A Chemical Biology Approach to Understanding the Basis of Voltage-Gated Sodium Channel Modulation

FOTEINI TZAKONIATI

Imperial College London
Department of Chemistry

Thesis submitted in candidature for the degree of
Doctor of Philosophy

June 2018
Declaration of Originality

This Thesis is my own work and reports the results of my original research. Where information derives from the work of others or in collaboration with others, this is acknowledged in the text and references.

Foteini Tzakoniati, June 2018

Copyright Declaration

The copyright of this thesis rests with the author and is made available under a Creative Commons Attribution Non-Commercial No Derivatives licence. Researchers are free to copy, distribute or transmit the thesis on the condition that they attribute it, that they do not use it for commercial purposes and that they do not alter, transform or build upon it. For any reuse or redistribution, researchers must make clear to others the licence terms of this work.
Abstract

The voltage-gated sodium channel, Nav1.7, is involved in the propagation of pain signals from the peripheral nervous system. Genomic data from individuals with non-functional Nav1.7 expression strongly suggest it has potential to be the target of novel analgesics; loss of Nav1.7 function completely abolishes pain sensations in otherwise healthy phenotypes.

The focus of this thesis is the development of chemical tools to elucidate mechanisms of Nav1.7 modulation in the cell. The design, synthesis, characterisation and potency data of photocrosslinking probes that target two distinct Nav1.7 domains is reported. Domain II is targeted by photoprobes derived from the spider venom inhibitory cystine knot peptide Huwentoxin-IV. Moreover, a photoprobe based on the novel family of Nav1.7-selective aryl sulfonamide inhibitors targets domain IV of Nav1.7.

Determining the binding sites that lead to modulation of gating was firstly attempted in bacterial/hNav1.7 chimeric proteins that have been purified and used for crystallographic and biophysical studies. According to gel shift assays, certain photoprobes exhibited efficient photocrosslinking capabilities and were taken forward to proteomic mass spectrometry analysis in pursuit of photocrosslinking sites. Additionally, a series of approaches were explored in order to optimise the identification of Nav1.7 by proteomic mass spectrometry in an engineered cell line. Finally, the maturation of induced pluripotent stem cells from patients that carry a Nav1.7 mutation was followed by quantitative proteomics as an initial approach to understand Nav1.7-related mechanisms in a disease model.
Acknowledgements

I would like to thank my supervisor Ed Tate for his support throughout this journey. Thank you for trusting in my abilities to contribute to Science as one of your PhD students, all the inspiring conversations, your optimism and enthusiasm. I have learned an immense amount from you and I am grateful for your mentorship and guidance. I could not have asked for a better and more talented supervisor, thank you.

I have been very fortunate to have worked with great collaborators. Firstly, I would like to thank Ian Storer and James Bilsland for their supervision in the first years of my PhD. Thank you for your interest and support, for providing the reagents and iPSC cell lines needed for this project even after your departure from Pfizer. I really appreciate it.

My time with Christopher Koth, Jian Payandeh and Hui Xu at Genentech has been an invaluable experience. I learned so much about Structural Biology and ion channels through eye-opening and inspiring conversations with you. Thank you for welcoming me in the membrane protein team, the XFEL adventures and endless support. It has been an absolute pleasure working with you.

I would also like to thank my fellow Tate group members for the methods developed in our group before and after I arrived, the willingness to help, train and proofread! Thank you all for being there, you have all made the lab a pleasant place to work in. Firstly, thank you Julia, Kate, Chiara and Remi for collecting MS data for me. My proofreaders: Anna, Julia, Kate, Masha, Scott, Theo. Julia, thank you for answering all my MS questions, you have been amazing. Monica, thanks for sharing your molecular biology wisdom. Rhiannon, thank you for teaching me everything around peptide synthesis and Chris, for your help with collecting peptide NMR spectra. Kwang, Monica, Soo Mei, and Julia, you have been great friends.

On a personal note, I would like to thank Alex for being so supportive through the last four years. Thank you for all the pep talks, always reminding me to ‘write a list’ and believing in me even more than I believe in myself. This journey would have been more challenging and definitely less fun without you.

Lastly, I am grateful for my family’s support. Κωνσταντίνα and Γιώργο, thank you for being amazing siblings and for looking up to me, it has given me a lot of strength. Θείε Μιχάλη, you have been so proud of me, I wish you were still with us.
Μαμά και Μπαμπά, words cannot express my gratitude... Thank you for your endless and unconditional love, for supporting me in every way possible, for inspiring me to be bold and pursue anything I am passionate for. I would not have achieved anything without you, your sacrifices and your optimism. I love you.

I would like to dedicate my thesis to my grandfather Γεώργιος Τσαγκανός for his love for Science and Philosophy. I truly believe I would not have pursued a PhD if I had not grown up under your influence (και λύνοντας μαθηματικά τα καλοκαίρια). I admire your thirst for knowledge and your wisdom. Thank you for all the invaluable lessons you have taught me.
Work contributed by collaborators

1. Patch-clamp assays either by Elisabeth Payne or Tianbo Li, detailed in the text
2. LC-MS/MS data collection by Julia Morales Sanfrutos, Remi Serwa, Kate Hadavizadeh and Chiara Fabbro
3. HEK Nav1.7β1β2 cell line generated by Peter Cox
4. Generation and differentiation of iPSCs until day 11 by Pfizer Neusentis
5. Compound PF-06456384 was provided by Pfizer Neusentis
6. Design of chimeras and purification scheme by Hui Xu, Jian Payandeh and Christopher Koth
7. Huwentoxin-IV 1D and 2D NMR data collection by Christopher Douse
Presentations arising from this thesis

Oral Presentations


- F. Tzakoniati et al. ‘A Chemical Biology Approach to Understand Binding Partners of Sensory Sodium Channels’, Royal Society of Chemistry Protein & Peptide Science Group Early Stage Researcher Meeting, University of Durham, St Mary’s College, Durham, U.K., November 2015.


† Awarded best speaker prize based on content and delivery

Poster Presentations


# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>δ</td>
<td>Chemical shift</td>
</tr>
<tr>
<td>$A_{280}$</td>
<td>Absorbance at 280 nm</td>
</tr>
<tr>
<td>A</td>
<td>Alanine</td>
</tr>
<tr>
<td>Ab</td>
<td><em>Arcobacter butzleri</em></td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
</tr>
<tr>
<td>AMBIC</td>
<td>Ammonium bicarbonate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>C</td>
<td>Cysteine</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementaty deoxyribonucleic acid</td>
</tr>
<tr>
<td>Cryo-EM</td>
<td>Cryo electron microscopy</td>
</tr>
<tr>
<td>CID</td>
<td>Collision-induced dissociation</td>
</tr>
<tr>
<td>CIP</td>
<td>Congenital indifference to pain</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>D</td>
<td>Domain</td>
</tr>
<tr>
<td>D</td>
<td>Aspartic acid</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DFF</td>
<td>Double flow focus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>DIC</td>
<td>Dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>DIPEA</td>
<td>N,N-diisopropylethylamine</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethyl formamide</td>
</tr>
<tr>
<td>DMP</td>
<td>Dimethyl pimelimidate dihydrochloride</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribose nucleic acid</td>
</tr>
<tr>
<td>DPN</td>
<td>Diabetic peripheral neuropathy</td>
</tr>
<tr>
<td>DRG</td>
<td>Dorsal root ganglion</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>E</td>
<td>Glutamic acid</td>
</tr>
<tr>
<td>EC$_{50}$</td>
<td>Half maximal effective concentration</td>
</tr>
<tr>
<td>EDTA</td>
<td>2,2',2'',2'''-(Ethane-1,2-diyldinitrilo)tetraacetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ee</td>
<td>Electric eel</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionisation</td>
</tr>
<tr>
<td>F</td>
<td>Phenylalanine</td>
</tr>
<tr>
<td>FA</td>
<td>Formic acid</td>
</tr>
<tr>
<td>FA3</td>
<td>Facade-EM</td>
</tr>
<tr>
<td>FASP</td>
<td>Filter-aided sample preparation</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FDR</td>
<td>False discovery rate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>Fmoc</td>
<td>Fluorenylmethyloxycarbonyl</td>
</tr>
<tr>
<td>G</td>
<td>Glycine</td>
</tr>
<tr>
<td>GABA&lt;sub&gt;A&lt;/sub&gt;</td>
<td>γ-aminobutyric acid type A</td>
</tr>
<tr>
<td>GDN</td>
<td>glyco-diosgenin</td>
</tr>
<tr>
<td>GDNF</td>
<td>Glial cell-derived neurotrophic factor</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione reduced</td>
</tr>
<tr>
<td>GSSG</td>
<td>Glutathione oxidised</td>
</tr>
<tr>
<td>H</td>
<td>Histidine</td>
</tr>
<tr>
<td>HATU</td>
<td>1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate</td>
</tr>
<tr>
<td>HBTU</td>
<td>2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate</td>
</tr>
<tr>
<td>HCD</td>
<td>Higher energy collision dissociation</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>High pressure liquid chromatography</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HwTx-IV</td>
<td>Huwentoxin-IV</td>
</tr>
<tr>
<td>I</td>
<td>Isoleucine</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Half-maximal inhibitory concentration</td>
</tr>
<tr>
<td>ICK</td>
<td>Inhibitory Cystine Knot</td>
</tr>
<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
</tr>
</tbody>
</table>
iPSCs  Induced pluripotent stem cells
IEM  Inherited erythromelalgia
K  Lysine
Kₜ  Equilibrium dissociation constant
L  Leucine
LC-MS  Liquid chromatography-mass spectrometry
LC-MS/MS  Liquid chromatography-tandem mass spectrometry
M  Methionine
MALDI  Matrix assisted laser desorption ionisation
MAPK  Mitogen activated protein kinase
MeCN  Acetonitrile
MeOH  Methanol
mRNA  Messenger ribonucleic acids
Ms  *Magnetococcus marinus*
MS  Mass spectrometry
MWCO  Molecular weight cut-off
N  Asparagine
nanoDSF  Nano-differential scanning calorimetry
Nav  Voltage-gated sodium channel
NGF  Nerve growth factor
NHS  N-hydroxysuccinimide
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMM</td>
<td>N-methyl morpholine</td>
</tr>
<tr>
<td>NMP</td>
<td>N-methyl pyrrolidone</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>NOESY</td>
<td>Nuclear Overhauser effect spectroscopy</td>
</tr>
<tr>
<td>NT-3</td>
<td>Neutrophin-3</td>
</tr>
<tr>
<td>P</td>
<td>Proline</td>
</tr>
<tr>
<td>PaS</td>
<td><em>Periplaneta americana</em></td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Pbf</td>
<td>2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PEPD</td>
<td>Paroxysmal extreme pain disorder</td>
</tr>
<tr>
<td>PF-384</td>
<td>PF-06456384</td>
</tr>
<tr>
<td>pI</td>
<td>Isoelectric point</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein Kinase A</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein Kinase B</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td>POPC</td>
<td>phosphatidylcholine</td>
</tr>
<tr>
<td>POPE</td>
<td>phosphatidylethanolamine</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>POPG</td>
<td>phosphatidylglycerol</td>
</tr>
<tr>
<td>ProTx-II</td>
<td>Protoxin-II</td>
</tr>
<tr>
<td>PTM</td>
<td>Post-translational modification</td>
</tr>
<tr>
<td>Q</td>
<td>Glutamine</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative Reverse Transcription Polymerase Chain Reaction</td>
</tr>
<tr>
<td>R</td>
<td>Arginine</td>
</tr>
<tr>
<td>RP</td>
<td>Reverse phase</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>Reverse phase high-performance liquid chromatography</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>S</td>
<td>Segment</td>
</tr>
<tr>
<td>S</td>
<td>Serine</td>
</tr>
<tr>
<td>SAR</td>
<td>Structure-activity relationship</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEC</td>
<td>Size-Exclusion Chromatography</td>
</tr>
<tr>
<td>S.E.M.</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SFN</td>
<td>Small fibre neuropathy</td>
</tr>
<tr>
<td>SLB</td>
<td>Sample loading buffer</td>
</tr>
<tr>
<td>SPE</td>
<td>Solid phase extraction</td>
</tr>
<tr>
<td>SPPS</td>
<td>Solid phase peptide synthesis</td>
</tr>
</tbody>
</table>
SPR  Surface Plasmon Resonance
Str  Streptavidin
SUMO  Small ubiquitin-like modified
T  Theanine
TAP  Tandem affinity purification
TB  Trypan blue
TBME  tert-butyl methyl ether
TBS  Tris-buffered saline
tBu  tert-butyl
TCEP  Tris(2-carboxyethyl)phosphine
TEAB  Triethylammonium bicarbonate
TFA  Trifluoro-acetic acid
TIPS  Trimethylsilylisopropane
Tm  Melting temperature
TMT  Tandem mass tag
Tni  *Trichoplusia* ni
TOCSY  Total Correlation Spectroscopy
Tris  Tris(hydroxymethyl)aminomethane
TTX  Tetrodotoxin
UPLC  Ultra-performance liquid chromatography
UV  Ultraviolet
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{1/2}$</td>
<td>Voltage of half-maximal activation</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular cell adhesion molecule</td>
</tr>
<tr>
<td>VGSC</td>
<td>Voltage-gated sodium channel</td>
</tr>
<tr>
<td>VSD</td>
<td>Voltage sensor domain</td>
</tr>
<tr>
<td>W</td>
<td>Tryptophan</td>
</tr>
<tr>
<td>XFEL</td>
<td>X-ray free electron laser</td>
</tr>
<tr>
<td>Y</td>
<td>Tyrosine</td>
</tr>
</tbody>
</table>
Contents

Declarations ......................................................... 2
Abstract ..................................................................... 3
Acknowledgements .................................................... 4
Presentations ............................................................. 7
Abbreviations ............................................................. 9

1 Introduction .......................................................... 22

1.1 Voltage-gated Sodium Channels .................................... 22
  1.1.1 Structure and Function ........................................... 22
  1.1.2 Modulation of VGSCs ............................................. 27

1.2 Nav1.7, a Voltage-Gated Sodium Channel ......................... 28
  1.2.1 Channelopathies and Involvement in Chronic Pain .......... 28
  1.2.2 Binders and Binding Sites ........................................ 31
    1.2.2.1 VGSCs: Mechanisms of Inhibition and Block ............ 31
    1.2.2.2 VGSCs: Binding Sites ....................................... 32
    1.2.2.3 Binders of Nav1.7 ........................................... 33
    1.2.2.4 Status of Inhibitors in Clinical Trials .................... 34
    1.2.2.5 Structural Studies of Binding Interactions ............... 36
  1.2.3 Inhibitory Cystine Knot Peptides that Target Nav1.7 ......... 37
    1.2.3.1 Huwentoxin-IV ............................................ 38
    1.2.3.2 Protoxin-II .................................................. 40
  1.2.4 Nav1.7 as a Novel Analgesic Target .......................... 41
  1.3 Chemical Biology Approaches ..................................... 43
CONTENTS

1.3.1 Photoaffinity Labeling .................................. 44
1.3.2 Mass Spectrometry Proteomics ............................. 46
1.4 Research Objectives ........................................ 49

2 Chemical Tools to Study Nav1.7 50

2.1 Photoaffinity Probes Based on Huwentoxin-IV ................. 50
  2.1.1 Introduction: Synthesis of ICK Peptides ..................... 50
  2.1.2 Photoaffinity Probe Design ............................... 52
  2.1.3 Solid Phase Synthesis and Oxidative Refolding ............... 54
  2.1.4 LC-MS Characterisation .................................. 55
  2.1.5 NMR Characterisation .................................. 56
  2.1.6 Patch-clamp Characterisation .............................. 60

2.2 Photoaffinity Probes Based on PF-06456384 ..................... 62
  2.2.1 Photoaffinity Probe Design and Synthesis .................... 62
  2.2.2 Patch-clamp Characterisation .............................. 64

2.3 Conclusions ............................................ 65

3 Mass Spectrometry Proteomics of Nav1.7 67

3.1 Challenges in Studying Membrane Proteins ...................... 67
3.2 Detection of Nav1.7 in an Engineered Cell Line ................. 69
  3.2.1 Total Lysate Proteomics .................................. 69
  3.2.2 Subcellular Fractionation Proteomics ....................... 70
  3.2.3 Immunoprecipitation ................................... 73
  3.2.4 Cell Surface Biotinylation ................................ 77
  3.2.5 Optimisation of Sample Preparation ......................... 79
  3.2.6 Conclusion ........................................ 82

3.3 Proteomics of iPSC-derived Neurons ............................ 84
  3.3.1 iPSCs in Nav1.7 Drug Discovery .......................... 84
  3.3.2 Neuronal Maturation ................................... 86
  3.3.3 Conclusion ........................................ 91
6.1.2.2 (S)-2-(((9H-fluoren-9-yl)methoxy)methyl)amino)-4-(3-methyl-3H-diazirin-3-yl)butanoic acid, Fmoc-L-photomethionine-OH ................. 122
6.1.2.3 (S)-3-(3-methyl-3H-diazirin-3-yl)-2-(5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)propanoic acid (4) ........ 123
6.1.2.4 Photoprobe PFDB (5) .................................... 124

6.2 Tissue Culture and Biochemical Methods ........................................ 125
6.2.1 General Methods ........................................................................ 125
6.2.2 Mammalian Cell Culture .......................................................... 126
6.2.3 Neuronal Maturation ................................................................. 126
6.2.4 qRT-PCR Analysis of Neuronal Maturation .................................. 127
6.2.5 Chimeric Expression in Insect Cells .......................................... 128
6.2.6 Nav1.7 Chimera Sequences ...................................................... 128
6.2.7 Purification of VSD Chimeras .................................................... 129
6.2.8 Co-crystallisation of VSD2 and Toxin Peptides ............................ 130
6.2.9 Differential Scanning Fluorimetry .............................................. 130

6.3 Patch-Clamp Electrophysiology ..................................................... 131
6.4 Chemical Biology and Proteomics ............................................... 131
6.4.1 Photocrosslinking of Purified Chimeras ..................................... 131
6.4.2 Streptavidin Shift Assay ............................................................. 132
6.4.3 Mammalian Cell Line Proteomics ............................................. 132
6.4.3.1 Protein Concentration Determination .................................. 132
6.4.3.2 Subcellular Fractionation ..................................................... 132
6.4.3.3 Surface Biotinylation .......................................................... 132
6.4.3.4 Immunoprecipitation ........................................................ 133
6.4.3.5 Detergent Screening for Nav1.7 Extraction .......................... 134
6.4.4 Filter-Aided Sample Preparation (FASP) ................................. 134
6.4.5 Biotinylated Peptide Pulldown ................................................ 135
6.4.6 Isobaric Mass Tagging in FASP (iFASP) .................................. 135
6.4.7 Proteomic Analysis ................................................................. 136
6.4.7.1 Sample Reconstitution ..................................................... 136
Chapter 1

Introduction

1.1 Voltage-gated Sodium Channels

1.1.1 Structure and Function

Voltage Gated Sodium Channels (VGSCs) are protein-complexes that allow the flux of Na\(^+\) ions in the cell in response to changes in the membrane potential.\(^1\) They are responsible for the initiation and propagation of action potentials in excitable cells including neuronal and muscle cells. VGSCs consist of an α subunit and β auxiliary subunits. The α subunit is functional without complexation with β subunits and consists of four homologous domains (DI to DIV).\(^1\) Each domain contains six α-helical transmembrane segments (S1 to S6) which are connected to each other through intracellular and extracellular linker loops, as shown in Figure 1.1. Segments S5 and S6 form the pore of the channel and segments S1-S4 within each domain form the voltage sensor domain (VSD).\(^1\) VGSCs can be found as either hetero-dimers or hetero-trimers, with the α subunit coupled with one or two β subunits respectively.\(^2\) The β subunits have a fold that resembles an immunoglobulin domain and a transmembrane helix and can bind to the α subunit either non-covalently or through a disulfide bond.\(^3\) There are four subtypes of Navβ subunits, β\(_1\) to β\(_4\), of which β\(_2\) and β\(_4\) are disulfide linked to the α subunit. The β subunits affect the localisation of VGSCs and play a role in modulation of their gating properties as will be discussed in this chapter. The nine mammalian isoforms of VGSCs (Nav1.1 to Nav1.9) share high sequence homology (over 50%) in the transmembrane and extracellular domains but are expressed in different tissues (Table 1.1). The gating properties of the nine isoforms vary, as does their sensitivity to a neurotoxin VGSC blocker, tetrodotoxin (TTX).\(^4\)
CHAPTER 1. INTRODUCTION

Figure 1.1: Representation of the domains of a VGSC. Three intracellular loops connect the VSD and the pore forming module PM (in green). The intracellular inactivation gate moves to physically block the pore during channel inactivation. Modulation of the channel is thought to be achieved through phosphorylation of serine residues 573, 610, 623, and 687 on the first intracellular loop (red circles).²,⁵

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Gene</th>
<th>Chromosome</th>
<th>Protein MW (kDa)</th>
<th>Tissue expression</th>
<th>TTX sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nav1.1</td>
<td>SCN1A</td>
<td>2q24</td>
<td>229</td>
<td>CNS neurons</td>
<td>yes</td>
</tr>
<tr>
<td>Nav1.2</td>
<td>SCN2A</td>
<td>2q23–24</td>
<td>228</td>
<td>CNS neurons</td>
<td>yes</td>
</tr>
<tr>
<td>Nav1.3</td>
<td>SCN3A</td>
<td>2q24</td>
<td>227</td>
<td>CNS neurons</td>
<td>yes</td>
</tr>
<tr>
<td>Nav1.4</td>
<td>SCN4A</td>
<td>17q23–25</td>
<td>208</td>
<td>skeletal muscle</td>
<td>yes</td>
</tr>
<tr>
<td>Nav1.5</td>
<td>SCN5A</td>
<td>3p21</td>
<td>227</td>
<td>heart</td>
<td>no</td>
</tr>
<tr>
<td>Nav1.6</td>
<td>SCN8A</td>
<td>12q13</td>
<td>225</td>
<td>CNS neurons</td>
<td>yes</td>
</tr>
<tr>
<td>Nav1.7</td>
<td>SCN9A</td>
<td>2q24</td>
<td>226</td>
<td>DRG, PNS neurons</td>
<td>yes</td>
</tr>
<tr>
<td>Nav1.8</td>
<td>SCN10A</td>
<td>3p22–24</td>
<td>220</td>
<td>DRG neurons</td>
<td>no</td>
</tr>
<tr>
<td>Nav1.9</td>
<td>SCN11A</td>
<td>3p21–24</td>
<td>205</td>
<td>DRG neurons</td>
<td>no</td>
</tr>
<tr>
<td>Navβ₁</td>
<td>SCN1B</td>
<td>19q13.11</td>
<td>25</td>
<td>CNS, heart, skeletal muscle, PNS</td>
<td>n/a</td>
</tr>
<tr>
<td>Navβ₂</td>
<td>SCN2B</td>
<td>11q23.3</td>
<td>24</td>
<td>CNS, heart, PNS</td>
<td>n/a</td>
</tr>
<tr>
<td>Navβ₃</td>
<td>SCN3B</td>
<td>11q24.1</td>
<td>25</td>
<td>CNS, heart, PNS</td>
<td>n/a</td>
</tr>
<tr>
<td>Navβ₄</td>
<td>SCN4B</td>
<td>11q23.3</td>
<td>25</td>
<td>CNS, heart, skeletal muscle, PNS</td>
<td>n/a</td>
</tr>
</tbody>
</table>

Table 1.1: The human VGSC α and β subunits.²,³,⁴
CHAPTER 1. INTRODUCTION

The structure of VGSCs can be described with the aid of the crystal structure of *Arcobacter butzleri* VGSC (NavAb). This was solved by Payandeh *et al.* and has since formed the basis of the hypotheses of the structure and gating of mammalian VGSCs.\(^6\) It was observed that the pore module of the channel is formed by S5 and S6 of each homologous domain and the VSD of one domain interacts closely with the pore forming segments of the adjacent domain (Figure 1.2). The ring that is on the outer side of the pore, formed by four negatively charged residues on the pore-helices, has been shown to play a role in the selectivity of ion conduction (Figure 1.2). This ring is called the ‘selectivity filter’ and is highly conserved among VGSCs, however, the four residues vary in channels that are not homotetrameric like NavAb.\(^2,6\)

Moving further into the pore, there is the ‘central cavity’ and at the intracellular end of the pore, there is the ‘activation gate’ which determines the opening and closing of the channel.\(^6,7\)

The function of VGSCs can be described by voltage-dependent activation and fast inactivation. During these events, the VGSCs can be in different states; the activated, inactivated and resting states (Figure 1.3).\(^6\) Voltage-dependent activation is explained using the ‘helical screw model of gating’,\(^1\) and it involves the S4 transmembrane segments. S4 is composed of motifs of a positively charged arginine residue (R1 to R4) followed by two hydrophobic residues. Stabilisation of this structure is achieved by interaction of the

**Figure 1.2:** Architecture of NavAb. **A**, Overall structure, a single domain is highlighted. The pore-helices are denoted as P and P2.\(^6\) **B**, Top view, the pore is located in the centre of the structure. **C**, Side view of the NavAb pore.\(^6\) The selectivity filter consists of one glutamate residue (purple sticks) from every domain. Figures were adapted, with permission, from Payandeh *et al.*\(^6\)
Figure 1.3: Simplified schematic of the function of a VGSC. The pore of the VGSC, as depicted, is closed in the resting state and intracellularly blocked by the inactivation gate upon fast inactivation.

positively charged residues with negatively charged residues in the neighbouring segments S1, S2, S3.\textsuperscript{9,10} At resting conditions, the membrane potential is approximately -80 mV with respect to the extracellular environment and the channel is in a closed conformation.\textsuperscript{9,10} The 'selectivity filter' motif of eukaryotic VGSCs consists of aspartate, glutamate, lysine and alanine (DEKA). Upon depolarisation of the membrane which is essentially a positive increase in the potential, the S4 helix 'slides' in an outward direction, resulting in a conformational change that allows the pore of the channel to open and the influx of Na\textsuperscript{+} to be activated.\textsuperscript{1} Stabilisation of this conformation is achieved again through neighbouring interactions. Fast inactivation of the channel happens through movement of the inactivation gate to block the intracellular side of the pore.\textsuperscript{2} This process happens within milliseconds and is referred to as the 'hinged-lid mechanism'. The inactivation gate is the loop linker between DIIIS6 and DIVS1 and it contains the important hydrophobic triad IFM (isoleucine, phenylalanine and methionine).\textsuperscript{1} Upon hyperpolarisation of the membrane, the inactivated channel transitions back to the resting closed state.\textsuperscript{10} The activation and inactivation of Na\textsuperscript{+} currents is important for initiation of action potentials in neurons and other excitable cell types. It is vital to note that there is more than one hypothesis on the mechanisms of activation and inactivation of VGSCs.\textsuperscript{2,8} Some structural data show the VSDs in different activation states,\textsuperscript{11} suggesting that activation might not be symmetrical throughout the tetramer. Other studies suggest that the activation of VSD4 is sufficient for the movement of the inactivation gate to inactivate the channel.\textsuperscript{8} Since the inactivation lid is not present in bacterial VGSCs, interrogation of these mechanisms is very challenging and this highlights the need for structural information on mammalian VGSCs.
The closest relative of the mammalian VGSC that has been used in structural studies is NavPaS from the American cockroach *Periplaneta americana*. Similar to the mammalian VGSCs, NavPaS is a single chain polypeptide that forms four homologous domains, with the DEKA selectivity filter motif, however, it lacks the IFM inactivation gate motif which is required for 'hinged-lid' fast inactivation. Cryo electron microscopy (Cryo-EM) was employed to elucidate an informative structure of full length NavPaS; the VSDs were at different activation states, interactions of S4-S5, S5 and S6 within and among domains were revealed and some extracellular loops of the channel appeared to be heavily glycosylated. Structural studies aiming to understand the elusive mechanisms of activation, inactivation, gating and ion selectivity are summarised in a review by Clairefeuille *et al.*

![Figure 1.4: Cryo-EM structure of eeNav1.4 in complex with β1 (PDB 3XSX).](image)

A. Surface representation of the α subunit, the residues on the extracellular loops of DI, DIII and DIV that participate in interactions with β1 (yellow) are highlighted. B. The transmembrane helix of β1 interacts with the transmembrane helices of S0 and S2 of DIII. Domains I, II, III, and IV are represented by the colours grey, dark yellow, green, and cyan, respectively.

Efforts to crystallise the extracellular part of β3 and β4 have been successful and have thus revealed the immunoglobulin-like fold of these subunits. In the case of β4, the cysteine residue that forms a disulfide bond with the α subunit of VGSCs was identified to be at position 58, conserved as Cys26 found in subunit β2. Interestingly, it was found that β4 binding via the disulfide bridge with Nav1.2 negatively affected the potency of ProToxin-II (ProTx-II) against Nav1.2. A detailed summary of toxin peptides as inhibitors and modulators of VGSCs is described in Section 1.2.3. Crystals of β3 contained three β3 molecules in the asymmetric unit, and further analysis by analytical ultracentrifugation and atomic force microscopy (AFM) in engineered cells expressing β3 further supports the hypothesis that β3 is able to form dimeric
and trimeric structures. This property of \( \beta_3 \) suggests that the \( \alpha \) subunits may form larger clusters in the cell membrane through interactions with \( \beta_3 \) subunits. Crystal structures of \( \beta_1 \) and \( \beta_2 \) have not yet been published. However, cryo-EM was employed to study the interaction of \( \beta_1 \) with electric eel eeNav1.4 (Figure 1.4). This structure demonstrated the numerous interactions between \( \beta_1 \) and the \( \alpha \) subunit of eeNav1.4 that span from the transmembrane helix of DIIIS2 to the extracellular loops of domain I, III and IV. Similarly to the structure of NavPaS, eeNav1.4 exhibits different conformations among the voltage sensors and interestingly, the inactivation gate is positioned away from the pore.

1.1.2 Modulation of VGSCs

Modulation of VGSCs is achieved through complexation with other proteins in the cell as well as post-translational modifications. Firstly, the trafficking and glycosylation of the \( \alpha \) subunit to the cell membrane is affected by co-expression with \( \beta \) subunits. Moreover, interaction with \( \beta \) subunits on the plasma membrane can affect the kinetics of activation and inactivation. \( \beta \) subunits do not only play a role in modulation of kinetics of VGSCs but also in their distribution in the neuronal cell; \( \beta \) subunits along with \( \alpha \) subunits form complexes with other molecules such as ankyrin G. The biophysical properties of VGSCs are affected by post translational modifications (PTMs). Briefly, phosphorylation is one of the major PTMs that alter the voltage dependence of these channels. Protein Kinase A (PKA) and Protein Kinase C (PKC) have been found to phosphorylate VGSCs as a result of the signaling cascade initiated by G protein coupled receptor (GPCR) activation. Protein lipidation has also been observed, with the example of palmitoylation of rNav1.2 and deacetylation of hNav1.5 by Sirt1 as reported in the literature. It is evident that the gating function of VGSCs is altered by various processes in the cell and it is important to note that the effects of certain PTMs or complexation with \( \beta \) subunits are not ubiquitous to all neuronal cells and to all subtypes of VGSCs. Finally, a recent study shed light on the function of the \( \alpha \) subunits of Nav1.5 in the cell, suggesting that they form functional dimers mediated by 14-3-3 proteins through their Loop 1 intracellular loops (Figure 1.1). This conclusion challenges the hypothesis that VGSCs are fully functional as monomers and stresses the complexity of their gating and localisation characteristics.
1.2 Nav1.7, a Voltage-Gated Sodium Channel

1.2.1 Channelopathies and Involvement in Chronic Pain

Nociceptive neurons sense noxious stimuli or damage in the peripheral nervous system (PNS). Propagation of pain signals originating in the periphery is achieved through depolarisation of the membrane of nociceptive Dorsal Root Ganglia (DRG) neurons. This process is a result of different pathways that, for example, involve G-protein coupled receptors, acid sensing- and ligand-gated ion channels. This membrane depolarisation is sensed by the VGSCs present in DRG which respond when the depolarisation reaches a certain threshold through activation coupled with fast inactivation. This response of VGSCs results in the initiation and propagation of action potentials (Figure 1.5). Nav1.7 is primarily expressed in small and large diameter DRG whereas Nav1.8 is preferentially found in small diameter DRG. These two VGSCs are therefore believed to play an important role in peripheral neuropathies and inflammatory pain.

Recently, the expression of Nav1.7 in hypothalamic neurons in the central nervous system (CNS) of mice has been reported, revealing the potential implication of Nav1.7 in weight regulation.

Certain inherited pain disorders involve mutations in SCN9A, the gene which encodes the α subunit of Nav1.7. Gain-of-function mutations in SCN9A result in painful conditions that are characterised by neuronal hyper-excitability. Neuronal hyperexcitability is due to either impairment of fast and slow inactivation of the channel, whereby the channel remains open for longer, or a hyperpolarising shift in the voltage of activation, which result in the channel activating more easily and frequently.

Inherited erythromelalgia (IEM) and paroxysmal extreme pain disorder (PEPD) are two disorders that result from gain-of-function mutations. IEM is usually caused by point mutations that result in lower threshold of activation of Nav1.7 and an increased ramp current in comparison with the wild type Nav1.7. A ramp current is the result of small and slow depolarisation of the cell membrane. On the other hand, PEPD-related mutations result in impaired fast inactivation; the channel remains open for longer causing a 'persistent current' during electrophysiological measurements. Additional gain-of-function mutations in SCN9A have been reported in patients with small fibre neuropathy (SFN), which is more prevalent than IEM and PEPD, and diabetic peripheral neuropathy (DPN). In general, DRG neurons that express gain-of-function mutants of Nav1.7 exhibit higher frequency of action potentials firing.

Interestingly, the only symptoms of patients suffering from congenital indifference to pain (CIP) are the inability to sense pain and anosmia; they are otherwise healthy. This disorder is described by loss-
Figure 1.5: Schematic of pain signaling in DRG neurons. The pain stimulus is transduced by channels and receptors into membrane depolarisation, for example, by acid-sensing ion channels (ASICs), G protein coupled receptors (GPCRs), P2X receptors, and transient receptor potential (TRP) channels. The depolarisation above a certain threshold causes activation of Nav1.7 and Nav1.8 which in turn amplify and propagate the signal through action potential generation along the nerve to the spinal cord. Figure adapted, with permission, from King and Vetter.26

of-function mutations in SCN9A resulting in functionally inactive Nav1.7. This extraordinary finding is the most compelling genetic evidence that Nav1.7 is directly involved in pain signaling and thus poses a very attractive target for novel analgesics that, if specific enough, would not exhibit the unfavourable side effects of current treatments that target the central nervous system. A more detailed discussion on the potential of Nav1.7 to be an excellent target for the new generation of analgesics is found in Subsection 1.2.4.

Chronic pain, whether it is a result of inflammation (inflammatory), injury or disease (neuropathic), involves dysregulated expression of VGSCs.27 The involvement of Nav1.7 in these phenotypes has been demonstrated in animal models of inflammatory and neuropathic pain through transcriptional and electrophysiological analysis; briefly, Nav1.7 levels are increased in inflammatory pain models and when it is knocked-down in mouse DRG neurons, the pain is ameliorated.27,30

As described in Subsection 1.1.2, post-translational modifications (PTMs) and protein-protein interacti-
CHAPTER 1. INTRODUCTION

ions of the α subunit contribute to its functional characteristics and trafficking in the cell and by extension, to neuronal firing. Firstly, the interaction of the α subunit with β subunits should be considered. Co-expression of α and β1 and β3 subunits resulted in different glycosylated forms of the α subunit but also in changes in the total Na+ current and the cell surface localisation of Nav1.7. More specifically, Laederman et al. describe the following model; β1 and β3 interact with Nav1.7 early in its synthesis in the endoplasmatic reticulum (ER) and Golgi, where they regulate its glycosylation and then escort it to the plasma membrane. On the other hand, it is hypothesised that β2 and β4 associate with Nav1.7 in much later stages, resulting in decreased glycosylation and translocation to the plasma membrane. Farmer et al. observed the effect of the β1 subunit on different splice variants of Nav1.7; the voltage of activation and inactivation was altered by different degrees among the variants. Once the channel localises into the plasma membrane, its endocytosis and degradation is regulated by ubiquitination. Upon ubiquitination by E3 ubiquitin ligase NEDD4-2 in mouse DRG neurons, the cell surface levels of Nav1.7 decrease and so does the excitability of the neurons. Interestingly, NEDD4-2 was found to be downregulated in a neuropathic pain model in mice which would suggest its involvement in neuronal hyperexcitability. Trafficking of Nav1.7 is also modulated by collapsin response mediator protein 2 (CRMP2); Dustrude et al. proposed a mechanism of endocytosis of Nav1.7. Briefly, small ubiquitin-like modified (SUMOylated) CRMP2 is required to maintain Nav1.7 surface levels, and non SUMOylated CRMP2 is responsible for recruiting NEDD4-2 that ubiquitinates Nav1.7 and initiates its endocytosis.

Upon triggering of inflammatory pathways, certain kinases are activated in peripheral neurons. Mitogen activated protein kinases (MAPK) pERK1/2 were shown to phosphorylate threonine 531 (T531), serine 535 (S535), S608 and S712 residues on the first intracellular loop of Nav1.7 leading to a hyperpolarising shift of the activation threshold (Figure 1.1). It was hypothesised that modulation of gating is due to structural changes occuring due to phosphorylation and not due to new binding interactions between the phosphorylated site and other proteins. It is important to mention that activation of MAPK occurs in response to other signalling events and might be associated with the hyperexcitability of diseased neurons. Studies on the effect of protein kinase A (PKA) on Nav1.7 revealed that PKA induces a decrease in the amplitude of Na+ current, but does not alter the other gating properties of Nav1.7. Protein kinase C (PKC) caused the same effect and is generally thought to phosphorylate residues on the inactivation gate of various VGSCs. Moreover, analysis of the four splice variants of Nav1.7 that are found in neurons, showed that certain variants can be phosphorylated by PKA which causes a depolarising shift in
the threshold of activation. These protein kinases do not have the same effects on the different subtypes of VGSCs and rationalisation of the basis of these effects is challenging as structural information is very limited and isolation of each individual subtype in physiologically relevant conditions is difficult. Most of the aforementioned studies are done in engineered cell lines that overexpress Nav1.7 which might not represent a good model for the neuronal cell environment. A detailed review of PTMs of Nav1.7 that are important in chronic pain conditions has been published.

Finally, Nav1.7 regulation was also observed in heat nociception; fibroblast growth factor 13 (FGF13) was found to interact with residues on the C-terminus and to stabilise it in the plasma membrane in a heat-dependent manner. This stabilisation resulted in increased levels of Nav1.7 on the cell surface and increased Na$^+$ current. Other FGFs, like FGF12 and FGF14 have also been implicated in regulation of other VGSCs.

1.2.2 Binders and Binding Sites

1.2.2.1 VGSCs: Mechanisms of Inhibition and Block

Binders of VGSCs can be classified as inhibitors or blockers depending on their effect on the activity of the channel. Blockers physically obstruct the pore and do not allow the influx of Na$^+$. Inhibitors, on the other hand, modulate the function of the channel by interacting with, for example, the voltage-sensor domain and thus altering the threshold of activation. An important contributor to the binding affinity of the binder is the state of the channel. As discussed above, there are three different states: the resting, the activated, and the fast inactivated states. The transition between these states involves conformational changes (for example, the ‘helical screw’ and ‘hinged lid’ mechanisms) and brings additional complexity to the rationalisation of the binding interactions. In order to gain insight into whether the binding affinity varies with the state of the channel, electrophysiological experiments are performed.

‘Whole cell patch clamp electrophysiology’ is employed to measure the binding affinity and its dependence on the membrane potential. By altering the applied potential, the response of the channel is induced and the effect of the binder can be quantified by measuring the Na$^+$ current. IC$\text{50}$ values are then determined by plotting the current against the concentration of the inhibitor. At a resting potential of -80 mV, the majority of the channels present in the cell are resting. By inducing pulses that depolarise the membrane (increasing the voltage to -20 mV, for example), the channels open and allow influx of Na$^+$ current. When
the membrane is hyperpolarised again, the ‘recovery time’ required to return to the resting state from the
inactivated state can be monitored.\(^9\)

Certain inhibitors exert a ‘state-dependent’ effect on the channel; they preferentially bind and stabili-
se the inactivated state.\(^9\) This property is useful for inhibitors that are developed for clinical use, as they
show selectivity for VGSCs that are frequently inactivated, usually present in damaged neurons that gen-
erate high frequency action potentials.\(^9\) ‘Frequency or use dependence’ of an inhibitor is observed when
inhibition of Na\(^+\) current increases as more depolarising pulses are imposed at the right frequency. The
inhibitor slowly dissociates as the channel transitions to the resting state. If the pulse frequency is higher
than the rate of dissociation, then use-dependent inhibition is observed.\(^9\) Finally, certain binders prefe-
rentially interact with the ‘slow inactivated’ state of VGSCs. This conformation is acquired after longer
depolarisations of the membrane and is thought to involve structural changes in the pore.\(^{47}\)

### 1.2.2.2 VGSCs: Binding Sites

As discussed in previous sections, VGSCs play a major role in the nervous system as they contribute to
action potential initiation. It is therefore not surprising that they are targeted by toxins found in animal
venom. These toxins proved very useful in enabling the study of VGSCs. Several binding sites of known
binders of VGSCs have been identified through mutagenesis and electrophysiological experiments using
toxin peptides.\(^4,9,48\) Overall, toxins that target binding sites 1, 2, and 5 interact with the pore, unlike the
toxins that interact with the voltage sensors in binding sites 3, 4 and 6. The receptor sites are summarised
below:

**Binding site 1** This site is at the extracellular side of the pore, composed of the S5-S6 linker loops in
all four homologous domains. The main binders that have been identified are tetrodotoxin (TTX), saxitoxin
from the spider venom family and \(\mu\)-conotoxins from cone snails. These molecules are pore-blockers as
they physically obstruct the influx of Na\(^+\) current. TTX is a potent inhibitor of most VGSCs, with IC\(_{50}\)
values of a few nM.\(^1\) Nav1.1-1.4, 1.6 and 1.7 are therefore called TTX-sensitive channels and Na\(^+\) currents
associated with these are termed TTX-S currents. On the other hand, Nav1.5, 1.8 and 1.9 are insensitive to
\(\mu\)M concentrations of TTX and are therefore classified as TTX-resistant (TTX-R) VGSCs.\(^1\)

**Binding site 2** Toxins such as batrachotoxin and veratridine bind to S6 of DI and DIV and affect the
function of the channel by slowing the fast inactivation. This interaction leads to prolonged activation.\(^9,48\)

**Binding site 3** This site, rich in acidic amino acids that are deprotonated at physiological pH, entails the

<table>
<thead>
<tr>
<th>Binding site</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site 1</td>
<td>Extracellular side of the pore, composed of S5-S6 linker loops in all four homologous domains. Main binders: TTX, saxitoxin, (\mu)-conotoxins. TTX is a potent inhibitor of most VGSCs.</td>
</tr>
<tr>
<td>Site 2</td>
<td>Toxins: batrachotoxin, veratridine, bind to S6 of DI and DIV, slowing the fast inactivation.</td>
</tr>
<tr>
<td>Site 3</td>
<td>Rich in acidic amino acids that are deprotonated at physiological pH, entailing structural changes.</td>
</tr>
</tbody>
</table>
extracellular loop connecting the S3-S4 segments in DIV. Consequently, peptides containing basic residues, such as sea anemone and α-scorpion toxins (e.g., AaH II), have been shown to be potent binding site 3 inhibitors. The main effect of this interaction is impairment of fast inactivation.

**Binding site 4** Binders that interact with this site (mainly S4 in DII) are ‘gate-modifiers’ as their binding changes the threshold of activation of the VGSC. A shift to more negative potentials leads to enhanced activation of the channel by β-scorpion toxins. Huwentoxin-IV (HwTx-IV) and Protoxin-II (ProTx-II) on the other hand, cause a positive shift of the threshold potential, and subsequent inhibition of the channel. These two toxins are discussed in the following Section 1.2.3 as they exhibit excellent potency and selectivity against Nav1.7.

**Binding site 5** This site involves S6 in DI and S5 DIV. An example of a binder is brevetoxin, which causes enhanced activation of the channel.

**Binding site 6** δ-conotoxins interact with the S3-S4 linker in DIV and result in delaying the process of fast inactivation.

**Local Anaesthetic/Small molecule binding site** Various small molecule inhibitors enter and block the cavity of the inner pore which is formed by the S6 of all domains DI–DIV. This cavity is highly conserved among VGSC subtypes, therefore these inhibitors are not particularly selective. In order to enter the cavity the size of these molecules needs to be sufficiently small. Examples of local anaesthetic and anticonvulsant small molecule binders are lidocaine, tetracaine, carbamazepine and lacosamide. These inhibitors exhibit state and use dependence.

### 1.2.2.3 Binders of Nav1.7

Table 1.2 contains selected potent binders of Nav1.7 and their IC$_{50}$ values as determined by electrophysiological studies. It is important to note that the patch clamp protocols employed to determine the potency of these binders are not always the same and that should be considered when comparing different studies. One of the major considerations when developing Nav1.7 inhibitors is the selectivity over the other eight VGSC subtypes. Molecules that have been developed recently, like PF-06456384 and aryl sulfonamide 3, in addition to some naturally occurring toxins like ProTx-II show strong selectivity for Nav1.7. Most of these inhibitors have been tested in vivo for their analgesic effects using models of inflammatory and neuropathic pain. Figure 1.6 contains the structures of the small molecule inhibitors listed in Table 1.2. The ‘new wave’ of small molecule inhibitors bear an aryl sulfonamide moiety and are more selective, unlike
XEN907, pyrrolopyrimidine 61 and biphenyl pyrazole 27. The binding site that attributes these characteristics to the aryl sulfonamide inhibitors is discussed in more detail in Subsection 1.2.2.5.

**Figure 1.6:** Structures of small molecule inhibitors of Nav1.7. Table 1.2 contains their selectivity and potency data.

### 1.2.2.4 Status of Inhibitors in Clinical Trials

Small molecule inhibitors of Nav1.7 that showed positive results in preclinical models have entered clinical development. Vixotrigine (BIIB074) is an inhibitor that targets the local anaesthetic binding site and is currently in phase 3 trials for trigeminal neuralgia. AZD3161 and other inhibitors such as NKTR-171 did not progress to phase 2 trials for neuropathic pain.\(^{75,76}\) In addition, inhibitors of the aryl sulfonamide class have been tested in a clinical environment. PF-05089771 was well tolerated but discontinued after phase 2 testing for dental pain and diabetic neuropathy due to lack of efficacy.\(^{67}\) Similarly, topical inhibitor TV-45070 showed no efficacy in a phase 2 study for postherpetic neuralgia.\(^{77}\) Currently, a phase 1 trial of sulfonamide inhibitor GDC-0310 is being carried out by Genentech and Xenon.\(^{78}\) Despite the genetic background supporting that Nav1.7 is implicated in pain signalling, there have been frequent disappointing results in...
<table>
<thead>
<tr>
<th>Compound</th>
<th>hNav 1.7 IC₅₀ (nM)</th>
<th>Selectivity - IC₅₀</th>
<th>In vivo Pharmacology</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PF-06456384*</td>
<td>0.01</td>
<td>all other subtypes: 3 – 26000 nM</td>
<td>No effect in mouse formalin model</td>
<td>51</td>
</tr>
<tr>
<td>ProTx-II</td>
<td>RS: 0.3</td>
<td>Nav1.2, 1.3, 1.4, 1.5, 1.6: 39 – 102 nM, Nav1.2, 1.3, 1.4, 1.5, 1.6: fold selectivity 79 – 1584</td>
<td>Rat formalin model</td>
<td>53, 54</td>
</tr>
<tr>
<td>GP-ProTx-II W7Q, W30L</td>
<td>0.8</td>
<td>Similar to Nav1.2 &gt; Nav1.6, other subtypes: &gt;1000 nM</td>
<td></td>
<td>55</td>
</tr>
<tr>
<td>GX-674*</td>
<td>0.1</td>
<td>Nav1.1, 1.2, 1.5, 1.6: 4.2 – 3080 nM</td>
<td>Complete Freund's adjuvant (CFA), acetic acid and formalin models</td>
<td>52</td>
</tr>
<tr>
<td>Aryl sulfonamide 3*</td>
<td>0.4</td>
<td>all other subtypes: 37 – 50000 nM</td>
<td>Mouse formalin and carrageenan models in synergy with opioids</td>
<td>56</td>
</tr>
<tr>
<td>Pn3a</td>
<td>RS: 0.9</td>
<td>Nav1.4: 0.2 μM, Nav1.5: &gt; 10 μM</td>
<td>OD1 induced pain model</td>
<td>58</td>
</tr>
<tr>
<td>GpTx-1</td>
<td>RS: 10</td>
<td>Nav1.4: 1.9 μM, Nav1.5: &gt; 10 μM</td>
<td>Mouse and rat formalin model</td>
<td>59, 60</td>
</tr>
<tr>
<td>A5, F6, L26, R28</td>
<td>1.6</td>
<td>Nav1.2, 1.3, 1.4: &gt;150 nM, Nav1.5: &gt;10 μM</td>
<td>OD1 induced pain model</td>
<td>61</td>
</tr>
<tr>
<td>HwTx-IV</td>
<td>RS: 17</td>
<td>Nav1.8, 1.9: no effect at 100 nM</td>
<td>Thermal and mechanical pain after injection in mouse paw</td>
<td>62</td>
</tr>
<tr>
<td>wildtype</td>
<td>10</td>
<td>Nav1.5: &gt;30000 nM</td>
<td>Mouse formalin and itch model</td>
<td>63</td>
</tr>
<tr>
<td>A5, F6, L26, R28</td>
<td>1.6</td>
<td>all other subtypes: &gt;119 – 10000 nM</td>
<td>Mouse formalin model</td>
<td>64</td>
</tr>
<tr>
<td>E1G, E4G</td>
<td>0.5</td>
<td>Mouse formalin model</td>
<td>No efficacy in clinical trial</td>
<td>65, 66</td>
</tr>
<tr>
<td>E1G, E4G, Y33W</td>
<td>0.4</td>
<td>Nav1.5: no inhibition at 1 μM</td>
<td>Mouse capsaicin and itch model</td>
<td>67</td>
</tr>
<tr>
<td>XEN907*</td>
<td>3</td>
<td>Nav1.1, 1.3, 1.4, 1.5: &gt;42 μM, Nav1.6: 0.65 μM</td>
<td>Mouse formalin and itch model</td>
<td>68</td>
</tr>
<tr>
<td>AaHII</td>
<td>(EC₅₀) 6.8</td>
<td>Nav1.5: &gt;1000 nM</td>
<td>Mouse formalin and itch model</td>
<td>69</td>
</tr>
<tr>
<td>N-linked arylsulfonamide 5*</td>
<td>8</td>
<td>all other subtypes: &gt;119 – 10000 nM</td>
<td>Mouse formalin model</td>
<td>70</td>
</tr>
<tr>
<td>PF-05089771*</td>
<td>11</td>
<td>Nav1.3, 1.4, 1.5: no effect</td>
<td>Mouse formalin model</td>
<td>71</td>
</tr>
<tr>
<td>AM-0466*</td>
<td>6 - 21</td>
<td>Nav1.1, 1.3, 1.4, 1.5: &gt;42 μM, Nav1.6: 0.65 μM</td>
<td>Mouse formalin model</td>
<td>72</td>
</tr>
<tr>
<td>HnTx-IV</td>
<td>RS: 21</td>
<td>Nav1.5: no inhibition at 1 μM</td>
<td>Mouse formalin model</td>
<td>73</td>
</tr>
<tr>
<td>μ-SLPTX-Ssm6a</td>
<td>RS: 25</td>
<td>Nav1.1, 1.2, 1.6: 4, 0.8, 15 μM</td>
<td>Mouse formalin model</td>
<td>74</td>
</tr>
<tr>
<td>PF-04856264*</td>
<td>28</td>
<td>Nav1.3, 1.5: &gt;10 μM</td>
<td>OD1 induced pain model</td>
<td>57</td>
</tr>
<tr>
<td>Pyrrolepyrimidine 61*</td>
<td>30</td>
<td>Nav1.5: 80 nM</td>
<td>Mouse formalin model</td>
<td>75</td>
</tr>
<tr>
<td>SVMab1*</td>
<td>31</td>
<td>all other subtypes: 5 – 9 μM</td>
<td>Mouse formalin model</td>
<td>76</td>
</tr>
<tr>
<td>Biphenyl pyrazole 27*</td>
<td>46</td>
<td>Nav1.3, 1.4, 1.5: no effect</td>
<td>Mouse formalin model</td>
<td>77</td>
</tr>
</tbody>
</table>

Table 1.2: A selection of the most potent Nav1.7 binders. Binders that are small molecules, antibodies and toxins with varying selectivity over other Nav subtypes are listed. Asterisks denote state-dependent inhibition. RS: at resting state of channel. Refer to Figure 1.6 for the structures of the small molecules.
clinical trials. The high complexity of the system that inhibitors aim to modulate makes research in this field challenging. High plasma protein binding has been a limiting factor for PF-05089771, but it is speculated that low nerve penetration additionally contributes to low target engagement in patients. This observation highlights that preclinical models that provide pharmacokinetic and pharmacodynamic properties do not guarantee a desirable outcome. Moreover, as pain sensation is subjective and there are no biomarkers that can be employed for the assessment of clinical drug candidates, the possible ways of designing and analysing the outcomes of these trials are limited.

1.2.2.5 Structural Studies of Binding Interactions

There have been numerous efforts to understand the pharmacological regulation of VGSCs with the employment of techniques such as X-ray crystallography and cryo-EM. The work of Bagneris et al. (2014), enabled the study of the local anaesthetic binding site in the pore cavity by X-ray crystallography. The pore of *Magnetococcus marinus* *Nav* (NavMs), which is strikingly similar to the pore of Nav1.1, was crystallised with brominated analogues of pore blockers lidocaine, lamotrigine and other blockers that belong in the local anaesthetic and anticonvulsant family. Aryl sulfonamide binding site on Nav1.7

Crystallographic data also revealed the binding interactions of aryl sulfonamide inhibitors with a NavAb/human Nav1.7 DIV voltage sensor chimera (VSD4). Aryl sulfonamide GX-936 which is structurally equivalent to GX-674, PF-06456384 and PF-05089771 (Table 1.2, Figure 1.6) was co-crystallised with the VSD4 chimera. It was found to trap VSD4 in an activated conformation by having direct interactions with an arginine residue (R4) on S4. This binding interaction opposes the deactivation of VSD4, and subsequently traps the channel in a non ion conducting state. This structure was of great importance as it explained the subtype selectivity of this family of inhibitors (Figure 1.7). Moreover, it revealed the participation of a phospholipid in the VSD4-GX-936 complex, suggesting the trimeric nature of this mode of inhibition.
1.2.3 Inhibitory Cystine Knot Peptides that Target Nav1.7

In addition to the aryl sulfonamide inhibitors, Table 1.2 contains peptides that are present in the venom of spiders and a centipede. These naturally occurring venom toxins have been developed to target insect VGSCs, but have also shown an effect on the nervous system of other organisms. As shown in Figure 1.8, all of them contain three disulfide bonds with C1-4, C2-5 and C3-6 connectivity. This motif is present in many venom peptides that exhibit high affinity towards VGSCs and is called the Inhibitory Cystine Knot (ICK). The properties of these toxin peptides are, among others, stability at high temperatures and pH, and resistance to proteases due to their compact fold. The structure of these peptides is amphipathic, consisting of a hydrophobic and a polar face. The potency and selectivity of certain neurotoxin peptides for Nav1.7 vary, some of them are very promiscuous whilst others are very potent and selective. Generally, they are gating modifiers and interact with the voltage sensors of the channels.

Pn3a (μ-TRTX-Pn3a) was isolated from the venom of tarantula *Pamphobeteus nigricolor* and it exhibits excellent potency (0.9 nM) and selectivity of 40-1000 fold over other subtypes. Binding of Pn3a causes a positive shift in the membrane potential activation threshold of Nav1.7 in engineered cell lines as determined electrophysiologically.
Another potent ICK peptide is GpTx-1 that was isolated from tarantula Grammostola porteri. Its potency against Nav1.7 is high (10 nM) and even more potent (1.6 nM) and selective derivatives have been reported.58 This peptide has been the focus of various studies; dimerisation through polyethylene glycol (PEG) conjugation resulted in reduction in the off rate of binding with Nav1.7.84 GpTx-1 reverses pain behaviours in vivo when administered intraplantarly during the novel OD1-induced spontaneous pain model in mice.57 OD1 is a scorpion toxin that induces pain behaviour in mice by enhancing Nav1.7 activation.57 In order to battle the quick renal clearance of GpTx-1, a property that most ICK peptides share, a derivative of GpTx-1 was conjugated to an antibody (Ab) through Huisgen cycloaddition and cysteine modification chemistry.85 The Ab-GpTx-1 conjugate exhibited 130-fold longer half life in vivo which is attributed to Fc recycling mechanisms. This tool compound was used to study the distribution of Nav1.7 in neurons through immunofluorescence and to inhibit Na⁺ currents in neurons, even though its potency was impaired.85

Two of the most studied hNav1.7 toxin inhibitors, HwTx-IV and ProTx-II, are discussed below. For a more detailed account of ICK spider toxins that target Nav1.7, one can refer to two excellent reviews.83,86

1.2.3.1 Huwentoxin-IV

Huwentoxin-IV (or μ-TRTX-Hh2a) is a 35 amino acid long peptide found in the venom of the Chinese tarantula Ornithoctonus huwena (Figure 1.9).49 Site directed mutagenesis studies firstly revealed that HwTx-IV interacts with binding site 4 and more specifically, with the S3-S4 linker in voltage sensor of DII of Nav1.7.49,81 It is thought to trap the voltage sensor in the inward conformation, impeding activation of the channel and increasing the activation potential threshold.81 Moreover, an important acidic residue of the linker was determined (E818), as affinity for the E818Q and E818C mutants was reduced by 63- and 400-
fold with respect to the wild-type Nav1.7.\textsuperscript{49,87} Mutagenesis analysis also pinpointed E753 in the DIIS1-S2 linker, and E811, L814, D816 in the DIIS3-S4 linker as important for the inhibitory activity of HwTx-IV.\textsuperscript{87}

![Figure 1.9: NMR Structure of HwTx-IV (PDB 1MB6). A, The β-sheet and four loops are colour-coded in the structure and sequence. B, Surface representation highlighting the residues that have the highest impact on the inhibitory activity against Nav1.7: F6, W30, K32, W33. Green represents hydrophobic residues, blue: basic residues, red: acidic and charged residues, yellow: cysteines.](image)

Revell \textit{et al.} synthesised wild-type and derivatives of HwTx-IV via solid phase peptide synthesis, substituting each residue with alanine but conserving the disulfide bonds.\textsuperscript{59} Circular dichroism spectroscopy was employed to ensure that the secondary structure of the derivatives did not significantly differ from the wild-type toxin. The activity of the derivatives was tested using patch clamp electrophysiology in HEK293 cells overexpressing Nav1.7. W30A and K32A showed no inhibitory effect, and these results agreed with the observations of Minassian \textit{et al.}, who obtained the derivatives using expression vectors in the HEK 293F cell line.\textsuperscript{59,81}

Following the use of NMR Spectroscopy studies to elicit the solution structure of the toxin (PDB 1MB6) and radiolabelled analogues to observe binding kinetics, it was concluded that the aromatic F6, W30, Y33 residues composed a hydrophobic surface of the toxin that, in conjunction with the basic K32, are important contributors to its affinity for Nav1.7.\textsuperscript{81} Derivatives with improved potency have also been developed. The ones that had a decreased net charge showed inhibition with IC\textsubscript{50} values of less than 1 nM (Table 1.2).\textsuperscript{59} One of these derivatives, HwTx-IV [E1G, E4G, Y33W], was recombinantly expressed and its solution NMR structure (PDB 5T3M) was found to correspond well with the structure of HwTx-IV.\textsuperscript{61} HwTx-IV [E1G, E4G, Y33W] had the same selectivity profile as the native peptide and exhibited an analgesic effect in a
mouse model of Nav1.7-mediated pain. The selectivity trend of HwTx-IV over the other VGSC subtypes is Nav1.7 > Nav1.6, Nav1.3 > Nav1.1 > Nav1.2 >> Nav1.4, Nav1.8, Nav1.5, and it has been suggested that the off-target effects when administered in vivo are due to Nav1.6 inhibition.

Some ICK peptides that target sodium channels have been suggested to partition into the cell membrane. The binding affinity of HwTx-IV for liposomes is weak, however, it increases with neutral and hydrophobic mutations like those in the [E1G, E4G, F6W, Y30W]HwTx-IV derivative. In parallel, this derivative exhibited higher potency in a fluorescent imaging plate reader (FLIP) calcium assay that measures changes in the membrane potential. These findings imply that there is a possible correlation between the membrane binding properties and the inhibitory activity of HwTx-IV.

### 1.2.3.2 Protoxin-II

This *Thrixopelma prurient* tarantula cystine knot peptide consists of 30 residues and is thought to bind to the same site of VGSCs as HwTx-IV. ProTx-II (β/ω-TRTX-Tp2a) has high affinity for TTX-S VGSCs but exhibits more than 70-fold specificity towards Nav1.7. Based on site directed mutagenesis experiments, its selectivity is thought to be due to the F813 in the DII S3-S4 linker, which is only present in Nav1.7 but a highly conserved G813 in the other subtypes. It was proposed that, unlike HwTx-IV, ProTx-II does not only impede activation of VGSCs, but also impairs fast inactivation by interaction with DIV S3-S4 linker similarly to site 3 α-scorpion toxins. This conclusion was drawn because a sustained Na+ current was induced in the presence of ProTx-II, but a definite explanation for the cause of this process was difficult to construct using solely electrophysiological and mutagenesis methods.

A recent extended biophysical investigation of ProTx-II revealed a solution NMR structure (Figure 1.10) and its lipid bilayer binding properties by Surface Plasmon Resonance (SPR). ProTx-II binds to different zwitterionic lipids exhibiting a fast on and a slow off rate. Mutagenesis analysis, activity assays and molecular dynamics simulations lead the authors to propose a model of ProTx-II binding to the cell membrane through Y1, K4, W5, M6, W7, W24 and W30. Membrane binding is highly responsible for ProTx-II’s Nav1.7 inhibitory activity, as it increases its concentration near the channel and also positions the toxin in a way that favours interactions with Nav1.7.
Figure 1.10: NMR Structure of ProTx-II (PDB 2N9T). A, Similar to HwTx-IV, ProTx-II folds in an ICK motif, without forming a β-sheet. B, Surface representation highlighting the proposed membrane binding face (Y1, K4, W5, M6, W7, W24 and W30). Green represents hydrophobic residues, blue: basic residues, red: acidic and charged residues, yellow: cysteines.

Efforts to develop ProTx-II variants that exhibit stronger selectivity for Nav1.7 yielded GP-ProTx-II [W7Q, W30L] which was more selective but, consistent with the membrane partitioning observations of Henriques et al., showed lower potency. Both ProTx-II and GP-ProTx-II [W7Q, W30L] were efficacious in rat pain models, with GP-ProTx-II [W7Q, W30L] not exhibiting motor impairment. 54

1.2.4 Nav1.7 as a Novel Analgesic Target

It is estimated that approximately 20% of the adult population suffers from chronic pain and the cost associated with it is extremely high. In the United States, the annual cost is ~ $600 billion if the treatments and loss of productivity of the affected individuals are considered. 94 Current pain treatments involve the use of drug molecules that target the central nervous system however, problems with resistance, addiction, and unwanted CNS side effects often arise with their use.

The discovery of the null mutation of SCN9A in patients with CIP generated the idea that drug molecules that specifically target and inhibit Nav1.7 can potentially have analgesic effects without undesired side effects. 34 The factors that should be considered when designing a Nav1.7 inhibitor are firstly, apart from high binding affinity to the channel, the location of the binding site. For example, molecules like local anaesthetics insert in the inner cavity of the pore and others interact with extracellular parts of the channel. The location of the binding site dictates the lipophilicity and size requirements of the target molecule. Secondly, the specificity of the binder should also be considered. Binders that non-specifically bind
to VGSCs of the CNS or, for example, Nav1.5 which is involved in the function of the heart muscle can cause undesired and potentially severe off-target effects. Another important consideration is the selectivity for Nav1.7 expressed in diseased neurons. This property is important for avoiding suppression of pain signaling throughout the body, but maintaining it in the neurons where it is relevant. Certain inhibitors have been shown to have a ‘state- or voltage-dependent’ effect on VGSCs, which is essentially higher affinity for the open state of the channel. The frequency of Nav1.7 changing conformations is related to the action potential generated and it differs between healthy neurons and injured more rapidly firing neurons. Moreover, the modulation of Nav1.7 by other proteins could lead to additional structural changes and consequently, possible variation in the affinity of the drug molecule. However, little research has been done in the area of neuromodulation and its effects on the structure of VGSCs.

Nav1.7 inhibitor development has been the focus of many pharmaceutical companies. The recent discovery of the aryl sulfonamide binding site on VSD4 has steered drug discovery efforts in a new direction. Some of the candidates that have been tested in a clinical environment face the problem of little to no efficacy. This could be attributed to poor in vivo target engagement and selectivity. Subtype-selective inhibitors are required for safety, however, certain inhibitors show potency differences across animal species in preclinical models. Furthermore, as various patch-clamp protocols are used to assess the potency and mode of inhibition, it is difficult to determine which protocol delivers the best prediction for efficacy in a physiologically-relevant environment. Similarly, there is a plethora of in vivo models that rely on observations of animal behaviour. As pain perception is subjective and challenging to assess even in clinical settings, there is concern that the current animal models equally deliver contradicting or inconclusive results. Efforts to address these issues include the development of induced pluripotent stem cells (iPSCs) as more predictive preclinical models. Patient-derived neurons can provide a step towards personalised medicine and assessment of differences in pain signaling among individuals. Finally, clinical experience has provided some insight into additional possible reasons for the failure of many inhibitors. In the case of PF-05089771, it was reported that there might be additional and more important contributors to pain signalling than Nav1.7 in the diseases tested (painful diabetic neuropathy and dental pain) as well as low nerve penetration of the candidate to achieve sufficient target engagement. Another study supports the hypothesis that loss-of-function of Nav1.7 leads to increased expression of enkephalin opioids, which significantly contributes to pain insensitivity thus stressing that Nav1.7 is not the only contributor to the CIP phenotype. It is widely agreed, however, that development of novel analge-
ics targeting Nav1.7 is a huge challenge and it will highly benefit from additional research on its binding interactions with toxins, small molecule modulators, and other proteins in the cell.

1.3 Chemical Biology Approaches

In line with other transmembrane proteins, human VGSCs have proven especially difficult to isolate and crystallise. As a consequence, crystal structures have not been reported to date, thus posing a great challenge in understanding the interaction of VGSCs with small molecules or peptides. Although cryo-EM has emerged as a very promising technique for structural studies of membrane proteins, and the bacterial-human chimeric approach has yielded valuable information on the binding of certain inhibitors through X-ray crystallography,\textsuperscript{55} it is still a very challenging field.

The primary method used to identify binding through blocking or modulation of the biophysical properties of VGSCs is patch clamp electrophysiology. However, electrophysiology does not provide specific information on the binding site, and thus is frequently coupled with site-directed mutagenesis. In this approach, the assumption that the overall structure and folding of the wild-type VGSC is not altered significantly by the mutations is made and possible amino acids that are located in the binding site can be identified. Moreover, additional patch-clamp experiments are done to characterise the gating mechanism of the mutant channels. All the above make the patch-clamp and mutagenesis approach very expensive and time consuming.

Molecular modelling of the structure based on homology modelling using the structure of NavAb or Kv1.2 has also been employed in order to identify binding interactions.\textsuperscript{48,81} However, the validity of these models and thus the significance of the obtained results, can only be confirmed using experimental data.

The high affinity of certain binders can be exploited in order to produce new chemical tools that will enable isolation of VGSCs. Such tools will allow for further study of their structure and facilitate understanding of how their function is modulated, potentially revealing new binding sites for drug development. Such an approach is discussed in this section; it involves photoaffinity labeling and proteomic mass spectrometry and we believe it would be very valuable as a complementary technique to the ‘golden standard’ of ion channel research, patch clamp electrophysiology.
1.3.1 Photoaffinity Labeling

This method is based on the use of a photo-reactive probe that has two main properties. Firstly, it is capable of forming non-covalent interactions with the protein target of interest and therefore has sufficiently high affinity for it. Secondly, after the formation of the probe-protein complex, upon irradiation with UV light, it becomes activated and able to covalently bind to the protein at its binding site. Consequently, the development of a photoaffinity probe is usually based on modification of known binders with the introduction of a photo-activating group. The main photo-activating groups that are usually employed in photoaffinity labeling experiments are benzophenones, diazirines and aryl azides. Upon excitation with UV light, these groups give rise to diradicals, carbenes, and nitrenes, respectively. The reactivated intermediates then attack C-H, N-H or O-H bonds in close proximity and form a covalent link between the label and protein (Figure 1.11). Desired properties of photoaffinity probes are the retention of high binding affinity and a high activation wavelength. As proteins absorb at ~200 and 280 nm, photo-activation should ideally be done at a wavelength that is not absorbed by the protein and does not damage its structure. Moreover, high specificity of attack of the reactive intermediate is desired, as reaction with other sites of the protein target or other proteins will complicate the interpretation of the results. Lastly, for effective incorporation of the photoprobe, the lifetime of the excited state should be long compared to its dissociation kinetics but not long enough to result in non-specific insertion.

A great amount of small molecules and natural products have been modified with photo-activating groups in order to photo-crosslink to their receptor proteins. The main concern with small molecule ligands is that introduction of a photophore will decrease its affinity dramatically. For this reason, less bulky diazirine photophores are usually preferred over benzophenone.

In the case of peptide ligands, diazirine-containing amino acids have been developed to synthesise peptide photoprobes. Two diazirine-containing examples are photomethionine and photoleucine (Figure 1.11). Photoleucine and photomethionine containing peptides have been used in photocrosslinking experiments that aim to identify protein targets or the binding site on a specific protein of interest. Photoleucine was used in the solution phase synthesis of a cyclodepsipeptide, replacing a leucine residue in the native peptide HUN-7293. HUN-7293 is a natural inhibitor of the expression of vascular cell adhesion molecule (VCAM). The diazirine-containing peptide showed similar inhibition of VCAM expression as HUN-7293 and it was used to identify the protein target of HUN-7293. Another example of synthetic
peptides that contain a photoreactive group includes the use of photomethionine analogue of a peptide that was hypothesised to be involved in the protein-protein interactions of the homo-trimer of the MH2 domain of Smad2. The peptide was selectively ligated to the truncated MH2 domain and enabled the identification of the transient MH2-MH2 interactions.\textsuperscript{107} Due to the structural similarity of photomethionine and photoleucine with methionine and leucine, they are effectively incorporated in newly synthesised proteins in cells growing in leucine and methionine free culture media. As an example, ultraviolet (UV) irradiation of the cells and immunoprecipitation revealed protein-protein interactions of membrane protein SCAP with Insig-1.\textsuperscript{109}

Photoaffinity labeling becomes more powerful when used in conjunction with affinity or visualisation tags for further manipulation of the protein of interest. In this case, the photoprobe contains both a photoactivating group, and a reporter group that can be bioorthogonally ligated with tags such as fluorophores and biotin.\textsuperscript{110} This method has given rise to linkers that contain a diazirine and an alkyne/azide allowing for probe modification by copper-catalysed ‘click’ reaction. The advances in ‘click chemistry’ have facilitated the incorporation of these tags in complex biological systems under non-denaturing conditions.\textsuperscript{105}

Usually, the copper-catalysed [3+2] cycloaddition of azides and alkynes is exploited for this purpose.\textsuperscript{111} In an interesting study by Parker et al., a small diazirine and alkyne linker was coupled to various drug fragments in order to identify their protein targets in cells. After ‘click’ incorporation of biotin-azide,
the crosslinked proteins were enriched using streptavidin agarose resin and identified. The HUN-7293-based diazirine probe that was discussed above contained a propargyl group at position 1, which served as a handle for tagging the photocrosslinked proteins with rhodamine-azide via ‘click’ chemistry. Tagging with rhodamine enabled in-gel fluorescence visualisation of the photo-crosslinked proteins and the estimation of % yield of photocrosslinking (23%). Finally, photocrosslinking and bioorthogonal ligation has been successful in identifying lipid-protein interactions in the cell. For this purpose, diazirine and alkyne analogues of various lipids were introduced to cells.

One of the major experiments that accelerated the discovery of VGSCs in the 1980s was the use of a azidonitrobenzoyl photoaffinity probe based on an α-scorpion toxin to crosslink VGSCs in neuroblastoma cells. Photoaffinity labels have also been used more recently for the study of other channels. For example, photoaffinity labeling of a voltage-gated potassium channel (Kv1.2) was achieved with the use of a diazirine analogue of general anaesthetic Sevoflurane. It enabled the identification of two allosteric binding sites; on the linker of the pore and voltage sensor domain, and also the extracellular part of the selectivity filter of the pore." Mapping of binding interactions between the photoprobes and proteins is a very challenging process and requires instrumentation that is very accurate and sensitive. Proteomic Mass Spectrometry (MS) lives up to this challenge and its applications will be discussed in the following Subsection 1.3.2.

1.3.2 Mass Spectrometry Proteomics

The focus of this section is 'bottom-up' proteomics. The term 'bottom-up' describes the process by which the sample of proteins is analysed. Firstly, proteins are digested by proteases into a complex mixture of peptides, which in turn is subjected to a high pressure liquid chromatography (HPLC) coupled to a high resolution electrospray ionisation (ESI) mass spectrometer (LC-MS). The peptide ions that are detected according to their ion intensity and retention time can be selected and subjected to collision-induced dissociation (CID) and mass-analysed again (MS/MS or MS²). This set up is referred to as LC-MS/MS and enables the identification of the peptide sequence using either de novo calculations or comparing the acquired spectra with predicted spectra based on the the amino acid sequence of the proteins of interest. LC-MS/MS is a powerful tool in proteomic analysis as modern instruments exhibit high sensitivity, mass accuracy, and allow for quantification of peptide levels in very complex samples.

When the protein of interest is membrane-bound, like an ion channel, the sample preparation for MS
analysis becomes more challenging as these receptors exhibit poor solubility and often low abundance in cells leading to little or no sequence coverage and challenges in quantification by LC-MS/MS. Another issue arising from the transmembrane domains of membrane proteins is the hydrophobic nature and low ionisation of the transmembrane proteolytic peptides. The first step is usually the isolation of plasma membranes in order to reduce the background introduced by the highly abundant proteins found in the cytoplasm and nucleus. Digestion can be done in-gel, in-solution and through filter-aided sample preparation (FASP). Chymotrypsin is usually employed as it cleaves at the C-terminus of hydrophobic amino acid residues, usually found in the transmembrane domains, and produces smaller and more likely to be soluble peptide fragments.

Photocrosslinking can be successfully coupled to bottom-up proteomics for a dual mission; the identification of the binding site of the photoprobe, and/or the photo-probe itself can be used as an enrichment tool for the identification of the target protein. In the case of membrane-bound proteins, photocrosslinking can potentially be achieved in isolated membranes, while the structure of the protein and its binding partners are conserved. An excellent example of photocrosslinking to identify the binding site of a common drug is described in this paragraph. Photocrosslinking of γ-aminobutyric acid type A (GABA\textsubscript{A}) receptors in intact membranes was successful using a diazirine analogue of propofol, a known binder that is used as an anaesthetic, and in another study, using a neurosteroid diazirine analogue, 6-Azi-Pregnanolone (6-AziP). The next step involved the solubilisation of the membrane-bound proteins with detergents; mild, non-ionic, as well as denaturing detergents can be used for this purpose. In the first study, the solubilised and photocrosslinked GABA\textsubscript{A} receptor subunits carried strep- and octahistidine-tags and were therefore run through affinity chromatography columns. The tagged protein was eluted, delipidated, reduced and alkylated in order to allow for efficient protease digestion using trypsin and chymotrypsin. Assuming that insertion of the photoprobe occurs at a single site of the receptor, the digestion peptide that contains the photoprobe will therefore have an increased mass compared to the non-crosslinked peptide. In order to facilitate the identification of this particular peptide, stable isotopes of the photoprobe were used by Yip et al.; a deuterated analogue of propofol-diazirine which would give an expected isotopic distribution in the mass spectra of the peptide digests. CID of the identified peptide enabled them to locate the exact residue that was modified by the photo-probe and to propose the binding site of propofol in GABA\textsubscript{A} receptors. However, in this approach, results should be interpreted with caution as the photo-probes might interact with a different site or multiple sites of the protein other than the known scaffold binder. There
have been some examples of photocrosslinking used to identify the binding site of inhibitors of proteins and their use has benefited from the development of cleavable and/or isotopically labeled linkers.\textsuperscript{117,124,125} The advantage of a cleavable linker that carries an affinity tag is the selective release of the crosslinked protein from the affinity resin, producing less background and enhancing its MS identification.

In the world of VGSC MS proteomics, Nav1.2 and Nav1.5 have received the most interest in recent years. The main methodology for enrichment of these channels has been immunoprecipitation using a pan-Nav antibody with applications in extracts of mammalian heart or brain tissue. Briefly, the interacting partners of rNav1.2 were identified by co-immunoprecipitation (co-IP) from membrane extracts of brain tissue and these included FGF12, β subunits, and ankyrin-3.\textsuperscript{46} Equally importantly, the authors managed to achieve a 70% sequence coverage using a combination of LysC and trypsin digestion of the immunoprecipitated proteins. MS proteomics has also been employed for the study of PTMs. Phosphorylationsites on Nav1.2 have been identified (41% sequence coverage),\textsuperscript{126} and the \textit{in vivo} analysis of the effect of brain seizures on mouse Nav1.2 PTMs such as phosphorylation and arginine methylation was achieved shortly after (50% coverage).\textsuperscript{127} A similar investigation was carried out in mouse cardiac ventricles to map the phosphorylation sites of Nav1.5 (25% coverage).\textsuperscript{128} Finally, another study focused on arginine methylation of hNav1.5 from cardiac tissue of patients (36% coverage).\textsuperscript{129}

In an attempt to characterise complexes of Nav1.7 \textit{in vivo}, Kanellopoulos \textit{et al.} developed a tandem affinity purification (TAP) construct of hNav1.7 that was used to generate knock-in mice. The TAP approach was combined with mass spectrometry and a plethora of potential binding partners of hNav1.7 in mice neurons were identified.\textsuperscript{130} To validate novel binders, the authors did co-IP experiments from engineered cell lines that expressed TAP-tag Nav1.7 and GPCR Gprin1, trafficking proteins synaptotagmin-2 and transmembrane P24-trafficking protein 10 (Tmed10), and CRMP2 which had previously been reported as a modulator of Nav1.7 activity.\textsuperscript{130,131} As far as we know, it is the only MS study of the Nav1.7 interactome and it is very possible that the extended washes during the double affinity purification have abolished a lot of transient interactions. The authors did not report a sequence coverage for Nav1.7. Nevertheless, this study has been a step forward for the analysis of binding partners of Nav1.7.
CHAPTER 1. INTRODUCTION

1.4 Research Objectives

This Thesis is focused on the development of novel chemical biology approaches to understand Nav1.7 interactions in simple as well as advanced in vitro disease model systems. There are many aspects of Nav1.7 modulation that are still poorly understood, including the structural determinants of inhibition by toxin peptides. The development of efficacious analgesics that inhibit Nav1.7 remains unsuccessful and this stresses the fact that potentially, high binding affinity is not the only property required for an inhibitor to become a successful clinical candidate. More information on the binding sites, and how these change in the various conformations of Nav1.7 due to complexation with other proteins in the cell or merely due to its gating activity, is needed. Moreover, increasing our understanding of the mode of inhibition by neurotoxins, which have been designed by nature to modulate these channels, will be an invaluable tool for subsequent drug discovery efforts.

To this end, the aim of this Thesis is to discuss:

- The design, synthesis and electrophysiological characterisation of photoaffinity probes based on HwTx-IV and an aryl sulfonamide inhibitor of Nav1.7
- The optimisation of proteomic MS analysis of Nav1.7 in an engineered cell line and efforts to detect Nav1.7 in induced pluripotent stem cells (iPSCs) of patients suffering from erythromelalgia
- Biophysical and structural studies of the binding interactions of modulators with chimeras of NavAb/Nav1.7 VSD2 and VSD4
- Photocrosslinking experiments to identify the binding sites of HwTx-IV on the VSD2 chimera
Chemical Tools to Study Nav1.7

This Chapter describes the efforts in the development of photoaffinity probes for Nav1.7. These probes are based on a toxin peptide and an aryl sulfonamide inhibitor. The design, synthetic routes and characterisation techniques of Nav1.7 photoprobes are discussed. Finally, potency data are reported based on electrophysiological assays.

2.1 Photoaffinity Probes Based on Huwentoxin-IV

2.1.1 Introduction: Synthesis of ICK Peptides

In Subsection 1.2.3, potent peptide modulators that belong to the Inhibitory Cystine Knot (ICK) family are discussed. The ICK structural motif is characterized by a triple- or double- stranded anti-parallel β-sheet connected via three disulfide bonds. The connectivity of the disulfide bonds forms a knotted core. This rigid fold results in increased thermal stability and resistance to proteases.

ICK peptides have received a lot of interest due to their increased stability and various efforts to synthesise them via solid phase peptide synthesis (SPPS) have been reported in the literature. Another approach that has been explored is the recombinant bacterial expression.

SPPS of long peptides that contain a significant amount of hydrophobic residues is challenging as aggregation and secondary structure formation can contribute to inefficient amide couplings and byproduct formation. However, the main challenge in synthesis of ICK peptides is the formation of the correct disulfide bonds. This process is called oxidative refolding and the six cysteine residues need to form disulfides in a specific connectivity in order to afford the correct ICK motif. Typically, the linear (reduced) peptide...
is purified and subjected to oxidation before the final purification of the oxidation product. For a peptide that contains six cysteine residues \( n = 6 \), the number of the possible isomers \( i \) that result from three \( p = 3 \) disulfide bridges is given by the equation:

\[
i = \frac{n!}{(n - 2p)!p!2^p}
\]  

(2.1)

Therefore, \( i = 15 \) isomers are expected from such a peptide and it is important to ensure that the refolding conditions of choice will yield the correct isomer. In nature, proteins that contain multiple disulfide bonds typically fold in the ER or mitochondria as a result of the redox environment. The redox environment in the cell is regulated by glutathione that can be found in reduced (GSH) and oxidised (GSSG) forms.

Figure 2.1 contains a schematic of the equilibria present in a glutathione redox system for the formation of a single intramolecular disulfide bond. Consequently, when there are six cysteine residues and the possibility of 15 combinations of intramolecular disulfide combinations, the representation of the equilibria becomes more challenging and the exact mechanism of disulfide formation is difficult to predict. Glutathione also serves as a reducing agent that continuously disrupts the formed disulfides (whether they are inter- or intra-molecular) until the most stable structure is adopted. The final connectivity should yield the correct, fully oxidised peptide which is resistant to further redox reactions.

An additional consideration in attempts to oxidatively refold ICK peptides is that cysteine residues can additionally undergo a variety of reactions that include the formation of sulfenic (-SOH), sulfinic (-SO\(_2\)H) and sulfonic acid (-SO\(_3\)H) before they form disulfide bonds.

Despite the complexity of the redox reactions that mediate correct oxidative refolding, synthesis of many ICK peptides has been successful. A review by Reinwarth et al. summarises various oxidative refolding procedures; these include exposure to air, dimethyl sulfoxide (DMSO), a GSH/GSSG or a cysteine/cystine redox system, and using orthogonal cysteine protecting groups during the SPPS. In some cases, there were two or more isomers at the end of the refolding process. For example, two disulfide connectivities in peptides KIIIA and KIIIB gave, as expected, rise to differences in potency. The analytical method used to identify the disulfide pairs was proteolysis coupled with MS/MS fragmentation and two dimensional (2D) Nuclear Magnetic Resonance Spectroscopy (NMR). A similar approach was adopted in the synthesis of Amaranthus \( \alpha \)-amylase inhibitor (AAI) in order to monitor the intermediates of the
Figure 2.1: The mechanism of intramolecular disulfide formation. It relies on disulfide exchange between the redox buffer (GSSG/GSH) and the thiols in the peptide. Normally, the formation of intramolecular disulfide is more favorable and the ‘mixed’ intermediates are not prevalent.  

refolding process. Studies on oxidative refolding of ProTx-II stress the need to establish a reliable and physiologically relevant refolding buffer in order to yield an active ProTx-II fold. Air oxidation yielded a mixture of incomplete oxidation products and as a result, the products did not exhibit any inhibitory activity against Nav1.7.  

Our interest was focused on Huwentoxin-IV as it is one of the most well studied ICK binders of Nav1.7, exhibiting high potency and selectivity over other VGSCs in the PNS. In the following subsections, SPPS of the native peptide and photoprobe analogues and validation of the oxidative refolding conditions are discussed. In addition, the analytical and electrophysiological methods used to determine that oxidative refolding was successful in yielding functional synthetic Huwentoxin-IV analogues are reported.

### 2.1.2 Photoaffinity Probe Design

Electrophysiological and mutagenesis experiments have pinpointed the extracellular loops of the voltage sensor of domain II (VSD2) of hNav1.7 as the binding site of HwTx-IV, as discussed in more detail in Subsection 1.2.3. Additionally, various reports have focused on the residues of HwTx-IV that participate in the binding interactions with VSD2. According to Revell et al., who performed alanine scanning mutagenesis on HwTx-IV and tested the potency against Nav1.7 by patch-clamp electrophysiology, some mutations to alanine resulted in peptides that were unable to fold. Despite this, 21 single residues were successfully
mutated to alanine, giving rise to peptides with no significant change, loss or gain in inhibitory activity (Figure 2.2A). The authors selected positions that exhibited the most striking effects (E1, E4, F6 and Y33) for further mutagenesis analysis. Interestingly, the Y33W analogue exhibited a 12-fold increase in potency, an effect attributed to the higher hydrophobicity of tryptophan.59 Moreover, a double mutant of HwTx-IV [E1G E4G] with lower net charge around loop 1 exhibited higher potency (IC$_{50}$ = 0.5 ± 0.1 nM). F6Y mutation resulted in a 24-fold decrease whereas F6W in no change in potency.59 However, the potency of the triple mutant [E1G E4G Y33W] did not significantly improve (IC$_{50}$ = 0.4 ± 0.1 nM) and it was hypothesised that binding is driven mainly by hydrophobic and not electrostatic interactions. In a different alanine scan study,81 it was concluded that F6, W30 and Y33 residues composed a hydrophobic surface that, in conjunction with basic K32, is important for HwTx-IV affinity for Nav1.7, results that agree with Revell et al.59

![Figure 2.2: Huwentoxin-IV photoprobe design. A, Colour-coded sequence of HwTx-IV. Each residue is coloured according to the effect its substitution with alanine had to the potency against Nav1.7. Reported native HwTx-IV IC$_{50}$ = 17 ± 2 nM59 (Green: unknown, not substituted, Blue: 2-3 fold increase, Grey: no change, Orange: 2-3 fold decrease, Red: over 4-fold decrease or compete loss of activity) B, The residues that were substituted with photomethionine in each photoprobe are depicted in yellow. The residues that are thought to form the face of interaction with Nav1.7 are in red. C, The structures of photomethionine, propargyl glycine and biotinylated lysine.](image-url)

The design of photoprobes was based on the effect alanine mutations had on the potency of the peptide. Residues whose substitution with alanine resulted in little or no change in the inhibitory activity were considered for the incorporation of a diazirine moiety and an affinity tag or 'clickable' handle. As far as the position of the photolabile diazirine is concerned, efforts to incorporate it in close proximity to the
binding interface were made. Despite the desire to only substitute residues that are not important in the inhibitory activity of the peptide with a diazirine-containing amino acid, we considered the possibility that these positions might just be solvent-exposed and not participating in binding. Figure 2.2B highlights the theoretical face of interaction of HwTx-IV in red and the six positions that were chosen for substitution with photomethionine (Mp) in yellow. Photomethionine was chosen as it contains a small diazirine group that would likely not affect the fold of the peptide and the interactions with Nav1.7. In addition, propargyl glycine (Gp) and biotinylated lysine (Kb) were incorporated at position 13, which is located in a flexible loop 2, away from the sites of interaction. Another aspect of HwTx-IV binding to Nav1.7 was the possibility of the participation of lipids present in the cell membrane. As reported in SPR studies, HwTx-IV is only weakly binding to model membranes, unlike other toxin peptides like ProTx-II. However, substitution of charged residues E1 and E4 with glycine in combination with F6W and Y33W mutations resulted in higher potency, suggesting that increasing the hydrophobicity and the net charge of the peptide might enhance its availability for binding. These studies further highlight the lack of knowledge in the exact mechanism of inhibition by HwTx-IV.

We believe that a photocrosslinking approach will enable the elucidation of the binding interactions that govern the strong inhibitory effect of HwTx-IV on Nav1.7. To this end, a variety of peptides were synthesised and refolded (Table 2.1). Additional analogues that cover a wider variety of positions for photocrosslinking were commercially obtained.

### 2.1.3 Solid Phase Synthesis and Oxidative Refolding

Table 2.1 contains the peptides that were synthesised by fluorenylmethyloxycarbonyl and tert-butyl (Fmoc/\textsuperscript{6}Bu) SPPS. E1G E4G was synthesised as it was reported to be more potent than the native HwTx-IV. Initially, position 7 was chosen for the incorporation of photomethionine. Gp- and Kb-containing analogues were also synthesised.

The synthesis was done on a 20 μmol scale on a Rink amide resin and it is described in more detail in Chapter 6. The cleavage and deprotection of the 35mers was performed paying special attention to possible side reactions of sensitive residues, like C and W. Cleavage of the peptides was initially done for 3 h with agitation but it was found that shorter reaction time (1.5 h) resulted in decreased formation of the main by-product corresponding to a mass shift of +56 Da, possibly due to reaction of the \textsuperscript{6}Bu cation to form N-alkylated tryptophan. The 'cleavage cocktail' consisted of scavengers such as thioanisole, phenol, water
and reducing dithiothreitol (DTT) to ensure the cysteine residues remained reduced after deprotection. Preparative LC-MS purification was needed to isolate the peptide before it was subjected to oxidative refolding. At this point, the isolated peptide yield was typically 5-10%.

In order to achieve successful oxidative refolding, small scale oxidative folding of the native HwTx-IV and E1G E4G mutant was investigated using conditions that were previously reported.59 However, as the refolding was not successful, additional conditions were investigated. A 3:1 mM mixture of GSH/GSSG, 0.1 mM EDTA in ammonium bicarbonate (pH 8), and 7% v/v isopropanol resulted in high conversion of the reduced peptide to fully oxidised, typically in 16 to 48 hours. EDTA was used as a chelating agent that prevents thioulates from binding to metals present in the mixture and isopropanol has been used in other refolding buffers in order to increase solubility of the peptides.132 This reaction was done at low peptide concentration (0.1 mg mL\(^{-1}\)) to avoid precipitation and intermolecular reactions. The folding was terminated by addition of formic acid to acidify to pH 3.

### 2.1.4 LC-MS Characterisation

The oxidative refolding reaction was monitored by LC-MS and generally, all peptides showed high conversion to the fully oxidised product and low amount of singly or doubly mixed disulphides with glutathione.
In order to confidently detect the fully oxidised peptides, an Ultra Performance Liquid Chromatography system coupled to a high resolution mass spectrometer (UPLC-MS) was used. As the molecular weight of HwTx-IV and the various analogues is >4000 Da, a Δm of -6 Da corresponding to the formation of three disulfides is difficult to detect in multiply charged species using a low resolution mass spectrometer.

In the case of HwTx-IV, the multiply charged ions in the linear peptide (7+ to 3+) and in the refolded peptide (6+ to 3+) are shown in Figure 2.3A and B. In order to validate the complete oxidation, the isotopic distribution of these ions was compared with the theoretical distribution of the reduced and oxidised species. Figure 2.3C and D show the good agreement of the observed isotopic distribution of the 3+ and 4+ ions with the prediction.

The chromatograms and mass spectra of all synthesised and commercially obtained peptides are included in the Appendix.

### 2.1.5 NMR Characterisation

Even though only one fully oxidised species was identified by UPLC-MS, the correct disulfide connectivity and fold obtained under the oxidative refolding conditions was confirmed by additional 1H and 2D NMR experiments. Due to the large quantity of peptide required for such an experiment, NMR analysis was only performed using native HwTx-IV.

Total correlation spectroscopy (TOCSY) and nuclear Overhauser effect spectroscopy (NOESY) experiments on the native HwTx-IV have been used to elucidate its solution structure (PDB 1MB6) and therefore, proton assignments have been published.81,147

Three spectra were recorded on a sample of ~1.5 mM refolded HwTx-IV in 10% v/v D2O with the help of Dr Christopher Douse. Firstly, the 1H spectrum indicated that the peptide had indeed adopted a fold, based on the wide dispersion in the amide proton region of the spectrum (Figure 2.4A). This observation suggests that the backbone amide protons reside in distinct chemical environments, rather than an average of many conformations that would be expected from an unfolded peptide (random coil). The cross-peaks in the 1H-1H TOCSY spectrum signify the J-coupling networks and along with the reported chemical shift values,147 they were used to identify the various side chains. Unfortunately, in our TOCSY spectrum the protons corresponding to S19, R26 and T28 were not identified.
Figure 2.3: Mass spectra of synthesised Huwentoxin-IV. A, Spectrum before refolding, all C residues are reduced. B, Spectrum after oxidative refolding. C, Comparison of the 3+ ion isotopic distribution before and after oxidative refolding. There was good agreement with the theoretical isotope model. D, Comparison of the 4+ ion with the predicted spectra.
The additional 2D experiment on the refolded HwTx-IV was NOESY. Figure 2.4B highlights the amide proton region and the through-space correlations with other protons. Such an experiment is useful in determining the fold of the peptide, as it reveals protons that are in close proximity despite belonging to residues that are far apart in the amino acid sequence. Through-space interactions in HwTx-IV due to the correct disulfide connectivity as found in the NMR solution structure were identified and highlighted in Tables 2.2 and 2.3. For instance, NOEs between protons in L3 and C16 could only be due to a fold that, as found in the NMR structure, brings them at a ~ 2 Å distance (Figure 2.5). Moreover, as the correctly folded HwTx-IV forms a double-stranded antiparallel β-sheet, NOEs between the side chains of residues participating in the β-sheet are highlighted in Table 2.3. For reference, L22–S25 and W30–Y33 form the two strands.
### Table 2.2: Refolded HwTx-IV NOEs indicative of the correct fold and the respective distance between the assigned protons as found in the NMR structure (PDB 1MB6).

<table>
<thead>
<tr>
<th>NOEs indicative of correct folding</th>
<th>Distance (^{147}) (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>residues</td>
<td>L3</td>
</tr>
<tr>
<td>(\delta (\text{ppm})/ ^{1}\text{H})</td>
<td>1.41</td>
</tr>
<tr>
<td></td>
<td>8.70</td>
</tr>
<tr>
<td>residues</td>
<td>L3</td>
</tr>
<tr>
<td>(\delta (\text{ppm})/ ^{1}\text{H})</td>
<td>0.88, 0.99</td>
</tr>
<tr>
<td>residues</td>
<td>L22</td>
</tr>
<tr>
<td>(\delta (\text{ppm})/ ^{1}\text{H})</td>
<td>0.83, 1.76</td>
</tr>
<tr>
<td>residues</td>
<td>S20</td>
</tr>
<tr>
<td>(\delta (\text{ppm})/ ^{1}\text{H})</td>
<td>3.67, 4.14</td>
</tr>
<tr>
<td>residues</td>
<td>C2</td>
</tr>
<tr>
<td>(\delta (\text{ppm})/ ^{1}\text{H})</td>
<td>3.24, 3.35</td>
</tr>
</tbody>
</table>

### Table 2.3: Refolded HwTx-IV NOEs between residues on the antiparallel \(\beta\)-strands and the respective distance between the assigned protons as found in the NMR structure (PDB 1MB6).

<table>
<thead>
<tr>
<th>Interstrand NOEs</th>
<th>Distance (^{147}) (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>residues</td>
<td>L22</td>
</tr>
<tr>
<td>(\delta (\text{ppm})/ ^{1}\text{H})</td>
<td>0.35</td>
</tr>
<tr>
<td>residues</td>
<td>K32</td>
</tr>
<tr>
<td>(\delta (\text{ppm})/ ^{1}\text{H})</td>
<td>9.45</td>
</tr>
<tr>
<td>residues</td>
<td>S25</td>
</tr>
<tr>
<td>(\delta (\text{ppm})/ ^{1}\text{H})</td>
<td>7.69</td>
</tr>
</tbody>
</table>

Finally, our findings based on TOCSY and NOESY spectra validated that the oxidative refolding conditions used afford the correct disulfide connectivity in HwTx-IV and we hypothesised that this would apply to the other analogues.
2.1.6 Patch-clamp Characterisation

The final step in validating our photoprobes is patch-clamp electrophysiology. As discussed in Subsection 1.2.2.1, patch-clamp electrophysiology is the ‘golden standard’ method for validation of ion channel inhibitors. Correctly folded peptides are expected to exhibit an inhibitory effect at low concentrations, typically at low micromolar or nanomolar if compared to native HwTx-IV (reported IC$_{50}$ = 26 nM$^8$). In this case, HEK293 cells expressing hNav1.7 were patched under different concentrations of our synthesised peptides and the effect on the Na$^+$ current was recorded over time. Initially, an automated platform was used to screen our peptides. Total current inhibition at 300 nM of all peptides over 17 minutes was observed (Ion-Flux, data not shown). This observation was quite encouraging as all peptides seemed to inhibit Nav1.7 in a similar fashion to HwTx-IV. However, this platform was not optimised for binders with slow on and off rates such as ICK peptides so the peptides were further subjected to manual patch-clamp experiments.

Due to limitations in time, our synthesised photoprobes Mp7 Kb13 and Mp7 Gp13 were validated at 30 and 100 nM. Figure 2.6 contains a graph of the fractional current over time; at 30 nM of Mp7 Gp13 and Mp7 Kb13, 42% and 52% of the maximum Na$^+$ current was observed. This result was encouraging as the reported IC$_{50}$ value of native HwTx-IV was 26 nM$^8$ as determined using a similar manual protocol; the double mutation in the sequence did not cause a significant loss in potency. Moreover, it is highly unlikely that an unfolded or misfolded peptide would exhibit such high inhibitory activity, therefore electrophysiology also validated the correct fold of the synthesised photoprobes.

Figure 2.5: Through-space interactions identified by NOESY mapped in the published structure of HwTx-IV (PDB 1MB6). A, Interstrand region B, Additional significant NOEs indicative of correct disulfide connectivity.
Figure 2.6: Inhibitory effect of Mp7 Kb13 and Mp7 Gp13 on Na⁺ currents. The potency does not differ significantly from native HwTx-IV with reported IC₅₀ = 26 nM. Data obtained from a manual patch-clamp assay by Elisabeth Payne.

As mentioned in Subsection 2.1.2, our photoprobe library included peptides that had been commercially obtained (Smartox Biotechnology, France). With the help of Dr Tianbo Li, IC₅₀ curves were obtained for all Mp probes in HEK293 cells expressing Nav1.7 (Figure 2.7). As a reference, HwTx-IV was also tested (IC₅₀ = 28 nM). Peptides Mp5, Mp7, Mp21, Mp27, Mp29 and Mp35 all exhibited IC₅₀ values in the range of 13-80 nM. We were pleased that the incorporation of photomethionine at these six positions did not significantly affect the potency; the photoprobes behaved as good mimics of HwTx-IV.

Figure 2.7: IC₅₀ curves of Mp probes as determined by manual patch-clamp electrophysiology. All Mp probes retain nM potency. Data obtained by Tianbo Li. (n = 3 and error bars represent S.E.M.). The residues that were substituted with photomethionine in each photoprobe are highlighted in yellow.
2.2 Photoaffinity Probes Based on PF-06456384

2.2.1 Photoaffinity Probe Design and Synthesis

Our strategy involved the development of a photoprobe that targets an alternative binding site on the voltage sensor of domain IV (VSD4) of Nav1.7. The aryl sulfonamide unique binding site was identified in a crystal structure by Ahuja et al. (further discussed in Subsection 1.2.2.5) and the determinants of the isoform selectivity were revealed by docking different aryl sulfonamide inhibitors in Nav1.7 VSD4-NavAb chimera.\(^5\)

![Structure of PF-06456384](image)

Figure 2.8: Structure of PF-06456384 developed and provided by Pfizer, Inc.

Our photoprobe was based on the aryl sulfonamide inhibitor PF-06456384 and in this Thesis it will be referred to as PF-384 or 1 (Figure 2.8). PF-384 was originally developed by Pfizer as a lead Nav1.7 inhibitor for intravenous administration. The reported IC\(_{50}\) was 0.01 nM in a HEK293 cell line expressing Nav1.7 and it is >300 times selective overall the other Nav subtypes.\(^5\) It exhibits high structural similarity with GX-936 and Storer et al. published a model of PF-384 binding to the VSD4-NavAb chimera based on the published crystal structure.\(^5\) This model is depicted in Figure 2.9 and it highlights a similar mode of binding, with the aryl sulfonamide buried within the \(\alpha\)-helices of the VSD and the piperidine moiety extending towards the extracellular loops that connect S1-S2 and S3-S4. It also forms a hydrogen bond with E1589. Finally, despite the high potency against Nav1.7, the compound failed to exhibit efficacy in a mouse formalin pain model. The authors stressed that PF-384 could be a valuable tool to better understand target engagement and reported the synthesis of a benzophenone and a fluorescent analogue.\(^5\)

PF-384 was provided by Dr Ian Storer and further functionalisation was pursued to introduce a linker that contains a diazirine photolabile group and biotin as an affinity tag. The choice of diazirine was mainly because of its small size and desirable UV activation properties. Diazirines require short irradiation time.
CHAPTER 2. CHEMICAL TOOLS TO STUDY NAV1.7

Figure 2.9: Model of PF-06456384 binding on hNav1.7/VSD4 chimera. This model is based on the crystal structure of GX-936 by Ahuja et al. The piperidine moiety is in close proximity with the extracellular S1-2 and S3-4 linkers of hNav1.7 (pink) whereas the aryl sulfonamide part of the inhibitor is located within the hNav1.7 sequence that forms the transmembrane segments (grey).

and the carbene intermediate formed is highly reactive thus minimising non-specific crosslinking. One of the disadvantages of diazirine photocrosslinking, however, is that the high reactivity of carbenes can lead to low photocrosslinking yields as they are rapidly quenched by water. Biotin was incorporated in order to enable enrichment of the crosslinked Nav1.7 if the crosslinking occurred in a complex system such as cell membranes. The biotin moiety can also be used in western blotting analysis with conjugated streptavidin-horseradish peroxidase or a fluorescent dye.

The photoprobe was synthesised through amide coupling on the piperidine moiety; based on the model of binding to VSD4, a long linker could prevent the diazirine moiety from attacking bonds on the extracellular loops. Therefore, a short linker that composed of L-photoleucine and biotin was selected (Scheme 2.1). The succinimide ester of biotin 2 and L-photoleucine 3 are commercially available, and reacted to afford linker 4 (76% yield). 4 was activated through reaction with N-hydroxy succinimide and EDC·HCl overnight. PF-384 1 was added to the reaction mixture along with triethylamine in order for the second amide coupling to take place. The overall reaction had a 43% yield and product 5 was purified by reverse-phase preparative LC-MS.
In order to confirm the formation of 5, the $^1$H NMR spectrum of the parent compound 1 was employed. TOCSY spectra of 1 and 5 were valuable for the assignment of the multiple aromatic and aliphatic protons in the product. Additionally, the $^1$H NMR assignment of 4 through TOCSY further validated the chemical shift assignment to the protons in the photoleucine-biotin portion of the molecule. Even though there are two secondary amines in 1, selective coupling took place. The amide bond formation on the piperidine was validated through $^1$H NMR. More specifically, the chemical shift of the $\sim$CH$_2$ protons adjacent to the pyridine ring was not affected in the product of the coupling reaction. Previous studies described functionalisation of 1 through selective amide bond formation on the piperidine without the use of protecting groups, presumably due to steric hindrance.\textsuperscript{51} The photoprobe PFDB (or 5) was used in photocrosslinking experiments as described in Chapter 4 after patch-clamp characterisation.

### 2.2.2 Patch-clamp Characterisation

Our photoprobe PFDB along with the parent compound PF-384 were evaluated by patch-clamp electrophysiology (Figure 2.10). The IC$_{50}$ value measured by Tianbo Li in this assay (217 nM) is a lot higher than the reported value (0.01 nM),\textsuperscript{51} highlighting the differences in measured potency that can be obtained.
by different patch-clamp protocols. In comparison with the measured IC$_{50}$ of PF-384, PFDB exhibits a 3-fold decrease in potency (688 nM) and still no effect on Nav1.5. The inhibitory activity was not abolished completely in PFDB, and the selectivity for Nav1.7 was retained, therefore it was taken forward to photocrosslinking experiments. The observed decrease in potency would enable competition experiments with the parent compound that would aid validation of selective photocrosslinking.

![Figure 2.10: Patch-clamp characterisation of PF-384 and PFDB against Nav1.5 and Nav1.7. The selectivity and potency for Nav1.7 is retained in the photoprobe PFDB. (PFDB IC$_{50}$ = 688 nM and 217 nM for PF-384). The compounds exhibit higher potency against inactivated channels at -50 mV. Data obtained by Tianbo Li. (n = 3 and error bars represent S.E.M.)](image)

### 2.3 Conclusions

This chapter has focused on the design of photoaffinity probes that target two different binding sites on hNav1.7. Firstly, photoprobes based on HwTx-IV were developed by incorporating photomethionine, a diazirine-containing amino acid, at positions in the sequence that are likely to be close to the face of interaction with VSD2 of hNav1.7. This design was based on an alanine scan and the solution structure of HwTx-IV. It aimed to maximise the probability of achieving successful photocrosslinking on VSD2 by exploring six different positions (Mp5, Mp7, Mp21, Mp27, Mp29, Mp35). In addition, analogues of Mp7 bearing biotin or an alkyne handle at position 13 were synthesised. Based on mass spectrometry and 2D NMR analysis of synthesised HwTx-IV, we were able to validate that the optimised oxidative refolding conditions afford correctly folded ICK peptides. These probes proved to be potent inhibitors of hNav1.7 in electrophysiological assays.
In addition, VSD4 of hNav1.7 is of interest as it is targeted by highly potent and selective aryl sulfonamide small molecules. To this end, synthesis of a biotin-diazirine analogue of preclinical candidate PF-384 was successful in yielding photoprobe PFDB that inhibits Na⁺ currents in a patch-clamp assay. The co-crystal structure with a relevant molecule aided the design of PFDB, as it revealed that the piperidine moiety in the molecule points away from the detrimental binding interactions between the aryl sulfonamide and the S4 helix of VSD4.

A more detailed discussion of the conclusions drawn from the development of the photoprobes is included in Chapter 5. The synthesised photoprobes were employed in photocrosslinking experiments described in Chapter 4.
Chapter 3

Mass Spectrometry Proteomics of Nav1.7

In Chapter 2, the design and synthesis of potent photocrosslinking probes for Nav1.7 is discussed. In order to identify the binding sites of these photoprobes, robust mass spectrometry-based detection of Nav1.7 is required. Therefore, the focus of this chapter is the optimisation of proteomic detection of Nav1.7 in an engineered cell line and nociceptors derived from iPSCs. Firstly, the challenges associated with the study of membrane proteins are highlighted (Section 3.1). A variety of approaches were employed to maximise the sequence coverage of Nav1.7 in an engineered cell line (Section 3.2). Finally, since iPSCs have been employed as a new model system in drug discovery efforts, maturation of neurons derived from inherited erythromelalgia patients with Nav1.7 mutations was attempted. The maturation process was monitored through a preliminary total proteome quantitative mass spectrometry analysis (Section 3.3).

3.1 Challenges in Studying Membrane Proteins

Membrane proteins such as Nav1.7 are intrinsically difficult to study as they are embedded in a lipid bilayer. Once extracted, they are unstable unless in the presence of detergent micelles, amphipols or styrene maleic acid lipid particles (SMALPs)\textsuperscript{149}. In many cases, the stability and function of a membrane protein becomes compromised soon after extraction from the lipid bilayer.

Although impressive advances in 'top-down' membrane proteomics have been reported,\textsuperscript{150,151} there are still many challenges in isolation and detection of membrane proteins in both 'top-down' and 'bottom-up' approaches. In 'bottom-up' proteomics,\textsuperscript{118} the proteins are proteolytically digested and the subsequent complex peptide mixture is analysed by LC-MS/MS. The expression levels of membrane proteins are low
compared to cytosolic proteins and as a result, cytosolic peptides dominate the MS/MS spectra. The digestion peptides of membrane-spanning regions are usually hydrophobic; issues with aggregation and low ionisation cause poor detection in the LC-MS/MS. Efforts to improve the analysis of membrane proteins involve membrane enrichment by subcellular fractionation. Various methods have been reported, including ultracentrifugation of mechanically lysed cells to obtain crude membranes and density gradient centrifugation to isolate different organelles according to their density.\textsuperscript{152} Although these techniques are very effective, abundant contaminating proteins are still present in the subcellular fractions.\textsuperscript{152}

If the protein of interest is a plasma membrane (PM) protein, there is an additional consideration; PM proteins are synthesised, glycosylated and trafficked to the plasma membrane through the Golgi apparatus and ER. Once localised in the PM, they can be endocytosed and cycled back to the PM. Approaches to enrich PM proteins that have extracellular presence involve surface lysine biotinylation with a cell impermeable biotinylation reagent or selective glycan biotinylation, and streptavidin affinity purification.\textsuperscript{153,154} Glycan biotinylation has been employed to monitor changes in the surface glycoproteome of embryonic stem cells differentiating to neurons.\textsuperscript{155} An additional enrichment method, which however does not ensure the enrichment of only PM-localised proteins, is immunoprecipitation using an antibody and protein A or G agarose resin to selectively capture the protein of interest. Immunoprecipitation of VGSCs is discussed in more detail in Subsection 3.2.3.

One of the main problems in proteomic analysis of detergent solubilised proteins is the incompatibility of most detergents with downstream LC-MS/MS. In order to circumvent this issue, a variety of approaches have been developed. One of these approaches is filter-aided sample preparation (FASP) which involves the use of a centrifugal spin filter that retains high molecular weight species and enables buffer-exchange in order to remove the detergent and exchange it with buffers of interest.\textsuperscript{121} Proteolysis is carried out in compatible buffer in the spin filter and the low molecular weight peptides are collected by centrifugation. Another approach is the use of MS-compatible detergents such as ProteaseMax (Promega) and RapiGest SF Surfactant (Waters). These detergents ensure the solubilisation of hydrophobic membrane proteins while degrading during digestion.

Various digestion additives and approaches have been explored in pursuit of better coverage of membrane proteins, and excellent publications summarise these attempts.\textsuperscript{119,153,156,157,158} It should be noted that while these approaches have increased the detection of certain membrane proteins in certain cell lines, protein-specific optimisation and protocols are required. Our efforts to maximise the sequence coverage
CHAPTER 3. MASS SPECTROMETRY PROTEOMICS OF NAV1.7

of Nav1.7 by LC-MS/MS proteomics are discussed in the Section 3.2.

3.2 Detection of Nav1.7 in an Engineered Cell Line

3.2.1 Total Lysate Proteomics

Our analysis started from a simpler system; a HEK293 cell line stably expressing the α subunit of Nav1.7 in addition to β₁ and β₂ subunits developed by Peter Cox at Pfizer Neusentis. Co-expression with β subunits was chosen due to their favourable role in trafficking Nav1.7 to the plasma membrane. The expression of functional Nav1.7 was validated by electrophysiological recordings that yielded Na⁺ current (average 2.35 nA with 64% of the cells exhibiting > 1 nA). This cell line will be referred to as HEK Nav1.7β₁β₂ in this Thesis. HEK293 cells are not known to express any relevant VGSCs, therefore they serve as an appropriate negative control cell line.

The first set of experiments involved a total proteome analysis by LC-MS/MS. The cells were lysed using a lysis buffer containing 1% v/v Triton X-100, 10% v/v glycerol, 50 mM HEPES pH 7.4, 150 mM NaCl, 1.5 mM MgCl₂, and protease inhibitors. This lysis buffer had been used before in glycosylation studies of Nav1.7 in overexpressing HEK293 cells.

Lysis buffers containing alternative detergents such as digitonin, n-Dodecyl β-D-maltoside (DDM), n-Octyl-β-D-Glucoside (OG), CHAPS were screened and the extraction efficiency of Nav1.7 was evaluated by western blotting densitometry using an α-potNav antibody. The relative intensity after centrifugation of the lysate to remove insoluble material with respect to the lysate before centrifugation was calculated (Figure 3.1). Triton X-100/glycerol extraction resulted in the highest levels of soluble Nav1.7 in membrane fractions therefore, for all subsequent experiments Triton X-100/glycerol was employed unless otherwise stated.

The sample preparation was performed following the FASP protocol as it is versatile and has been used before for whole proteome analysis with a focus on membrane proteins. Following washes with urea, the detergent was removed and replaced with ammonium bicarbonate buffer that is the buffer of choice for trypsin digestion. In parallel, a sample of HEK293 lysates was digested as a negative control. The peptides were desalted following the StageTip method.

After LC-MS/MS and MaxQuant data analysis, 2020 proteins in HEK Nav1.7β₁β₂ and 1845 proteins in HEK293 samples were identified. Considering the challenges in detecting membrane proteins, it was
not surprising that Nav1.7 was not one of the identified proteins in the overexpressing cell line. Na⁺/K⁺-ATPase which is an abundant plasma membrane protein was identified in both cell lines (17 and 13 unique peptides), unlike β₁ and β₂ subunits that were identified through a considerable number of unique peptides only in the HEK Nav1.7β₁β₂ samples (Table 3.1).

<table>
<thead>
<tr>
<th>Protein ID</th>
<th>HEK Nav1.7β₁β₂</th>
<th>Sequence Coverage (%)</th>
<th>Unique peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nav1.7</td>
<td>---</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>β₁</td>
<td>37</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>β₂</td>
<td>43</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Na⁺/K⁺-ATPase</td>
<td>25</td>
<td>17</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.1: Sequence coverage and unique peptides obtained by total lysate proteomic analysis.

### 3.2.2 Subcellular Fractionation Proteomics

Since Nav1.7 was not identified by the total lysate proteomics, subcellular fractionation was chosen in order to enrich membrane proteins. Cells were subjected to hypotonic shock and mechanically lysed through sheering. Firstly, a low speed centrifugation (500g, 5 min) was performed to separate the nuclear fraction.
CHAPTER 3. MASS SPECTROMETRY PROTEOMICS OF NAV1.7

The crude membrane fraction was collected as a pellet after ultracentrifugation of the post-nuclear fraction (100,000g, 1 h). The supernatant was the cytosolic fraction. The membrane fraction was solubilised in lysis buffer and both membrane and cytosolic fractions were digested in FASP spin filters.

Figure 3.2: Proteomic results in membrane and cytosolic fractions of HEK Nav1.7β1β2. A, Heat map representing the ion intensities corresponding to membrane proteins of interest in triplicate samples. Higher abundance is observed in the membrane fractions (grey represents non quantified proteins). B, Venn diagram highlighting the number of proteins identified in each fraction. There is ~ 10% overlap between cytosolic and membrane samples.

<table>
<thead>
<tr>
<th>Protein ID</th>
<th>HEK Nav1.7β1β2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sequence Coverage (%)</td>
</tr>
<tr>
<td>Nav1.7</td>
<td>6</td>
</tr>
<tr>
<td>β1</td>
<td>34</td>
</tr>
<tr>
<td>β2</td>
<td>43</td>
</tr>
<tr>
<td>Na+/K+-ATPase</td>
<td>37</td>
</tr>
</tbody>
</table>

Table 3.2: Sequence coverage and unique peptides obtained in the membrane fraction.

Figure 3.2A contains a heat map representing the ion intensity of peptides that correspond to identified proteins of interest. The membrane proteins identified in total lysate proteomics (Table 3.1) were also identified in the membrane fraction with high intensity. Moreover, Nav1.7 was identified only in the membrane fraction. Figure 3.2B depicts a Venn diagram of the protein identifications in both fractions, highlighting a small overlap of commonly identified proteins. This sample preparation method is never free of crosscontaminants between fractions therefore this result was expected. Nav1.7 detection in the HEK
Nav1.7β1β2 membrane fraction samples was achieved through the identification of 10 unique peptides, giving rise to 6% sequence coverage (Table 3.2). More unique peptides were identified in the case of the control protein, however, the number of β1 and β2 unique peptides did not improve significantly with respect to the previous total lysate proteomic experiment. It was evident that optimisation was required as the sequence coverage of the protein of interest was still low. The difficulty in detecting Nav1.7 peptides is also a result of various post-translational modifications, such as glycosylation that contribute to a complex mixture of Nav1.7 glycosylation states. In our experiments, deglycosylation was not attempted in order to improve the sequence coverage.

Figure 3.3: Western blotting of HEK Nav1.7β1β2 and HEK293 cell lines. Membrane proteins of interest are enriched in the membrane (M) fraction compared to the cytosolic (C) and total lysate (TL) samples. PNGase F treatment revealed the high glycosylation state of β2 and the non specific interaction of the α-β2 antibody present in the HEK293 cell line and the cytosolic fractions (~ 40 kDa). Deglycosylation did not seem to affect the detection of Nav1.7 and Na+/K⁺-ATPase. Arrows indicate the expected molecular weight of unglycosylated Nav1.7 (230 kDa) and β2 (24 kDa).

Our proteomic MS analysis was also complemented with western blotting analysis. In Figure 3.3, α-pan Nav, α-Na⁺/K⁺-ATPase and α-β2 antibodies were employed to detect the distribution of the respective proteins in the membrane and cytosolic fractions of HEK293 and HEK Nav1.7β1β2. As expected, Nav1.7 and β2 were not detected in HEK293 cells, and all membrane proteins were enriched in the membrane fraction.
with respect to the total lysate (TL), with no presence in the cytosolic fraction. Treatment with amidase PNGase F was explored in order to improve detection of heavily glycosylated \( \beta_2 \). However, it revealed many bands that probably correspond to incomplete deglycosylated states of \( \beta_2 \) and a non-specific binder of the antibody found only in the total lysate and cytosolic samples of both cell lines. The antibody against \( \beta_1 \) revealed even more non-specific interactions and was therefore not used.

### 3.2.3 Immunoprecipitation

As discussed in Subsection 1.3.2, immunoprecipitation (IP) is the enrichment method of choice for studying PTMs and binding partners of VGSCs. Immunoprecipitation of Nav1.2 and Nav1.5 by various groups has achieved sequence coverage in the 30-50% range,\(^{46,126,127,128,129} \) highlighting the difficulty in detecting specific parts of the sequence despite high levels of enrichment. No similar studies on Nav1.7 have been reported according to our knowledge, therefore IP was explored in order to increase the sequence coverage of Nav1.7.

Immunoprecipitation is based on the strong and specific antibody-protein binding interaction. The method generally involves chemically crosslinking an antibody to protein G or A agarose resin. Cell lysate is pre-cleared via incubation with protein G or A resin in order to remove non-specific binders for the IP experiment. The pre-cleared lysate is incubated with the Ab-crosslinked resin and mild washes are performed to preserve protein complexes and remove non binders. Finally, the protein of interest and its binders are eluted in denaturing or non-denaturing conditions for further analysis including western blotting or FASP digestion. In order to identify the binding partners that have co-immunoprecipitated with the protein of interest, an additional control experiment is performed; a non-specific IgG is crosslinked to a different sample of protein G or A resin and IP is performed. Quantitative and statistical analysis of the proteins identified in both IP experiments reveals the confident binding partners of the protein of interest. Figure 3.4 depicts the experimental design in order to enrich Nav1.7 from HEK Nav1.7\( \beta_1\beta_2 \) and analyse the potential co-eluting binding partners. The experiment was performed in three biological replicates.

The eluted proteins were processed using the FASP protocol for LC-MS/MS analysis. Protein quantification was performed by Label Free Quantification (LFQ) where the peak ion intensities of peptides that correspond to the protein sequence are employed. The ion current therefore provides an approximation of protein abundance in the sample.\(^{160} \) LFQ was performed using the built-in algorithm found in MaxQuant data analysis software and the data was processed on Perseus. For data analysis, only proteins with at
least two unique peptides in at least two out of three replicate samples were considered. Two sample \( t \)-test analysis was performed in order to identify significantly enriched proteins in the pan Nav IP with respect to the control IgG IP (Figure 3.5).

The statistical analysis pinpointed a small number of significantly enriched proteins in the pan Nav IP sample. As expected, Nav1.7 was enriched in the pan Nav IP samples. Interestingly, oxysterol-binding proteins 9, 10 and 11 were also enriched. Oxysterol-binding proteins are involved in trafficking of lipids between membranes and changing their composition (bending). They are able to bind both specific lipids and membranes.\(^{161} \) It is not a surprise, therefore, that other membrane-bound proteins were enriched. Another prominent protein was E3 ubiquitin ligase HERC2 which has not been reported to interact with VGSCs. However, endocytosis of Nav1.7 has been hypothesised to be regulated by E3 ubiquitin ligase NEDD4-2 in neurons (Subsection 1.2.1).\(^{38,40} \) It is not therefore unlikely that another E3 ligase can interact with Nav1.7. Interestingly, while investigating the possible relationship of neuralised like protein 4 (NEURL4) with Nav1.7, a published immunoprecipitation study revealed that HERC2 and NEURL4 form a complex in centrosomes, thus regulating their architecture via promotion of CP110 ubiquitination.\(^{162} \) IP of CP110 in HEK293 cells contained HERC2 and NEURL4 as observed by western blotting. An unexpected
Figure 3.5: LFQ of pan Nav IP. Two-sample t-test was performed in Perseus (permutation-based FDR, 250 permutations, FDR 0.01, $S_0 = 0.5$) and the $-\log p$ value was plotted against the $\log_2$ difference in the mean ion intensity of proteins identified in pan Nav IP versus control IgG IP. Proteins of interest are highlighted in green. As expected, Nav1.7 is highly enriched in the pan Nav IP sample. Potential interactors include oxysterol-binding proteins and HERC2. β subunits are not significantly enriched.

observation was the poor enrichment of β subunits. β2 is expected to be covalently bound to Nav1.7 via a disulfide bond and therefore issues with transient complex IP should not apply in this case. One of the major limitations of this experiment is the use of an engineered cell line that does not express Nav1.7 endogenously. In this system, even though the statistical analysis highlighted non-artefact from artefact binders, it is expected that Nav1.7 will form non-physiologically relevant complexes with proteins that it does not normally associate with in neurons. However, this experiment was a step forward in the initial proteomic analysis of this VGSC in a simple system.

In order to answer some of the questions regarding the lack of β subunit enrichment, the immunoprecipitation experiment was visualised by western blotting (Figure 3.6). The enrichment of Nav1.7 is evident, however, a significant amount of β2 remains in the supernatant. The observation that the low MW band corresponding to β2 is not detected in the input or the supernatant samples generated the hypothesis that only a small subpopulation of β2 actually binds to Nav1.7 or that the interactions are very transient (lack of
disulfide bond) to be maintained during the IP experiment. It should be considered that a large proportion of Nav1.7 and β2 in this overexpressing cell line are not expected to reside in the plasma membrane, but to be embedded in intracellular membranes such as the ER and Golgi. It is likely that the intracellular pools of these proteins do not form complexes unless they are correctly folded in the plasma membrane. This hypothesis can help explain the co-IP of oxysterol-binding proteins that are involved in trafficking mechanisms and the possible ubiquitination by HERC2, marking Nav1.7 for endocytosis. Further investigation is required to confidently elucidate the complexes Nav1.7 participates in. Immunoprecipitation of Nav1.7 from a more physiologically relevant system such as neuronal cells will be highly beneficial. This was attempted through a TAP-tag approach by Kanellopoulos et al. with the limitation that weaker interactions are disrupted by the extensive washes in this approach (discussed in more detail in Subsection 1.3.2).\textsuperscript{130} It should be noted that there was no overlap between the proteins identified by the TAP-tag approach in neurons and our IP experiment in HEK Nav1.7β1β2 cells.

In order to trap transient interactions, chemical crosslinking can be employed. It is a technique that has been used extensively in the literature.\textsuperscript{163,164,165,166} However, the data analysis of crosslinked peptides is more demanding and challenging.\textsuperscript{167} An additional control for future experiments should be pan Nav immunoprecipitation of the control HEK293 cell lysate in order to confirm that there is no overlap with the potential interactors as identified in the HEK Nav1.7β1β2 IP. Finally, our interest is mainly focused on

**Figure 3.6:** Western blotting of HEK Nav1.7β1β2 immunoprecipitated with α-pan Nav. β2 co-immunoprecipitates with the α subunit. Further investigation is required to understand the reason the lower MW band of β2 is not identified in the input sample. Sup\textsuperscript{n} refers to supernatant.
CHAPTER 3. MASS SPECTROMETRY PROTEOMICS OF NAV1.7

the functional population of Nav1.7, therefore it would be beneficial to distinguish the intracellular from
the plasma membrane-bound population as functional Nav1.7 is expected to form more physiologically
relevant complexes.

Despite the limitations in our system, immunoprecipitation of Nav1.7 achieved a good coverage of 36%
by identifying 69 unique peptides (89 peptides overall) by PEAKS data analysis. To our knowledge, LC-
MS/MS coverage data on Nav1.7 have not been reported in the literature therefore this is the first report
of significant Nav1.7 detection in an engineered cell line.

3.2.4 Cell Surface Biotinylation

In order to increase the sequence coverage of Nav, plasma membrane bound proteins were enriched by
cell surface biotinylation. HEK Nav1.7β1β2 and HEK293 cells were treated with the highly reactive sulfo-
NHS-Biotin reagent (Figure 3.7). This reagent has dual properties; it is an activated analogue of biotin
which can react with N-terminal or lysine amines to form a stable amide bond as well as it bears a charged
sulfo moiety that prevents cell membrane diffusion. Sulfo-NHS-Biotin is therefore a cell surface, non-
specific, amine labelling reagent. In the following experiments, the cells were kept on ice in order to
reduce any intracellular uptake of the biotinylation reagent. The reaction was quenched with glycine and
the unreacted Sulfo-NHS-Biotin was removed by washing.

![Figure 3.7: Structure of Sulfo-NHS-Biotin](image)

Cell surface labelled cells were lysed and a neutravidin pull-down was performed in order to enrich the
biotinylated proteins. After stringent washes using buffers that contain 4 M urea and 1% v/v SDS in order
to remove the non-biotinylated background proteins, on-bead digestion of the proteins was performed
using trypsin. After desalting, peptides were analysed by LC-MS/MS. A database search using MaxQuant
revealed 1602 proteins in HEK Nav1.7β1β2 and HEK293 samples combined. Unfortunately, Nav1.7 was
not identified in this experiment, however, β1 and β2 subunits were the most enriched proteins in HEK
Nav1.7β1β2 by a log2 difference of 8.0 and 9.9, respectively. The α subunit of Na’/K’-ATPase was not
significantly enriched in either sample.

As the pull-down on the protein level did not enable the detection of Nav1.7, pull-down of biotinylated peptides was explored. This method has been reported to increase the number of protein IDs in NHS-Biotin labelled samples by ten times compared to pull-down on the protein level.\textsuperscript{168} Additionally, the fraction of proteins that were identified through biotin-modified peptides was considerably higher.\textsuperscript{168} This method exploits the favourable properties of tryptic peptides; fewer non-specific interactions with neutravidin agarose beads, less steric hindrance, more availability to form neutravidin-biotin complexes and stability in organic solvents that disrupt the biotin-neutravidin interactions.

\textbf{Figure 3.8:} Sequence coverage obtained by surface biotinylation, membrane enrichment and peptide pull-down. Only one peptide on Nav1.7 is found in an extracellular loop.

After surface biotinylation and total cell lysis of a HEK Nav1.7β₁β₂ sample, 1076 proteins were identified by PEAKS proteomic analysis software. Even though lysine biotinylation was set as a variable modification, practically all identified peptides were biotinylated (3406 out of 3418 peptide-spectrum matches).
The experiment was repeated with subcellular fractionation. In the crude membrane fraction of surface biotinylated HEK Nav1.7β1β2, 2692 proteins were identified with 10435 out of 10513 peptide-spectrum matches exhibiting biotin modifications.

This experiment highlighted some faults in our sample preparation. Firstly, the majority of identified proteins are not localised in the cell surface as identified using the cell surface atlas. Secondly, most identified β1, β2 and Nav1.7 biotinylated peptides correspond to intracellular parts of the sequence (Figure 3.8). In spite of the experiment being carried out on ice, there was significant uptake of the biotinylation reagent into the cell, and cell surface specific labelling was not achieved.

In addition, immunoprecipitation of surface biotinylated HEK Nav1.7β1β2 cells using the pan Nav antibody resulted in the maximum coverage of Nav1.7 in our studies (42%) with detection of 85 unique peptides (111 in total). Perhaps the higher sequence coverage in this experiment is a result of better performance of the mass spectrometer and not related to biotinylation of Nav1.7. Figure 3.9 contains a detailed scheme of the sequence and identified peptides.

3.2.5 Optimisation of Sample Preparation

In the efforts to increase the coverage of Nav1.7, different digestion enzymes and additives were explored. Firstly, chymotrypsin which is a protease that cleaves at the C-terminus of hydrophobic amino acids (F, Y, L, M, W) could be beneficial for the coverage of hydrophobic transmembrane regions. An additional protease that was employed was LysC. LysC cleaves at the C-terminus of K residues and it is active in denaturing conditions (up to 8 M urea) making it useful for digestion of proteins that tend to aggregate. It was used in combination with trypsin, after dilution to <2 M urea in order to achieve higher digestion efficiency.

The samples were prepared by solubilising the crude membrane fraction of HEK Nav1.7β1β2 with 4% v/v SDS, as reported in the literature. According to the FASP protocol, the samples were treated with trypsin (control), chymotrypsin or LysC for 16 hours at 37 °C. The following day, LysC samples were diluted and further trypsin digestion was performed for 4 hours. The samples were desalted by StageTipping and analysed by LC-MS/MS. The peptide identification was performed with PEAKS and the results are shown in Figure 3.10A and B. Trypsin digestion outperformed chymotrypsin and LysC/trypsin in the case of Nav1.7 and β2 with 6 and 14 identified unique peptides, respectively. Chymotrypsin enabled the identification of only 3 unique peptides for Nav1.7 which corresponded to parts of the sequence that had not been
Figure 3.9: Sequence coverage of Nav1.7. 111 peptides were identified, out of those, 85 were unique and 42% coverage was obtained. It was achieved through immunoprecipitation of Sulfo-NHS-Biotin labelled HEK Nav1.7β1β2. The transmembrane segments for each domain are underlined. The extracellular and intracellular sequences are in dark green and black, respectively.

identified before. LysC/trypsin resulted in lower sequence coverage for Nav1.7 (3%) which could be due to lower trypsin efficiency in 2 M urea and shorter incubation time (4 h).

As it had enabled the highest sequence coverage, trypsin digestion was selected for the next optimisation experiment. The purpose of this enhancement was to optimise the digestion of membrane proteins that has been reported in the literature.171,172 More specifically, organic solvents such as acetonitrile and methanol, chaotropes such as urea and guanidine HCl, detergents that can be removed by precipitation, or MS-compatible surfactants have been explored. In this experiment, three additives were chosen; 1% v/v sodium deoxycholate (SDC), 20% v/v acetonitrile and 0.05% w/v ProteaseMax (Promega) for the digestion of Triton X-100-solubilised cell membranes. SDC is a detergent that does not affect trypsin digestion
Figure 3.10: Optimisation of sequence coverage and peptide identification. **A**, Number of peptide identifications and **B**, Sequence coverage upon digestion with different enzymes. Trypsin exhibited the best results for Nav1.7 and β2, whereas LysC/trypsin improved the detection of β1. **C**, Effect of additives on sequence coverage and number of peptide identifications for Nav1.7. Surfactant ProteaseMax was marginally superior whereas SDC and acetonitrile did not improve peptide detection. **D**, Coverage of Nav1.7 obtained through standard StageTip versus three-step StageTip fractionation. All samples were analysed in triplicate except for the StageTip fractionation of 0.05% ProteaseMax (n = 1). Error bars represent SD and asterisks indicate $p < 0.05$ in paired $t$-test of the means.
even at high concentrations and can be removed by precipitation with trifluoroacetic acid. ProteaseMax degrades during digestion but enhances the solubility of hydrophobic proteins and peptides. Figure 3.10C contains the sequence coverage of Nav1.7 obtained from these conditions and a control sample (no additive). Acetonitrile and SDC did not improve the detection of Nav1.7 peptides. However, ProteaseMax increased the coverage of Nav1.7 (18%) through 27 unique peptide IDs. This result was encouraging as no previous enrichment of Nav1.7 was performed.

Finally, in order to reduce the complexity of the crude membrane peptide mixture, StageTip fractionation was investigated. A published protocol on poly(styrenedivinylbenzene) reverse phase sulfonate (SDB-RPS) fractionation was followed. The fractions were collected with buffers at different pH values and acetonitrile concentrations (Subsection 6.4.4). StageTip fractionation indeed increased the coverage of Nav1.7 under all conditions (Figure 3.10D). The highest coverage achieved by StageTip fractionation was 26% in the control and ProteaseMax sample. It should be noted that only one replicate of the ProteaseMax sample was analysed, unlike the triplicate analysis of all other samples. This was a great step forward from the initial crude membrane digestion coverage of 6%.

To sum up, optimisation experiments designated ProteaseMax and StageTip fractionation as the way forward in trypsin digestion of HEK Nav1.7β1β2. Overall, through the investigation of digestion enzymes, additives and StageTip fractionation; 56 unique peptide identifications of Nav1.7 and 30% sequence coverage were achieved. The next steps in the optimisation of sequence coverage of Nav1.7 would be to combine ProteaseMax and StageTip fractionation in immunoprecipitated Nav1.7.

### 3.2.6 Conclusion

MS-based detection of membrane proteins is very challenging. Our first experiments involved a model system, an engineered cell line that expresses Nav1.7, β₁ and β₂. Nav1.7 detection was only possible in crude membrane fractions with low (6%) sequence coverage. With the help of controls such as monitoring an abundant plasma membrane protein (Na⁺/K⁺-ATPase) and in parallel analysis of HEK293 cells, optimisation of the peptide detection was achieved. Membrane fractionation of HEK293 cells did not yield Nav protein identifications which increased our confidence in the identified Nav1.7 peptides.

Immunoprecipitation had been successful in the MS-based analysis of PTMs and binding partners of other VGSCs, therefore it was subsequently explored. A pan Nav and control IgG IP from HEK Nav1.7β₁β₂ in combination with LFQ revealed potential interactors of Nav1.7. The potential interactors did not in-
clude β subunits with statistical significance whereas they included other membrane-bound intracellular proteins such as oxysterol-binding proteins. Moreover, an E3 ligase and NEURL4 which have been shown to associate in centrosomes were significantly enriched. This experiment highlighted the limitations of the engineered system used. Overexpression most likely leads to a big proportion of Nav1.7, β1 and β2 that do not make it to cell membrane and are probably misfolded, bound to intracellular membranes. Moreover, these proteins are naturally expressed in neuronal cells, therefore the physiological relevance of these potential binders is questionable. Future experiments should involve physiologically relevant neurons in order to investigate neuromodulation of Nav1.7 in healthy and disease states. Elucidation of meaningful binders from immunoprecipitation requires the optimisation of washes, incubation time, chemical crosslinking and appropriate controls.

Despite these limitations, the sequence coverage (42%) achieved by immunoprecipitation was in the range of the reported sequence coverage of other VGSCs. Another aspect that was investigated was the use of different digestion enzymes. Trypsin proved to be superior and it was employed in additional optimisation experiments involving digestion additives. The coverage of Nav1.7 obtained from trypsinisation of membranes and three-step fractionation on the peptide level was significantly improved (30%) and remarkable considering that no other enrichment tool was used.

We believe that a combination of immunoprecipitation, MS-compatible surfactants and fractionation will lead to even higher sequence coverage. An aspect that is not covered in this Section is the topology of the identified peptides. Even though the transmembrane regions are hard to identify, the most interesting parts of the sequence as far as modulation by PTMs or binders is concerned, are the intracellular and extracellular loops. Not many extracellular loops were detected in our experiments with mostly intracellular regions dominating the sequence coverage. In photocrosslinking experiments, one can focus on maximising the coverage of the domain that is specifically targeted by the photoprobes. To this end, a selection of digestion enzymes can be employed. Enzymes that have shown success in membrane protein 'bottom-up' approaches are elastase, Proteinase K and pepsin, but there are many more proteases that have emerged. Achievement of high coverage of the binding site along with IP of photocrosslinked Nav1.7 is the next step forward for this approach. In Chapter 5, the future steps in order to elucidate the binding sites of Nav1.7 modulators are discussed in more detail.
3.3 Proteomics of iPSC-derived Neurons

3.3.1 iPSCs in Nav1.7 Drug Discovery

In order to investigate modulation mechanisms of Nav1.7 in a more physiologically relevant system, an emerging approach has been employed with considerable success. It involves the generation of sensory neurons from induced-pluripotent stem cells (iPSCs) of human donors.

Human stem cells can be reprogrammed into iPSCs by exposure to certain transcription factors. In order for differentiation into neuronal cells to take place, a small molecule approach was developed by Chambers et al. Exposure to three inhibitors (SU5402, CHIR99021 and DAPT) of WNT, Notch and FGF tyrosine kinase signalling led to neuronal cells in 10 days. Figure 3.11A contains a detailed workflow. The process can be monitored by transcriptomic analysis in order to identify key gene markers by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Maturation of the neurons in the presence of neutrophins is required in order to express nociceptor markers such as SCN9A (gene encoding Nav1.7). After a few days, the nociceptors are organised into ganglia-like clusters. Moreover, immunofluorescence is employed (Figure 3.11B) to further validate the expression of markers such as transcription factors BRN3A (encoded by POU4F1 gene) and ISL1, and neuronal filament protein peripherin (encoded by PRPH gene).

The maturation process was monitored for six weeks by single cell qRT-PCR analysis of 48 marker genes at five time points. Moreover, electrophysiological validation of the expression of certain ion channels was described in a subsequent study. The study firstly revealed the heterogeneity within the sensory neuron population in terms of transcription profiles but also the resemblance to DRG neuronal profiles. Overall, SCN9A transcription was significantly increased on day 32 and 39, reaching DRG neuronal levels.

A proof-of-principle study employed the same approach in order to assess the effect of potent aryl sulfonamide inhibitors on nociceptors that carry disease-relevant SCN9A mutations. Inherited erythromelalgia is associated with symptoms that include pain sensations triggered by temperature increase. Individuals that suffer from erythromelalgia as a result of different point mutations and healthy individuals (no SCN9A mutations) donated iPSCs that were differentiated and matured into nociceptors for 8 weeks. Robust expression of Nav1.7 with equivalent sodium currents in the control and erythromelalgia (EM) neurons was observed. The in vivo painful phenotype was complemented with more depolarised mean
Figure 3.11: Differentiation and maturation of iPSCs to nociceptors. A, Initially, inhibition of SMAD and TGF-β signaling is required in order to maintain the expression of pluripotent marker genes OCT4 and NANOG and prevent differentiation to non-neural ectoderm or mesendoderm types. Differentiation to neural crest cells is achieved with treatment using CHIR99021, SU5402 and DAPT inhibitors of WNT, FGF and Notch signalling, respectively. WNT inhibition from day 4 promotes differentiation of immature sensory neurons. Maturation of sensory neurons to nociceptors requires three neurophins (GDNF, BDNF, NGF beta) and is monitored through biomarker gene expression (ISL1, PRPH, BRN3A etc) from day 11. Figure adapted, with permission, from Chambers et al.\textsuperscript{174} B, Immunofluorescence is employed to visualise marker expression in EM neurons. Brn3a and Islet1 are neuronal transcription factors and peripherin is an abundant neuronal filament protein. Figure adapted, with permission, from Cao et al.\textsuperscript{175}

resting potential and lower current threshold required for action potential generation in the EM neurons in comparison with the healthy donor neurons. Additionally, all EM neurons exhibited more frequent spontaneous firing. EM1 neurons expressing S241T Nav1.7 were associated with the mildest clinical phenotype and showed less excitability whereas EM2 (I848T) that were associated with the most painful phenotype exhibited higher excitability. The effect of an aryl sulfonamide inhibitor after a heat-stimulus on patient-derived EM neurons was successfully investigated.\textsuperscript{175} EM1 showed no response whereas EM2 showed significant decrease in firing frequency. These results were also observed in the clinic, highlighting the success of this disease model.

These studies have stressed the utility of iPSCs in elucidating disease mechanisms and the effect of potential drug molecules in painful conditions. Precision medicine is likely to be the answer for the next generation pain therapeutics and it will definitely benefit from the development of iPSC platforms.\textsuperscript{30} In this Section, the maturation and proteomic characterisation of EM1 and EM2 neurons that were provided
by Dr James Bilsland (Pfizer Neusentis) are described. Unfortunately, due to the closure of Pfizer Neusentis early in this project, the analysis of this system was stopped abruptly.

3.3.2 Neuronal Maturation

The immature neurons were thawed on day 11 of the protocol described in Figure 3.11A and were treated with brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF), neutrophin-3 (NT-3), beta-nerve growth factor (NGF) until day 53. The maturation time was selected according to the reports that highlighted the transcription profiles of nociceptor markers such as Nav1.7. Figure 3.12 displays brightfield images of the neurons on day 12 and 39. It is evident that the morphology changes over time, with the mature neurons comprising of somata and dendritic structures which are bundled or connected.

![Figure 3.12: Optical microscope images of EM1 and EM2 during neuronal maturation. The difference in the morphology is evident as somata and dendritic structures are formed.](image)

In addition to the morphology study, three time points (day 15, 32, and 53) during maturation were selected in order to monitor the mRNA levels of key gene markers by qRT-PCR. Four genes were selected; POU4F1 and ISL1 encoding transcription factors, markers of sensory neurons, TAC1 precursor to Substance P, and SCN9A, markers of nociceptors. The housekeeping genes were HRPT encoding hypoxanthine guanine phosphoribosyltransferase and GAPDH encoding glyceraldehyde 3-phosphate dehydrogenase. Our
analysis was performed using the $2^{\Delta\Delta CT}$ method\textsuperscript{177}, $\Delta CT$ was calculated for each gene by subtracting the average cycle threshold (CT) from the CT of HRPT ($n = 2$). In order to monitor the variation in expression within EM1 or EM2, $\Delta\Delta CT$ was calculated by subtracting the $\Delta CT$ values between the first and subsequent time points. Figure 3.13 contains the fold change in transcription of the genes of interest in both cell lines. On average, the transcription of neuronal markers POU4F1 and ISL1 decrease on day 53 in both EM1 and EM2 cells. This trend was observed during maturation into nociceptors in a study by Young et al.\textsuperscript{176} TAC1 transcription exhibits a significant difference between EM1 and EM2, with almost no change during EM1 maturation and significant increase in EM2. Finally, expression of Nav1.7 (SCN9A gene) increased 8-fold in EM2 but not significantly in EM1. This experiment should be repeated with more replicates in order to draw any conclusions with confidence. However, when the average SCN9A and HRPT CTs of EM1 and EM2 on day 53 were compared to the published EM1 and EM2 data,\textsuperscript{175} it is evident that maturation in our hands resulted in somewhat decreased expression of Nav1.7.

Despite the limited number of EM1 and EM2 neuronal cells at our disposal and the ambiguity in the key gene expression, this interesting system was also analysed by quantitative proteomics. Analysis by
Figure 3.14: TMT experimental workflow. Two time points (day 15 and 53) were chosen to analyse EM1 and EM2 neuronal maturation by TMT quantification. The FASP protocol is followed and the peptides are labelled using four TMT labels. Equal amounts of the labelled peptides are combined, and LC-MS/MS analysis is performed. The TMT labels are isobaric but upon MS/MS the labels are fragmented into reporter ions of different $m/z$. The intensity of these peaks is used for quantification among the combined samples.

qRT-PCR provides information on mRNA transcripts, but not necessarily on protein levels and how these change during maturation. Figure 3.14 describes the workflow chosen to monitor differences in the whole cell proteome of EM1 and EM2 over two time points; day 15 and 53. The chosen proteomic quantification method was Tandem Mass Tag (TMT) labeling. The proteomic analysis of EM neurons involved FASP in combination with TMT labeling, adapted from the literature (iFASP). Briefly, the cells were collected, lysed in Triton-X100 buffer and trypsinised by FASP. Due to the small number of cells, subcellular fractionation was not attempted. The recovered peptides were labelled using the TMT reagents that correspond to reporter ions of $m/z$ 127, 128, 130 and 131. The TMT reaction involves amide bond formation on the N-termini and K side chains of the tryptic peptides. Equal amounts of the samples are mixed into one tube and analysed by LC-MS/MS. The unique property of the TMT labels is that although they are isobaric, therefore a specific TMT-labelled peptide in all four samples will have the same $m/z$ in MS$^1$; upon CID fragmentation they give rise to fragments of different $m/z$ values. Quantification of a specific peptide among the different samples is therefore based on the ion intensities of the individual TMT reporter ion peaks in MS/MS spectra.
Figure 3.15: \( t \)-test analysis based on TMT quantification. A, Proteins significantly upregulated on day 53 versus day 15 of EM1 maturation (109 in total) and B, EM2 maturation (162 in total). C, Common proteins (gene names) upregulated in EM1 and EM2 (day 53). The classification of the most upregulated proteins was performed using PANTHER.\(^{180}\) Two-sample \( t \)-test was performed in Perseus (permutation-based FDR, 250 permutations, FDR 0.001, \( S_0 = 0.5 \)).

The total proteome analysis of EM1 and EM2 on day 15 and 53 of maturation by TMT quantification yielded 1778 protein IDs in duplicate. The Nav proteins of interest were not identified, but this was not unexpected as no enrichment was performed. In order to compare the protein expression during maturation, a two-sample \( t \)-test between the mean TMT-intensity that corresponds to day 15 and day 53 was carried out in Perseus (Figure 3.15). The focus was on proteins that are significantly upregulated on day 53. In the EM1 sample, the expression levels of 39 proteins increased (\( \log_2 \) difference in the mean >2) on day 53. Moreover, 29 EM2 proteins were upregulated (\( \log_2 \) difference >2) on day 53. Figures 3.15A and
3.15B contain the plots of the differential protein expression between the two time points in EM1 and EM2. PANTHER classification was employed in order to identify the classes of proteins whose expression is increased on day 53. A significant proportion of these proteins are involved in cell adhesion and filament structure. Moreover, several membrane proteins such as proteins involved in trafficking, ion channels and transporters were upregulated. Similarly, the expression of certain signalling molecules, a G-protein and neuropeptides was increased on day 53.

There is significant overlap between day 53 EM1 and EM2 proteins (listed in Figure 3.15C and Table 3.3). VGF is one of the neuropeptides that were identified by proteomic quantification, in addition, increased VGF gene expression was also measured by qRT-PCR during sensory neuronal maturation by Young et al. Additional protein IDs that agree with qRT-PCR data of sensory neuron maturation in the literature are neuromodulin (GAP43) and alpha-internexin (INA). Neuromodulin is a membrane protein involved in axonal growth, whereas alpha-internexin is a neuronal filament protein. Other neuronal filament proteins that were upregulated on day 53 correspond to NEFL and NEFM genes that have previously been identified by proteomics in oligodentrocyte and motor neuron differentiation. NCAM2 is associated with neurogenesis in other studies, and \( G_o \) protein is widely expressed in sensory neurons. Finally, CD166 and CD59 have previously been identified by LC-MS/MS in a study monitoring glycosylation during neuronal differentiation. The confidence in the observed proteins is boosted by their implication in neuronal maturation and differentiation as described in the literature.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein name</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>VGF</td>
<td>nerve growth factor inducible</td>
<td>overexpressed during neuronal maturation&lt;sup&gt;176&lt;/sup&gt;</td>
</tr>
<tr>
<td>NCAM2</td>
<td>neuronal cell adhesion molecule 2</td>
<td>neuronal marker&lt;sup&gt;183&lt;/sup&gt;</td>
</tr>
<tr>
<td>NEFL</td>
<td>neurofilament light polypeptide</td>
<td>identified in oligodentrocyte differentiation&lt;sup&gt;181&lt;/sup&gt;</td>
</tr>
<tr>
<td>NEFM</td>
<td>neurofilament medium polypeptide</td>
<td>identified in motor neuron differentiation&lt;sup&gt;182&lt;/sup&gt;</td>
</tr>
<tr>
<td>GNAO1</td>
<td>guanine nucleotide-binding protein G0 subunit alpha</td>
<td>signalling in sensory neurons&lt;sup&gt;184&lt;/sup&gt;</td>
</tr>
<tr>
<td>GAP43</td>
<td>neuromodulin</td>
<td>overexpressed during neuronal maturation&lt;sup&gt;174&lt;/sup&gt;</td>
</tr>
<tr>
<td>ALCAM</td>
<td>CD166 antigen</td>
<td>identified in neural stem cells&lt;sup&gt;185&lt;/sup&gt;</td>
</tr>
<tr>
<td>INA</td>
<td>alpha-internexin</td>
<td>overexpressed during neuronal maturation&lt;sup&gt;174&lt;/sup&gt;</td>
</tr>
<tr>
<td>VAT1L</td>
<td>synaptic vesicle membrane protein VAT-1 homologue-like</td>
<td>not mentioned in relevant studies</td>
</tr>
<tr>
<td>CD59</td>
<td>CD59 glycoprotein</td>
<td>identified in neural stem cells&lt;sup&gt;185&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Table 3.3: Upregulated proteins (maturation day 53) as quantified in both EM1 and EM2 samples.
3.3.3 Conclusion

The development of Nav1.7 inhibitors is currently hindered by, among other reasons, lack of efficacy in the clinic despite high \textit{in vitro} potency. New pain models such as patient-derived iPSCs provide more physiologically relevant systems that can be exploited to study the effect of inhibitors but also the differences in disease and healthy states. Our collaborators provided us with day 11 neurons expressing mutant Nav1.7 (EM1 and EM2) that were previously characterised by qRT-PCR, immunofluorescence, and patch-clamp electrophysiology.\textsuperscript{175} The maturation time required for maximal expression of nociceptor channels such as Nav1.7 and Nav1.8 was 6-8 weeks. The neurons formed evident ganglia-like structures a few days into the maturation process. Analysis of mRNA levels by qRT-PCR revealed variation on days 15, 32 and 53 and no consensus in the trends of SCN9A and TAC1 nociceptor markers between the two samples. The two mutations exhibit different phenotypes, EM1 (S241T) having the mildest and EM2 (I848T) the most severe effects in the respective patients. Therefore, it is possible that the qRT-PCR trends represent this difference. However, it is more likely that the limited amount of RNA extract from a small number of cells contributed to this apparent difference between EM1 and EM2 cells. The transcriptomic analysis should be repeated with more replicates, and with iPSC-derived neurons that carry no mutation as a control in order to fully assess the differences in transcription.

Even though the preliminary proteomic experiment did not enable the identification of the Nav proteins of interest, it set the basis for further analysis of iPSC-derived neurons. The increase in expression of filament proteins, neuropeptides, transporters, cell adhesion molecules and signalling compounds is in line with the morphological changes observed during maturation. Identification of many membrane proteins was successful in this total proteome approach, but further work is required in order to identify Nav1.7. For this purpose, the optimisation results in the HEK Nav1.7β1β2 cell line proteomics will prove useful. Techniques such as surface lysine or glycan biotinylation and enrichment can shed light on trafficking mechanisms in disease and healthy states. Insight into the localisation, protein binding partners, and the PTM profile of Nav1.7 in iPSC-derived neurons will provide answers to questions regarding the differential effect of inhibitors against different mutants of Nav1.7, as observed by Cao \textit{et al.},\textsuperscript{175} and assist the development of more selective inhibitors.
Despite the successful proteomic characterisation of hNav1.7 and the insights into its binding partners in an engineered cell line (Chapter 3), efforts to photocrosslink hNav1.7 in cell membranes, in detergent or in cells were unsuccessful. A great amount of complexity arises from experiments on the full length hNav1.7, for example, the localisation and correct folding of Nav1.7 in the cell, the strong complexation with chaperones, tendency to precipitate, difficulty in determining whether it remains functional in detergent and general instability issues. Consequently, our focus turned towards an even simpler system for validation of the photoprobes. This system involves purified chimeras of voltage sensors of hNav1.7 with the well studied homotetrameric bacterial NavAb.6,7

Two excellent examples of VSD-NavAb chimeras have been reported in the literature in recent years.55,186 Firstly, a groundbreaking study by our collaborators at Genentech revealed the binding interactions of aryl sulfonamides on voltage sensor domain IV (VSD4) highlighting the key determinants of the selectivity of this class of gating modifiers over other Nav subtypes. The voltage-sensor ‘trapping mechanism’ and the participation of a phospholipid in the complex were also discussed.55 In this study, a NavAb/Nav1.7 VSD4 FLAG-tagged chimera was developed and used for co-crystallisation with aryl sulfonamide GX-936 (Subsection 1.2.2.5) in a robust phosphatidylycholine-based bicelle crystallisation system.55 The chimera was developed based on NavAb as a scaffold that carried hNav1.7 sequences for part of S1, S2, S3, S4 of VSD4 and their extracellular linker loops S1-S2, S3-S4. At the N-terminus, it carries the FLAG
affinity tag along with a thrombin cleavage site (Figure 4.1A and B). In this chapter, this chimera will be referred to as VSD4.

**Figure 4.1:** Schematic of the chimeric approach. **A.** NavAb is the scaffold that bears part of the S1-S4 hNav1.7 sequence of either VSD2 or VSD4. As a consequence, the functional channels are homotetrameric comprising of a NavAb pore and partly hNav1.7 voltage sensors. **B.** Sequence of VSD2 and VSD4 chimeras. The FLAG affinity tag and thrombin protease cleavage site are underlined. The parts of hNav1.7 origin are highlighted in green.

The same approach was later used to develop a NavAb/Nav1.7 voltage sensor domain II (VSD2) chimera. It was expressed in Sf9 insect cells that were able to generate sodium currents; this construct was the first Nav1.7 chimera that has been functionally characterised by electrophysiology. Interestingly, the half maximal activation potential of VSD2 was +52 mV, significantly right shifted with respect to the na-
tive hNav1.7 (-19 mV) and NavAb (-93 mV). Moreover, the VSD2 Sf9 sodium currents were sensitive to ProTx-II inhibition and the purified VSD2 demonstrated binding to ProTx-II and HwTx-IV by radioligand binding and Surface Plasmon Resonance (SPR).

4.1 Purification of VSD2 and VSD4

Our collaborators Dr Christopher Koth, Dr Jian Payandeh and Dr Hui Xu at Genentech provided a *Trichoplusia ni* (Tni) cell line expressing the VSD4 chimera described by Ahuja *et al.*, \(^{55}\) and a Tni cell line expressing a VSD2 chimera. Figure 4.1B depicts the amino acid sequence of the VSD2 and VSD4 chimeras used in this Thesis. More detail on the sequences originating from hNav1.7 is given in Figure 4.2.

![Figure 4.2: The hNav1.7 sequence in VSD2 and VSD4 chimeras. The extracellular linker loops are highlighted in yellow.](image)

The purification of VSD2 homotetramers served two purposes; the co-crystallisation with toxins of interest in order to aid the structural efforts of our collaborators, and photocrosslinking with our HwTx-IV photoprobes. In membrane protein crystallisation, one of the important elements that affect the quality of the crystals is the detergent. A thorough screening of the stability and diffraction quality of VSD2 complexes with various toxins by Hui Xu pointed towards Facade-EM (FA3) detergent in combination with lipids phosphatidylcholine (POPC), phosphatidylethanolamine (POPE) and phosphatidylglycerol (POPG). The purification workflow developed by Hui Xu involves lysis of Tni cells using a microfluidiser and solubilisation of the membrane proteins in glyco-diosgenin GDN, a detergent that is structurally similar to digitonin. After clarification by ultracentrifugation, the VSD2 tetramers were purified using an anti-FLAG affinity resin. At this point, the detergent can be exchanged with FA3 and the lipid mixture. The VSD2 tetramers are then concentrated and purified to homogeneity using Size-Exclusion Chromatography (SEC). Figure 4.3 contains representative SDS-PAGE stain-free gels after FLAG purification, the size exclusion chromatogram of VSD2 in FA3 and lipid mixture and the fractions that are collected for crystallisation.
Figure 4.3: Purification of VSD2 chimera in FA3/lipid mixture. A. SDS-PAGE gel of the FLAG purification of VSD2 tetramers. B. SEC profile of VSD2 (top) and molecular weight marker (bottom). Unfortunately, it is very prone to aggregation, therefore a stringent selection of the peak highlighted in blue is needed to ensure a good quality sample for further applications. The molecular weight marker trace is shown at the bottom and can be used as a reference for all the chromatograms of this chapter. C. SDS-PAGE gel of the fractions collected after SEC. The selected fractions for crystallisation are highlighted in blue. SEC buffer: 10 mM Tris pH 8.0, 100 mM NaCl, 0.06% (w/v) FA3 and 0.1 mg mL^{-1} lipid mixture POPC:POPG:POPE at 3:1:1 mass ratio.

Fluorescence-detection Size-Exclusion Chromatography (FSEC) analysis of VSD chimeras in different detergents pinpointed GDN, a detergent very structurally similar to digitonin, as the detergent that offers the most stability, and it was therefore chosen for the subsequent photocrosslinking experiments. The relevant purification and SEC profiles of VSD2 and VSD4 in GDN are show in Figures 4.4 and 4.5, respectively.

4.2 Co-Crystallisation of VSD2 with ICK Peptides

The overlapping interest in identifying the binding sites of VSD2 gating modifiers (toxins) with hNav1.7 was reinforced by the complementary approaches undertaken by ourselves and our collaborators; crystallography and photoaffinity labeling. Microcrystal samples of complexes of VSD2 with two toxin peptides of interest; ProTx-II and GsAf-1187 were prepared by Hui Xu and myself in order to obtain crystallographic data at the Coherent X-ray Imaging end station of the Linac Coherent Light Source (LCLS). The X-ray Free
Figure 4.4: Purification of VSD2 chimera in GDN. A. SDS-PAGE gel of the FLAG purification of VSD2 tetramers. B. SDS-PAGE gel of VSD2 after SEC, pooled fractions denoted in blue. C. SEC profile of VSD2. SDS-PAGE gel of the fractions collected after SEC. SEC buffer: 10 mM Tris pH 8.0, 100 mM NaCl, 0.042% (w/v) GDN.

Figure 4.5: Purification of VSD4 chimera in GDN. A. SDS-PAGE gel of the FLAG purification of VSD4 tetramers. B. SDS-PAGE gel of VSD4 after SEC, pooled fractions denoted in blue. C. SEC profile of VSD4. SDS-PAGE gel of the fractions collected after SEC. SEC buffer: 10 mM Tris pH 8.0, 100 mM NaCl, 0.042% (w/v) GDN.
CHAPTER 4. VOLTAGE SENSOR DOMAIN CHIMERAS OF NAVAB AND NAV1.7

Electron Laser (XFEL) at the LCLS has been very successful in numerous crystallographic experiments focusing on membrane proteins. Briefly, an XFEL offers high frequency, femtosecond pulses of very powerful X-ray microbeams (\( \sim 1 \mu m \)). In some cases, it has enhanced the diffraction quality of crystals that are poorly diffracting at a classic synchrotron. The sample preparation was very time consuming as microcrystals (5-20 \( \mu \)m) were produced in large quantities, typically a few mLs of dense crystal slurry per toxin complex.

Figure 4.6: Microcrystal slurry of VSD2 and ProTx-II. Size range 5-20 \( \mu \)m. A few millilitres of microcrystal slurry was pooled in batches according to their mean size and introduced to the XFEL through a double flow focus (DFF) nozzle.

The XFEL data collection requires so much sample because of the delivery method to the beam; a double flow focus (DFF) nozzle was used in this case. DFF sample delivery is a new technique that involves the generation of a jet of crystal slurry in a sheath alcoholic solution through a 40 \( \mu \)m capillary at room temperature. The jet is positioned in front of the XFEL beam and the diffraction of passing microcrystals is detected. In the case of our microcrystals, the optimum flow rate was 20 \( \mu \)L min\(^{-1}\) with a successful diffraction hit rate of 2.5–5%. XFEL data collection requires thousands of diffraction patterns of single microcrystals to enable structure determination. Figure 4.6 contains images of ProTx-II-VSD2 microcrystals in ammonium sulfate conditions. A dataset for each toxin peptide was successfully collected, however, the resolution was disappointingly low (\( > 5 \) Å) and further analysis of the results was not attempted.
4.3 Photocrosslinking of VSD2 with HwTx-IV Photoprobes

4.3.1 Gel Shift Assay

Our subsequent efforts focused on the use of our photoaffinity labeling peptides that are based on HwTx-IV. Initially, a screening of six Mp probes was done by incubation of purified VSD2 in GDN-containing buffer with 100 and 300 μM photoprobes in ice. After 1 hour, the mixture was irradiated at 365 nm for 10 minutes and loaded on an SDS-PAGE gel. Evidently, the covalent modification due to photocrosslinking caused the crosslinked VSD2 to migrate higher in gel (Figure 4.7, top) and the higher molecular weight band was verified by anti-FLAG western blotting (Figure 4.7, bottom). We observed that the crosslinking was UV-dependent in the cases of Mp7, Mp27 and Mp29 but the concentration dependence was not very obvious, suggesting that the amount of photoprobe had exceeded a useful range. Photoprobes Mp5, Mp21 and Mp35, even though they were very potent hNav1.7 inhibitors in the patch-clamp experiments, exhibited hardly any photocrosslinking capabilities in the gel shift assay.

<table>
<thead>
<tr>
<th>peptide</th>
<th>-</th>
<th>Mp5</th>
<th>Mp7</th>
<th>Mp21</th>
<th>Mp29</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>[peptide] (μM)</td>
<td>100</td>
<td>300</td>
<td>300</td>
<td>100</td>
<td>300</td>
</tr>
</tbody>
</table>

![Screening of all Mp photoprobes in an SDS-PAGE gel shift assay (stain free). The Mp-crosslinked VSD2 has a higher apparent molecular weight in gel due to a ~4 kDa MW increase. Anti-FLAG western blotting validated the nature of the higher MW band in samples crosslinked with Mp7, Mp27 and Mp29.](image_url)

**Figure 4.7:** Screening of all Mp photoprobes in an SDS-PAGE gel shift assay (stain free). The Mp-crosslinked VSD2 has a higher apparent molecular weight in gel due to a ~4 kDa MW increase. Anti-FLAG western blotting validated the nature of the higher MW band in samples crosslinked with Mp7, Mp27 and Mp29.
CHAPTER 4. VOLTAGE SENSOR DOMAIN CHIMERAS OF NAVAB AND NAV1.7

Figure 4.8: Concentration dependence of Mp7, Mp27 and Mp29 photocrosslinking and competition with HwTx-IV (stain free gel). The band that is due to the crosslinked VSD2 looks like a doublet and is likely due to insufficient disulfide reduction or an artifact. A, Crosslinking is abolished by excess of HwTx-IV and lack of UV irradiation. B, Titration of HwTx-IV in a competition study against Mp29 photocrosslinking.

The concentration dependence and specificity of photocrosslinking was examined in the case of Mp7, Mp27 and Mp29 photoprobes (Figure 4.8). Mp27 and Mp29 showed crosslinking capability even at 10 μM, whereas Mp7-dependent crosslinking started at 50 μM. Pre-incubation with native HwTx-IV abolished crosslinking at the highest range of concentrations of these three photoprobes. The concentrations that were selected for further photocrosslinking experiments were the following: 200 μM for Mp5, Mp21 and Mp35, 100 μM for Mp7 and Mp27, and 50 μM for Mp29 photocrosslinking. Figure 4.8B contains the concen-
tration dependence of competition of Mp29 (50 μM) with HwTx-IV. This gel was used in order to quantify the inhibition of photocrosslinking by HwTx-IV using tryptophan fluorescence (stain free gel imaging) and led to the rough estimation of an IC₅₀ of 39 μM which is not surprising considering that the lack of a lipid bilayer and the properties of the detergent might affect the VSD2 binding ability.

### 4.3.2 Proteomic Mass Spectrometry Analysis

The next step towards the validation of the HwTx-IV Mp probes was identification of the sites of modification on VSD2. Firstly, a selection of digestion conditions were screened with the aim of achieving a high sequence coverage, and particularly focusing on the human Nav1.7 sequence coverage of the chimera. Even though chymotrypsin digestion gave the highest coverage, trypsin enabled sufficient detection of the hNav1.7 sequences of interest. Figure 4.9 contains representative coverage using trypsin digestion and a list of the other enzymes that were explored.

![Sequence coverage of VSD2: Trypsin digestion](image)

**Figure 4.9:** Sequence coverage of VSD2 (1% FDR). Different digestion enzymes were employed, with trypsin exhibiting the highest coverage of the hNav1.7 sequence of the chimera (highlighted in orange). Results obtained through FASP sample preparation.

Even though trypsin enabled the identification of the extracellular loops of S1-2 and S3-4, it still missed a considerable portion of the α-helical segments. Optimisation of the digestion conditions in order to improve the coverage involved techniques such as in gel digestion,¹⁰⁰ FASP,¹²¹ and methanol/chloroform precipitation¹⁶⁰ in order to ensure the removal of the detergent and the highest digestion efficiency. The
use of ProteaseMax surfactant was also employed. Unfortunately, FASP digestion marginally gave the best results and the addition of ProteaseMax did not provide a considerable advantage.

As the coverage could not be improved further, photocrosslinking site identification was explored. All photoprobbers were taken forward with careful prediction of their own digestion products using in silico digestion and considering full and partial digestion (missed cleavages). It was evident that identification of a crosslinked peptide would be challenging due to low abundance and complicated MS2 spectra. To this end, tables containing the predicted b and y ions resulting from Collision Induced Dissociation of the photoprobe peptides in MS2 were generated. The identification workflow initially consisted of two steps: search for modification sites based on a Δm of VSD2 peptides that corresponds to the respective predicted Mp digested peptide, and validation of the modification site by identification of the ‘diagnostic’ fragment ion peaks for each Mp peptide. The diagnostic fragment ion peaks were also validated through the behaviour of the intramolecular crosslinks under MS2. Unfortunately, most of the VSD2-Mp hits in our searches could not be validated by the existence of the diagnostic peaks and other crosslinked peptides were missed by the search engine but identified manually. The second identification strategy involved the use of StavroX3.6.6 and MeroX1.6.6 developed by Michael Goetze (University of Halle-Wittenberg). Both pieces of software have been developed for the identification of peptide crosslinks. 164,191 In this case, the sequence of VSD2 and HwTx-IV photoprobe is provided, the theoretical crosslinking sites and the crosslinked peptide masses are identified by the programme before comparing the MS2 data with theoretical fragmentation spectra.

VSD2 was treated with Mp probes at concentrations determined by the gel shift assay and irradiated at 365 nm. The most promising photoprobbers (Mp7, Mp27 and Mp29) were employed. The control samples were not irradiated but kept in the dark. FASP sample preparation and trypsin, chymotrypsin, GluC/LysC and GluC/trypsin proteolysis was explored. MeroX analysis of the LC-MS/MS data revealed a peptide on VSD2 S1-S2 as a modification site by Mp29 (Figure 4.10). This peptide was identified with a high confidence score in chymotrypsin and trypsin treated samples. Unfortunately, Mp7 gave no confident crosslinking hits and Mp27 seemed to have crosslinked to the coiled-coil C-terminus of the VSD2 construct that is part of the NavAb sequence (Figure A.21). Unfortunately, due to time limitations, the exploration of sites of crosslinking was stopped at this point.
CHAPTER 4. VOLTAGE SENSOR DOMAIN CHIMERAS OF NAVAB AND NAV1.7

4.3.3 Discussion

When the VSD2 chimera was irradiated in the presence of photomethionine-containing HwTx-IV analogues, photocrosslinking was assessed by a gel shift assay and Mp7, Mp27 and Mp29 probes seemed to yield considerable a amount of crosslinked VSD. Figure 4.11 highlights these three positions on HwTx-IV with respect to the residues that have the most prevalent effect on the inhibitory activity. Even though the excess of photoprobes with respect to VSD2 is quite high, the photocrosslinking efficiency is low as determined by the gel shift assay. Increasing the concentration of photoprobe even more resulted in doubly labeled VSD2 and not in an increase in photocrosslinking, therefore we believe that the limit of crosslinking efficiency had been reached.
Photocrosslinking is not an efficient way of modifying proteins according to other publications and our observations.\textsuperscript{102,103,105} The main reason for this is quenching of the reactive intermediate by reacting intramolecularly or with solvent molecules. A variety of products are generated by photolysis of aliphatic diazirinines. This is partly due to the different reactive intermediates that can be formed upon UV irradiation; singlet, triplet carbenes and diazo species.\textsuperscript{104,192} Singlet carbenes have been shown to react rapidly with alcohol, carboxylic acid and aromatic groups. Triplet carbenes, whose unpaired parallel spin electrons are in different orbitals, are more stable, and unable to insert into O-H bonds.\textsuperscript{104} The diazo intermediate can further form a carbene under UV or other reactive species such as carbocations. Figure 4.12 provides an overview of the possible reactions of aliphatic diazirines; a lot of the possibilities do not yield a covalent bond to the protein of interest through e.g. C-H or O-H insertion. The excited state rearrangements, the side reactions with solvent molecules or intramolecular insertions contribute to the low efficiency of photocrosslinking.

Peptides resulting from intramolecular attack were identified in every Mp probe in UV-irradiated samples by LC-MS/MS.\textsuperscript{193,104,191} In addition, peptide hits corresponding to the product of photomethionine crosslinking to water molecules were identified. The most confident hits of photocrosslinked VSD2 were identified by the dedicated identification software MeroX.\textsuperscript{191} MeroX has been successfully used for the identification of sites of modification due to diazirine photolysis and chemical crosslinking. Its success relies on the ability to detect characteristic ions that result from cleavage of crosslinked peptides during MS\textsuperscript{2}. Some applications of MeroX include identification of multiple sites of intramolecular crosslinking in photomethionine-bearing proteins CaM and GCAP-2.\textsuperscript{191} MeroX enabled the identification of
enzyme–substrate interactions between arginine methyl transferase Hmt1p and nucleolar protein Npl3p through crosslinking with disuccinimido dibutyric urea. A similar crosslinking and analysis approach was employed for the detection of binding interactions in kinetochore subcomplexes.

In the case of Mp29-VSD2, the photocrosslinking site was localised in the S1-2 loop and specifically in the TEEF portion of the linker. It should be noted that the crosslinked peptide identified in the trypsin digestion sample is a result of non specific cleavage at the C-terminus of M757. The theoretical cleavage product of trypsin digestion is a very long peptide (Figure 4.10). We hypothesise that this and other non specific cleavages observed in the rest of the VSD2 sequence could be due to chymotrypsin contamination in the purchased samples of trypsin. This issue has been reported before and in our hands, it did not seem to change dramatically with the use of trypsin from another vendor. Despite the ambiguity in the tryptic peptide identification, the crosslinking site has also been identified in chymotryptic samples; in this case, the expected cleavage sites on both Mp29 and VSD2 loops were detected (Figure 4.10). According to both digestion methods, the crosslinked peptide is quite short (TEEF) but at this point of the analysis, conclusions on the exact residue that gets modified could not be drawn with confidence. According to MeroX, the likelihood of E759 being the site of crosslinking is 65%, followed by T758 (20%). Additional MS
experiments and especially MS3 analysis, need to be done in order to more confidently assign the site of modification.

In Subsection 1.2.2.2, the binding sites of HwTx-IV are discussed; mutagenesis analysis pointed towards one hydrophobic and four acidic residues on S3-4 (E811, L814, D816 and E818) and S1-2 (E753) on VSD2.\textsuperscript{81,87} According to these observations, we hypothesised that the photomethionine in the photoprobes would likely crosslink to S1-2 and S3-4, depending on its position on the HwTx-IV sequence and its orientation with respect to the VSD. Therefore, the observation of Mp29 crosslinking to \textsuperscript{758}TEEF\textsuperscript{761} does not conflict with the existing hypothesis regarding HwTx-IV binding.

Even though we were unable to identify crosslinking of Mp27 to the hNav1.7 sequence of the VSD2 chimera, we identified a peptide crosslink to the C-terminus coiled coil, which seems to be an event of non-specific binding (Appendix A). It should be noted that the confidence score of this identification was not as high as the crosslinked peptides of Mp29-VSD2. It is very possible that the specific site of modification by Mp7 and Mp27 is missed in the MS analysis as site specific photocrosslinking was validated by competition with the native peptide HwTx-IV (Figure 4.8).

Other photoprobes did not yield any interesting crosslinking MS data. It is expected that some positions on HwTx-IV would not be in close proximity to VSD2 to allow photocrosslinking. However, lack of detection could also be a consequence of the challenges in identifying crosslinked peptides and poor crosslinking efficiency. More proteases could be explored in order to increase the likelihood of generating peptides that have properties favourable for MS identification. An alternative approach would be to utilise biotinylated analogues such as Mp7 Kb13 in order to enrich the successfully crosslinked VSD2. In an attempt to analyse an enriched sample of crosslinked VSD2, in gel digestion of the higher molecular weight band was explored. Unfortunately, it did not improve the detection of peptides of interest. In conclusion, experiments described in this section have demonstrated the successful use of HwTx-IV photoaffinity labeling probes Mp7, Mp27 and Mp29 and created the foundation for further validation experiments and application to more complex systems such as hNav1.7 in cell membranes.

4.4 Photocrosslinking of VSD4 with a Small Molecule Photoprobe

In addition to VSD2 toxin modulators, our interest in VSD4 modulators led to photocrosslinking experiments of aryl sulfonamide probe PFDB 5 (Figure 4.13) with the VSD4 chimera that had been crystallised.
in complex with aryl sulfonamides by Ahuja et al. 55

**Figure 4.13:** The structures of 1 (PF-06456384) and the diazirine-biotin probe 5 (PFDB). The synthesis of 5 is described in Chapter 2.

### 4.4.1 Differential Scanning Fluorimetry

Due to low aqueous solubility of PFDB, the photocrosslinking experiments of VSD4 had to be done in buffer containing 6% v/v dimethyl sulfoxide (DMSO). In order to determine whether the chimera was stable and folded in 6% DMSO, nano-differential scanning fluorimetry (nanoDSF) was employed. NanoDSF records changes in the emission of tryptophan fluorescence at 330 nm indicative of protein unfolding. 197 A plot of the derivative of the tryptophan fluorescence intensity against increasing temperature is used to determine the melting temperature ($T_m$) of a protein. The $T_m$ of VSD4 ($\sim 52 ^\circ C$) did not vary significantly between the SEC buffer and SEC containing 6% DMSO buffer (mean $\Delta T_m = -1.2 ^\circ C$) (Figure A.1). We next tested the effect of our inhibitors on VSD4. The presence of either the parent compound PF-384 or the photoprobe PFDB caused an increase in the $T_m$ of VSD4, suggesting that the molecules indeed bind to VSD4 forming a stabilised complex of mean $T_m$ of $\sim 59$ and $60 ^\circ C$, respectively. (Figure 4.14). The overall tryptophan fluorescence intensity decreased in the samples containing the inhibitors, suggesting a quenching effect upon binding. As a control, VSD2 ($T_m \sim 42 ^\circ C$) incubation with PFDB showed no effect, supporting the VSD4-selectivity of the aryl sulfonamide class of inhibitors. Unfortunately, nanoDSF was not employable to study the binding of HwTx-IV probes as there was an overlap between the melting properties of VSD2 and the peptide photoprobess.
Figure 4.14: NanoDSF analysis of VSD chimeras. A, T_m curves of VSD4 and VSD2 in the presence of PFDB. Measurements were recorded in duplicate. B, Graph representing the mean T_m as measured by the curves from A (n = 2). Similar to PF-384, PFDB shifted the mean T_m of VSD4 by + 8.4 °C whereas it did not have a significant effect on VSD2 stability.

4.4.2 Streptavidin Gel Shift Assay

In the case of a small molecule probe, the gel shift assay employed in Subsection 4.3.1 is not informative. Instead, a gel shift assay that relies on the binding interactions between biotin and streptavidin was developed. PFDB is a biotin-cotaining probe and will therefore form a stable complex with streptavidin (K_d ~ 10^{-15} M)\(^\text{198}\). In our hands, when transferred into sample loading buffer, the complex is stable at room temperature. Therefore, mixtures of PFDB and VSD4 (or VSD2 as a control) that had been UV-irradiated were incubated with streptavidin and separated by SDS-PAGE electrophoresis. Interestingly, as expected, bands that correspond to streptavidin-free biotinylated probe and streptavidin-biotinylated VSD4 emerged. The VSD-streptavidin band emerged in a UV-dependent manner and not in the case of VSD2 (Figure 4.15). Moreover, competition with the parent compound PF-384 was observed at low concentrations (Figures 4.15...
and 4.16). Based on this assay, 60 μM treatment was chosen for subsequent MS sample preparation as the photocrosslinking efficiency at 100 μM was not significantly higher.

![Figure 4.15](image1.png)

**Figure 4.15:** Assessment of PFDB photocrosslinking by stain free SDS-PAGE. Streptavidin shift assay exploits the biotin-streptavidin interaction to shift the migration of crosslinked VSD4 in gel. VSD2 did not get crosslinked by PFDB and VSD4 labeling was outcompeted by PF-384.

![Figure 4.16](image2.png)

**Figure 4.16:** Streptavidin shift assay to assess the competition effect of PF-384 against PFDB crosslinking (60 μM). Complete loss of labeling from 10 μM of PF-384 was observed.
4.4.3 Proteomic Mass Spectrometry Analysis

Similarly to VSD2, VSD4 sample preparation procedure was optimised by screening different digestion enzymes and techniques (FASP, in gel digestion and protein precipitation). VSD4 proved to be more challenging as most of the S1 and S2 hNav1.7 sequences were not identified even after Proteinase K digestion, which was the best performing protease in terms of overall coverage (Figure 4.17). Photocrosslinking of PFDB to VSD4 was not detected in GluC/trypsin treated samples and due to limitations in time, Proteinase K digestion has not been tested yet.

![Sequence coverage of VSD4: Proteinase K digestion](image)

**Figure 4.17:** Sequence coverage of VSD4 (1% FDR). Proteinase K resulted in the highest coverage of the hNav1.7 sequence of the chimera (highlighted in purple). Samples prepared by FASP.

4.4.4 Discussion

The diazirine-biotin probe based on PF-384 showed successful and specific photocrosslinking to VSD4 in our streptavidin shift assay. A model of PF-384 binding to VSD4 based on crystallographic data of the aryl sulfonamide binding site on VSD4 was reported by Storer et al. and represented in Figure 2.9. PFDB
was expected to crosslink to the extracellular loops of S1-2 or S3-4 as the diazirine/biotin containing linker extends from the piperidine moiety of the molecule.

Our main challenge in identifying the crosslinking site was the poor sequence coverage of VSD4 until Proteinase K was explored. However, at least in the case of potential crosslinking to S3-4, the lack of detection of the PFDB-modified peptide could be a result of poor solubility and ionisation ability of PFDB. In our sample preparation procedure, the dried digested peptides are resuspended in 2% v/v MeCN, 0.5% v/v TFA in water, conditions under which the modified peptides might not be successfully resolubilised. One future direction would be to use matrix assisted laser desorption ionisation (MALDI) coupled to MS/MS to circumvent this solubility issue. The MALDI sample preparation involves mixing the analyte in a solid matrix instead of resuspension in aqueous buffers. Finally, streptavidin enrichment could also improve the detection of the crosslinked VSD4 peptides.
Chapter 5

Conclusions and Outlook

The aim of this Thesis is to elucidate the basis of modulation of Nav1.7 by toxins and small molecule inhibitors. The development of photocrosslinking probes is discussed in Chapter 2. The photoprobes based on Huwentoxin-IV aim to interrogate the binding interface on VSD2 of Nav1.7. Moreover, a photoprobe based on aryl sulfonamide PF-384 was developed in order to label VSD4. Chapter 3 discusses the proteomic LC-MS/MS analysis of hNav1.7 in a recombinant cell line; significant optimisation was required in order to achieve confident detection and quantification of Nav1.7. In addition, a total proteome analysis of iPSC-derived neurons during maturation into nociceptors is reported. Finally, the photoprobes were employed in photocrosslinking experiments of purified chimeras of Nav1.7 with gel shift assays and LC-MS/MS in order to identify sites of interaction.

Below, a more detailed account of the conclusions and suggestions for future work are provided.

5.1 Probe Design and Synthesis

A large number of ICK toxins are interesting modulators of Nav1.7 because they exhibit high inhibitory activity in combination with elusive mechanisms of binding. The tertiary structure of these peptides is characterised by amphipathic properties and a rigid fold. Interestingly, ICK peptides have been shown to associate with liposomes with high or moderate affinity, highlighting the implication of the plasma membrane in modulation of Nav1.7. In order to increase our understanding of the basis of modulation by ICK peptides that bind to VSD2 of Nav1.7, photocrosslinking probes based on HwTx-IV were designed and synthesised.
Six positions on HwTx-IV were chosen in order to incorporate photomethionine, a diazirine-containing amino acid. The choice of six positions was made in order to increase the likelihood of successful crosslinking and in the case of a large binding interface, to probe interactions at various sites. The selection of these positions was based on an alanine scanning mutagenesis study. Firstly, SPPS of various analogues of HwTx-IV was completed successfully. Oxidative refolding of these peptides was achieved by incubation in a glutathione-based redox buffer. It is of vital importance to develop an analytical method in order to characterise the products of refolding. As discussed in a recent study describing the synthesis of ProTx-II, insufficient oxidation and misfolding was confirmed very late in the process by the toxin’s inability to inhibit Na⁺ current. In our experiments, the fully oxidised, refolded product was purified from any glutathione-peptide mixed disulfides and validated by high resolution MS. The isotopic distribution of the product was compared to predicted spectra in order to accurately assess the level of oxidation. In addition, the native peptide was subjected to NOESY in order to validate that the obtained disulfide connectivity under the specific refolding conditions was correct. To this end, correlations due to through-space interactions were identified and compared to the published solution NMR-based structure of HwTx-IV (PDB: 1MB6). Certain correlations strongly suggested the correct fold and the rest of the analogues were oxidatively refolded under the same conditions. Moreover, patch-clamp electrophysiology revealed that all peptides retained high inhibitory activity (13-80 nM), similar to native HwTx-IV. This observation was partly in agreement with the alanine scanning study, however, due to the larger side chain of photomethionine, the effect of Mp incorporation could not have been predicted with certainty. Revell et al. reported no change in the potency of the A7, A13, A21, A29, A35 mutants and a 2-fold increase for A5, and decrease for A27. Out of our probe library, the least potent probe was Mp27 (80 nM) in line with findings that this position was unfavourably sensitive to changes by Revell et al. Double mutants Mp7 Kb13 and Mp7 Gp13 exhibited half-maximal inhibition in a similar range of concentrations.

The aryl sulfonamide class of inhibitors were chosen in order to probe VSD4. Aryl sulfonamide inhibitors exhibit very high selectivity for Nav1.7 over other VGSC subtypes, and have participated in preclinical and clinical studies towards novel analgesics. PF-384, an inhibitor provided by Dr Ian Storer, structurally resembles GX-936 which was crystallised in complex with a VSD4 chimera in a pioneering study. This structure enabled the photoprobe design of PFDB; the piperidine moiety of PF-384 was functionalised with a biotin and diazirine-containing linker through N-hydroxy succinimide ester activation and coupling. This photoprobe, PFDB, exhibited a 3-fold decrease in potency by patch-clamp electrophysiology, however, lo-
wer potency is not necessarily unfavourable as it enables competition with the parent compound PF-384 which is necessary in order to validate specific photocrosslinking.

Overall, the development of ICK photocrosslinking probes is synthetically challenging and requires careful characterisation of the products. However, this Thesis has demonstrated that it is feasible to develop potent probes based on previous structural and mutagenesis studies. Initially, there were concerns that the incorporation of unnatural amino acids would negatively affect the complex oxidative refolding process but that was not observed. There are various reports on the synthesis and derivatisation of ICK peptides thus development of photocrosslinking probes based on other toxins that exhibit higher selectivity or complex inhibitory activity can be pursued with a similar strategy. Furthermore, in order to assess the binding affinity of the photoprobes to a VSD2 chimera for initial photocrosslinking experiments, future work would benefit from the use of a reported chimera that is successfully used in SPR studies. Additionally, this reported VSD2 chimera is electrophysiologically active and therefore a correlation between binding affinity to the detergent-solubilised protein and Na\(^+\) current inhibition in cells will be possible.

### 5.2 Mass Spectrometry Proteomics

#### 5.2.1 Nav1.7

Proteomic analysis of hNav1.7 began by subcellular fractionation in order to enrich membrane-bound proteins from HEK Nav1.7β1β2. Proteins that were readily identified through a high number of unique peptides were β1, β2 and a sodium-potassium pump that was used as a membrane protein control. However, little coverage of Nav1.7 was achieved and it was not significantly improved by Sulfo-NHS-Biotin surface biotinylation. Considerable leaking of the biotinylation reagent was suggested by the identification of intracellular biotinylated peptides. Therefore, this approach did not provide information on the level of plasma membrane localisation of Nav proteins. Affinity purification of the biotinylated peptides was performed both on the protein level followed by on-bead digestion, and on the tryptic peptide level. Detection of Nav1.7 peptides was achieved only through the latter. This method was very powerful as it allowed the exclusive detection of biotinylated peptides that under on-bead digestion conditions would not be released for further analysis.

The highest number of unique Nav1.7 peptides (85) was achieved through immunoprecipitation. In addition, optimisation of Nav1.7 detection from membranes without an additional form of enrichment yielded
30% sequence coverage (56 unique peptides). Given the challenges in LC-MS/MS analysis of membrane proteins, this is an encouraging result and future work should encompass a selection of MS-compatible detergents and proteases that will enable identification of different parts of the sequence such as the extracellular loops of the voltage-sensors. As the sample preparation optimisation of VSD chimeras highlighted less specific enzymes such as proteinase K and elastase, these enzymes could be initially explored in future optimisation experiments. Moreover, as significant uptake of the Sulfo-NHS-Biotin was observed, less stringent washing of the cells and lower reaction times should be attempted.

In the pan Nav immunoprecipitation experiment, β₁ and β₂ were not significantly enriched as it would be expected. The prominent enriched proteins mostly reside intracellularly and this observation additionally highlights issues with the localisation of Nav1.7 in an overexpressing cell line. In order to address these issues, an alternative approach that should be explored is glycan biotinylation. In a similar manner to lysine biotinylation, sialic acid oxidation to form an aldehyde and reaction with amino-xy biotin or biocytin hydrazide has also been used in plasma membrane protein enrichment studies. Further experiments could include immunofluorescence in order to localise Nav1.7 in cells and neurons. For this purpose, more specific antibodies are required and this problem can potentially be tackled with the development of highly specific tool molecules that carry a fluorescent tag or a ‘clickable’ handle.

Finally, photocrosslinking probes exhibit capabilities beyond identification of binding sites. Selective labeling of functional, plasma membrane-bound Nav1.7 by non cell-permeable photocrosslinking probes provides a lot of prospects for interrogation of Nav1.7 modulation by other proteins, trafficking and degradation. This can be achieved by quantitative mass spectrometry proteomics and immunofluorescence studies.

5.2.2 iPSCs in Erythromelalgia Disease Models

As discussed in Chapter 3, there has been increasing interest in patient-derived neurons as disease models. Neurons from erythromelalgia patients that carry different mutations and exhibit varied phenotypes and response to aryl sulfonamide treatment were allowed to mature for 53 days. The initial quantitative whole proteome analysis during neuronal maturation was successful in identifying upregulated proteins that are relevant to axonal growth and signaling. In parallel, qRT-PCR enabled the detection of changes in transcription of marker genes such as SCN9A, the gene encoding Nav1.7. In order to gain insights into the maturation process as well as Nav1.7 expression and modulation in disease-states, future experiments...
should include more biological replicates and control neurons expressing non mutant Nav1.7. A whole proteome analysis did not enable identification of Nav1.7, therefore immunoprecipitation and proteaseMax digestion will be employed in the future. Successful proteomic MS detection of Nav1.7 in patient-derived neurons will lead the way for further studies aiming to pinpoint the causes behind disease phenotypes. For instance, a comprehensive analysis of macromolecular complexes of Nav.7 that might contribute to the regulation of its biophysical properties or trafficking will be invaluable. Additionally, this approach could shed light on whether higher order structures are formed, as it has been proposed in the case of β3 and Nav1.5,24199 and any changes in post-translational modifications in disease states.

5.2.3 VSD Chimeras

The photocrosslinking probes were initially validated in experiments involving NavAb/Nav1.7 chimeras VSD2 and VSD4. The homotetrameric pores were FLAG-purified from insect cells. VSD2 was co-crystallised with ProTx-II and GsAf-1. Binding site identification through photocrosslinking will complement structural studies through orthogonal validation or if used in parallel, potential enhancement of the complex stability through a covalent bond formation.

Photocrosslinking was assessed by gel shift assays; in the case of peptide photoprobes, an apparent molecular weight shift in SDS-PAGE was observed in cases of successful photocrosslinking. VSD2 photoprobes that exhibited the significant photocrosslinking were Mp7, Mp27 and Mp29. Crosslinking dependent on concentration, UV irradiation and competition with HwTx-IV was observed. All six Mp probes surrounded the proposed binding site of HwTx-IV according to the published solution structure but successful crosslinking was observed only by residues that are closer to W30 in space. In the case of PFDB crosslinking to VSD4, a streptavidin shift assay revealed crosslinking that was competed by low concentrations of the parent compound. This was expected from the observed lower potency of PFDB with respect to PF-384. In addition, binding VSD4 to the aryl sulfonamide compounds in detergent was assessed by nanoDSF with significant (over 8 °C) increase in T_m.

Photocrosslinking experiments in HEK Nav1.7β1β2 membranes were unsuccessful in enriching Nav1.7 or yielding modified peptides therefore the photoprobes were tested on VSD chimeras. Initially, optimisation of sample preparation led to 64% sequence coverage of VSD2 (chymotrypsin) and 58% VSD4 (protease K). The parts of the sequence that are of interest belong to the hNav1.7 sequence; HwTx-IV has been suggested to interact with S1-S2 and S3-S4 and the piperidine moiety of PF-384 to point towards the same
site on voltage sensor domain IV. Chymotrypsin provided sufficient coverage of hNav1.7 on VSD2 chimera, whereas proteinase K exhibited the best hNav1.7 coverage on VSD4 however still lacking S2 coverage.

The encouraging result from proteinase K digestion was obtained late in the project, therefore PFDB photocrosslinking was only assessed through GluC/trypsin digestion. No significant crosslinking was detected and despite the lower coverage provided by GluC/trypsin, it is likely that properties such as hydrophobicity and low ionisation of the probe are not favourable for robust MS detection of crosslinked peptides. Future steps in pursuit of photocrosslinking sites should include MALDI analysis and proteinase K digestion as it results in peptides with different properties. In the case of low abundance of crosslinked peptides, streptavidin pull-down on the peptide level will likely favour detection by decreasing the background.

A piece of software dedicated to the identification of crosslinked peptides was employed for the analysis of Mp photocrosslinking. No significant Mp7-crosslinked peptides and only low confidence Mp27 crosslinking to the coiled-coil of NavAb were identified. Mp29 crosslinking to the S1-2 loop was identified in both trypsin and chymotrypsin digestion conditions. This observation supports mutagenesis studies that highlight the extracellular loops as the binding site of HwTx-IV (Subsection 1.2.2.2). MS validation of crosslinking in combination with the gel shift assays are a significant step forward for the characterisation of HwTx-IV binding. As the exact binding interface is elusive, the initial aim was to explore diverse positions on HwTx-IV that are available for photocrosslinking. Identification of crosslinking sites with the remaining Mp probes can be pursued with additional proteases. In order to tackle the issue of challenging identification of crosslinked peptides, the development of photoprobes that carry an affinity tag will be beneficial. Additional validation of MS hits will be performed through orthogonal pull-down assays. Successful validation of these chemical tools in experiments with purified chimeras will represent the starting point for further development of photoprobes for Nav1.7 in disease-relevant systems such as neurons.

5.3 Future Directions - Probing Binding Sites

General considerations regarding the elucidation of binding sites through crosslinking are described in this final section. In this Thesis, diazirine photocrosslinking dominates the probe design due to its short lifetime and small size. The suitability and success of the photolabile moieties are mostly system-dependent and other groups such as benzophenone could be explored in the future. Different activation properties
could provide additional information on binding sites as preference for crosslinking to certain amino acid side chains varies.

Orthogonal approaches that could be explored involve experiments such as hydroxyl radical footprinting. The basis of this approach is that formation of a complex ‘shields’ parts of the protein from, for example, oxidation events caused by radicals.\textsuperscript{200,201,202} The level of modification due to exposure is quantified and compared to the apo state of the protein allowing investigation of conformational changes and the binding site. Recently, a carbene footprinting approach that involves a diazirine-containing molecule and UV irradiation has been employed to map ligand binding sites and trimer formation in both soluble and membrane proteins.\textsuperscript{203,204}

Since a significant number of toxin peptides have been suggested to partition into the lipid bilayer, the mechanism of modulation of Nav1.7 is still unclear. Implementation of a photocrosslinking approach involving various positions on the photoprobe can shed light on this aspect. For example, in the case of HwTx-IV it is unclear whether mutations that promote hydrophobicity cause an increase in potency through beneficial interactions with Nav1.7 or lipids in the membrane. VSD chimeras serve as good models for Nav1.7 for crystallographic studies and similarly, photocrosslinking studies. Engineering additional constructs that exhibit various biophysical properties, such as high $V_1/2$ promoting a closed pore conformation can be beneficial to explore binding at different states. Moreover, photocrosslinking could be applied in the emerging field of cryo-EM. Specific covalent bond formation in the toxin-channel complex could increase the uniformity of the sample in order to battle dissociation and instability issues.
Chapter 6

Materials and Methods

The materials and apparatus are described in this chapter. The procedures are divided into four sections: Chemical Synthesis, Tissue Culture and Biochemical Methods, Patch-Clamp Electrophysiology, Chemical Biology and Proteomics.

6.1 Chemical Synthesis

6.1.1 Peptide synthesis

6.1.1.1 Reagents and Apparatus

All peptides were prepared by automated Solid Phase Peptide Synthesis using Intavis ResPep SL synthesiser (Intavis Bioanalytical Instruments, Germany) and the Fmoc/\textsuperscript{Bu} protocol. Rink amide Tentagel SRAM resin (RAPP Polymere) was used as the solid support. N-\textalpha-Fmoc amino acid derivatives were obtained from Novabiochem, UK. Cysteine (Cys), asparagine (Asn) and glutamine (Gln) were trityl (Trt)-protected. Serine (Ser), threonine (Thr) and tyrosine (Tyr) were incorporated as sidechain \textsuperscript{Bu} ethers, and aspartate (Asp) and glutamate (Glu) as \textsuperscript{O}Bu esters. Tryptophan (Trp) and lysine (Lys) sidechains were Boc-protected. Finally, arginine (Arg)-Pbf was used. H-L-photomethionine-OH was purchased from Thermo Scientific, Fmoc-L-Lysine(biotinyl)-OH and Fmoc-L-propargylglycine from Novabiochem UK. The reagents diisopropylethylamine (DIPEA), 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethylenaminium hexafluorophosphate (HBTU) and 2- (7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) were purchased from Novabiochem UK and Sigma-Aldrich. Dimethylformamide (DMF) for synt-
thesis, Dichloromethane (DCM), N-methyl pyrrolidone (NMP), N-methyl morpholine (NMM), methanol, piperidine, t-butylmethylether (TBME), trifluoroacetic acid (TFA), dithiolthreitol (DTT), phenol solution (80% v/v in ethanol), trimethylsilylisopropane (TIPS), and thioanisole were purchased from Sigma-Aldrich and Fluka.

Low resolution Mass Spectrometry: A Waters RP-HPLC system (Waters 2767 autosampler for sample injection and collection; Waters 515 HPLC pump to deliver the mobile phase to the source; XBridge C18 column 19 mm x 100 mm for preparative runs and 4.6 mm x 100 mm for analytical runs) coupled to a Waters 3100 ESI mass spectrometer and a Waters 2998 Photodiode Array were used for analytical and preparative runs. Samples were eluted with a linear gradient from 5-98% or 20-95% MeOH in H2O over 12 min at a flow rate of 1.2 mL min⁻¹ for analytical and 20 mL min⁻¹ for preparative runs; both solvents were supplemented with 0.1% v/v formic acid.

High resolution Mass Spectrometry: In order to monitor the oxidative refolding of the peptides, peptides were analysed by ultra-performance liquid chromatography (UPLC) coupled to a mass spectrometer (Waters LCT 50 Premier/Acquity-iClass) and a PDA detector UPLC LG 500 nm. This instrument has high mass resolution of 10,000. A C18 column (Acquity BEH 1.7 μm, 2.1 mm x 50 mm at 40.0 °C) with a linear gradient of 5-95% v/v MeCN in H2O (both containing 0.1% formic v/v acid) over 3.30 min and flow rate of 0.5 mL min⁻¹ were used. For the commercially available peptides, an Agilent LC-TOF-MS instrument comprising of an Agilent PLRP-S column (PLRP-S, 1.0 mm x 50 mm, 8 μm, 1000 Å) with a linear gradient of 15-60% v/v MeCN in H2O (both containing 0.05% v/v TFA) over 6 min and flow rate of 0.5 mL min⁻¹.

NMR Spectroscopy: A solution of refolded HwTx-IV (1.5 mM in 10% v/v D2O) was used to record ¹H NMR, TOCSY and NOESY spectra were recorded on a Bruker 800 MHz spectrometer at 300 K. The TOCSY mixing time was 73 ms over 64 scans. NOESY was recorded with 200 ms mixing time over 24 scans.

6.1.1.2 Synthesis of Linear Peptides

Each peptide was synthesised on a 20 μmol scale at RT. Coupling of natural amino acids was performed using 5-fold excess in NMP or DMF and HBTU for 40 min. The unnatural amino acids were coupled twice, at 2.5-fold excess and the presence of HATU. Coupling of Fmoc-L-photomethionine-OH was performed manually for 1.5 h to ensure high coupling efficiency.
6.1.1.3 Cleavage and Purification of Linear Peptides

Cleavage of the peptides was performed with agitation for 1.5 h. The ‘cleavage cocktail’ contained % v/v of TFA (87.5%), TIPS (2.5%), water (2.5%), phenol (2.5%), thioanisole (2.5%) and 1,4-dithiothreitol (2.5%, w/v). Crude peptides were precipitated by addition of cold TBME (x 2), the solids were isolated by centrifugation at 4000 rpm at 4 °C for 5 min and then dried under a flow of dry nitrogen, reconstituted in methanol/water (maximum 20% v/v) and filtered through a 0.45 μm filter. Crude peptides were purified by chromatography using the RP-HPLC system described previously. Purification of the crude peptide was performed at 20 mL min⁻¹ with a linear solvent gradient of 5–98% methanol (0.1% v/v FA) in water (0.1% v/v FA) over 18 min monitoring the UV absorption at 280 nm. The desired fractions containing the linear peptide, as identified by MS, were concentrated and lyophilised prior to refolding.

6.1.1.4 Oxidative Refolding of Linear Peptides

The oxidant used was a mixture of reduced and oxidised glutathione (GSH, GSSG) and the ‘refolding mixture’ contained 3:1 mM ratio of GSH/GSSG, 0.1 M NH₄HCO₃ pH 7-8, 0.1 mM EDTA and 7% v/v isopropanol. The final concentration of the peptide in the mixture was 0.1 mg mL⁻¹ in order to minimise intermolecular disulfide formation that can lead to aggregation or polymerisation. Termination of the reaction was done at various time points, after a minimum of 16 h using formic acid to acidify to pH 3. The refolded peptides were subsequently desalted and separated from glutathione adducts on a Sep-Pak tC18 Plus cartridge (Waters).

6.1.1.5 Characterisation data for VSD2 peptide probes

Tables 6.1 and 6.2 contain the sequence, retention time and deconvoluted molecular weight of each HwTx-IV analogue that was either synthesised or commercially obtained. It is worth noting that the commercially obtained peptides were analysed by the Agilent LC-MS under linear gradient conditions: 15-60% v/v MeCN in H₂O (both containing 0.05% TFA) over 6 min. The in-house synthesised peptides were analysed in the Waters LC-MS with a linear gradient of 5-95% v/v MeCN in H₂O (both containing 0.1% v/v formic acid) over 3.30 min.
6.1.2 Small molecule synthesis

6.1.2.1 Reagents and Apparatus

All general reagents and solvents were purchased from Sigma-Aldrich Ltd and VWR International and were used without further purification. The parent compound PF-06456384 was provided as a tris HCl salt by Dr Ian Storer, Pfizer Neusentis, UK. The synthesis of PF-06456384 has been described before.\textsuperscript{51} H-L-photoleucine-OH was purchased from Thermo Scientific.

Flash Column Chromatography: It was performed using silica gel (Merck, Geduran 560, 40-63 μm).
CHAPTER 6. MATERIALS AND METHODS

TLC analysis was performed using aluminium backed silica gel sheets (Merck, TLC Silica Gel 60, F254) with detection by UV absorption at 254 and 365 nm or chemical staining.

NMR Spectroscopy: \(^1\)H and \(^{13}\)C NMR spectra were recorded on 400 MHz and 101 MHz Bruker AV spectrometers, respectively. All spectra were assigned using the following reference solvent peaks for residual non-deuterated solvent in the \(^1\)H NMR spectra and for the deuterated solvent in the \(^{13}\)C NMR spectra: CDCl\(_3\) (7.26 ppm for \(^1\)H NMR; 77.16 ppm for \(^{13}\)C NMR), CD\(_3\)OD (3.31 ppm for \(^1\)H NMR; 49.00 for \(^{13}\)C NMR); DMSO-d\(_6\) (2.50 ppm for \(^1\)H NMR, 39.52 ppm for \(^{13}\)C NMR). All spectra were recorded at room temperature in deuterated solvents, as stated, and referenced to the solvent residual peak. Chemical shifts are given in parts per million. For \(^1\)H NMR the coupling constants are assigned in Hz, where possible.

6.1.2.2 (S)-2-((((9H-fluoren-9-yl)methoxy)methyl)amino)-4-(3-methyl-3H-diazirin-3-yl)butanoic acid, Fmoc-L-photomethionine-OH

![Chemical structure](image)

Fmoc-L-photomethionine-OH was synthesised following an adapted published method.\(^{107}\) Fmoc N-hydroxysuccinimide ester (Fmoc-OSu, 316 mg, 0.94 mmol) was dissolved in 1,4-dioxane (6 mL) and this solution was added dropwise over 15 min to a mixture of H-L-photomethionine-OH (100 mg, 0.64 mmol) and NaHCO\(_3\) (84 mg, 1.0 mmol) in H\(_2\)O (5 mL) at RT. The reaction mixture was stirred for 20 h, acidified to pH 3 with HCl, and extracted with ethyl acetate (3 x 10 mL). Organic layers were combined, dried over Na\(_2\)SO\(_4\), filtered and concentrated under reduced pressure. Column chromatography (Biotage Isolera SNAP 10 g cartridge with a gradient of 2-4% MeOH in DCM substituted with 0.1% v/v acetic acid) afforded Fmoc-L-photomethionine-OH as white crystals (183 mg, 76%). R\(_f\) 0.13 (2.5% v/v MeOH in DCM with 0.1% v/v acetic acid).

\(^1\)H NMR (400 MHz, CDCl\(_3\)): \(\delta\) 10.24 (s, 1H), 7.76 (d, J = 7.5 Hz, 2H), 7.62 – 7.51 (m, 2H), 7.40 (t, J = 7.5
Hz, 2H), 7.31 (td, J = 7.4, 1.3 Hz, 2H), 5.46 – 5.40 (m, 1H), 4.58 – 4.51 (m, 1H), 4.47 – 4.33 (m, 2H), 4.21 (t, J = 6.6 Hz, 1H), 1.91 – 1.77 (m, 1H), 1.62 – 1.24 (m, 3H), 0.98 (d, J = 23.2 Hz, 3H).

$^{13}$C NMR (101 MHz, CDCl$_3$): δ 176.88, 176.02, 156.51, 144.22, 144.03, 141.81, 128.27, 127.58, 125.49, 120.52, 67.60, 53.57, 47.59, 30.85, 27.49, 25.68, 20.14.

6.1.2.3 (S)-3-(3-methyl-3H-diazirin-3-yl)-2-(5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)propanoic acid (4)

A solution of N-hydroxysuccinimide ester of D-biotin 2 (69 mg, 0.2 mmol) in DMF (2 mL) was added dropwise to a mixture of H-L-photoleucine-OH 3, 23 mg, 0.2 mmol) and triethylamine (34 μL, 0.3 mmol) in DMF/H$_2$O (1:3 v/v, 3 mL) at RT for 1h. The product was purified by LC-MS, using a 12 min 5-98% linear gradient of MeCN in H$_2$O, both containing 0.1% v/v formic acid. The product was lyophilised and collected as a white solid (45.6 mg, 76%). Rt = 5.21 min, HRMS (ESI$^+$, m/z): found 370.1537. C$_{15}$H$_{24}$N$_5$O$_4$S ([M+H]$^+$) requires 370.1549.

$^1$H NMR (400 MHz, DMSO-d$_6$): δ 12.74 (s, 1H, -COOH), 8.15 (d, J = 8.3 Hz, 1H, NH), 6.42 (s, 1H, NH of biotin), 6.36 (s, 1H, NH of biotin), 4.32 – 4.28 (m, 1H, gg), 4.15 – 4.07 (m, 2H, ff and w), 3.13 – 3.06 (m, 1H, ee), 2.87 – 2.77 (m, 1H, hh), 2.57 (d, J = 12.5 Hz, 1H, hh$'$), 2.14 (t, J = 7.4 Hz, 2H, aa and aa$'$), 1.89 (ddd, J = 14.8, 4.5, 1.5 Hz, 1H, x), 1.69 – 1.27 (m, 7H, bb, bb$'$, cc, cc$'$, dd, dd$'$, x$'$), 1.01 (d, J = 1.5 Hz, 3H, y).

$^{13}$C NMR (101 MHz, DMSO-d$_6$): δ 172.84, 172.08, 162.70, 61.04, 59.19, 55.45, 47.79, 35.76, 34.97, 28.12, 28.05, 25.19, 24.46, 19.49.
A solution of (S)-3-(3-methyl-3H-diazirin-3-yl)-2-(5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)propanoic acid 4 (18.6 mg, 0.050 mmol), N-hydroxysuccinimide (6.1 mg, 0.053 mmol) and EDC.HCl (10.0 mg, 0.053 mmol) in dry DMF (1.8 mL) was left to stir at RT for 16 h. To the reaction mixture, a solution of PF-06456384 tris hydrochloride salt51 (41.7 mg, 0.054 mmol) and triethylamine (42.7 μL, 0.30 mmol) in dry DMF (0.8 mL) was added dropwise. The final mixture was heated to 50 °C and stirred for 2.5 h. The product was purified by LC-MS, using a 12 min 30-98% linear gradient of MeCN in water (0.1% v/v formic acid). The product was lyophilised and collected as a white solid (24.8 mg, 43%). \( \text{Rt} = 8.94 \text{ min, UV 254 nm 99\% purity, HRMS (ESI\', m/z): found 1071.3491. C}_{50}\text{H}_{54}\text{F}_{3}\text{N}_{12}\text{O}_{6}\text{S}_{3} ([M+H]\text{')} requires 1071.3403.} \\

\[^{1}\text{H NMR (500 MHz, CD}_{3}\text{OD):} \delta 8.55 (d, J = 5.0 \text{ Hz, 1H, l}), 8.02 (d, J = 1.4 \text{ Hz, 1H, b}), 7.98 (s, 1H, h), 7.97 (d, J = 1.7 \text{ Hz, 1H, i}), 7.94 (s, 1H, a), 7.91 (dd, J = 8.4, 2.3 \text{ Hz, 1H, f}), 7.87 (dd, J = 9.0, 1.8 \text{ Hz, 1H, c}), 7.85 (d, J = 2.4 \text{ Hz, 1H, g}), 7.73 - 7.66 (m, 2H, j and k), 7.63 (s, 1H, n), 7.55 (d, J = 5.2 \text{ Hz, 1H, m}), 7.50 (d, J = 8.4 \text{ Hz, 1H, e}), 6.82 (d, J = 9.0 \text{ Hz, 1H, d}), 4.78 (dd, J = 10.7, 4.8 \text{ Hz, 1H, w}), 4.52 - 4.42 (m, 2H, gg and v), 4.38 (d, J = 3.8 \text{ Hz, 2H, o and o'}), 4.30 (dt, J = 7.8, 4.8 \text{ Hz, 1H, ff}), 4.05 (dd, J = 32.4, 14.2 \text{ Hz, 1H, t}), 3.22 - 3.11 (m, 4H, p, p', t', ee), 2.91 (ddd, J = 12.8, 4.8, 2.5 \text{ Hz, 1H, hh}), 2.74 - 2.62 (m, 2H, hh' and v'), 2.27 (tt, J = 8.6, 6.2 \text{ Hz, 2H, aa and aa'}), 1.98 - 1.40 (m, 13H, q, q', r, s, x, x', bb, bb', dd, dd', cc, cc'), 1.21 (dd, J = 56.2, 19.3 \text{ Hz, 2H, s' and u'}), 1.04 (d, J = 8.4 \text{ Hz, 3H, y}). \]
13C NMR (126 MHz, CD3OD): δ 187.24, 175.39, 170.94, 166.11, 162.25, 159.41, 152.89, 152.09, 150.87, 147.12, 141.75, 139.97, 133.74, 132.94, 131.99, 131.21, 131.05, 125.67, 125.08, 124.78, 124.22, 124.15, 116.92, 116.02, 103.52, 63.26, 61.64, 57.02, 51.70, 49.51, 47.11, 46.65, 46.27, 43.82, 43.52, 41.06, 38.02, 37.69, 36.36, 34.89, 34.62, 33.49, 33.31, 32.69, 32.49, 29.63, 29.40, 26.81, 26.70, 24.82, 20.10.

### 6.2 Tissue Culture and Biochemical Methods

#### 6.2.1 General Methods

**SDS-PAGE gels.** 5-10 μg of protein samples were incubated with 2x Laemmli sample loading buffer (SLB), supplemented with 10% v/v β-mercaptoethanol for 30 min with shaking. The samples were then loaded into 4–15% polyacrylamide Mini-PROTEAN TGX protein gels (Bio-Rad) and the electrophoresis was done using Tris/Glycine/SDS running buffer and Precision Plus protein standard (Bio-Rad) at 200 mV for 45 min.

1-5 μg of VSD samples were incubated with 1x Laemmli SLB supplemented with 10% v/v β-mercaptoethanol and loaded into 4–20% polyacrylamide Mini-PROTEAN TGX gels (200 mV for 45 min).

**Western Blotting.** Gels were briefly washed with deionised water and the proteins were transferred to a 0.45 μm Nitrocellulose membrane (GE Healthcare) in transfer buffer containing 25 mM Tris, 190 mM glycine and 20% v/v methanol. TBST buffer (20 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween-20) was used for all washes and resuspension of blocking reagents and antibodies. Membranes were blocked at RT for 1 h or at 4 °C overnight with skim milk powder (5 % w/v) and then washed with TBST (3 x 10 min). For biotinylated protein detection, milk was replaced with bovine serum albumin (BSA) and membranes were then incubated with Avidin NeutrAvidin-HRP in 0.3% BSA/TBST (Invitrogen) for 1 h and washed again with TBST (3 x 10 min). For specific target protein blots, membranes were incubated with the primary antibody overnight at 4 °C in blocking solution, washed (3 x 10 min), incubated with secondary antibody for 1 h in blocking solution and washed again. Detection was carried out using Luminata Crescendo Western HRP substrate (Millipore) according to the manufactures instructions and on a ImageQuant LAS4000.
6.2.2 Mammalian Cell Culture

HEK Nav1.7β1β2 cells were obtained from Dr Peter Cox (Pfizer Neusentis) and were cultured in T75 culture flasks at 37 °C in a humidified incubator with 5% CO2. The culture media consisted of high glucose and pyruvate-containing Dulbecco Modified Eagle Medium (Life Technologies, catalogue number: 41966029) supplemented with 10% fetal bovine serum (Life Technologies), 1x MEM Non-essential Amino Acid Solution, 1 mM HEPES, 1 μg mL⁻¹ puromycin and 500 μg mL⁻¹ G-418. Cells were passaged by trypLE treatment (Life Technologies, catalogue number: 12604013) for 2 min, seeded 48-72 h prior to experiments and used between passage 5 and 20. Cells were harvested at 70% confluence for further experiments. HEK293 cells were cultured under the same conditions using standard DMEM.

6.2.3 Neuronal Maturation

Frozen immature neurons EM1 and EM2 at day 11 of the differentiation protocol205,174 were obtained from Pfizer Neusentis.

Differentiated neurons were seeded at 100,000 cells cm⁻² in 6 well plates coated with growth factor reduced Matrigel (Corning) maintained for 8 weeks in Neurobasal medium containing the supplements and growth factors detailed in table 6.4. Upon thawing, cells were treated with ROCK Y27632 (10 μM) for 24 h (day 11). On day 13 and 20, the cells were treated with mitomycin-C (1 μg mL⁻¹) for 2 h. The growth medium was changed twice weekly.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Vendor</th>
</tr>
</thead>
<tbody>
<tr>
<td>pan Nav K58/35</td>
<td>mouse monoclonal</td>
<td>Sigma S8809</td>
</tr>
<tr>
<td>α-tubulin</td>
<td>mouse monoclonal</td>
<td>Santa Cruz sc-8035</td>
</tr>
<tr>
<td>α-Navβ₁</td>
<td>rabbit polyclonal</td>
<td>Alomone ASC-041</td>
</tr>
<tr>
<td>α-Navβ₂</td>
<td>rabbit polyclonal</td>
<td>Alomone ASC-007</td>
</tr>
<tr>
<td>α-Na⁺/ K⁺ ATPase</td>
<td>mouse monoclonal</td>
<td>Abcam ab7671</td>
</tr>
<tr>
<td>α-FLAG M2-HRP</td>
<td>mouse monoclonal</td>
<td>Sigma A8592</td>
</tr>
<tr>
<td>α-mouse-HRP</td>
<td>goat monoclonal</td>
<td>Advansta R-05071-500</td>
</tr>
<tr>
<td>α-rabbit-HRP</td>
<td>goat monoclonal</td>
<td>Advansta R-05072-500</td>
</tr>
<tr>
<td>normal IgG</td>
<td>mouse</td>
<td>Santa Cruz sc-2025</td>
</tr>
</tbody>
</table>

Table 6.3: Information on antibodies used for Western Blotting.
## CHAPTER 6. MATERIALS AND METHODS

### 6.2.4 qRT-PCR Analysis of Neuronal Maturation

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPRT</td>
<td>Forward: 5'-GCTTACTAAATTCTTTTGCTTGACCTGCTG-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-AATTACTTTTTATGTCCCCTGTTGACTTG-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward: 5'-CAACTTTGGTATCCTGGAAGGAC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-AAGTGCTCTGTTGCGGACTG-3'</td>
</tr>
<tr>
<td>SCN9A</td>
<td>Forward: 5'-AGAGGGGTACACTCGTGGAA-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-CCCAGGAAAATCAGATCAG-3'</td>
</tr>
<tr>
<td>SCN10A</td>
<td>Forward: 5'-ATGACCCGAAACTCTTCCC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-TGAGCGTAAATCCAGCCAG-3'</td>
</tr>
<tr>
<td>TAC1</td>
<td>Forward: 5'-ACTGCTCCGTCGAAAATC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-GGGCCACTTTTTTCAA-3'</td>
</tr>
<tr>
<td>POU4F1</td>
<td>Forward: 5'-GATACCCAGATCAACGC-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-AGGAGATGTGCTCCAGAC-3'</td>
</tr>
<tr>
<td>ISL1</td>
<td>Forward: 5'-CAACTGGTCATTTTTCAGAAGGA-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-TTGAGAACATTTGACCTTCCAC-3'</td>
</tr>
</tbody>
</table>

Table 6.5: Primers used for qRT-PCR analysis of neuronal maturation.
26 ng of total RNA derived from EM neurons at different time points was isolated using RNase minikit (Qiagen) and reverse-transcribed into cDNA using Superscript III (Life Technologies) and oligo d(T). SYBR Green PCR Master mix (Applied Biosystems) was performed according to the manufacturer’s protocol using primers to SCN9A, SCN10A, TAC1, ISL1, POU4F1, HPRT and GAPDH (Table 6.5). Relative expression of the target gene was calculated using the comparative ΔΔCT method, where CT is the cycle threshold. Expression of the test gene was compared with that of HPRT measured on the same sample, giving a CT difference (ΔCT) for HPRT minus the test gene. ΔCT values of the different time points were compared to ΔCT of the first time point (15 days), giving ΔΔCT values for each specific time point (15, 35 and 53 days) and relative expression was calculated as $2^{-\Delta\Delta CT}$ using mean ΔCT values.

6.2.5 Chimeric Expression in Insect Cells

The insect cells expressing VSD4 and VSD2 chimeras were cultured at Genentech. Recombinant baculovirus was generated using the Baculogold system (BD Biosciences) following standard protocols. *Trichoplusia ni* (Tni) cells were infected for protein production and harvested 48 h post-infection.

6.2.6 Nav1.7 Chimera Sequences

The sequence and molecular weight information of the hNav1.7/NavAb chimeras VSD2 and VSD4 are given below. The FLAG affinity tag and thrombin protease cleavage site are underlined and the human Nav1.7 is highlighted in green.

**VSD2 chimera sequence:**

```
MDYKDDDKGSLVPRGSMSNLRTINVESQFTFIYLIYLIYLNTLFMAMEHHPMTE
EFKNVLAIGNLVFTGIFAIIBIIILRIVHRSFSSFKDPWSLDLIVTLSLVELFLAD
VEGLSVLRSPRLRVFRLLVTAVPQMRTIVSALISVIPGLMLSVIALMTLFFYIFAIM
ATQIFGGERFPWEFGTLGESFYTLFQMTLEWSMGIVRPLMVEYPAWVFFIFIF
VTTFVMINLVAICVDAAILNQKEEQIIDEVQSHEDINNEIIKLREEIEVELKE
LIKTSKLN
```

Molecular Weight: 33425 Da, length: 288 residues, pI: 5.30
CHAPTER 6. MATERIALS AND METHODS

VSD4 chimera sequence:

MDYKDDDKGSLVPGRSHMYLRTNIVESSFFTIIYLIYLNVMVTMVEKEGSQHMTEVLWVINVFIILFTIEIILRIVYHRISFFKDPWSLFDVFVIISIVGFLADLIETYFVSPSLFRVRLARTIGRLRLVTAQMPRKIVSAISVIGMSLVIALMTLFYIFAIMATQLGFGERFPEWFGTQGEOFTQVMTLESWSMGIVRPLMEVYPYAWVFFIPFIFVTVNFMINLVAIIVDAMAILNQKEEQHIIDEVQSHEDNINNEIKLREEIVELKELIKTLSKN

Molecular Weight: 34589 Da, length: 296 residues, pl: 5.43

6.2.7 Purification of VSD Chimeras

Tn1 cells expressing VSD4 or VSD2 channels were resuspended in 50 mM Tris pH 8.0, 200 mM NaCl (Buffer A) supplemented with EDTA-free complete protease inhibitors (Roche). Following cell lysis, GDN (Anatrace) was added to 2% (w/v) and solubilisation was carried out with gentle agitation for 2 h at 4 °C. The cell lysate was then clarified under 50,000 g for 1 h and the supernatant was mixed gently with anti-FLAG M2 agarose resin (Sigma Aldrich) that had been pre-equilibrated with Buffer A for 2 h at 4 °C. For purification of VSD2 for crystallisation, the FLAG resin was collected under gravity flow and washed with 4 x 5 column volumes of Buffer B (Buffer A supplemented with 0.042% (w/v) GDN), 1 column volume of buffer C (Buffer A supplemented with 0.042% (w/v) GDN, 0.08% (w/v) FA3 (Facade-EM, Anatrace) and 0.13 mg mL⁻¹ lipid mixture POPC:POPG:POPE at 3:1:1 mass ratio. Finally, it was washed with 5 column volumes of Buffer D (Buffer A supplemented with 0.06% (w/v) FA3 and 0.1 mg mL⁻¹ lipid mixture of phosphatidylcholine (POPC), phosphatidylethanolamine (POPE) and phosphatidylglycerol (POPG) (3:1:1 mass ratio). The protein was eluted with 5 x 1 column volumes of Buffer D supplemented with 150 μg mL⁻¹ FLAG peptide.

For the purification of VSD4 or VSD2 for photocrosslinking experiments, the same procedure was followed with all washes and elution performed using Buffer B and adding the FLAG peptide when necessary.

The eluate was passed over a Superose 6 Increase column (GE Healthcare) in 10 mM Tris pH 8.0, 100 mM NaCl and 0.042% (w/v) GDN for VSD4 and VSD2, or 0.06% (w/v) FA3 and 0.1 mg mL⁻¹ lipid mixture POPC:POPG:POPE at 3:1:1 ratio for VSD2 for crystallisation and peak fractions were concentrated using a Vivaspin concentrator (100 KDa MWCO).
6.2.8 Co-crystallisation of VSD2 and Toxin Peptides

A mixture of purified VSD2 chimera (20 mg mL⁻¹, 0.3 mM) and toxin peptide (0.6 mM) in 100 mM NaCl and 10 mM Tris pH 8.0, 0.06% (w/v) FA3 and 0.1 mg mL⁻¹ lipid mixture POPC:POPG:POPE at 3:1:1 mass ratio. The mixture was left to equilibrate on ice for 1 h and crystallisation drops were set up in a sitting drop vapour diffusion format over well solutions containing ammonium sulfate (2.55 M) in sodium cacodylate trihydrate (pH 5.6 or pH 6.2, 100 mM). Microcrystal growth was allowed for 5 days at 13°C. On the day of the data collection, the microcrystals were pooled in batches of similar size (5-20 μm) (ZEISS SteREO Discovery.V8 microscope) and the slurry was allowed to settle at RT. Part of the supernatant was removed and the concentrated crystal slurry was introduced in a reservoir connected to the Double Flow Focused liquid injector at the Coherent X-ray Imaging end station of the Linac Coherent Light Source (LCLS). The flow rate (20 μL min⁻¹) was adjusted in order to achieve a jet of crystal slurry with acceptable hit rate by the beam.

6.2.9 Differential Scanning Fluorimetry

Purified VSD4 or VSD2 (0.9 mg mL⁻¹) in buffer (10 mM Tris pH 8.0, 100 mM NaCl and 0.042% (w/v) GDN) was incubated with an inhibitor (100 and 300 μM) or DMSO by addition of a stock solution so that the final concentration of DMSO was 6%. After incubation for 1 h on ice, the mixture was loaded into nanoDSF Grade Standard Capillaries (NanoTemper Technologies) in duplicate and transferred to a Prometheus NT.48 nanoDSF device (NanoTemper Technologies). Thermal unfolding was detected during heating in a linear thermal ramp (1 °C min⁻¹; 15 °C to 80 °C) with an excitation power of 15%. Unfolding transition points were determined from changes in the emission wavelengths of tryptophan fluorescence at 330 nm, 350 nm and their ratios. Data was analyzed with the Prometheus PR. Control software (NanoTemper Technologies).

6.3 Patch-Clamp Electrophysiology

All recordings were carried out at RT (22-23 °C) using a HEK293 stable cell line expressing hNav1.7 (Millipore). Cells were plated out onto 12 mm coverslips coated with poly-D-lysine the day prior to patching. Currents were recorded using whole cell recording methods (Axopatch 200B), digitised at 50 kHz and low pass filtered at 5 kHz.

The voltage protocol used to measure current inhibition (V₁/₂ protocol) was as follows: hold at -110 mV
and step to $V_{1/2}$ inactivation for 5s. Recovery was for 100 ms at -110mV and followed by a test step to -10 mV (peak IV) for 20 ms. A sweep was carried out every 15 s. The $V_{1/2}$ protocol described above was used to evoke currents until the peak current recorded was stable. Following this, a steady state inactivation curve was run to re-check the $V_{1/2}$ inactivation determined at the beginning of the experiment. The cell was then held at this $V_{1/2}$ inactivation value for 2 min during which time the compound was applied. The $V_{1/2}$ protocol was immediately run following the 2 min hold and current inhibition assessed. Wash was measured stepping to the peak of the IV from -120 mV. Following this the current was re-measured following wash using the $V_{1/2}$ protocol. $V_{1/2}$ inactivation value used was the same as at the beginning of the experiment.

Standard extracellular solutions (ECS) contained (mM): 60 NaCl, 70 CsCl, 5.4 KCl, 1.8 CaCl$_2$, 0.8 MgCl$_2$, 10 HEPES, 5 Glucose (pH 7.4; 290 mOsm). The pipette solution contained (mM): 100 CsF, 5 NaCl, 45 CsCl, 10 HEPES, 5 EGTA, (pH 7.3; 284 - 290 mOsm).

6.4 Chemical Biology and Proteomics

6.4.1 Photocrosslinking of Purified Chimeras

The purified VSD2 chimera (0.3 mg mL$^{-1}$, 8.6 μM in 10 mM Tris pH 8.0 and 100 mM NaCl and 0.042% (w/v) GDN) was incubated with the photocrosslinking probes based on Huwentoxin-IV: Mp5 (200 μM), Mp7 (100 μM), Mp21 (200 μM), Mp27 (100 μM), Mp29 (50 μM) and Mp35 (200 μM) on ice for 1 h. The samples were transferred to a transparent 96 well plate (Nunc, Thermo Scientific) and irradiated at 365 nm in a UV box (Spectrolinker XL1500, Spectronics Corporation) for 10 min. In a similar manner, the VSD4 chimera was incubated with PFDB (60 μM) on ice for 1 h. The irradiation was done under the conditions described above. The competition with the parent inhibitor PF-06456384 in the case of VSD4 or native Huwentoxin-IV in the case of VSD2 was done with pre-incubation for 30 min at various concentrations on ice.
6.4.2 Streptavidin Shift Assay

After UV irradiation, protein samples (5 μL, 0.3 mg mL\(^{-1}\)) were incubated with 4 μL streptavidin (Jackson Immunoresearch, 100 μM in water) in 1 x Laemml SLB supplemented with 10% v/v β-mercaptoethanol at RT for 10 min. The samples were then loaded on a SDS-PAGE gel (4–20% Stain Free Mini-PROTEAN TGX™ precast polyacrylamide protein gels, Bio-Rad) and electrophoresis was performed in a Tris/Glycine/SDS system at 200 mV for 45 min. The gels were visualised using a Bio-Rad Gel Doc EZ System.

6.4.3 Mammalian Cell Line Proteomics

6.4.3.1 Protein Concentration Determination

Cell lysates were clarified by centrifugation at 16000g at 4 °C for 15 min and the protein concentration was estimated using the DC protein assay (Bio-Rad) and a standard curve generated using BSA concentrations. All lysates were stored at -80 °C.

6.4.3.2 Subcellular Fractionation

Mammalian cells (HEK293 and HEK293 Nav1.7β\(_1\) β\(_2\)) at 70% confluence were washed with buffer containing 150 mM NaCl, 50 mM HEPES pH 7.4 and harvested using non-enzymatic cell dissociation buffer (Life Technologies) for 5 min. The cell pellet was incubated in hypotonic buffer (0.6 mL, 10 mM NaCl, 1.5 mM MgCl\(_2\), 10 mM HEPES pH 7.4, 1 x complete EDTA-free inhibitor (Roche)) on ice for 30-40 min. The cells were then sheered with 21G (15 strokes), followed by 25G (25 strokes) and 30G (10 strokes) needles. The homogenate was centrifuged (800g) at 4 °C for 5 min. The pellet obtained was the nuclear fraction, and the supernatant was taken forward to ultracentrifugation (100,000g) at 4 °C for 1 h. The crude membrane fraction was the pellet collected after ultracentrifugation, and it was washed with buffer (0.5 mL, 150 mM NaCl, 50 mM HEPES pH 7.4) twice before addition of lysis buffer 1 (1% v/v Triton, 150 mM NaCl, 1.5 mM MgCl\(_2\), 10% v/v glycerol, 50 mM Tris pH 7.4, 1 x complete EDTA-free Protease Inhibitor) on ice for 1 h with occasional vortexing. The lysate was clarified by centrifugation (16,000g, 15 min).

6.4.3.3 Surface Biotinylation

Mammalian cells (HEK293 and HEK Nav1.7β\(_1\) β\(_2\)) at 70% confluence were washed with cold PBS three times before being harvested using non-enzymatic cell dissociation buffer for 5 min. The cells were then...
centrifuged at 800 rpm for 5 min. The cell pellet was incubated with a solution of EZ-link Sulfo-NHS-
Biotin (Thermo Scientific, 1 mL, 2 mM in PBS) and gently agitated on a rocking platform at 4 °C for 30
min. Glycine (0.5 mL, 0.2 M in PBS) was added to quench any unreacted biotinylation reagent. The cells
were then centrifuged at 1000 rpm for 3 min and the supernatant was discarded. Three washes of PBS (5
mL) were performed before lysis of the cells with lysis buffer 1 on ice for 1 h with occasional shaking. The
solubilised proteins were clarified by centrifugation (16,000g, 15 min).

6.4.3.4 Immunoprecipitation

Mammalian cells (HEK293 and HEK Nav1.7β₁ β₂) at 70% confluence were washed with cold PBS and har-
vested with non-enzymatic cell dissociation buffer for 5 min. The cells were then centrifuged at 800 rpm
for 5 min. The cell pellet was lysed with lysis buffer 1 for on ice for 1 h with occasional shaking. The lysate
was clarified by centrifugation (16,000g, 15 min).

Dynabeads Protein G (Life Technologies) were washed with PBS twice (10 x volume) by vortexing for 1-
2 min and centrifuging to remove the supernatant. The antibody (pan Nav, K58/35) was added to the beads
in PBS pH 7.4 supplemented with 0.02% v/v Tween-20. After a 10 min incubation at RT, the beads were
washed twice with PBS + 0.02% v/v Tween-20 and twice with triethanolamine pH 8.2 (0.2 M). Crosslinking
of the antibody to the beads was done with freshly made dimethyl pimelimidate dihydrochloride (DMP)
solution (50 mM in 0.2 M triethanolamine pH 8.2) and vortexing gently for 30 min. The crosslinking was
quenched by washing with 50 mM Tris pH 7.5 for 15 min. The beads were then washed with PBS + 0.02% v/v
Tween-20 twice before elution of non-crosslinked antibody by glycine pH 3 (1 M) treatment with shaking
for 10 min. The beads were washed twice with PBS + 0.02% v/v Tween-20 and stored overnight at 4 °C.

Lysate pre-clearing was achieved by incubating lysates from HEK293 and HEK Nav1.7β₁ β₂ cells (400
μg) with Dynabeads Protein G, previously washed with lysis buffer 1 for 1 h at 4 °C. The pre-cleared lysate
was incubated with the antibody-crosslinked beads for 2 h at 4 °C. The beads were then washed three
times with lysis buffer 1 (10 x volume). For gel and western blotting analysis, the bound proteins were
eluted from the beads using 2 x SLB and vortexing for 30 min. For further proteomic analysis, the proteins
were eluted by incubation with glycine pH 3 (0.2 M) for 15 min at RT. The sample pH was then neutralised
by addition of Tris pH 9.5 (1/4 of the eluent volume, 1 M).
6.4.3.5 Detergent Screening for Nav1.7 Extraction

HEK Nav1.7β1β2 cell membranes were solubilised using different buffers (Table 6.6) on ice for 1 h. A sample was collected before centrifugation at 17,000g for 1 h. Before and after centrifugation samples were loaded into SDS-PAGE gels and western blotting against a pan Nav antibody was performed. The intensity of the bands was quantified using ImageJ and the ratio before and after centrifugation was calculated.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triton</td>
<td>50 mM HEPES pH 7.5, 150 mM NaCl, 1% v/v Triton-X100</td>
</tr>
<tr>
<td>Triton + glycerol</td>
<td>50 mM HEPES pH 7.5, 150 mM NaCl, 1% v/v Triton-X100, 10% v/v glycerol</td>
</tr>
<tr>
<td>DDM</td>
<td>50 mM HEPES pH 7.5, 150 mM NaCl, 1% v/v DDM</td>
</tr>
<tr>
<td>DDM + cholesterol</td>
<td>50 mM HEPES pH 7.5, 150 mM NaCl, 1% v/v DDM, 0.2% w/v cholesterol</td>
</tr>
<tr>
<td>DDM + cholesterol + glycerol</td>
<td>50 mM HEPES pH 7.5, 150 mM NaCl, 1% v/v DDM, 0.2% w/v cholesterol, 10% v/v glycerol</td>
</tr>
<tr>
<td>OG</td>
<td>50 mM HEPES pH 7.5, 150 mM NaCl, 7% v/v OG</td>
</tr>
<tr>
<td>Digitonin</td>
<td>100 mM Tris pH 8, 200 mM NaCl, 1% v/v digitonin</td>
</tr>
<tr>
<td>Digitonin + glycerol</td>
<td>100 mM Tris pH 8, 200 mM NaCl, 1% v/v digitonin, 10% v/v glycerol</td>
</tr>
<tr>
<td>CHAPS</td>
<td>50 mM HEPES pH 7.5, 150 mM NaCl, 9% v/v CHAPS</td>
</tr>
</tbody>
</table>

Table 6.6: Composition of buffers used for extraction of Nav1.7 from cell membranes.

6.4.4 Filter-Aided Sample Preparation (FASP)

The FASP protocol described in this section was employed to prepare proteomics samples from cell lysates in lysis buffer 1, neutralised immunoprecipitated samples, purified and photocrosslinked chimeras. The samples were reduced with DTT (10 mM) for 30 min at 55 °C and then transferred to a 30 kDa MWCO Vivacon 500 concentrator (Sartorius). Urea solution (200 μL, 8 M urea in 100 mM Tris pH 8.5) was added and the samples were centrifuged at 14000g for 10 min. Iodoacetamide was added to a final concentration of 50 mM and the mixture was vortexed for 1 min before incubation in the dark for 20 min. The concentrators were washed three times with urea solution and three times with AMBIC solution (100 μL, 50 mM NH₄HCO₃ pH 8.5). Digestion was performed using sequencing grade modified trypsin (Promega), Glu-C (Promega), Proteinase K (New England Biolabs), elastase (Promega) or chymotrypsin (Promega or
Roche) with either a 1:50 or 1:100 w/w enzyme/protein ratio and 0.05% w/v ProteaseMAX (Promega). All samples were shaken overnight at 37 °C with the exception of Proteinase K (37 °C, 3 h) and chymotrypsin (25 °C, overnight). Digestion peptides were collected into a clean Vivacon tube with 40 μL washes of AMBIC (50 mM) and NaCl (0.5 M). The peptides were acidified with 0.5% v/v trifluoroacetic acid (TFA) and desalted using StageTip method. Elution from the sorbent (SDB-XC poly(styrene/divinylbenzene) copolymer, from 3M) with 79% v/v MeCN in water was followed by speed-vac assisted solvent removal. Three-step StageTip fractionation was performed using poly(styrenedivinylbenzene) reverse phase sulfonate SDB-RPS (3M). Three elution buffers were used to collect three samples of the fractionated peptides; Buffer 1 (0.1 M ammonium formate, 40% v/v MeCN, 0.5% v/v formic acid), Buffer 2 (0.15 M ammonium formate, 60% v/v MeCN, 0.5% v/v formic acid) and Buffer 3 (5% v/v NH₃(aq) in 80% v/v MeCN). The dried peptides were stored at -80 °C until LC-MS/MS analysis.

6.4.5 Biotinylated Peptide Pulldown

Biotinylated samples were transferred to a 30 kDa MWCO Vivacon 500 concentrator and the FASP protocol was performed as described in Subsection 6.4.4 with modifications based on a published procedure. The digested peptides were collected from the concentrator with two washes of PBS (100 μL). TFA was added to the peptides to a final concentration of 0.1% v/v. Agarose Neutravidin beads (Invitrogen) were washed three times by vortex mixing and centrifuging with PBS (10 x volume) and incubated with the acidified peptides at RT for 2 h. The non-biotinylated peptides were removed by washing with PBS (x 4) and water (x 1). The biotinylated peptides were eluted from the agarose beads by incubation with % (v/v): 80% MeCN, 0.1% TFA, 0.2% FA in water for 10 min. Speedvac removal of the solvents was followed by storage of the dried biotinylated peptides at -80 °C.

6.4.6 Isobaric Mass Tagging in FASP (iFASP)

Lysates of EM1 and EM2 were reduced with tris(2-carboxyethyl)phosphine (TCEP, 10 mM) at 55 °C for 1 h. The reduced samples were then transferred into a 10 kDa MWCO Vivacon 500 concentrator and the FASP protocol was followed as previously described. Instead of AMBIC washes, three washes with triethylammonium bicarbonate (TEAB) were performed (100 μL, 100 mM) before trypsin digestion (1:50 w/w enzyme/protein ratio) at 37 °C for 16 h with shaking at 600 rpm. The peptide concentration was estimated using NanoDrop A₂₈₀, assuming 1 Abs unit corresponds to 1 mg mL⁻¹ peptide concentration.
(0.1% w/v). 12 μg from each sample were transferred in clean tubes and the volume was adjusted to 50 μL with TAEB (100 mM, pH > 7.8). Immediately before use, TMT Label Reagents (Thermo Scientific, cat. no. 90061) were equilibrated to RT. To the vials containing 0.8 mg TMT Label reagent, anhydrous MeCN (41 μL) was added to dissolve the contents. The solution of each TMT Label Reagent (20.5 μL) was added to each peptide sample and the reaction was allowed to proceed at RT for 1 h. The reaction was quenched by addition of 5% v/v hydroxylamine (4 μL), vortexing at 600 rpm for 30 min. The labelled peptides were collected from the spin column with TAEB (100 mM, 40 μL) and NaCl (500 mM, 50 μL). The samples were finally combined into one tube. The organic solvent was evaporated in the Speedvac and TFA was added to the sample (1% v/v). SDB-RPS StageTip fractionation was performed to collect three fractions of the TMT-labelled peptide samples. Speedvac removal of the solvents was followed by storage of the dried biotinylated peptides at -80 °C.

6.4.7 Proteomic Analysis

6.4.7.1 Sample Reconstitution

Prior to LC-MS/MS analysis, dried peptide samples were resuspended in 0.5% v/v trifluoroacetic acid and 2% v/v MeCN in water. Insoluble material was removed by centrifugation (17,000 g, 10 min).

6.4.7.2 LC-MS/MS Analysis

The analysis was performed using an Acclaim PepMap RSLC column 50 cm x 75 mm inner diameter (Thermo Fisher Scientific) using a 2 h MeCN gradient in 0.1% aqueous formic acid at a flow rate of 250 nl min⁻¹. For VSD samples, a 1 h MeCN gradient was used instead (2 – 55% v/v in 0.1% v/v aqueous formic acid). Easy nLC-1000 was coupled to a Q Exactive mass spectrometer via an easy-spray source (all Thermo Fisher Scientific). The Q Exactive was operated in data-dependent mode with survey scans acquired at a resolution of 75,000 at m/z 200 (transient time 256 ms). Up to 10 of the most abundant isotope patterns with charge 2+ or higher from the survey scan were selected with an isolation window of 3.0 m/z and fragmented by higher energy collision dissociation (HCD) with normalized collision energies of 25. The maximum ion injection times for the survey scan and the MS/MS scans (acquired with a resolution of 17,500 at m/z 200) were 20 and 120 ms, respectively. The ion target value for MS was set to 10⁶ and for MS/MS to 10⁵, and the intensity threshold was set to 8.3 x 10².
6.4.7.3 MaxQuant Data Analysis

The mammalian cell experimental data were processed using MaxQuant software v1.5.2.8 or v1.6.0.1. The peptides were identified from the MS/MS spectra searched against the complete reviewed human proteome (Uniprot) using the Andromeda search engine. Cysteine carbamidomethylation was selected as a fixed, and methionine oxidation and N-terminal acetylation as variable modifications. The selected digestion enzymes were trypsin which allows cleavage at the C-terminus of R and K residues and LysC which allows cleavage after K residues. Up to two missed cleavages were allowed. The false discovery rate was set to 0.01 for peptides, proteins and sites. Other parameters were used as pre-set in the software. 'Unique and razor peptides' mode was selected to allow identification and quantification of proteins in groups (razor peptides are uniquely assigned to protein groups and not to individual proteins), and all identifications were based on at least 2 unique peptides. Label Free and TMT quantification was performed using the built-in algorithms.

6.4.7.4 Statistical Analysis on Perseus

The data were analyzed using Perseus v1.5.0.9 and filtered to remove proteins categorized as 'Only identified by site', 'Reverse' and 'Potential contaminant'. Replicates of each sample were grouped. The intensities were log2-transformed.

**Label Free Quantification:** Further filtering required at least three valid intensity values in at least one sample for each identified protein. In the case of non-valid intensity values, the values were imputed from a normal distribution with imputation criteria width 0.3 and down shift 1.8. A two-sample t-test was performed (permutation-based FDR). The number of permutations was 250, FDR was set to 0.01 and s0 parameter was set to 0.5.

**TMT Quantification:** EM1 and EM2 samples at two time points were analysed in duplicate. At least 6 valid intensity values among all samples were required for a protein identification to be considered. The data were arranged by columns containing the intensities for each time point, cell line and replicate and rows of protein identifications corresponding to these values. Normalisation of the data was firstly performed by subtraction of the mean intensity of a certain protein ID within each replicate. The second normalisation procedure was subtraction of the mean intensity within columns. A two-sample t-test was performed (permutation-based FDR). The number of permutations was 250, FDR was set to 0.001. s0 para-
mometer was set to 0.5.

### 6.4.7.5 PEAKS Studio Data Analysis

For the identification of modified peptides, PEAKS Studio 7, 8 or 8.5 were used. Where appropriate, the complete reviewed human proteome (UniProt) or the sequence of VSD2, VSD4 and of any Huwentoxin-IV-based probes were employed for the peptide identification search. Cysteine carboxymethylation was selected as a fixed, and methionine oxidation as a variable modification. Table 6.7 contains the search parameters as set for each digestion enzyme and different probes.

### 6.4.7.6 MeroX Data Analysis

Photocrosslinking experiments were additionally analysed using MeroX 1.6.6. The search database contained the VSD2 sequence and the respective Mp probe sequence. For each enzyme predicted cleavage site, three missed cleavages were allowed and the option for semi-unspecific digestion was selected. Carboxymethylation was set as a fixed modification and represented as B. Methionine oxidation (m) was set as variable modification with a maximum of two occurrences per peptide. The photoprobe sequence in the provided database contained photomethionine represented by p. In the search, the crosslinker was set as p (C6H9N3O) requiring loss of N2 and all amino acids were considered as potential crosslinking sites. Modification of fragments of the photomethionine-containing peptide was set to -28.0061 for loss of N2 and -9.9956 for loss of N2 and addition of H2O. The precursor and fragment ion tolerance was 10 ppm and the FDR cut-off for reported spectra was set to 0.05. The peptide crosslink identifications that were considered as significant always had a higher score than the decoy hits.
### General Parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precursor mass tolerance</td>
<td>5 ppm</td>
</tr>
<tr>
<td>Fragment ion mass tolerance</td>
<td>0.01 Da</td>
</tr>
<tr>
<td>Maximum allowed variable PTMs</td>
<td>5</td>
</tr>
</tbody>
</table>

### Digestion Enzyme Specific Parameters

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Cleavage site</th>
<th>Maximum missed cleavages</th>
<th>Non-specific cleavage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin</td>
<td>R/K</td>
<td>5</td>
<td>both ends of peptide</td>
</tr>
<tr>
<td>LysC</td>
<td>K</td>
<td>5</td>
<td>one end of peptide</td>
</tr>
<tr>
<td>GluC/Trypsin</td>
<td>R/K/D/E</td>
<td>5</td>
<td>one end of peptide</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>F/L/M/W/Y</td>
<td>5</td>
<td>one end of peptide</td>
</tr>
<tr>
<td>GluC/LysC</td>
<td>K/D/E</td>
<td>5</td>
<td>one end of peptide</td>
</tr>
<tr>
<td>Proteinase K</td>
<td>A/C/G/M/F/S/Y/W</td>
<td>5</td>
<td>one end of peptide</td>
</tr>
<tr>
<td>Elastase</td>
<td>A/V/G/L/I</td>
<td>5</td>
<td>one end of peptide</td>
</tr>
</tbody>
</table>

### Modification Search Parameters for Trypsin Digestion

<table>
<thead>
<tr>
<th>Probe</th>
<th>Modification Chemical formula and sequence</th>
<th>Monoisotopic mass (Da)</th>
<th>Site of modification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfo-NHS-Biotin</td>
<td>C10H14N2O2S</td>
<td>226.07759</td>
<td>K</td>
</tr>
<tr>
<td>PFDB</td>
<td>C50H53F3N10O6S3</td>
<td>1042.32638</td>
<td>any residue</td>
</tr>
<tr>
<td>Mp5</td>
<td>C42H65N9O13S1 for ECLEMpFK</td>
<td>935.44226</td>
<td>any residue</td>
</tr>
<tr>
<td></td>
<td>C91H143N27O33S4 for ECLEIFMpACNPSNDQCCK</td>
<td>2245.91160</td>
<td>any residue</td>
</tr>
<tr>
<td>Mp7</td>
<td>C91H142N26O33S4 for ECLEIFMpACNPSNDQCCK</td>
<td>2254.91160</td>
<td>any residue</td>
</tr>
<tr>
<td>Mp21</td>
<td>C37H60N10O9S1 for MpTR</td>
<td>704.33158</td>
<td>any residue</td>
</tr>
<tr>
<td></td>
<td>C38H61N12O9S1 for KMPWCK</td>
<td>832.42654</td>
<td>any residue</td>
</tr>
<tr>
<td></td>
<td>C41H75N15O12S1 for LVCSRMPTR</td>
<td>1001.54403</td>
<td>any residue</td>
</tr>
<tr>
<td>Mp27</td>
<td>C16H30N6O6 for YQMp</td>
<td>420.20089</td>
<td>any residue</td>
</tr>
<tr>
<td></td>
<td>C42H59N10O10S1 for WCKYQMp</td>
<td>895.41364</td>
<td>any residue</td>
</tr>
<tr>
<td>Mp29</td>
<td>C32H48N8O8S1 for TMRWCK</td>
<td>704.33158</td>
<td>any residue</td>
</tr>
<tr>
<td></td>
<td>C38H60N10O9S1 for KTMPWCK</td>
<td>832.42654</td>
<td>any residue</td>
</tr>
<tr>
<td></td>
<td>C52H76N12O13S1 for TMRWCK</td>
<td>1108.53755</td>
<td>any residue</td>
</tr>
<tr>
<td>Mp35</td>
<td>C20H28N4O6 for YQMp</td>
<td>420.20089</td>
<td>any residue</td>
</tr>
<tr>
<td></td>
<td>C42H59N10O10S1 for WCKYQMp</td>
<td>895.41364</td>
<td>any residue</td>
</tr>
</tbody>
</table>

Table 6.7: Search parameters used for PEAKS Studio data analysis of proteomic data. All C residues are expected to be carboxamidomethylated.
Bibliography


Appendix A

The appendix contains additional data in the following categories: nanoDSF, NMR spectra of small molecules, LC-MS characterisation of peptides and proteomic LC-MS/MS data.

Figure A.1: Tm curves of VSD4 in size exclusion buffer with and without 6% v/v DMSO.
Figure A.2: $^1$H NMR spectrum of Fmoc-L-photomethionine-OH
Figure A.3: $^{13}$C NMR spectrum of Fmoc-L-photomethionine-OH
Figure A.4: $^1$H NMR spectrum of (S)-3-(3-methyl-3H-diazirin-3-yl)-2-(5-((3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)propanoic acid 4
Figure A.5: $^{13}$C NMR spectrum of (S)-3-(3-methyl-3H-diazirin-3-yl)-2-(5-((3aS,4S,6aR)-2-(oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)propanoic acid 4
Figure A.6: $^1$H NMR spectrum of PFDB 5
Figure A.7: $^{13}$C NMR spectrum of PFDB 5
Figure A.8: Huwentoxin-IV LC trace and MS data.

Figure A.9: E1G E4G LC trace and MS data.
Figure A.10: Kb13 LC trace and MS data.

Figure A.11: Gp13 LC trace and MS data.
Figure A.12:Mp7 LC trace and MS data.

Figure A.13:Mp7 Kb13 LC trace and MS data.

Figure A.14:Mp7 Gp13 LC trace and MS data.
Figure A.15: LC traces and MS data of commercial Mp probes.
Figure A.16: LC traces and MS data of commercial Mp probes.
Figure A.17: LC trace and MS data of commercial HwTx-IV.
Figure A.18: Coverage of Nav1.7 obtained through StageTip fractionation and additive screening.

Figure A.19: Coverage of VSD2 with various digestion enzymes.
Figure A.20: Coverage of VSD4 with various digestion enzymes.
<table>
<thead>
<tr>
<th>Gene names</th>
<th>t-test</th>
<th>(log t-test p value)</th>
<th>log2 t-test Diff.</th>
<th>Majority Protein ID</th>
<th>Majority Protein names</th>
<th>Intensity</th>
<th>MS/MS Count</th>
<th>Unique peptides</th>
<th>Unique peptides Nav IP A</th>
<th>Unique peptides Nav IP B</th>
<th>Unique peptides Nav IP C</th>
<th>Unique peptides IgG IP A</th>
<th>Unique peptides IgG IP B</th>
<th>Unique peptides IgG IP C</th>
<th>Unique peptides IgG IP C</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCN9A</td>
<td>3.965953083</td>
<td>8.929679644</td>
<td>2.1929056</td>
<td>Sodium channel protein type 9 subunit alpha</td>
<td>Q01585 Sodium channel protein type 9 subunit alpha</td>
<td>2.447000000</td>
<td>217 52 42 44 8 0 0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HERC2</td>
<td>3.823740776</td>
<td>5.477698958</td>
<td>1.6540829</td>
<td>E3 ubiquitin-protein ligase HERC2</td>
<td>O95714 E3 ubiquitin-protein ligase HERC2</td>
<td>1.933000000</td>
<td>152 48 48 44 0 0 0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OSRPL6</td>
<td>2.773676172</td>
<td>0.921627388</td>
<td>0.6420486</td>
<td>Oxysterol-binding protein-related protein 6</td>
<td>Q96U14 Oxysterol-binding protein-related protein 6</td>
<td>0.986000000</td>
<td>0.9 0 0 17 0 0 0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ODE7L1</td>
<td>2.741625957</td>
<td>0.927652764</td>
<td>0.8142378</td>
<td>Oysterol-binding protein-related protein 11</td>
<td>Q96U14 Oxysterol-binding protein-related protein 11</td>
<td>2.095000000</td>
<td>1.9 13 23 18 0 0 0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCN9B</td>
<td>3.912791292</td>
<td>4.064932176</td>
<td>0.1521353</td>
<td>Sodium channel subunit beta-3</td>
<td>Q96U14 Oxysterol-binding protein-related protein 11</td>
<td>2.414000000</td>
<td>4.6 4 4 7 1 0 0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NEU1A</td>
<td>3.661798717</td>
<td>3.150563155</td>
<td>0.5112328</td>
<td>Neuronal acetylcholine receptor protein 4</td>
<td>Q96U14 Oxysterol-binding protein-related protein 11</td>
<td>2.393000000</td>
<td>5.8 11 24 13 0 0 0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAVMA5B</td>
<td>3.901239280</td>
<td>0.923864556</td>
<td>2.9783651</td>
<td>Oxysterol-binding protein-related protein 10</td>
<td>Q96U14 Oxysterol-binding protein-related protein 10</td>
<td>2.861000000</td>
<td>2.0 4 11 13 0 0 0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JUP</td>
<td>1.102261671</td>
<td>2.146729767</td>
<td>1.0445272</td>
<td>Junction plakoglobin</td>
<td>P14923 Junction plakoglobin</td>
<td>1.129200000</td>
<td>0.9 11 20 3 2 4 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B.CAN13.OLOGA</td>
<td>1.085733594</td>
<td>2.115576930</td>
<td>1.0298233</td>
<td>Brownian movement 3</td>
<td>Q96U14 Oxysterol-binding protein-related protein 10</td>
<td>2.313000000</td>
<td>1.9 11 2 4 1 0 0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTP4A5.CB1</td>
<td>2.738279297</td>
<td>0.311031618</td>
<td>2.4272476</td>
<td>Very low density lipoprotein receptor-related 15</td>
<td>Q96U14 Oxysterol-binding protein-related protein 10</td>
<td>1.203000000</td>
<td>1.8 2 2 4 0 0 0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RPERURBANZ</td>
<td>3.337929326</td>
<td>1.905295575</td>
<td>1.4339328</td>
<td>Ubiquitin-40S ribosomal protein S27a</td>
<td>P62979 Ubiquitin-40S ribosomal protein S27a</td>
<td>2.044000000</td>
<td>2.6 6 3 5 0 2 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C3P</td>
<td>2.610389284</td>
<td>1.941679874</td>
<td>0.6687027</td>
<td>Desmoplakin</td>
<td>Q96U14 Oxysterol-binding protein-related protein 10</td>
<td>3.104000000</td>
<td>0.6 24 27 5 4 4 4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCN9B</td>
<td>1.627823136</td>
<td>1.935597102</td>
<td>1.6177676</td>
<td>Sodium channel subunit beta-1</td>
<td>Q07699 Sodium channel subunit beta-1</td>
<td>2.079000000</td>
<td>0.4 7 8 7 3 5 8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table A.1:** List of proteins that are enriched in pan Nav IP with respect of IgG IP. FDR-based t-test was performed with FDR set to 0.05, - log p value > 1.5 was considered significant.
<table>
<thead>
<tr>
<th>Gene names</th>
<th>log(p value)</th>
<th>log2 t-test difference</th>
<th>Majority protein ID's</th>
<th>Panther classification</th>
<th>Protein names</th>
<th>Score</th>
<th>Intensity</th>
<th>MS/MS Count</th>
<th>Unique peptides</th>
<th>Peptides A</th>
<th>Peptides B</th>
</tr>
</thead>
<tbody>
<tr>
<td>VGF</td>
<td>2.560167032</td>
<td>-2.756205559</td>
<td>O1524</td>
<td>neuropeptide</td>
<td>Neurosecretory protein VGF</td>
<td>32.56</td>
<td>146000000</td>
<td>13</td>
<td>4</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>NCAM2</td>
<td>4.068533006</td>
<td>-3.299933493</td>
<td>O15394</td>
<td>cell adhesion molecule</td>
<td>Neural cell adhesion molecule 2</td>
<td>14.06</td>
<td>247000000</td>
<td>8</td>
<td>3</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>EDIL3</td>
<td>3.301392581</td>
<td>-2.496914387</td>
<td>O43854</td>
<td>cell adhesion molecule</td>
<td>EGF-like repeat and discoidin I-like domain-containing protein 3</td>
<td>52.37</td>
<td>603000000</td>
<td>24</td>
<td>17</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>NPTN</td>
<td>2.920154289</td>
<td>-3.474053015</td>
<td>P09675</td>
<td>receptor</td>
<td>Nectin-2</td>
<td>62.96</td>
<td>699000000</td>
<td>24</td>
<td>17</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>NCOL1</td>
<td>-3.68981567</td>
<td>2.709077083</td>
<td>P04548</td>
<td>membrane trafficking</td>
<td>Synaptotagmin-1</td>
<td>77.36</td>
<td>170000000</td>
<td>24</td>
<td>17</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>INSC</td>
<td>3.638342843</td>
<td>-2.185501642</td>
<td>P12532</td>
<td>cellular nucleic acid</td>
<td>Creatine kinase U-type, mitochondrial</td>
<td>56.67</td>
<td>328570000</td>
<td>21</td>
<td>8</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>SCG2</td>
<td>2.170114201</td>
<td>-3.291224897</td>
<td>P13521</td>
<td>neuropeptide</td>
<td>Secretogranin-2; Secretoneurin</td>
<td>12.28</td>
<td>615420000</td>
<td>9</td>
<td>4</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>NCAM1</td>
<td>3.548175646</td>
<td>-3.284169719</td>
<td>P13591</td>
<td>cell adhesion molecule</td>
<td>Neural cell adhesion molecule 1</td>
<td>150.69</td>
<td>2307000000</td>
<td>92</td>
<td>24</td>
<td>20</td>
<td>21</td>
</tr>
<tr>
<td>ATP1A3</td>
<td>2.63496412</td>
<td>-2.399813086</td>
<td>P13637</td>
<td>cation transporter</td>
<td>Sodium/potassium-transporting ATPase subunit alpha-3</td>
<td>221.99</td>
<td>2651300000</td>
<td>130</td>
<td>14</td>
<td>25</td>
<td>32</td>
</tr>
<tr>
<td>SYN1</td>
<td>4.244980477</td>
<td>-2.478060812</td>
<td>P17600</td>
<td>membrane trafficking</td>
<td>Synapsin-1</td>
<td>7.48</td>
<td>738150000</td>
<td>5</td>
<td>4</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>GNAO1</td>
<td>3.305569424</td>
<td>-3.288210869</td>
<td>P09471;P59215</td>
<td>G-protein</td>
<td>Guanine nucleotide-binding protein G(o) subunit alpha</td>
<td>59.25</td>
<td>241920000</td>
<td>2</td>
<td>19</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>MAPT</td>
<td>1.895854013</td>
<td>-2.397436209</td>
<td>P10636;P10637;P19332</td>
<td>Microtubule-associated protein tau</td>
<td>Microtubule-associated protein tau</td>
<td>17.01</td>
<td>214400000</td>
<td>16</td>
<td>8</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>SYP;Syp</td>
<td>2.49989545</td>
<td>-3.863188386</td>
<td>P08247;P07825</td>
<td>membrane trafficking</td>
<td>Synaptophysin</td>
<td>9.02</td>
<td>378790000</td>
<td>19</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>ENO2;Eno2</td>
<td>2.69283843</td>
<td>-2.699045569</td>
<td>P09104;P17183</td>
<td>lyase</td>
<td>Gamma-enolase</td>
<td>9.54</td>
<td>662600000</td>
<td>57</td>
<td>9</td>
<td>12</td>
<td>25</td>
</tr>
<tr>
<td>GNAO1</td>
<td>3.305569424</td>
<td>-3.288210869</td>
<td>P09471;P59215</td>
<td>G-protein</td>
<td>Guanine nucleotide-binding protein G(o) subunit alpha</td>
<td>59.25</td>
<td>241920000</td>
<td>2</td>
<td>19</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>MAPT</td>
<td>1.895854013</td>
<td>-2.397436209</td>
<td>P10636;P10637;P19332</td>
<td>Microtubule-associated protein tau</td>
<td>Microtubule-associated protein tau</td>
<td>17.01</td>
<td>214400000</td>
<td>16</td>
<td>8</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>SYP;Syp</td>
<td>2.49989545</td>
<td>-3.863188386</td>
<td>P08247;P07825</td>
<td>membrane trafficking</td>
<td>Synaptophysin</td>
<td>9.02</td>
<td>378790000</td>
<td>19</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>ENO2;Eno2</td>
<td>2.69283843</td>
<td>-2.699045569</td>
<td>P09104;P17183</td>
<td>lyase</td>
<td>Gamma-enolase</td>
<td>9.54</td>
<td>662600000</td>
<td>57</td>
<td>9</td>
<td>12</td>
<td>25</td>
</tr>
<tr>
<td>GNAO1</td>
<td>3.305569424</td>
<td>-3.288210869</td>
<td>P09471;P59215</td>
<td>G-protein</td>
<td>Guanine nucleotide-binding protein G(o) subunit alpha</td>
<td>59.25</td>
<td>241920000</td>
<td>2</td>
<td>19</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>MAPT</td>
<td>1.895854013</td>
<td>-2.397436209</td>
<td>P10636;P10637;P19332</td>
<td>Microtubule-associated protein tau</td>
<td>Microtubule-associated protein tau</td>
<td>17.01</td>
<td>214400000</td>
<td>16</td>
<td>8</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>SYP;Syp</td>
<td>2.49989545</td>
<td>-3.863188386</td>
<td>P08247;P07825</td>
<td>membrane trafficking</td>
<td>Synaptophysin</td>
<td>9.02</td>
<td>378790000</td>
<td>19</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>ENO2;Eno2</td>
<td>2.69283843</td>
<td>-2.699045569</td>
<td>P09104;P17183</td>
<td>lyase</td>
<td>Gamma-enolase</td>
<td>9.54</td>
<td>662600000</td>
<td>57</td>
<td>9</td>
<td>12</td>
<td>25</td>
</tr>
</tbody>
</table>

Table A.2: List of EM1 proteins that are significantly enriched on day 53 (w.r.t. day 15) and their classification. FDR-based t-test was performed with FDR set to 0.001, S0 = 0.5.
### Table A.3: List of EM2 proteins that are significantly enriched on day 53 (w.r.t. day 15) and their classification. FDR-based t-test was performed with FDR set to 0.001, S0 = 0.5.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Log p-value</th>
<th>Log 2 t-diff.</th>
<th>Majority protein IDs</th>
<th>Panther class ID</th>
<th>Protein names</th>
<th>%Prot</th>
<th>Intensity</th>
<th>MS/MS Count</th>
<th>Unique peptides</th>
<th>Peptide A</th>
<th>Peptide B</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASAH1</td>
<td>-2.35</td>
<td>2.70</td>
<td>A5A6P2</td>
<td>lysosomal degradation</td>
<td>Acid ceramidase</td>
<td>72.34</td>
<td>11441 00000</td>
<td>69</td>
<td>2</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>NCGA1</td>
<td>-1.90</td>
<td>2.33</td>
<td>Q13054</td>
<td>cell adhesion molecule</td>
<td>Neural cell adhesion molecule 2</td>
<td>14.03</td>
<td>24578 00000</td>
<td>8</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>CRKBP1</td>
<td>-2.09</td>
<td>2.47</td>
<td>P12951</td>
<td>cholesterol</td>
<td>Apolipoprotein B chain</td>
<td>42.86</td>
<td>10239 00000</td>
<td>29</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>BCO1</td>
<td>1.88</td>
<td>-0.52</td>
<td>P06728</td>
<td></td>
<td>Cholesterolase</td>
<td>11.37</td>
<td>517890000</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>STX1A</td>
<td>-1.80</td>
<td>2.15</td>
<td>P10533 P14069</td>
<td>signaling molecule</td>
<td>Protein S100A10</td>
<td>8.439</td>
<td>2111900000</td>
<td>19</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>NDEL1</td>
<td>-1.90</td>
<td>2.73031578</td>
<td>R07196</td>
<td>filament structural protein</td>
<td>Neurofilament light polypeptide</td>
<td>7.175</td>
<td>1746700000</td>
<td>92</td>
<td>21</td>
<td>21</td>
<td>23</td>
</tr>
<tr>
<td>PICALM</td>
<td>-2.09</td>
<td>2.70</td>
<td>P03402</td>
<td>lysosomal degradation</td>
<td>Phosphatidylinositol-4-phosphate 5-kinase II</td>
<td>23.746</td>
<td>8794300000</td>
<td>171</td>
<td>79</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>CTSB</td>
<td>1.90</td>
<td>2.93031578</td>
<td>R07196</td>
<td></td>
<td>Cathepsin B; Cathepsin B light chain</td>
<td>84.953</td>
<td>1077900000</td>
<td>42</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>RNF155</td>
<td>1.70</td>
<td>2.33</td>
<td>R07196</td>
<td></td>
<td>Noncollagenous Nematode protein</td>
<td>36.777</td>
<td>2019700000</td>
<td>31</td>
<td>9</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>PRKPH</td>
<td>-1.80</td>
<td>2.73027194</td>
<td>R12191</td>
<td>filament structural protein</td>
<td>Protein</td>
<td>119.2</td>
<td>2017700000</td>
<td>102</td>
<td>22</td>
<td>19</td>
<td>25</td>
</tr>
<tr>
<td>CTSF</td>
<td>2.42</td>
<td>2.93086452</td>
<td>Q10295</td>
<td></td>
<td>Di-N-acetylated lysozyme</td>
<td>7.987</td>
<td>149720000</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>ADCAM1</td>
<td>2.41</td>
<td>2.70</td>
<td>Q17132 Q18031</td>
<td>cell adhesion molecule</td>
<td>alpha-crystallin B chain</td>
<td>72.73</td>
<td>292700000</td>
<td>43</td>
<td>10</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>OPNMB</td>
<td>-1.70</td>
<td>2.730262912</td>
<td>Q10296</td>
<td></td>
<td>Transmembrane glycoprotein NMB</td>
<td>8.014</td>
<td>252610000</td>
<td>10</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>IRAK</td>
<td>1.88</td>
<td>-1.60</td>
<td>Q10296</td>
<td></td>
<td>Chaperone protein</td>
<td>77.29</td>
<td>145710000</td>
<td>81</td>
<td>18</td>
<td>17</td>
<td>20</td>
</tr>
<tr>
<td>ETOR1</td>
<td>-1.80</td>
<td>2.33</td>
<td>Q10296</td>
<td></td>
<td>Neuroendocrine protein</td>
<td>5.093</td>
<td>72849000</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>UCP4</td>
<td>1.97</td>
<td>2.70715757</td>
<td>Q10296</td>
<td></td>
<td>Neuroendocrine protein</td>
<td>32.069</td>
<td>140790000</td>
<td>13</td>
<td>4</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>HSPA1A</td>
<td>-1.70</td>
<td>2.33</td>
<td>Q10296</td>
<td></td>
<td>Heat shock B-box protein 1</td>
<td>39.19</td>
<td>65654000</td>
<td>12</td>
<td>8</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>NDE1</td>
<td>2.27</td>
<td>2.93086452</td>
<td>R12191</td>
<td></td>
<td>Neurofilament medium polypeptide</td>
<td>195.8</td>
<td>175180000</td>
<td>79</td>
<td>30</td>
<td>17</td>
<td>25</td>
</tr>
<tr>
<td>CTNO2</td>
<td>2.41</td>
<td>2.93086452</td>
<td>R12191</td>
<td></td>
<td>Neuroendocrine protein</td>
<td>119.94</td>
<td>309710000</td>
<td>130</td>
<td>14</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>VMD2</td>
<td>1.90</td>
<td>2.70715757</td>
<td>Q10296</td>
<td></td>
<td>Chaperone protein</td>
<td>6.015</td>
<td>32900000</td>
<td>23</td>
<td>6</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>LCCAS1</td>
<td>-1.80</td>
<td>2.33</td>
<td>Q10296</td>
<td></td>
<td>Chaperone protein</td>
<td>56.58</td>
<td>34734000</td>
<td>96</td>
<td>9</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>QNAT1</td>
<td>-1.55</td>
<td>2.93086452</td>
<td>P04877 P95512</td>
<td></td>
<td>G protein</td>
<td>99.246</td>
<td>24130000</td>
<td>22</td>
<td>8</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>CSN4</td>
<td>1.90</td>
<td>2.70715757</td>
<td>Q10296</td>
<td></td>
<td>Chaperone</td>
<td>119.37</td>
<td>32830000</td>
<td>11</td>
<td>3</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>QPRT3</td>
<td>2.40</td>
<td>2.70471786</td>
<td>P17877</td>
<td></td>
<td>Neuraminidase</td>
<td>46.35</td>
<td>47280000</td>
<td>51</td>
<td>7</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>NPC1</td>
<td>2.33</td>
<td>2.93086452</td>
<td>P11730</td>
<td></td>
<td>Neuroendocrine protein</td>
<td>135.7</td>
<td>47280000</td>
<td>66</td>
<td>38</td>
<td>23</td>
<td>19</td>
</tr>
<tr>
<td>SDC1</td>
<td>2.77</td>
<td>2.93086452</td>
<td>P11730</td>
<td></td>
<td>Protein SDC1</td>
<td>38.22</td>
<td>168010000</td>
<td>19</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>TRIM26</td>
<td>1.95</td>
<td>2.33</td>
<td>Q09719</td>
<td></td>
<td>Ubiquitin E3 ligase</td>
<td>6.5043</td>
<td>30200000</td>
<td>8</td>
<td>3</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>VAT1L</td>
<td>2.33</td>
<td>2.93086452</td>
<td>Q10296</td>
<td></td>
<td>Membrane trafficking</td>
<td>48.87</td>
<td>30200000</td>
<td>17</td>
<td>6</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>VAPB</td>
<td>2.43</td>
<td>2.50</td>
<td>Q10296</td>
<td></td>
<td>Sapophilin</td>
<td>164.94</td>
<td>6793000</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

**Table A.3**: The table lists the EM2 proteins that are significantly enriched on day 53 (w.r.t. day 15) and their classification. FDR-based t-test was performed with FDR set to 0.001, S0 = 0.5.
**Figure A.21:** MS identification of the photocrosslinking site between Mp27 and VSD2 C-terminal coiled-coil.
Appendix B

Permissions to reproduce published figures and data are included in this Appendix.
This Agreement between Ms. Foteini Tzakoniati ("You") and Springer Nature ("Springer Nature") consists of your license details and the terms and conditions provided by Springer Nature and Copyright Clearance Center.

License Number 4344220776760
License date May 08, 2018
Licensed Content Publisher Springer Nature
Licensed Content Publication Nature
Licensed Content Title The crystal structure of a voltage-gated sodium channel
Licensed Content Author Jian Payandeh, Todd Scheuer, Ning Zheng, William A. Catterall
Licensed Content Date Jul 10, 2011
Licensed Content Volume 475
Licensed Content Issue 7356
Type of Use Thesis/Dissertation
Requestor type academic/university or research institute
Format print and electronic
Portion figures/tables/illustrations
Number of figures/tables/illustrations 2
High-res required no
Will you be translating? no
Circulation/distribution <501
Author of this Springer Nature content no
Title A Chemical Biology Approach to Understanding the Basis of Voltage-Gated Sodium Channel Modulation
Instructor name Foteini Tzakoniati
Institution name Imperial College London
Expected presentation date Jun 2018
Portions Figure 1a (page 354)
            Figure 2d (page 355)
Requestor Location Ms. Foteini Tzakoniati
                    Imperial College London
                    Department of Chemistry
                    Frankland road
                    London, SW7 2AZ
                    United Kingdom
                    Attn: Ms. Foteini Tzakoniati
Billing Type Invoice
Billing Address Ms. Foteini Tzakoniati
              Imperial College London
              Department of Chemistry
              Frankland road
              London, United Kingdom SW7 2AZ
              Attn: Ms. Foteini Tzakoniati
Total 0.00 USD
Terms and Conditions

Springer Nature Terms and Conditions for RightsLink Permissions

Springer Customer Service Centre GmbH (the Licensor) hereby grants you a non-exclusive, world-wide licence to reproduce the material and for the purpose and requirements specified in the attached copy of your order form, and for no other use, subject to the conditions below:

1. The Licensor warrants that it has, to the best of its knowledge, the rights to license reuse of this material. However, you should ensure that the material you are requesting is original to the Licensor and does not carry the copyright of another entity (as credited in the published version).

If the credit line on any part of the material you have requested indicates that it was reprinted or adapted with permission from another source, then you should also seek permission from that source to reuse the material.

2. Where print only permission has been granted for a fee, separate permission must be obtained for any additional electronic re-use.

3. Permission granted free of charge for material in print is also usually granted for any electronic version of that work, provided that the material is incidental to your work as a whole and that the electronic version is essentially equivalent to, or substitutes for, the print version.

4. A licence for ‘post on a website’ is valid for 12 months from the licence date. This licence does not cover use of full text articles on websites.

5. Where 'reuse in a dissertation/thesis' has been selected the following terms apply: Print rights for up to 100 copies, electronic rights for use only on a personal website or institutional repository as defined by the Sherpa guideline (www.sherpa.ac.uk/romeo/).

6. Permission granted for books and journals is granted for the lifetime of the first edition and does not apply to second and subsequent editions (except where the first edition permission was granted free of charge or for signatories to the STM Permissions Guidelines http://www.stm-assoc.org/copyright-legal-affairs/permissions/permissions-guidelines/), and does not apply for editions in other languages unless additional translation rights have been granted separately in the licence.

7. Rights for additional components such as custom editions and derivatives require additional permission and may be subject to an additional fee. Please apply to Journalpermissions@springernature.com/bookpermissions@springernature.com for these rights.

8. The Licensor’s permission must be acknowledged next to the licensed material in print. In electronic form, this acknowledgement must be visible at the same time as the figures/tables/illustrations or abstract, and must be hyperlinked to the journal/book’s homepage. Our required acknowledgement format is in the Appendix below.

9. Use of the material for incidental promotional use, minor editing privileges (this does not include cropping, adapting, omitting material or any other changes that affect the meaning, intention or moral rights of the author) and copies for the disabled are permitted under this licence.

10. Minor adaptations of single figures (changes of format, colour and style) do not require the Licensor’s approval. However, the adaptation should be credited as shown in Appendix below.

Appendix — Acknowledgements:

For Journal Content:
Reprinted by permission from [the Licensor]: [Journal Publisher (e.g. Nature/Springer/Palgrave)] [JOURNAL NAME] [REFERENCE CITATION (Article name, Author(s) Name), [COPYRIGHT] (year of publication)
This Agreement between Ms. Foteini Tzakoniati ("You") and Springer Nature ("Springer Nature") consists of your license details and the terms and conditions provided by Springer Nature and Copyright Clearance Center.

<table>
<thead>
<tr>
<th>License Number</th>
<th>4344220078315</th>
</tr>
</thead>
<tbody>
<tr>
<td>License date</td>
<td>May 08, 2018</td>
</tr>
<tr>
<td>Licensed Content Publisher</td>
<td>Springer Nature</td>
</tr>
<tr>
<td>Licensed Content Publication</td>
<td>Nature Biotechnology</td>
</tr>
<tr>
<td>Licensed Content Title</td>
<td>Combined small-molecule inhibition accelerates developmental timing and converts human pluripotent stem cells into nociceptors</td>
</tr>
<tr>
<td>Licensed Content Author</td>
<td>Stuart M Chambers, Yuchen Qi, Yvonne Mica, Gabsang Lee, Xin-Jun Zhang et al.</td>
</tr>
<tr>
<td>Licensed Content Date</td>
<td>Jul 1, 2012</td>
</tr>
<tr>
<td>Licensed Content Volume</td>
<td>30</td>
</tr>
<tr>
<td>Licensed Content Issue</td>
<td>7</td>
</tr>
<tr>
<td>Type of Use</td>
<td>Thesis/Dissertation</td>
</tr>
<tr>
<td>Requestor type</td>
<td>academic/university or research institute</td>
</tr>
<tr>
<td>Format</td>
<td>print and electronic</td>
</tr>
<tr>
<td>Portion</td>
<td>figures/tables/illustrations</td>
</tr>
<tr>
<td>Number of figures/tables/illustrations</td>
<td>1</td>
</tr>
<tr>
<td>High-res required</td>
<td>no</td>
</tr>
<tr>
<td>Will you be translating?</td>
<td>no</td>
</tr>
<tr>
<td>Circulation/distribution</td>
<td>&lt;501</td>
</tr>
<tr>
<td>Author of this Springer Nature content</td>
<td>no</td>
</tr>
<tr>
<td>Title</td>
<td>A Chemical Biology Approach to Understanding the Basis of Voltage-Gated Sodium Channel Modulation</td>
</tr>
<tr>
<td>Instructor name</td>
<td>Foteini Tzakoniati</td>
</tr>
<tr>
<td>Institution name</td>
<td>Imperial College London</td>
</tr>
<tr>
<td>Expected presentation date</td>
<td>Jun 2018</td>
</tr>
<tr>
<td>Portions</td>
<td>Figure S15, Supplementary information.</td>
</tr>
<tr>
<td>Requestor Location</td>
<td>Ms. Foteini Tzakoniati</td>
</tr>
<tr>
<td></td>
<td>Imperial College London</td>
</tr>
<tr>
<td></td>
<td>Department of Chemistry</td>
</tr>
<tr>
<td></td>
<td>Frankland road</td>
</tr>
<tr>
<td></td>
<td>London, SW7 2AZ</td>
</tr>
<tr>
<td></td>
<td>United Kingdom</td>
</tr>
<tr>
<td></td>
<td>Attn: Ms. Foteini Tzakoniati</td>
</tr>
</tbody>
</table>

Billing Type: Invoice
Billing Address: Ms. Foteini Tzakoniati
Imperial College London
Department of Chemistry
Frankland road
London, United Kingdom SW7 2AZ
Attn: Ms. Foteini Tzakoniati
Spranger Nature Terms and Conditions for RightsLink Permissions

Spranger Customer Service Centre GmbH (the Licensor) hereby grants you a non-exclusive, world-wide licence to reproduce the material and for the purpose and requirements specified in the attached copy of your order form, and for no other use, subject to the conditions below:

1. The Licensor warrants that it has, to the best of its knowledge, the rights to license reuse of this material. However, you should ensure that the material you are requesting is original to the Licensor and does not carry the copyright of another entity (as credited in the published version).

If the credit line on any part of the material you have requested indicates that it was reprinted or adapted with permission from another source, then you should also seek permission from that source to reuse the material.

2. Where print only permission has been granted for a fee, separate permission must be obtained for any additional electronic re-use.

3. Permission granted free of charge for material in print is also usually granted for any electronic version of that work, provided that the material is incidental to your work as a whole and that the electronic version is essentially equivalent to, or substitutes for, the print version.

4. A licence for ‘post on a website’ is valid for 12 months from the licence date. This licence does not cover use of full text articles on websites.

5. Where ‘reuse in a dissertation/thesis’ has been selected the following terms apply:
   Print rights for up to 100 copies, electronic rights for use only on a personal website or institutional repository as defined by the Sherpa guideline (www.sherp.as.ac.uk/romeo/).

6. Permission granted for books and journals is granted for the lifetime of the first edition and does not apply to second and subsequent editions (except where the first edition permission was granted free of charge or for signatories to the STM Permissions Guidelines http://www.stm-assoc.org/copyright-legal-affairs/permissions/permissions-guidelines/), and does not apply for editions in other languages unless additional translation rights have been granted separately in the licence.

7. Rights for additional components such as custom editions and derivatives require additional permission and may be subject to an additional fee. Please apply to Journalpermissions@springernature.com/bookpermissions@springernature.com for these rights.

8. The Licensor’s permission must be acknowledged next to the licensed material in print. In electronic form, this acknowledgement must be visible at the same time as the figures/tables/illustrations or abstract, and must be hyperlinked to the journal/book’s homepage. Our required acknowledgement format is in the Appendix below.

9. Use of the material for incidental promotional use, minor editing privileges (this does not include cropping, adapting, omitting material or any other changes that affect the meaning, intention or moral rights of the author) and copies for the disabled are permitted under this licence.

10. Minor adaptations of single figures (changes of format, colour and style) do not require the Licensor’s approval. However, the adaptation should be credited as shown in Appendix below.

Appendix — Acknowledgements:

For Journal Content:
Reprinted by permission from [the Licensor]: [Journal Publisher (e.g. Nature/Springer/Palgrave)] [JOURNAL NAME] [REFERENCE CITATION (Article name, Author(s) Name), [COPYRIGHT] (year of publication)
PERMISSION/LICENSE IS GRANTED FOR YOUR ORDER AT NO CHARGE

This type of permission/license, instead of the standard Terms & Conditions, is sent to you because no fee is being charged for your order. Please note the following:

- Permission is granted for your request in both print and electronic formats, and translations.
- If figures and/or tables were requested, they may be adapted or used in part.
- Please print this page for your records and send a copy of it to your publisher/graduate school.
- Appropriate credit for the requested material should be given as follows: "Reprinted (adapted) with permission from (COMPLETE REFERENCE CITATION). Copyright (YEAR) American Chemical Society." Insert appropriate information in place of the capitalized words.
- One-time permission is granted only for the use specified in your request. No additional uses are granted (such as derivative works or other editions). For any other uses, please submit a new request.

If credit is given to another source for the material you requested, permission must be obtained from that source.
Pharmacological reversal of a pain phenotype in iPSC-derived sensory neurons and patients with inherited erythromelalgia


Publication: Science Translational Medicine
Publisher: The American Association for the Advancement of Science
Date: Apr 20, 2016
Copyright © 2016, American Association for the Advancement of Science

Order Completed
Thank you for your order.

This Agreement between Ms. Foteini Tzakoniati ("You") and The American Association for the Advancement of Science ("The American Association for the Advancement of Science") consists of your license details and the terms and conditions provided by The American Association for the Advancement of Science and Copyright Clearance Center.

Your confirmation email will contain your order number for future reference.

printable details

License Number 4344710612293
License date May 09, 2018
Licensed Content The American Association for the Advancement of Science
Licensed Content Science Translational Medicine
Licensed Content Title Pharmacological reversal of a pain phenotype in iPSC-derived sensory neurons and patients with inherited erythromelalgia
Licensed Content Date Apr 20, 2016
Licensed Content Volume 8
Licensed Content Issue 335
Type of Use Thesis / Dissertation
Requestor type Scientist/individual at a research institution
Format Print and electronic
Portion Figure
Number of figures/tables 1
Order reference number
<table>
<thead>
<tr>
<th><strong>Title of your thesis / dissertation</strong></th>
<th>A Chemical Biology Approach to Understanding the Basis of Voltage-Gated Sodium Channel Modulation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Expected completion date</strong></td>
<td>Jun 2018</td>
</tr>
<tr>
<td><strong>Estimated size(pages)</strong></td>
<td>1</td>
</tr>
<tr>
<td><strong>Attachment</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Requestor Location</strong></td>
<td>Ms. Foteini Tzakoniati</td>
</tr>
<tr>
<td></td>
<td>Imperial College London</td>
</tr>
<tr>
<td></td>
<td>Department of Chemistry</td>
</tr>
<tr>
<td></td>
<td>Frankland road</td>
</tr>
<tr>
<td></td>
<td>London, SW7 2AZ</td>
</tr>
<tr>
<td></td>
<td>Attn: Ms. Foteini Tzakoniati</td>
</tr>
<tr>
<td><strong>Billing Type</strong></td>
<td>Invoice</td>
</tr>
<tr>
<td><strong>Billing address</strong></td>
<td>Ms. Foteini Tzakoniati</td>
</tr>
<tr>
<td></td>
<td>Imperial College London</td>
</tr>
<tr>
<td></td>
<td>Department of Chemistry</td>
</tr>
<tr>
<td></td>
<td>Frankland road</td>
</tr>
<tr>
<td></td>
<td>London, United Kingdom SW7 2AZ</td>
</tr>
<tr>
<td></td>
<td>Attn: Ms. Foteini Tzakoniati</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>0.00 GBP</td>
</tr>
</tbody>
</table>

**ORDER MORE**  **CLOSE WINDOW**

Copyright © 2018 Copyright Clearance Center, Inc. All Rights Reserved. Privacy statement. Terms and Conditions. Comments? We would like to hear from you. E-mail us at customercare@copyright.com