2003).

S. cerevisiae cells overexpressing TRPV1 variations to be used in ligand binding experiments were grown as for small scale expression trials, except for the lactate back dilution a 100 mL culture was incubated in a 250 mL flask at 30°C. When it had been 24 hours since protein induction, expression was checked by measuring the fluorescence in a plate reader and cells were spun down in 50 mL conical tubes at 4000g for 10 minutes. The supernatant was removed and the pellet resuspended in 1x PBS, normalising OD₆₀₀ to 75 for each sample. Samples were then aliquoted into 5 x 300 μL aliquots for storage at -80°C or for shipment on dry ice to the lab of Peter Blumberg, NCI, NIH for subsequent radioligand binding assays.

The 300 μL aliquot was then transferred to a 15 mL conical tube and 3mL cold 1x DPBS + 0.25 mg/mL Bovine Serum Albumin (BSA) was added and kept on ice. For experiments conducted on CHO cells, the pellet from two plates of CHO cells (as described above) is resuspended with 3 mL cold 1x DPBS + 0.25 mg/mL BSA in a 15mL conical tube and kept on ice. 'Cold' (non-radioactive) and 'hot' (radioactive) resiniferatoxin (RTX) solutions were

then made in glass tubes on ice, these were made at 7x concentration, such that 50 μ L could be added to a total of 350 μ L in each assay tube and result in the desired concentration. The 'cold' solution resulted in saturating concentration of 100 nM final concentration, and the 'hot' solution was a serial dilution of 6 or 7 concentrations, ranging from either .001 nM to 3 nM or .01 nM to 10 nM final concentration. The 'cold' solution was made by adding 1.05 μ L 'cold' RTX (in 2 mM in DMSO) to 3 mL DPBS + 0.25 mg/mL BSA. The 'hot' solution was made by, for example, adding 4.025 μ L 'hot' RTX (5.22 μ M in EtOH) to 1 mL DBPS + 0.25 mg/mL BSA, and then serially diluting this in glass tubes to correspond to the subsequent 7x concentrations of 1 nM, .03 nM, .01 nM, .003 nM, and .001 nM.

Once the cells had been resuspended in 1x DPBS + 0.25 mg/mL Bovine Serum Albumin (BSA) and the 'cold' and 'hot' RTX solutions made, the assay tubes were setup to allow for triplicate measurements of each 'hot' experiment (1-3, 5-7, 9-11, 13-15, 17-19, 21-23) and a 'cold' experiment for each 'hot' concentration (4, 8, 12, 16, 20, 24) (Figure 2.2). In the 'hot' assay tubes 200 μ L DBPS + 0.25 mg/mL BSA was added followed by 50 μ L of appropriate concentration 'hot' solution followed by 100 μ L of the cell solution. In the 'cold' assay tubes 150 μ L DBPS + 0.25 mg/mL BSA was added followed by 50 μ L of the 'cold' solution followed by 50 μ L of the appropriate concentration 'hot' solution followed by 100 μ L of the cell solution.

The tubes were then vortexed and incubated for 1 hour in a 37°C water bath with gentle shaking, the tubes were then transferred to ice and 100 μ L 2 mg/mL α -glycoprotein (AGP) was added. Each tube was then vortexed and allowed to incubate on ice for 10-15 minutes. The samples were then centrifuged 15 minutes at 12,200 rpm ($\sim$ 12,400g) in Beckman bowl centrifuge. Then 200 μ L supernatant from each tube was transferred to a scintillation vial, and the remaining supernatant was aspirated. The pellet of each tube was then cut from $\sim$ 2 cm of the end of the tube and added to a scintillation vial. Then 3 mL scintillation liquid was added each to each vial, which were then capped, labeled, and vortexed vigorously. The

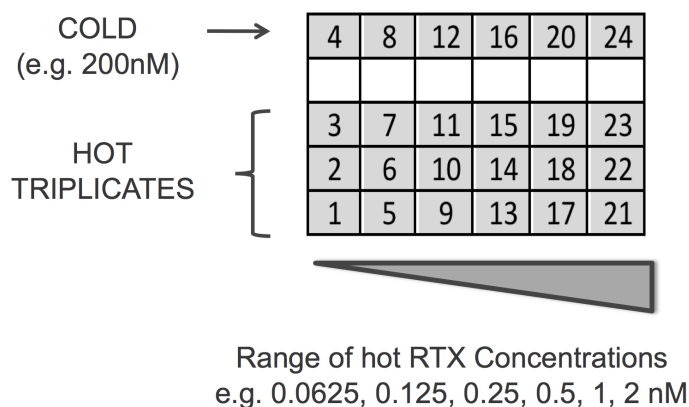


Figure 2.2: **Radioligand binding assay setup.**

racks of scintillation vials were then placed in the scintillation counter, allowing 4 hours of dead time and the tritium radiation was counted for 5 minutes for each vial.

2.1.5.3 Adding allosteric modulators

Experiments performed to investigate modulators of TRPV1 and their effect on RTX binding were performed on membranes prepared by Dmitriy Krepiy in the Swartz Lab in an extra-large scale protein preparation (100 L) in *S. cerevisiae* of rat TRPV1 with a 1D4 tag expressed using a constitutive promoter as in [183].

The TRPV1-activating Double-knot toxin (DkTx) [28], was produced to high purity by HPLC purification in the lab of Kenton J. Swartz by Jeet Kalia and Tsg-Hui 'Helena' Chang. Yeast membranes were diluted in DPBS + BSA and 100 μ L of 100 μ M DkTx was added to 4 mL of these to make a final concentration of 2.5 μ M DkTx. This membrane-DkTx solution, as well as 4 mL of membranes without DkTx, was then incubated for 1 hour at 4°C with gentle agitation. Radioligand binding affinities were then assessed according to the protocol above, adding 100 μ L of these membranes to each assay tube. This assay was then repeated with different concentrations of DkTx.

Experiments assessing the effects of Ca^{2+} /calmodulin-dependent protein kinase (CaMK), ATP and Mg^{2+} were performed in a similar manner. Membranes were incubated with 10 mM Mg^{2+} , 10 mM ATP + 10 mM Mg^{2+} , 10 mM ATP + 10 mM Mg^{2+} , 2 nM CaMK + 10 mM ATP + 10 mM Mg^{2+} , or nothing at all, and RTX binding was determined as described above. Note that the DPBS buffer in which all assays are performed already contains 0.5 mM MgCl_2 .

2.1.5.4 Calcium imaging

Calcium imaging experiments were conducted in smaller plates with small circular slides on the bottom that will fit on the microscope stand, and therefore smaller amounts of all reagents were used, but otherwise the protocol was identical to previously described transient CHO cell expression. The two solutions in this case thus contained (1) 1.4 μg DNA with 10 μL PLUSTM reagent and 100 μL Opti-MEM[®], and (2) 100 μL Opti-MEM[®] with 4 μL Lipofectamine[®]. This mixture was then added to 600 μL Opti-MEM[®] before being incubated with the plate of cells for 4 hours at 37°C. The transfection mix was then replace with CHO cell medium before the cells were returned to 37°C. One day later the cells were washed twice with 1 mL DPBS before the cells were loaded with Loading Solution and incubated at room temperature in the dark for 1 hour and 15 minutes. The Loading Solution consists of adding 200 μL of DMSO to a tube of 1mg of Fura-2AM, and then adding 80 μL of this to 40 mL PBS + 0.25 mg /mL to total 0.01 mg / mL of Fura-2AM. After the cells have been incubating for the allotted time, the Loading Solution is removed and the cells washed with PBS and non-fluorescent media added. The slide on which the cells are attached inside the dish is then placed on the microscope and the ratio of fluorescent emission at 340 and 380 nm from the Fura-2AM dye was then recorded using the InCytIm2 program. After 7.5 minutes 1 μM of capsaicin was added. This was done from by adding 500 μL of 2 μM capsaicin (1 μL of 1mM initially added to 500 μL media) to the 500 μL

media. Fluorescence continued to be recorded for 10 more minutes after that.

2.2 Computational materials and methods

The molecular dynamics (MD) simulations described in this thesis have been conducted using the resources of the Structural Bioinformatics and Computational Biochemistry (SBCB) Unit of the Department of Biochemistry at the University of Oxford.

2.2.1 Instruments

Simulation setup and analysis have been conducted on:

- Workstation ‘indy’: Intel® Core™ i7-2600 3.40GHz (4 cores /8 threads) running Ubuntu 12.04.2 LTS with 32GB memory and a GTX580 graphics card.

Production simulations have been conducted on the larger capacity hardware below, using a Sun Grid Engine (SGE) batch-queueing system and the Probable Batch System (PBS) queuing and job submission system:

- Cluster ‘astrocyte’: 60 nodes with between 8 and 64 cores per node: 8 cores nodes are Quad-Core AMD Opteron™ Processor 2378 with 16 GB or 32 GB memory, 32 core nodes are AMD Opteron™ Processor 6136 with 126 GB memory, and 64 core nodes are AMD Opteron™ Processor 6274 with 126 GB memory (purchased from and maintained by Clustervision).
- Cluster ‘neuron’: 30 node dual core machine, with 4 core (Intel® Xeon® E5410 2.33GHz nodes with 4 GB memory and 8 core Dual Core AMD Opteron™) Processor 280 nodes with 8 GB memory.

2.2.2 Programs

Simulations were performed using versions 4.5.5 and 4.6 of the Gromacs open-source software package [23]. Gromacs comes with a variety of ready-made setup and analysis tools that have been used throughout this thesis [138, 301]. Other analysis tools from the VMD software [109], PyMol [244], MDAnalysis [176], and in house scripts have been used when more flexible or usable tools were necessary. In addition JalView, MODELLER and PSICOV were used for sequence visualisation, homology modelling and covariance analysis respectively [49, 75, 119]. Many thanks are due to Jean Hélie, Phill Stansfeld, Caroline Dahl, and Joseph Goose for scripts used in the setup and analysis of simulations and models described herein.

2.2.3 Molecular dynamics simulations

2.2.3.1 The classical approach

With the advent of quantum mechanics, it became possible to completely describe the motions of particles in space and time from first principles. However, to fully describe these probabilities for a molecule, for example of interest to a chemist or biologist, quickly becomes complicated. The number of elementary particles in any molecule more complicated than a hydrogen atom renders the calculations computationally intensive and often intractable. While methods are being developed to increase the efficiency of calculating more accurate quantum-like descriptions of molecules [195], the standard method for systems of biological relevance is a classical method based on the Born-Oppenheimer approximation [29]. In the Born-Oppenheimer approximation, the large mass difference between the electrons and the nuclei is taken advantage of to assume that the electronic configuration can instantaneously adjust to the nuclear position, which allows the position of each atom to be described by a single set of Cartesian coordinates.

It is worth noting that the classical treatment of molecular dynamics (MD) results in a variety of limitations, mostly related to polarisation. For example, charged molecules are modelled by a static partial charge added during the initial parameterisation of the molecule and protonation is not an observable phenomenon. While a variety of techniques have been developed to impose polarisability within a classical force field, these are not standard practice [223, 230]. Despite this caveat, the classical approximation in MD has provided effective and informative descriptions of a variety of systems for over half a century: a first description of the motions of hard spheres appeared in 1959 [4], followed by a study of liquid Argon in 1964 [226], and in 1977 the first MD simulation study of a protein was conducted [171].

This classical treatment allows for the motions of the particles to be described simply by Newton’s familiar equations of motion (eq. 2.1), which can also be written as a potential energy function (U) that depends on the positions of all N particles in the simulation (eq. 2.2). Where $\mathbf{F}_i$ is the force on the i th particle, $\ddot{\mathbf{r}}_i(t)$ is the acceleration and m_i is the mass of each particle.

$$\mathbf{F}_i = m_i \ddot{\mathbf{r}}_i \quad (2.1)$$

$$\mathbf{F}_i = -\nabla_i U(\mathbf{r}_1, \dots, \mathbf{r}_i, \dots, \mathbf{r}_N) \quad (2.2)$$

2.2.3.2 The force field

The ‘force field’ in MD simulations is comprised of this potential energy function (U) in combination with the parameters for standard atom types. The classical approximation of MD defines atoms not by their wave function, but as charged mass points that are bonded to other atoms via a harmonic force, termed the ‘ball and spring’ model. A given MD force

field defines its U with two categories of interactions: (1) bonded interactions between covalently bound atoms and (2) non-bonded interactions between all atoms (eq. 2.3).

$$U = U_B + U_{NB} \quad (2.3)$$

Several force fields are commonly used for biochemical applications: the Chemistry at Harvard Molecular Mechanics (CHARMM) force field [37], the Assisted Model Building with Energy Refinement (AMBER) force field[41], the Groningen Molecular Simulation (GROMOS) force field [278], and the Optimised Potentials for Liquid Simulations (OPLS) force field [122]. The following sections provide an overview of bonded and non-bonded interactions and how their treatment differs within these common forcefields.

Bonded interactions

The contributions to bonded interactions can be divided into four groups: (1) bond stretch, (2) bond angle bending, (3) dihedral angle rotations and (4) improper dihedrals (eq. 2.4) (Figure 2.3).

$$U_B = U_{bonds} + U_{angles} + U_{dihedrals} + U_{impropers} \quad (2.4)$$

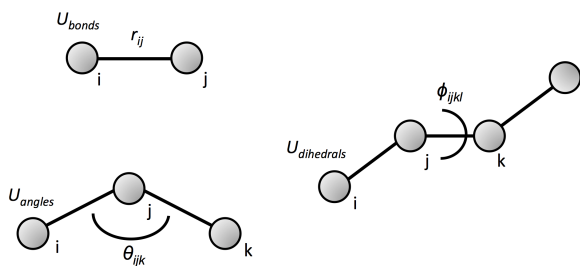


Figure 2.3: Bonded interactions.

The bond stretch potential is defined by a classic harmonic oscillator, in which the atoms make small deviations from their equilibrium bond length (b_{ij}) according to equation 2.5), where k_{ij} is the spring constant of the bond and r_{ij} is the distance between the two atoms, i and j .

$$U_{bonds}(r_{ij}) = \frac{1}{2}k_{ij}(r_{ij} - b_{ij})^2 \quad (2.5)$$

The bond angle bending potential is defined by harmonic angular vibrations according to equation 2.6, in which θ^0 is the equilibrium angle between atoms i , j and k and θ is the angle between atoms i , j and k .

$$U_{angles}(\theta_{ijk}) = \frac{1}{2}k_{ijk}(\theta_{ijk} - \theta_{ijk}^0)^2 \quad (2.6)$$

The potential describing the dihedral rotations, or bond torsions, are described according to equation 2.7, in which ϕ^0 is the phase shift, n the multiplicity and k_ϕ the dihedral force constant.

$$U_{dihedrals}(\phi_{ijkl}) = k_\phi(1 + \cos(n\phi_{ijkl} - \phi_{ijkl}^0)) \quad (2.7)$$

A different form of the dihedral potential, used in the OPLS-AA force field, is a variant on the Ryckaert-Bellemans function and is described by equation 2.8.

$$U_{dihedrals-OPLS}(\phi_{ijkl}) = U_0 + \frac{1}{2} \sum_{n=1}^3 U_n(1 + \cos(n\phi_{ijkl})) \quad (2.8)$$

Improper dihedrals restrict are described similarly to equation 2.6, and are used to keep aromatic molecules planar or prevent flipping of chiral molecules.

Non-bonded interactions

Quantum mechanically, non-bonded interactions are defined only by the Coulomb potential. However, to accurately describe non-bonded interactions in a classical potential it is necessary to define two non-bonded interactions: (1) those defined by the Lennard-Jones potential and (2) electrostatic interactions. The Lennard-Jones potential combines a description of the strong repulsion of atoms whose atomic (Van der Waals) radii are overlapping and the weak attraction of atoms by Van der Waals forces into one equation (eq. 2.9), where ϵ_{ij} is the depth of the attractive well and σ_{ij} is the distance at which the potential energy vanishes.

$$U_{LJ}(r_{ij}) = 4\epsilon_{ij} \left(\left[\frac{\sigma_{ij}}{r_{ij}} \right]^{12} - \left[\frac{\sigma_{ij}}{r_{ij}} \right]^6 \right) \quad (2.9)$$

The electrostatic interactions between two particles, i and j are described by Coulomb's law (eq. 2.10), where q_i and q_j are the partial charges of the atoms, and ϵ_0 is the permittivity of free space. The assignment of the partial charges q to atoms in a force field, is a key element of force field parameterisation.

$$U_C(r_{ij}) = \frac{q_i q_j}{4\pi\epsilon_0 r_{ij}} \quad (2.10)$$

Describing electrostatic interactions by Coulomb's law, however, rapidly becomes a computational burden, even in classical MD, as the system size increases. This is because, unlike bonded interactions where computational complexity increases with the number of atoms, N , the computational complexity in calculating non-bonded interactions increases on the order of N^2 , since all atoms effect all other atoms of the system. This requires some form of cut-off scheme or other method for reducing the computational burden of long-range non-bonded interactions. However, unlike for the Lennard-Jones potential, introducing a

cut-off for the Coulomb potential would introduce severe inaccuracies, since it only decays as r^{-1} . One widely used method in MD for getting around this problem is the Particle Mesh Ewald (PME) summation method, which splits the Coulomb energy into two parts using the identity

$$\frac{1}{r} = \frac{f(r)}{r} + \frac{1-f(r)}{r}. \quad (2.11)$$

By carefully choosing $f(r)$, generally the complementary error function $\text{erfc}(r)$, the Coulomb potential can be evaluated using a discrete fast Fourier transform provided a grid has been assigned on which the forces can be assigned back to the particles.

Force field parameterisation

With these interactions defining the definition of classical MD, parameterising these force fields can be done in a variety of ways. The two force fields used in this thesis are the OPLS force field for capsaicin simulations, and the GROMOS 53a6 force field for the majority of protein simulations. The GROMOS 53a6 forcefield is the most recent update of the GROMOS forcefield originally used with the first implementation of the Gromacs software. Its parameters were optimised to the thermodynamic properties of liquids and small molecules as well as the solvation free enthalpies of amino acid analogs in cyclohexane [204]. The recommended water molecule for use with the GROMOS forcefield is the simple point charge (SPC) water model, which is composed of the three simple point charges, positive for the two hydrogens and negative for the oxygen, at a 109.47° angle. The hydrogens have no Lennard-Jones component, and the dipole is slightly enhanced from experimental observations to account for polarisation effects. The OPLS all atom force field was parametrised by ab initio molecular orbital calculations of more than 50 molecules that were then compared to experimental data for heats of vaporisation and densities [122]. The

OPLS forcefield recommends the TIP4P water model which is similar to the SPC water model, but breaks the oxygen atom into two particles, one of which is a negatively charged virtual site.

While both these forcefields are atomistic, some molecules are parametrised as united-atom molecules where hydrogen atoms are not explicitly defined. For example, in this thesis the 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) lipid that makes up the simple bilayer is a united-atom molecule in both forcefields. This is effective because 79 of 131 total atoms of POPC are non polar hydrogens in an all-atom model, but when converted to a united-atom representation the molecule becomes only 52 atoms, reducing computational cost with minimal effects on the result. The coarse-grained (CG) MARTINI force field was also used for the initial bilayer self-assembly process [165, 257]. CG forcefields lump many atoms together into one particle to speed up computational efficiency, for example alanine is one particle and no amino acid is more than four particles. More on the benefits and parameterisation of the MARTINI force field can be found a recent review [166].

2.2.3.3 Simulation techniques

With the force field defining the potentials that describe the molecular motions of a system, next are the factors taken into account in implementing these equations.

Equations of motion and time integration

With Equation 2.2 describing the motions of atoms in an MD system, it must now be integrated numerically in time. This is performed with a finite difference method using the time step Δt . The Verlet algorithm is a simple and robust scheme derived using the Taylor expansion, commonly used in MD for the integration of Newton's equations [283] (eq. 2.12).

$$\begin{aligned}
r_i(t + \Delta t) &= r_i(t) + \Delta t \dot{r}_i(t) + \frac{\Delta t^2}{2!} \ddot{r}_i(t) + \frac{\Delta t^3}{3!} \dddot{r}_i(t) + \mathcal{O}(\Delta t^4) \\
\Rightarrow r_i(t + \Delta t) &= 2r_i(t) - r_i(t - \Delta t) + \frac{\mathbf{F}_i(t)}{m} (\Delta t)^2 + \mathcal{O}(\Delta t^4)
\end{aligned}
\tag{2.12}$$

One of several versions of the Verlet algorithm is the leap-frog algorithm, used in this thesis, which calculates velocities at half time-step intervals from the time at which positions are defined (eq. 2.13).

$$\begin{aligned}
\mathbf{r}_i(t + \frac{\Delta t}{2}) &= \mathbf{r}_i(t) + \mathbf{v}_i(t + \frac{\Delta t}{2}) \Delta t \\
\mathbf{v}_i(t + \frac{\Delta t}{2}) &= \mathbf{v}_i(t - \frac{\Delta t}{2}) + \frac{\mathbf{F}_i(t)}{m_i} \Delta t
\end{aligned}
\tag{2.13}$$

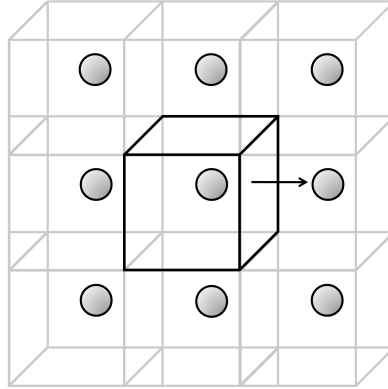


Figure 2.4: **Periodic Boundary Conditions.**

The simulation box and periodic boundary conditions

While a given simulation will have a finite size, denoted by the simulation box, and number of atoms, imposing a firm boundary of a vacuum, infinite potential or any other boundary type would cause severe artifacts in the simulation. This problem is addressed

by the presence of periodic boundary conditions (PBC), which replicate the system to itself on all sides of the simulation box (Figure 2.4). This means that both bonded and non-bonded interactions are effective across boundary conditions, making approximations like PME crucial to both calculation efficiency and reducing artifacts [212]. Several types of simulation boxes can be used, depending on the types of artifacts from PBC one seeks to reduce. The most common box type is the cubic-rectangular box, used in all simulations in this thesis.

Temperature and pressure control

In essence a simulation box is a microcanonical ensemble of constant number of particles (N), volume (V) or pressure (P), and temperature (T), defined according to standard statistical mechanics. To maintain constant energy MD programs must implement one of a range of available barostats or thermostats, that mimic coupling to a heat bath with a given temperature T_0 . This also helps to overcome any fluctuations in the total energy that might come about due to integration errors. To achieve this the temperature, T , is computed from the velocities of the particles in the system by equating two equivalent definitions of the kinetic energy (eq. 2.14), where k_B is the Boltzmann constant.

$$\frac{1}{2} \sum_{i=1}^N m_i v_i^2 = \frac{1}{2} N k_B T \quad (2.14)$$

The Berendsen thermostat facilitates this internal coupling by scaling the velocities at each time step according to a velocity scaling parameter (eq. 2.15), where τ_T is the coupling parameter and T_0 is the reference temperature. In the simulations in this thesis a thermostat similar to Berendsen's was used that has an extra stochastic parameter that ensures a proper canonical ensemble is generated [22].

$$\lambda^2 = 1 + \frac{\Delta t}{\tau_T} \left(\frac{T_0}{T(t)} - 1 \right) \quad (2.15)$$

The Berendsen barostat, used here for pressure coupling, operates under a similar concept, but with more complex mathematics relating the pressure tensor to the kinetic energy to adjust the box vectors and scaling coordinates in relation to a preset τ_{au_p} and p_0 . In this case, semiisotropic pressure coupling was applied, keeping the xy -plane of the simulation box at a constant size. This method allows the system to fluctuate as needed while keeping the effects on the lipid bilayer in the xy -plane to a minimum.

2.2.3.4 Free energy calculations

Generally, MD simulations are allowed to progress freely without constraints and it is assumed molecules will tend toward conformations of minimum free energy over time. In theory an MD simulation is ideal for measuring contributions to changes in free energy for a given system, as the conformations of all components of a state space are available. However, in practice, the sampling available from equilibrium MD simulations is not adequate for direct measurements of free energy. This is due both to the small timescales available using the current computational resources and that high energy states will be scarcely sampled in a simulation quickly converging toward its lowest energy state. Enhanced sampling methods have been developed to address this issue, including free energy perturbation methods [314], thermodynamic integration [260], steered MD [210], umbrella sampling [273], metadynamics [139], adaptive biasing force [59], and markov state models [225]. In Chapter 6, the umbrella sampling method, described below, is used to calculate estimated free energy barriers of capsaicin bilayer penetration.

The potential of mean force

The potential of mean force (PMF) is defined as the change in free energy as a function of a chosen reaction coordinate (χ) [143]. PMF calculations have been used extensively to characterise ion permeation through channels [18, 9, 24]. These studies are likely to accurately reflect the free energy wells and barriers because the reaction coordinate is confined to the one dimensional path of a single particle with few degrees of freedom to explore. Generally, the PMF, $W(\chi)$, will be defined from the average distribution function $\langle\rho(\chi)\rangle$ using the reaction coordinate and a chosen reference point along that reaction coordinate, (χ^*) (eq. 2.16).

$$W(\chi) = W(\chi^*) - k_B T \ln \left[\frac{\langle\rho(\chi)\rangle}{\langle\rho(\chi^*)\rangle} \right] \quad (2.16)$$

Umbrella sampling

While it is technically possible to calculate a PMF from an equilibrium simulation, in practice the sampling of high energy states will be poor, so one of the methods for enhanced sampling mentioned above are required. Here umbrella sampling is used, which breaks the reaction coordinate up into N_w windows, and applies a harmonic potential, $\omega_i(\chi)$, with a force constant, K , to constrain the molecule within that window, χ_i (eq. 2.17).

$$\omega_i(\chi) = \frac{1}{2} K (\chi - \chi_i)^2 \quad (2.17)$$

Simulations of each window then produces a local, biased probability distribution (eq. 2.18). From each of these, a cumulative unbiased probability distribution can be produced when taking into account the free energy required to introduce the biasing, F_i .

$$\langle\rho(\chi)\rangle_i = \frac{e^{-\omega_i(\chi)/k_B T}}{\langle e^{-\omega_i(\chi)/k_B T} \rangle} \langle\rho(\chi)\rangle \quad (2.18)$$

$$W_i(\chi) = W(\chi^*) - \frac{1}{k_B T} \ln \left[\frac{\langle \rho(\chi) \rangle_i}{\langle \rho(\chi^*) \rangle} \right] - \omega_i(\chi) + F_i \quad (2.19)$$

The Weighted Histogram Analysis Method

The Weighted Histogram Analysis Method (WHAM), is one of several methods to produce an unbiased profile from umbrella sampling by iteratively determining appropriate values for F_i [236, 138]. The WHAM method uses all data from the N_w windows to create a χ -dependent weighted sum of the unbiased distribution functions, explicitly using the number of data points (n_i) with which the distribution function was calculated for each window (eq. 2.20). It then uses this unbiased distribution function to iteratively solve for F_i , starting from an initial guess, until the maximum difference between $F_i^{current}$ and F_i^{last} converges to a defined, preset tolerance.

$$\begin{aligned} \langle \rho(\chi) \rangle &= \frac{\sum_{i=1}^{N_w} n_i \langle \rho(\chi) \rangle_i}{\sum_{j=1}^{N_w} n_j e^{-[(\omega_j(\chi) - F_j)/k_B T]}} \\ e^{-F_i/k_B T} &= \int e^{-\omega_i(\chi)/k_B T} \langle \rho(\chi) \rangle dx \end{aligned} \quad (2.20)$$

2.2.3.5 Practical choices for simulating TRPV1 agonists in a lipid bilayer

A range of choices is available in setting up an MD simulation, many of which are included in the initial choice of the force field. In addition, parameters for pressure and temperature coupling and non-bonded interaction cut-offs are required. For example, in the simulations of small molecules with a simple bilayer using the OPLS-AA force field, PME was chosen as the non-bonded interaction type and the parameters $\tau_p = 1$ ps, $p_0 = 1$ bar, $\tau_T = 0.1$ ps and $T_0 = 323$ K were chosen. In addition, parameters for non-standard molecules must be defined. All such molecules were parametrised in the OPLS force field using

Marvin to draw and characterise chemical structures (Marvin 5.10.1, 2012, ChemAxon, <http://www.chemaxon.com>), topolbuild version 1.3 to perform initial parameterisation, and manual curation as well as the program ‘topfill,’ written locally by James Dunbar, were used to clean up parameter sets. A complete set of parameters produced can be found in the Appendix.

2.2.4 Homology modelling

The reliability of MD simulations depends on the reliability of the input structures and parameters chosen at the outset. To be able to approach TRP channels via atomistic simulation methods, a homology model was built. Homology modelling allows for an atomistic prediction of a protein structure using the structure of a related protein. The method relies on the assumption that structural identity is generally more conserved than sequence identity. In the case of TRP channels, where sequence identity is $\sim 16\%$ at best, other promising studies of homology models with similar sequence identity such as of the Hv1 proton channel and a couple of the family of Major Facilitator Superfamily (MFS) of transporters show that even this low sequence identity can result in useful models [227, 107]. Membrane proteins, especially, show high structural conservation in the transmembrane domain [201].

In this thesis the program MODELLER, a widely adopted and effective tool for comparative protein modelling, was used to create homology models of TRP channels [75]. The program imposes restraints on the sequence from the target protein, adjusts the protein such that it conforms to basic peptide chemistry, and then performs an iterative energy minimisation procedure, with force fields similar to those described above. Often, the region of least sequence identity in membrane proteins are ‘loop’ regions, for which the challenge of predicting a proper structure are on par with a small protein-folding simulation. Modeller has a tool build specifically to address these loops, which performs loop modelling with a force field based on the CHARMM force field [76].

2.2.5 Covariance analysis

While homology modelling can provide an excellent basis for understanding the secondary, tertiary and quaternary structure of a protein when high resolution biophysical data is not available, another computational method was used in this thesis in the hopes of improving the understanding of the spatial distribution of amino acids in TRP channel quaternary structure. This method is covariance analysis, which predicts contacting residues by identifying evolutionary covarying amino acids in provided sequence alignments. These predicted contacts can then be simply plotted to determine their general distribution across the protein. The majority of predicted contacts will likely be along the diagonal, which indicates the interaction of proximal residues. The most interesting interactions for adding constraints to homology models, however, are those that are off-diagonal and therefor might predict inter-domain interactions.

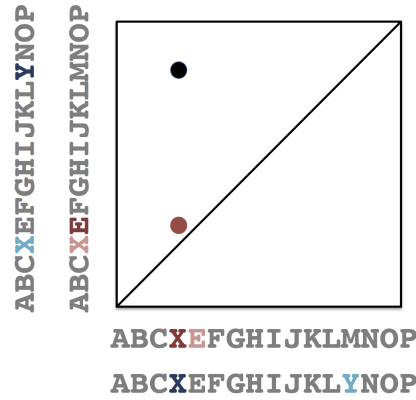


Figure 2.5: **Evolutionary covariance analysis.**

While several methods can be used to identify co-evolved residues [235, 263], the PSICOV software was used in this thesis [119]. This software has improved discrimination between directly and indirectly coupled mutants from previous methods by using the graphical Lasso procedure [79] to calculate the final PSICOV score for positions i and j (eq. 2.21) from the initial covariance matrix (eq. 2.22), where $S_{ij}^{contact}$ is the raw precision norm,

$\bar{S}_{i-}^{contact}$ and $\bar{S}_{j-}^{contact}$ are the mean precision norms between that alignment column and all other alignment columns, $\bar{S}^{contact}$ is the mean precision norm across the whole alignment, n is the number of rows in the alignment, and x_i^{ak} and x_j^{bk} are the binary variables ($x \in \{0,1\}$) indicating the presence of absence of the amino acid a or b at column i or j in row k .

$$PC_{ij} = S_{ij}^{contact} - \frac{\bar{S}_{i-}^{contact} \bar{S}_{j-}^{contact}}{\bar{S}^{contact}} \quad (2.21)$$

$$S_{ij}^{ab} = \frac{1}{n} \sum_{k=1}^n (x_i^{ak} - \bar{x}_i^a)(x_j^{bk} - \bar{x}_j^b) \quad (2.22)$$

This method has been successfully incorporated into the FILM3 modelling suite, which uses secondary structure prediction, covariance analysis and fragment libraries to predict the 3D structure of membrane proteins [200]. In this thesis the JACKHMMER suite, which uses hidden Markov models to search for relevant sequences from a large database and align them iteratively [118], was used to create the alignment inputted into the PSICOV software, and predicted interactions with greater than 60% probability of interacting were plotted as in Figure 2.5.

Evaluating expression systems for TRP channel overexpression

3.1 Introduction

Despite a wide interest in the medical and biophysical properties of TRP channels, their mechanisms remain poorly understood. One reason for this is a lack of structural characterisation. Two reasons a structural understanding of TRP channels has eluded the field so far are:

- ▷ A lack of prokaryotic homologues, which currently constitute the majority of structurally characterised membrane proteins;
- ▷ An inherent flexibility may be functionally required to facilitate the known polymodal sensitivities of TRP channels.

Membrane protein structural biology, however, is rapidly developing techniques with the potential to tackle these challenges. In addition to structural characterisation, a firm understanding of which properties are intrinsic to TRP channels similarly requires efficient and reliable methods for obtaining pure protein. This chapter addresses promising avenues

for approaching TRP channel, most specifically TRPV1 from *Rattus norvegicus*, overexpression, detergent extraction and functional characterisation.

3.1.1 Expression and purification of TRP channels to date

Since the initial discovery that the TRP gene codes for ion channels similar to the voltage-gated family of channels [186], little direct structural information of the transmembrane region of any members of the seven TRP subfamilies has been determined. Successful characterisation of the overall structures of some TRP channels has been achieved via electron cryomicroscopy [248, 58, 168, 180, 183], revealing large cytoplasmic domains and confirming the expected tetrameric assembly. These low resolution structures span almost the entire range of TRP subfamilies with TRPC, TRPM, TRPV, and TRPA all represented (Figure 3.1). While the transmembrane regions are present in these structures, the highest resolution achieved so far is 15Å and the technique is known to represent distorted transmembrane regions due to the presence of the lipid bilayer.

In addition to these successes of TRP channel structural biology, several important functional studies have also been carried out on purified, reconstituted TRP channels. The first of these studies solubilised TRPC1 from CHO cells using the detergent OG to study the channel's mechanosensitive properties [163]. Subsequently, a study on TRPM8 determined that functional channels could be successfully expressed and reconstituted from both HEK-293 and *E. coli* expression systems using DDM as a detergent for extraction [309]. A couple of studies were performed on TRPV1 expression and purification [136, 182] from *S. cerevisiae* and Sf9 insect cells with minimal functional studies before a recent study reconstituted TRPV1 from Sf9 cells to confirm several intrinsic properties of TRPV1, such as capsaicin and heat sensitivity [40]. This TRPV1 work from the David Julius lab at UCSF exemplifies why biophysical studies of purified TRP channels are crucial to grasping the intricacies of these multi-faceted proteins. Before this paper it was not clear that the

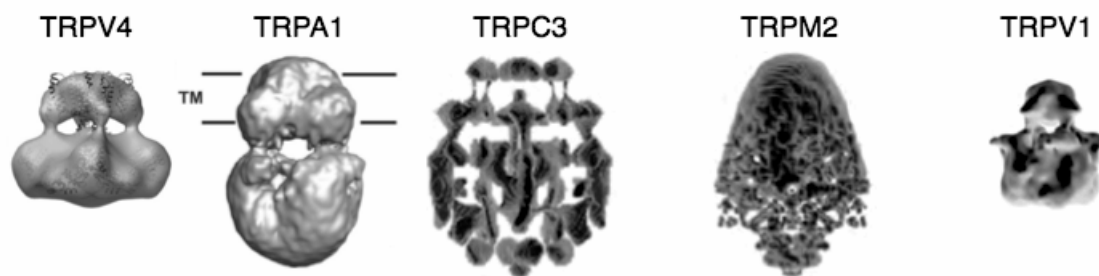


Figure 3.1: EM structures of TRP channels. From left to right: the 98.3 kDa rat TRPV4, a 'warm' and osmosensitive channel, solved to 35Å resolution from an Sf9 expression system [248]; the 127.5 kDa mouse TRPA1, a putative cold and allyl isothiocyanate sensitive channel, solved to 16Å resolution from an *emphS. cerevisiae* expression system [58]; the 95.7 kDa mouse TRPC3, a canonical TRP channel found to be involved in cerebellar ataxia mouse models, solved to 15Å resolution from a HEK-293 cell expression system [180]; the 171.2 kDa human TRPM2, known to be highly expressed in the brain but not well-characterised physiologically, solved to 28Å resolution from a HEK-293 cell expression system [168]; the 94.9 kDa rat TRPV1, the heat and capsaicin sensitive channel, solved to 19Å resolution from an *S. cerevisiae* expression system [183]. Figure adapted from [184], [248], and [58].

chemical and thermal sensitivities of TRPV1 were intrinsic properties of the protein itself rather than the result of some aspect of the cellular environment. However, this work did not explicitly state the yield of purified TRPV1 and the assays presented could be accomplished with relatively small amounts of protein compared to what is necessary for other biophysical techniques such as NMR or crystallography. Additionally, it is mentioned that the maltose binding protein (MBP) tag used to stabilise the protein during expression and purification procedures is not cleavable, indicating a possible protein instability upon purification, though this does not seem to have negatively effected functionality. With this summary it is clear that while progress is being made in TRP channel characterisation, establishing a reliable overexpression system remains vital.

3.1.2 Advantages of *S. cerevisiae* for TRPV1 overexpression

Of the five electron microscopy structures on the previous page, two have been solved using an *S. cerevisiae* expression system, however it remains unclear how far this expression system will take structural studies on TRP channels. In the last decade, more eukaryotic integral membrane protein structures are being solved than ever before, mostly from either yeast (both *Pichia pastoris* and *Saccharomyces cerevisiae*) or insect cells [26] (Figure 3.2). The yeast systems have a distinct advantage over insect cells, being almost as cheap as *E. coli* expression systems in terms of both time and money. *S. cerevisiae* in particular has its own specific advantages, the most notable being that it is amenable to functional screens [189], has an extensively characterised genome [300], and a GFP-tagged screening method for eukaryotic membrane proteins has been established [64, 194]. Additionally, it has already been shown to be a successful system in which to express rat TRPV1 [189, 182, 183]. Previous studies of TRPV1 expressed in *S. cerevisiae* have measured functionality, but only with either calcium imaging [182] or visually observing a cell-death phenotype when plated on media laced with capsaicin [189]. With the other successes of the *S. cerevisiae* as a TRP channel expression system, further characterisation of the structural and functional integrity of the protein it produces is a valuable addition to the assessment of cell types with potential to overexpress TRP channels, an essential first step before entering the structural era for TRP channels [85].

3.2 Overexpression of TRPV1 in a variety of systems

To obtain pure protein for subsequent structural and functional characterisation, an optimal expression system must be identified for protein overexpression. When possible *E. coli* is a preferred expression system due to the relatively small amount of time, money and overall effort that is required to maintain reliable overexpression. However, *E. coli* lacks

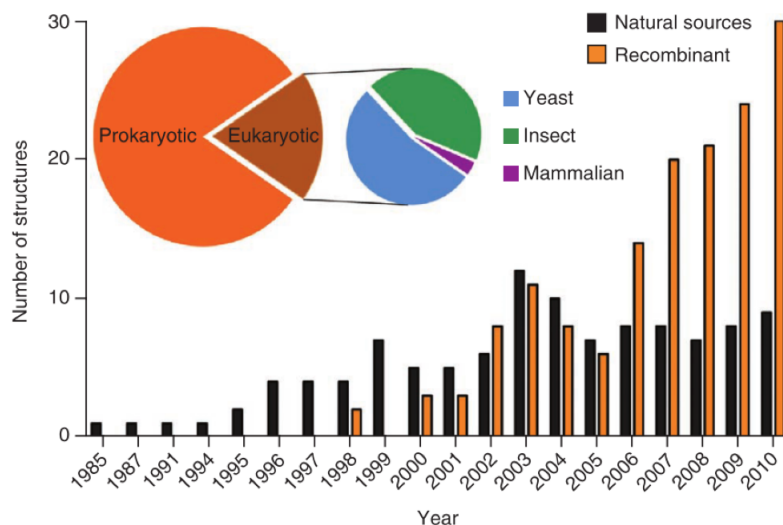


Figure 3.2: **Progress in solving structures of membrane proteins.** Yeast contributes over half of the successes in recombinant expression of eukaryotic membrane proteins. Image adapted from [26].

much of the cellular machinery required for successful production of eukaryotic proteins. For this reason eukaryotic yeast, usually *Pichia pastoris* or *Saccharomyces cerevisiae* are often considered a first choice for eukaryotic protein overexpression. In addition, the range of yeast strains available provides a variety of possible mechanisms to facilitate overexpression of the protein of choice. Crucial to the purification procedure as well are the tags used for either metal affinity or immunoaffinity purification procedures, the anticipation of which is also a key part in screening protein overexpression levels.

3.2.1 Overexpression of TRPV1 in *S. cerevisiae*, *E. coli* and CHO cell overexpression systems

A recent advance in membrane protein expression and purification is the discovery that a C-terminal green fluorescent protein (GFP) tag provides an effective indicator of the amount of protein that has been successfully expressed and inserted into the cell membrane [64, 194, 131]. With this method real-time assessment of overexpression levels is possible by

measuring the whole-cell fluorescence of cells expressing the protein of interest. Additionally, in later steps of protein purification this tag allows for the assessment of detergent solubility and the stability of the protein before approaching the affinity purification steps.

The GFP tag also facilitates visualisation via fluorescence microscopy of how and where the membrane protein is being expressed in the cell. Best case, the protein expresses in the plasma membrane creating a 'halo' effect around the outside of the cell. Worst case, the protein aggregates in the vacuoles of the cell. Fluorescence microscopy of two different cell types, Chinese Hamster Ovary (CHO) and *S. cerevisiae*, expressing the TRPV1 gene from *R. norvegicus* with a GFP tag shows expression profiles between these best and worst cases (Figure 3.3). In the CHO cells, the expression pattern is indicative of canonical endoplasmic reticulum expression [203]. In the yeast cells, however, it is hard to identify the organelles that the TRPV1 seems to most preferentially localise in. However, it primarily seems to localise in the vacuoles, which may be related to the fact that the endogenous yeast TRP channel, YVC1, is a vacuolar protein [208]. Upon further examination of the yeast expression profiles, the protein is also notably expressed in the plasma membrane, though the fluorescence of the protein expressed in the vacuoles masks this in most cases.

Fluorescence imaging and measuring whole-cell fluorescence with a fluorescence plate reader allow initial qualification of protein overexpression, however, much more information is available. In-gel fluorescence (not shown) clearly shows TRPV1 expression as a single, albeit faint, band on the gel. However, to compare whether the GFP tag effects expression over wild-type (WT) protein, Western blots remain essential. These gels also provide an avenue to assess protein quality. Western blots of TRPV1 expression between two overexpression systems resulted in clear bands for TRPV1 with GFP tag (a second band can be seen, likely due to glycosylation), but showed significant degradation for protein from CHO cells (Figure 3.4).

In the *S. cerevisiae* system described here, cells overexpressing GFP-tagged TRPV1

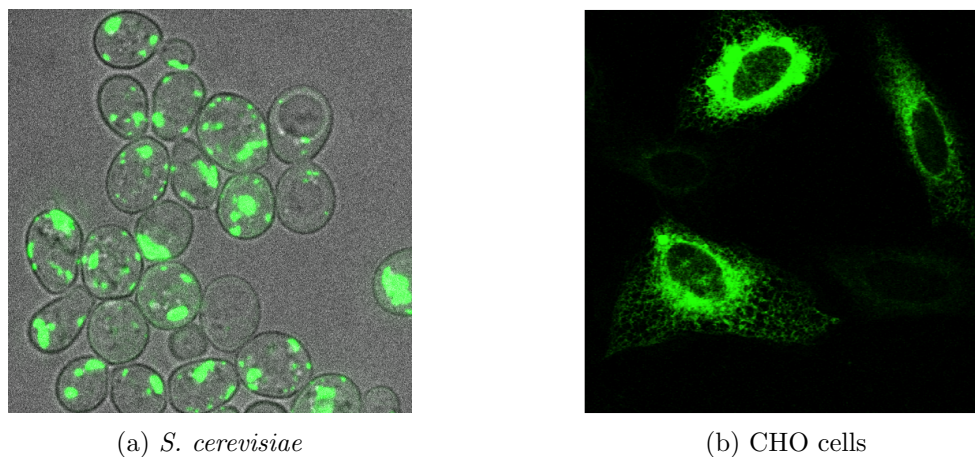


Figure 3.3: **GFP-tagged TRPV1 overexpression in two cell types.** (a) In *S. cerevisiae* though some 'halo'ing can be seen, it seems that TRPV1 expresses mainly in the vacuoles, which may be related to the fact that the endogenous yeast TRP channel, YVC1, is a vacuolar protein [208]. (b) In CHO cells, the expression pattern is indicative of canonical endoplasmic reticulum expression.

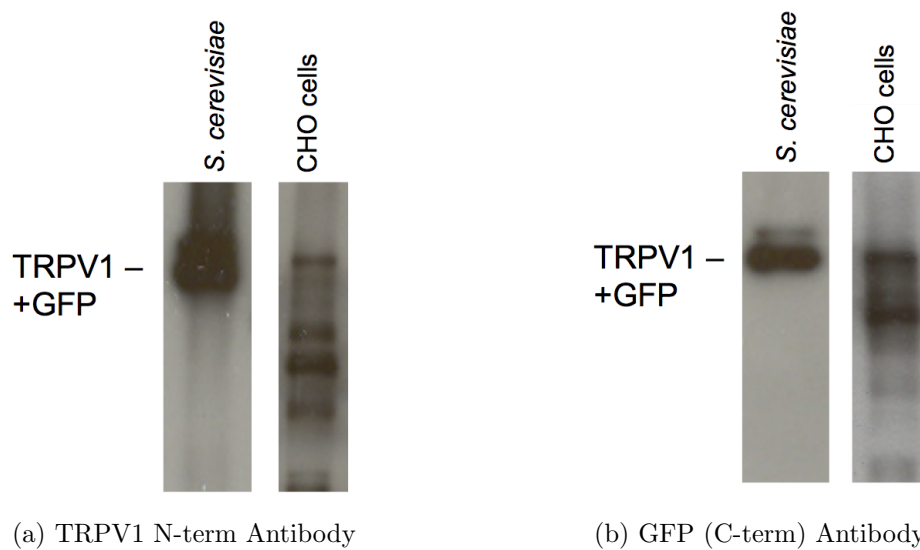


Figure 3.4: **Degradation of overexpressed TRPV1 in three expression systems.** Western blots of TRPV1 expression in *S. cerevisiae* and CHO cells using both an (a) N-terminal and (b) C-terminal antibody show degradation of TRPV1 in CHO cells and an undegraded TRPV1 in *S. cerevisiae*, that possibly also shows a double-band consistent with known glycosylation.

averaged a whole-cell fluorescence of 8 236 relative fluorescence units (RFU) / 10 mL while at an OD₆₀₀ of 12.7. Using the fact that 11 300 RFU corresponds to 0.03 mg GFP and that 1.0 OD₆₀₀ corresponds to approximately 2×10^7 yeast cells / mL, equation (3.1) gives an approximate prediction of protein yield of $1.6 \mu\text{g TRPV1} / 10^9$ cells.

$$\frac{\text{RFU with protein of choice}}{11\,300 \text{ GFP RFU}} \times 0.03 \text{ mg GFP} \times \frac{\text{MW of protein of choice}}{28 \text{ kDa (MW of GFP)}} \times \frac{1}{\text{OD}_{600} \times 2 \times 10^7 \text{ cells}} \quad (3.1)$$

Before this study, expression of TRPV1 in an *S. cerevisiae* overexpression system has previously proved successful [183], with a yield of $2.5 \mu\text{g} / 10^9$ cells (1 mg of protein harvested from 16L of cells at an OD₆₀₀ 1-1.5). Comparing initial rough estimates of TRPV1 yield achievable from initial trials in the GFP-based system to this previously obtained figure gives comparable protein yields (Figure 3.5).



Figure 3.5: **Comparing expression levels: literature vs. initial trials with GFP-tagged system.** Initial expression trials of TRPV1 in *S. cerevisiae* achieved expression levels on par with those seen previously, specifically in successful characterisation of the protein by electron microscopy.

3.2.2 Improving TRPV1 overexpression by changing *S. cerevisiae* strain

While this level of expression proved sufficient for electron microscopy studies, this yield is relatively poor compared to previous successes in membrane protein production using the GFP-based *S. cerevisiae* system. Thus a short project to optimise the *S. cerevisiae* expression system itself ensued, as both useful for TRPV1 expression and to increase the overall expected yield of the system for any eukaryotic membrane protein. As mentioned above the well-characterised genetics of *S. cerevisiae* provide a useful background for such a project. In this case, both a new expression strain and a new expression plasmid were used to increase protein expressed per cell. The original system uses a yeast strain unable to produce uracil without the expression plasmid inserted into the genome via homologous recombination. Inserting a leucine synthesis gene into the original plasmid and switching to a yeast strain that also cannot produce leucine, mandated that the yeast can only survive in leucine drop-out media (-LEU) with high copy number of the plasmid, which in turn produces a high copy number of any of the other genes on the plasmid, in this case the GFP-tagged membrane protein. This method did indeed produce a much higher amount of protein/cell, measured approximately by RFU per OD₆₀₀. However, with this method the overall cells per volume (measured via OD₆₀₀) was lower than with the previous yeast strain. In order to increase the overall cell mass, while keeping the desired high RFU/OD₆₀₀, experiments were conducted to replacing various sugars within the growth media in sequence. The 2% glucose, originally used in starter culture, inhibited the GAL1 promoter used to induce protein expression. Instead 2% lactate, which would keep the yeast nourished but not inhibit the GAL1 promoter, was used in starter culture increasing cell density markedly. After further optimisation of, for example, induction and harvesting times, this protocol proved to reliably increase protein production per cell, and is the standard method used in the rest of this thesis and continues to be used on a regular basis in the lab of Dr. Newstead for a wide range of eukaryotic membrane proteins (Figure 3.6).

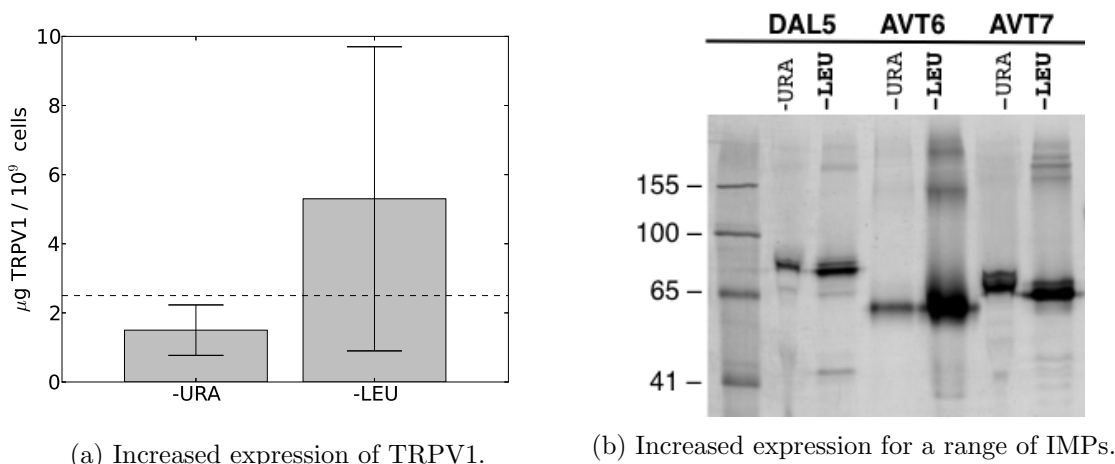


Figure 3.6: **Comparing expression levels: -URA vs. -LEU.** (a) Further optimisation of the *S. cerevisiae* system for eukaryotic membrane protein overexpression achieved increased levels of protein expressed per cell for TRPV1. (b) In-gel fluorescence shows increased expression for a variety of membrane proteins expressed in the -LEU system over -URA.

3.3 Assessing functionality of TRPV1 determined by [^3H]-labeled resiniferatoxin binding assays

While before this study, the *S. cerevisiae* expression system had shown promise, most notably resulting in an EM structure [183], prior to this work radioligand binding assays on TRPV1 from this expression system had not been performed. Radiolabeled-resiniferatoxin has long been used as technique for addressing the presence and activity of TRPV1. While capsaicin is the canonical ligand for TRPV1, its lipophilicity results in high non-specific binding to membranes that masks the specific binding signal making kinetic analysis of TRPV1 binding difficult. Resiniferatoxin (RTX) was discovered in 1990 as an ultrapotent TRPV1 ligand notable for its measurably specific binding to TRPV1 expressed in dorsal root ganglion (DRG) neurons [268]. Additionally capsaicin was found to inhibit RTX binding to TRPV1, encouraging the model that RTX and capsaicin have a common binding site in TRPV1. Here we use [^3H]-RTX binding assays to assess the functionality of TRPV1 from

Rattus norvegicus overexpressed in *Saccharomyces cerevisiae*. Previous studies of TRPV1 expressed in *S. cerevisiae* have measured channel functionality using either calcium imaging [182] or visually observing a cell-death phenotype when plating cells on media laced with capsaicin [189], but not with rigorous binding assays in which a K_d could be measured.

3.3.1 Saturation binding of [^3H]-labeled resiniferatoxin to TRPV1 expressed in CHO cells

[^3H]-RTX binding to TRPV1 from *R. norvegicus* has been well characterised in a variety of experiments on DRG neurons and Chinese Hamster Ovary (CHO) cells in the lab of Dr. Peter M. Blumberg [86], in whose lab the experiments in this section were performed. Though the bulk of the experiments in this thesis focus on TRPV1 overexpressed in *S. cerevisiae*, CHO cells expressing TRPV1 were used as initial controls. Since binding assays are performed on cells the data analysis requires subtraction of non-specific [^3H]-RTX binding to the cell membrane from the total bound [^3H]-RTX. It is useful to show the full binding data to CHO cells, stably expressing TRPV1 and those that are not (Figure 3.7). After subtracting the non-specific binding (dashed) from the specific binding (red), the final curve (black) is used to calculate K_d and $B_{\max}$ by fitting Equation 3.2 to the data. Performing the experiment in triplicate on CHO cells stably expressing rat TRPV1 resulted in a K_d of 105.3 ± 59.6 pM ($n=3$), corresponding to previous experiments.

$$\text{Bound Ligand} = \frac{B_{\max} \times \text{Free Ligand}}{K_d + \text{Free Ligand}} \quad (3.2)$$

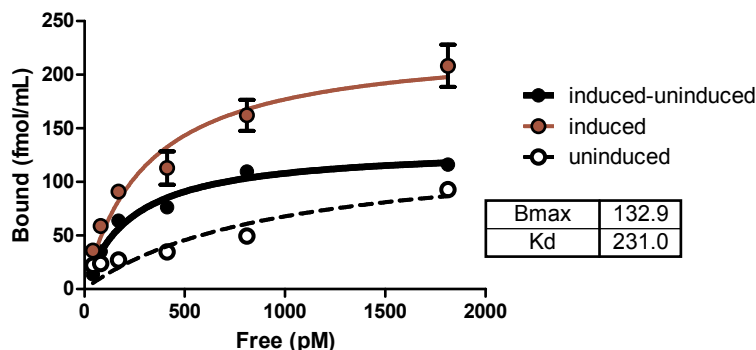


Figure 3.7: **RTX binding to TRPV1 stably expressed in CHO cells.** After subtracting the non-specific binding to uninduced cells (dashed) from the specific binding to induced cells (red), the final curve (black) is used to calculate K_d and B_{max} by fitting equation 3.2 to the data.

3.3.2 Saturation binding of [3 H]-labeled resiniferatoxin to TRPV1 expressed in *Saccharomyces cerevisiae*

Following the same experimental procedure, the functionality of TRPV1 overexpressed in *S. cerevisiae* was confirmed (Figure 3.8). However, the final calculated K_d of 2.82 ± 0.38 nM ($n=3$) was 20-fold higher than what was measured for TRPV1 in CHO cells. Additional controls were performed as well: (1) to ensure that the GFP tag had no effect on binding and (2) to ensure specific binding (Figure 3.8). And indeed the Y511A mutant ablated binding and the TRPV1 without a GFP-tag exhibited a similar K_d of 2.63 ± 1.2 nM ($n=3$).

Combining estimates of protein expression from GFP fluorescence of the GFP-tagged TRPV1 with the B_{max} calculated in these saturation binding experiments gives an estimate of <10% functional TRPV1. This calculation corresponds to results discussed in Section 3.5 in which the stability of the protein comes into question. However, even with a modest amount of functional protein, this observed saturable binding provides a positive control for testing other TRPV1 constructs within the context of a *Saccharomyces cerevisiae* over-expression system.

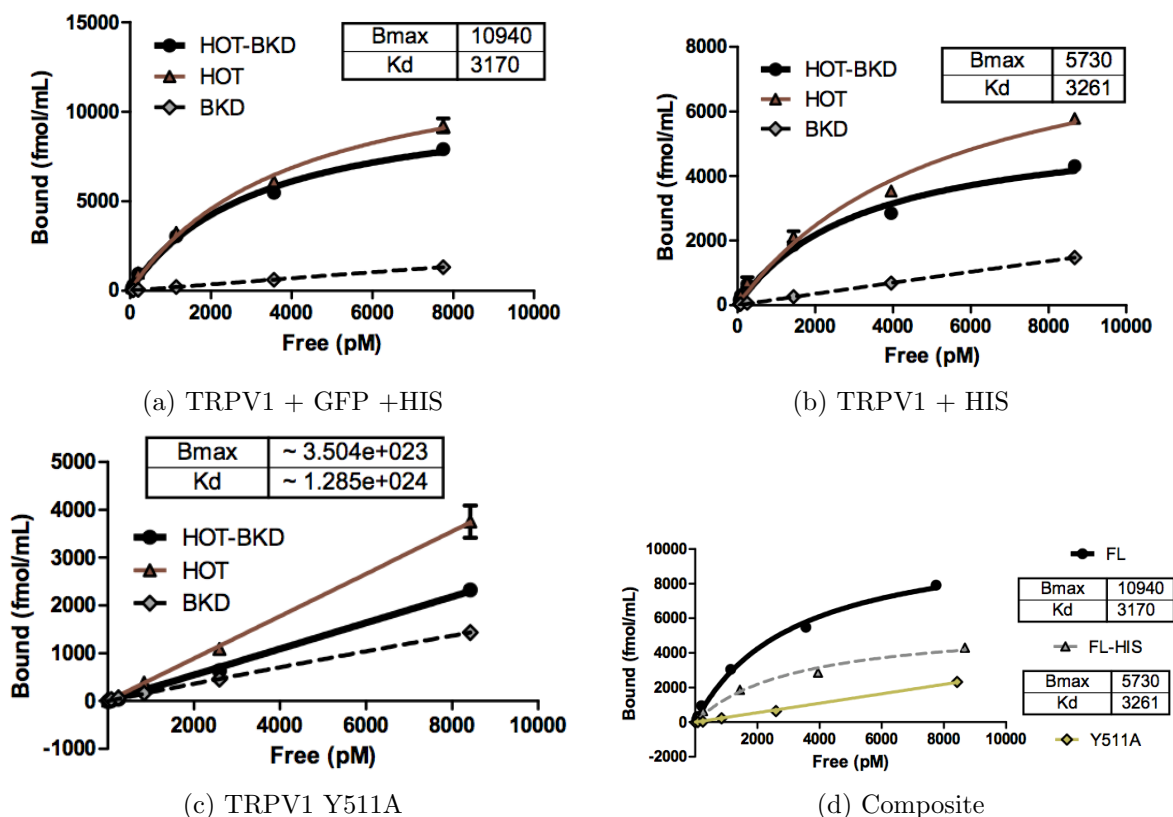


Figure 3.8: **RTX binding to TRPV1 overexpressed in *S. cerevisiae*.** The functionality of TRPV1 overexpressed in *S. cerevisiae* is confirmed here via [3 H]-RTX binding assays. (a) For GFP-tagged TRPV1 a K_d of 2.82 ± 0.38 nM ($n=3$) was calculated. (b) For HIS-tagged TRPV1 a K_d of 2.63 ± 1.2 nM ($n=3$) was calculated. (c) And limited specific binding was observed for the Y511A mutant of GFP-TRPV1. (d) A composite of all three binding curves shows these data with respect to one another.

3.3.3 K_d variability dependent on TRPV1 overexpression

Two possible explanations for the difference in calculated K_d between TRPV1 expressed in stable CHO cells and *S. cerevisiae* are that cell-specific translational modifications likely vary between the two systems and that the cell wall of *S. cerevisiae* might effect access of RTX to TRPV1. However, subsequent experiments with a transient expression of TRPV1 in CHO cells rather than TRPV1 expression in a stable CHO cell line resulted in evidence for a variability in K_d even within the same expression system. It was found that performing

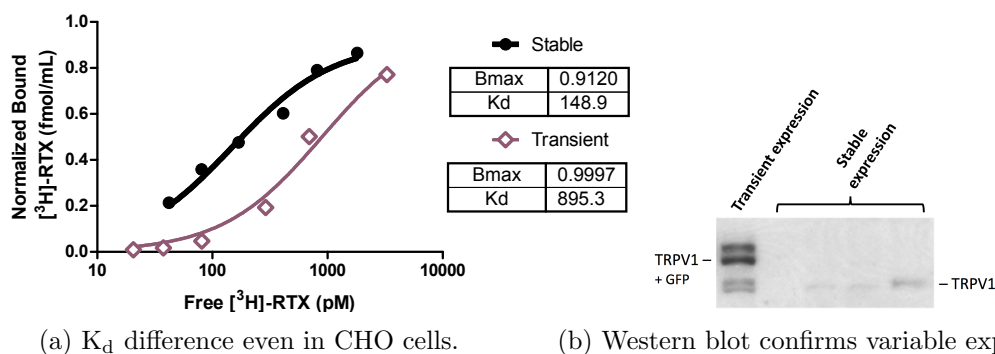


Figure 3.9: **Differences in K_d within one expression system.** (a) Saturation binding curves normalised to B_{max} show that TRPV1 expressed in transient CHO cells (purple) rather than stable CHO cells (black) exhibit a higher K_d , much like that observed in TRPV1 overexpressed using the *S. cerevisiae* system. (b) CHO cells stably expressing TRPV1 do so in much smaller quantities than transient CHO cells, indicating a possible explanation for the difference in K_d .

[^3H]-RTX binding assays on transient CHO cells resulted in a higher K_d , much like that observed in TRPV1 overexpressed in *S. cerevisiae* system (Figure 3.9). The most apparent difference between these two types of CHO cell systems is the level of expression of TRPV1, with the transient cells expressing much more TRPV1 into the membrane than the stable CHO cells. This was confirmed with Western blot analysis (Figure 3.4).

Motivated by the observation that the TRPV1-RTX interaction seemed to display variable K_d perhaps correlated to channel overexpression, subsequent experiments were performed in which a variable amount of DNA was transfected into the CHO cells, resulting in a corresponding variability in TRPV1 expression. In these experiments the variability of the K_d did seem to show a linear relationship to both the amount of DNA transfected and the calculated B_{max} , the maximum number of functional receptors present (Figure 3.10). Additional controls for this experiment were performed in which not only the amount of TRPV1 DNA that was transfected varied, but also amounts of non-TRPV1 DNA or even the DNA for the Y511A mutant of TRPV1. In this way we expected to control for non-specific effects of, for example, the protein production machinery of the transient CHO cells,

however TRPV1 expression still varied among these cells and the non-specific effects proved difficult to control for. However these experiments continued to add weight to the K_d vs $B_{\max}$ trendline (Figure 3.10).

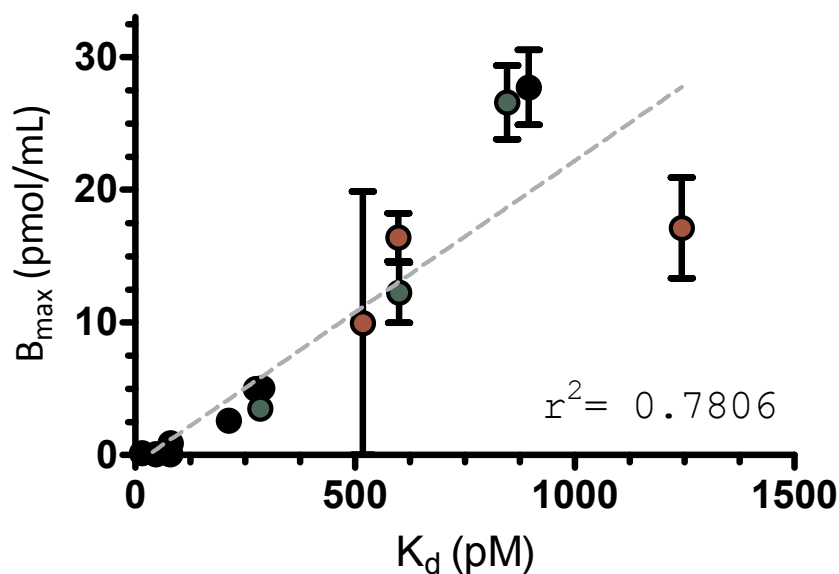


Figure 3.10: **Plot of K_d vs $B_{\max}$.** An approximately linear relationship between K_d and $B_{\max}$ can be observed when plotting these calculated values for fourteen separate experiments in which varying amounts of DNA was transfected into CHO cells. Orange points represent points from saturation curves calculated in an experiment to determine the effect of adding TRPV1-Y511A DNA. Green points represent points from saturation curves calculated in an experiment to determine the effect of adding pKc-delta red cherry DNA.

It is unclear what this result might mean to the physical state of the TRPV1-RTX interaction upon overexpression. One interpretation, supported by the Western blots in Figure 3.4 is that significant degradation is taking place at these levels of expression in CHO cells. However, it remains unclear why this would cause a linear change in K_d , when one would expect protein degradation to be characterised simply by a decrease in $B_{\max}$. While on the surface it seems odd to propose a binding site with a variable binding affinity, another instance of expression-dependent binding affinity has been noted for a pore blocker of TRPV1 [233]. In this case, a pore blocker with a variable affinity can perhaps be rationalised by the known pore-dilation properties of TRPV1. The P2X receptor, for

instance, is another channel found to show expression-dependent binding affinities [81], and is also known to have pore dilation properties. Since RTX is thought to bind intracellularly to transmembrane helices in the S1-S4 region, explaining the variable affinity as an artifact of pore dilation is not as clear as for a pore-blocker. Though one could consider it as an indication that RTX must therefore interact significantly with the pore. Overall correlations between binding affinity and overexpression have no clear explanation in any system for any protein as of yet.

In an attempt to further address the putative relationship between the K_d of RTX and TRPV1, confocal microscopy and Ca^{2+} -imaging assays were performed on CHO cells transfected with various amounts of TRPV1 DNA. Microscopy images show that higher amounts of TRPV1 expression resulted in differences in localisation, aggregation, cell size, and frequency of apoptosis (Figure 3.11). When selecting for cells visibly expressing TRPV1 via fluorescence microscopy, Ca^{2+} -imaging experiments show calcium leakage in these cells over those not visibly seen to be expressing TRPV1 (Figure 3.12). These results indicate that perhaps it is either TRPV1 aggregation or a certain amount of active, leaky channels that could be responsible for the variability in K_d . The second of these mechanisms, leaky channels, is more in line with the idea of pore dilation somehow being related to this phenomenon. While a clear explanation remains elusive these results warn of the possibility that overexpression in itself can effect the physical state of a protein, be it by aggregation, cell metabolism, pore-dilation, co-factor depletion, or some other effect yet to be fully described.

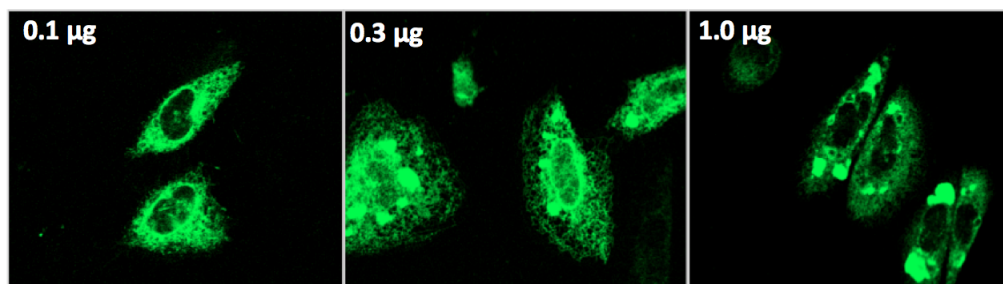


Figure 3.11: **Confocal microscopy overexpressed TRPV1 in CHO cells.** Microscopy shows that with higher amounts of TRPV1 expression differences in localisation, aggregation, cell size, and frequency of apoptosis can be seen in CHO cells.

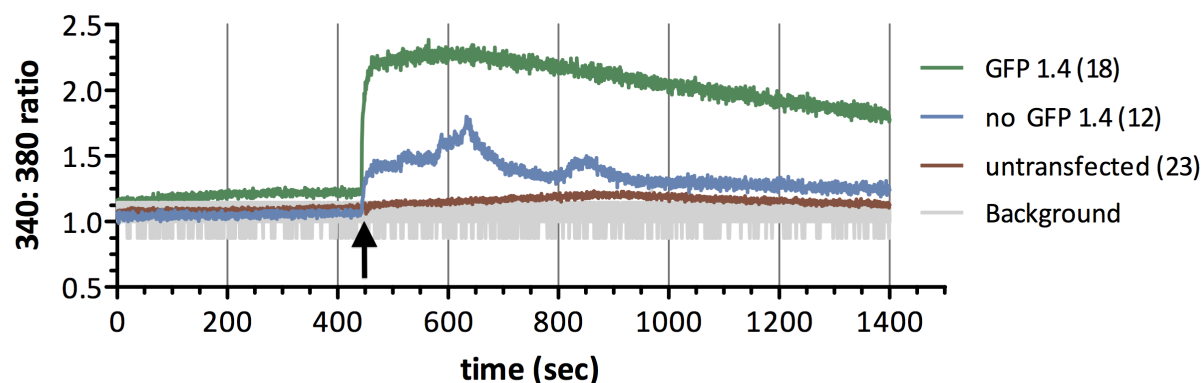


Figure 3.12: **Ca²⁺-imaging experiments of transiently-transfected CHO cells expressing.** Ca²⁺-imaging experiments of transiently-transfected CHO cells expressing and not expressing TRPV1, as chosen from visual appearance of GFP fluorescence. Leakage is evident in cells visibly expressing TRPV1 even before application of 1 μ M capsaicin, indicated by the arrow.

3.4 Effects of other modulators on [³H]-labeled resiniferatoxin binding to TRPV1

TRP channels and TRPV1 are well known for being sensitive to multiple stimuli and being able to integrate these signals into properties of channel opening and sensitisation. Section 3.3 established that TRPV1 is a functional protein when overexpressed in *Saccharomyces cerevisiae*, which makes a variety of experiments accessible to address intrinsic properties of the protein in the absence of a native mammalian cell environment.

3.4.1 Effects of the double-knot toxin (DkTx) on saturation binding of [^3H]-labeled resiniferatoxin to TRPV1

In 2010, the “double knot” toxin (DkTx), a tarantula toxin, was found to activate TRPV1, providing a potentially valuable tool for studying TRP channel structure function relationships [28]. It was found that mutants in the pore region between the S5 and S6 helices eliminated the toxin’s ability to open the channel. To examine the possibility of DkTx having an effect on RTX binding affinity, assays were performed on the membranes of *S. cerevisiae* overexpressing TRPV1 that had been previously incubated with 2.5 μM DkTx. This resulted in a reproducible difference in the K_d of RTX binding (Figure 3.13). This indicates that at least the functional fraction of protein produced from *S. cerevisiae* can bind two separate toxins, at least one of which allosterically modulates the others binding. Since both RTX and DkTx cause channel opening, the resulting increased affinity for RTX can be explained by DkTx stabilising a more open state channel, reducing the free energy barrier to binding for RTX.

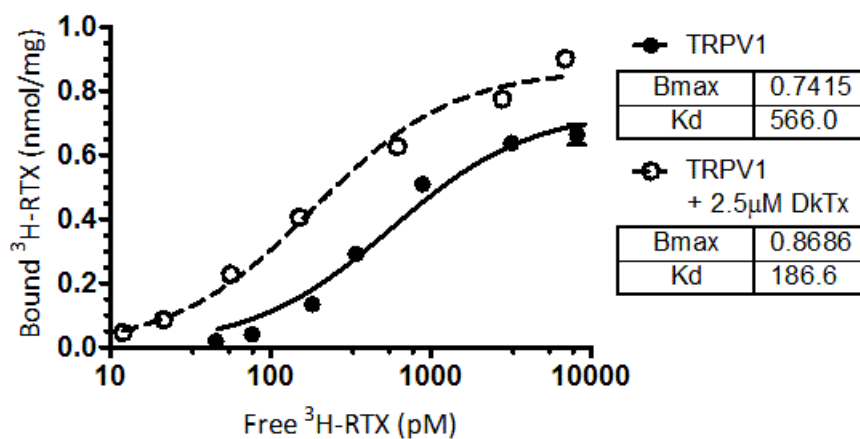


Figure 3.13: **DkTx effects the K_d of RTX.** Saturation binding of [^3H]-RTX in the presence (dashed line) and absence (solid line) of 2.5 μM DkTx.

To further address whether this change in K_d was the result of a positive allosteric effect of a saturating concentration of DkTx on the RTX binding site rather than a non-specific

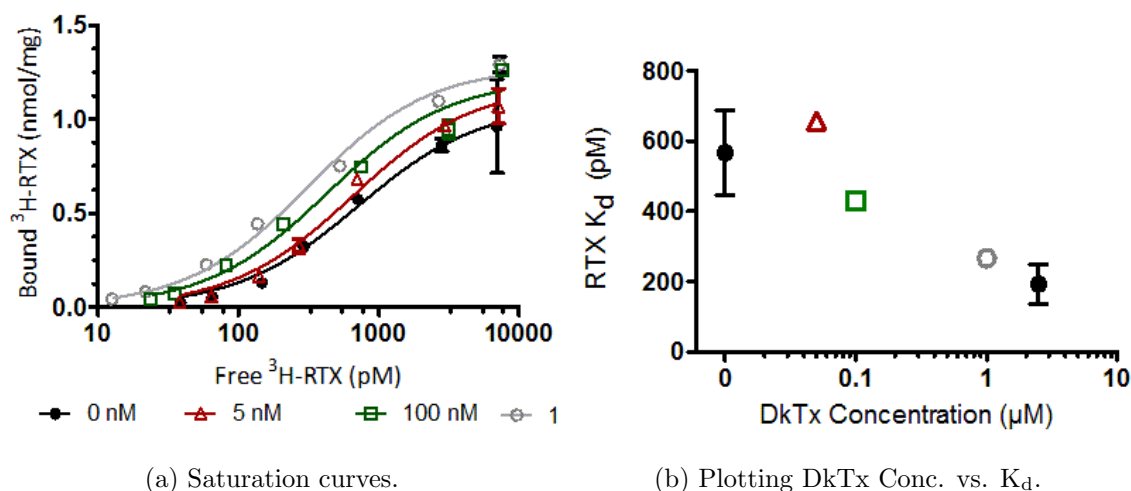


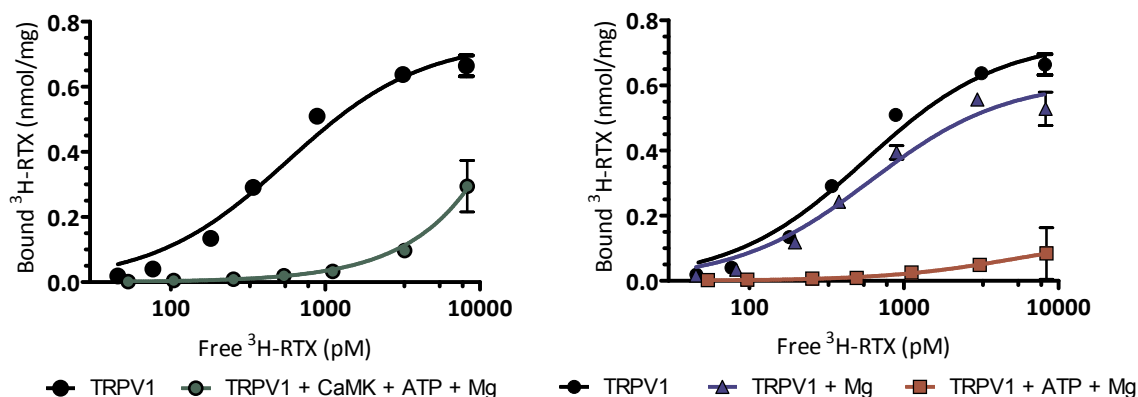
Figure 3.14: **DkTx effect on RTX binding is concentration-dependent.** (a) Saturation curves from a series of experiments show that varying the concentration of DkTx in the assay solution during RTX binding assays has a concentration-dependent effect on the K_d of RTX binding to TRPV1. (b) Plotting the relationship between the K_d and the concentration of DkTx used in the experiment shows this effect most clearly. Triplicate experiments were only performed for the first and last points in this data set.

effect in some other aspect of the experiment, experiments were conducted to confirm that the effect was truly concentration dependent. Using concentrations of DkTx ranging from 5 nM up to the original 2.5 μM a concentration dependent effect was confirmed (Figure 3.14). Thus, these experiments demonstrate the functional coupling of DkTx and RTX binding to TRPV1. Since DkTx is predicted to bind to the pore S5-S6 helices and RTX to the S1-S4 helices, this hints at a stronger coupling between the two transmembrane regions than merely allowing the movement of the S1-S4 helices to couple to the pore via the S4-S5 linker.

3.4.2 Effects of adenosine triphosphate (ATP) on saturation binding of [³H]-labeled resiniferatoxin to TRPV1

As noted in section 3.3 one initial avenue of study for why a variability in K_d was observed between expression systems was that post-translational modifications differs between yeast and mammalian cells. Since there is a known phosphorylation site near the expected RTX binding site, it was expected that perhaps different phosphorylation machinery in yeast and mammalian cells was the cause of the difference in affinity. Thus an experiment was conducted to see whether addition of Ca^{2+} /calmodulin-dependent protein kinase (CaMKII) and Mg^{2+} -ATP as a substrate for it would allow for an increase in RTX binding affinity. Surprisingly, the experiment showed the opposite: that adding these elements at concentrations of 10 mM and 2 nM for Mg^{2+} -ATP and CaMKII, respectively, eliminated high-affinity RTX binding (Figure 3.15). However when the experiment was repeated with individual components it became clear that, in fact, only the Mg^{2+} -ATP was necessary for the negative effect on RTX binding to TRPV1 (Figure 3.15).

This effect of Mg^{2+} -ATP inhibiting RTX binding was unexpected and so far remains without a firm explanation. Previous studies have suggested that ATP sensitises TRPV1 activation by capsaicin, and does so via a binding site in the N-terminal Ankyrin Repeat Domain (ARD) [152]. A crystal structure of the complex between the rat TRPV1 ARD and ATP has even been solved (Figure 3.16). In these experiments, the presence of ATP seems to have no effect on the initial capsaicin-induced currents, but only on the subsequent desensitisation. To explain why RTX binding would be inhibited by Mg^{2+} -ATP, but in previous experiments would not have an effect on capsaicin-induced currents is not straightforward, but may somehow relate to either differences in reagent concentrations used between the two experiments, the different expression systems, or intrinsic differences between RTX and capsaicin binding.



(a) Effect of CaMK on RTX binding?

(b) Effect of Mg^{2+} -ATP on RTX binding.

Figure 3.15: **Preliminary results toward an effect of Mg^{2+} -ATP on RTX binding to TRPV1.** The presence of ATP, Mg^{2+} -ATP, and Ca^{2+} /calmodulin-dependent protein kinase (CaMKII) in the binding assay solution resulted in an unexpected negative effect of ^3H -RTX binding to TRPV1. However this loss of binding is also seen with just Mg^{2+} -ATP and not CaMK in the assay solution. Mg^{2+} alone has a minimal effect, if any. This indicates that the loss of RTX binding seen in the presence of CaMK is due to the presence of Mg^{2+} -ATP and not CaMK, ATP, or Mg^{2+} on their own. (Binding results with ATP alone agree with this interpretation but are not shown.)

These results on modulators of the TRPV1-RTX interaction provide evidence that continuing to investigate TRPV1 in the context of a *S. cerevisiae* expression system remains promising for future functional work. Performing experiments on cells or membranes as in this thesis remains viable, however extending the same methods to TRPV1 in detergent or reconstituted into liposomes would provide even more insight into the intrinsic properties of TRPV1.

3.5 Stability of detergent-extracted TRPV1

The previous sections have shown that functional rat TRPV1 can be expressed in relatively high quantities in a *Saccharomyces cerevisiae* system which uses GFP to track the overexpression. However, for structural studies it is necessary to purify the protein by

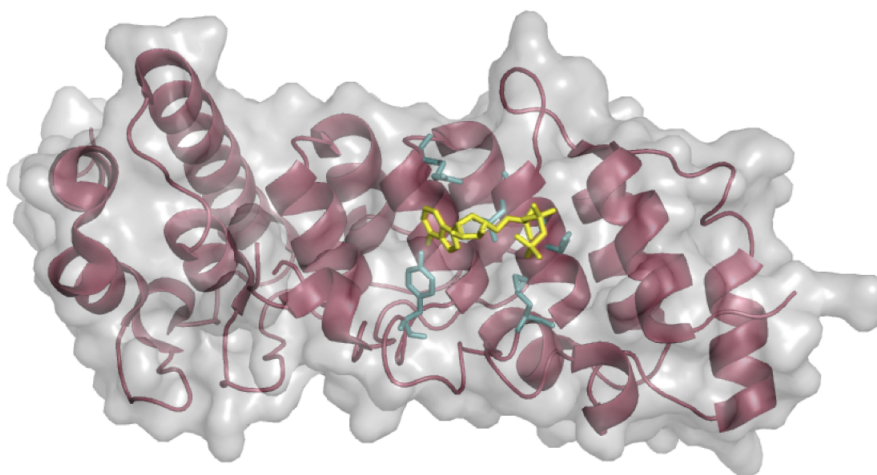


Figure 3.16: **Proposed ATP-binding site in the N-terminus of TRPV1.** Previous experiments, including this crystal structure (PDB ID: 2NYJ) of ATP bound to the N-terminal Ankyrin Repeat Domain (ARD) of TRPV1 from *Rattus norvegicus*, have shown that ATP interacts with TRPV1.

extracting it from the cell membrane. To determine which detergents are most likely to extract the protein in such a way as it maintains structural and functional integrity, the GFP tag continues to be useful to measure solubilisation efficiency and in the technique of fluorescence-coupled size exclusion chromatography (FSEC).

3.5.1 Detergent solubility of GFP-tagged TRPV1

A range of detergents have shown success for solubilising eukaryotic membrane proteins in the past, as referred to in the Introduction. However, in this case few detergents showed much promise for detergent-extraction of TRPV1 from the yeast membranes (Figure 3.17). Using the GFP tag, the solubilisation efficiency can be calculated from the amount of fluorescence of the supernatant before and after ultracentrifugation of yeast membranes once incubated with 1% detergent for an hour. Protein that the detergent was able to solubilise will be in detergent micelles and remain in the supernatant after the membranes pellet in multi-lamellar vesicles during ultracentrifugation.

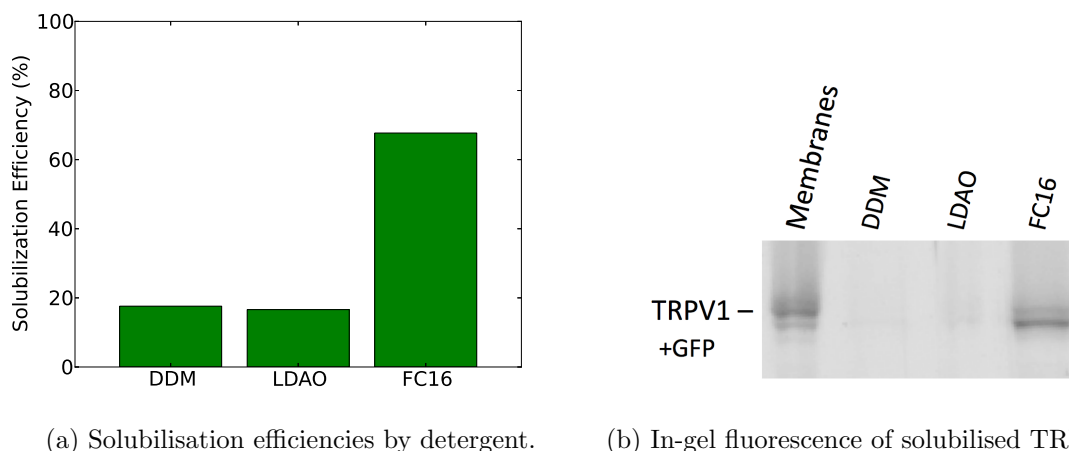


Figure 3.17: **Solubilisation efficiency of TRPV1 in different detergents.** Standard detergents like DDM and LDAO proved unsuccessful for extracting TRPV1 from the yeast membranes.

3.5.2 Fluorescence-coupled size exclusion chromatography of GFP-tagged TRPV1

While detergent solubility was overall poor in detergents other than the harsh Fos Choline 16 (Figure 3.17), it has been shown that solubilisation efficiency does not necessarily correlate with the stability of the protein upon solubilisation [194]. The integrity of solubilised TRPV1 in various detergents was thus determined via fluorescence-coupled size-exclusion chromatography (FSEC). Two values are useful to look at in chromatography traces: (1) the amount of protein in the void volume and (2) the sharpness and monodispersity of the peak that corresponds to the protein. The resulting peaks seen in FSEC traces of GFP-tagged TRPV1 had large amounts of aggregate in the void volume and very little protein elsewhere (Figure 3.18). In correlation with the solubilisation efficiency results, the only detergent to show any promise is Fos-Choline 16. While this detergent is known to be harsh, and therefore indicates that a certain amount of protein unfolding occurred in order to solubilise TRPV1, its use could remain a possible avenue forward in TRPV1 purification, for example detergent exchange into a less-harsh detergent after initial purification

with Fos-Choline 16, though it is likely the protein being extracted will be of very poor quality.

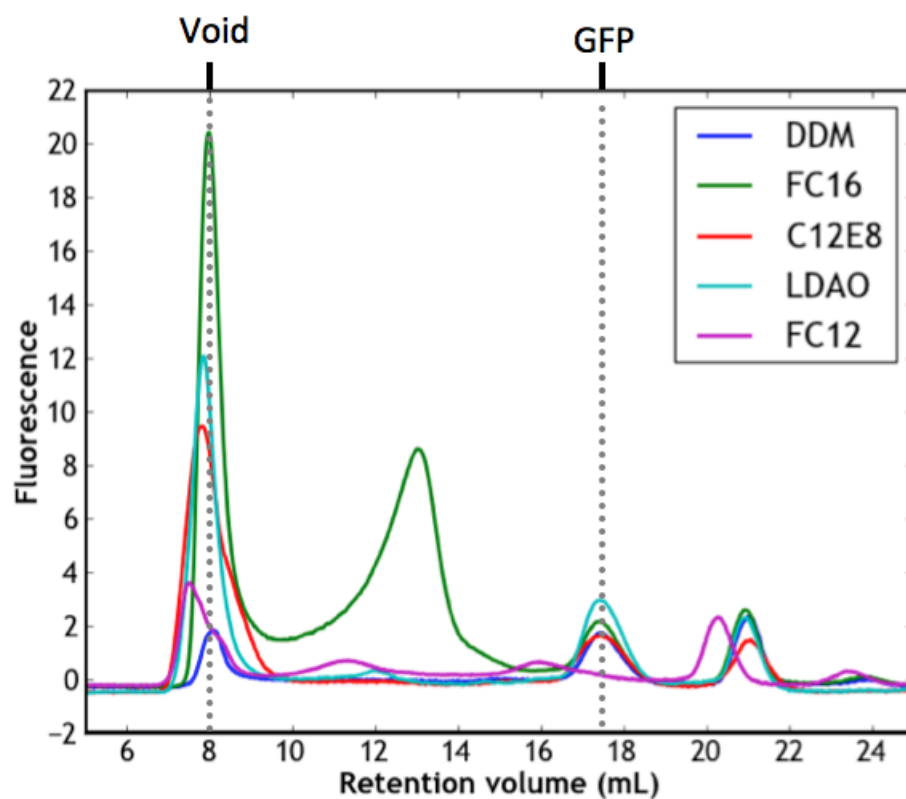


Figure 3.18: **Rat TRPV1 aggregates in most detergents.** Fluorescence size exclusion chromatography showing a lack of promising leads for wild-type rTRPV1. Only Fos-Choline 16, known to be very harsh and probably denaturing detergent, was seen to solubilise TRPV1 to any extent.

3.6 Conclusions and future directions

3.6.1 Functional studies to clarify modulator effects on RTX binding to TRPV1

The functional read-out of [^3H]-RTX binding is a direct measurement of the quality of the binding site. Interpretation of the experiments does not require consideration of coupling to the pore, as is the case for capsaicin-induced currents. In this way, one can directly deduce structural effects on the binding site from experiments with mutants or modulators. With the experiments described here as the starting point, subsequent studies can be pursued to clarify their possible explanations and implications. For the DkTx experiments, mutating the residues between the S5 and S6 helices that effect DkTx-induced currents, and observing if the effect of DkTx is eliminated, would be a good control, for example. Additionally, DkTx (the ‘double-knot’ toxin) has two knots: the K1 and K2, which can be isolated and have slightly different effects on TRPV1 function. The same experiment can be conducted with individual knots to assess if they have variable effects on RTX affinity, or if the whole toxin is needed. A perhaps more risky experiment than either of these, is to attempt to decouple the RTX-DkTx interaction by mutating residues presumably at the interface between the S1-S4 helices where RTX is predicted to bind and the S5-S6 pore helices where DkTx binds. This idea is possibly flawed, in that some studies indicate that RTX might bind at the interface between these two domains [144], but this has yet to become a common assumption.

In a similar vein to mutating the pore as a control for the DkTx experiment, a mutant is known that removes ATP binding (K155A) to the ARD. This mutant could be cloned into TRPV1, and the same experiment repeated to see if this ARD binding site is responsible for the effect of Mg^{2+} -ATP on RTX binding.

3.6.2 *Saccharomyces cerevisiae* remains a promising overexpression system

In section 3.1 the previous successes of TRP channel purification and structural characterisation were illustrated. *Saccharomyces cerevisiae* had been previously successful as a system for expressing and purifying qualitatively functional TRPV1 in adequate amounts for a low resolution EM structure. However, attempts at increasing protein yield seemed a valuable approach in hopes of further structural studies. Additionally quantitatively assessing the functionality of TRPV1 expressed in *S. cerevisiae* to be able to compare expression systems as well as to assess the percent of functional protein produced had never been addressed.

These studies observed an increased yield from that previously attained, though problems with TRPV1 toxicity to *S. cerevisiae* and a large variability in yield per experiment remain. Saturable binding to TRPV1 overexpressed in *S. cerevisiae* using [³H]-RTX comparable to that seen in CHO cells was seen. Additionally when overexpressed in *S. cerevisiae* TRPV1 exhibited less degradation than when expressed to similar amounts in CHO cells. Thus these results initially confirmed that *S. cerevisiae* could produce TRPV1 in amounts greater than previously, with a certain amount of structural integrity, and with quantifiable binding affinity to RTX. However, calculations using the estimated amounts of TRPV1 in the cell (confirmed separately with purified TRPV1 from a 100 L growup) to the B_{max} estimated from the RTX binding curves give estimates that only <10% of the TRPV1 produced is functional. This prediction of a small amount of functional, correctly folded protein correlates with the difficulties in detergent extraction.

Using the [³H]-RTX binding assay with cells or membranes from *S. cerevisiae* coupled with the fluorescence methods available when adding a C-terminal GFP tag to TRPV1, further studies of mutants or other species can assess the overexpression, stability, and % functional protein with small scale cultures before investing the resources for large scale

growups, detergent extraction and purification.

3.6.3 Shifting focus from wild-type TRPV1 from *Rattus norvegicus* is encouraged

While expression of TRPV1 in *S. cerevisiae* proved functional and not degraded, the amount of functional protein and its ultimate stability indicate that shifting the focus of structural biology studies to other TRP channel constructs is perhaps a more constructive avenue of study. Initial expression trials of rat TRPV1 mutants, other species of TRPV1, and other species of TRPM8 indicate that these are potentially promising alternatives (Figure 3.19).

One of the big hurdles for TRP channel structural biology is a lack of prokaryotic homologues. However, recently the first crystal structure of a eukaryotic transporter was obtained of a phosphate transporter from the yeast *Piriformospora indica* [216]. This indicates that yeast, while single-celled eukaryotes and not prokaryotes, could be a promising source for relatively well-behaved membrane proteins. As mentioned in section 3.2 yeast have a TRP channel homologue, for example the YVC1 in *Saccharomyces cerevisiae*. Targeting the TRP channels of single-celled eukaryotes may be an even more favorable path than the proteins screened in Figure 3.19. A BLAST search indicates that *Piriformospora indica* has a TRP channel (UniProt ID: Q12324) similar in sequence to the *S. cerevisiae* YVC1 (Figure 3.20). Even more promising would be to target the TRP channels of thermophilic single-celled eukaryotes, and while the relevant genomes are scarce, there is likely to be a TRP channel of high homology to YVC1 in the thermophilic yeast *Candida thermophila* [249].

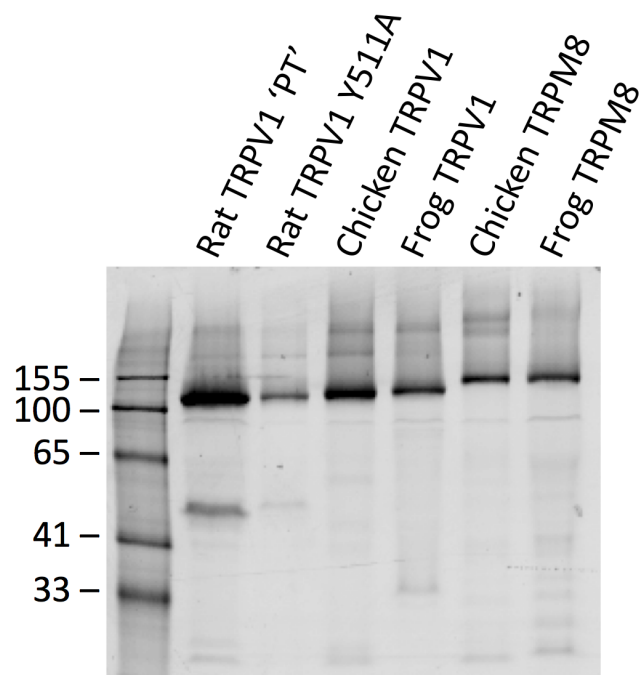


Figure 3.19: **Small scale screen of other TRPs.** In-gel fluorescence of yeast membranes after small scale screens of a variety of species of TRPV1 and TRPM8 show promising levels of expression.

```

G4T9Y7_PIRID      1 --MADPEEASL ISTRSIQ P-----APATLTKLIKRIALTFKLLPVQ 40
YVC1_YEAST        1 MVSANGDLHLP ISNEQCM PENNGSLGF EAPT PRQILRVTLNLKY LIDKVVPIV 53

G4T9Y7_PIRID      41 VNLEELAEPT SRVIT PKVINAYAKAAG-----DFGEALPYCLLRARQT 83
YVC1_YEAST        54 YDPNDIVCDHSEILSPKVVKLAYEACG GNPDKKANKRKYQSVIIFSLLKVC EW 106

G4T9Y7_PIRID      84 FIWDANHSAADYDEHMGRA IACEVLARRIVHN----APQERLKSIMSTRYRHK 132
YVC1_YEAST       107 YSILAATMEVHNAKLYETRNLSAQQLCKLLIEREETRDLOFLFMQLLLRRYV IN 159

G4T9Y7_PIRID     133 EWDDSSLSALELAIDQHCTIFLSSNEAQFVIDSLWSGTWVQNNED----- 181
YVC1_YEAST       160 ENDEDQEP LN-ALELATDMHCTTVIGSSGFQRCCLKWIWRG-WIVQNGLDPTTF 210

G4T9Y7_PIRID     182 -----HDIDFRQP L EK-----VDPH 196
YVC1_YEAST       211 IKDDSLAEVSLISHFNVR LKAPVYQNYLQMIFSFLFLGLYTLVNVNGKDSERV 263

G4T9Y7_PIRID     197 HDFSFFEGMLYTMALAFSFEETHRLYTTLRYFTWRAFGFWTIIGLITNGLLIA 249
YVC1_YEAST       264 QSFDDLLESIFYVFNTGFILDELT KLY----YIGYAHLSFWNLFNDDTYLIITF 312

G4T9Y7_PIRID     250 AFVLRIMG IASHDQTKS-DNWHFRSFQVLACVR-----VITVLDGFKYIGTM 295
YVC1_YEAST       313 AMGFRAMSVTPLNAKYSSEDWDKISYRVLSCAAPFVWSRLLLYLESQRFIGIM 365

G4T9Y7_PIRID     296 QICVARMMQESTIFFVLLSILLIGFYQGMYALDAADGVTDKGGVVMHSL LQAL 348
YVC1_YEAST       366 LVILKHMMKESIVFFFLFLFLIMIGFTQGFLGLDSADGKRDI TGPILGNLTITV 418

G4T9Y7_PIRID     349 LQAPDFNATEG---AFGLLLYYMWCLVTTIVLLNVLISLFSSAYEDVVGAAES 398
YVC1_YEAST       419 LGLGSFEDVFE EFAPPYAAILYYGYFIVSVILLN ILIALYSTAYQKV IDNADD 471

G4T9Y7_PIRID     399 HYLA FVAGKTVSMIRAPDSYVYPAPFN LIEIFFIAPLEPCMPADTYAKLNRAI 451
YVC1_YEAST       472 EYMAALMSQKT LRYIRAPDEDVYVSPNLN LIEVFMTPIFR-ILPPKRAKDL SYTV 523

G4T9Y7_PIRID     452 MRTLFWIP LTCIALFEA-----TLDPRKNEFTKAWFEANEEEA DDN 492
YVC1_YEAST       524 MTIVYSPFLLLISVKETREARRIKYNRMKR LNDDANEYDTPWDLTDGYLD DDD 576

G4T9Y7_PIRID     493 PEYQDPD VDSGEPGVITKTKFEDLVREFP----- 521
YVC1_YEAST       577 GLFS DN RNSGM RATQLKNSRSLK LQRTAEQEDVHFVKVPKKWYKNVKKCSPSFE 629

G4T9Y7_PIRID     522 ----NTSQSMEATIINEIQKL RQR LDELTA KMT EP SDRVG----- 557
YVC1_YEAST       630 QYDND DTEDDAGEDKDEVKELTKKVENLTAVITD LLEKLDIKDKKE 675

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Figure 3.20: **An alignment of TRP channels from yeast.** A BLAST search indicates that *Piriformospora indica* has a TRP channel (UniProt ID: Q12324) similar in sequence to the *S. cerevisiae* YVC1.

Addressing modularity in TRP channel transmembrane domains

4.1 Introduction

While TRP channels have the same six transmembrane helix architecture as the rest of the voltage-gated-like (VGL) channel family, it is unclear whether the transmembrane domain is similarly composed of two distinct domains: the voltage-sensor-like domain and the pore domain. This chapter attempts to address this question of TRP channel modularity by investigating if the S1-S4 helices, the putative voltage-sensor-like domain, of several TRP channels show biochemical evidence for structural modularity and by determining functional modularity by ligand-binding to TRPV1 when truncated around these transmembrane domains.

4.1.1 The S1-S4 helices as modular domains in voltage-sensitive proteins

The S1-S4 helices of voltage-gated K^+ , Na^+ and Ca^{2+} channels have been shown in a variety of experiments to exhibit both functional and structural modularity, such that the term voltage-sensor domain (VSD) has become common-place. Structures have been solved

of the KvAP VSD independently from the pore domain [38, 116], and similarly pore domain structures have been solved independently of the VSD [173]. Additionally structures of the tetrameric assembly of the S1-S6 helices, the full transmembrane domain, have been solved separately of, for example, bacterial voltage-gated Na^+ channel, which have limited N- and C- terminal domains [214, 215]. The first strong indication of the VSD forming a proper structural domain was with the determination that VsTx, known to be similar to toxins such as hanatoxin that bind to the Kv channel voltage sensor, was able to bind and pull down the VSD of KvAP when expressed separate from the rest of the channel [116]. Since then, the functional modularity of the VSD has been illustrated by numerous functional chimeras of voltage-gated channels in which voltage-sensitive properties are transferred with the VSD [158, 157, 3]. In addition to the modularity of the VSD within voltage-sensitive channels, the four helix domain has been found as a voltage-sensor to a voltage-sensitive enzyme [112] and as a stand-alone voltage-sensitive proton channel, which forms its channel without the additional S5 and S6 helices of the pore domain [228]. The S1-S4 domains of both of these have been expressed and purified [149, 147].

While this voltage-sensor domain is a characteristic element of the VGL channel super-family, in some members of the family it does not act as a canonical voltage sensor despite containing the characteristic string of positive residues along the S4 helix. The cyclic nucleotide-modulated channels are the most striking example of this. Cyclic nucleotide-gated (CNG) channels, for example, only respond weakly to changes in membrane potential despite having three or four positively charged residues in their S4. Instead they are gated by the binding of cyclic nucleotides (cAMP and CGMP) to the cytosolic nucleotide binding domains in their C-terminus [308]. The hyperpolarisation-activated cyclic nucleotide-gated (HCN) channels are also an example of an atypical voltage sensor, as while they are responsive to changes in membrane potential, they have a reverse voltage-dependence and are activated upon hyperpolarisation. While this may be related to the existence of up to ten positively charged residues in their S4, as compared to the five to seven charges of most

voltage-gated channels, the mechanisms of this reverse voltage dependence remain unclear [161]. Similarly, one of the four unique crystal structures of an S1-S6 tetramer (see section 1.2 for more details) is a CNG channel from the bacteria *M. loti*. While this channel remains poorly characterised its lack of positive charges on the S4, as well as tighter packing between the S1-S4 helices and the S5-S6 helices, remains a curiosity.

4.1.2 Evidence for the significance of the S1-S4 helices in TRP channel function

While cyclic-nucleotide modulated channels have shown some peculiarities regarding their VSD, no VGL channel has a VSD as enigmatic as the TRP channels. As TRP channels lack the common string of charged residues in their S4 helix while some members maintain degrees of voltage-sensitivity it is unclear how to interpret the function of their VSD. The strongest case for a functional sensory domain in the S1-S4 of TRP channels is a series of mutational studies that have identified this region as key to capsaicin and menthol binding of TRPV1 and TRPM8, respectively [116, 307]. Though other regions, in the case of TRPV1, have been implicated in capsaicin binding, it is largely unclear whether these regions are directly or allosterically effecting binding or effecting the channel gating process after binding [137, 38], though the same could be said for the S1-S4 mutants. This chapter aims to address the question of whether the transmembrane domains of TRP channels form two distinct structural and functional domains as in other VGL channels through expression and purification trials and ligand binding assays. This hypothesis has two parts: (1) that the TRP channel S1-S6 is a structural and functional domain that can be (2) divided into the two domains of the pore domain and the voltage-sensor-like domain (Figure 4.1).

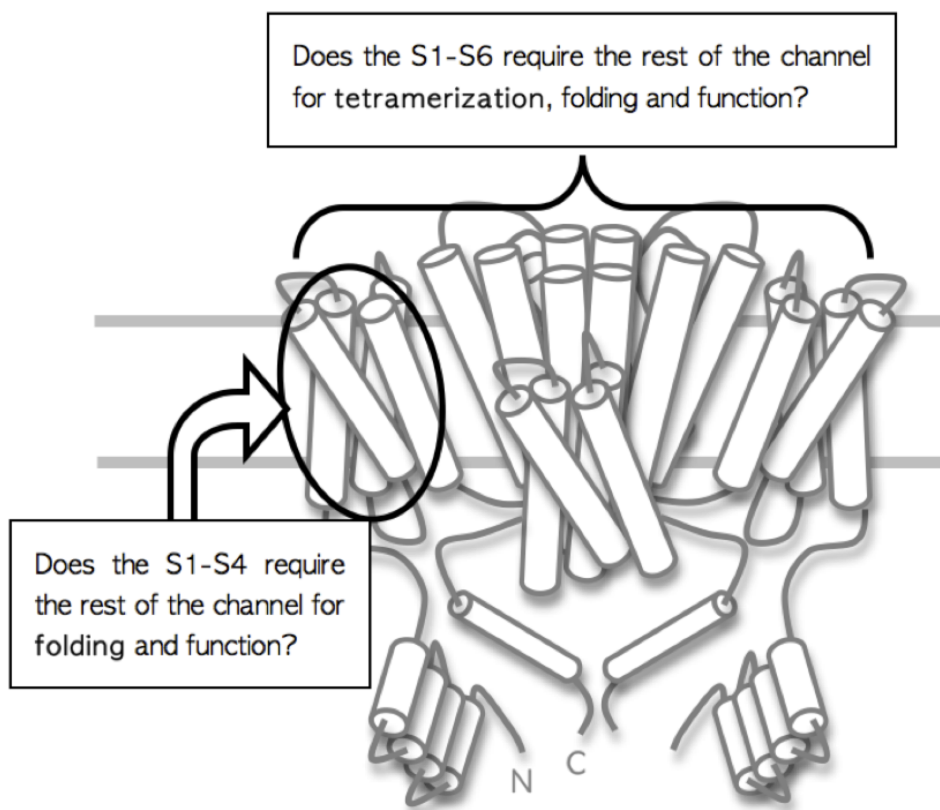


Figure 4.1: **TRP transmembrane divisions.** What are the significant structural and functional divisions within TRP channel transmembrane domain?

4.2 Overexpression of TRPV1 transmembrane-based constructs in *S. cerevisiae*

With the successful expression and characterisation of functional rat TRPV1 from *Saccharomyces cerevisiae* of the previous chapter (Chapter 3), a system was established in which to address the modularity of TRP channel transmembrane domains. Thus expression trials of various TRPV1 transmembrane constructs in the *S. cerevisiae* system were conducted to assess whether this system would be appropriate for the production and characterisation of TRPV1 transmembrane modular domains.

4.2.1 Expression trials of transmembrane-centric constructs of TRPV1 from *Rattus norvegicus*

Using the expression system described in Chapter 3, clones of TRPV1 transmembrane-centric constructs were transformed into *S. cerevisiae*. In small-scale 10 mL expression trials, all constructs showed measurable expression within the system (Figure 4.2). Although expression levels measured as protein per cell were less for the S1-S4 constructs, due to the decreased cell density from the toxicity of the wild-type (WT) TRPV1 (Figure 4.3), overall protein yield per volume of cells was ultimately higher than WT for all constructs. Despite removing the soluble portions of TRPV1, protein localisation remained heterogeneous and the ideal ‘halo’ing effect that occurs when the protein localises predominantly in the plasma membrane was not visible (Figure 4.4).

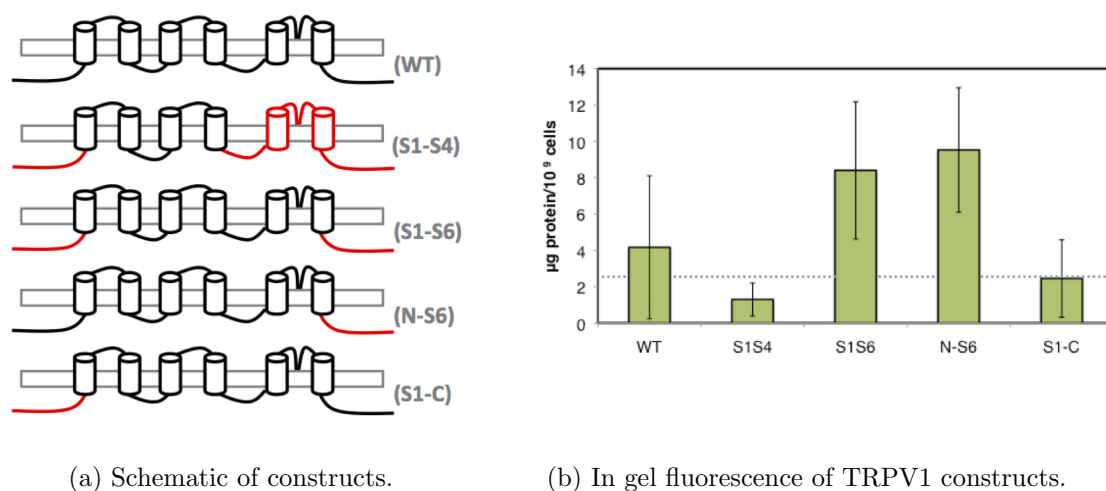


Figure 4.2: **Small scale expression of TRPV1 truncations.** (a) Schematic of rat TRPV1 truncations tested. (b) Expression data of membrane-centric constructs, calculated from measurements of whole-cell fluorescence, show the increase of protein expression achieved by removing soluble regions. The dotted line indicates previously published levels of expression for rat TRPV1 in *S. cerevisiae*.

Crude membrane preparations of small scale trials yielded clean, prominent bands in Tris-Glycine gels imaged with in-gel fluorescence, indicating low levels of aggregation and

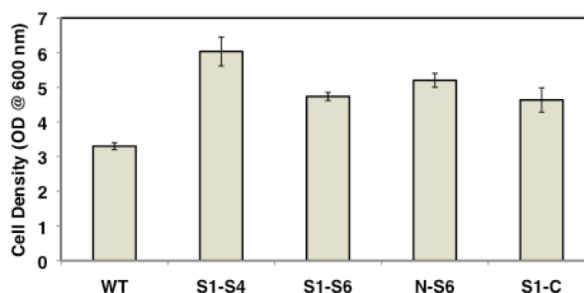


Figure 4.3: **Yeast cell density upon overexpression of TRPV1 truncations.** Measurements of yeast cell density using optical density at 600 nm, show evidence for less toxicity in cells expressing truncations. Data were calculated from final measurements of cell density via optical density at 600 nm over three separate small-scale trials.

degradation, promising for further investigation via large scale preparations (Figure 4.5). Similar results were also seen for S1-S6 and S1-S4 constructs of rat TRPM8 (Figure 4.5).

4.2.2 Optimising expression of the S1-S4 helices from *Rattus norvegicus*

Even with this promising start, protein yield was not yet at an ideal level for purification within the context of previously successful projects within the *S. cerevisiae* system, so we chose to further optimise the expression system itself, as described in Chapter 2 and used in Chapter 3. As with the WT channel, this yielded higher protein expression levels for the truncations. Additional optimisation of the S1-S4 helices was also conducted. Since the definition to date of TRPV1 and TRPM8 alpha helices are defined only by hydrophobicity prediction, the N- and C-terminal cut-offs were explored with modest variations. The initial constructs were the longest constructs used, but then shortening either end resulted in three more possible constructs: short-short (SS), short-long (SL), and long-short (LS). These variations, though small, resulted in a further increase in expression (Figure 4.6).

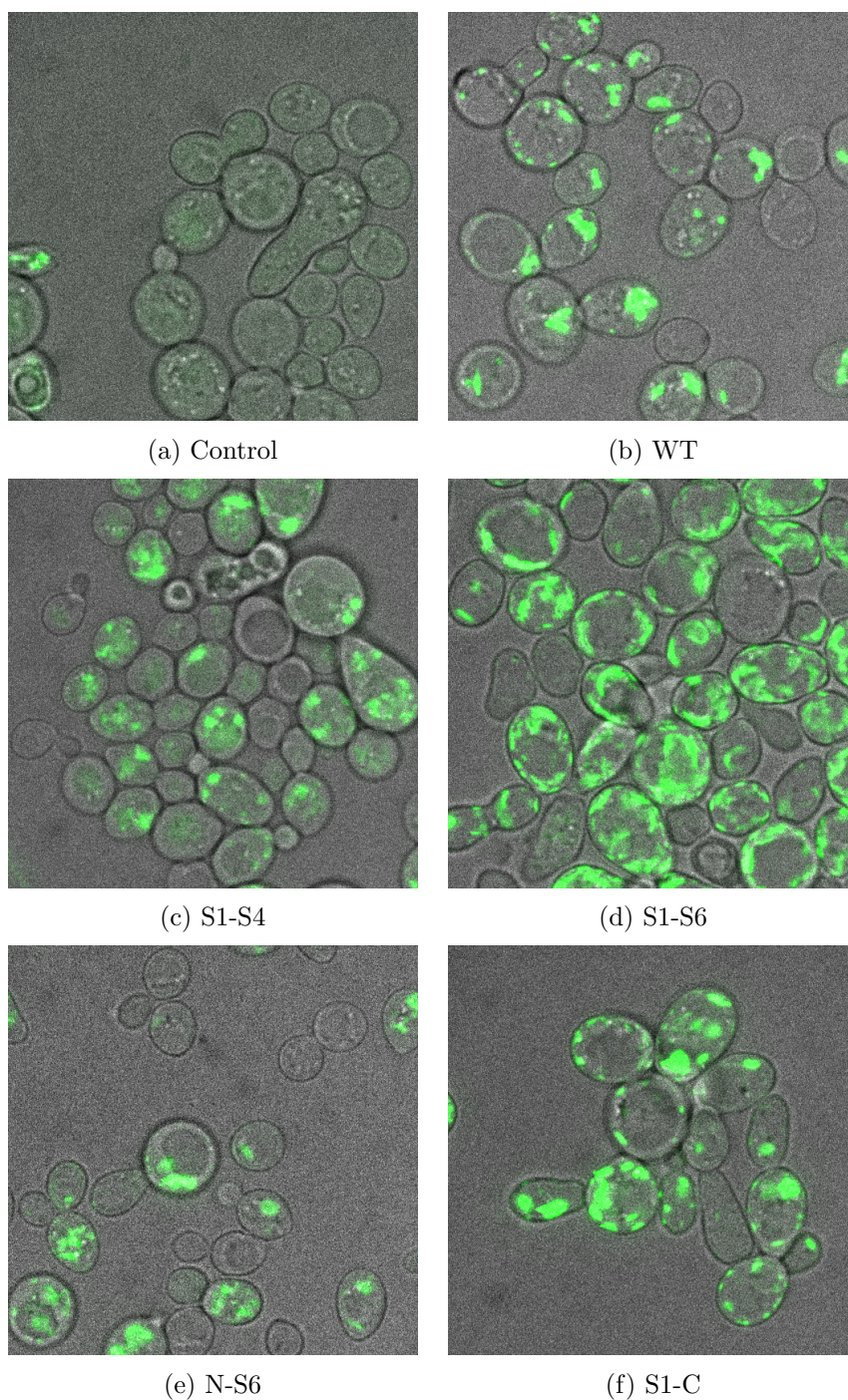
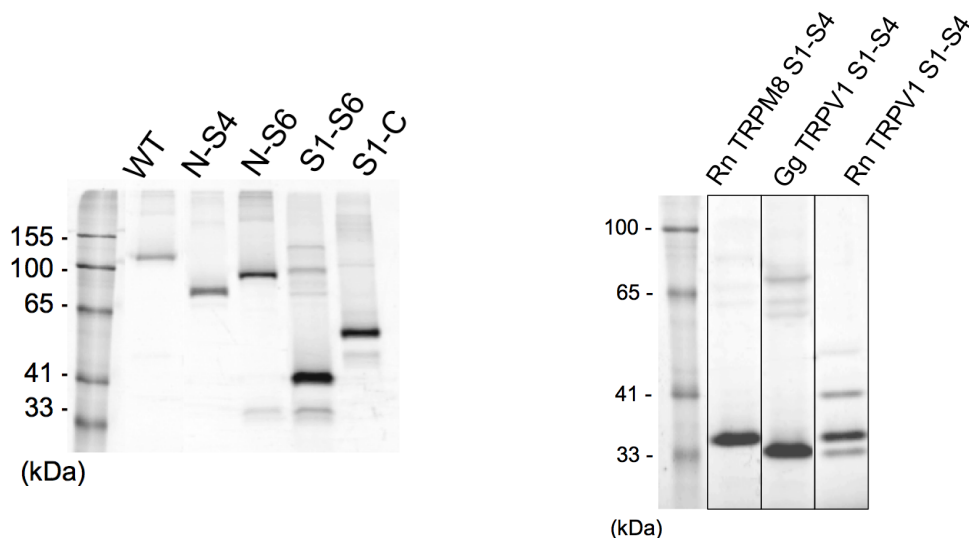


Figure 4.4: **Microscopy of overexpressed GFP-tagged TRPV1 constructs in *S. cerevisiae*.** (a) Control cells with empty plasmid. (b) Cells expressing WT rat TRPV1, as in Figure 3.3. (c) Cells expressing the S1-S4 helices of rat TRPV1. (d) Cells expressing the S1-S6 helices of rat TRPV1. (e) Cells expressing rat TRPV1 from the N-terminus to the end of the S6 helix. (f) Cells expressing rat TRPV1 from the beginning of the S1 helix to the C-terminus.



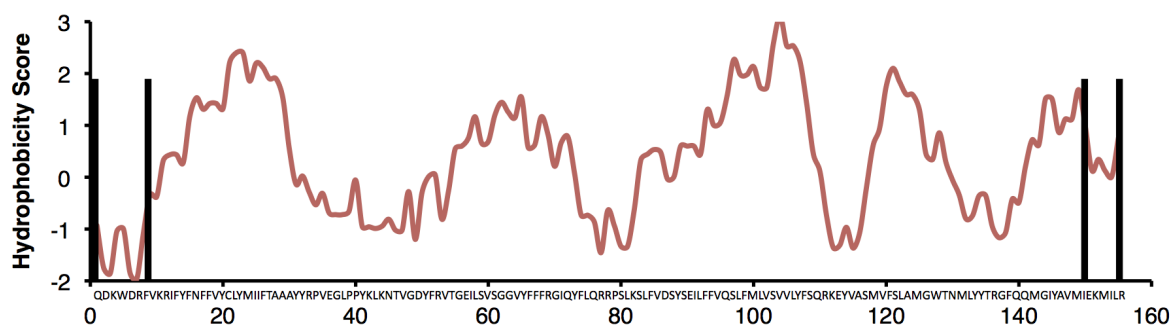
(a) In-gel fluorescence of TRPV1 truncations. (b) In-gel fluorescence of TRP S1-S4 helices.

Figure 4.5: In-gel fluorescence of TRP membrane-centric constructs. (a) In-gel fluorescence of small scale membrane preparations of yeast cells overexpressing GFP-tagged truncations of rat TRPV1 show promising expression with single bands indicating relatively low levels of aggregation and degradation. (b) In-gel fluorescence of small scale membrane preparations of yeast cells overexpressing GFP-tagged S1-S4 'domains' of rat (Rn) TRPM8, chicken (Gg) TRPV1 and rat (Rn) TRPV1.

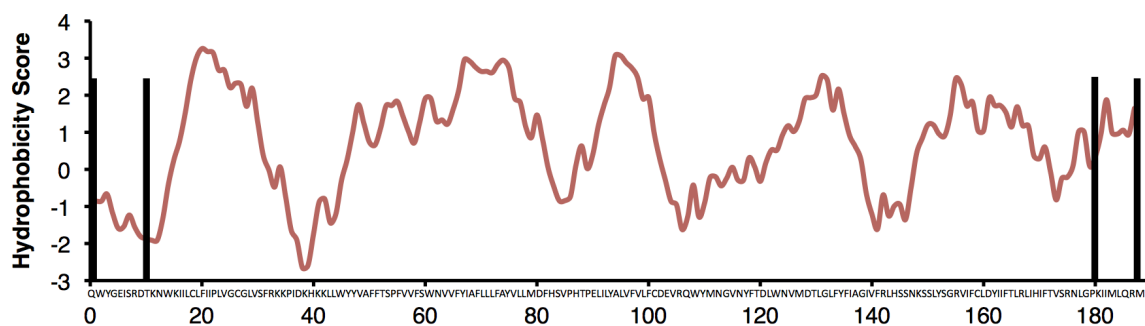
4.3 Assessing functionality of TRPV1 transmembrane regions determined by [^3H]-labeled resiniferatoxin binding assays

With this promising batch of initial expression trials and a working functional assay for WT rat TRPV1 expressed in the same *S. cerevisiae* system, the door was open to study TRPV1 transmembrane modularity via [^3H]-RTX binding assays.

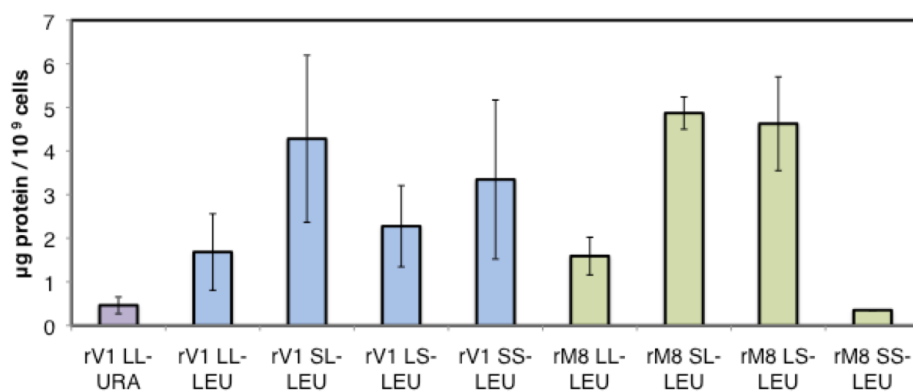
Comparable binding, however, was not seen to cells expressing any of the constructs missing either the N- or C-terminal domains (Figure 4.7), including the S1-S6 and S1-S4 regions of TRPV1. This result is in line with previous experiments performed in HEK 293



(a) TRPV1 S1-S4 hydrophobicity



(b) TRPM8 S1-S4 hydrophobicity



(c) Expression improvement by varying helix lengths

Figure 4.6: Varying construct length improves expression. (a) Hydrophobicity plot calculated via the Kyte-Doolittle scale via the ExPASy server of the rat TRPV1 S1-S4 region. Black lines indicate chosen cut-offs marked as Long (L) and Short (S). (b) Same as A for rat TRPM8. (c) Expression levels of S1-S4 constructs of TRPV1 and TRPM8 as calculated from whole cell fluorescence.

cells [124]. It also supports the idea that despite the topological similarity between TRP and voltage-gated channels, the insights that can be gleaned from this similarity may be limited regarding the functional significance of TRP channel S1-S4 helices [126]. Notably, it is possible that ‘partial binding’ is occurring to these truncated TRPV1 constructs, but just not at affinities detectable in this assay. However that high-affinity WT-like [^3H]-RTX binding requires both the N- and C-terminal domains raises questions about the exact nature of this binding site: does it require three distantly related regions of the protein to come together or indeed is TRPV1 so delicately tuned that allosteric modulation can result from mutants on opposite ends of the protein?

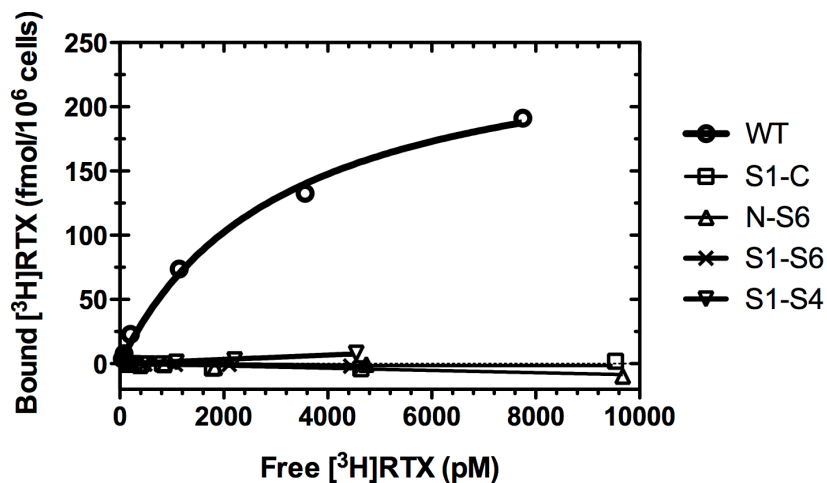


Figure 4.7: -

RTX binding to TRPV1 transmembrane-centric constructs. [^3H]-RTX binding to TRPV1 transmembrane-centric constructs. A comparison of [^3H]-RTX binding of the wild-type rat TRPV1 to transmembrane-centric constructs missing N- or C- terminal regions.

4.4 Stability of detergent-extracted TRPV1 transmembrane regions

With small scale trials indicating successful overexpression of stable, non-degraded S1-S4 and S1-S6 helices of TRPV1 and TRPM8 into *S. cerevisiae* membranes, further investigation followed of the detergent solubility and monodispersity of the protein to determine the quality of the protein as well as determine optimal conditions for large scale production and purification.

4.4.1 Fluorescence-coupled size exclusion chromatography of the GFP-tagged S1-S4 helices

After optimising both the expression system and the construct length for TRPV1 S1-S4 expression 2-3 L cultures were grown to perform full scale membrane preparations to test solubilisation efficiency and determine protein monodispersity with fluorescence-coupled size exclusion chromatography (FSEC) traces of membranes solubilised in 1% detergent.

Increasing overexpression with the -LEU system over the -URA system seems to have decreased the relative amount of protein aggregation, when comparing just the traces of S1-S4 (LL) (Figure 4.8 and Figure 4.9). When comparing FSEC profiles of the various lengths of S1-S4, it can be noted that cutting off the last few amino acids of the S4 seems to have negative effects on protein stability. Oddly, retention volumes of individual S1-S4 constructs seem to be inconsistent, most notably with the SL and LL constructs (Figure 4.9). This was found even with the same construct and the same detergent, and has been attributed to a natural variability in overexpression levels in the yeast, which might lead to variable lipid:protein and lipid:detergent ratios upon solubilisation, though a consistent volume of membranes and percentage of detergent was used.

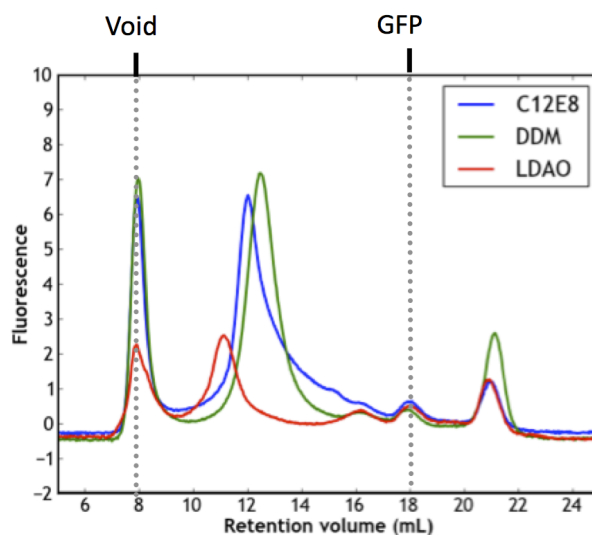


Figure 4.8: **Initial FSEC traces of TRPV1 S1-S4 (LL) from the -URA system.** Initial FSEC traces of the solubilised *S. cerevisiae* membranes with the S1-S4 helices from rat TRPV1 in the original -URA system.

After these results, two conditions were deemed most viable to bring to large scale production and purification trials: TRPV1 S1-S4 (SL) in DDM and LDAO (Figure 4.10).

While a range of conditions were screened to assess stability in rat TRPV1 S1-S4 helices in various detergents, screens were also conducted for the stability of the S1-S4 helices of a couple other TRP channels. While the peak:void ratio was not as favorable for these, the S1-S4 of both rat TRPM8 and chicken TRPV1 showed hints of monodispersity (Figure 4.11).

Overall these traces indicate that yeast can not only synthesise, properly fold, and insert the S1-S4 helices of TRPM8 and TRPV1 into membrane, but that the protein produced is monodisperse in DDM and LDAO.

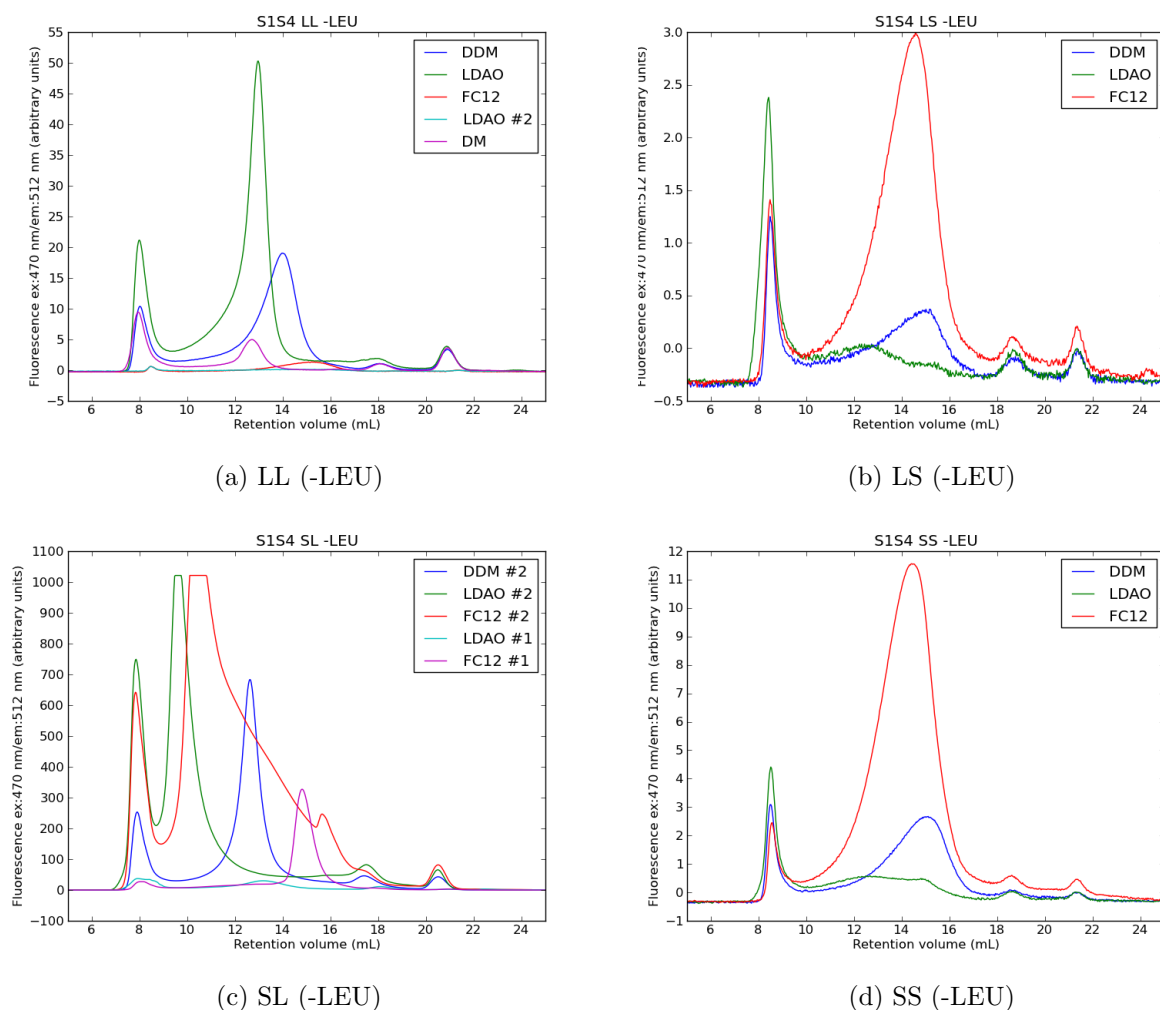


Figure 4.9: Comparing FSEC traces among TRPV1 S1-S4 constructs. Differences in solubilisation, stability and peak migration seen even with small construct changes. (a) Rat TRPV1 S1-S4 (LL) solubilised indicates most promise in DDM and LDAO. (b) Rat TRPV1 S1-S4 (LS) shows poor stability, and a broad peak even in the best detergent, Fos Choline 12. (c) Rat TRPV1 S1-S4 (SL) shows the most promising indications of protein monodispersity in DDM and LDAO, but with significant differences in retention volume. (d) Rat TRPV1 S1-S4 (SS), similar to (LS), solubilised shows poor stability, and a broad peak even in the best detergent, Fos Choline 12.

4.4.2 Fluorescence-coupled size exclusion chromatography of the GFP-tagged S1-S6 helices

From Figure 4.2b one can see that initial expression of rat TRPV1 S1-S6 was already at the level of S1-S4 after optimisation. While the S1-S6 did not exhibit as promising solubility

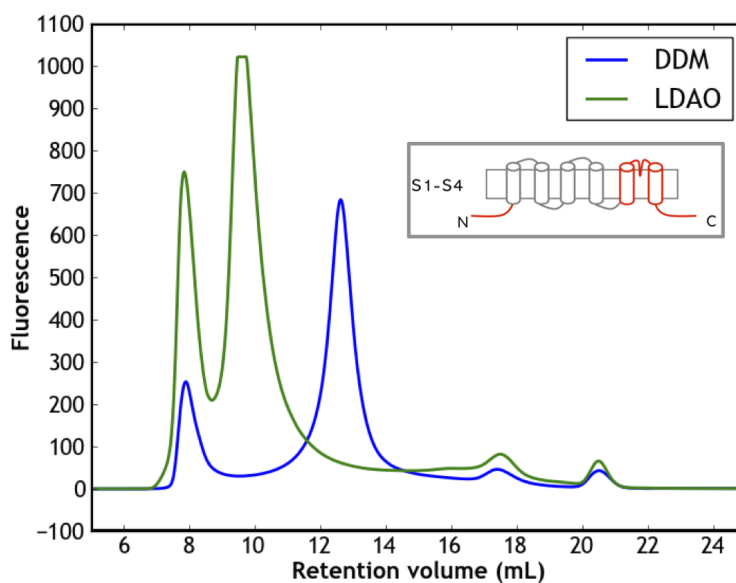
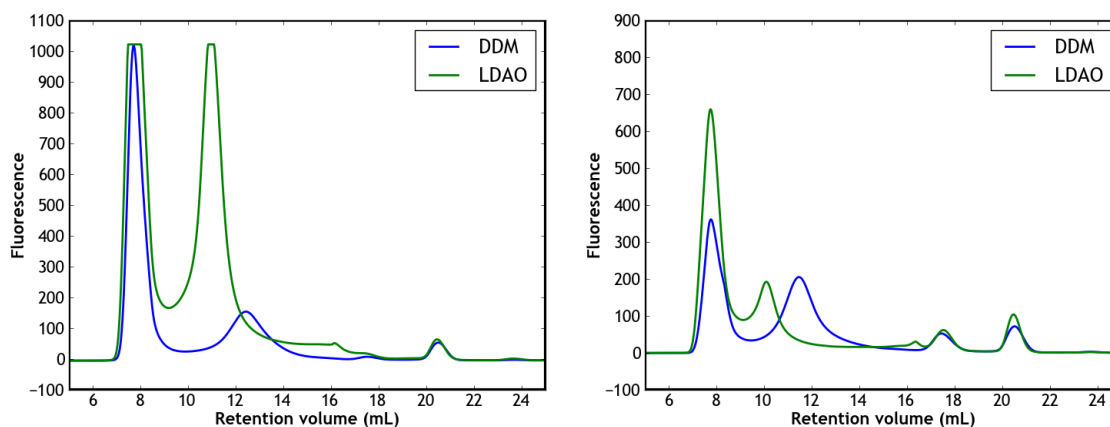


Figure 4.10: **Best FSEC traces of TRPV1 S1-S4 (SL).** FSEC traces chosen as most promising and favored for further large scale preparations were the rat TRPV1 S1-S4 in DDM and LDAO.



(a) FSEC of S1-S4 helices from chicken TRPV1. (b) FSEC of S1-S4 helices from rat TRPM8.

Figure 4.11: **Chicken TRPV1 and rat TRPM8 also show a stable domain-like S1-S4.** (a) The S1-S4 helices of the chicken TRPV1 shows promise in LDAO. (b) The S1-S4 helices of the rat TRPM8 show peaks with sloping sides and large aggregation, but still with some indication of monodispersity.

and evidence of stability in as wide a range of detergents as the S1-S4 constructs, its FSEC profile when solubilised in LDAO showed some promise for further large scale production (Figure 4.12).

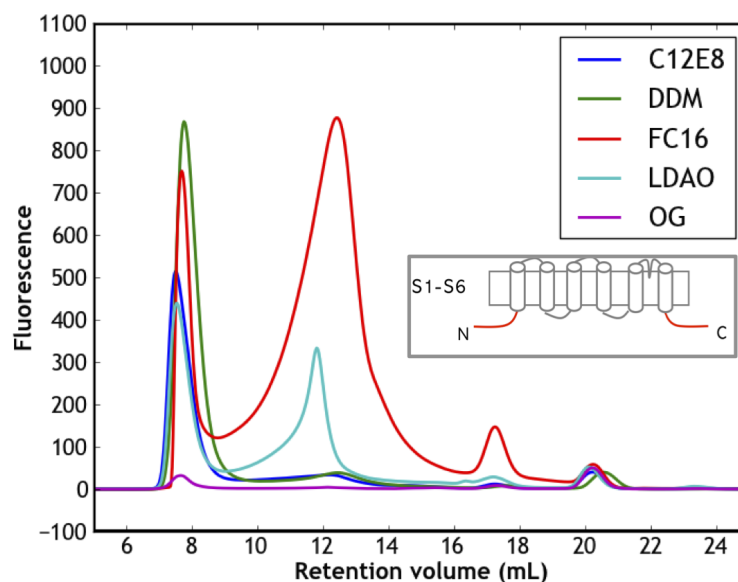


Figure 4.12: **FSEC traces of TRPV1 S1-S6 in various detergents.** FSEC profiles of rV1 S1-S6 in various detergents. Here, as with the wild-type, we disconcertingly see the best solubilisation with fos choline 16 which is extremely harsh. However, unlike with the wild-type protein, we see a promising profile with LDAO. Also, the retention volume is reasonably more consistent here than for S1-S4 profiles.

4.4.3 Fluorescence-coupled size exclusion chromatography of the GFP-tagged N- and C- terminal deletions from TRPV1

Unlike the solubility and monodispersity of the membrane-only constructs, the stability of the constructs with either N- or C- termini intact was limited in any detergent besides Fos Choline 16, similar to the full length protein (Figure 4.13).

Table 4.1: Overview of FSEC profiles of TRP membrane-centric constructs.

Construct	Variant	Detergent	Score	Solubilization Efficiency
S1-S4 Rn TRPV1	S1-S4 LL	DDM	*	-
		DM	*	-
		LDAO	*	-
		LDAO	X	49.3%
		FC12	*	86.6%
		2DPC:1LDAO	*	-
	S1-S4 SL	DDM	*	35.7%
		LDAO	X	57.6%
		FC12	*	-
		2DPC:1LDAO	*	-
		LDAO	*	-
		FC12	*→	76.6%
		DDM	*→	26.2%
		LDAO	*→	43.0%
		FC12	X	83.6%
	S1-S4 LS	D7PC	*	23.5%
		DDM	X	23.9%
		LDAO	X	27.1%
	S1-S4 SS	FC12	*	81.1%
		DDM	*	29.7%
		LDAO	X	11.8%
		FC12	*	63.6%
		2DPC:1LDAO	*	-
S1-S4 Gg TRPV1	S1-S4 LL	DDM	*	-
		LDAO	*	50.4%
		DDM	*→	19.9%
S1-S4 Rn TRPM8	S1-S4 LL	DDM	*	-
	S1-S4 SL	DDM	*	57.2%
	S1-S4 LS	DDM	*	51.6%
	S1-S4 LL	LDAO	*	54.4%
	S1-S4 LL	DDM	*	51.8%
S1-S6 Rn TRPM8	S1-S6	DDM	X	23.3%
S1-S6 Rn TRPV1	S1-S6	DDM	X	19.5%
		LDAO	*→	19.5%
		FC16	*	61.4%
		C12E8	X	18.8%
		OG	*	6.8%
N- C- Variants Rn TRPV1	S1-C	DDM	X	-
		LDAO	X	-
	N-S6	DDM	X	50.1%
		FC16	*	-
		LDAO	X	-

KEY

Detergents used:

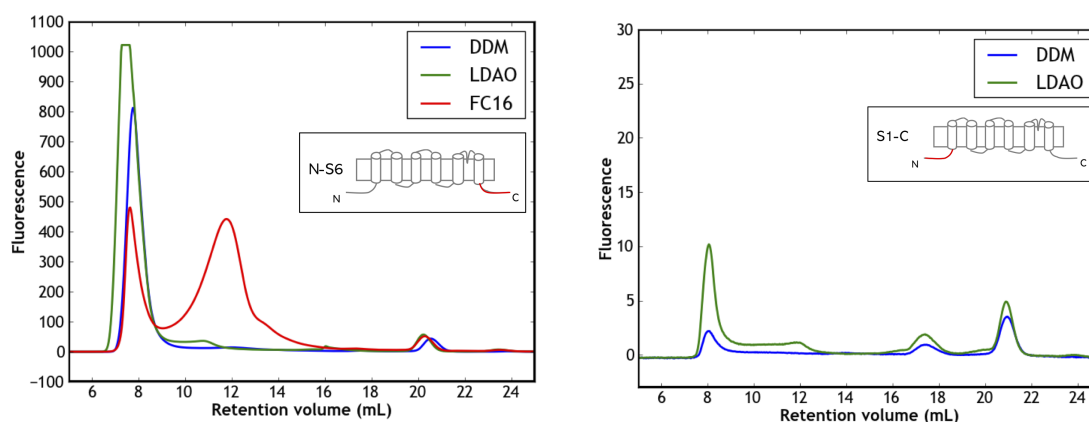
DDM, LDAO, DM, FC12, FC16, C12E8, D7PC, OG, 2DPC:1LDAO

* = Promising FSEC Profile with peak/void

* = Mediocre FSEC Profile with peak/void

X = Poor FSEC Profile

→ = Conditions chosen for purification trials



(a) FSEC of TRPV1 with C-terminus truncated. (b) FSEC of TRPV1 with N-terminus truncated.

Figure 4.13: FSEC profiles when leaving N- and C- termini intact. (a) FSEC profiles from the N-S6 construct in DDM, LDAO and Fos Choline 16 show only a modicum of solubility in the last, harsh detergent. (b) FSEC profiles from the S1-C construct in DDM and LDAO show now evidence of stable, soluble protein upon detergent extraction.

4.4.4 Overview of FSEC profiles of TRP membrane-centric constructs

After the success of the small-scale overexpression trials, the FSEC profiles of these proteins indicated only a few promising leads for stable detergent-solubilised membrane-centric TRP constructs. An overview of the FSEC results is presented in Table 4.1, and the construct/detergent combinations chosen for further large scale prep are indicated with bold arrows.

4.5 Purifications of transmembrane domains of TRPV1

After indications in the above data that the S1-S4 and S1-S6 helices of rat TRPV1 show reasonable stability when detergent-solubilised with DDM or LDAO, came the next steps of large scale protein production and purification.

4.5.1 Promising purification conditions for the S1-S4 helices of TRPV1.

Initial purification trials of the long construct expressed in the original -URA system in DDM yielded poor results as some protein was visible in both in-gel fluorescence and coomassie stained gels, but was largely masked by other protein impurities. Solubilisation efficiency of protein from cells to detergent-solubilisation was 50%, which is respectable. Size exclusion results were similarly not promising, and this was partial motivation for continuing with optimising the new yeast system and length variants.

The first purification attempt of the new SL construct of S1-S4 overexpressed in the -LEU system was performed in Fos Choline 12. Solubilisation efficiency rose to 85-90%. Results were initially promising with clear protein bands visible in elution during the first step of purification (Figure 4.14). However after dialysis with TEV protease, GFP cleavage was not seen. Additionally concentrating the protein was unsuccessful as it would consistently stick to the filters of both the 50 kD and 100 kD MWCO VivaSpin Filters. Running the 'concentrated' protein down a Superdex 200 10/300 size exclusion chromatography column, however, resulted in some promising elution profiles with two peaks perhaps indicating oligomerisation and a distinct peak eluting around 13 mL (Figure 4.14). The contaminant and TEV, however remained.

With these problems with GFP cleavage and concentration, DDM was then approached as another possibly promising detergent from FSEC screens. With this purification, GFP cleavage was seen upon dialysis with TEV protease, visible by the simultaneous appearance

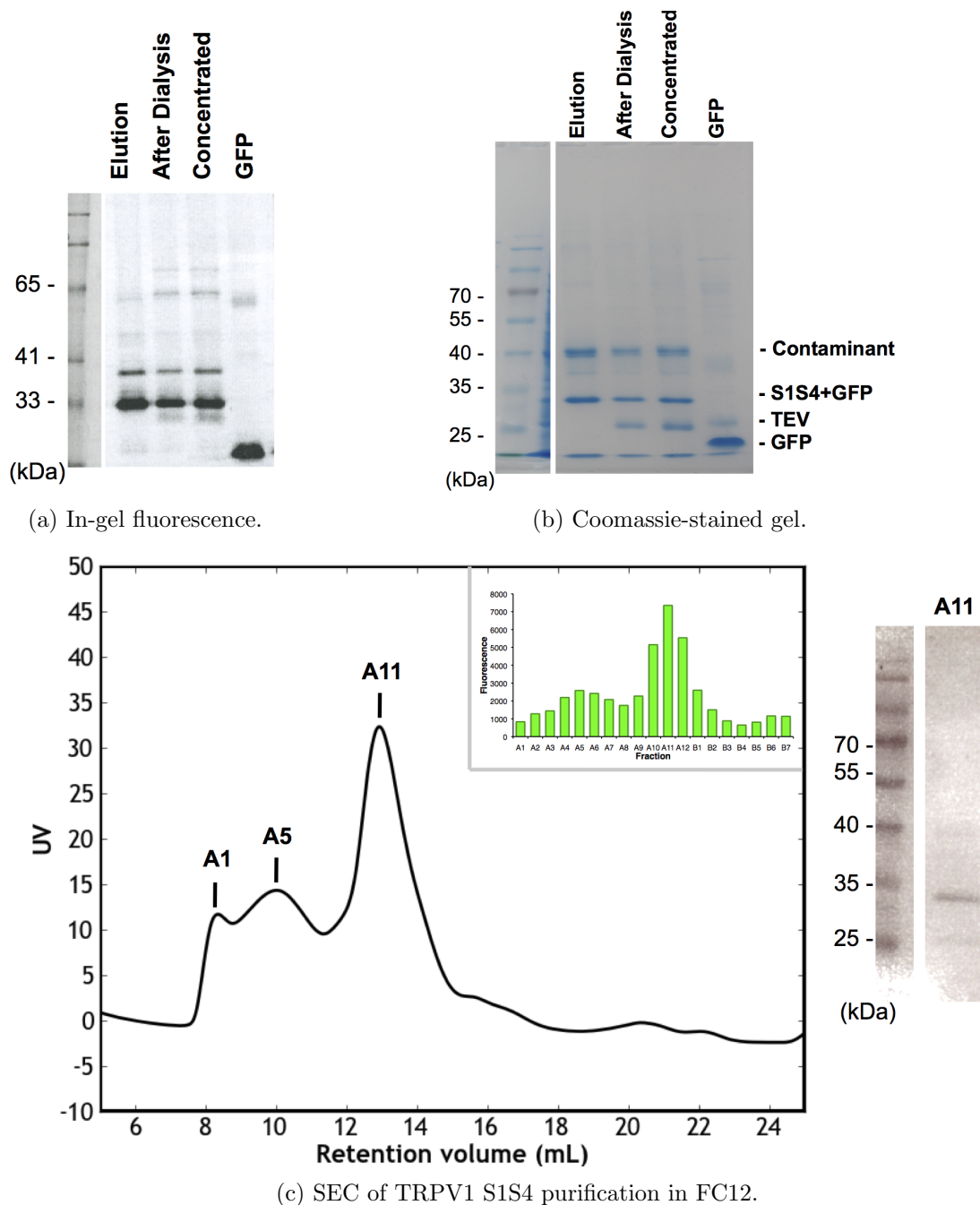


Figure 4.14: **Gels and SEC of TRPV1 S1-S4 (SL) purification with solubilisation in FC12.** (a) In-gel fluorescence of the Elution in 250 mM Imidazole from first step of purification, Elution after overnight Dialysis with TEV protease of a trial purification of TRPV1 S1-S4 in FC12. Notice no GFP appears after incubated with TEV protease. (b) Coomassie-stained gel same as (a). Notice the presence of TEV protease. (c) Size exclusion chromatography profile of the concentrated Elution using the Superdex 200 10/300 size exclusion chromatography column. Notice the protein does not show up in the void volume, but instead elutes in two peaks, the second of which could represent a monodisperse portion of the protein. An inset shows the fluorescence of each fraction, and to the left is the coomassie stained gel of the peak fraction.

of a GFP band in in-gel fluorescence and disappearance of the S1-S4 in the coomassie-stained gel upon dialysis (Figure 4.15). During the second step of purification, however, the concentration of the flowthrough, which is supposed to contain a GFP-less protein has no S1-S4 to be seen.

Similar results were seen for LDAO and a trial purification of the TRPV1 S1-S4 of chicken in DDM (Figure 4.16). A consistent band was appearing around the expected size of a dimer in both in-gel fluorescence before purification and coomassie-stained gels after TEV-cleavage and concentration, hinting that perhaps the S1-S4 was dimerising upon TEV cleavage. When this band was extremely prominent in three subsequent purifications it was sent off for analysis via mass spec. Unfortunately this was determined to be a bacterial protein that was later discovered to be a contaminant from the in-house TEV protease production.

4.5.2 Promising purification conditions for the S1-S6 helices of TRPV1

A similar result was seen in purifications with the rat TRPV1 S1-S6 in LDAO: while pure GFP-tagged protein was seen after the first step of purification (Figure 4.17), further purification either via GFP cleavage or size exclusion chromatography proved prohibitive. A final purification attempt conducted in hopes to characterise the protein without removing the GFP-tag, resulted in the same inability to concentrate the protein as seen with the S1-S4 construct, due to its propensity to stick to the filters of both the 50 kD and 100 kD MWCO VivaSpin concentrators, resulting in the concentration of the contaminants at the expense of the TRP protein itself (Figure 4.18). Additionally, attempts to separate the S1-S6 from the contaminants via SEC resulted in a broad elution profile (Figure 4.18), making any attempts toward purification of the protein without removing the GFP tag futile. Additionally, this broadness indicates that the protein would likely be difficult to characterise by biophysical methods even in the event of purification.

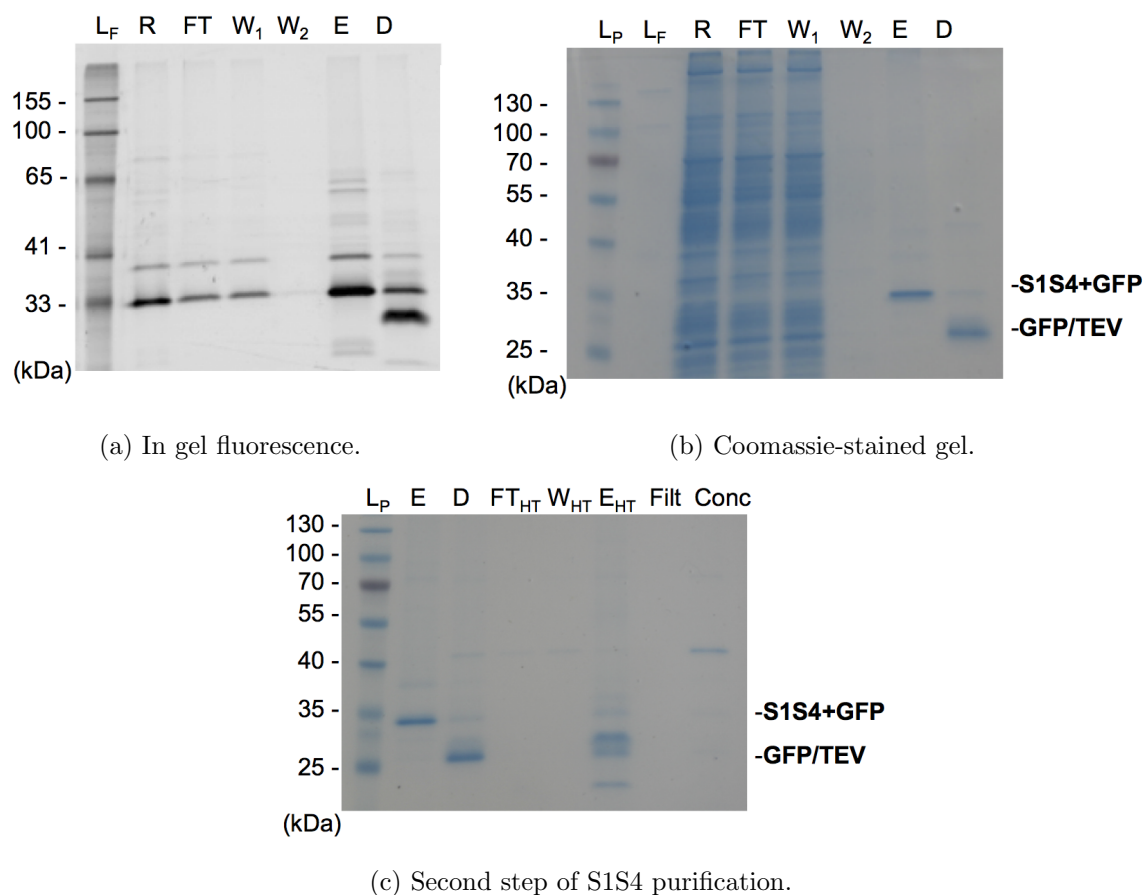
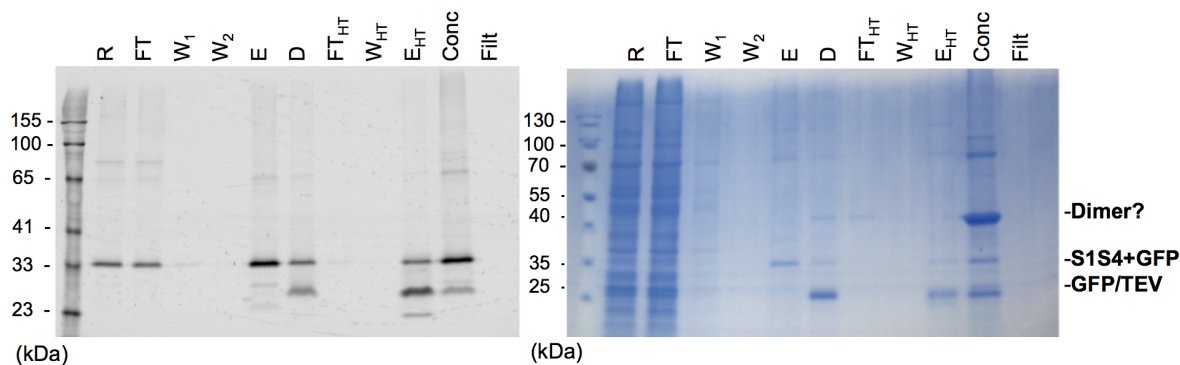


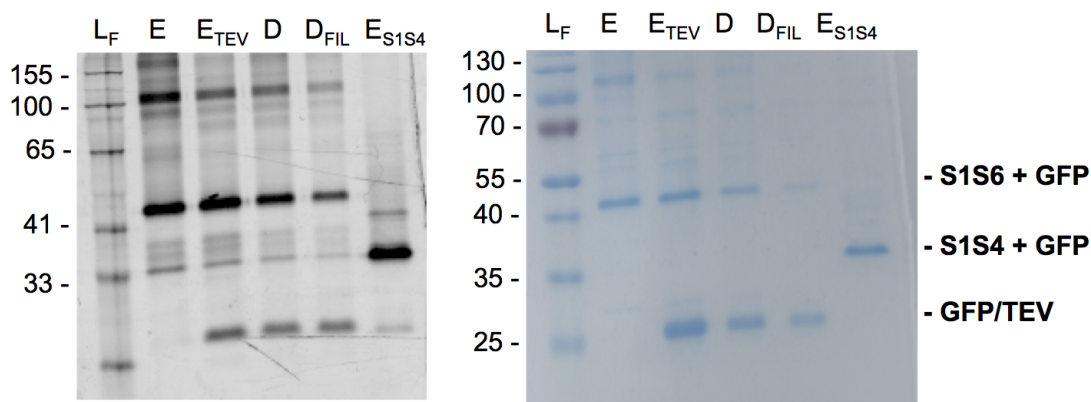
Figure 4.15: Purification of TRPV1 S1-S4 in DDM. (a) In-gel fluorescence image of the first step of purification of S1-S4 (SL) in DDM. It is clear to see the S1-S4 with GFP tag in the flowthrough, wash and elution. It is also clear to see the appearance of a GFP band after TEV cleavage. (b) However in this Coomassie-stained gel, it is not clear where the band for the S1-S4 with GFP removed is. (c) In the second step of purification, the GFP should stick to the column, while the protein remains in the flow through. The flow through can then be concentrated, but here in the Concentrate (Con) no bands of appropriate size remain, and the one that could possibly be a dimer (45 kDa) has been identified as a contaminant. Lanes: L_F and L_P - Fluorescent or pre-stained ladder, R and FT resin and pre-flow through or flow through, W₁ and W₂ washes, E Elution, D sample after Dialysis with TEV protease, FT_{HT} Flow through for second step of purification via his-trap column, W_{HT} Wash for second step, E_{HT} Elution for second step, Fil and Con the filtrate and concentrate of the FT_{HT} using a 50 MWCO VivaSpin filter.



(a) In-gel fluorescence.

(b) Coomassie-stained gel.

Figure 4.16: **Purification of chicken TRPV1 S1-S4 in DDM.** In-gel fluorescence (a) and Coomassie-stained gel (b) of the entire two step purification of chicken TRPV1 S1-S4 in DDM. Lanes are defined as in previous figure. Note the appearance of GFP in lane D in the in-gel fluorescence image. The possible dimer in the concentration of the His-Trap Flow Through was seen in this purification as well as DDM and LDAO purifications of rat TRPV1 S1-S4. However it was also seen in the purification of a completely unrelated protein and was discovered to be a contaminant.



(a) In-gel fluorescence.

(b) Coomassie-stained gel.

Figure 4.17: **Purification of TRPV1 S1-S6 in LDAO.** (a) In-gel fluorescence image of rat TRPV1 S1-S6 purification in LDAO. We see a similar problem as in S1-S4 purifications: we see GFP cleavage, but cannot identify the TRP protein of choice sans GFP. (b) Coomassie staining of same gel. Lanes: LF and LP - Fluorescent or pre-stained ladder, E Elution, E_{TEV} E + TEV without Dialysing, D sample after dialysis + TEV, D_{FIL} D after filtration with 22 μ M filter, E_{S1S4} E from Figure 4.15.

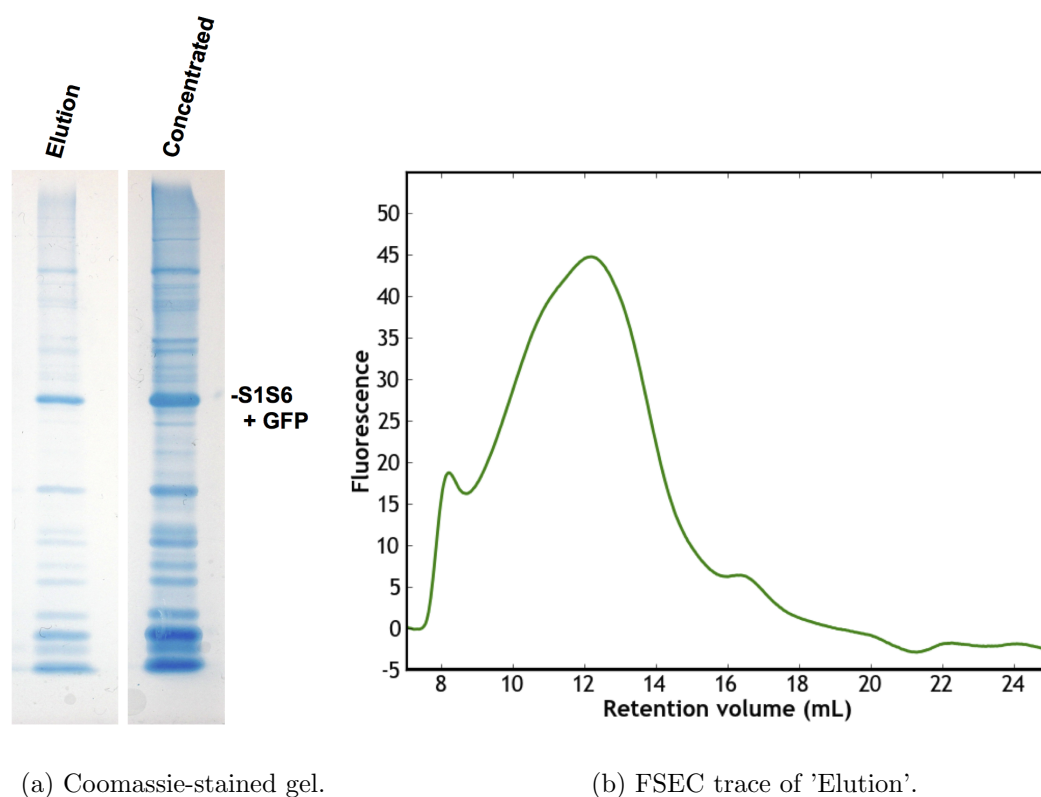


Figure 4.18: Retaining GFP in TRPV1 S1-S6 purification. (a) Coomassie-stained gel after a one step purification of GFP-tagged S1-S6 in LDAO of the Elution and of the Elution concentrated from 25 mL down to 1 mL in a 100 kD MWCO VivaSpin Concentrator. (b) The size exclusion chromatography trace of the fluorescent protein in the "Elution" sample shows a broad peak indicative of unstable protein, which could explain why it was sticking to the filter of the concentrator.

4.5.3 Interpreting and addressing problems with TRPV1 transmembrane domain purification

In all the purifications performed, no matter the species (chicken or rat), detergent (FC12, LDAO, or DDM) or construct (S1-S4 or S1-S6) the same problems arose in GFP-cleavage and sticking to concentration filters. In an attempt to address this issue, several approaches were taken to assess TEV cleavage efficiency and protein aggregation. While there has been some evidence that DDM inhibits TEV-protease activity [181], we ruled this out by performing the TEV cleavage in a variety of detergents and did not see obvious differences in cleavage efficiency as related to detergent choice (Figure 4.19). Additionally, we saw clear evidence of protein aggregation after the overnight dialysis with TEV protease (Figure 4.19). That this aggregation occurred only during overnight dialysis with TEV and not the same overnight dialysis procedure without TEV is evidence that this is due somehow to the TEV cleavage process and not the overnight dialysis itself (data not shown).

Since protein production for these constructs remained promising, with the main obstacle being methods of further concentrating and purifying the protein, an exploration of construct expression without a GFP tag began. These results were initially promising in small scale trials (Figure 4.20), and a purification trial of the rat TRPV1 S1-S4 helices revealed positive peptide identification upon mass spec analysis of the concentrated result of a two step purification (Figure 4.20).

4.6 Conclusions and future directions

From a promising start in small scale expression trials through evidence of monodispersity in FSEC screens to as-of-yet unsuccessful large scale purifications, there remain indications that the biophysical characterisation of a purified TRP channel transmembrane domain remains on the horizon.

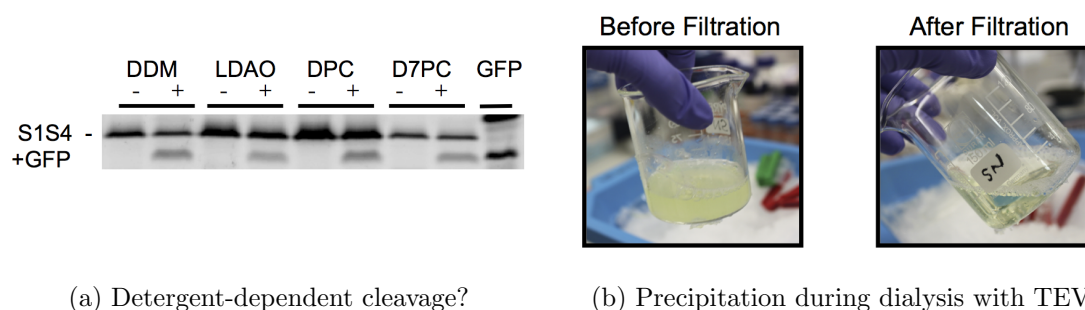


Figure 4.19: **Investigating problems with GFP cleavage.** (a) Small scale membranes of S1-S4 (SL) expressing yeast, were solubilised for an hour with 1% detergent and then incubated overnight at 4°C with (+) or without (-) TEV protease. An obvious band for GFP appears upon TEV cleavage, but efficiency is poor in all detergents. (b) In this purification of S1-S6 in LDAO, it is clear to see the aggregate that forms during overnight dialysis with TEV. The bottom picture is the same sample after passing through 22 μ M filter.

4.6.1 Possible avenues to successful purification

The most promising avenues for the purification of the TRPV1 S1-S4 or S1-S6 are likely those that either do not require a GFP cleavage step or do not incorporate GFP in the first place. For example, the size exclusion trace for the GFP-tagged rat TRPV1 S1-S4 purified in Fos Choline 12 shows a promising monodispersity, despite the fact that it was initially dismissed due to exhibiting 0% cleavage efficiency. Additionally, further optimisation of the HIS-only tagged constructs may prove to be a successful approach. If not, higher affinity tags such as a FLAG tag or 1D4 tag could improve protein purity in this situation [69, 183]. Also available, is a wider range of detergents to screen, such as the amphipols recently seen to be successful in solubilising TRPA1 [58]. Additionally, stabilising mutant screens and drug screens can be used to stabilise these membrane domains, such as has been successful in the structural characterisation of GPCRs [271].

Similarly, not fully tested in this chapter are the constructs of other species of both TRPM8 and TRPV1, which may lead to a stable protein domain without the addition of expensive detergents or time-consuming mutant screens to the protocol. Currently readily

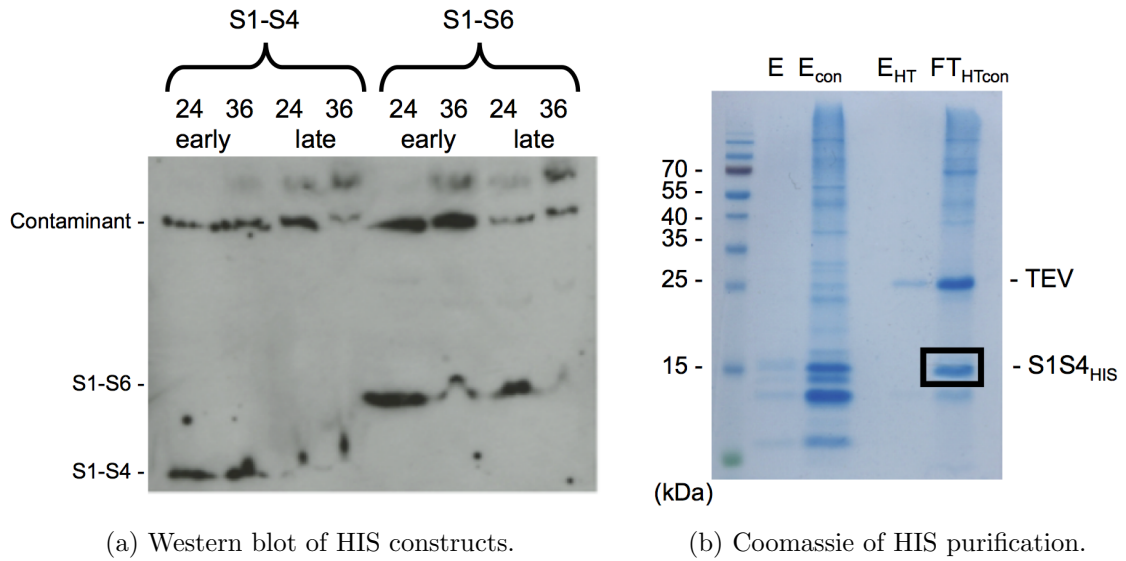


Figure 4.20: **Successes in leaving off the GFP tag.** (a) A Western blot using His-tag-directed antibodies of membranes of a small-scale expression trial of rV1 S1-S4 and S1-S6 expressed in yeast without GFP tags. Cultures were induced either early or late (OD600 0.9 or 1.2 for S1-S4, and 0.6 or 1.0 for S1-S6), and harvested either 24 or 36 hours after induction. (b) Coomassie-stained Tris-Glycine gel with band sent for mass spectrometry analysis highlighted. Lanes are E - Elution from first step of purification from the Nickel Column. E_{con} - This elution concentrated. E_{HT} - Elution from the second step of purification after TEV cleavage. FT_{HTcon} Concentration of flow through from the second step of purification. The band at 25 kDa is likely TEV protease used to cleave off the His-Tag. (c) The rV1 S1-S4 (SL) sequence with the peptides identified from mass spec analysis of the boxed band in (b) highlighted in red.

available are the three species rat, frog and chicken of both TRPV1 and TRPM8. If one takes the same protocol as described above with four length variants for each S1-S4 (SS, SL, LS, LL), that is a total of 24 constructs, only 9 of which have been tested here to any extent.

4.6.2 Structural modularity without functional modularity?

While these studies have not yielded pure protein, the successes in purification and the monodispersity of some constructs indicates that *S. cerevisiae* is indeed able to produce and insert high quantities of both the S1-S4 and S1-S6 helices of TRPV1 and TRPM8 into the membrane. Though the characterisation of these proteins as having the expected α -helical fold has proved elusive, the successes seen indicate that this path is not ill-conceived and that the S1-S4 is likely a structural domain in TRP channels. In further support of this conclusion are the notable negative FSEC results of not only the S1-C and N-S6 constructs of TRPV1, but also the S1-S4 domains with shortened S4 helix.

While evidence points toward the S1-S4 helices as being a structural domain in TRP channels, the functional assay used to characterise rat TRPV1 is evidence that this domain is not necessarily a separate functional domain, in the sense of being sufficient for ligand binding. There are several interpretations available to why RTX would not bind to the isolated S1-S4 helices. Two key pieces of experimental evidence can help interpret this. The first is an experiment that implicates the N- and C- termini in capsaicin sensitivity [124]. The second is that it is predicted that the C-terminus is needed for TRPV1 to oligomerise, perhaps via a coiled-coil domain [83]. Thus, it is possible that either these domains are required for the stable assembly of the TRPV1 such that the capsaicin/RTX binding pocket itself is stable. Conversely, the N- or C-termini could be directly involved in the RTX binding site. The experiments here cannot distinguish between these two reasons for a lack of WT-like binding to either the S1-S4 or S1-S6 helices of TRPV1.

While interpreting FSEC traces and expression levels of TRP channel S1-S4 domains has its limits as an approach, combining this with functional data has been able to provide insights into how these proteins might be behaving. A study of TRP/Kv chimera's indicated that the S3-S4 'paddle motif', known to have few contacts to other parts of the protein in

Kv channels, was not transferable to TRP channels [126]. Indicating that perhaps the S1-S4 and S5-S6 pore helices of TRP channels are more tightly packed than in Kv channels, further supported by the lack of binding to the S1-S4 helices in the experiments presented here. This is also in line with the ‘stickiness’ seen upon protein purification. While evidence for a domain exists, as discussed in the paragraphs above, perhaps the increased number of contacts between the S1-S4 and pore helices adds that little bit of instability that causes the protein to stick to concentration filters or aggregate upon GFP-cleavage.

Bioinformatics and modelling of TRP channels

Remember that all models are wrong; the practical question is how wrong do they have to be to not be useful.

–George E.P. Box, *Empirical Model-Building and Response Surfaces*, 1987

5.1 Introduction

The preceding two chapters address structural and functional questions of TRP channel modularity via wetlab methods, however with the difficulties inherent in purifying eukaryotic membrane proteins, approaches to these same questions by computational methods using the resources of TRP channel sequence data and the template structures of other voltage-gated-like (VGL) channels becomes natural.

5.1.1 The Voltage-Gated-Like ion channel superfamily: a sound foundation for TRP channel studies

While TRP channels were first discovered in *Drosophila melanogaster* by the 1970s [55], their relationship to voltage-gated potassium (Kv) and voltage-gated sodium channels

(Nav) channels was not determined until the 1980s [186], and what are now the most well-known subtypes, the heat sensitive Thermo-TRPs, did not begin to be discovered until the late 1990s [44]. Now TRP channels number as the second largest member of the voltage-gated-like (VGL) channelome, a term originally proposed in a review by William Catterall in 2004, which includes the Nav, Kv, voltage-gated calcium (Cav), calcium-gated potassium (CaK), cyclic-nucleotide-gated potassium (CNG), inward-rectifying potassium (Kir), two-pore potassium (K2P), and TRP channels, whose similar tetrameric pore domains, voltage-sensor domains and selectivity filters allow them to be grouped together (Figure 5.1) [307].

While the VGL channel superfamily provides a fertile background from which to approach TRP channel structure-function relationships, including the over 100 individual crystal structures of members of the superfamily starting with the bacterial KcsA in 1998 (listed in Chapter 1), only a handful of these contain the S1-S4 voltage-sensor-like domain and of these only one is eukaryotic. With this pattern, and the TRP family devoid of prokaryotic homologues, the lack of high resolution TRP channel structures is not surprising (Figure 5.1).

5.1.2 Molecular dynamics studies of voltage-gated channels

A range of studies incorporating molecular dynamics simulations of crystal structures of voltage-gated channels as well as homology models based on these structures have been conducted. In line with when structures became available, these simulations initially focused on the selectivity filter, and indeed ion conduction remains a topic of active study, and extended to properties of the voltage sensor. While there is a broad range of literature on the selectivity filter and ion conduction, these will not be addressed at length, allowing the focus to remain instead on studies of the voltage-sensor and voltage-sensor-like helices.

Molecular dynamics studies of structures of the S1-S4 helices, initially showed an intrinsic flexibility in the voltage sensor helices of the crystal structure of the isolated KvAP

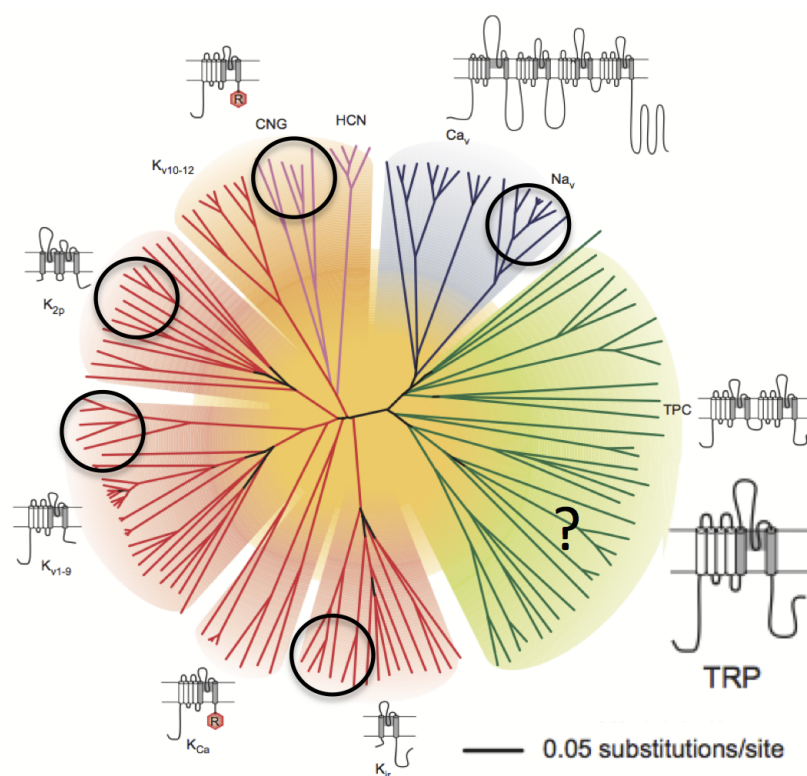


Figure 5.1: **The voltage-gated-like channel superfamily.** Transient receptor potential (TRP) channels are the second largest family in the voltage-gated-like (VGL) channel superfamily. TRP and TRP-related channels are represented in green in this phylogenetic tree adapted from Yu & Catterall (2004), which was generated by analysis of the pore domains of the VGL channels. Families for which structures have been solved are highlighted with black circles.

[239]. Then a clearer picture started to appear regarding the importance of membrane deformation to voltage-sensor function [39, 137, 240]. This membrane deformation was consistently seen in simulations of the available structures for the full 6 TM tetrameric channels as well as homology models of the S1-S4 of Hv1 and Ci-VSP[185]. Notably this deformation was even seen for the structure of the *M. loti* bacterial CNG channel, which does not contain a full set of positive residues in the S4 helix. In line with membrane deformation, the voltage-sensor has also been seen to form a vestibule in which water can penetrate into the bilayer, a characteristic which is predicted to facilitate proton transport

in experiments and simulation of an Hv1 homology model [227]. In addition, simulation studies have been conducted to describe how a voltage-sensor toxin interacts with the lipid bilayer to facilitate its interaction the voltage sensor [295]. Altogether these simulations form a respectable backdrop for addressing both modularity and the bilayer-mediated toxin / ligand interactions of TRP channel S1-S4 domains.

5.1.3 Current state of structural understanding of TRP channels

The relationship of TRP channels to the larger superfamily of the VGL-channome allows for the broad hypothesis of TRP channel structure: a 6-TM tetrameric ion channel (Figure 5.2) with two helices (the S5 and S6 helices) forming the cation-selective pore. This hypothesis has since seen experimental support with evidence of tetramerization [133, 7] and the evidence for a selectivity filter between the S5 and S6 helices [206, 288]. The most conserved secondary structure element of TRP channels is their transmembrane region, and the most universal features of this region are the selectivity filter between the S5 and S6 and the TRP Box at the C-terminal end of the S6 (Figure 5.2). Even now knowing the 28 mammalian TRP channels and their seven subgroups (A, C, M, ML, N, P, V), these conserved regions are conserved most strongly within specific TRP subfamilies, thus making conjectures of TRP-wide mechanisms difficult.

Outside these regions, even the transmembrane regions of TRP channels remain poorly understood. When TRP channels were initially cloned, their number of transmembrane α -helices was unclear, with a first prediction of eight rather than the currently accepted six [186]. A study in which glycosylation sites were engineered into TRPC3 helped clarify that the first helix of the predicted seven potential transmembrane alpha-helices most likely did not cross the membrane [281], confirming the existence of an S0 helix in many TRP channels, similar to the S0 in BK potassium channels [175]. While the current general consensus is that TRP channels have the same architecture as other VGL channels, it remains unclear

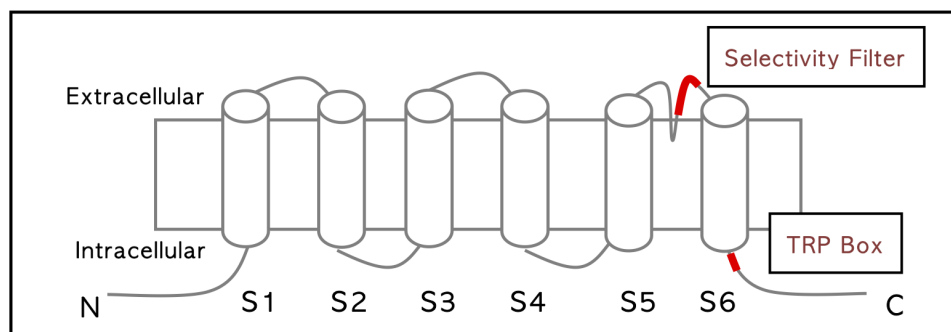


Figure 5.2: **TRP channel schematic.** While TRP channels have variable N- and C-termini (see Introduction for further details), the transmembrane domain across all sub-families is predicted to contain a 6-TM tetrameric ion channel with two helices (the S5 and S6 helices) forming the cation-selective pore.

whether the mechanisms known to be involved in channel activation are conserved as well. For example, the functionality of the S1-S4 voltage-sensing domain and the role of the S4-S5 linker in coupling voltage-sensor movement to channel gating have been well-studied and characterized in Kv channels, but have yet to be thoroughly investigated in TRP channels.

Homology modelling remains the only structural resource available for the transmembrane domains of TRP channels. Homology modelling of membrane proteins has shown a range of successes depending on sequence identity and the lengths of helices and loops [306, 77]. One of the earliest attempts at TRP channel modelling was a homology model used to address PIP₂ binding to TRPV1 in 2007 [32]. This model included the C-terminal domains, and was simulated in an explicit lipid bilayer in the presence of PIP₂. Another approach was the modelling of the full-length TRPV1, including all cytosolic domains, to attempt to draw an initial picture of what the field could expect, however at the time, the information available was far from sufficient to create a reliable picture [74]. Then, most recently a model was built to address capsaicin and RTX binding to the transmembrane domains of TRPV1 [144].

5.2 Understanding trends in VGL channel transmembrane domains to build TRP channel homology models

With a handful of available structures containing the same 6 TM architecture predicted of TRP channels, building an informative homology model seems feasible. While TRP channels have low sequence identity to the available templates, so too do the templates to each other but they display a remarkable structural similarity (illustrated in detail in Figure 1.15). Additionally the success of simulations of the homology model of the Hv1 S1-S4 on the Kv S1-S4, encourages a push toward homology models of, at the very least, the TRP S1-S4 helices.

5.2.1 Comparing TM helices between TRPs and the rest of the VGL channome

While searching for the most appropriate template for a homology model for the TRPV1 transmembrane helices, a couple of characteristics surfaced as being most significant. The templates available are the bacterial KvAP channel [116], the rat Kv 1.2 / 2.1 ‘paddle’ chimera [156], a bacterial CNG channel from the bacteria *M. loti* [52], and a voltage-gated sodium channel from bacteria [215]. While other variants on these structures are available they are generally not ostensibly different for the purposes of building a homology model, for example a structure of the rat Kv 1.2 S1-S6 tetramer is largely similar to the structure of that of the same protein with the S3-S4 ‘paddle’ of the Kv 2.1, used here, and is of lower resolution [155]. As TRP channels do not have the regularly spaced charged residues in their S4 helices characteristic of canonical voltage-sensitive channels, simple calculations of the isoelectric points of each TM helix were conducted to attempt to identify any similarly characteristic elements (Figure 5.3). While these are approximate calculations that do not take the lipid or protein environment into account, they replicate the expected outliers in

the charged S4 of the Nav and Kv structures, 3RVY and 2R9R respectively. One of the key factors taken into account in judging the most appropriate templates for TRP homology models was predicted TM lengths (Figure 5.4). This proved to be a problem, discussed more fully later, when attempting to model on the NavAb structure, despite having the best sequence identity of any alignment.

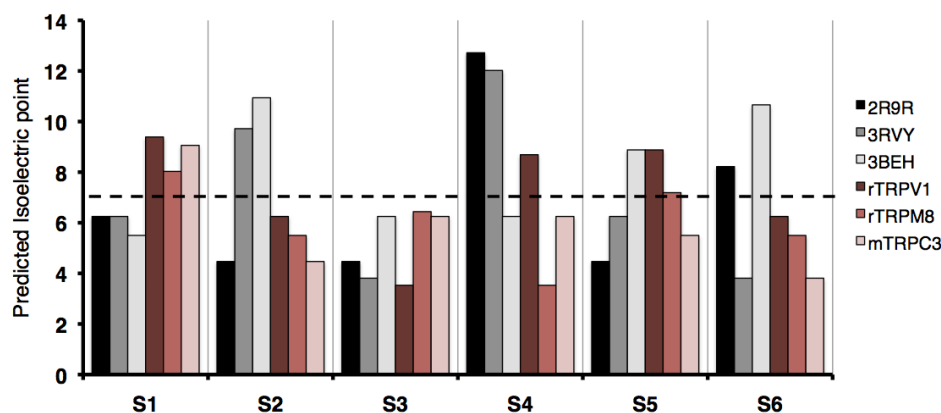


Figure 5.3: **Comparing theoretical pIs of the TMs of TRP channels and available templates.** The isoelectric point of the helices as calculated using the Scripps Protein Calculator at <http://www.scripps.edu/~cdputnam/protcalc.html> shows that the charge distribution across TM helices in TRP channels is not as obviously skewed as in Nav and Kv channels, 3RVY and 2R9R respectively. Note that these are not meant to be physiological pIs but are a reasonable first approximation.

5.2.2 Homology models of the TRPV1 S1-S4

Homology models of the TRPV1 S1-S4 were originally built on a range of templates. An alignment of the KvAP, the Kv Chimera, and the NavAb structure sequences with rat and chicken TRPV1 was made using the MAFFT sequence alignment algorithm with added manual adjustments [129] (Figure 5.5). The resulting alignments resulted in sequence identities of 9.5% to KvAP, 10.2% to the Chimera and 17.5% to the NavAb structure. With such a high sequence identity between the TRPV1 and NavAb S1-S4 helices, it was predicted this template would provide the best homology model. However, the S1 helix of

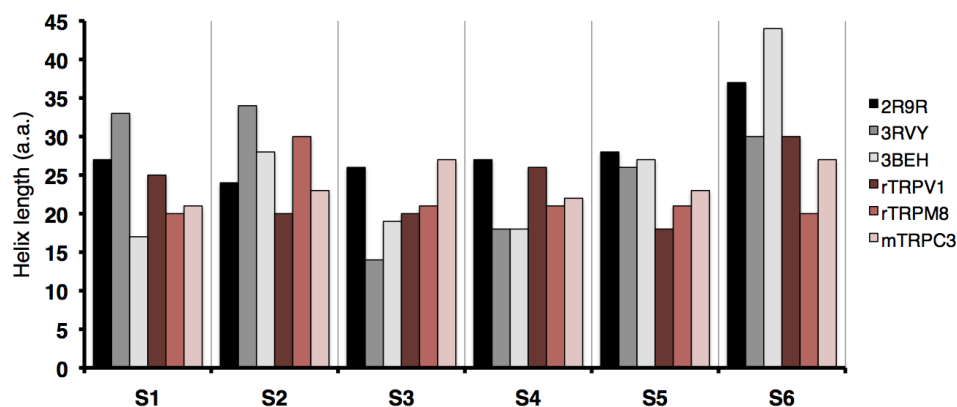


Figure 5.4: **Comparing TM lengths of TRP channels and available templates.** One of the key factors taken into account in judging the most appropriate templates for TRP homology models, was predicted TM lengths. TM lengths for templates are taken from the structures, which matched well with hydrophobicity predictions. TM lengths for TRP channels are taken from hydrophobicity predictions.

the NavAb was noticeably shorter than that predicted for the TRPV1, so an additional model was proposed that might combine the best of the Chimera structure and NavAb structure alignments (Figure 5.6).

Using these sequence alignments, homology models were made using Modeller 9.8 and the loop model function, with the best of 10 models with three iterations of loop model chosen as the final structures. The top three structures for the Chimera, NavAb, and loop model structures are shown (Figure 5.7). Discrete optimized protein energy (DOPE) scores of the representative of each type of homology model show that the model based on the Chimera structure and the combined model structure are the most promising, so only these were used later in bilayer simulations (Figure 5.8).

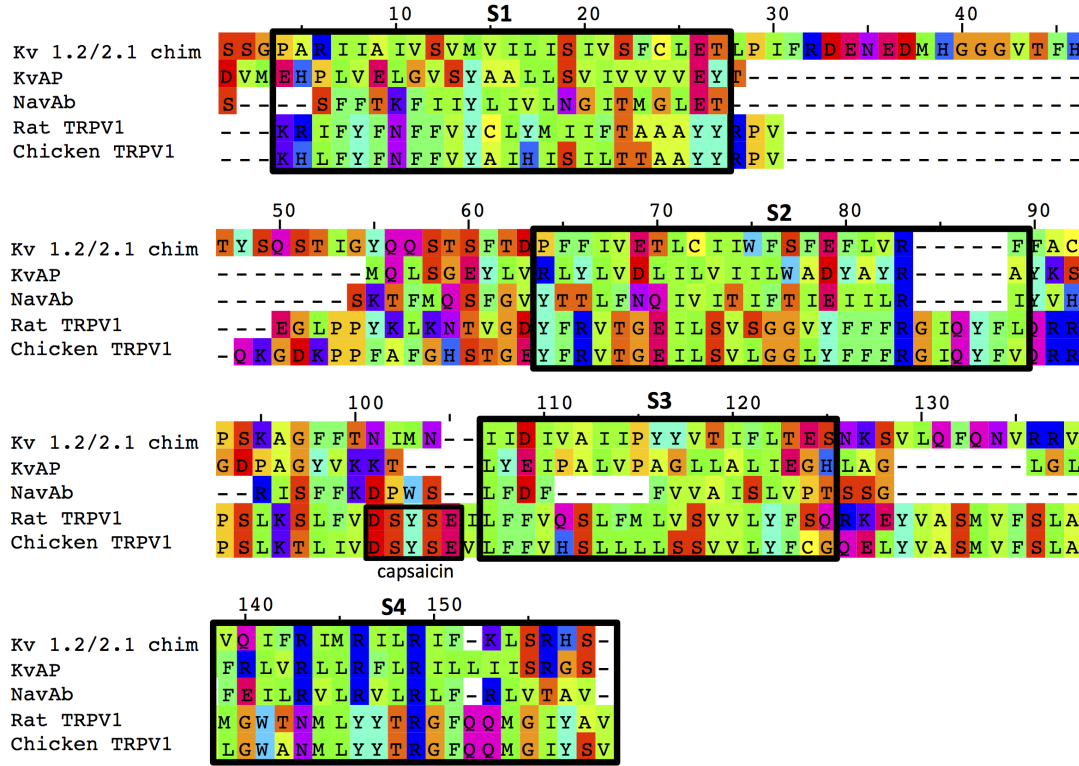


Figure 5.5: **Sequence alignment of the rat TRPV1 S1-S4 with structural templates.** An alignment of the KvAP, the Kv Chimera, and the NavAb structure sequences with rat and chicken TRPV1 was made using the MAFFT sequence alignment algorithm plus minor manual adjustments [129]. This sequence is visualised using the Taylor colour scheme.

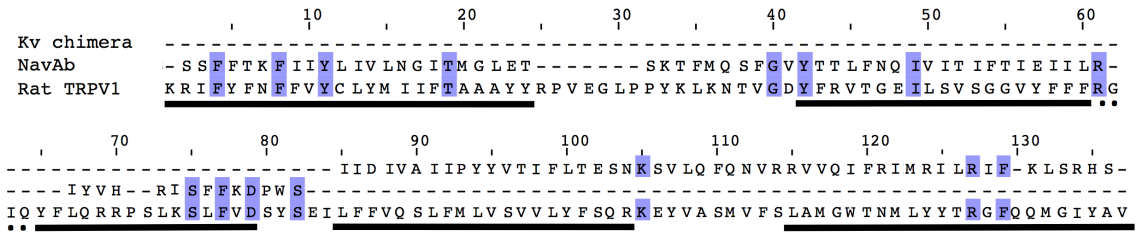


Figure 5.6: **Alignment for 'combined model'.** With such high sequence identity between the TRPV1 the NavAb S1-S4 helices, though the S1 helix of the NavAb was noticeably shorter than that predicted for the TRPV1, an additional model was proposed that might combine the best of the Chimera structure and NavAb structure alignments

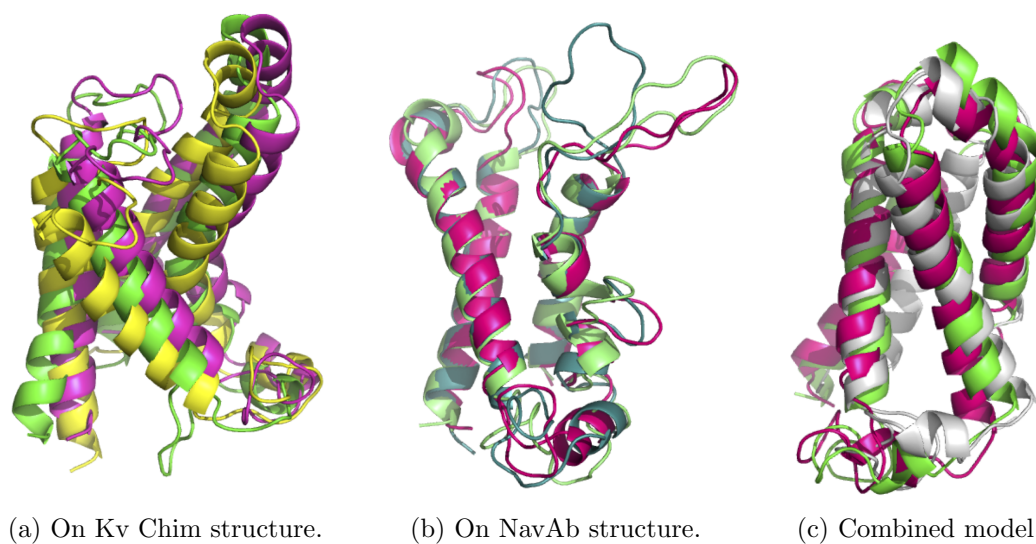


Figure 5.7: Models of the TRPV1 S1-S4 helices. (a) Best models of the rat TRPV1 S1-S4 helices using the Kv Chimera structure as a template. (b) Best models of the rat TRPV1 S1-S4 helices using the NavAb structure as a template. (c) Best models of the rat TRPV1 S1-S4 helices using the combined templates of the Kv Chimera and NavAb.

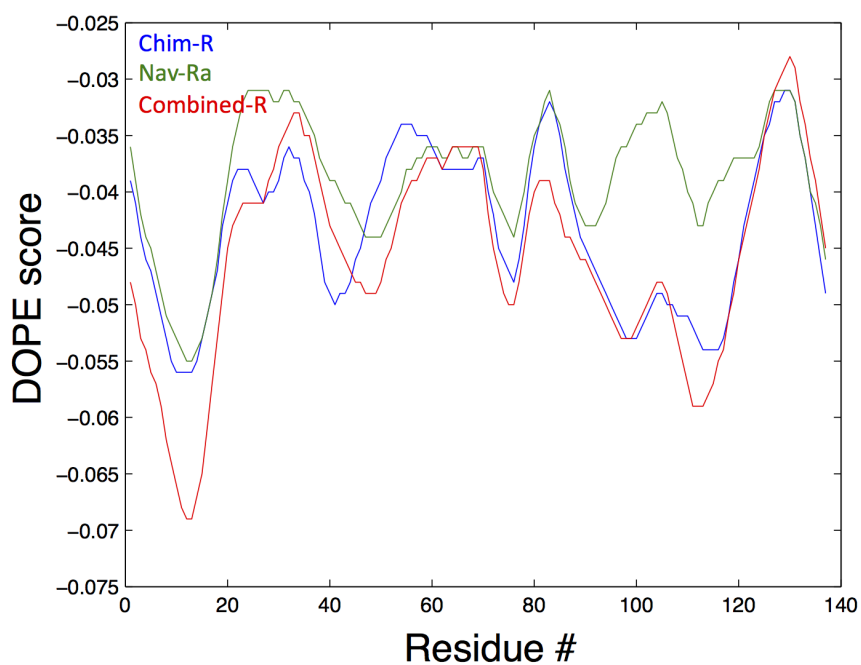


Figure 5.8: **Model scores by residue.** DOPE scores by residue of the representative of each type of homology model indicate the 'combined' model with the lowest score should be a preferred model.

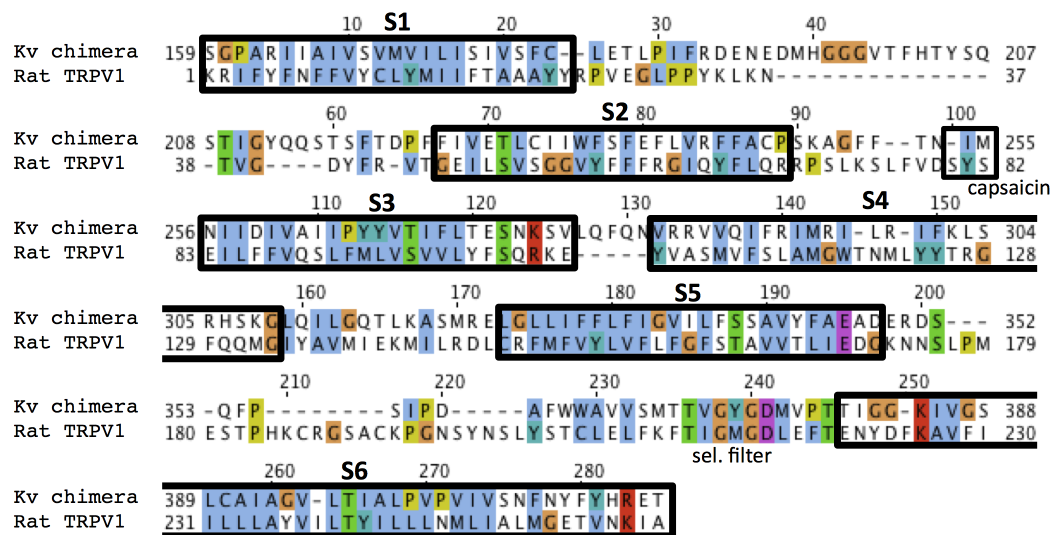


Figure 5.9: **Alignment of the rat TRPV1 S1-S6 with the Kv Chimera sequence.** The alignment of TRPV1 to the Kv Chimera used to build homology models. This alignment was previously used in reference [144].

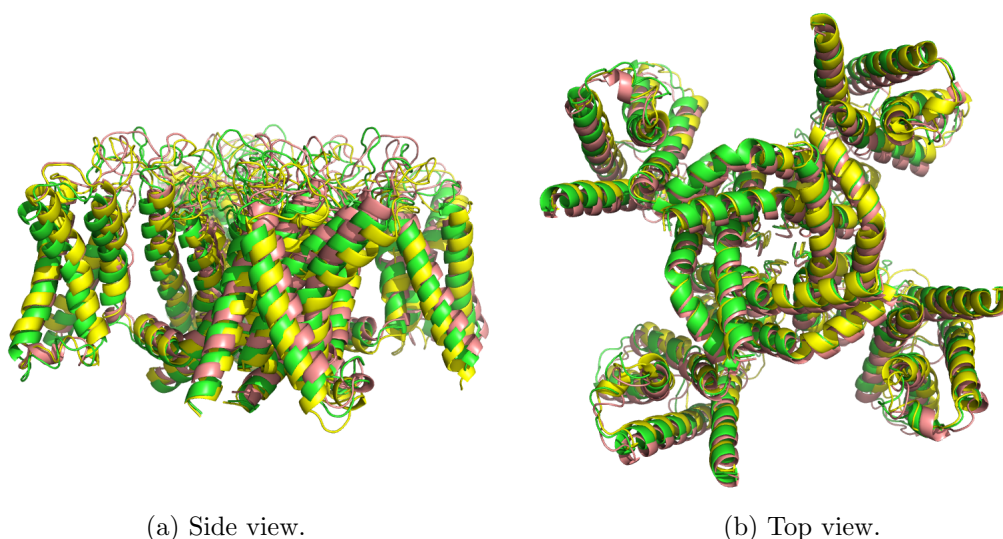


Figure 5.10: **Best models of rat TRPV1 S1-S6 using the Kv chimera as a template.** Three best models of the TRPV1 S1-S6 shown in (a) side view and (b) top view.

5.2.3 Homology models of the TRPV1 S1-S6 tetramer

With the difficulties in using the NavAb template for modelling the longer helices of TRPV1 in the S1-S4 model, the Kv Chimera structure was used exclusively to model tetramer-forming S1-S6 helices of TRPV1 (Figure 5.9). These models were made with Modeller 9.10, and similarly 10 models were made with three iterations of loop model (Figure 5.10).

5.3 Molecular dynamics simulations of TRP models and their templates

With homology models of the S1-S4 and the tetrameric S1-S6 helices of rat TRPV1, molecular simulations were used to test the integrity and relevance of these models toward making physiologically relevant predictions.

5.3.1 Molecular dynamics of TRP S1-S4 helices in a lipid membrane

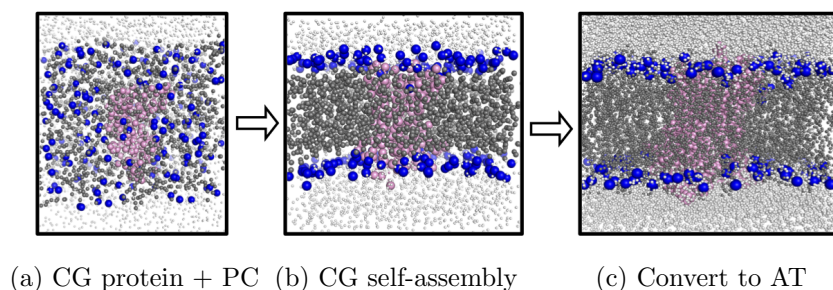


Figure 5.11: **Simulation setup.** (a) First a hydrated box of randomly placed CG POPC molecules around the CG protein model was generated, with gaps on opposite sides of the z-axis of the box to facilitate bilayer formation. (b) This lipid-protein system was then simulated for 100ns to allow for the self-assembly of a stable protein-bilayer system. (c) This lipid-protein system was then converted from CG to AT.

Homology models of the TRPV1 S1-S4 were coarse-grained and inserted into a simple

POPC lipid bilayer by randomly placing lipids around the protein and allowing the bilayer to self-assemble[258] (Figure 5.11). The S1-S4 model that combined templates was pushed out of the bilayer before it could stably form (Figure 5.12). This was taken as an indication of poor model quality due to lack of contacts between the S1/S2 and S3/S4 carried over from the models, and this combined model was not pursued further.

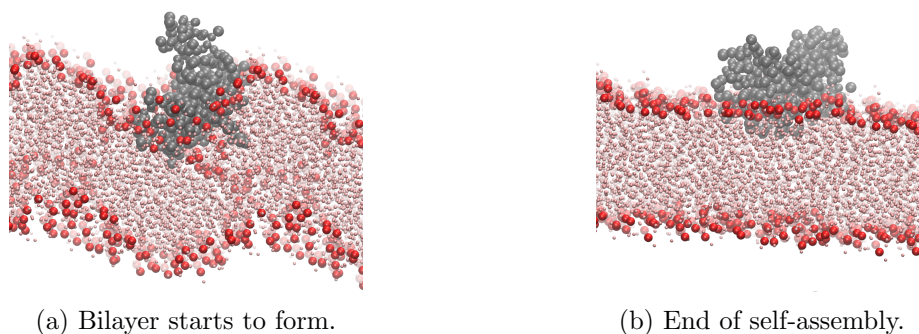


Figure 5.12: TRP S1-S4 model made by combining templates gets pushed out of the bilayer. (a) A snapshot of the CG self-assembly at 3.4 ns shows the bilayer forming with the TRP S1-S4 model inside. (b) A snapshot of the end of the 100ns CG self-assembly shows the model at the water-bilayer interface.

The model based on the Kv Chimera structure, however, was stably inserted into the bilayer during self-assembly, and converted into atomistic representation for a 100 ns simulation. The same simulation procedure was conducted for the Kv Chimera S1-S4 itself. The homology model exhibited poor stability with an RMSD reaching 8 Å compared to 5 Å for the crystal structure (Figure 5.13a). This was likely related to the observation that the helices themselves were not stable, and seen to unravel (Figure 5.13b).

Despite these indications of the poor quality of the homology model, a few basic analyses were conducted. Previous literature on S1-S4 domains have focused on the importance of their ability to distort the bilayer and allow water penetration. Looking at these propensities with this homology model, the bilayer is indeed observed to distort around the TRP S1-S4 domain, almost more so than for the Kv S1-S4 (Figure 5.14). This however, is hard to separate from both the general instability of the model and the seemingly randomly

penetrating waters into the model (Figure 5.15). While water penetration is not a surprising characteristic of an S1-S4 domain, notice how for the Kv S1-S4 model it is ordered forming a vestibule with an obvious hydrophobic block midway through the bilayer.

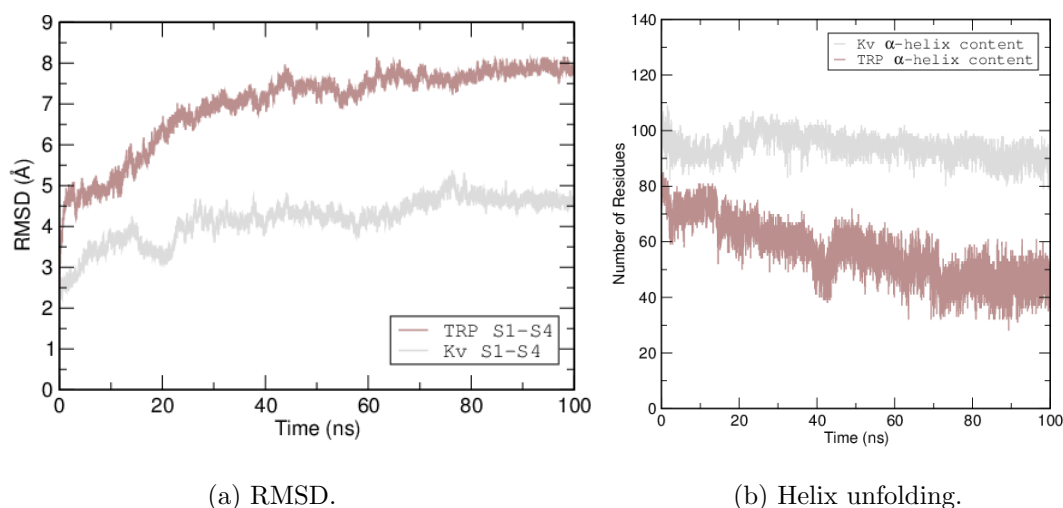
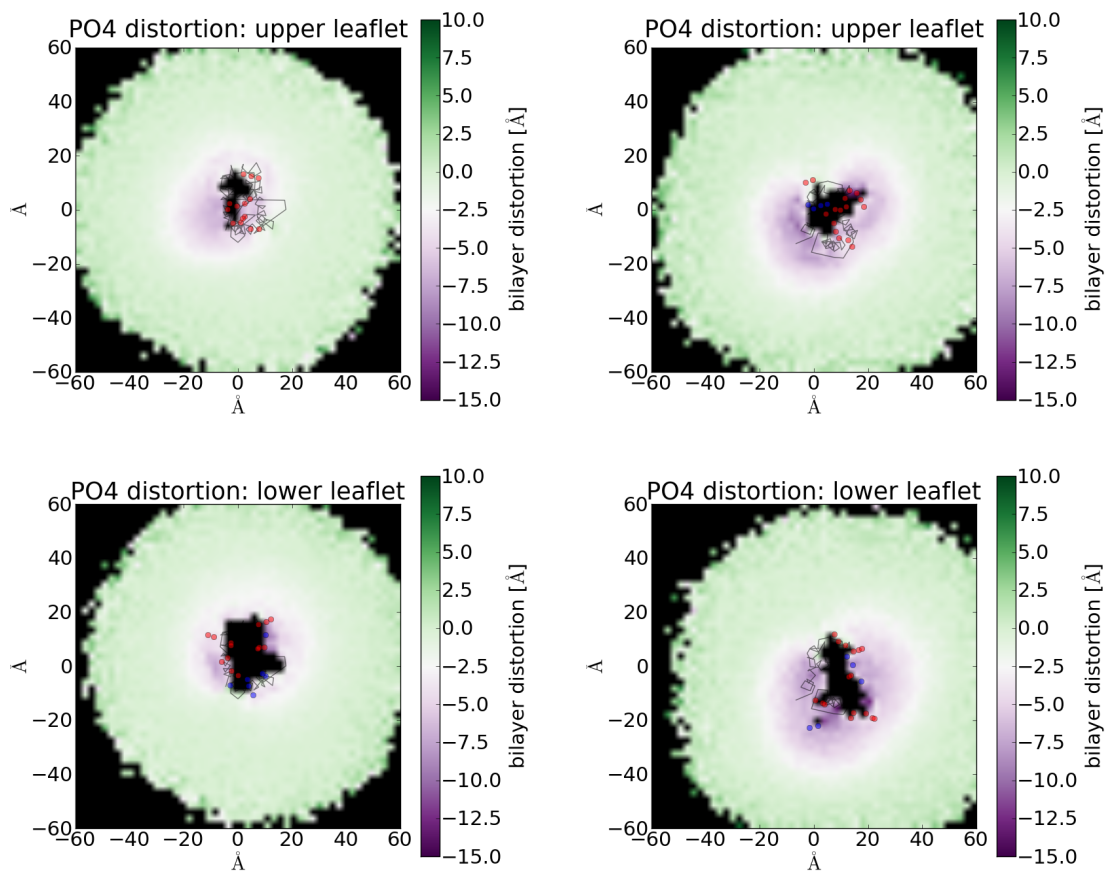


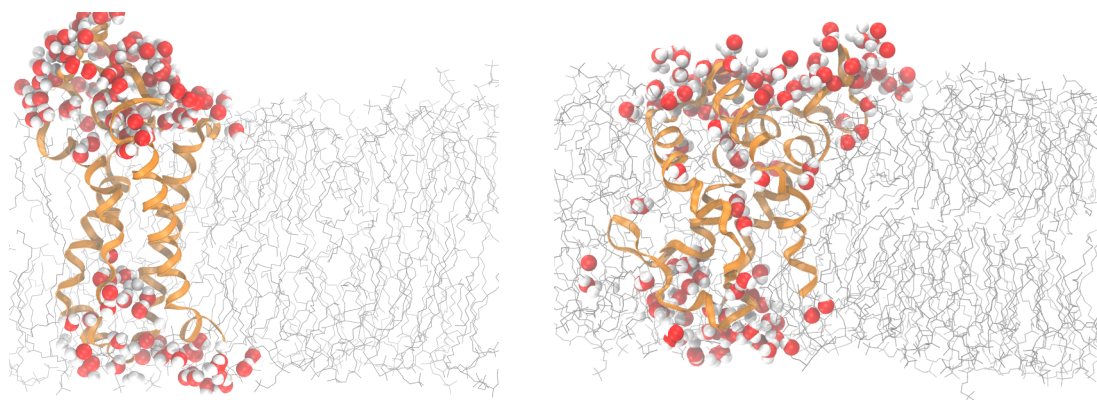
Figure 5.13: **Evidence of pore model quality for S1-S4.** (a) Comparing the RSMD of the S1-S4 of the Kv Chimera structure (gray) to that of the rat TRPV1 S1-S4 homology model (brown) shows that the model quickly and dramatically diverges from its original structure, unlike the crystal structure. (b) Similarly the number of residues that maintain their α -helical conformation in the Kv Chimera S1-S4 helices (gray) remains stable, while this same analysis on the homology model shows a consistent drop in the number of α -helix-forming residues, indicating the unfolding of the protein in simulation.



(a) Kv Chimera S1-S4.

(b) TRPV1 S1-S4.

Figure 5.14: Bilayer thickness around S1-S4 domains. (a) Simulation of the Kv Chimera S1-S4 in a POPC bilayer show local bilayer distortion around the domain, similar to what has been seen before [240]. The protein has been centered in the simulation box and purple indicates a decrease in bilayer thickness. (b) Simulation of the TRP S1-S4 homology model in a POPC bilayer show an even greater local bilayer distortion around the domain, but perhaps with less physiological relevance due to the noted unfolding of the model.



(a) Kv Chimera S1-S4.

(b) TRPV1 S1-S4.

Figure 5.15: Water penetration into S1-S4 domains. (a) Water penetration into S1-S4 domains has previously been seen both in experiment and simulation [240, 137], here it can be seen again with the Kv Chimera S1-S4. The POPC bilayer is shown as gray sticks, waters within 3 Å of the protein are shown as red and white spheres, and the protein is shown as orange ribbon. (b) However, while there is water penetration into the TRPV1 S1-S4 homology model, it is unclear whether this is due to the poor quality of the model.

5.3.2 Molecular dynamics of the full S1-S6 tetrameric transmembrane domain in a lipid membrane

Simulations of the tetramer of the rTRPV1 S1-S6 were conducted as with the S1-S4, with an initial CG self-assembly step followed by conversion to atomistic (Figure 5.11). While it was thought that the instability of the S1-S4 model was due to the lack of the pore domain, simulations of the tetramer remained unstable with the RMSD of the tetramer stabilizing only 1 Å lower than the S1-S4 helices (Figure 5.17). This however, may be due to an inherent instability in the pore domain in this particular simulation, as it is known that placing ions in the pore of simulations of Kv channels is required to maintain structural stability, and this was not part of the simulation procedure in this case. While TRPV1 has comparatively high sequence identity to the Kv in the selectivity filter region, it is not thought to have comparable mechanisms of ion coordination in this region, as the channel is not K⁺ selective. This is just another factor to add to the difficulties of creating a reliable and physiologically insightful TRP channel model.

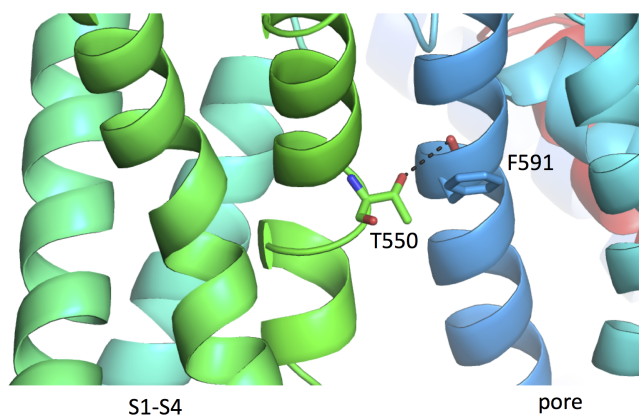


Figure 5.16: **A possible interaction between the two transmembrane domains.** In looking for or against coupling between the S1-S4 voltage-sensor-like and S5-S6 pore regions of the TRPV1 S1-S6 homology model, one possible hydrogen bonding interaction was found between the T550 of the S4 of one subunit to the F591 of the S5 of another. While the threonine keeps facing the pore region, the interaction is not stable through the simulation.

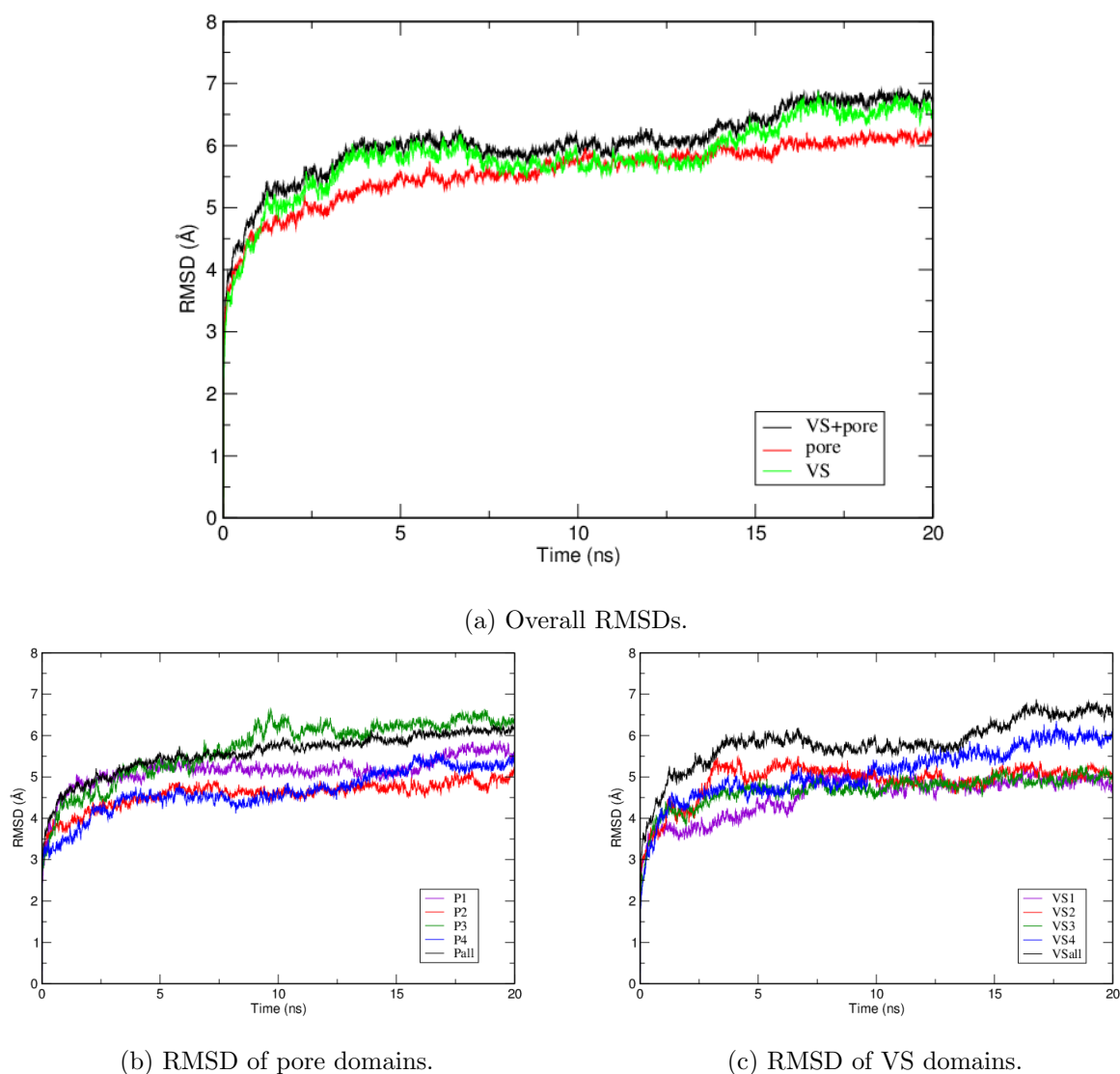


Figure 5.17: RMSD of the TRPV1 S1-S6 homology model in a simple bilayer. (a) Cumulative RMSDs of the whole protein and all the pore and VS-like domains. The high RMSD of the pore domain might be related to the relaxation of the selectivity filter over the course of the simulation. (b) The RMSD of each individual pore domain. (c) The RMSD of each individual voltage-sensor-like domain.

In an attempt to look at clues toward or against transmembrane domain modularity in the context of this TRPV1 model, an analysis of the interactions between the S1-S4 and S5-S6 pore region was conducted. In the starting model for the simulations, very few

interactions are seen except one threonine in the S4 is seen to make a hydrogen bond with the backbone carbonyl of a phenylalanine in the S5 (Figure 5.16). With two threonines in the TRP S4 and none in the charged residue-riddled S4 of the Kv Chimera, this is a potentially interesting observation. However, with difficulties in determining the helix register, it is difficult to say whether the placement of this threonine toward the pore domain is accurate or not. By the end of the simulation, a range of hydrogen bonds can be seen between the extracellular loops near the S1 and S5 helices, and this threonine interaction continues with a now distorted S5, though the interaction shifted to a different residue. All of these observations are, however, extremely tentative regarding their physiological significance, and this paragraph serves mostly to illustrate the difficulty with which any conclusions can be made from this homology model.

5.4 Conclusions and future directions

While the experimental structures of TRP channels remain elusive, homology modelling of TRP channels remain the best method for understanding their structure-function relationships. However, with low sequence identity and very few common motifs to match and ensure confident placement of helix register, homology modelling of TRPV1 transmembrane domains have not provided an easy out in the understanding of TRP channel structure.

5.4.1 Progress in homology modelling of TRP channels

While other homology models have been created of TRPV1, and indeed molecular dynamics simulations of one of these have been conducted in an explicit lipid bilayer [32], these models have not focused on aspects of S1-S4 helix modularity and carefully scrutinized model integrity. That being said, there are still measures that can be taken to improve model quality, while carefully taking into account the stability of the models as described

here. Two major methods that come to mind are (1) to focus on the structure of the *M. loti* cyclic-nucleotide gated K^+ (PDB ID: 3BEH) as a template [52], which similarly has no charges in its S4 and (2) to conduct more rigorous bioinformatics searches to develop a better idea of conserved TRP motifs also similar in VGL templates to help align helix register.

5.4.2 Comparing modularity of TRP and Kv channels in simulation

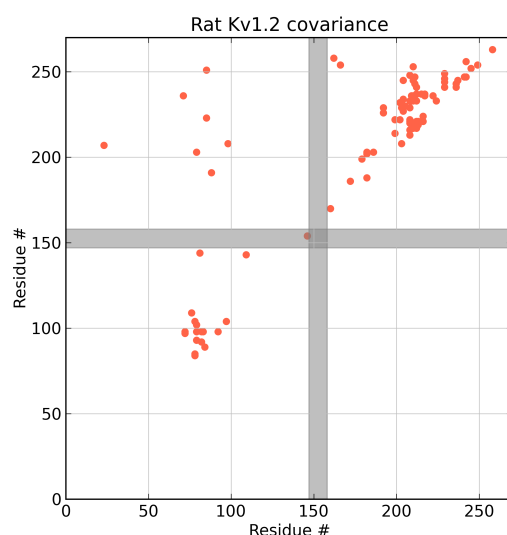
One hope in embarking on TRPV1 homology modelling in this chapter was to gain some insights toward the function of the S1-S4 helices of TRP channels as a modular domain as in Kv Channels, perhaps either through suggestions of contacts between the two transmembrane domains to contradict the modularity hypothesis or by observing similar behaviors of the S1-S4 helices in simulation. While the models currently available were not able to give confident suggestions toward either conclusion, a bioinformatics method that did not require accurate homology modelling became a possible avenue to gain computational insights to TRP transmembrane domain modularity.

A paper from the MacKinnon lab in 2009 [145], investigated contacts between the S1-S4 voltage-sensor domain and the S5-S6 pore domain by looking at evolutionarily covarying amino acid residues that would predict contacts between the region. Their analysis of 360 eukaryotic and prokaryotic Kv channel sequences showed very few covarying residues, in agreement with the interpretation of the S1-S4 as a functionally and structurally modular domain, except one contact between the tops of the S1 and pore helices. Along these lines, a similar approach was suggested to investigate possible evolutionary covarying residues in TRP channels to lend evidence for or against the hypothesis of the S1-S4 as a modular domain in TRP channels.

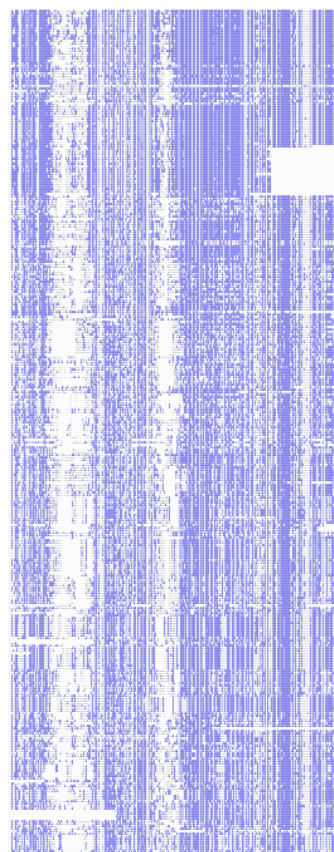
The method of the PSICOV algorithm was adopted to analyze covariance within TRP sequences for this purpose [119]. The analysis was performed using the rat TRPV1 S1-S6

sequence as well as an additional analysis with the rat Kv1.2 S1-S6 sequence to reduce the likelihood of errors and misguided conclusions. The JACKHMMER suite, which uses hidden Markov models to search for relevant sequences from a large database and aligns them iteratively [23], built a sequence alignment of >1000 sequences from the TRP seed and >5000 sequences from the Kv seed (Figures 5.18b and 5.19b). Performing the covariance analysis using the PSICOV algorithm resulted in 95 and 22 contacts with 60% or greater predicted probability of interacting, for the Kv and TRP respectively (Figures 5.18a and 5.19a).

It is promising that the majority of predicted contacts fall along the diagonal, which indicates that, as one would expect, most of the highly predicted contacts are close to each other. Another indication that these results are somewhat meaningful is that an interaction is predicted between the S1 and the pore helix, as seen in the previous Kv covariance analysis work, though the interaction is not reproduced residue for residue. Despite these promising beginnings, the few contacts predicted between the S1-S4 and the S5-S6 from both analyses map back to physically unreasonable locations on the structures. This is an indication that the sequences need to be manually curated, since perhaps the sequences chosen, though at first glance there don't seem to be major problems, are not physiologically relevant enough to be included in the covariance analysis. Additionally it is possible that upon this manual curation, it will be found that not enough TRP channel sequences are available for this type of analysis, as they lack the large number of prokaryotic homologues that are a resource to the same type of calculations of Kv channel sequences.

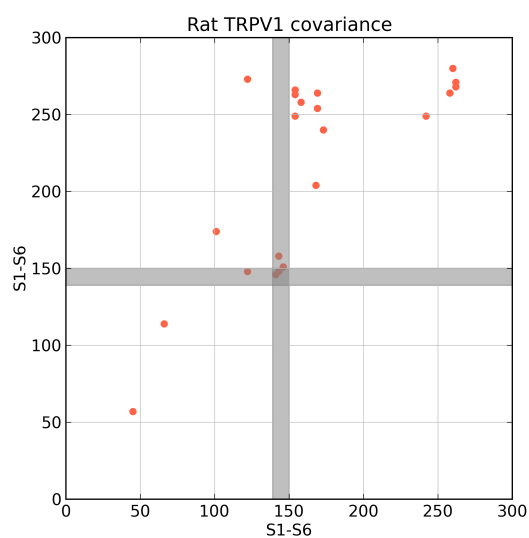


(a) Predicted contacts of Kv TMs.

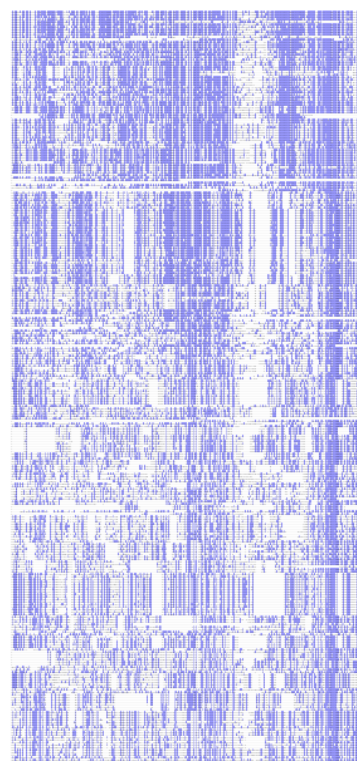


(b) Representative sequences from the 5333 sequences used in Kv alignment.

Figure 5.18: Covariance analysis of Kv TMs. (a) Predicted contacts with greater than 60% predicted probability of interacting. The S4-S5 linker is represented as a gray bar and separates the plot into quadrants. The predicted contact in the first quadrant on the far left, predicts a contact between the S1 and the pore helix. (b) Representative sequences from the 5333 sequences used in Kv alignment. Notice several gaps in what are the S5-S6 helices at the top and the S1 helix at the bottom.



(a) Predicted contacts of TRP TMs.



(b) Representative sequences from the 1297 sequences used in TRP alignment.

Figure 5.19: **Covariance analysis of TRP TMs.** (a) Predicted contacts with greater than 60% predicted probability of interacting. The S4-S5 linker is represented as a gray bar and separates the plot into quadrants. (b) Representative sequences from the 1297 sequences used in TRP alignment.

5.5 CODA: mTRPC3 modelling to understand a mouse model of cerebellar ataxia

While homology models are no substitute to crystal structures, occasionally a question particularly suited to the available model will come along. While for TRPV1 homology modelling proved to be of limited use, using a similar approach to model TRPC3 provided some valuable insight into experimental results in a collaboration on the moonwalker (Mwk) mutant in mouse TRPC3.

5.5.1 Background

In a forward genetic screen to identify a cerebellar ataxic mouse by N-ethyl-N-nitrosourea (ENU)-mutagenizing a large cohort of mice, the moonwalker (Mwk) mouse was identified [16]. Genotyping this mouse revealed that a threonine to alanine mutant in the S4-S5 linker of the TRPC3 channel was the cause (Figure 5.20a). Overexpressing this Mwk mutant TRPC3 in NSC-34 cells caused markedly increased cell death compared to NSC-34 cells overexpressing the WT TRPC3 (Figure 5.20b). This threonine was identified as a putative phosphorylation site for PKC- γ , which has been seen to negatively regulate TRPC3 [282]. One hypothesis was that the Mwk phenotype is the result of removing this phosphorylation-mediated inhibition, pushing the channel equilibrium towards a conducting state, which then leads to increased cell death.

5.5.2 Sequence alignment and modelling of mTRPC3 to relevant templates

This linker region remains remarkably conserved across TRPC channels, which being the ‘canonical TRP’ subfamily are expressed in the largest range of species of any TRP

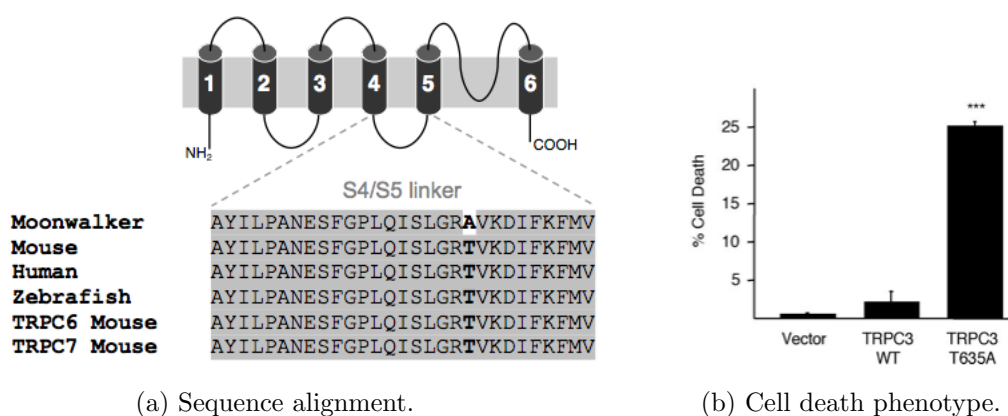


Figure 5.20: **The moonwalker (Mwk) mouse mutant in TRPC3.** (a) The threonine to alanine mutation in the Mwk mouse is at the S4-S5 linker. (b) NSC-34 cell death when overexpressing MWK TRPC3. Image adapted from Becker PNAS (2009).

channel subtype, for example they are the only TRP channel subtype found in *Drosophila* and unicellular *S. cerevisiae* (Figure 5.21). While the S4-S5 linker region is highly conserved across all of these TRPCs, the T561 (numbered according to the mouse TRCP3 sequence) mutated in the MWK mouse is not conserved, and is a methionine instead in four out of the seven TRPCs (Figure 5.21). Worth noting is that the trend observed in by Hofmann et al as published in PNAS in 2002 regarding the ability of TRPC channels to form functional hetero-oligomers follows the identity of this residue exactly: TRPC3, TRPC6, and TRPC7, which all have a threonine at this position, can form functional hetero-oligomers with each other, but not with TRPC1, TRPC4, or TRPC5, which have a methionine instead, and vice versa [106]. However, whether this threonine would be responsible for this trend is not clear, since TRPC3, TRPC6, and TRPC7 have other common, divergent residues.

To address a possible mechanism involved in the phenotype of the moonwalker mouse, models were made of mouse TRPC3 using the alignment shown in Figure 5.22. As shown in Figure 5.21, the S4-S5 linker of TRPC3 aligns remarkably well with the Kv channel sequence, without aligning particularly well to any other TRP channels or VGL templates. Surprisingly this residue is remarkably conserved across Kv channels, even more so than in

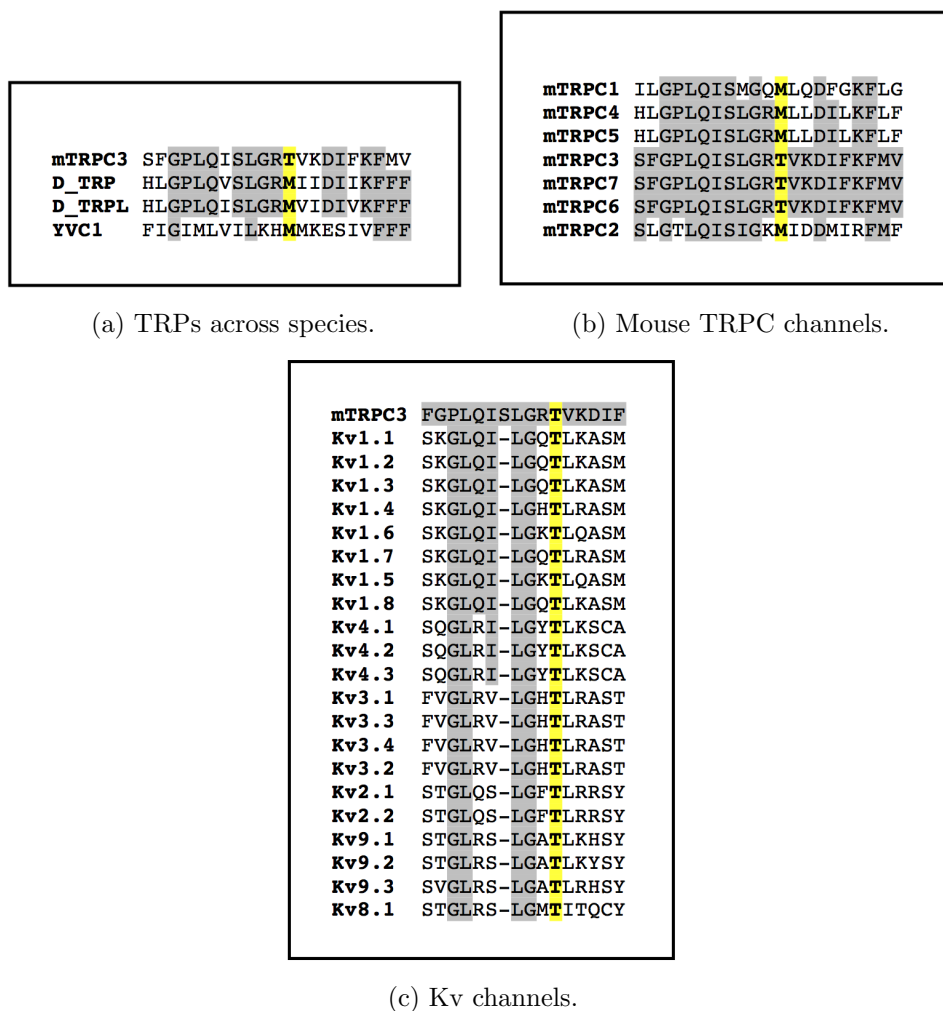


Figure 5.21: **Sequence alignments of the mTRPC3 S4-S5 linker region.** . (a) Sequence alignment of the S4-S5 linkers of mouse TRPC3, *Drosophila melanogaster* TRP and TRPL, and *Saccharomyces cerevisiae* YVC1. (b) Sequence alignment of all 7 mouse TRPC S4-S5 linkers. (c) Alignment of human the Kv channel S4-S5 linkers to the mouse TRPC3. This alignment does not include Kvs 5.x, 6.x, 7.x, 8.2, 10.x, 11.x and 12.x.

TRPC channels (Figure 5.21).

To align other regions of the channel outside the S4-S5 linker, transmembrane helix and disorder prediction provided useful guidelines. Notably, transmembrane helix prediction programs predicted a transmembrane helix at the S0, which is found in the TRPC, TRPM,

and TRPV family, but is thought to remain at the water/bilayer interface rather than cross the membrane. The best evidence for this is from glycosylation site analysis of TRPC3 that informs the placement of the intracellular and extracellular loops behind TM helices [281]. Confirming the prediction of this TM helix organization, the S0 did not align with the S1 of the VGL structural templates using any alignment algorithm. Disorder prediction proved useful in choosing a region to remove from the model in order to improve S1-S3 alignment. Both the Regional Order Neural Network (RONN, <http://www.strubi.ox.ac.uk/RONN>) and the disEMBL (<http://dis.embl.de>) servers predicted the sequence QESDLSEVTLP of mTRPC3 between the S3 and S4 to be disordered. Informing the removal of this region and the remaining PEVQYFTYARDKWLP of the loop to facilitate homology model building. Additionally, this region did not align well with TRPV1 or TRPM8 sequences, with the tendency for a gap in these two channels in the location of the mTRPC3 the QESD sequence.

With this sequence alignment on the structure of the Kv 1.2 / Kv 2.1 Chimera, a tetrameric model was made using Modeller 9v9. The top five models of 100 were chosen (more details are available in the Materials and Methods), and were inserted into a bilayer and simulated for 20ns to further assess their quality. Since the Kv 1.2 / 2.1 Chimera structure is in an open state [156], and the hypothesis of the TRPC3 Mwk mechanism is that the threonine to alanine mutation causes a constitutively active channel, the model was then used to understand any possible interactions that would be missing in the T561A mutant.

5.5.3 Modelling predicts a hydrogen-bonding interaction

To address the hypothesis of whether phosphorylation of the Mwk threonine, and the loss of this phosphorylation upon the mutation to alanine, was the mechanistic cause of the phenotype, a series of mutations were made in the Becker lab to address this hypothesis.

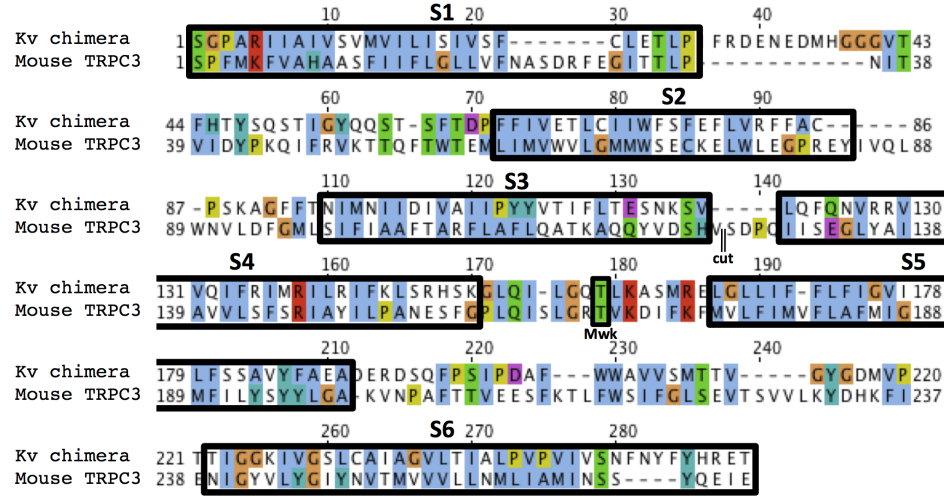


Figure 5.22: **mTRPC3 alignment with Kv channel template.** Sequence alignment of mouse TRPC3 S1-S6 helices with the S1-S6 helices of the sequence of the crystal structure of the Kv 1.2 / 2.1 chimera. A region between the S3 and S4 loop has been removed due to disorder prediction. Sequence alignment was made using the MAFFT alignment algorithm with manual adjustment. Coloring is according to the ClusatlX coloring scheme.

Mutating the threonine to an aspartate, a residue, which would mimic a phosphorylated threonine, maintained the WT phenotype. However, an asparagine in the same place, which would not mimic phosphorylation also shows a low, WT-like rate of cell death. This resulted in an unclear interpretation of what mechanism was being disrupted in the Mwk mouse. Using the mTRPC3 homology model on the Kv chimera structure, it was clear that the threonine on the S4-S5 linker pointed inward, toward the pore domain, in a prime position to hydrogen bond with residues at the end of the S6 helix (Figure 5.24). That asparagine and aspartate maintain a wild type phenotype makes sense in this framework. To test this hypothesis, the Becker lab performed a Neuro2a cell death experiment (similar to the NSC-34 experiment) with the threonine to valine mutant, which since valine is isosteric to threonine but lacks a hydrogen bonding group, would not be able to form hydrogen bonds but should have no other effect on channel structure. Indeed, mutating the threonine to valine reproduced the Mwk mutant cell death phenotype (Figure 5.23).

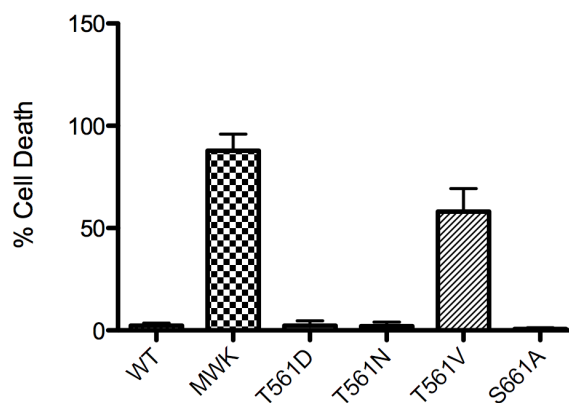


Figure 5.23: **Cell death experiments show T561V mutant replicates Mwk phenotype.** In experiments performed by collaborator Dr. Becker on Neuro2a cells, only the T561V mutant mimicked the Mwk phenotype. This suggests a role for hydrogen bonding in the mechanism leading to Mwk constitutive channel activity.

Further analysis of the mTRPC3 models and template structures suggested an additional detail to this hydrogen bonding interaction. In over half of the models created, a hydrogen bonding interaction was observed between the Mwk threonine and the backbone carbonyl of a serine (S661) at the end of the S6 helix. Surprisingly, a similar interaction was seen to be stable in 100ns simulations of the Kv 1.2 / 2.1 chimera structure in a simple POPC bilayer. From these observations it was suggested that the S661A mutant might also mimic the Mwk phenotype (Figure 5.24), but results indicate that this potential hydrogen-bonding group is not as significant as the T561 (Figure 5.23).

Even with questions remaining, two phenomena of interest have been described here: the significant conservation between the TRPC and Kv linker region and the recreation of the Mwk phenotype by a valine mutation, likely due to a loss of hydrogen-bonding interactions.

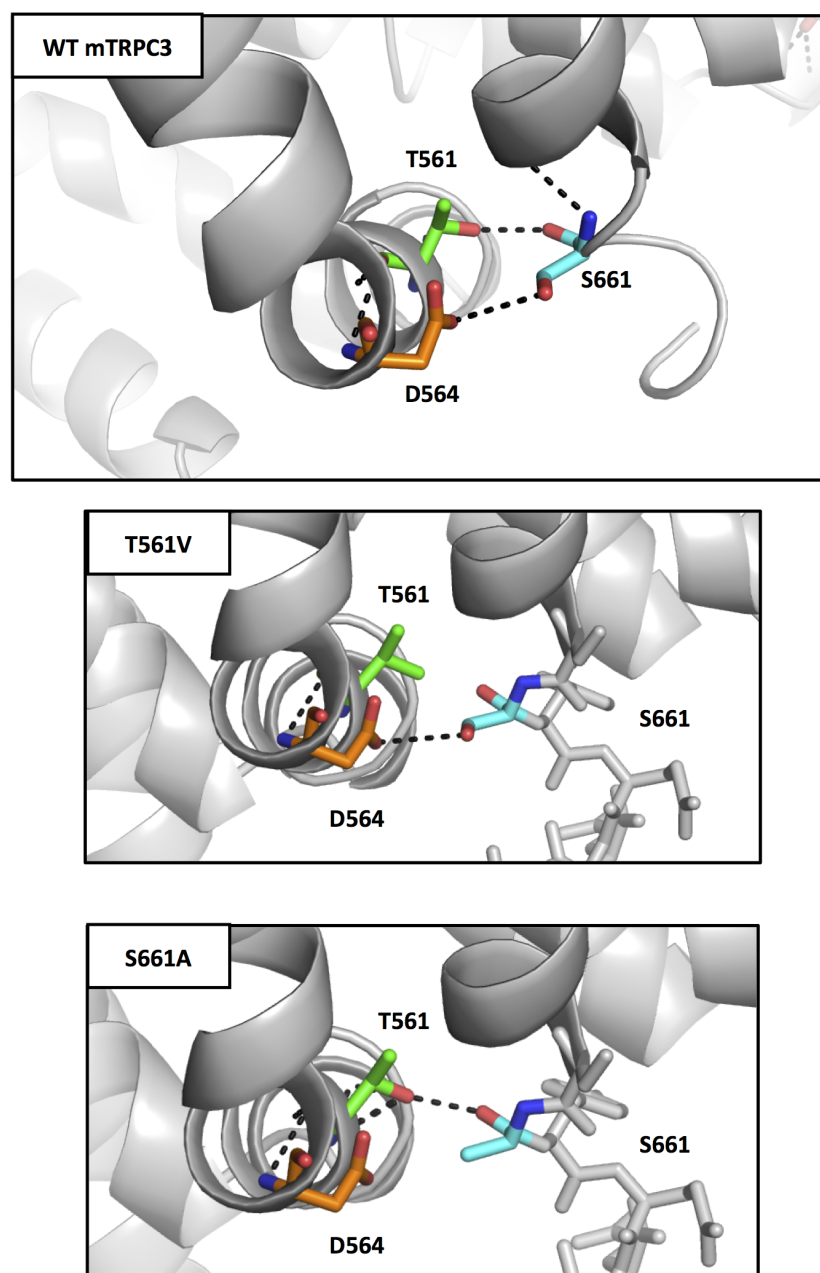


Figure 5.24: **Possible hydrogen bonds between the S4-S4 linker and the end of the S6 helix.** In wild-type mTRPC3 a hydrogen bond to the backbone carbonyl is predicted to stabilize an interaction between T561 and the backbone carbonyl of S661. This interaction is interrupted in the MwK mutant (T561A) and the valine mutant (T561V). It is to be seen of this interaction is also interrupted when mutating the serine to a non-hydrogen bonding alternative. Though this would not interrupt the interaction with the backbone-carbonyl it would likely interrupt other stabilizing interactions of the same residue.

Molecular dynamics simulations of TRPV1 ligands

6.1 Introduction

While ligand activation of TRP channels has been one of the greatest tools for their characterisation, the mechanisms of this ligand binding event through the membrane have not been well-characterised. Even for capsaicin, which is by far the most well-characterised of TRP ligands, very little is defined, and in fact many experiments have come to conflicting conclusions. This chapter addresses the interactions of capsaicin and its analogues with a lipid bilayer using atomistic molecular dynamics simulation techniques to gain insights toward possible mechanisms and kinetics of channel activation.

6.1.1 Ambiguities in the capsaicin binding site of TRPV1

While TRP channels have the same six transmembrane helix, pore-forming, tetrameric architecture as other members of the VGL superfamily [141] and have been shown to have voltage-sensitive properties [170], the S4 helix of TRP channels is missing the conserved

string of positively charged amino acids responsible for the voltage-sensitivity of canonical voltage-gated channels. The discovery that residues crucial to capsaicin-dependent activation of TRPV1 and menthol-dependent activation of TRPM8 are within the four transmembrane helices (S1-S4) of the voltage-sensor-like domain suggests that this domain could still be functionally significant in TRP channels, even if not for voltage-dependent gating. However, mutants key to capsaicin-dependent activation have been identified in the N- and C-termini [124] as well as the pore domain [297] (Figure 6.1a).

While these ambiguities are worrying, the dominant view remains that the S1-S4 contains the primary capsaicin binding site while other mutants are allosterically involved or contribute to pore opening. However, regardless of the binding site itself, it remains unclear if capsaicin preferentially activates TRPV1 when applied intracellularly versus extracellularly [291, 53] (Figure 6.1b). With the main cluster of key residues for capsaicin binding located at the intracellular loop between the S2 and S3 helices, the preferred interpretation is that the binding site is intracellular [86, 121]. Nevertheless, to rationalise some of the confusion caused by conflicting experimental data it has been proposed that capsaicin could have two binding sites [269], but as experiments and their interpretation remain difficult due to the lipophilic nature of capsaicin and its analogues, this hypothesis has yet to be clearly addressed. Similarly, the direction from which capsaicin accesses the bilayer has an effect on which orientation it would be most likely to bind TRPV1. The models presented in the papers implicating the S1-S4 in capsaicin binding differ as to whether the hydrophobic tail is intracellular or extracellular to the vanilloid group, though both predict capsaicin to be perpendicular to the bilayer in its binding pose [86, 121].

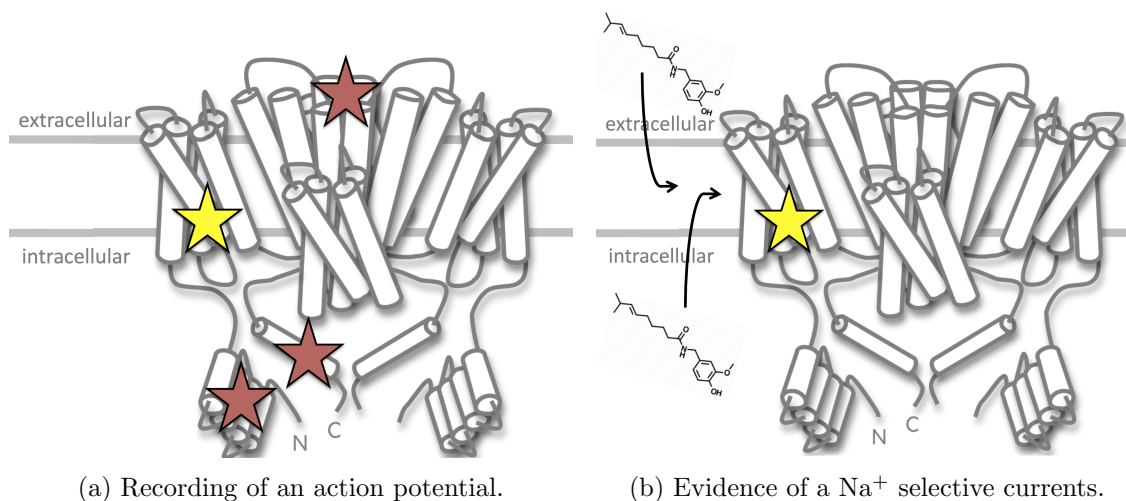


Figure 6.1: **Disputed mechanisms of capsaicin activation of TRPV1.** (a) Mutants besides those in the S1-S4 helices have been found to be crucial for capsaicin activation [124, 297]. (b) Experiments also disagree regarding from which side of the bilayer capsaicin activates TRPV1 [123, 291].

6.1.2 Simulations as an ideal approach for addressing these ambiguities

This conflicted understanding of the capsaicin-TRPV1 and capsaicin-bilayer interactions encourage a closer look at the rationale behind our current models. The extensive data concerning capsaicin analogues collected and capsaicin interactions with the bilayer since even before the discovery of TRPV1, encourages a closer look at the simple ligand-bilayer system. However, with a water solubility of 10.3 mg/L at 25°C, experiments with capsaicin remain difficult. Instead a computational technique is encouraged, with the increased success of molecular dynamics (MD) simulations of lipid bilayers and that they allow for direct estimates of partition coefficients [267]. While other simulations of capsaicin have been conducted, the only published results only address the interactions of capsaicin with the model hydrophobic system of an octanol bilayer [140].

MD simulations and related approaches have been used to look at permeation of lipid bilayers by drugs and related molecules have been increasingly widespread [267]. The free

energy profiles of bilayer penetration have been calculated for a variety of functional groups, providing insight into the preferential bilayer localisation that is not straightforward by other methods [20]. Another interesting example of the use of MD to address questions of bilayer, small molecule interactions are a range of studies on cholesterol flip-flop and spontaneous lipid flip-flop from one leaflet of the bilayer to the other [92, 21].

In addition, the increasing computational power available for molecular simulations is allowing for the observation of spontaneous drug binding events rather than relying on docking algorithms [65]. Other similar MD simulation studies have seen specific binding of lipid molecules, cardiolipin and PIP₂ to membrane proteins [12, 243]. Other molecular dynamics studies have looked at the effects of proteins, bilayer asymmetry, and lipid heterogeneity on bilayer fluidity, which is relevant to studies on capsaicin that suggest its presence in the cell membrane changes bilayer fluidity [160].

6.2 Capsaicin interactions with a lipid bilayer

The lipophilic nature of capsaicin may be a primary cause of the experimental ambiguities that exist regarding the nature of capsaicin-bilayer and capsaicin-TRPV1 interactions. Studying how capsaicin molecules might interact with a lipid bilayer using molecular dynamics simulations can provide insights into this interaction that are otherwise unavailable.

6.2.1 Capsaicin parametrisation and simulation setup

While proteins, water, and ions have well-developed parameters for a variety of force fields, ensuring parameters for small molecules are compatible is less straightforward. Since a library of atom types exists for the Optimised Potentials for Liquid Simulations (OPLS) force field that can be used to parametrise small molecules, capsaicin parameters were developed using this force field (Figure 6.2). OPLS parameters are created by using a set of

lookup tables for atom types, bond types, angle types, and dihedral types that have been optimised mostly for peptides and other commonly used molecules. The AMBER force field similarly has a set of tools, the generalised AMBER force field or GAFF, for parametrising small molecules that instead of using lookup tables, recalculates the parameters for each molecule individually. Due to the similarity of the chemical elements of capsaicin to those of peptides, capsaicin parameters obtained using more advanced tools than the OPLS lookup method was not deemed necessary.

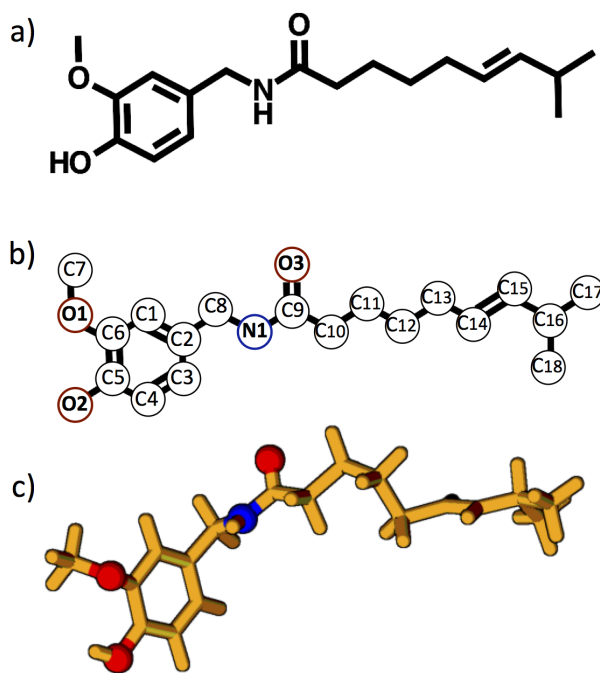


Figure 6.2: **Capsaicin parameterisation.** (a) The chemical structure of capsaicin. (b) Using the tools Marvin Sketch and topolbuild this drawing can be made into a PDB file. Note that hydrogens are left out for simplicity, but are represented in the PDB file. (c) The tool topolbuild and manual use of available OPLS lookup tables allow for the parameterisation of capsaicin into a simulation-ready molecule.

A phosphatidylcholine (POPC) bilayer was chosen as a simple bilayer that has been used successfully as a useful first approximation of a eukaryotic membrane in a variety of studies [90]. The bilayer was initially created using coarse-grained self-assembly methods [258], to

allow the bilayer to form naturally in solution, and then it was converted to a united-atom atomistic representation in the same OPLS force field as the capsaicin molecule (Figure 6.3). The final simulation box was of dimensions 10 nm^3 with 256 POPC molecules forming the bilayer.

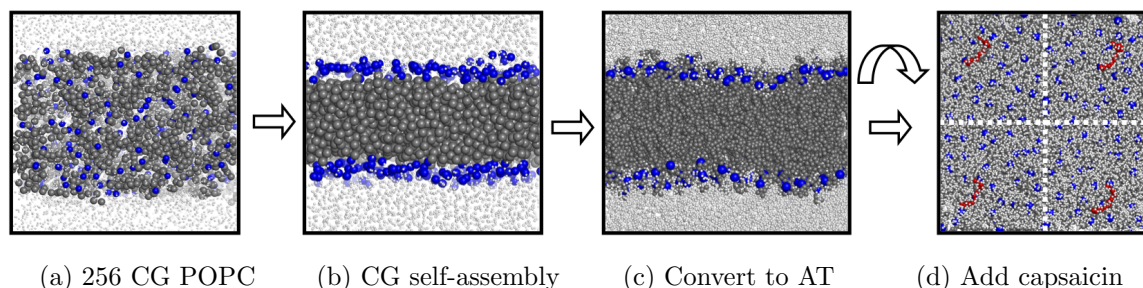


Figure 6.3: Capsaicin simulation setup. (a) First a hydrated box of 256 randomly placed coarse-grained (CG) phosphatidylcholine (POPC) molecules, with gaps on opposite sides of the z-axis of the box to facilitate bilayer formation, was generated. (b) This system was then simulated for 100ns to allow for the self-assembly of a stable bilayer. (c) This bilayer was then converted from CG to atomistic (AT), and (d) four capsaicin molecules were added at the same z-coordinate (either at the center of mass of the bilayer or in the solute, at $30\text{\AA}$ from the center of mass of the bilayer) and at the center of the four x-y quadrants of the simulation box.

After the bilayer was determined to be stable and equilibrated, four capsaicin molecules were added to the system. The molecules were placed at the center of each the x-y quadrant of the simulation box, and either in the solute at $30\text{\AA}$ from the center of the bilayer or at the center of the bilayer itself (Figure 6.3). This placement was done using a script and overlapping water molecules were removed from the box if they interfered with energy minimisation. After energy minimisation of the system, the capsaicin molecules were held in place for 500 ps as the charges were gradually added to capsaicin, allowing the solute and lipids around it to adjust. After this setup procedure $3 \times 100\text{ns}$ simulations were ran for the system in which four capsaicin molecules started in the solute and $1 \times 100\text{ns}$ simulation was run for the system in which the initial position of the molecules was at the center of the lipid bilayer. The exact parameters of the simulation and setup are discussed in more

detail in Chapter 2.

6.2.2 Penetration of capsaicin into a POPC bilayer

Throughout the course of these simulations, capsaicin penetrated the lipid bilayer spontaneously, as expected. Ten capsaicin molecules out of twelve total penetrated the bilayer and then maintained a consistent distribution near the water/bilayer interface for the rest of the simulation (Figure 6.7). One exception to this trend is discussed further in section 6.2.4. Despite the molecules being set within 10 Å of the bilayer surface, every molecule diffused significantly in the solute before penetrating the bilayer. Sometimes the molecules would indeed, diffuse off the bilayer several times before the penetration event, indicating an energetically preferred mechanism of penetration. Before entering the bilayer several capsaicin aggregates were observed, perhaps unsurprisingly. Due to the proximity to the bilayer that the capsaicin molecules began their trajectory it is likely that aggregation is even more prevalent physiologically. While the capsaicin molecules that entered the bilayer individually tended to do so with their hydrophobic tails first, the fact that capsaicin aggregated in solute allowed for more diverse mechanisms of bilayer penetration (Figure 6.5).

6.2.3 Distribution of capsaicin within a POPC bilayer

After penetrating the bilayer, the capsaicin molecules maintained a consistent distribution for the remainder of the simulations (Figure 6.6). While an initial hypothesis was that the oxygens of the aromatic A-region might interact with the phosphate headgroups of the lipid, the resulting density profile shows that the capsaicin molecules preferred a location slightly deeper within the bilayer, often interacting with the carbonyls of the lipid. This enabled the hydrophobic tail of the capsaicin to fully penetrate the core of the bilayer.

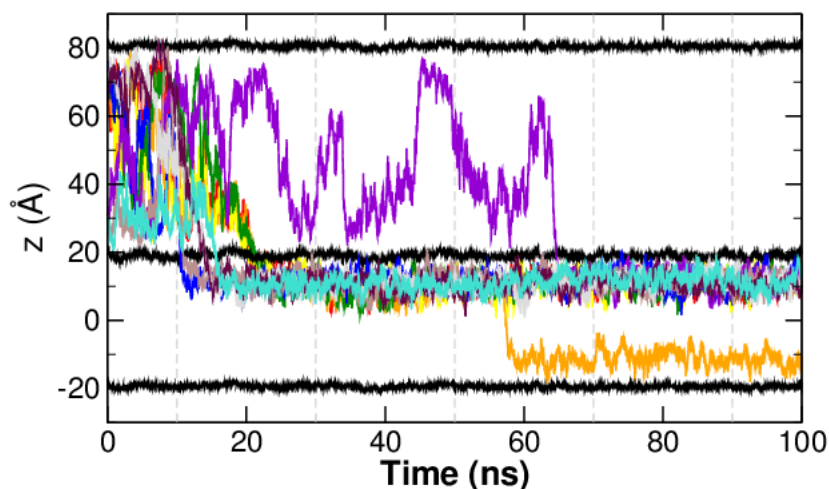


Figure 6.4: **Capsaicin molecules spontaneously penetrate the lipid bilayer.** Center of mass of ten of the twelve capsaicin molecules plotted over the course of three 100 ns simulations. Each capsaicin molecule is shown as a different colour of the rainbow. The two capsaicin molecules that did not penetrate the bilayer are not shown. The center of mass of the phosphate headgroups of both leaflets of the bilayer are shown in black. The center of the bilayer is at $z = 0$ Å (or 100 Å when considering periodic boundary conditions).

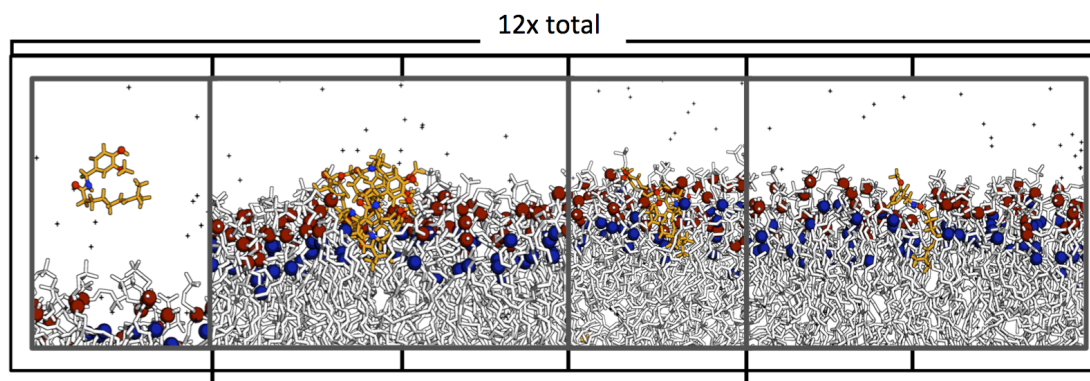


Figure 6.5: **Capsaicin aggregation in solute effects penetration modes.** Of the twelve capsaicin molecules, ten of the twelve penetrated the bilayer while the remaining two diffused in the solute for the full 100ns. Of these ten, four penetrated individually, and six penetrated in aggregates. Of the six that penetrated in aggregates there was one aggregate of four and one aggregate of two capsaicin molecules.

6.2.4 Capsaicin flip-flops between bilayer leaflets

As visible in Figure 6.7, a plot of the center of mass of capsaicin molecules, one capsaicin molecule, plotted in orange, diffuses from one leaflet of the bilayer to the other. A zoom

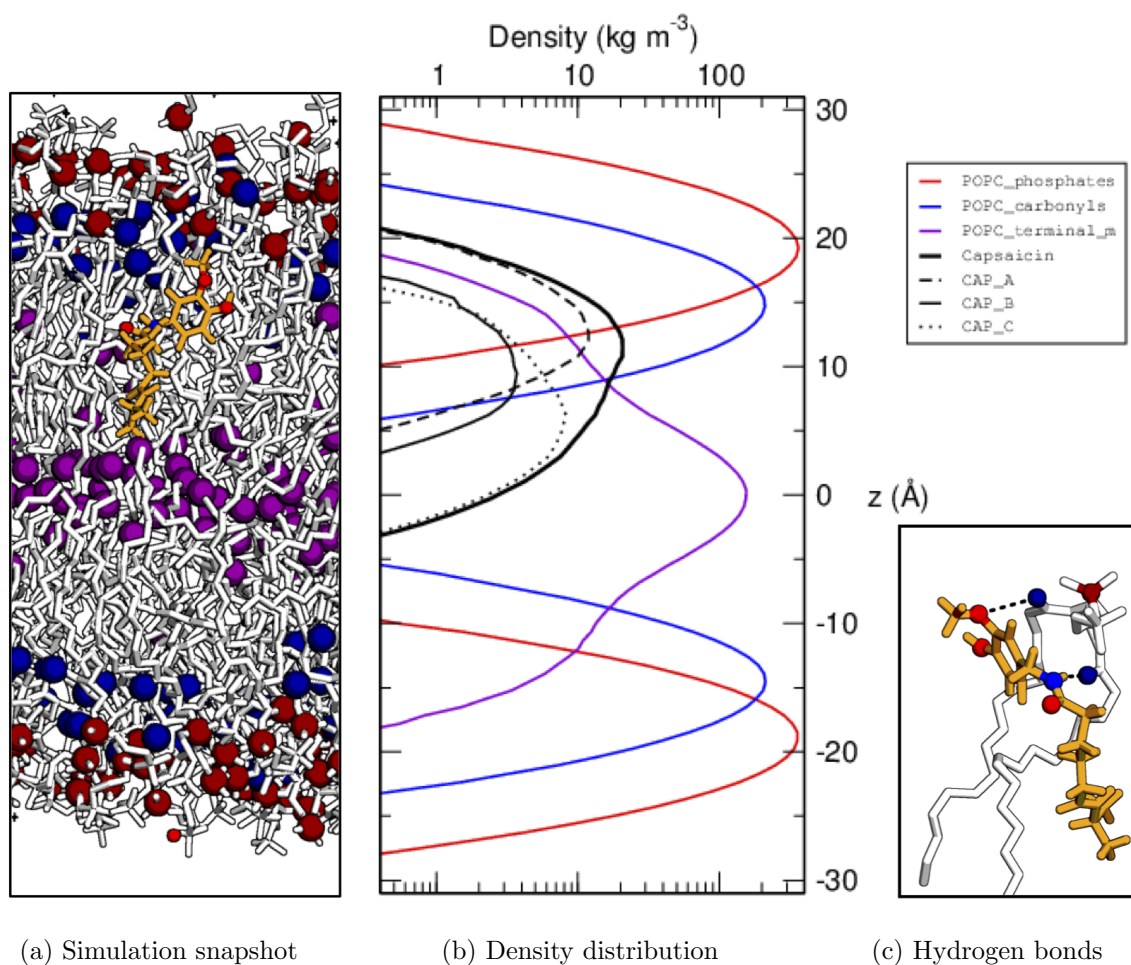
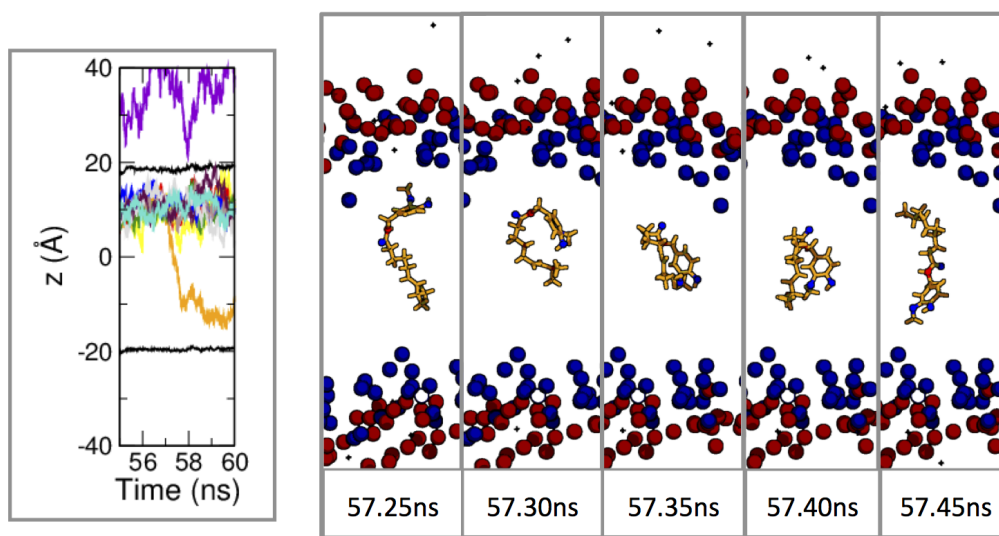


Figure 6.6: **Capsaicin distribution within the lipid bilayer.** (a) A snapshot of a capsaicin molecule after penetrating the bilayer. The capsaicin molecule is shown in orange. POPC phosphates are shown in dark red. POPC carbonyls are shown in dark blue. POPC terminal carbonyls are shown in purple. (b) The density distribution of a selection of chemical groups of capsaicin and POPC. (c) An example of a hydrogen-bonding interaction seen between a capsaicin and a POPC molecule.

of this event focuses on the short timescale during which this occurs (Figure 6.7). In order to increase the likelihood of observing another such event, two simulations were extended to 500ns, resulting in a total of over 4 μ s of individual capsaicin trajectories within a bilayer. The phenomenon, however, was not seen again. Additional simulations restarted at different timepoints of this flip-flop event indicated that even when the molecule had past the minima of the non-flip-flopping molecules, it could still return unaided to the original side of the bilayer. This result did not help to clarify why this particular capsaicin had crossed the bilayer while the 4 μ s of simulation showed no other capsaicin molecules leaving the water-bilayer interface at which the penetration event had occurred.



(a) Center of mass plot.

(b) Snapshots as capsaicin crosses the bilayer.

Figure 6.7: A capsaicin flip-flop event. (a) A zoom of the center of mass plot of the flip-flop of a capsaicin molecule from one leaflet of the bilayer to the other. Black indicates POPC phosphate head groups, and the multiple capsaicin molecules are in an assortment of colours. (b) Snapshots of this capsaicin molecule as it crosses the bilayer at 50 ps intervals. POPC carbonyls are in blue while POPC phosphates are in red.

6.3 Calculating the free energy profile of capsaicin crossing a lipid bilayer

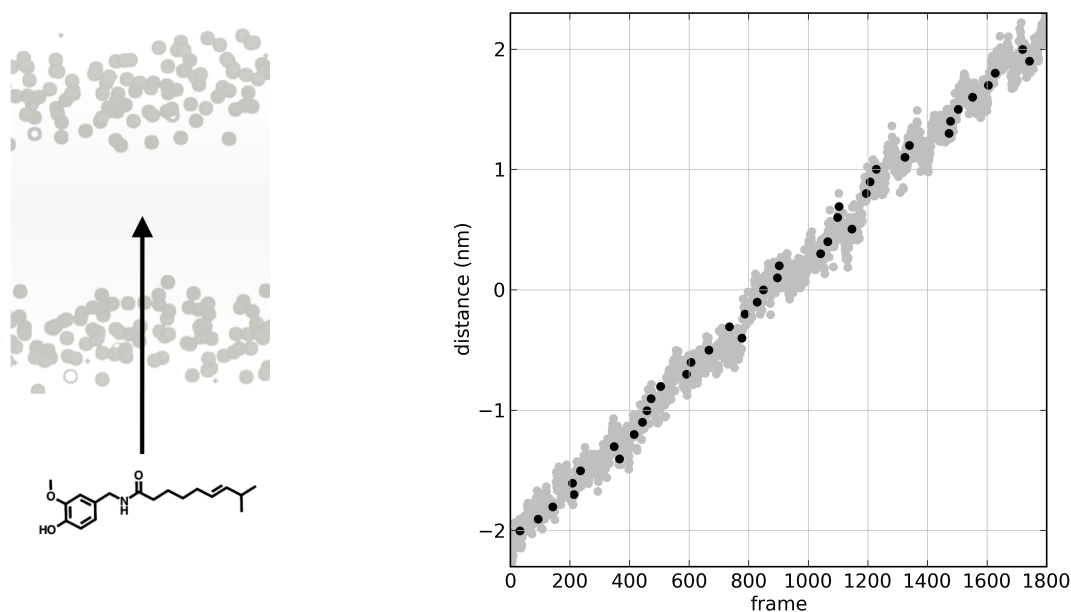
While equilibrium simulations provided an insightful initial look at the possible qualitative interactions between capsaicin and a POPC bilayer, questions remained regarding the driving forces behind these observations. In order to obtain a quantitative understanding of both the partition coefficient of capsaicin into the bilayer and the flip-flop of capsaicin from one leaflet to the other, a free energy profile was calculated by umbrella sampling methods of capsaicin restrained in simulation windows distributed across the bilayer.

6.3.1 System setup

Calculating the potential of mean force (PMF) using umbrella sampling methods in MD simulations have been widely used to study the energetic barriers to solute penetration of bilayers and pores [5, 17]. The method calculates the potential that gives the average force acting on a molecule as a function of a selected parameter, by sampling a set of semi-overlapping windows (details in Materials and Methods). In this case, the parameter used is the distance of the capsaicin center of mass from the center of the bilayer along the bilayer norm (z coordinate of the simulation box) (Figure 6.8a). To create appropriate windows to use for umbrella sampling, the capsaicin molecule was pulled across the POPC bilayer using steered MD. A set of frames from this steered MD simulation were selected at 1 Å intervals spanning the entire bilayer to setup up the PMF calculation (Figure 6.8b). Each window was simulated for 10ns to create a set of overlapping, biased probability distributions.

6.3.2 Free energy calculations

From these 10ns windows the weighted histogram analysis method (WHAM) can be used to calculate the PMF for capsaicin at different distances, z , across the bilayer [236].



(a) Pulling capsaicin across the bilayer.

(b) Frames chosen for umbrella sampling.

Figure 6.8: **Setting up umbrella sampling.** (a) Using steered MD, the capsaicin was pulled across the bilayer. (b) From the steered MD simulation, frames were chosen from which to start the PMF calculations.

The PMF can be determined from this set of simulations because the free energy, $\Delta G(z)$, is directly proportional to the probability distribution, $\rho(z')$ (Equation 6.1). While the umbrella sampling gives a set of biased probability distributions centered around the windows of choice, the WHAM method calculates an unbiased distribution from these (see Materials and Methods for more details).

$$\Delta G(z) = - \int_{\text{solute}}^z \langle \mathbf{F}(z') \rangle_t dz' = - \int_{\text{solute}}^z k_B T \ln \rho(z') dz' \quad (6.1)$$

In this case, the windows give the set of biased distributions in Figure 6.9, which shows mostly overlapping umbrellas with a few exceptions. From these windows using boot strapping methods and symmetrising the free energy profile around $z=0$, a prediction of the PMF profile of capsaicin distribution across a lipid bilayer was calculated (Figure 6.10).

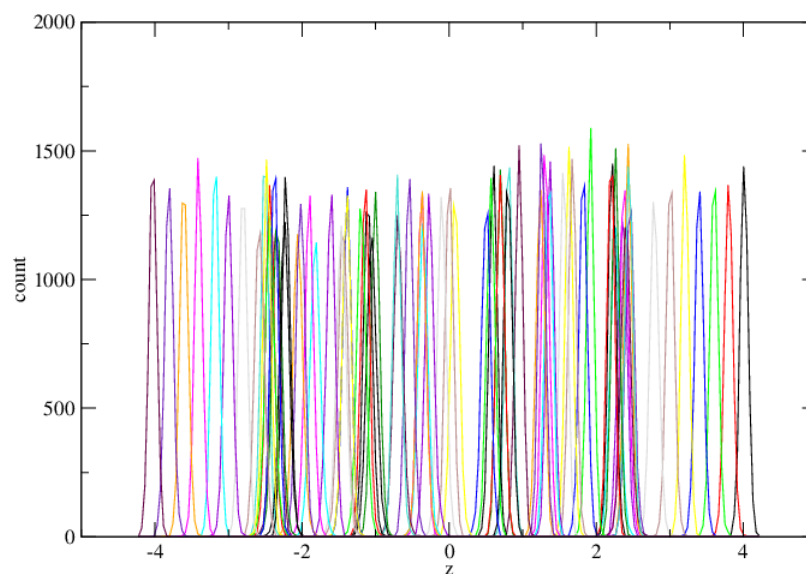


Figure 6.9: **Histogram of sampled regions.** This histogram shows the counts of sampled regions in each umbrella. Most windows show reasonable overlap, with some notable exceptions such as the lack of a window near 0.4 nm from the center.

The reference coordinate here (where kcal/mol=0) has been set to the capsaicin in solute. Checks of convergence indicate that the general shape of this curve is maintained, though not necessarily the magnitude. In addition, when comparing this profile to profiles of common functional groups, this profile is most similar to that of methyl acetate, which has both hydrophobic and polar groups, similar to capsaicin [19]. Additionally the energy minima correspond to the maxima of the capsaicin density profile observed in ‘equilibrium’ simulations near the carbonyls of the lipid bilayer (Figure6.6).

6.3.3 Physiological implications

While 10ns is likely not enough to achieve complete convergence of either capsaicin or the lipid tails of the bilayer, this method gives a first approximation of the energy barriers involved in the two processes of bilayer penetration and bilayer flip-flop. Notably, some research suggests that longer umbrella simulations for the calculation of PMF profiles does

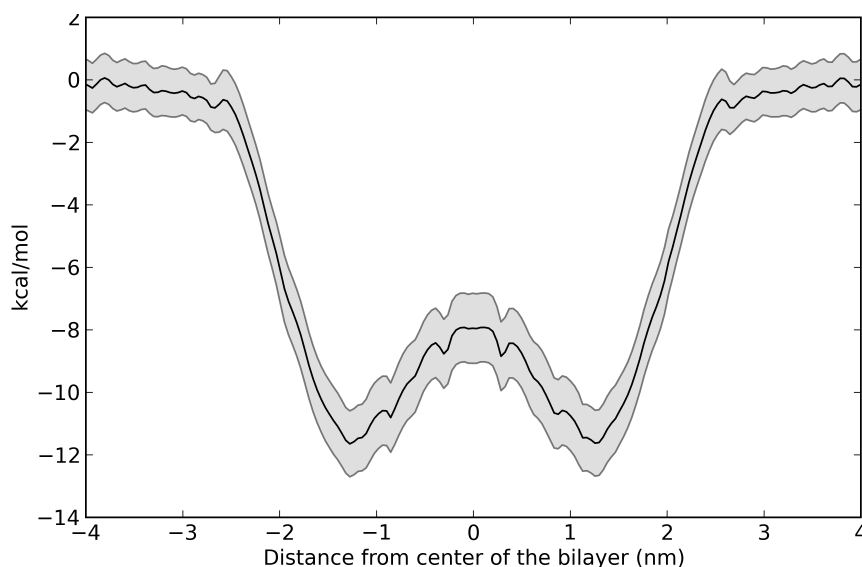


Figure 6.10: **Free energy profile of capsaicin in a POPC bilayer.** The free energy profile of capsaicin in a POPC bilayer indicates no barrier to bilayer penetration and shows large wells at the bilayer interface, corresponding to that seen in the density profiles seen in the previous section. Also notable is a large free energy barrier to bilayer crossing.

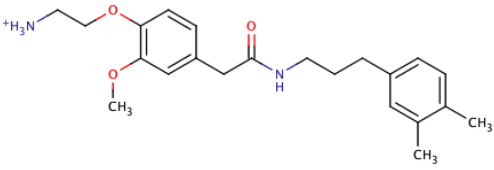
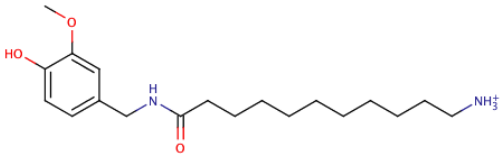
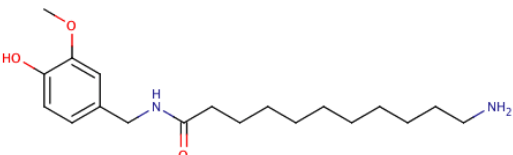
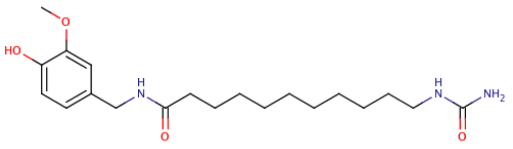
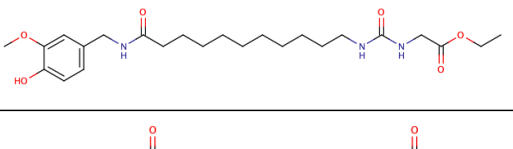
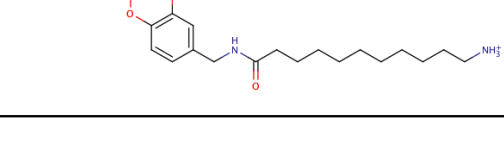
not comparatively increase the estimates of partition coefficients [267]. The fact that this PMF indicates such a large free energy barrier to flip-flop agrees with the low frequency of this event in equilibrium simulations. In the context of a predicted intracellular binding site to capsaicin this suggests one of three things are happening: (1) the protein is facilitating capsaicin movement across the bilayer or (2) capsaicin flip-flop is a rate-limiting step to TRPV1 activation, or (3) capsaicin is actively transported across the bilayer.

6.4 Interactions of other TRPV1 ligands with a lipid bilayer

While developing both a qualitative and quantitative understanding of the interactions of capsaicin with a lipid bilayer in the previous two sections has added to the understanding of capsaicin bilayer localisation and the energetic requirements for flip-flop, some compelling questions have been raised in experiment with the use of capsaicin analogues. Charged

analogues, for example, have been a key contributor to studies of the asymmetric activation of capsaicin intracellularly versus extracellularly.

Table 6.1: Capsaicin analogues chosen for further investigation.

Name	Molecular Structure
DA-5018	
Conway #2	
Conway #2d	
Conway #3	
Conway #4	
Conway #4a	
Conway #5	

6.4.1 Ligands chosen to investigate

The range of analogues selected for further simulations was based on those used in experimental studies (Table 6.1). The first simulations of this range of molecules were conducted with the charged capsaicin analogue, DA-5018, featured in the experiments showing that this ‘membrane impermeable’ ligand could only activate capsaicin when applied intracellularly [123]. In collaboration with Dr. Stuart Conway from the Oxford Chemistry Department, a set of charged capsaicin analogues used in experiments assessing the asymmetry of capsaicin TRPV1 activation through a bilayer were parametrised and addressed in parallel here via MD simulation.

The parameters for these ligands were determined as for capsaicin (see Materials and Methods for more details), and the simulations were setup as stated previously. All simulations were of four analogues in solution and run for 100ns.

6.4.2 Charged analogues readily penetrate the bilayer

Charged capsaicin analogues are used due to their reduced lipophilicity compared to capsaicin to be able to address the location and accessibility of the binding site on TRPV1. In these simulations, however, the explicitly charged molecules readily penetrate the bilayer, much like capsaicin (Figure 6.11).

6.4.3 Patterns of bilayer localisation of charged analogues

This brings up the question of why charged capsaicin molecules have differential effects on TRPV1 activation when applied extracellular vs. intracellularly. Looking at the density distribution, and keeping capsaicin flip-flop from the previous sections in mind, it seems like it is this step that would determine the propensity of charged analogues to activate TRPV1. While the peaks of the density profile were not much changed, how far toward the center

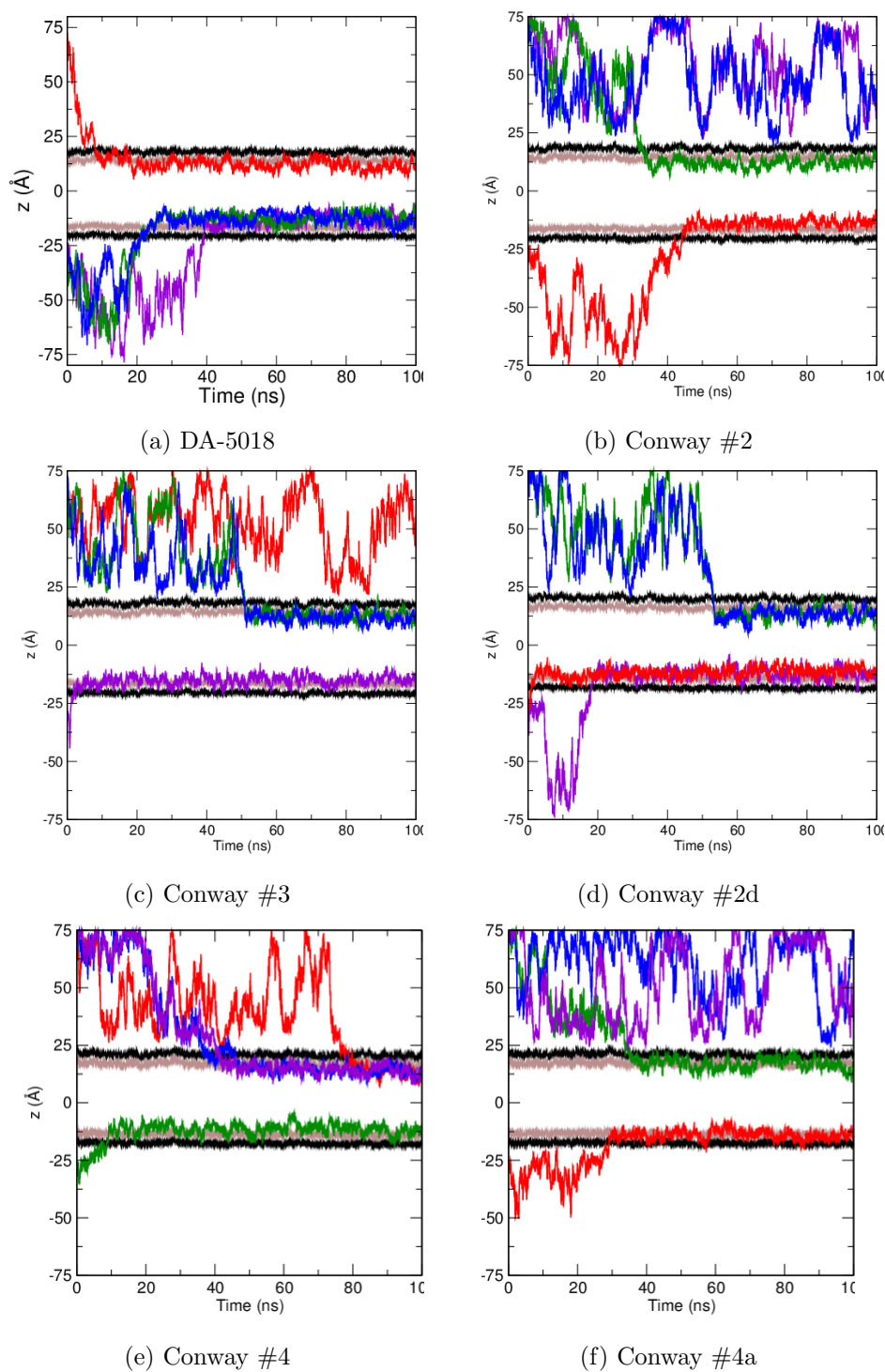


Figure 6.11: **Charged analogues readily penetrate the bilayer.** Each of these figures tracks the center of mass of the phosphate head groups (black) and carbonyls (brown) of the POPC molecules in both leaflets, as well as the four analogue molecules in red, green, blue and purple. (a) DA-5018, an analogue with a large charge on the vanilloid A group penetrates the bilayer. (b) As does the analogue Conway #2, which has charge on the end of the hydrophilic C group, which is similar to the (c) deprotonated version of this molecule. (d) Adding a urea group to the hydrophobic tail replicates similar behaviour, even with (e) an additional polar group (f) and an acid that is predicted to a metabolite of this.

of the bilayer the tails would penetrate showed marked differences for some of the charged molecules. This resulted in differential partial density profile at the center of the bilayer for these molecules (Figure 6.12). While some charged molecules still showed capsaicin-like density at the center of the bilayer, what was most notable was that differences between the charged and uncharged versions of a single compound (2 and 2d, and 4 and 4a) did follow this pattern. While speculative, this distribution could indicate their propensity to flip-flop, just as the PMF should be directly related to the density distribution. This would be interesting to study more rigorously with PMF profiles of charged and uncharged analogues.

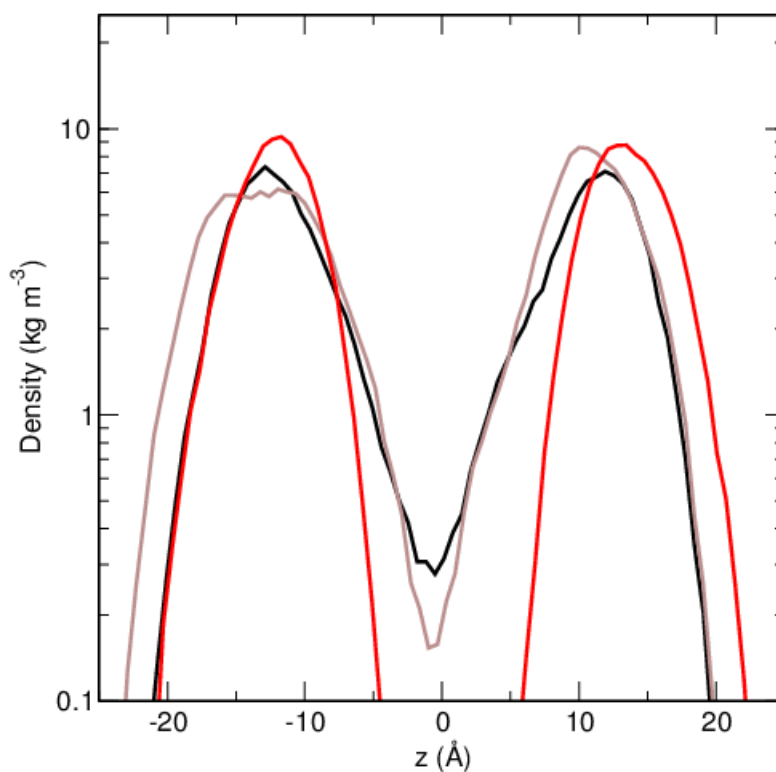


Figure 6.12: **Partial density distribution of analogues.** This partial density distribution displays the overall densities within the bilayer of capsaicin (black), DA-5018 (pink), and Conway #2 (red). This trend of charged ligands having less density at the center of the bilayer, could be a clue to their propensity to flip-flop.

6.5 Capsaicin interaction with a TRPV1 homology model in a simple bilayer

It is clear that the TRPV1-capsaicin interaction is at least somewhat bilayer-mediated, and little previous to this work has addressed the relatively simple molecular mechanisms between ligand and bilayer that likely contribute immensely to this three way interaction. That being said, this section returns to the more obviously relevant system of the TRPV1 protein and capsaicin ligand in a POPC bilayer.

6.5.1 Control for non-specific protein interactions

To look at the non-specific interactions of capsaicin with protein, four simple model TM helices, the α -helical GWALP peptide, were added, evenly spaced, to the capsaicin/bilayer system (Figure 6.13a) [94]. In these simulations, capsaicin interactions with the model α -helix were observed, but were never long-lasting (Figure 6.13b).

6.5.2 Simulation setup

Simulations were set up using the homology models described in Chapter 5. The simulation described here is with a model of only the S1-S4 α -helices of rat TRPV1. The method of bilayer self-assembly previously mentioned in section 6.1, was also used here to build a bilayer around the homology model in coarse-grained before converting to an atomistic system to a stable protein-bilayer system (Figure 6.14). As in the capsaicin simulations, two sets of starting conditions were studied: four capsaicin molecules evenly spaced in solute, either at the z center of the bilayer or at z of 30 Å from this center. Simulations were run for 100ns, 1 $\times$ with the starting configuration of capsaicin in solute and 2 $\times$ in the starting configuration with capsaicin at the center of the bilayer.

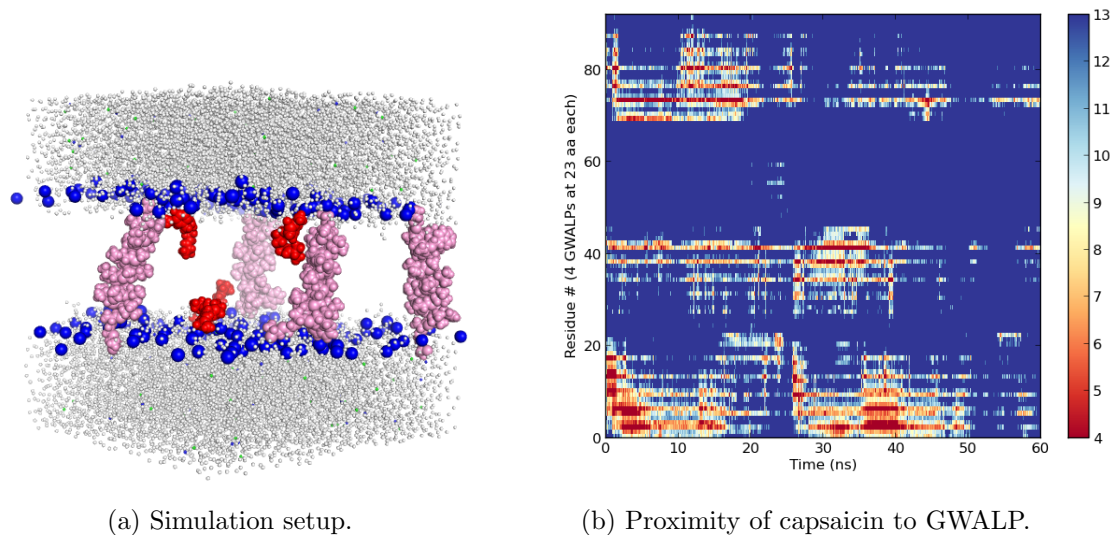


Figure 6.13: **Testing for non-specific interactions with a model TM helix.** (a) Four GWALP peptides were placed at the center of the x-y quadrants of the simulation box. Four capsaicin molecules were then added to the simulation box, either in the center of the membrane or in the solute. This snapshot is a few ns after the former. (b) While interactions between capsaicin and GWALP occurred, they were transient, and disappeared within the expect time of lipid diffusion, shown by this heatmap of distance of any GWALP peptide (numbered 1-92, with each GWALP at 23 amino acids) to any capsaicin molecule.

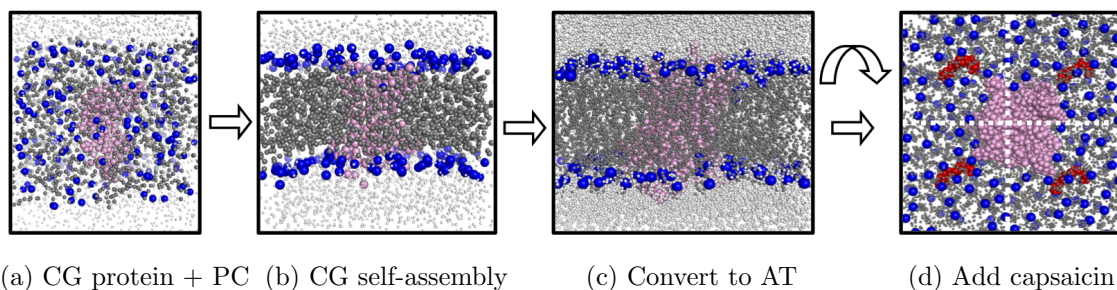


Figure 6.14: **Cap+protein simulation setup.** (a) First a hydrated box of randomly placed CG POPC molecules around the CG protein model was generated, with gaps on opposite sides of the z-axis of the box to facilitate bilayer formation. (b) This lipid-protein system was then simulated for 100ns to allow for the self-assembly of a stable protein-bilayer system. (c) This lipid-protein system was then converted from CG to AT, and (d) four capsaicin molecules were added at the same z-coordinate (either at the center of mass of the bilayer or in the solute, at 30Å from the center of mass of the bilayer) and at the center of the four x-y quadrants of the simulation box.

6.5.3 Observing ‘binding’ between protein and ligand within the bilayer

Over the course of the simulations in which capsaicin started in the bilayer, it immediately diffused to the bilayer interface, as seen in the previous simulations. In one case out of the eight capsaicin molecules. A concerted interaction with the model was observed (Figure 6.15). To test if this interaction could be termed a proper ‘binding’ event, rare to see in simulations due to the small timescales available, analysis was conducted to see if the interaction was specific to a certain set of residues and the simulation was extended. While the interaction seemed to center around the residues in the S2-S3 loop predicted to be significant to capsaicin binding to TRPV1 from mutational studies [86, 121] the interaction was not maintained when the simulation was extended (Figure 6.16).

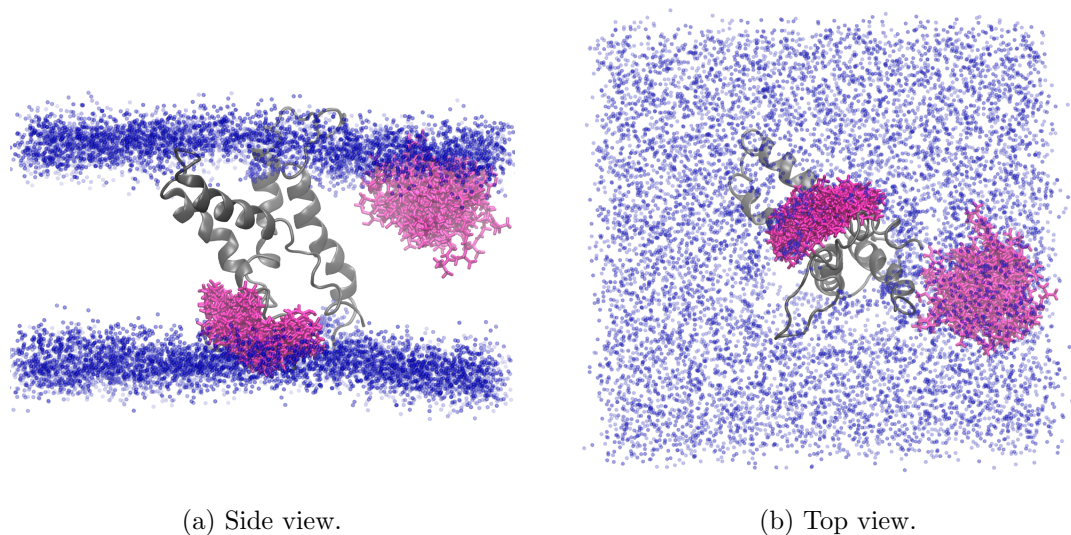


Figure 6.15: **3D plot of capsaicin near TRPV1.** One capsaicin molecule can be seen to interact concertedly with this TRPV1 model at the intracellular side of the bilayer while another molecule diffuses normally. These figures are an overlay over 50-100ns of two capsaicin trajectories, one of which is interacting with the homology model and one of which isn’t. Both are coloured in magenta and showed as sticks, while the POPC phosphate headgroups are shown as blue circles. This is viewed from the (a) side and (b) top.

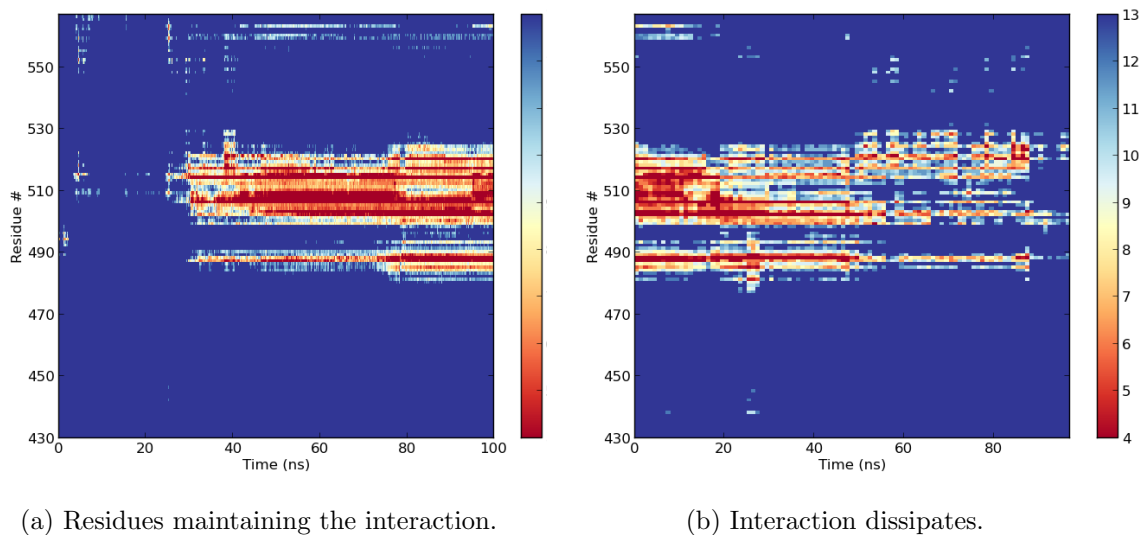


Figure 6.16: **Capsaicin 'binding' to TRPV1.** (a) Here we see a capsaicin concertedly interact with the TRPV1 homology model, at the region near the S2-S3 loop, here residue 81 is Y511, which is known to remove capsaicin affinity when mutated to an alanine. Red indicates residues that are close (within 4 Å). (b) However, when this simulation is extended, the interaction quickly dissipates.

6.5.4 Physiological relevance

Since the question of whether this interaction is indeed specific or non-specific remains unclear despite the GWALP control, the physiological significance of observing this interaction in simulation remains unclear. Perhaps this is a clue that non-specific interactions between capsaicin and proteins within the bilayer are common, but perhaps it is an insight into the beginnings of how capsaicin could activate TRPV1. That capsaicin 'binds' to the exact region shown to be important in mutational studies and that the very experiments illustrated previously in this thesis indicate that capsaicin does not bind the S1-S4 helices to the same affinity as the full length protein, suggests the latter. To this extent, perhaps this could be the beginnings of a binding event that requires the S5-S6 helices, N-terminal, or C-terminal regions to acquire physiological specificity. While this is perhaps too much to conclude from a simulation of a homology model whose problems were illustrated in

the previous chapter, it is at least a possible model of the mechanisms taking place during TRPV1 activation by capsaicin, that takes into account the real complexities of drug binding through a bilayer.

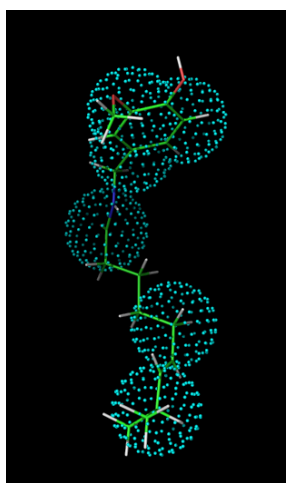
6.6 Conclusions and future directions

6.6.1 Further simulations and experiments could help clarify some remaining ambiguities

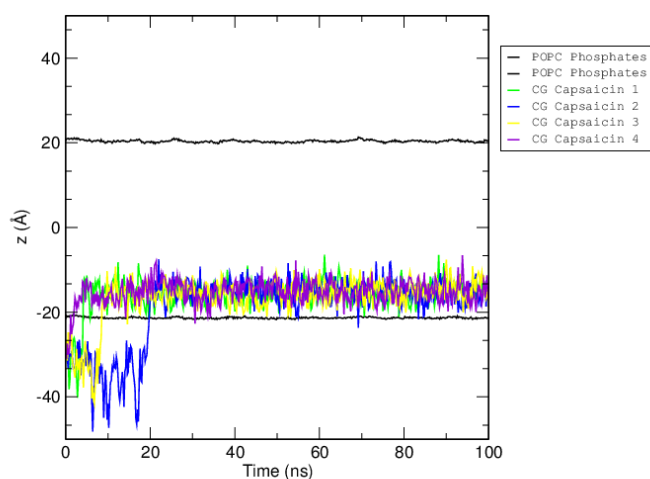
With the results in this chapter raising some interesting questions about the capsaicin-TRPV1 interaction through the lipid bilayer, more rigorous and directed questions are now open to exploration. One avenue of study, already mentioned, is calculating PMFs of analogues to approach a rigorous determination of their bilayer-crossing propensities. PMFs of these analogues could also allow for the direct calculation of the contributions of desolvation energies to the partition coefficients of the molecules. Not only would further study of the analogues already studied within this chapter be worthwhile, but there remain a diverse range of other TRP agonists and antagonists, not only to TRPV1, with a variety of activities and hydrophobicities that could be studied and reveal unexpected insights in the manner described above.

In addition to extending the range of ligands studied, addressing properties and contributions of the lipid bilayer would also be a valuable addition to this work. Preliminary studies, parametrising a coarse-grained capsaicin for the purpose of both extending the timescales accessible and addressing the contributions of bilayer asymmetry and heterogeneity to capsaicin dynamics have already been conducted (Figure 6.17). One key motivation here, is the existence of cholesterol within eukaryotic bilayers, which has much higher rates of flip-flop than phospholipids and could considerable influence how capsaicin behaves in a lipid bilayer. Another aspect of the lipid bilayer that could be studied in more detail is whether

in simulations capsaicin effects bilayer fluidity, has been previously suggested. While the simulations in this chapter are too small and too short to make accurate predictions of lipid diffusion, this would be an area worth further study, not only for capsaicin but any small molecule lipophilic drug.



(a) Building CG capsaicin..



(b) CG capsaicin with a CG POPC bilayer.

Figure 6.17: A coarse-grained capsaicin. (a) A coarse-grained capsaicin was parametrised by comparison to previous parameters for PIP₂ and tyrosine. In green is the atomistic capsaicin, while in blue are the coarse-grained particles. (b) Center of mass plot of POPC headgroups (black) and the CG capsaicin molecules over the course of a short MD simulation. The particles seem to behave similar to atomistic capsaicin, though are seen to be more hydrophobic when comparing the speed of bilayer penetration, a 'stickiness' that has been seen for other coarse-grained models.

Finally, confirming and extending these results with experimental techniques would be ideal. While the mechanisms proposed here have not suggested any mutants that would effect TRPV1 activation by capsaicin, experiments in NMR [177], neutron scattering [177] and ITC [188] could be directly compared to the simulation results. For example, neutron scattering results return partial density profiles, much like those used to analyse simulation results in this chapter.

6.6.2 Progress toward a multi-step model of capsaicin binding to TRPV1

While the key events addressed within this chapter have been sampled only briefly, they allow for a first guess at the steps involved in TRPV1 activation by capsaicin: bilayer partitioning, flip-flop, and protein binding. While further calculations and simulations are ideal to achieve rigorous estimates of the rates we have seen just a glimpse of here, it would not be unreasonable to consider creating kinetic model of these steps to address which of these steps are rate-limiting, which can then be tested and compared with experiment.

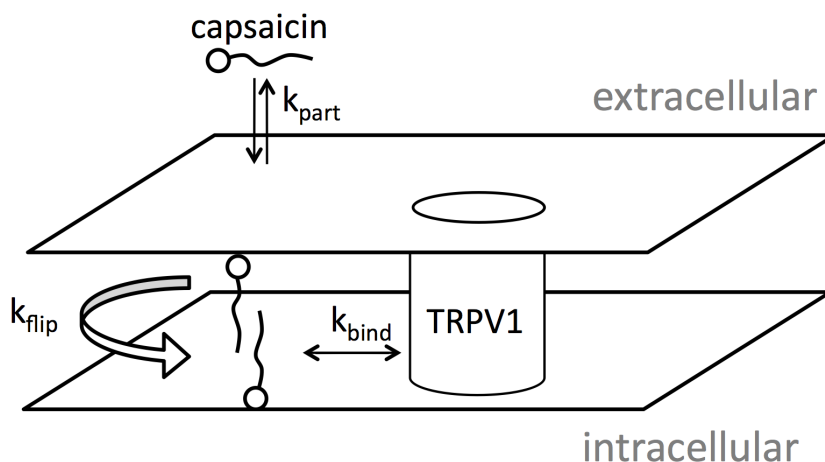


Figure 6.18: **Proposed mechanism for capsaicin binding within a bilayer.** These simulations are the start of understanding the processes necessary for capsaicin binding to TRPV1 through the bilayer. Each of the simulations described in this chapter can contribute to estimations for rates of each step. Initially capsaicin must diffuse in solute before penetrate the bilayer (k_{part}), then flip across the bilayer (k_{flip}) to be able to access the TRPV1 binding site (k_{bind}).

Summary and future directions

This thesis describes a multidisciplinary attempt toward understanding the structure and function of TRP channel transmembrane domains by focusing on the S1-S4 helices of the TRPV1 as a possible structural and functional ligand-binding domain. While the *S. cerevisiae* expression system proved to produce functional, RTX-binding and DkTx-binding WT rat TRPV1 as well as the membrane-inserted S1-S4 helices of several TRP channels, the domain did not prove to be a functional RTX-binding domain itself. Addressing the same issues of the structural and functional modularity of the TRPV1 S1-S4 via computational modelling and simulation yielded few reliable hints. However, simulations of the TRPV1 ligand, capsaicin, in a lipid bilayer began to reveal molecular insight into a possible mechanism of capsaicin binding through the lipid bilayer.

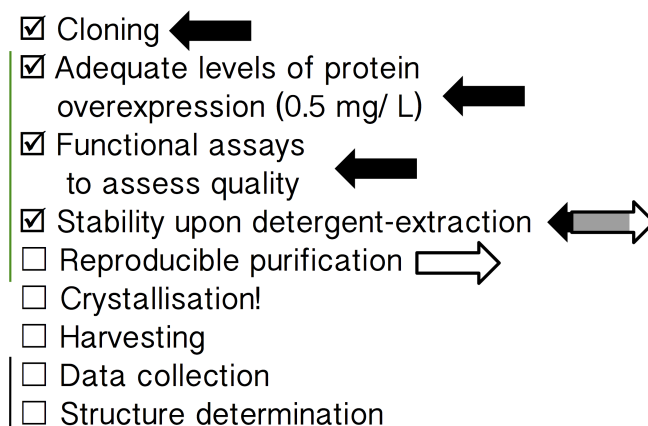
7.1 TRP channel transmembrane domains as a target for biophysical characterisation

This project began with the goal of biophysically characterising the S1-S4 ‘domain’ of a TRP channel with the idea that the data could be used to refine a homology model or maybe even solve a crystal structure, however it was not clear that these helices formed a

domain in the formal sense in the first place. This then became the first aim of the project, and to a certain extent is still a question without a firm answer. The clearest result from this thesis is that the S1-S4 and S1-S6 helices of rat TRPV1 do not bind resiniferatoxin (RTX), indicating that these transmembrane regions are not functional domains in the sense of ligand binding. (Even this result is not novel as previous work has shown that point mutants in the N- and C-terminal domains remove both capsaicin-induced currents and RTX binding [124].) However, these experiments do not address the functionality of these domains on other terms. For example, it is still possible that there is a ‘minor’ binding site of lower affinity than is observable in the experiments presented here. This is even suggested by the ‘binding event’ seen between capsaicin and a TRPV1 S1-S4 homology model in the simulations presented in Chapter 6.

Thus, despite the negative results of functional experiments, the S1-S4 domains themselves may still be a valuable target for biophysical characterisation. This is further encouraged by the degrees of success achieved in attempts toward purification of the TRPV1 S1-S4 domain of both rat and chicken. Fluorescence-coupled Size Exclusion Chromatography (FSEC) traces in FC12, DDM, and LDAO showed strong evidence for monodispersity, in some cases even after large-scale detergent solubilisation and purification. In addition, the S1-S6 of TRPV1 exhibited consistently higher levels of overexpression and showed promise in FSEC traces once solubilised in LDAO. However, upon purification the S1-S6 helices tended to precipitate more and show extremely wide SEC traces upon large scale solubilisation and purification.

While the experiments presented here have yet to lead to the quality and quantity of protein needed for biophysical characterisation (solid-state or solution NMR, circular dichroism (CD) spectroscopy, dynamic light scattering (DLS), crystallography, analytical ultracentrifugation (AUC)), they have established that the GFP-based *S. cerevisiae* system would be more than adequate to continue screens of the transmembrane domains of a wider

Figure 7.1: **Crystallography pipeline.**

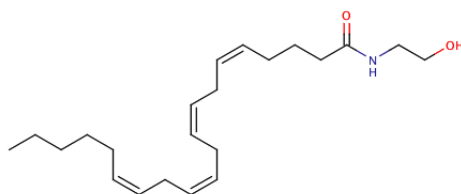
range of species of TRP channels with varieties of mutants, detergents and affinity tags (Figure 7.1). It is also clear, however, that indications of monodispersity indicated by FSEC traces are not always accurate, and that the GFP may be stabilising the protein, as indicated by the problems illustrated in Chapter 4 with GFP-cleavage upon purification. However, while promising FSEC traces are not a 100% guarantee of protein stability upon GFP cleavage, they help to narrow constructs taken to purification trials and point toward constructs which may be beneficial to explore in affinity tag-only vectors.

7.2 Insights toward a mechanism of ligand binding to TRPV1 inside the lipid membrane

While key residues to capsaicin and menthol binding to the S1-S4 helices of TRPV1 and TRPM8, respectively, have been an encouraging clue toward the modularity of TRP channel voltage-sensor-like domains, the binding site and mechanisms of binding through a lipid bilayer remain poorly characterised. Additionally, data regarding the bilayer asymmetry of capsaicin activation of TRPV1 remains inconsistent, and the reasons for this are unclear [53, 123, 291]. Some accounts claim it is due to a lack of complete washout of capsaicin from

the bilayer, while others suggest it may be due to the presence of two separate capsaicin binding sites in TRPV1 [291]. However, the consensus remains that a string of residues around the intracellular S2-S3 loop of TRPV1 is a key region for capsaicin binding. Since this loop likely has cytosolic elements, it could be true that capsaicin does not bind within the bilayer at all, and instead travels through a pore or transporter to reach the intracellular binding site. However, the lipophilicity of capsaicin and the predicted placement along the TM helices of some of the residues implicated in binding suggests this is unlikely. Thus, the bilayer localisation of capsaicin at equilibrium, presented via simulation in Chapter 6, is likely extremely important to its activation of TRPV1.

Ideally, it is possible to experimentally observe average bilayer depth of capsaicin chemical groups using neutron spectroscopy in a Dipalmitoylphosphatidylcholine (DPPC) bilayer, to corroborate computational results [177]. However, even with these results the molecular view available in simulation is unique, especially with regards to capsaicin flip-flop. While only one flip-flop event was seen in the over 4 μ s of simulation, this is still an extremely short time-scale with respect to experimental measurements, and the molecular mechanism of capsaicin flip-flop in order to reach its binding site remains valid. The exploration of charged capsaicin analogues helps emphasise the physiological relevance of this study, such that for charged analogues it can be speculated that it is not the bilayer penetration, but the flip-flop event itself that is significant to a varied ability to activate TRPV1. Additionally, it emphasises the extendability of such a study to the wide variety of TRPV1 ligands. For example, an extended study could provide insight into the mechanisms of the regulation of TRPV1 by the endogenous cannabinoid neurotransmitter anandamide (Figure 7.2). In addition, the wide variety of pungent TRP channel ligands described in Chapter 1 could be approached with a similar methodology to address similarities and differences among bilayer distributions and to find potential correlations to which TRP channel they are known to activate.

Figure 7.2: **Anandamide.**

As a general note, very few simulation studies have been conducted of drug binding events to membrane proteins within the lipid membrane. However, it is becoming increasingly clear that the membrane plays a critical role. Though membrane permeability is a standard criteria taken into account in drug development through assays such as the parallel artificial membrane permeability assay (PAMPA) and the carcinoma colorectal cell-based (CaCo-2) assay [267], these do not directly account for the involvement of the membrane in the drug binding event itself. Observing a drug binding event within an ‘equilibrium’ MD simulation, is rare, and leaves an open question about whether observing such an event in the simulations presented in Chapter 6 is physiologically relevant or somehow an artifact. One of the most promising examples of using MD to predict drug binding interaction was the prediction of the PIP₂ interaction with a Kir channel. With the parameterisation of CG-capsaicin at the end of Chapter 6, the same methodology becomes accessible for TRPV1. While the poor quality of the homology models has posed a problem for atomistic simulation, approaching a CG simulation of the same system could help elucidate broad trends in bilayer and ligand interactions. This may seem surprising, but since proteins in CG are held together via an imposed elastic network model, homology models will not be as susceptible to the small inaccuracies of the model.

7.3 An increase in computational power will increase sampling and encourage the use of state-of-the-art computational tools

Since the beginning of this project a new version of the gromacs open-source software (version 4.6) was released. This new version takes advantage of graphics processing units (GPUs), which are highly parallelised circuits used to speed up the manipulation and production of graphics, to calculate non-bonded interactions. This incorporation of GPU processors into gromacs, previously only available via the OpenMM toolkit [68], allows for a significant speed up of atomistic simulations. Benchmarks of this new implementation of gromacs were performed on the capsaicin-bilayer system, and the speed up is quantified in Figure 7.3, showing a three-fold speed-up on 4 cores. Simulations used in the PMF profile calculated in Chapter 6 were performed with this speed up, and would likely have not been completed in time to appear in this thesis without it.

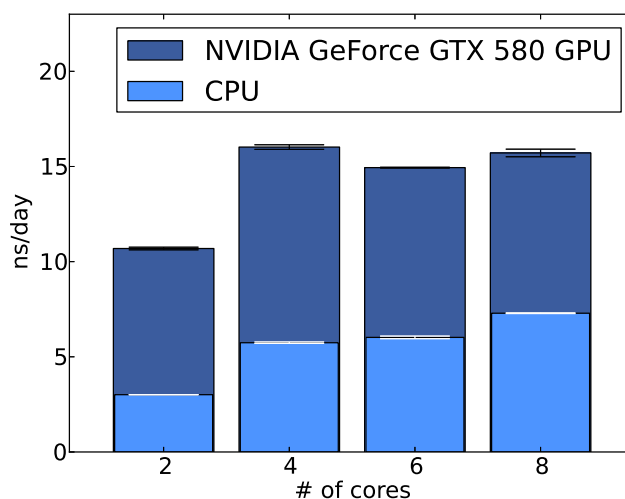


Figure 7.3: **Benchmarking capsaicin simulations.**

This increase in computational power implies not only that larger and longer simulations can be run in the same amount of time, enhancing the amount of conformational space that can be sampled, but also makes the more state-of-the-art computational tools more accessible. In this category are a range of free energy calculation methods like the PMF calculated in Chapter 6, polarisable force fields, and quantum mechanics/molecular mechanics (QM/MM) calculations. The parameters for capsaicin and analogues calculated here were done strictly using OPLS look-up tables, which match atom types to those already parameterised, for example of amino acids, rather than using a program, such as Gaussian [80] to recalculate the parameters from first principles. In the case of capsaicin and its analogues it might be especially interesting to perform the calculation in solute, and again in the bilayer, and again in the bilayer near the protein to see what effect this has on the polar groups of the molecule.

7.4 Looking to other VGL channels for clues to TRP channel structure-function relationships

The dominant model used in this thesis to address modularity in TRP channel transmembrane domains has been the voltage-gated potassium (Kv) channel. This model was chosen for a variety of reasons: (1) both TRPs and Kvs are homotetramers rather than one protein with four distinct S1-S6 domains like the Nav and Cav channels, (2) the Kv 1.2 channel is the only eukaryotic crystal structure of TRP homologues, and (3) there are peculiar regions of high sequence identity between TRPs and Kvs such as the selectivity filter of TRPVs and the S4-S5 linker of TRPCs. However, these factors do not necessarily make Kv the BEST model for TRPs.

As discussed at the end of Chapter 5, of the currently available S1-S6 tetramer structures, the best template for the TRP channel might be the CNG channel from the *M. loti* bacteria,

as it has no charged residues in its S4 helix. This channel, though, is slightly enigmatic, as most CNG channels do have these canonical charged residues. But similarly, the CNG channels in general seem to have atypical voltage-sensitive properties, hinting that perhaps these two atypical families in the VGL superfamily might share a non-canonical voltage-sensing mechanism. It would be worth, addressing, in this light, if a peculiar tighter packing seen in the *M. loti* crystal structure could be relevant to packing of TRP S1-S4 helices with the pore domain (Figure 7.4). In addition, the closely related two-pore (TPC) channels, which unlike either the Kv, Nav or Cav channels have two S1-S6 domains per protein. TRPP1, similarly, is structured in this way, and it could be worth investigating whether this architecture has functional significance to the more common homotetramerising TRP channels.

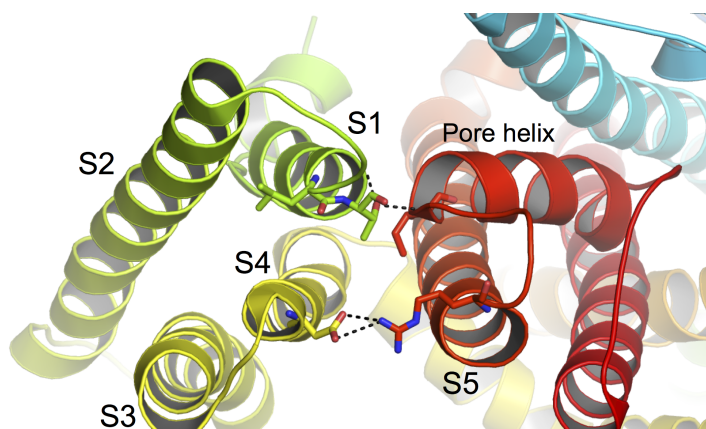


Figure 7.4: **Contacts between the S1-S4 and S5-S6 pore helices in the MlotiK crystal structure.**

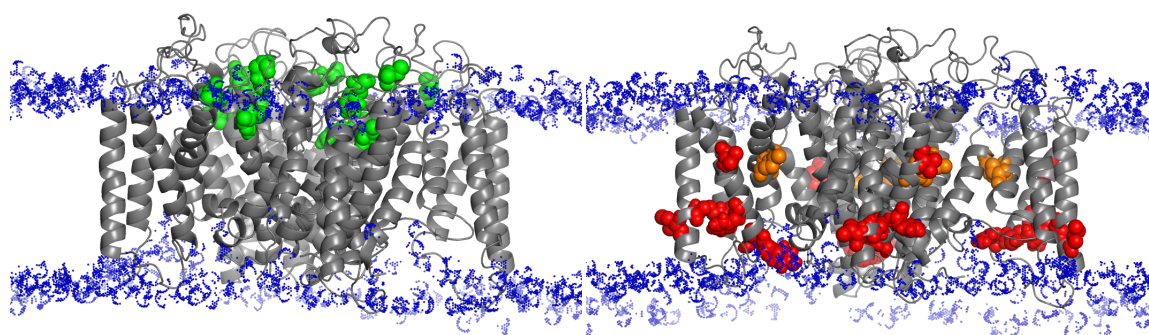
Another interesting and likely fruitful avenue to pursue is work on the evolutionary footprint of TRP channels. Not much has been done in this area, but one example of its power is a study addressing the divergence of reptilian TRP channels, which have 4 TRPV4 and 2 TRPM8 isoforms, since they require more accurate temperature sensing abilities [237]. More significantly to the hypothesis of this thesis, however, it remains unclear whether TRP channels initially evolved from voltage-gated channels, and whether the voltage-sensor-like

domain could be an artifact due to the family appearing relatively late in evolutionary history.

7.5 Refining the question of transmembrane domain modularity in TRP channels

While this thesis has presented experiments and simulations towards the answer, a small cloud still hangs over the major question of this thesis: do TRP channels have a structurally and functionally modular S1-S4 domain like canonical voltage-gated channels? Chapter 4 showed that the S1-S4 of TRPV1 does not bind RTX, but TRP channels are more multifaceted proteins, with more than just this function that could be mediated by the S1-S4 helices. Chapter 4 also discussed that the S1-S4 helices could fold, be stably inserted into the membrane, and be detergent solubilised as a monodisperse protein in FSEC with GFP attached. This is strong indication of an S1-S4 ‘domain’ of TRP channels in a structural sense, which is why in several places in subsequent chapters it has been referred to as such. However, it is not clear, yet, if this membrane-inserted domain maintains a ‘physiological’ fold and how one would determine this lacking a working assay such as the RTX-binding assay.

Whether or not the TRPV1 S1-S4 forms a physiologically folded structural domain, another avenue of addressing transmembrane domain modularity is through the packing interactions between the S1-S4 domain and the S5-S6 pore domain. In Kv channels, there are very few contacts between the two domains, limited to a contact at the top of the S1 with the pore helix and the S4-S5 linker [145]. One indication in this work that this might not be the case in TRPV1 is the allosteric coupling between DkTx and RTX binding, when one is predicted to bind in the pore domain and the other within the S1-S4 helices (Figure 7.5). In addition, the computational methods of covariance analysis and homology modelling in



(a) DkTx binding site.

(b) Capsaicin and RTX binding site.

Figure 7.5: DkTx and Capsaicin/RTX binding sites. (a) Residues in green spheres are those that remove DkTx activation. (b) Residues in red spheres are those that are implicated in capsaicin binding. Residues in oranges spheres are those additionally implicated in RTX binding. Figure made using PyMol and the TRPV1 homology model based on the Paddle-Chimera structure (PDB ID: 2R9R) from Chapter 5.

Chapter 5 were the beginnings of an attempt to address this question, and the idea should be taken further both experimentally and computationally. Looking at conserved contacts between the two domains and the effects they have in functional experiments would be a viable method for addressing the modularity of TRP channels that would require less investment and have a higher likelihood of success than the biophysical characterisation of the purified protein. For example, the threonine identified in Chapter 5 as a putative contact between the S1-S4 and S5-S6 pore domain of TRPV1 could be mutated to observe possible changes in the capsaicin activation. In one scenario it would effect channel activation by capsaicin, but not [^3H]-RTX binding, indicating that it would be a crucial residue in functionally connecting the S1-S4 to the pore domain. With this mindset, the question of modularity becomes instead, the imminently more answerable: are the S1-S4 helices of TRP channels more tightly packed to the S5-S6 helices of the pore domain than in Kv channels?

Another key issue to not overlook in breaking TRP channels into domains, especially in the transmembrane region, is that experimental evidence suggests (see Section 1.1.4) that large conformational changes are expected upon channel opening. This is also in line with

evidence that the pore is likely NOT like a Kv or NaV channel pore, at least not all the time, as not only is not selective, but has been shown to be able to become large enough to allow for the passage of small molecule drugs [272]. This may be related to the specialty of TRP channels to be molecular integrators of chemical and physical stimuli, able to incorporate many signals at once to regulate pore properties. Perhaps, indeed, this requires domains to be somewhat stable in a variety of conformations, making the question of whether the S1-S4 expressed and assayed in this thesis is ‘physiologically folded’ decidedly more complex.

A

APPENDIX A: Capsaicin parameters

```
[ moleculetype ]
; name nrexcl
CAP 3

[ atoms ]
; nr type resnr residu atom cgnr charge mass
1 op1s_145 1 CAP C1 1 -0.11500 12.01100 ; -0.1150000
2 op1s_146 1 CAP H12 1 0.11500 1.00800 ; 0.0000000
3 op1s_145 1 CAP C2 2 -0.11500 12.01100 ; -0.1150000
4 op1s_145 1 CAP C3 3 -0.11500 12.01100 ; -0.2300000
5 op1s_146 1 CAP H13 3 0.11500 1.00800 ; -0.1150000
6 op1s_145 1 CAP C4 4 -0.11500 12.01100 ; -0.2300000
7 op1s_146 1 CAP H14 4 0.11500 1.00800 ; -0.1150000
8 op1s_166 1 CAP C5 5 0.15000 12.01100 ; 0.0350000
9 op1s_166 1 CAP C6 6 0.15000 12.01100 ; 0.1850000
10 op1s_179 1 CAP O1 6 -0.28500 15.99940 ; -0.1000000
11 op1s_157 1 CAP C7 7 0.13000 12.01100 ; 0.0450000
12 op1s_156 1 CAP H17 7 0.04000 1.00800 ; 0.0850000
13 op1s_156 1 CAP H18 7 0.04000 1.00800 ; 0.1250000
14 op1s_156 1 CAP H19 7 0.04000 1.00800 ; 0.1650000
15 op1s_167 1 CAP O2 5 -0.58500 15.99940 ; -0.4200000
16 op1s_168 1 CAP H20 5 0.43500 1.00800 ; 0.0150000
17 op1s_244 1 CAP C8 8 0.08000 12.01100 ; 0.0950000
18 op1s_140 1 CAP H10 8 0.06000 1.00800 ; 0.1550000
19 op1s_140 1 CAP H11 8 0.06000 1.00800 ; 0.2150000
20 op1s_238 1 CAP N1 8 -0.50000 14.00670 ; -0.2850000
21 op1s_241 1 CAP H21 8 0.30000 1.00800 ; 0.0150000
22 op1s_235 1 CAP C9 9 0.50000 12.01100 ; 0.5150000
23 op1s_136 1 CAP C10 10 -0.12000 12.01100 ; 0.3950000
24 op1s_140 1 CAP H8 10 0.06000 1.00800 ; 0.4550000
25 op1s_140 1 CAP H9 10 0.06000 1.00800 ; 0.5150000
26 op1s_236 1 CAP O3 9 -0.50000 15.99940 ; 0.0150000
27 op1s_136 1 CAP C11 11 -0.12000 12.01100 ; -0.1050000
28 op1s_140 1 CAP H7 11 0.06000 1.00800 ; -0.0450000
29 op1s_140 1 CAP H16 11 0.06000 1.00800 ; 0.0150000
30 op1s_136 1 CAP C12 12 -0.12000 12.01100 ; -0.1050000
31 op1s_140 1 CAP H5 12 0.06000 1.00800 ; -0.0450000
32 op1s_140 1 CAP H6 12 0.06000 1.00800 ; 0.0150000
33 op1s_136 1 CAP C13 13 -0.12000 12.01100 ; -0.1050000
34 op1s_140 1 CAP H4 13 0.06000 1.00800 ; -0.0450000
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36 op1s_142 1 CAP C14 14 -0.11500 12.01100 ; -0.1000000
37 op1s_144 1 CAP H3 14 0.11500 1.00800 ; 0.0150000
38 op1s_142 1 CAP C15 15 -0.11500 12.01100 ; -0.1000000
39 op1s_144 1 CAP H1 15 0.11500 1.00800 ; 0.0150000
40 op1s_137 1 CAP C16 16 -0.06000 12.01100 ; -0.0450000
41 op1s_140 1 CAP H2 16 0.06000 1.00800 ; 0.0150000
42 op1s_135 1 CAP C17 17 -0.18000 12.01100 ; -0.1650000
43 op1s_140 1 CAP H25 17 0.06000 1.00800 ; -0.1050000
44 op1s_140 1 CAP H26 17 0.06000 1.00800 ; -0.0450000
```

```

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46   opls_135   1      CAP   C18   18  -0.18000   12.01100   ;  -0.1650000
47   opls_140   1      CAP   H22   18   0.06000   1.00800   ;  -0.1050000
48   opls_140   1      CAP   H23   18   0.06000   1.00800   ;  -0.0450000
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    3    4    1    0.14000   392459.    ;   C2-   C3
    4    6    1    0.14000   392459.    ;   C3-   C4
    6    8    1    0.14000   392459.    ;   C4-   C5
    8    9    1    0.14000   392459.    ;   C5-   C6
    9   10    1    0.13640   376560.    ;   C6-   O1
   10   11    1    0.14100   267776.    ;   O1-   C7
    8   15    1    0.13640   376560.    ;   C5-   O2
    3   17    1    0.15100   265266.    ;   C2-   C8
   17   20    1    0.14490   282002.    ;   C8-   N1
   20   22    1    0.13350   410032.    ;   N1-   C9
   22   23    1    0.15220   265266.    ;   C9-   C10
   22   26    1    0.12290   476976.    ;   C9-   O3
   23   27    1    0.15290   224262.    ;   C10-  C11
   27   30    1    0.15290   224262.    ;   C11-  C12
   30   33    1    0.15290   224262.    ;   C12-  C13
   33   36    1    0.15100   265266.    ;   C13-  C14
   36   38    1    0.13400   459403.    ;   C14-  C15
   38   40    1    0.15100   265266.    ;   C15-  C16
   40   42    1    0.15290   224262.    ;   C16-  C17
   40   46    1    0.15290   224262.    ;   C16-  C18
   38   39    1    0.10800   307106.    ;   C15-   H1
   40   41    1    0.10900   284512.    ;   C16-   H2
   36   37    1    0.10800   307106.    ;   C14-   H3
   33   34    1    0.10900   284512.    ;   C13-   H4
   30   31    1    0.10900   284512.    ;   C12-   H5
   30   32    1    0.10900   284512.    ;   C12-   H6
   27   28    1    0.10900   284512.    ;   C11-   H7
   23   24    1    0.10900   284512.    ;   C10-   H8
   23   25    1    0.10900   284512.    ;   C10-   H9
   17   18    1    0.10900   284512.    ;   C8-   H10
   17   19    1    0.10900   284512.    ;   C8-   H11
    1    2    1    0.10800   307106.    ;   C1-   H12
    4    5    1    0.10800   307106.    ;   C3-   H13
    6    7    1    0.10800   307106.    ;   C4-   H14
   33   35    1    0.10900   284512.    ;   C13-   H15
   27   29    1    0.10900   284512.    ;   C11-   H16
   11   12    1    0.10900   284512.    ;   C7-   H17
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   20   21    1    0.10100   363171.    ;   N1-   H21
   46   47    1    0.10900   284512.    ;   C18-   H22
   46   48    1    0.10900   284512.    ;   C18-   H23
   46   49    1    0.10900   284512.    ;   C18-   H24
   42   43    1    0.10900   284512.    ;   C17-   H25
   42   44    1    0.10900   284512.    ;   C17-   H26
   42   45    1    0.10900   284512.    ;   C17-   H27

```

[constraints]
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[pairs]

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9	17	1	;	C6-	C8
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2	17	1	;	H12-	C8
3	8	1	;	C2-	C5
3	10	1	;	C2-	O1
2	8	1	;	H12-	C5
2	10	1	;	H12-	O1
1	6	1	;	C1-	C4
1	5	1	;	C1-	H13
17	6	1	;	C8-	C4
17	5	1	;	C8-	H13
3	7	1	;	C2-	H14
5	8	1	;	H13-	C5
5	7	1	;	H13-	H14
4	15	1	;	C3-	O2
7	9	1	;	H14-	C6
7	15	1	;	H14-	O2
6	10	1	;	C4-	O1
15	1	1	;	O2-	C1
15	10	1	;	O2-	O1
1	11	1	;	C1-	C7
8	11	1	;	C5-	C7
9	12	1	;	C6-	H17
9	13	1	;	C6-	H18
9	14	1	;	C6-	H19
6	16	1	;	C4-	H20
9	16	1	;	C6-	H20
1	20	1	;	C1-	N1
1	18	1	;	C1-	H10
1	19	1	;	C1-	H11
4	20	1	;	C3-	N1
4	18	1	;	C3-	H10
4	19	1	;	C3-	H11
3	22	1	;	C2-	C9
3	21	1	;	C2-	H21
18	22	1	;	H10-	C9
18	21	1	;	H10-	H21
19	22	1	;	H11-	C9
19	21	1	;	H11-	H21
17	23	1	;	C8-	C10
17	26	1	;	C8-	O3
21	23	1	;	H21-	C10
21	26	1	;	H21-	O3
20	27	1	;	N1-	C11
20	24	1	;	N1-	H8
20	25	1	;	N1-	H9
26	27	1	;	O3-	C11
26	24	1	;	O3-	H8
26	25	1	;	O3-	H9
22	30	1	;	C9-	C12
22	28	1	;	C9-	H7
22	29	1	;	C9-	H16
24	30	1	;	H8-	C12
24	28	1	;	H8-	H7
24	29	1	;	H8-	H16
25	30	1	;	H9-	C12

25	28	1			H9-	H7				
25	29	1			H9-	H16				
23	33	1			C10-	C13				
23	31	1			C10-	H5				
23	32	1			C10-	H6				
28	33	1			H7-	C13				
28	31	1			H7-	H5				
28	32	1			H7-	H6				
29	33	1			H16-	C13				
29	31	1			H16-	H5				
29	32	1			H16-	H6				
27	36	1			C11-	C14				
27	34	1			C11-	H4				
27	35	1			C11-	H15				
31	36	1			H5-	C14				
31	34	1			H5-	H4				
31	35	1			H5-	H15				
32	36	1			H6-	C14				
32	34	1			H6-	H4				
32	35	1			H6-	H15				
30	38	1			C12-	C15				
30	37	1			C12-	H3				
34	38	1			H4-	C15				
34	37	1			H4-	H3				
35	38	1			H15-	C15				
35	37	1			H15-	H3				
33	40	1			C13-	C16				
33	39	1			C13-	H1				
37	40	1			H3-	C16				
37	39	1			H3-	H1				
36	42	1			C14-	C17				
36	46	1			C14-	C18				
36	41	1			C14-	H2				
39	42	1			H1-	C17				
39	46	1			H1-	C18				
39	41	1			H1-	H2				
38	43	1			C15-	H25				
38	44	1			C15-	H26				
38	45	1			C15-	H27				
46	43	1			C18-	H25				
46	44	1			C18-	H26				
46	45	1			C18-	H27				
41	43	1			H2-	H25				
41	44	1			H2-	H26				
41	45	1			H2-	H27				
38	47	1			C15-	H22				
38	48	1			C15-	H23				
38	49	1			C15-	H24				
42	47	1			C17-	H22				
42	48	1			C17-	H23				
42	49	1			C17-	H24				
41	47	1			H2-	H22				
41	48	1			H2-	H23				
41	49	1			H2-	H24				
[angles]										
; ai aj ak funct th0 cth										
9	1	3	1	120.000	527.1840		C6-	C1-	C2	
2	1	3	1	120.000	292.8800		H12-	C1-	C2	
1	3	4	1	120.000	527.1840		C1-	C2-	C3	
1	3	17	1	120.000	585.7600		C1-	C2-	C3	

2	1	9	1	120.000	292.8800	;	H12-	C1-	C6
1	9	8	1	120.000	527.1840	;	C1-	C6-	C5
1	9	10	1	120.000	585.7600	;	C1-	C6-	01
17	3	4	1	120.000	585.7600	;	C8-	C2-	C3
3	4	6	1	120.000	527.1840	;	C2-	C3-	C4
3	4	5	1	120.000	292.8800	;	C2-	C3-	H13
5	4	6	1	120.000	292.8800	;	H13-	C3-	C4
4	6	8	1	120.000	527.1840	;	C3-	C4-	C5
4	6	7	1	120.000	292.8800	;	C3-	C4-	H14
7	6	8	1	120.000	292.8800	;	H14-	C4-	C5
6	8	9	1	120.000	527.1840	;	C4-	C5-	C6
6	8	15	1	120.000	585.7600	;	C4-	C5-	02
15	8	9	1	120.000	585.7600	;	02-	C5-	C6
8	9	10	1	120.000	585.7600	;	C5-	C6-	01
9	10	11	1	111.000	627.6000	;	C6-	01-	C7
10	11	12	1	109.500	292.8800	;	01-	C7-	H17
10	11	13	1	109.500	292.8800	;	01-	C7-	H18
10	11	14	1	109.500	292.8800	;	01-	C7-	H19
8	15	16	1	113.000	292.8800	;	C5-	02-	H20
3	17	20	1	109.700	669.4400	;	C2-	C8-	N1
3	17	18	1	109.500	292.8800	;	C2-	C8-	H10
3	17	19	1	109.500	292.8800	;	C2-	C8-	H11
18	17	20	1	109.500	292.8800	;	H10-	C8-	N1
19	17	20	1	109.500	292.8800	;	H11-	C8-	N1
17	20	22	1	121.900	418.4000	;	C8-	N1-	C9
17	20	21	1	118.400	317.9840	;	C8-	N1-	H21
21	20	22	1	119.800	292.8800	;	H21-	N1-	C9
20	22	23	1	116.600	585.7600	;	N1-	C9-	C10
20	22	26	1	122.900	669.4400	;	N1-	C9-	03
26	22	23	1	120.400	669.4400	;	03-	C9-	C10
22	23	27	1	111.100	527.1840	;	C9-	C10-	C11
22	23	24	1	109.500	292.8800	;	C9-	C10-	H8
22	23	25	1	109.500	292.8800	;	C9-	C10-	H9
24	23	27	1	110.700	313.8000	;	H8-	C10-	C11
25	23	27	1	110.700	313.8000	;	H9-	C10-	C11
23	27	30	1	112.700	488.2730	;	C10-	C11-	C12
23	27	28	1	110.700	313.8000	;	C10-	C11-	H7
23	27	29	1	110.700	313.8000	;	C10-	C11-	H16
28	27	30	1	110.700	313.8000	;	H7-	C11-	C12
29	27	30	1	110.700	313.8000	;	H16-	C11-	C12
27	30	33	1	112.700	488.2730	;	C11-	C12-	C13
27	30	31	1	110.700	313.8000	;	C11-	C12-	H5
27	30	32	1	110.700	313.8000	;	C11-	C12-	H6
31	30	33	1	110.700	313.8000	;	H5-	C12-	C13
32	30	33	1	110.700	313.8000	;	H6-	C12-	C13
30	33	36	1	111.100	527.1840	;	C12-	C13-	C14
30	33	34	1	110.700	313.8000	;	C12-	C13-	H4
30	33	35	1	110.700	313.8000	;	C12-	C13-	H15
34	33	36	1	109.500	292.8800	;	H4-	C13-	C14
35	33	36	1	109.500	292.8800	;	H15-	C13-	C14
33	36	38	1	124.000	585.7600	;	C13-	C14-	C15
33	36	37	1	117.000	292.8800	;	filled		
37	36	38	1	120.000	292.8800	;	H3-	C14-	C15
36	38	40	1	124.000	585.7600	;	C14-	C15-	C16
36	38	39	1	120.000	292.8800	;	C14-	C15-	H1
39	38	40	1	117.000	292.8800	;	filled		
38	40	42	1	111.100	527.1840	;	C15-	C16-	C17
38	40	46	1	111.100	527.1840	;	C15-	C16-	C18
38	40	41	1	109.500	292.8800	;	C15-	C16-	H2
46	40	42	1	112.700	488.2730	;	C18-	C16-	C17
41	40	42	1	110.700	313.8000	;	H2-	C16-	C17

40	42	43	1	110.700	313.8000	;	C16-	C17-	H25
40	42	44	1	110.700	313.8000	;	C16-	C17-	H26
40	42	45	1	110.700	313.8000	;	C16-	C17-	H27
41	40	46	1	110.700	313.8000	;	H2-	C16-	C18
40	46	47	1	110.700	313.8000	;	C16-	C18-	H22
40	46	48	1	110.700	313.8000	;	C16-	C18-	H23
40	46	49	1	110.700	313.8000	;	C16-	C18-	H24
35	33	34	1	107.800	276.1440	;	H15-	C13-	H4
32	30	31	1	107.800	276.1440	;	H6-	C12-	H5
29	27	28	1	107.800	276.1440	;	H16-	C11-	H7
25	23	24	1	107.800	276.1440	;	H9-	C10-	H8
19	17	18	1	107.800	276.1440	;	H11-	C8-	H10
13	11	12	1	107.800	276.1440	;	H18-	C7-	H17
14	11	12	1	107.800	276.1440	;	H19-	C7-	H17
14	11	13	1	107.800	276.1440	;	H19-	C7-	H18
48	46	47	1	107.800	276.1440	;	H23-	C18-	H22
49	46	47	1	107.800	276.1440	;	H24-	C18-	H22
49	46	48	1	107.800	276.1440	;	H24-	C18-	H23
44	42	43	1	107.800	276.1440	;	H26-	C17-	H25
45	42	43	1	107.800	276.1440	;	H27-	C17-	H25
45	42	44	1	107.800	276.1440	;	H27-	C17-	H26

[dihedrals]

;	ai	aj	ak	al	funct	c0	c1	c2	c3	c4	c5
	9	1	3	4	3	30.33400	0.00000	-30.33400	0.00000	0.00000	; dih C6- C1- C2- C3
	3	1	9	8	3	30.33400	0.00000	-30.33400	0.00000	0.00000	; dih C2- C1- C6- C5
	1	3	4	6	3	30.33400	0.00000	-30.33400	0.00000	0.00000	; dih C1- C2- C3- C4
	3	4	6	8	3	30.33400	0.00000	-30.33400	0.00000	0.00000	; dih C2- C3- C4- C5
	4	6	8	9	3	30.33400	0.00000	-30.33400	0.00000	0.00000	; dih C3- C4- C5- C6
	6	8	9	1	3	30.33400	0.00000	-30.33400	0.00000	0.00000	; dih C4- C5- C6- C1
	1	9	10	11	3	12.55200	0.00000	-12.55200	0.00000	0.00000	; dih C1- C6- O1- C7
	9	10	11	12	3	1.58992	4.76976	0.00000	-6.35968	0.00000	; dih C6- O1- C7-H17
	6	8	15	16	3	7.03749	0.00000	-7.03749	0.00000	0.00000	; dih C4- C5- O2-H20
	1	3	17	20	3	0.00000	0.00000	0.00000	0.00000	0.00000	; dih C1- C2- C8- N1
	3	17	20	22	3	-4.70700	2.92044	1.78656	0.00000	0.00000	; dih C2- C8- N1- C9
	17	20	22	23	3	30.28798	-4.81160	-25.47638	0.00000	0.00000	; dih C8- N1- C9-C10
	20	22	23	27	3	4.83252	-7.65254	1.68196	1.13805	0.00000	; dih N1- C9-C10-C11
	22	23	27	30	3	-4.96013	6.28646	1.30959	-2.63592	0.00000	; dih C9-C10-C11-C12
	23	27	30	33	3	2.92880	-1.46440	0.20920	-1.67360	0.00000	; dih C10-C11-C12-C13
	27	30	33	36	3	2.92880	-1.46440	0.20920	-1.67360	0.00000	; dih C11-C12-C13-C14
	30	33	36	38	3	0.52719	-6.39734	-1.69452	7.56467	0.00000	; dih C12-C13-C14-C15
	33	36	38	40	3	58.57600	0.00000	-58.57600	0.00000	0.00000	; dih C13-C14-C15-C16
	36	38	40	42	3	0.52719	-6.39734	-1.69452	7.56467	0.00000	; dih C14-C15-C16-C17
	38	40	42	43	3	0.76567	2.29701	0.00000	-3.06269	0.00000	; dih C15-C16-C17-H25
	38	40	46	47	3	0.76567	2.29701	0.00000	-3.06269	0.00000	; dih C15-C16-C18-H22

[dihedrals]

;	ai	aj	ak	al	funct	phi0	cp	mult				
	1	3	9	2	1	180.000	4.602	2	; imp	C1-	C2-	C6- H12
	3	1	4	17	1	180.000	4.602	2	; imp	C2-	C1-	C3- C8
	4	3	6	5	1	180.000	4.602	2	; imp	C3-	C2-	C4- H13
	6	4	8	7	1	180.000	4.602	2	; imp	C4-	C3-	C5- H14
	8	6	9	15	1	180.000	4.602	2	; imp	C5-	C4-	C6- O2
	9	1	8	10	1	180.000	4.602	2	; imp	C6-	C1-	C5- O1
	22	20	23	26	1	180.000	43.932	2	; imp	C9-	N1-	C10- O3
	36	33	38	37	1	180.000	62.760	2	; imp	C14-	C13-	C15- H3
	38	36	40	39	1	180.000	62.760	2	; imp	C15-	C14-	C16- H1

B

APPENDIX B: Figure permissions

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Figure 1.2 **Hodgkin and Huxley prove ion selectivity in squid axon in 1950s.**

Reference [103] B. Hille. Ionic basis of resting and action potentials. In J. Brokhart, V. Mountcastle, E. Kandel, and S. Geiger, editors, *Handbook of Physiology. The Nervous System I.*, pages 99-136. American Physiological Society, Washington, DC, 1977.

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Figure 1.3 **Bertil Hille's schematic of ion channel understanding circa 1977.**

Reference [102] B. Hille. Ion channels of nerve: questions for theoretical chemists. *BioSystems*, 8:195- 199, 1977.

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Figure 1.10 **The first voltage-sensitive TRP.** and Figure 1.11 **Cooperativity between voltage sensitivity and other activating stimuli in TRPs.**

Reference [197] B. Nilius, K. Talavera, G. Owsianik, J. Prenen, G. Droogmans, and T. Voets. Gating of TRP channels: a voltage connection? *J. Physiol.*, 567(1):35-44, 2005.

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Figure 1.17a **Insights from simulation toward VGL channel function.**

Reference [250] I. H. Shrivastava and M. S. Sansom. Simulations of ion permeation through a potassium channel: molecular dynamics of KcsA in a phospholipid bilayer. *Biophys. J.*, 78(2):557-70, Feb. 2000.

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Figure 1.17b **Insights from simulation toward VGL channel function.**

Reference [240] Z. A. Sands and M. S. P. Sansom. How does a voltage sensor interact with a lipid bilayer? Simulations of a potassium channel domain. *Structure*, 15(2):235-44, 2007.

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Figure 1.19a **MD predicts PIP₂ binding site.**

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Figure 1.19b **MD predicts PIP₂ binding site.**

Reference [95] S. B. Hansen, X. Tao, and R. MacKinnon. Structural basis of PIP₂ activation of the classical inward rectifier K⁺ channel Kir2.2. *Nature*, 477(7365):495-8, Sept. 2011.

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Figure 1.21b **The discovery of RTX.**

Reference [269] A. Szallasi and P. M. Blumberg. Specific binding of resiniferatoxin, an ultrapotent capsaicin analog, by dorsal root ganglion membranes. *Brain Res.*, 524:106-111, 1990.

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Figure 1.22 **The S1-S4 voltage-sensing domain.**

Reference [265] K. J. Swartz. Sensing voltage across lipid membranes. *Nature*, 456(7224):891-7, 2008.

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Figure 3.1 **EM structures of TRP channels.**

Reference [58] T. L. Cvetkov, K. W. Huynh, M. R. Cohen, and V. Y. Moiseenkova-Bell. Molecular architecture and subunit organization of TRPA1 channel revealed by electron microscopy. *J. Biol. Chem.*, 286(44):38168-38176, Sept. 2011.

And reference [248] H. Shigematsu, T. Sokabe, R. Danev, M. Tominaga, and K. Nagayama. A 3.5-nm structure of rat TRPV4 cation channel revealed by Zernike phase-contrast cryoelectron microscopy. *J. Biol. Chem.*, 285(15):11210-8, Apr. 2010.

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Reference [184] V. Y. Moiseenkova-bell and T. G. Wensel. Hot on the Trail of TRP Channel Structure. *J. Gen. Physiol.*, 133(3):239-244, 2009.

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Figure 3.2 **Progress in solving structures of membrane proteins.**

Reference [26] R. M. Bill, P. J. F. Henderson, S. Iwata, E. R. S. Kunji, H. Michel, R. Neutze, S. Newstead, B. Poolman, C. G. Tate, and H. Vogel. Overcoming barriers to membrane protein structure determination. *Nat. Biotechnol.*, 29(4):335-340, Apr. 2011.

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Figure 5.1 **The voltage-gated-like channel superfamily**

Reference [307] F. H. Yu and W. A. Catterall. The VGL-chanome: a protein superfamily specialized for electrical signaling and ionic homeostasis. *Science's STKE*, 2004(253):re15, Oct. 2004.

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