The cloning and functional characterisation of a family of human stargazin-like genes

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“Opportunity is missed by most people because it is dressed in overalls and looks like work.”

*Thomas Alva Edison (1847-1931)*

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**To Mum and Dad**

Thanks to the many opportunities you presented to me early in life, I soon learned to recognise others whenever they appeared. No matter how hard the resulting work, achieving my goals has always been possible because of the boundless support you give.

Thank you.
Abstract

1) Stargazin, the product of the gene mutated in the epileptic mouse Stargazer, is hypothesised to be the first example of a neuronal voltage-dependent calcium channel (VDCC) \( \gamma \) subunit. The aim of this thesis project was to identify, clone and characterise the functional properties of a family of human stargazin-like genes when expressed as part of a VDCC complex.

2) A family of five human stargazin-like genes were identified and cloned, including the human orthologue of stargazin itself.

3) The \( \gamma_2, \gamma_3 \) and \( \gamma_4 \) subunits are closely related to each other and are encoded by four exon genes. The \( \gamma_5 \) and \( \gamma_7 \) subunits display much lower homology to stargazin and are encoded by five exon genes, indicating they form a distinct subfamily of stargazin-like proteins.

4) The human \( \gamma_2, \gamma_3, \gamma_4, \gamma_5 \) and \( \gamma_7 \) subunit proteins share a conserved predicted tetra-spanning transmembrane topology. However, whilst the \( \gamma_2, \gamma_3 \) and \( \gamma_4 \) subunits localise specifically to the plasma membrane upon heterologous expression, \( \gamma_7 \) is expressed throughout the cell with little insertion into the membrane.

5) The \( \gamma_2, \gamma_3, \gamma_4 \) and \( \gamma_7 \) subunits are selectively expressed in brain with varying distributions in the different brain regions. More detailed distribution and cellular localisation data for the \( \gamma_2, \gamma_3, \) and \( \gamma_4 \) subunits in human cerebellum and hippocampus are reported.

6) Co-expression of either the \( \gamma_2 \) or \( \gamma_4 \) subunits failed significantly to modulate the biophysical properties of Cav2.1/\( \beta_4 \) VDCCs in the absence or presence of an \( \alpha 2\delta \) subunit. However, N-type current through Cav2.2 channels was almost abolished when co-expressed transiently with \( \gamma_7 \) in either Xenopus oocytes or COS-7 cells. Furthermore, \( \gamma_7 \) has this effect by causing a large reduction in
Abstract

expression of Cav2.2 protein rather than by interfering with trafficking or biophysical properties of the channel.
Contents

ABSTRACT 3

CONTENTS 5

1 Introduction 22
1.1 Ion channels: A brief introduction 23
1.1.1 The ionic hypothesis and channel doctrine 23
1.2 Voltage Dependent Calcium Channels (VDCCs) 25
1.2.1 Functional characterisation of VDCCs 26
1.2.1.1 Voltage classification 26
1.2.1.2 Pharmacological classification 28
1.2.1.2.1 T-type 28
1.2.1.2.2 L-type 28
1.2.1.2.3 N-type 29
1.2.1.2.4 P/Q-type 29
1.2.1.2.5 R-type 30
1.2.2 Molecular structure of VDCCs 31
1.2.2.1 The \( \alpha_l \) subunit 32
1.2.2.1.1 L-type \( \alpha_l \) subunit genes (Cav1.1-Cav1.4) 34
1.2.2.1.1.1 Cav1.1 (\( \alpha_{l1S} \)) 34
1.2.2.1.1.2 Cav1.2 (\( \alpha_{l1C} \)) 37
1.2.2.1.1.3 Cav1.3 (\( \alpha_{l1D} \)) 38
1.2.2.1.1.4 Cav1.4 (\( \alpha_{l1F} \)) 40
1.2.2.1.2 DHP-insensitive neuronal \( \alpha_l \) subunit genes (Cav2.x) 41
1.2.2.1.2.1 Cav2.1 (\( \alpha_{l1A} \)) 41
1.2.2.1.2.2 Cav2.2 (\( \alpha_{l1B} \)) 43
1.2.2.1.2.3 Cav2.3 (\( \alpha_{l1E} \)) 45
1.2.2.1.3 Low voltage activated T-type \( \alpha_l \) subunits (Cav3.1-Cav3.3) 47
1.2.2.2 Structural and functional properties integral for VDCC function. 52
1.2.2.2.1 The channel pore and selectivity filter 52
1.2.2.2.2 Activation and the voltage sensor 57
Contents

1.2.2.2.3 Inactivation 57
  1.2.2.2.3.1 Voltage dependent 58
  1.2.2.2.3.2 Ca^{2+}-dependent 61
1.2.2.2.4 Agonist/antagonist binding sites 62
  1.2.2.2.4.1 A multisubsite domain in L-type channels 62
  1.2.2.2.4.2 ω-conotoxin and ω-agatoxin binding sites 65
1.2.2.2.5 G-protein modulation of VDCCs 66
1.2.2.2.6 VDCC phosphorylation 68
1.2.2.2.7 Association of presynaptic VDCC with the SNARE complex 71
1.2.2.3 The β subunit 72
  1.2.2.3.1.1 Post-translational modification 75
  1.2.2.3.2 Functional properties 77
    1.2.2.3.2.1 β subunit is obligatory for surface expression and correct functional assembly of VDCCs 77
    1.2.2.3.2.2 Setting of proper kinetics of activation and voltage dependence of activation. 78
  1.2.2.3.2.3 Inactivation 78
  1.2.2.3.2.4 Prepulse facilitation 79
1.2.2.4 The α2δ subunit 80
1.2.3 The VDCC γ subunit 84
  1.2.3.1 The skeletal muscle γ1 subunit. 84
  1.2.3.2 Ca^{2+} channelopathies: Insights into channel structure and function from nature’s mistakes 86
  1.2.3.3 Channelopathies causing absence epilepsy in mice are linked to mutations in VDCC subunits. 89
  1.2.3.4 The stargazer mouse (stg) 91
    1.2.3.4.1 Aetiology 91
    1.2.3.4.2 Identification of a putative neuronal VDCC γ subunit (γ2) in stargazer mice 93
1.3 Stargazer foretells a constellation of γ subunits: Aims of the investigation 94
1.4 Epilogue to introduction 95

2 Materials & Methods 97
Contents

2.1 Cloning on a computer 98
   2.1.1 An introduction to “in silico” cloning 98
   2.1.2 In silico cloning of a family of human VDCC γ subunits 99
      2.1.2.1 Identification of human ESTs related to the mouse cacng2 by in silico analysis 99
      2.1.2.2 Identification and analysis of a human genomic sequence related to CACNG7 100
      2.1.2.3 Identification and analysis of a mouse genomic sequences related to CACNG5 and CACNG7 100
2.2 Standard molecular biology procedures 100
   2.2.1 cDNA sources and synthesis 100
      2.2.1.1 Concentration and purity of extracted mRNA 101
      2.2.1.2 First strand cDNA synthesis from Total RNA 102
         2.2.1.2.1 Priming with Random hexamers 102
         2.2.1.2.2 First strand synthesis reaction conditions 103
         2.2.1.2.3 Marathon ready cDNA 103
         2.2.1.2.4 SMART cDNA synthesis 104
            2.2.1.2.4.1 SMART first strand cDNA synthesis protocol 105
      2.2.2 Polymerase Chain Reaction (PCR) 106
         2.2.2.1 Taq polymerase 107
         2.2.2.2 Pfu and Pfu Turbo polymerases 108
         2.2.2.3 “Touchdown” PCR 109
         2.2.2.4 “Splice-overlap” PCR 110
      2.2.3 DNA isolation and purification 111
         2.2.3.1 Agarose gel electrophoresis 111
            2.2.3.1.1 Purification of cDNA from agarose gels 111
            2.2.3.1.2 Purification of cDNA from solutions 112
      2.2.4 Cloning cDNA 112
         2.2.4.1 pCR-TOPO 112
         2.2.4.2 Transformation of competent cells 115
         2.2.4.3 Bacterial culture 115
            2.2.4.3.1 Small-scale 115
            2.2.4.3.2 Large-scale 115
Contents

2.2.4.4 Plasmid purification 116
   2.2.4.4.1 Miniprep 116
   2.2.4.4.2 Maxi-prep 117
2.2.5 Subcloning cDNA into expression vectors 118
   2.2.5.1 Restriction Digest and ligation 118
      2.2.5.1.1 Restriction digest 118
      2.2.5.1.2 Ligation 118
   2.2.5.2 pMT2 118
   2.2.5.3 pcDNA3.1-myc/his 119
2.2.6 Automated DNA sequencing 120
2.3 Isolation and cloning of human γ subunit cDNAs 121
   2.3.1 Isolation and cloning of the CACNG2 cDNA 121
   2.3.2 Isolation and cloning of the CACNG3 cDNA 122
   2.3.3 Isolation and cloning of the CACNG4 cDNA 123
      2.3.3.1 SMART amplification of missing 5' end 123
      2.3.3.2 Amplification of complete CACNG4 cDNA 124
   2.3.4 Isolation and cloning of the CACNG7 subunit cDNA 125
      2.3.4.1 Cloning human CACNG7 cDNA 125
         2.3.4.1.1 Amplification of CACNG7 487bp cDNA predicted by in silico cloning 125
         2.3.4.1.2 Elucidation of unknown 5' and 3' sequence by Marathon RACE 125
      2.3.4.1.3 Assembly of a full CACNG7 ORF cDNA 127
   2.3.4.2 Cloning mouse cacng7 cDNA 128
2.4 Subcloning into expression vectors 128
   2.4.1.1 pMT2 128
   2.4.1.2 pcDNA3.1 myc/his 128
2.5 Expression Analysis 129
   2.5.1 Northern Blot 129
      2.5.1.1 Human blots 129
      2.5.1.2 COS-7 and Xenopus oocyte blot 129
         2.5.1.2.1 RNA purification 129
         2.5.1.2.2 Blot creation 130
## Contents

2.5.1.3  Probe design and synthesis 131  
  2.5.1.3.1  cDNA probes 131  
  2.5.1.3.2  Oligonucleotide probes 132  
2.5.1.4  Hybridisation and washing 133  
  2.5.1.4.1  Blot pre-hybridisation 133  
  2.5.1.4.2  Hybridisation 133  
  2.5.1.4.3  Washes 133  
2.5.1.5  Exposure, developing and image processing 134  

2.5.2  Antibodies 134  
  2.5.2.1  Design 134  
  2.5.2.2  Peptide Synthesis 135  
  2.5.2.3  Antibody production and affinity purification 135  
    2.5.2.3.1  Column Construction 135  
    2.5.2.3.2  Antibody affinity purification 136  
  2.5.2.4  Antibody specificity 136  

2.5.3  Immunocytochemistry 137  
  2.5.3.1  Cell culture 137  
    2.5.3.1.1  COS-7 flask culture 137  
    2.5.3.1.2  Plating for cytochemistry on coverslips 137  
  2.5.3.2  Transfections 138  
    2.5.3.2.1  Geneporter transfections 138  
    2.5.3.2.2  cDNA mixes 139  
    2.5.3.2.3  Non-\(\gamma\) subunit cDNAs used 139  
  2.5.3.3  Fixing and staining 139  
    2.5.3.3.1  Organelle Markers 140  
    2.5.3.3.1.1  Plasma Membrane 140  
    2.5.3.3.1.2  Nucleus 141  
  2.5.4  Confocal laser scanning microscopy 142  

2.5.5  Human Brain Immunohistochemistry 143  
  2.5.5.1  Tissue sources 143  
  2.5.5.2  Tissue sectioning 144  
  2.5.5.3  Tissue processing and staining 144  
  2.5.5.4  Control Procedures 145
## Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5.5.5 Microscopy and image analysis</td>
<td>145</td>
</tr>
<tr>
<td>2.5.6 Mouse cerebellum immunocytochemistry</td>
<td>146</td>
</tr>
<tr>
<td>2.5.6.1 Paraformaldehyde perfusion and cryo-sectioning of mouse cerebellum</td>
<td>146</td>
</tr>
<tr>
<td>2.5.6.2 Staining of cerebellar sections</td>
<td>146</td>
</tr>
<tr>
<td>2.6 Xenopus oocyte two electrode voltage clamp (TEVC)</td>
<td>147</td>
</tr>
<tr>
<td>2.6.1 Preparation of oocytes</td>
<td>147</td>
</tr>
<tr>
<td>2.6.2 Two-electrode voltage clamp</td>
<td>147</td>
</tr>
<tr>
<td>2.6.3 Data analysis and presentation</td>
<td>149</td>
</tr>
<tr>
<td>2.6.3.1 Equations</td>
<td>149</td>
</tr>
<tr>
<td>2.6.3.1.1 Current-voltage (I-V) relation</td>
<td>150</td>
</tr>
<tr>
<td>2.6.3.1.2 Steady-state inactivation</td>
<td>150</td>
</tr>
<tr>
<td>2.6.3.1.3 Inactivation time-course</td>
<td>150</td>
</tr>
<tr>
<td>3 Identification and cloning of human stargazin-related genes</td>
<td>151</td>
</tr>
<tr>
<td>3.1 Introduction</td>
<td>152</td>
</tr>
<tr>
<td>3.2 Results</td>
<td>154</td>
</tr>
<tr>
<td>3.2.1 Identification and virtual cloning of a family of human stargazin-related genes by electronic database mining</td>
<td>154</td>
</tr>
<tr>
<td>3.2.2 Amplification and cloning of cDNAs encoding novel human stargazin like proteins</td>
<td>159</td>
</tr>
<tr>
<td>3.3 Discussion</td>
<td>165</td>
</tr>
<tr>
<td>3.3.1 Identification and cloning of human putative VDCC ( \gamma_2, \gamma_3 ) and ( \gamma_4 ) subunits</td>
<td>165</td>
</tr>
<tr>
<td>3.3.2 Predicted structure and motifs of potential functional significance</td>
<td>166</td>
</tr>
<tr>
<td>4 In vitro expression and differential tissue distribution of the putative ( \gamma_2, \gamma_3 ) and ( \gamma_4 ) subunits</td>
<td>172</td>
</tr>
<tr>
<td>4.1 Introduction</td>
<td>173</td>
</tr>
<tr>
<td>4.2 Results</td>
<td>174</td>
</tr>
<tr>
<td>4.2.1 Transient expression of cloned ( \gamma ) subunit cDNAs in COS-7 cells</td>
<td>174</td>
</tr>
<tr>
<td>4.2.2 Probing for endogenous ( \gamma ) subunit mRNAs in <em>Xenopus</em> oocytes and COS-7 cells</td>
<td>176</td>
</tr>
<tr>
<td>4.2.3 Northern Blot analysis of mRNA distribution</td>
<td>177</td>
</tr>
</tbody>
</table>
4.2.3.1 \( \gamma_2 \) 178
4.2.3.2 \( \gamma_3 \) 178
4.2.3.3 \( \gamma_4 \) 179
4.2.4 Immunohistochemical analysis of neuronal \( \gamma \) subunits 181
4.2.4.1 Anatomy of the cerebellum 181
  4.2.4.1.1 Purkinje cells 182
  4.2.4.1.2 Granule cells 183
  4.2.4.1.3 Inhibitory inter-neurones 183
  4.2.4.1.4 Afferent connections – Mossy and climbing fibres 183
  4.2.4.1.5 Efferent pathways of the cerebellum 185
4.2.4.2 Immunohistochemistry of the putative neuronal \( \gamma \) subunits in the cerebellum 185
4.2.4.3 The localisation of the \( \gamma_2 \) subunit in human cerebellum 185
4.2.4.4 The localisation of the \( \gamma_3 \) subunit in human cerebellum 188
4.2.4.5 The localisation of the \( \gamma_4 \) subunit in human cerebellum 189
4.2.4.6 The localisation of the \( \gamma_2, \gamma_3, \) and \( \gamma_4 \) subunit in mouse cerebellum 190
4.2.4.7 Outline of the anatomy of the hippocampus 192
  4.2.4.7.1 Dentate gyrus 193
  4.2.4.7.2 Hippocampus 193
  4.2.4.7.3 Subiculum 194
4.2.4.8 Immunohistochemistry of the putative neuronal \( \gamma \) subunits in the hippocampus 194
4.2.4.9 The localisation of the \( \gamma_2 \) subunit in human hippocampus 195
4.2.4.10 The localisation of the \( \gamma_3 \) subunit in human hippocampus 200
4.2.4.11 The localisation of the \( \gamma_4 \) subunit in human hippocampus 203
4.3 Discussion 205
4.3.1 In vitro expression of neuronal \( \gamma \) subunits and antibody specificity 205
4.3.2 Northern blot distribution 206
4.3.3 Expression of \( \gamma \) subunits in cerebellum and hippocampus 209
  4.3.3.1 The distribution of the putative VDCC \( \gamma \) subunits in cerebellum 212
  4.3.3.2 The distribution of the putative VDCC \( \gamma \) subunit in hippocampus 214
4.4 Future work 216
Contents

4.4.1 In vitro expression of γ subunits 216
4.4.2 Differential tissue distribution 216

5 The functional properties of a family of human stargazin-like genes when co-expressed with neuronal voltage-dependent calcium channels 218
5.1 Introduction 219
5.2 Results 221
  5.2.1 Selection of a suitable VDCC complex with which to characterise the human γ2 and γ4 subunits 221
  5.2.2 Influence of the α2δ2 subunit upon the biophysical properties of Cav2.1/β4 currents expressed in *Xenopus* oocytes 222
    5.2.2.1 Activation 223
    5.2.2.2 Inactivation 224
  5.2.3 The effects of γ2 and γ4 subunits upon the biophysical properties of Cav2.1/β4 currents ± the α2δ2 subunit, expressed in *Xenopus* oocytes. 226
    5.2.3.1 Activation 226
    5.2.3.2 Steady-state inactivation 228
    5.2.3.3 Inactivation kinetics 231
5.3 Discussion 234
  5.3.1 Selection of a suitable channel complex with which to characterise the novel neuronal VDCC γ subunits 234
  5.3.2 The α2δ2 increases maximum conductance of Cav2.1/β4 channel expressed in *Xenopus* oocytes 235
  5.3.3 Activation properties of Cav2.1/β4 channels are unaltered by the γ2 and γ4 subunits in the presence or absence of α2δ2 subunits 237
  5.3.4 Modulation of VDCC inactivation by γ2 or γ4 subunits 237
  5.3.5 Comparison of data with results from other laboratories investigating the influence of putative neuronal γ subunit on VDCC electrophysiology 238
    5.3.5.1 Activation 238
    5.3.5.2 Inactivation 240
  5.3.6 γ2, γ3 and γ4 are important chaperones in the trafficking of AMPA receptor subunits. 242
  5.3.7 Are the γ2, γ3 and γ4 proteins VDCC subunits? 243
6 Identification, cloning and functional characterisation of a pair of low-homology stargazin-related proteins 245

6.1 Introduction 246

6.2 Results 247

6.2.1 The identification and cloning of a pair of putative γ subunits with low homology to stargazin. 247

6.2.2 Tissue distribution 256

6.2.2.1 γ7 mRNA distribution 256

6.2.2.2 γ5 mRNA distribution 256

6.2.3 Influence of the γ7 subunit on heterologous expression of VDCCs 260

6.2.3.1 Electrophysiology 260

6.2.4 Immunocytochemical analysis of the effects of γ7 267

6.3 Discussion 283

6.3.1 Identification and cloning of splice variants of putative human γ subunits. 283

6.3.2 Tissue distribution of the γ7 subunit 285

6.3.3 Tissue distribution of the γ5 subunit 286

6.3.4 Co-expression of the γ7 subunit almost totally abolishes heterologous Cav2.2 currents by reducing protein expression. 287

6.4 Direction of future research to determine the true identity and functional properties of the γ7 and γ5 subunits 291

6.4.1 Cloning the human γ5 subunit and determining functional properties 291

6.4.2 Distribution of the γ7 and γ5 subunits 292

6.4.3 Binding partners for γ7 and γ5 293

6.4.4 Functional importance of critical residues and motifs of the γ5 and γ7 proteins 294

6.4.5 Identifying the mechanism underlying the suppression of Cav2.2 expression 295

6.4.6 Cloning of γ5 and γ7 and investigation of the predicted intron retention events underlying their potential expression 295

Supplementary data for Chapter 6 regarding the functional characterisation of the γ7 subunit 299
Contents

6.5 Introduction 300
6.6 Conjoint experiments 300
  6.6.1 Influence of the γ subunit upon the heterologous expression of Kv3.1b in Xenopus oocytes (A. C. Dolphin & F. J. Moss) 300
    6.6.1.1 Methods 300
    6.6.1.2 Results 301
  6.6.2 Influence of the γ subunit upon Cav2.2 VDCC currents expressed in mammalian COS-7 cells (P. Viard and F. J. Moss) 301
    6.6.2.1 Methods 302
    6.6.2.2 Results 303
  6.6.3 Influence of the γ subunit on endogenous Ca\(^{2+}\) currents recorded from cultured sympathetic neurones (F. Bertaso and F. J. Moss) 304
    6.6.3.1 Methods 304
    6.6.3.2 Results 305
  6.6.4 Effect of co-expression of the γ subunit on the Cav2.2 protein levels (A. Davies and F. J. Moss) 308
    6.6.4.1 Methods 308
    6.6.4.2 Results 309

7 General Discussion 311
  7.1 Identification and cloning of proteins related to stargazin 312
  7.2 Tissue distribution and subcellular localisation 316
  7.3 Functional properties of the neuronal γ subunits 319
  7.4 Proposed Future Research 322
  7.5 Concluding remarks 323

ACKNOWLEDGEMENTS 325

PUBLICATIONS AND MEETING ABSTRACTS 327

GLOSSARY OF TERMS 328

REFERENCES 335
Figures

Figure 1.1 - The action potential and its contributory ionic conductances ........................................ 25
Figure 1.2 - LVA and LVA VDCC currents ........................................................................................ 27
Figure 1.3 - Schematic diagram of the molecular organisation of a VDCC ........................................ 32
Figure 1.4 - Nomenclature and phylogenetic relationship of the ten VDCC α\textsubscript{i} subunits .................................................................................................................................................... 34
Figure 1.5 - The archetypal pore structure of a voltage dependent cation channel predicted by the crystal structure of the KcsA K\textsuperscript{+} channel .............................................................................................................................................. 53
Figure 1.6 - Sequence of the P-loop SS2 segment in HVA and LVA VDCCs in all four conserved motifs ....................................................................................................................................................... 56
Figure 1.7 - Properties that facilitate cation permeation through the pore ........................................ 56
Figure 1.8 - Ca\textsuperscript{2+} channel state transitions during an action potential ................................ 58
Figure 1.9 - Possible model of fast inactivation of high voltage-activated calcium channels .............................................................................................................................................................................. 61
Table 1.3 and Figure 1.10 - A multisubsite domain in the pore of L-type VDCC is critical in the binding of DHPs, PAAs and BTZs ........................................................................................................................................... 65
Figure 1.11 - Prepulse facilitation of Ca\textsubscript{v}2.2 currents modulated by co-expressed Gβγ. ................................................................................................................................................................................... 68
Figure 1.12 - Regions of the VDCC α\textsubscript{i} subunit of functional importance in channel activation, inactivation and interaction with cytosolic proteins ........................................................................................................................................ 72
Figure 1.13 - Structure of the α\textsubscript{i}-interaction domain (AID) and a functional map of the β subunit. ........................................................................................................................................................................... 74
Figure 1.14 - Functional properties of the skeletal muscle γ\textsubscript{i} subunit in vivo ................................ 86
Figure 1.15 - The stargazer mouse ........................................................................................................ 92
Figure 1.16 - Properties of stargazin that identify it as a candidate neuronal VDCC γ subunit ......... 94
Figure 2.1 - The templates and primers used in Marathon RACE reactions ...................................... 104
Figure 2.2 - Mechanism of SMART cDNA synthesis ....................................................................... 105
Figure 2.3 - The splice overlap PCR principle ...................................................................................... 110
Figure 2.4 - Invitrogen pCR-TOPO vector maps and multiple cloning sites .................................... 114
Figure 2.5 - The pcDNA3.1/myc-His vector map ............................................................................... 120
Figure 2.6 - 5’RACE to amplify unknown portion of CACNG4 from SMART human brain cDNA .............................................................. 124
Figure 2.7 - Apparatus for transfer of RNA from denaturing gel to nitrocellulose membrane using an electroblotter

Figure 2.8 - Diagram of the Illumination Path in a Confocal Microscope

Figure 2.9 - Schematic of the two-electrode voltage clamp circuit

Figure 3.1 - A line up of the predicted protein sequences of human genes identified *in silico* by their homology to *caen2*

Figure 3.2 - Chromosomal location of *CACNG2*

Figure 3.3 - Chromosomal location of *CACNG3*

Figure 3.4 - Multiple alignments of the predicted protein sequences of human γ1,4 subunits

Figure 3.5 - *CACNG4* ORF cDNA sequence compared to the partial *in silico* prediction

Figure 3.6 - Chromosomal location of *CACNG1* and *CACNG4*

Figure 3.7 - Predicted transmembrane topology of the VDCC γ subunits

Figure 3.8 - Regions of the γ2, γ3 and γ4 subunits exhibit structural similarity to a group of integral membrane proteins

Figure 4.1 - Transient expression and sub-cellular localisation of cloned neuronal γ subunits in COS-7 cells

Figure 4.2 - Co-expression of neuronal γ subunits with VDCC CaV2.1 and β4 subunits in COS-7 cells

Figure 4.3 - Northern blot to probe for endogenous γ2, γ3 and γ4 subunit RNAs in *Xenopus* oocytes and COS-7 cells

Figure 4.4 - Northern blot analysis of the expression of the mRNA transcripts of the γ2, γ3, and γ4 subunits

Figure 4.5 - Location and structure of neurones in the cerebellar cortex

Figure 4.6 - Schematic of cerebellum circuitry

Figure 4.7 - γ2 subunit immuno-reactivity in human cerebellum

Figure 4.8 - γ3 subunit immuno-reactivity in human cerebellar folia

Figure 4.9 - γ4 subunit immuno-reactivity in human cerebellum

Figure 4.10 - Distribution of the neuronal γ2, γ3 and γ4 subunits in mouse cerebellum

Figure 4.11 - Schematic of a coronal section of the hippocampal formation

Figure 4.12 - Macroscopic view of human hippocampus immuno-reactivity with the γ2 subunit specific antibody
Figure 4.13 – low power magnification of \( \gamma_2 \) Ab staining of the hippocampus .......... 197
Figure 4.14 – 10 \( \times \) magnification of hippocampal regions stained with the \( \gamma_2 \) Ab...... 198
Figure 4.15 – High power images (\( \times40 \)) of cellular \( \gamma_2 \) staining in the hippocampal regions ...................................................................................................................................... 199
Figure 4.16 – Specific staining of human hippocampus with an anti \( \gamma_3 \) subunit antibody. .................................................................................................................................. 200
Figure 4.17 – Low power magnification images of \( \gamma_3 \) Ab immuno-reactivity in human hippocampus .................................................................................................................................. 201
Figure 4.18 – Investigating the \( \gamma_3 \) subunit somatic immunoreactivity in human hippocampus .................................................................................................................................. 202
Figure 4.19 – Macroscopic \( \gamma_4 \) immuno-reactivity in human hippocampus ................. 203
Figure 4.20 – \( \gamma_4 \) immuno-reactivity in pyramidal cells and dentate gyrus ................. 204
Figure 5.1 - Influence of the \( \alpha_2 \delta_2 \) subunit upon activation of a \( \text{Cav2.1}/\beta_4 \) VDCC expressed in \textit{Xenopus} oocytes............................................................................................223
Figure 5.2 - Effects of the \( \alpha_2 \delta_2 \) subunit upon steady state inactivation properties of \( \text{Cav2.1}/\beta_4 \) VDCCs expressed in \textit{Xenopus} oocytes..........................................................225
Figure 5.3 - The influence of the \( \alpha_2 \delta_2 \) subunit on the time course of \( \text{Cav2.1}/\beta_4 \) current inactivation expressed in \textit{Xenopus} oocytes..........................................................225
Figure 5.4 - Influence of the \( \gamma_2 \) and \( \gamma_4 \) subunits on the I-V relationship of \( \text{Cav2.1}/\beta_4 \) VDCCs......................................................................................................227
Figure 5.5 - Influence of the \( \gamma_2 \) and \( \gamma_4 \) subunits on the I-V relationship of \( \text{Cav2.1}/\beta_4/\alpha_2 \delta_2 \) VDCCs ..............................................................227
Figure 5.6 - Influence of the \( \gamma_2 \) and \( \gamma_4 \) subunits upon the steady state inactivation properties of \( \text{Cav2.1}/\beta_4/\alpha_2 \delta_2 \) channels expressed in \textit{Xenopus} oocytes. ...............229
Figure 5.7 - Influence of the \( \gamma_2 \) and \( \gamma_4 \) subunits upon the steady state inactivation properties of \( \text{Cav2.1}/\beta_4 \) channels expressed in \textit{Xenopus} oocytes.................230
Figure 5.8 - The influence of co-expression of the \( \gamma_2 \) and \( \gamma_4 \) subunit on the inactivation kinetics of \( \text{Cav2.1}/\beta_4/\alpha_2 \delta_2 \) VDCCs expressed in \textit{Xenopus} oocytes.................232
Figure 5.9 - The influence of co-expression of the \( \gamma_2 \) and \( \gamma_4 \) subunit on the inactivation kinetics of \( \text{Cav2.1}/\beta_4 \) VDCCs expressed in \textit{Xenopus} oocytes.................233
Figure 6.1 – A 487bp \textit{in-silico} sequence displayed 26% identity to \textit{stargazin} ...............248
Figure 6.2 – The 487bp in silico prediction of a partial stargazin-like gene was amplified from human brain total RNA by RT-PCR...................................................248
Figure 6.3 – 5' and 3' RACE on human brain cDNA generated bands containing sequence predicted to complete the ORF of a low-homology stargazin-like gene. ........................................................................................................249
Figure 6.4 – Splice overlap PCR generated an 828bp complete ORF..........................249
Figure 6.5 – 828bp five-exon mouse γ7 band can be amplified in a single RT-PCR reaction from mouse cerebellum total RNA ..................................................................251
Figure 6.6 – Nucleotide alignment of five-exon human and mouse γ7 nucleotide sequences. ..........................................................................................................................252
Figure 6.7 - 828bp mouse γ5 band can be amplified in a single PCR reaction from mouse brain cDNA...........................................................................................................253
Figure 6.8 – Amino acid alignment of human and mouse γ5 sequences..................253
Figure 6.9 - Protein sequences of a family of low homology stargazin related genes. 254
Figure 6.10 – Hydropathy plots comparing five-exon human γ7 and γ5 to the previously published predicted four-exon human γ7 and γ5 amino acid sequences........255
Figure 6.11 – Northern blot analysis of the tissue distribution of the γ7 subunit ..........258
Figure 6.12 – Northern blot analysis of the mRNA distribution of the human γ5 subunit. .................................................................................................................................259
Figure 6.13 - Peak current amplitude of neuronal voltage dependent calcium channels is reduced upon co-expression of the γ7 subunit......................................................261
Figure 6.14 - Peak current amplitude of CaV2.2/β1δ/α2δ voltage dependent calcium channels is reduced upon co-expression of the γ7 subunit.........................................263
Figure 6.15 – Properties of the γ7 subunit upon the steady state inactivation properties of VDCCs expressed in Xenopus oocytes.........................................................264
Figure 6.16 – The γ7 subunit accelerates the inactivation of CaV2.2 Ba2+ currents but not CaV2.1 or CaV1.2 currents............................................................266
Figure 6.17 - Sub-cellular distribution of the γ7 subunit transiently expressed in COS-7 cells.........................................................................................................................267
Figure 6.18 – Detection of the subcellular distribution of the γ7 subunit using an antibody directed to the loop linking transmembrane sements 1 and 2..........268
Figure 6.19 – Expression of N-terminal GFP tagged CaV2.2 subunit in COS-7 cells.. 270
Figure 6.20 – Co-expression of the γ7 subunit almost abolishes GFP-Cav2.2 fluorescence when expressed transiently in COS-7 cells. ............................................271

Figure 6.21 – The effect of co-expression of γ7 upon the expression of GFP-Cav2.2 in COS-7 cells ....................................................................................................................................272

Figure 6.22 - Cav-β1b-GFP fluorescence is reduced in the majority of cells co-expressing γ7 ...........................................................275

Figure 6.23 - The γ7 subunit does not reduce staining for γ2-myc/his but alters its subcellular distribution ........................................................................276

Figure 6.24- Subcellular distribution of the γ7-myc/his and Kv3.1b subunits transiently expressed alone in COS-7 cells ........................................................................278

Figure 6.25 – Staining of the Kv3.1b subunit is not reduced by the co-expression of the γ7-myc/his subunit ................................................................................279

Figure 6.26 - Reduction in GFP-Cav2.2 fluorescence is dependent upon the concentration of γ7 subunit cDNA with which it is co-transfected .......................281

Figure 6.27 - Effects of the concentration of γ2 subunit cDNA in the transfection mix upon GFP-Cav2.2 fluorescence when it is co-transfected in COS-7 cells ..........282

Figure 6.28 – A schematic diagram of the four/five exon γ7/γ7 gene structure. ...............284

Figure 6.29 – Association of biotinylated methanethiosulfonate (MTS) to free sulphydryl groups of proteins .................................................................................295

Figure 6.30 – RNase protection strategy to determine splicing or retention of intron 4 of the genes CACNG5 and CACNG7 .................................................................297

Figure 6.31 - Co-expression of the γ7 subunit with Kv3.1b in Xenopus oocytes does not modulate expressed peak current amplitudes ...........................................301

Figure 6.32 - Heterologously expressed VDCC current density is reduced by γ7 in COS-7 cells ........................................................................................................304

Figure 6.33 - The endogenous VDCC current from sympathetic neurons is unaltered by γ7 expression ............................................................307

Figure 6.34 - Acute expression of the γ7 subunit in sympathetic neurones from the SCG .................................................................................................307

Figure 6.35 - The effect of co-expression of γ7 subunit on the expression of Cav2.2 in COS-7 cells ..........................................................................................310

Figure 7.1 – Phylogenetic relationship of human γ subunits ........................................................................................................313

Figure 7.2 – Evolutionary replication of γ subunit genes by tandem or chromosomal duplication .......................................................................................315
Tables

Table 1.1 - Biophysical and pharmacological classification of VDCCs ......................... 31
Table 1.2 - The cloned VDCC α₁ subunits and most widely studied alternate splice forms, presented together with their primary tissue distribution, biophysical properties and pharmacological antagonists ...................................................... 51
Table 1.3 and Figure 1.10 – A multisubsite domain in the pore of L-type VDCC is critical in the binding of DHPs, PAAs and BTZs ......................................................... 65
Table 1.4 – Summary of the VDCC auxiliary subunit genes, chromosomal location, splice-variants and primary tissue distribution ................................................. 83
Table 1.5 – Human Disorders arising from VDCC channelopathies .......................... 87
Table 1.6 – Mutant mouse models of absence epilepsy: Mutations and physiological consequences ................................................................................................. 90
Table 2.1 - RNA/primer mixtures for 1st strand cDNA synthesis .............................. 103
Table 2.2 - RT reaction mixture ................................................................................... 103
Table 2.3 - RNA/primer mixtures for SMART 1st strand cDNA synthesis ............... 105
Table 2.4 - Standard Taq polymerase reaction mix for a 50μl PCR reaction .............. 107
Table 2.5 - Taq polymerase protocols ......................................................................... 108
Table 2.6 - Standard Pfu and Pfu Turbo polymerase reaction mix for a 50μl PCR reaction ................................................................................................................. 109
Table 2.7 - Pfu and Pfu Turbo polymerase protocols ................................................. 109
Table 2.8 – Separation range in TAE gels containing different concentrations of agarose .................................................................................................................. 111
Table 2.9 – Plasmid purification kit buffer compositions ............................................. 117
Table 2.10 – The pMT2LR multiple cloning site ....................................................... 119
Table 2.11 – 5’ RACE primers for CACNG4 .............................................................. 123
Table 2.12 – Marathon RACE primers for CACNG7 ............................................... 125
Table 2.13 – Constituents of a 1×MOPS denaturing agarose gel .............................. 130
Table 2.14 – RNA sample preparation for denaturing gel ....................................... 130
Table 2.15 - γ subunit northern probe primers ......................................................... 131
Table 2.16 – Northern blot wash solutions .................................................................... 133
Table 2.17 – Sequences of peptides designed to raise rabbit polyclonal anti γ subunit antibodies .............................................................................................................. 134
Table 2.18 - Primary and secondary Abs used in immunocytochemistry experiments 141
Contents

Table 2.19 – Organelle markers and streptavidin-conjugated fluorphores used in immunocytochemistry experiments ................................................................. 142
Table 2.20 – Working dilutions for primary Abs in immunohistochemistry experiments .................................................................................................................. 145
Table 3.1– Summary of human genes identified in silico by their homology to murine cacng2 ................................................................................................................ 155
Table 4.1 – Differential distribution of VDCC subunits in the brain ................................ 208
Table 4.2 - The relative expression levels of VDCC subunits expressed in cerebellum ......................................................................................................................... 211
Table 4.3 - The relative expression levels of VDCC subunits expressed in hippocampus .................................................................................................................. 215
Table 5.1 - Characteristics of I{Ba via Cav2.1/β4 channels alone or co-expressed with α2δ2 subunits in Xenopus oocytes ........................................................................ 224
Table 5.2 - Parameters of the voltage-dependence of steady state inactivation of I{Ba via Cav2.1/β4 channels alone or co-expressed with the α2δ2 subunit in Xenopus oocytes .................................................................................................................. 224
Table 5.3 - Characteristics of I{Ba via Cav2.1/β4 or Cav2.1/β4/α2δ2 channels alone or co-expressed with γ2 or γ4 subunits in Xenopus oocytes ........................................ 228
Table 5.4 - Influence of the γ2 and γ4 subunits upon the steady state inactivation properties of Cav2.1/β4/α2δ2 channels ........................................................................ 229
Table 5.5 - Influence of the γ2 and γ4 subunits upon the steady state inactivation properties of Cav2.1/β4 channels ........................................................................ 231
Table 5.6 - Data to compare the influence of co-expression of the γ2 or γ4 subunit on the inactivation kinetics of Cav2.1/β4/α2δ2 VDCCs ........................................ 232
Table 5.7 - Data to compare the influence of co-expression of the γ2 or γ4 subunit on the inactivation kinetics of Cav2.1/β4 VDCCs ........................................ 233
Table 6.1 - Influence of the γ7 subunit on the activation properties of VDCC expressed in Xenopus oocytes determined from the I-V plots ........................................ 262
Table 6.2 – Average steady-state inactivation data from recordings displayed in Figure 6.15 .................................................................................................................. 264
Table 6.3 – Modulation of inactivation kinetics by the γ7 subunit ................................ 266
Introduction
1.1 Ion channels: A brief introduction

1.1.1 The ionic hypothesis and channel doctrine

The function of the nervous system is to rapidly transmit information throughout the body via a highly organised network of neurones, the excitable cells that operate as functional signalling units of a system that communicates by means of both electrical and chemical transmission. What in particular sets the nervous system apart from other physiological systems is its unique ability to coincidently evaluate information from multiple sources and then take a swift and suitable course of action based upon that assessment. The physiological process that allows such a rapid and discriminatory process to occur is synaptic transmission, where an electrical signal arriving at the pre-synaptic terminal triggers the release of chemical transmitter that interacts with the postsynaptic target to produce a synaptic response.

The conserved mechanism for signalling within virtually all neurones is the action potential (Cole and Curtis, 1939; Hodgkin and Huxley, 1939). The action potential is the result of a change in ionic conductance of the membrane resulting in all or none regenerative electrical episode that propagates without fail from the initial segment of the axon to the presynaptic terminal. These days, it is taken for granted that rapid, voltage dependent changes in the ion permeability of the membrane are mediated by molecules known as voltage dependent ion channels that selectively control the flow of ions through an aqueous pore in response to changes in the local membrane potential.

In a series of seminal papers, Hodgkin, Huxley and Katz formulated a set of principles referred to today as the ionic hypothesis (Hodgkin and Huxley, 1952a; Hodgkin and Huxley, 1952b; Hodgkin and Huxley, 1952c; Hodgkin and Huxley, 1952d; Hodgkin et al., 1952). In their experiments performed on the squid giant axon preparation (Young, 1936), they were able to break down the action potential into its discrete components, which represented the ionic conductance of Na$^+$ and K$^+$ ions across the plasma membrane. In doing this, they not only established the principles explaining the existence of resting membrane potential and how alterations in this potential difference reflects changes in membrane Na$^+$ and K$^+$ permeability, but also provided a framework
for modern biophysics, predicting the existence of discrete channels with voltage
sensors and gating particles that selectively control the flow of ions through a water-
filled pore. Shortly afterwards, Hodgkin and Keynes (Hodgkin and Keynes, 1955) gave
what can be considered the first description of an ion selective channel when they stated
the $K^+$ flux of a giant nerve fibre could be explained by assuming:

"Ions tend to move through the membrane in narrow
channels, or along a chain of sites such that might be
provided by the negatively charged groups of a cation
exchange resin...ions should be constrained to move in
single file (with) several ions in the channel at any moment".

Despite this remarkable piece of insight, over the following 25 years, the debate raged
over the nature of the mechanism of rapid ion transduction across the plasma
membrane. However, Bertil Hille and Clay Armstrong were the chief proponents of the
dogma that ion channels, rather than transporters, selectively control the flux of ions in
excitable membranes (Armstrong and Hille, 1998; Hille et al., 1999). Over the course
of two decades, their research predicted the size and shape of $Na^+$ and $K^+$ pores, defined
the nature of the "selectivity filter" of each channel type, and elucidated their gating
mechanisms in response to fluctuations in membrane potential. These concepts were
reinforced with the advent of patch-clamp electrophysiology, which permitted the
characterisation of the minute currents that flow upon the opening of a single channel
and determined that the flux of a particular ion was dependent upon the probability of a
channel being in an open or closed state (Hamill et al., 1981; Neher and Sakmann,
1976).

Final vindication of the ion channel concept came with the molecular cloning of both
voltage- and ligand- gated ion channels in the 1980's (Ballivet et al., 1982; Betz, 1990;
Hollmann and Heinemann, 1994; Noda et al., 1984; Noda et al., 1982; Papazian et al.,
1987; Tanabe et al., 1987; Tempel et al., 1987). Analysis of the primary amino acid
sequences of the principal subunit of $Na^+$, $Ca^{2+}$, and $K^+$ channels revealed that all the
voltage dependent channels were part of a superfamily of proteins with the same overall
structure, which indicated that they should also share mechanistic similarities as
predicted by the biophysical research of the previous decades.
Chapter 1  

Introduction

Figure 1.1 - The action potential and its contributory ionic conductances.

Changes in Na⁺ and K⁺ conductance during an action potential (a). When depolarisation exceeds threshold levels, Na⁺ channels open allowing rapid flow of Na⁺ inward along its electrochemical gradient. Membrane potential moves towards the equilibrium potential for Na⁺ (E_{Na}) but is halted prematurely because rapid inactivation of the Na⁺ channels decreases membrane Na⁺ permeability. Shortly afterwards, K⁺ channels open in a slower and more prolonged manner, the net efflux of positive charge as K⁺ moves down its electrochemical gradient repolarising the cell. However, the slow closure of K⁺ channels results in the after-hyperpolarisation (E_{K} = equilibrium potential for K⁺). b) The simplified shape of an action potential generated by the ventricular cardiomyocyte. The action potential of the heart muscle cell lasts a great deal longer than that of the neurone. The Ca²⁺ plateau phase is also clearly visible in this cell type, a result of the inward flow of Ca²⁺ ions equalling the outward flow of K⁺ ions.

Repolarisation eventually continues because as the membrane potential falls, Ca²⁺ permeability decreases and K⁺ permeability increases. Figures were reproduced and modified from Ganong, (1993) and http://library.thinkquest.org/203758/Function/UnderstandingActionPotential.htm.

1.2 Voltage Dependent Calcium Channels (VDCCs)

In most excitable cells the action potential consists of three phases. A rapid depolarisation phase mediated by voltage-dependent Na⁺ channels, a plateau phase, caused by the inward movement of Ca²⁺ ions through voltage-dependent calcium channels (VDCCs), and termination phase that commences on activation of voltage-dependent K⁺ channels that mediate the outward movement of K⁺ ions to repolarise the cell (Figure 1.1). The Ca²⁺ entering the cell during the second plateau phase of the action potential serves as the primary intracellular second messenger from the electrical signals generated in the plasma membrane and initiates vital Ca²⁺ dependent processes including, excitation contraction coupling in muscle cells, excitation-secretion coupling in neuronal synapses and neurosecretory tissue, Ca²⁺ activated second messenger pathways and initiation of gene transcription.
This chapter describes the biophysical and molecular characteristics of the VDCCs, a highly diverse family of ion channels, which underlie this plethora of indispensable somatic functions.

1.2.1 Functional characterisation of VDCCs

1.2.1.1 Voltage classification

Originally it was assumed that there was only one type of VDCC. However, the first indication that there existed more than one species of VDCC came from recordings of the egg cell membrane of the starfish (Hagiwara et al., 1975). The currents could be distinguished by their different voltage thresholds for activation, a result that was also later observed in multiple cell types from different organisms (Bean, 1985; Bean et al., 1986; Carbone and Lux, 1984; Nilius et al., 1985; Nowycky et al., 1985; Reuter et al., 1982). On the basis of channel biophysics, VDCC currents were individually classified according to:

1) The magnitude of the depolarisation step required to activate the channel.
2) The time-course of inactivation of the channel following its activation.
3) The magnitude of the single channel conductance, which is unique to each channel subtype.

The first of these parameters categorises VDCC into two broad groups according to their voltage dependence of activation. Low voltage activated (LVA) or T-type (transient) channels activate at potentials slightly above the resting potential, gate fast, rapidly inactivating transient currents with a typical single channel conductance of ~8pS, exhibit Ca\(^{2+}\) currents of equal or greater magnitude than Ba\(^{2+}\) currents and are typically more sensitive to block by Ni\(^{2+}\) than Cd\(^{2+}\). These properties suggested that these channels were involved in the pacemaker activity in cardiac myocytes and neurones (Hagiwara et al., 1988; Huguenard, 1996; Jahnsen and Llinas, 1984; Llinas and Yarom, 1981).

The high voltage activated (HVA) channels have a threshold for activation substantially above the resting potential (towards 0mV), larger single channel conductances than T-type (Figure 1.2), display larger Ba\(^{2+}\) than Ca\(^{2+}\) conductances, are highly sensitive to
Cd$^{2+}$ block and inactivate more slowly. Early biophysical analysis identified two distinct components of HVA current. The L-type was so called because of its “long-lasting”, slowly inactivating current and possessed a single channel conductance of approximately 25pS. Another property peculiar to the L-type current is that it is both voltage and calcium dependently inactivated (see page 57). The functional importance of calcium-induced inactivation is that it provides a negative feedback mechanism that limits calcium entry into the cell.

The biophysical properties of N-type current make it very difficult to identify from other current types. Named N-type because its intermediate nature was “neither” L- or T-type, it displayed an average single channel conductance of 13pS (later modified to ~18pS (Wakamori et al., 1998)), activation over a range of potentials between those of T- and L-type channels, and inactivation over an extremely broad range (-80 to -20 mV) (Fox et al., 1987a; Fox et al., 1987b; Nowycky et al., 1985). These multiple degrees of inactivation, broad activation ranges and the propensity to remain available for activation at relatively positive holding potentials could be explained by different gating modes (Plummer and Hess, 1991b; Rittenhouse and Hess, 1994), the expression of different channel splice-variants (Lin et al., 1997), or differential association with an auxiliary subunit (Scott et al., 1996).

Application of organic reagents and peptide toxins developed a pharmacological basis for VDCC current classification. These studies clarified the ambiguity surrounding the nature of N-type current, and identified additional current types according to their sensitivity to selective pharmacological agents.

**Figure 1.2 - LVA and LVA VDCC currents**

Single channel currents flowing through a) LVA T-type channels and b) HVA L-type channels from a cell-attached patch on a guinea pig cardiac ventricular myocyte. Ba$^{2+}$ has been used as the permeant ion. Upper current traces were elicited by a single depolarisation, whereas bottom traces represent the average of ~280 such traces. Reproduced and modified from Nilius et al. (1985).
1.2.1.2 Pharmacological classification

1.2.1.2.1 T-type

T-type currents can be clearly identified by their biophysical properties alone, which is fortunate for researchers since a pharmacologically selective compound was only recently discovered. The application of Ni$^{2+}$ or amiloride (Hirano et al., 1989; Tytgat et al., 1990) has often been used to reduce T-type currents, however neither the cation nor drug have ever proven particularly selective. It was proposed that a novel Ca$^{2+}$ channel antagonist, mibefradil, could selectively block T-type current (Mishra and Hermesmeyer, 1994). However this compound has been shown to block HVA VDCC types, and displays an especially high-affinity binding for VDCCs when in the inactivated state (Bezprozvanny and Tsien, 1995; Jimenez et al., 2000; McDonough and Bean, 1998; Viana et al., 1997). Kurtoxin, isolated from the venom of the South African scorpio Parabuthus transvaalicus, is potentially the first truly selective T-type antagonist (Chuang et al., 1998). This peptide demonstrates over 600-fold selectivity for block of LVA current over HVA currents and works by modifying the channel gating properties, as demonstrated by shifting the voltage dependence of activation of cloned T-type channels to more depolarised voltages rather than blocking the channel pore. Nevertheless, kurtoxin also causes pronounced slowing of rat brain type IIA Na$^{+}$ channel current activation and inactivation, indicating that although it is selective for a particular VDCC current, it is not exclusively a Ca$^{2+}$channel antagonist and that T-type VDCCs and voltage-dependent Na$^{+}$ channels probably, both of which control membrane excitability, share a common toxin-binding motif (Chuang et al., 1998).

1.2.1.2.2 L-type

The L-type current is sensitive to modulation by the organic reagents called dihydropyridines (DHPs) (Hess et al., 1984) whereas the LVA T-type channels are not (Bean, 1985). Nitrendipine, and nifedipine have both been shown to be L-type antagonists whilst (-)-BayK8644, another dihydropyridine enhances L-type current (Almers and McCleskey, 1984; Bean, 1985; Sanguinetti and Kass, 1984a). However, it has become apparent that the DHP agonists and antagonists also act upon other VDCC
Chapter 1

Introduction

currents, albeit with less potency (Furukawa et al., 1999; Lacinová et al., 2000; Stephens et al., 1997; Uneyama et al., 1999). In addition to the DHPs L-type current is modulated by phenylalkyamines (PAA e.g. verapamil) and benzothiazepines (BTZ e.g. diltiazem) (Mitterdorfer et al., 1998), which act at allosterically linked receptor sites to block Ca\(^{2+}\) current (Glossmann et al., 1987; Mitterdorfer et al., 1998). L-type channels are probably the primary sites of action for BTZs and PAAs, but these compounds are also non-selective, inhibiting Ca\(^{2+}\) currents from other channel types (Diochot et al., 1995).

1.2.1.2.3 N-type

N-type current is insensitive to DHP modulation but is instead blocked by toxins extracted from the venom of the fish-hunting snail Conus geographus (Olivera et al., 1994). In mammalian neurones, the toxin fraction ω-conotoxin GVI A (ω-ctx GVI A) is a highly selective and irreversible blocker of only N-type VDCCs (Sher and Clementi, 1991). The sensitivity of VDCC current to this particular toxin is now the standard defining criterion of N-type current. A second toxin fraction from the venom of the Conus magnus snail, ω-conotoxin MVII A (ω-ctx MVII A) blocks N-type current with similar potency and efficiency as ω-ctx GVI A, but binds reversibly (Stoehr and Dooley, 1993). The synthetic version of this toxin (SNX-111) has entered clinical trials for the control of pain and as a neuroprotective reagent (Bowersox et al., 1996; Brose et al., 1997).

1.2.1.2.4 P/Q-type

The predominant VDCC current in cerebellar Purkinje cell bodies (>80%) was designated the P-type. It could be separated from L- and N-type currents because it was insensitive to block by DHPs or ω-ctx GVI A, but was specifically blocked by the polyamine-containing fraction of funnel web spider toxin venom, FTX (Olivera et al., 1994). Subsequent investigations used a more refined peptide venom fraction ω-Agatoxin IVA (ω-AgaIVA), which proved to be a more potent and selective P-type inhibitor (IC\(_{50}\) ~2 nM)(Mintz et al., 1992a; Mintz et al., 1992b). P-type current is slow
to inactivate during prolonged depolarisations and exhibits multiple conductance levels of 9, 14, and 19 pS (in 110 mM Ba\(^{2+}\)), which could be attributable to molecular channel diversity or differential subunit associations (Pichler et al., 1997; Scott et al., 1996).

A similar current identified in rat cerebellar granule cells was called Q-type (Randall et al., 1993). Like P-type current, Q-type is HVA, DHP and \(\omega\)-ctx GVIA insensitive, and potently inhibited by the \(\omega\)-conotoxin MVIIC. It can however be pharmacologically distinguished from P-type by its lower sensitivity to \(\omega\)-AgaIVA (IC\(_{50}\) ~200mM (Sather et al., 1993)). It also displays an inactivating waveform even in the presence of Ba\(^{2+}\) (Randall et al., 1993; Zhang et al., 1993). Despite these differences, the similarity between P- and Q- type currents has often led to the two classes being grouped into a single P/Q-class. However, recent identification of some of the molecular determinants for P- and Q-type current has allowed researchers to be less ambiguous in their current classification ((Bourinet et al., 1999; Hans et al., 1999) & page 41).

1.2.1.2.5 R-type

If L-, N-, P-, and Q- currents comprised the total VDCC current in a neurone, application of a cocktail of DHPs, \(\omega\)-ctx GVIA and \(\omega\)-AgaIVA, each at a sufficient but non-toxic concentration, would completely abolish all Ca\(^{2+}\) current stimulated by a prolonged depolarisation (this would eliminate the T-type component). This does not occur in the majority of neurones due to the presence of a "resistant" R-type current. First classified as another component of cerebellar granule cell current, much controversy surrounds the R-type channels and their molecular components (see page 45). Their threshold for activation is usually at potentials between those for LVA or HVA channels, they inactivate rapidly like T-type channels and are more sensitive to block by Ni\(^{2+}\) than Ca\(^{2+}\) (Ellinor et al., 1993; Randall and Tsien, 1995; Randall et al., 1993; Zhang et al., 1993).
Chapter 1

Introduction

Table 1.1 - Biophysical and pharmacological classification of VDCCs

<table>
<thead>
<tr>
<th>Current type</th>
<th>Single channel conductance</th>
<th>Inactivation</th>
<th>Pharmacology</th>
</tr>
</thead>
<tbody>
<tr>
<td>L</td>
<td>25pS</td>
<td>Slow (&gt; 500 ms)</td>
<td>1,4-Dihydropyridines $^\S$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Intermediate (50-500 ms)</td>
<td>$\omega$-conotoxin GVIA</td>
</tr>
<tr>
<td>N</td>
<td>13-20pS</td>
<td>Slow (&gt;500ms)</td>
<td>$\omega$-Agatoxin IVA</td>
</tr>
<tr>
<td>P</td>
<td>9-20pS</td>
<td>Intermediate (&gt;100ms)</td>
<td>(IC$_{50}$ ~2 nM)</td>
</tr>
<tr>
<td>Q</td>
<td>9-20pS</td>
<td>Intermediate/ (IC$_{50}$ ~200 nM)</td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>15pS</td>
<td>Fast</td>
<td>Ni$^{2+}$ (IC$_{50}$ ~50µM) $^*$</td>
</tr>
<tr>
<td>T</td>
<td>8pS</td>
<td>Fast</td>
<td>SNX-482 (IC$_{50}$ 15-30nM) $^*$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Kurtoxin (&lt;350nM)</td>
</tr>
</tbody>
</table>

Summary table of parameters used to classify VDCC currents according to single channel conductance (100mM Ba$^{2+}$), rate of inactivation and selective pharmacology. Although DHPs are the standard pharmacological reagent used to modulate L-type channels, $^\S$ denotes that non-L-type channels are also modulated by DHPs. $^*$ Low concentrations of Ni$^{2+}$ are reported to block R-type current, but this is a non-selective effect. $^\dagger$ SNX-482 reduces the R-type current in rat nerve endings, but not in soma (Wilson et al., 2000). Indicates heterogeneous population of differentially expressed VDCC types may underlie R-type current.

1.2.2 Molecular structure of VDCCs

Research into the molecular composition of VDCCs was coincident with their biophysical and pharmacological classification. The initial purification of L-type VDCCs from the transverse tubule membranes of rabbit skeletal muscle took advantage of their high-density in this tissue and their high-affinity binding of the DHP antagonists (Curtis and Catterall, 1984). The major components of this “DHP-receptor” were named $\alpha$ (200 kilo Daltons, kDa), $\beta$ (50 kDa), and $\gamma$ (33 kDa) and were proposed to associate through non-covalent interactions as they separated under non-reducing sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) conditions. In the same investigation, under reducing conditions designed to disrupt any covalent interactions, the $\alpha$ subunit migrated as a 160/130 kDa doublet. In a subsequent investigation, the same laboratory cleaved all the disulphide bonds present in the purified L-type DHP-sensitive Ca$^{2+}$ channel complex to reveal that the $\alpha$ subunit doublet represented two distinct polypeptides whilst also detecting an additional $\delta$ component (Takahashi et al., 1987). The authors concluded that the skeletal muscle
calcium channel is a heteroligomeric complex of a principal $\alpha_1$ polypeptide (175 kDa), the auxiliary $\alpha_2\delta$ subunit, which is a composite of the $\alpha_2$ glycoprotein (143kDa) and the much smaller $\delta$ subunit (24-27kDa) that are linked via disulphide bonds, the $\beta$ subunit (54kDa) and the $\gamma$ subunit (30kDa). These are arranged in a 1:1:1:1 stoichiometry (Figure 1.3). Shortly afterwards the cDNA encoding the $\alpha_1$ subunit of the rabbit skeletal muscle was cloned (Tanabe et al., 1987) and paved the way for the identification of the superabundance of VDCC genes and their splice variants that are differentially expressed and assembled to control the multitude of signalling functions required in different neural networks.

**Figure 1.3 – Schematic diagram of the molecular organisation of a VDCC.**

The principal $\alpha_1$ subunit gates the flow of calcium ions across the plasma membrane in a voltage-dependent manner. It consists of four homologous domains (I-IV) arranged around a central aqueous pore. Each conserved domain contains six transmembrane (TM) segments S1-S6. The cytoplasmic loops of the $\alpha_1$ subunit are named according to the domains that they link. These interact with many intracellular species, including the auxiliary $\beta$ subunit, whose $\alpha$-helices are shaded black to denote the subunit’s hydrophilic nature, rather than the grey hydrophobic helices of the other subunits. The hydrophobic $\delta$ subunit is inserted into the membrane and anchors the highly glycosylated extracellular $\alpha_2$ subunit (denoted by forked structures) via disulphide bonds (S-S). The $\gamma$ subunit is a 4TM species that is only lightly glycosylated.

### 1.2.2.1 The $\alpha_1$ subunit

Similar to the $Na^+$ channel pore forming proteins (Noda et al., 1984), the VDCC $\alpha_1$ subunit is a single polypeptide containing approximately 2000 amino acids arranged in four repeating domains (I-IV). These domains contain 300-400 amino acids and share 50\% sequence identity with one another. Each domain consists of six hydrophobic segments (S1-S6) with the potential to form membrane spanning $\alpha$ helices. Domains I-IV are arranged around a central pore that is formed by a region of the extracellular loop between S5 and S6 of each domain. Termed the “P-loop”, this region contains the acidic residues required for divalent cation selectivity and permeability (Figure 1.6 & page 52). S4 of each domain contains a number of charged residues at every third amino acid along the
length of its $\alpha$-helix, and by analogy with the structure of Na$^+$ and K$^+$ channel structure (Catterall, 1993; Noda et al., 1984; Papazian et al., 1987), acts as the voltage sensor (see page 57 & Figure 1.12). These motifs and the structural organisation of the $\alpha_1$ pore-forming subunit in the membrane make it indispensable for the normal functioning of the channel. Indeed, heterologous expression of cloned $\alpha_1$ subunits reveal it is able to gate the voltage-activated flux of Ca$^{2+}$ ions when expressed in the absence of any of the auxiliary subunits (Perez-Reyes et al., 1989; Singer et al., 1991), which serve only to modulate the biophysical properties of the $\alpha_1$. The $\alpha_1$ subunit also contains the binding sites for channel modulators like the dihydropyridines, PAAs, BTZs, or $\omega$-conotoxins (Mitterdorfer et al., 1998; Olivera et al., 1994) & page 62) and has the potential to interact with non-calcium channel subunit proteins including GTP-binding proteins (G-proteins) (Dolphin, 1998) and synaptic proteins involved in neurotransmitter release (Catterall, 1998; Meir et al., 1999). Furthermore the subunit is a substrate for post-translational modification by PKA, protein kinase C (PKC) and Ca$^{2+}$-calmodulin dependent protein kinase (Gao et al., 1997a; Mikala et al., 1998; Werz et al., 1993).

To date, ten genes have been identified that encode VDCC $\alpha_1$ subunits. Extensive characterisation of the functional properties of these genes has determined that the majority of isoforms can be assigned as the molecular correlate, or one of the molecular correlates of the biophysically and pharmacologically defined L-, N-, P-, Q-, R-, and T-types currents. Fine-tuning of the current resulting from an expressed $\alpha_1$-subunit occurs as the result of alternative splicing of the gene transcript and/or differential association with auxiliary subunits depending on the cell type in which the VDCC complex is expressed (Bourinet et al., 1999; Hans et al., 1999; Reimer et al., 2000; Scott et al., 1996). The ten proteins encoded by the $\alpha_1$ subunit genes were historically designated $\alpha_{1A-1}$ and $\alpha_{1S}$ (Bech-Hansen et al., 1998; Birnbaumer et al., 1994; Lee et al., 1999d; Perez-Reyes, 1998). However, as new genes encoding ion channel subunits continued to be identified, it became apparent that the molecular and biophysical nomenclatures would soon overlap, with the likelihood that should a hypothetical $\alpha_{1L}$ subunit be identified, it would be unlikely to gate L-type VDCC current. More importantly, the alphabetical molecular nomenclature did not reveal the phylogenetic relationships between all the $\alpha_1$ subunits. Therefore, based on a system previously developed for K$^+$ channel nomenclature (Chandy, 1991), a systematic numerical nomenclature has been
developed that allows Ca\(^{2+}\) channel proteins to be named according to the principal permeating ion (Ca) with the major physiological regulator (voltage) indicated as a subscript (Cav)(Ertel et al., 2000). The VDCC \(\alpha_1\) subunits can be grouped into three families; Cav1 includes channel that mediate L-type Ca\(^{2+}\) currents (Cav1.1- Cav1.4), Cav2 includes the neuronal VDCCs mediating P/Q-, N-, and R-type currents (Cav2.1- Cav2.3) and Cav3 includes channels that mediate T-type currents (Cav3.1- Cav3.3). This thesis will use the new nomenclature when describing any VDCC compositions.

![Diagram](image-url)

**Figure 1.4 - Nomenclature and phylogenetic relationship of the ten VDCC \(\alpha_1\) subunits**

According to phylogenetic relationships the VDCC \(\alpha_1\) subunits have been classified into three families, Cav1, Cav2, and Cav3. These families encode HVA DHP sensitive L-type channels, the HVA neuronal DHP insensitive non-L-type channels and the LVA T-type respectively. (*) Denotes that although CavL3 and CavL4 are clustered as part of the HVA group of channels in this scheme, recent evidence suggests that they are could be grouped separately as channels activated at voltages intermediate to the threshold values for LVA or HVA channels (See sections CavL3 (a1p) & CavL4 (a1f) pages 38 & 40). Interfamily sequence identities are approximately 52% (Cav1 versus Cav2) and 28% (Cav3 versus Cav1 or Cav2). The official human gene names are listed to the right and mimic the previous alphabetical nomenclature. Figure reproduced and modified from Ertel et al. (2000).

1.2.2.1.1 **L-type \(\alpha_1\) subunit genes (Cav1.1-Cav1.4)**

1.2.2.1.1 **Cav1.1 (a1s)**

The identification and purification of the \(\alpha_1\) subunit of the DHP receptor from skeletal muscle transverse tubule membranes facilitated the isolation and cloning of the subunit’s cDNA, Cav1.1 (a1s) (Tanabe et al., 1987). The 6083 nucleotide (nt) cDNA sequence contained an open reading frame that encoded a polypeptide of 1873 amino acids with a predicted molecular mass of 212 kDa. This was somewhat larger than the
175-kDa α₄ band resolved by SDS-PAGE in previous investigations (Curtis and Catterall, 1984; Takahashi et al., 1987). These differences were attributed to proteolytic modification in vivo or cleavage during the purification process. Both the long and short forms were subsequently purified from skeletal muscle and their correct molecular masses determined by Ferguson analysis to be 214 kDa and 193 kDa respectively (De Jongh et al., 1989; De Jongh et al., 1991). Experimental findings that reconstitution of the DHP receptor into phospholipid bilayers (Flockerzi et al., 1986) or injection of the DHP receptor cDNA into dysgenic murine myotubes or L-cells resulted in expression of DHP-sensitive voltage-dependent Ca²⁺ current (Hosey and Lazdunski, 1988; Perez-Reyes et al., 1989) coupled with the striking similarity of its predicted structure with the voltage dependent Na⁺ channel (Noda et al., 1984), suggested that the DHP receptor formed a functional VDCC. However, recordings from skeletal muscle suggested that only a tiny proportion of the DHP receptors are involved in ion conductance, whilst the majority act as the voltage sensor for excitation contraction coupling (ECC) (Rios and Brum, 1987; Schwartz et al., 1985). Although the DHP receptor had this dual function, only the one gene was cloned from skeletal muscle (Tanabe et al., 1987) that is required for both functional activities in mice (Tanabe et al., 1988). It was hypothesised that the larger 214 kDa polypeptide, which represents only a small fraction of the α₄ subunit purified from skeletal muscle, retained its C-terminal amino acids that contain PKA phosphorylation sites (Tanabe et al., 1988) whose modification could possibly be required in order to activate ion conductance (Hymel et al., 1988; Nunoki et al., 1989).

The 193 kDa α₄ isoform was the more prevalent species purified from skeletal muscle and is the product of post-translational cleavage of the C-terminal fragment at a site between residues 1685-1699 of the 214 kDa primary translation product (De Jongh et al., 1991). It was suggested that it therefore lacked the domain that may have been necessary to stimulate ion-conductance, suggesting a functional role as the voltage sensor in ECC (De Jongh et al., 1989). This hypothesis was refuted when a clone that expressed solely the truncated Cav1.1 isoform fully restored both ECC and Ca²⁺ current when transfected into dysgenic myotubes, implying that a single class of DHP receptor performs both functions (Beam et al., 1992). Moreover, in a much later study, both the full-length and truncated Cav1.1 formed functional calcium channels in Xenopus oocytes, although the truncated version did produce significantly larger currents (Ren and Hall, 1997). However, the roles of complete and truncated Cav1.1 remain
contentious because insertion of the complete Cav1.1 into the junctional domains of skeletal muscle was recently demonstrated to be independent of post-translational cleavage of the C-terminus (Flucher et al., 2000) and is likely to be at least in part, dependent upon a motif between residues 1543-1647 which are present in the C-termini of both the complete and truncated Cav1.1 (Proenza et al., 2000).

Whichever form of Cav1.1 controls muscle contraction, during ECC, the movement of gating charges of the Cav1.1 subunit is transmitted via a physical interactions of the II-III loop of the α1 polypeptide with the ryanodine receptor (RyR) of the sarcoplasmic reticulum (SR), ultimately opening the RyR and releasing SR-stored Ca^{2+} (Grabner et al., 1999; Lu et al., 1995; Nakai et al., 1998; Tanabe et al., 1990). The Ca^{2+} release from RyRs occurring either spontaneously or during ECC has also been shown to promote the long-term expression of L-type channels in skeletal muscle myotubes (Avila et al., 2001).

The L-type current gated by Cav1.1 is poorly characterised because reconstitution of Cav1.1 has proven difficult in non-muscle cells (Dascal et al., 1992; Nargeot et al., 1992). This was believed to be because Cav1.1 lacked an interaction with the RyR in non-muscle cells (Fleig et al., 1996; Nakai et al., 1996). In spite of this, large, slowly activating L-type currents have been recorded from Xenopus oocytes expressing Cav1.1, but only on co-expression with the neuronal β_{1b} auxiliary subunit (Ren and Hall, 1997). The β_{1a} subunit is the predominant β subunit expressed in skeletal muscle and was the standard auxiliary subunit with which Cav1.1 was co-expressed in previous investigations (Hosey and Lazdunski, 1988; Perez-Reyes et al., 1989). The β_{1b} subunit is the neuronal splice variant of the β1 gene, but was detected at 2% of the levels of β_{1a} in skeletal muscle (Ren and Hall, 1997). Whether β_{1b} is able to out-compete the β_{1a} for association with Cav1.1 in skeletal muscle, and the precise molecular determinants for such an action remains to be determined. Nonetheless, one could hypothesise that association of Cav1.1 with β_{1a} is important in trafficking the DHP receptor complex to the membrane to act as the voltage sensor for ECC (Krizanova et al., 1995), whilst β_{1b} association is required for the gating of voltage-dependent Ca^{2+} current in myotubes (Ren and Hall, 1997).
Using sequences from the skeletal muscle Ca_v1.1 subunit as a probe, the cDNAs encoding additional α_1 subunit genes were identified in cardiac and neuronal tissues (Koch et al., 1990; Mikami et al., 1989; Perez-Reyes et al., 1990; Snutch et al., 1990).

1.2.2.1.1.2 Ca_v1.2 (α_1C)

Ca_v1.2 is the primary HVA VDCC α_1 subunit expressed in the heart and the molecular correlate of cardiac L-type VDCC current but is also widely expressed in other tissues. Originally cloned from rabbit cardiac tissue (Mikami et al., 1989) where its principal role is to control the Ca^{2+} flux regulating ECC in cardiac muscle, Ca_v1.2 is also found throughout the nervous system (Snutch et al., 1991). It is usually localised in the cell bodies and proximal dendrites of neurones, where its role is to regulate calcium dependent functions including protein phosphorylation, enzyme activation and gene expression (Ahlijanian et al., 1990; Catterall, 2000; Hell et al., 1993), although it has also been implicated in the control of neuroendocrine secretions (Wiser et al., 1999). Similarly to the Ca_v1.1 subunit, two size forms are usually expressed, a 210-240 kDa polypeptide corresponding to the complete translation product, and a 190-210 kDa form resulting from the proteolytic cleavage of part of the C-terminus (De Jongh et al., 1996; Hell et al., 1993). Multiple splice forms of Ca_v1.2 also exist, expressed in a tissue specific manner (Biel et al., 1991; Koch et al., 1990; Perez-Reyes et al., 1990), but in general it is 66% identical to the Ca_v1.1 subunit, with particularly high sequence conservation in the transmembrane regions (Mikami et al., 1989). Sequence is less well conserved in the loops linking the conserved transmembrane domains, which contain sites of predicted post-translational modification by protein kinases on the cytoplasmic side (Gerstin et al., 1998; Yoshida et al., 1992b) or N-linked glycosylation on the extracellular surface (Mikami et al., 1989). Homology is also low in the N- and C-termini, the latter of which is involved in multiple events regulating channel function, including channel phosphorylation (De Jongh et al., 1996; Gao et al., 1997a; Mitterdorfer et al., 1996a), binding of the Ca^{2+}/calmodulin complex resulting in Ca^{2+}-dependent inactivation or facilitation (Peterson et al., 1999; Qin et al., 1999; Zuhlke et al., 1999; Zuhlke and Reuter, 1998)(see page 61), autoinhibition of Ca^{2+} current by C-terminal motifs (Wei et al., 1994) and targeting of the channel to the plasma membrane (Gao et al., 2000b). Initial investigations reported Ca_v1.2 mRNA injected alone into
Chapter 1

Introduction

Xenopus oocytes was sufficient to direct the formation of functional DHP-sensitive L-type currents (Mikami et al., 1989). However, later research has shown that an endogenous Xenopus β subunit (β_{ex}) associated with the exogenous Cav1.2 and suppression of the β_{ex} expression obliterates VDCC currents that arise after injection of mammalian α_{i} cRNAs (Tareilus et al., 1997). Furthermore, the formation of a Cav1.2/β subunit complex is required to target the channel to the plasma membrane of mammalian tsA-201 cells (Gao et al., 1999). Nevertheless, co-expression of recombinant auxiliary β, α2δ or γ subunits, individually or as a complete complex with Cav1.2 enhances recorded VDCC activity (Mikami et al., 1989; Singer et al., 1991; Wei et al., 1991), and in the case of the β subunit, also modulates the channels biophysics, shifting its voltage dependence of activation to more negative potentials and accelerating its rate of activation (Singer et al., 1991; Wei et al., 1991).

1.2.2.1.3 Cav1.3 (α_{ID})

Polymerase chain reaction (PCR) with primers based upon conserved regions of the skeletal muscle Cav1.1 and cardiac Cav1.2 subunits, identified a third molecular correlate of L-type current, Cav1.3 (α_{ID}) (Hui et al., 1991; Perez-Reyes et al., 1990; Seino et al., 1992; Williams et al., 1992b; Yaney et al., 1992). Referred to as the neuroendocrine L-type channel because it is localised both in neurones and endocrine cells (Perez-Reyes et al., 1990; Seino et al., 1992; Yaney et al., 1992), four splice variants of Cav1.3 have been identified in mammalian tissues and cell lines (Hui et al., 1991; Perez-Reyes et al., 1990; Williams et al., 1992b). The subunit represents only 20% of the L-type channel solubilised from rat cerebral cortex and hippocampus and is principally localised in the soma and proximal dendrites of these neurones (Hell et al., 1993). It is however, strongly expressed in pancreatic β-cells (Seino et al., 1992; Yaney et al., 1992), where it may be the principal VDCC isoform regulating insulin secretion (Ihara et al., 1995) and is required for proper cell generation in postnatal pancreas islets (Namkung et al., 2001), although glucose metabolism is not disturbed the Cav1.3 knockout mice (Platzer et al., 2000). The human neuronal Cav1.3 subunit displays 79.7% and 50% identity with the rabbit cardiac Cav1.2 and rabbit brain Cav2.1 (see page 41) channel respectively (Williams et al., 1992b), however the splice variant cloned from human pancreatic β-cells is less homologous, being 68%, 64% and 41% identical to the rabbit
cardiac $\text{Ca}_v1.2$, skeletal muscle $\text{Ca}_v1.1$ and neuronal $\text{Ca}_v2.1$ channel respectively (Seino et al., 1992). The subunit possesses sites for potential post-translational modification including multiple intracellular PKA and PKC sites, which may be involved in enhancement of $\beta$-cell L-type VDCC currents (Henquin and Meissner, 1984), and 3 extracellular N-linked glycosylation sites (Seino et al., 1992; Williams et al., 1992b).

The current gated by cloned $\text{Ca}_v1.3$ was originally classified as L-type, however functional studies of recombinant $\text{Ca}_v1.3$ were limited and the cell types in which the subunit was expressed, the splice variant expressed and the concentration of external cations in the recording solutions varied greatly between laboratories (Bell et al., 2001; Ihara et al., 1995; Williams et al., 1992b; Xu and Lipscombe, 2001). Expression of the $\text{Ca}_v1.3$ subunit alone in *Xenopus* oocytes is insufficient to generate a detectable $\text{Ba}^{2+}$ current (in 40mM $\text{Ba}^{2+}$). However, co-expression of a $\beta$ subunit or $\beta$ and $\alpha2\delta$ subunit results in sustained $\text{Ba}^{2+}$ currents (162 nA), which showed little inactivation during test pulses ranging from 140-700ms. In 40mM $\text{Ba}^{2+}$, the threshold for $\text{Ca}_v1.3$ current activation occurred at approximately $-30$ mV, and peaked at approximately 0mV (Williams et al., 1992b), a current-voltage relation that was shifted approximately 20mV more hyperpolarized compared to cardiac/lung $\text{Ca}_v1.2$ expressed in oocytes (Biel et al., 1990; Mikami et al., 1989). The current was also sensitive to DHP modulation, was blocked completely by 50$\mu$M $\text{Cd}^{2+}$ but poorly inhibited by 100$\mu$M $\text{Ni}^{2+}$, leading to its classification as an L-type channel. Later investigation of the $\text{Ca}_v1.3$ subunit stably expressed in a mammalian HEK-293 cell-line recorded classical HVA L-type currents that peaked at +20mV (20mM $\text{Ba}^{2+}$), showed little inactivation in external $\text{Ba}^{2+}$ recording solution, but inactivated rapidly when $\text{Ca}^{2+}$ was the charge carrier and were sensitive to DHP modulation (Bell et al., 2001). Another, interesting feature of this study was that in contrast to *in vivo* neuroendocrine L-type currents, $\text{Ca}_v1.3$ currents were not modulated by a variety of GTP-binding (G-) protein pathways, (Degtiar et al., 1997; Gilon et al., 1997; Haws et al., 1993; Hernandez-Guijo et al., 1999). The role of G-protein modulation of VDCCs is expanded in more detail from page 66.

Recently, it has been proposed that neuronal $\text{Ca}_v1.3$ subunits gate currents that do not conform to the classical biophysical and pharmacological criteria for an L-type channel
Transient expression of this neuronal Cav1.3 isoform in *Xenopus* oocytes and mammalian tsa-201 cells produced currents in 5mM Ba^{2+} that like classical L-type channels, did not inactivate rapidly during prolonged depolarisation, but unlike L-type channels activated at unexpectedly hyperpolarized potentials (an effect repeated in 5mM Ca^{2+}), but not in the range of T-type channels. Voltage dependent modulation by DHPs remained a characteristic of the Cav1.3; however, sensitivity to inhibition by the DHP antagonists was greatly reduced compared to Cav1.2. The difference in the voltage dependence of activation was due to an intrinsic difference between the Cav1.3 and Cav1.1 or Cav1.2 genes because it was independent of the auxiliary subunits with which Cav1.3 associates, the splice variant of Cav1.3 expressed and the expression system used to study channel properties. It would appear that the higher extracellular divalent cation concentrations used in previous studies masked the low threshold of activation of Cav1.3 (Bell *et al.*, 2001; Ihara *et al.*, 1995; Williams *et al.*, 1992b). Cav1.3 may therefore be the molecular correlate of endogenous low-threshold L-type currents recorded from pyramidal neurones (Avery and Johnston, 1996) or Purkinje cells of the cerebellum (Liljelund *et al.*, 2000).

### 1.2.2.1.1.4 Cav1.4 (α_{1F})

Loss-of-function mutations in a human gene consisting of 48 exons encoding a 1966 amino acid protein showing high homology to L-type calcium channel α_{1} subunits were shown to underlie the recessive disorder X-linked congenital stationary night blindness (CSNB2). Located on human chromosome Xp11.23, Cav1.4 (α_{1F} – gene CACNA1F) is exclusively expressed in the retina, and truncations, nonsense and frameshift mutations all result in the disease phenotype (Bech-Hansen *et al.*, 1998; Strom *et al.*, 1998). Immunolocalisation studies detected Cav1.4 in rod photoreceptor active zones in the outer plexiform layer and bipolar cell active zones in the inner plexiform layer (Morgans, 2001; Morgans *et al.*, 2001), consistent with a synaptic localisation, and indicating that that Ca^{2+} influx via Cav1.4 is possibly involved in glutamate release form rod photo-receptors. It has been previously reported that bipolar cells express at least two types of VDCC, a LVA T-type current and a sustained current with properties similar to L-type (de la Villa *et al.*, 1998). This latter current is probably mediated by Cav1.4 and recordings from the bi-polar cell in 10mM Ba^{2+} show the Cav1.4 current.
activates -45mV, peaks at about -25mV and is suppressed ~50% by 10μM nifedipine (Morgans et al., 2001). These properties are similar to those recently reported for the Cav1.3 L-type channel, with the threshold of activation for Cav1.4 being more hyperpolarised than the classical HVA channels and the channel having a poor sensitivity to DHP block (Xu and Lipscombe, 2001).

1.2.2.1.2 DHP-insensitive neuronal α, subunit genes (Cav2.x)

1.2.2.1.2.1 Cav2.1 (α1A)

In vivo expression of Cav2.1 underlies the native P- and Q-type currents, which are commonly grouped together as the P/Q-type current due to their common genetic identity and sensitivity to ω-AgaIVA block (Mintz et al., 1992b; Pinto et al., 1998a; Randall and Tsien, 1995). Originally cloned from rat and rabbit brain (Mori et al., 1991; Starr et al., 1991), the Cav2.1 subunit possesses approximately 40% homology with the Cav1 L-type channels, is widely distributed throughout the nervous system with particularly high concentrations in the cerebellum (Craig et al., 1998; Day et al., 1996; Stea et al., 1994; Westenbroek et al., 1995), and has a principal role in the orchestration of fast neurotransmitter release at both excitatory and inhibitory synapses (Meir et al., 1999; Takahashi and Momiyama, 1993). Characterisation of the ω-AgaIVA sensitive currents resulting from expression of Cav2.1 in different expression systems allowed the distinct biophysical and pharmacological properties of P- and Q-type current to be elucidated. The Cav2.1 currents recorded when the protein was expressed in Xenopus oocytes were resistant to DHPs and ω-ctx GVIA, but were blocked by ω-AgaIVA with an IC50 ~ 100nM, and displayed profound voltage dependent inactivation (Sather et al., 1993). This is in contrast to the sustained, non-inactivating highly ω-AgaIVA sensitive (IC50 ~1nM) P-type current recorded from cerebellar Purkinje neurones (Mintz et al., 1992b; Regan, 1991; Usowicz et al., 1992). Recombinant Cav2.1 currents expressed in oocytes bore a closer resemblance to the Q-type current of cerebellar granule cells (Randall and Tsien, 1995; Randall et al., 1993). Expression of Cav2.1 in the mammalian COS-7 cells resulted in a current that inactivated more rapidly than native P-type current but was 9-fold more sensitive to ω-AgaIVA blockade than native Q-type currents, although 2-fold less sensitive than native
Chapter 1

Introduction

P-type currents (Berrow et al., 1997). These intermediate properties witnessed by many groups in heterologous expression systems lead to confusion about the source of the current diversity produced by Cav2.1 (Berrow et al., 1997; De Waard and Campbell, 1995; Moreno et al., 1997; Mori et al., 1991; Sather et al., 1993; Stea et al., 1994). Was this due to the different expression systems used in each study, alternative splicing of the α1 subunit, differential association with auxiliary subunits, or a combination of some or all of these factors?

Although differential association of auxiliary subunits can to some extent explain the broad range of currents described as P/Q-type (De Waard and Campbell, 1995; Moreno et al., 1997), it has been demonstrated that the principal factor controlling identity of current gated by Cav2.1 is alternative splicing. Insertion of valine at position 421 (V421) of II-III linker (Bourinet et al., 1999) and an asparagine-proline (NP) insertion (Bourinet et al., 1999; Hans et al., 1999) in the IVS3-IVS4 extracellular linker of the Cav2.1α variant significantly alter the biophysical and pharmacological properties of the channel. The insertion of V421 promotes the expression of non-inactivating responses, which when co-expressed with the β2a subunit, an isoform found abundantly in cerebellar Purkinje cells (Volsen et al., 1997), is extremely similar to the P-type currents native to these neurones (Llinas et al., 1992). The insertion of NP in the IVS3-IVS4 linker lowers ω-AgaIVA affinity, suggesting it is present in channels that gate the less ω-AgaIVA sensitive Q-type current. It is therefore possible to generate a broad range of phenotypes of the Cav2.1 subunit by alternative splicing (Bourinet et al., 1999), which can be further modulated by the differential association of β subunits in different tissues (Berrow et al., 1997; Moreno et al., 1997).

It is now beyond all reasonable doubt that Cav2.1 is the molecular correlate of both P- and Q-type currents. Auto-antibodies generated by Lambert-Eaton myasthenic syndrome (LEMS) patients caused a specific 75% reduction of current gated by heterologous Cav2.1 subunit stably expressed in HEK-293 cells (Pinto et al., 1998b) and when applied to cultured cerebellar Purkinje neurones or granule cells, significantly and specifically reduced the low- and high- sensitivity ω-AgaIVA components of whole cell current (Pinto et al., 1998a). Furthermore, the generation of a Cav2.1 knockout mouse resulted in a progressively ataxic animal devoid of P-type current in cerebellar...
Chapter 1

Introduction

Purkinje neurones and P- and Q-type current in cerebellar granule cells (Jun et al., 1999). Not only does this study prove the molecular identity of P- and Q-type currents, but it also highlights the importance of the Cav2.1 subunit in the control of motor function in the synapses of the cerebellum (although synaptic transmission was not lost due to concomitant increases in expression of N- and R-type currents).

1.2.2.1.2.2 Cav2.2 (α1B)

Unlike the P- and Q-type currents, little confusion surrounded the identity of the α1 subunit gating N-type current. ω-ctx GVIA is a selective and potent N-type channel antagonist. A photo-affinity derivative of ω-ctx GVIA (N-hydroxysuccinimidyl-4-azido-benzoate-125I-ω-conotoxinGVIA) was utilised to purify N-type channels from rat and rabbit brain (McEnery et al., 1991; Witcher et al., 1993). The N-type channel was shown to have a similar molecular composition as the L-type channels of skeletal, cardiac and neuronal tissue (Ahlijanian et al., 1990; Mikami et al., 1989; Takahashi and Catterall, 1987). The 230 kDa α1 polypeptide contained the ω-ctx GVIA binding site, and was associated with auxiliary β and α2δ subunits, but like the neuronal L- and P/Q-type channels, no γ subunit (Ahlijanian et al., 1990; Mori et al., 1991). The cDNAs encoding rat (Dubel et al., 1992), rabbit (Fujita et al., 1993) and two splice variants of the human brain Cav2.2 gene (Williams et al., 1992a) were cloned and the human Cav2.2 gene was localised to chromosome 9q34 (Diriong et al., 1995; Kim et al., 1997). The predicted Cav2.2 amino acid sequence displayed 65% identity with Cav2.1, but 41%, 40%, and 40% identity with the Cav1.1, Cav1.2 and Cav1.3 subunits respectively (Fujita et al., 1993), indicating a closer evolutionary link to the neuronal Cav2.1 subunit than the L-type Cav1 family. Investigations into the differential tissue expression of Cav2.2 revealed high, and almost exclusive expression in brain (Day et al., 1996; Dubel et al., 1992; Fujita et al., 1993) but can also be found in peripheral nervous tissue (Day et al., 1997; Lin et al., 1997; Lin et al., 1999). An abundance of evidence that N-type channels were a major component of the neurotransmitter release pathways had already been assembled (Dunlap et al., 1994; Meir et al., 1999), utilising the pharmacological selectivity of ω-ctx GVIA to localise N-type channels to neuromuscular and hippocampal synaptic membranes (Jones et al., 1989; Robitaille et al., 1996) where post-synaptic responses are reduced following block of presynaptic N-type channels by
Chapter 1  
Introduction

ω-ctx GVIA (Ohno-Shosaku et al., 1994). Cloning of the Cav2.2 facilitated the
generation of specific antibodies against the α₁ subunit, which were employed in the
analysis of the tissue and sub-cellular distribution of Cav2.2 (Day et al., 1996; Day et
al., 1997; Volsen et al., 1995). Staining in hippocampal neurones revealed a low
density of Cav2.2 in cell somata but stronger staining of neuropil, especially in certain
regions of high synaptic density such as the polymorphic layer of the dentate gyrus and
the stratum lucidum and radiatum of the CA regions (Day et al., 1996) reinforcing the
opinion that N-type/Cav2.2 channels play a principal role in neuronal transmission.
However in the periphery, whilst it would appear that although not the principal VDCC
isoform involved in neurotransmission at the neuromuscular junction, Cav2.2 may play
an important role modulating Ca²⁺ dependent processes in the axon-associated Schwann
cells at this synapse (Day et al., 1997) although to date, only only T- and L-type
currents have been detected in mammalian Schwann cells (Amedee et al., 1991).

Transient and stable expression of the Cav2.2 subunit in mammalian cells (Bleakman et
al., 1995; Fujita et al., 1993; Williams et al., 1992a) together with auxiliary β and α2δ
subunits generated HVA, DHP-insensitive, ω-ctx GVIA-sensitive currents with
appreciable biophysical similarity to the N-current originally described in the chick
dorsal root ganglion (Fox et al., 1987b; Nowycky et al., 1985). Furthermore, the
observation that recombinant N-type currents could inactivate in a biphasic manner
(Williams et al., 1992a) highlighted the existence of mixed populations of N-type
channels, which was consistent with the earlier observations that N-type channels can
switch between transient and long-lasting modes of gating (Plummer and Hess, 1991a).

N-type currents are modulated by both phosphorylation and activated G-proteins, two
processes that are interlinked in vivo. Recordings of N-type currents from neurons in
culture show that they are sensitive to modulation by intracellular protein kinases, but
this is normally manifest as relief of G-protein mediated inhibition (Dolphin, 1991;
Dolphin et al., 1989; Swartz, 1993; Swartz et al., 1993). Purified Cav2.2 α₁ subunits
are readily phosphorylated (Ahlijanian et al., 1991; Hell et al., 1994) and transiently
expressed recombinant Cav2.2 channels exhibit a 30-40% increase in peak currents
upon activation of PKC (Stea et al., 1995). Nevertheless, a significant, but always
partial inhibition of heterologous Cav2.2 current activation occurs upon activation of
tonic or transiently expressed G-protein (Dolphin, 1998; Meir et al., 2000). The cross
talk between phosphorylation and G-protein inhibition of VDCCs seen in neuronal
culture has been reconstituted in vitro (Hamid et al., 1999; Zamponi et al., 1997) and
the primary site of action of both modulatory molecules were identified as the \( \text{Ca}_\text{v}2.2 \)
\( \alpha_i \) subunit. The mechanisms and interactions underlying this process and will be
discussed in more detail in section 1.2.2.2.5.

As witnessed in the characterisation of recombinant \( \text{Ca}_\text{v}2.1 \) channels, splice variations
(Ghasemzadeh et al., 1999; Lin et al., 1997; Lin et al., 1999; Stea et al., 1999),
differential subunit interactions (Scott et al., 1996; Stea et al., 1993) and the combined
effects of splice variations on the association of auxiliary subunits (Pan and Lipscombe,
2000), cause the current heterogeneity that probably represents a mechanism for
controlling the plasticity of excitation-secretion coupling at different synapses.
Interestingly, the insertion of a short exon encoding glutamate-threonine (ET) motif into
the IVS3-S4 linker of \( \text{Ca}_\text{v}2.2 \) slows its activation kinetics (Lin et al., 1999) and was
shown to correspond to the insertion of NP into the same region of \( \text{Ca}_\text{v}2.1 \) (Bourinet et
al., 1999; Hans et al., 1999). Site-directed mutagenesis showed that insertion of the NP
cassette of \( \text{Ca}_\text{v}2.1 \) was able to functionally substitute for ET splice insertion in \( \text{Ca}_\text{v}2.1 \)
(Lin et al., 1999). This presence of the NP cassette in \( \text{Ca}_\text{v}2.1 \) lowers sensitivity to \( \omega-\text{AgaIVA} \)
(Bourinet et al., 1999). It will be interesting to discover if the insertion of the
ET motif into \( \text{Ca}_\text{v}2.2 \) similarly lowers the sensitivity of the channel to \( \omega-\text{ctx GVIA} \).

1.2.2.2.3 \( \text{Ca}_\text{v}2.3 (\alpha_{1E}) \)

The third DHP-insensitive VDCC \( \alpha_i \) subunit clone from brain is \( \text{Ca}_\text{v}2.3 \) (Niidome et
al., 1992; Schneider et al., 1994; Soong et al., 1993; Williams et al., 1994). It
possesses the archetypal predicted \( \alpha_i \) subunit structure, shares approximately 40%
identity with the \( \text{Ca}_\text{v}1 \) family of channels, but is phylogenetically most closely related
to the \( \text{Ca}_\text{v}2.1 \) and \( \text{Ca}_\text{v}2.2 \) subunits with which it shares approximately 58% identity
(Fujita et al., 1993). It also shares close homology to the doe-1 \( \alpha_i \) subunit cloned from
the ray Discopyge ommata (Ellinor et al., 1993; Horne et al., 1993). In common with all
the VDCC \( \alpha_i \) subunits, it possesses regions for post-translational modification by N-
linked glycosylation and protein kinases PKA and PKC and like the \( \text{Ca}_\text{v}2.1 \) and \( \text{Ca}_\text{v}2.2 \)
Early recordings of the rat brain isoform transiently expressed in *Xenopus* oocytes, demonstrated biophysical properties very similar to neuronal LVA channels (Fox et al., 1987b; Nowycky et al., 1985). Recorded currents were of a transient nature, activating and peaking at much more negative membrane potentials than for the previously characterised HVA VDCCs (Soong et al., 1993).
subunits possesses the motif in the I-II linker postulated to be the site of interaction with activated G-proteins (Dolphin, 1998). Investigation of the G-protein dependent inhibition of Cav2.3 containing channels highlighted that the originally reported rat brain sequence was incomplete (Soong et al., 1993). The currents gated by this clone were not modulated by activated G-proteins (Page et al., 1997; Toth et al., 1996), but subsequent identification of an extra fifty amino acids in the N-terminus of the complete rat brain Cav2.3 cDNA, revealed their additional presence was a critical determinant for G-protein modulation of Cav2.3 (Page et al., 1998). In situ hybridisation (Soong et al., 1993) and immunohistochemistry (Day et al., 1996; Volsen et al., 1995) revealed that Cav2.3 was expressed widely throughout the CNS, and is also detected at neuromuscular junctions (Day et al., 1997).

The functional characteristics of the Cav2.3 subunit are a unique with properties common to both high and low voltage activated channels (Schneider et al., 1994; Soong et al., 1993; Williams et al., 1994). (Early recordings of the rat brain isoform transiently expressed in Xenopus oocytes, demonstrated biophysical properties very similar neuronal LVA channels (Fox et al., 1987b; Nowycky et al., 1985); transient currents, activating and peaking at much more negative membrane potentials than for the previously characterised HVA VDCCs (Soong et al., 1993) However, three genes were recently identified that encode classical LVA T-type channels (Perez-Reyes, 1998), and the consensus of opinion arising from several studies characterising Cav2.3 current, suggested it was more similar to HVA R-type current found in cerebellar granule neurones (Jouvenceau et al., 2000; Piedras-Renteria and Tsien, 1998; Schneider et al., 1994; Stephens et al., 1997; Tottene et al., 1996; Wakamori et al., 1994; Williams et al., 1994; Zhang et al., 1993). Heterologous expression of Cav2.3 generates currents with a single channel conductance of 12-14pS (Schneider et al., 1994; Wakamori et al., 1994) sensitive to block by both Ni^{2+} and by Cd^{2+} (Jouvenceau et al., 2000; Soong et al., 1993; Zamponi et al., 1996) but insensitive to DHP antagonists, or ω-conotoxin GVIA and ω-AgaIvA (Schneider et al., 1994; Zhang et al., 1993). For a long period of time, the lack of a selective pharmacological agent for Cav2.3 prohibited the identification of the current types gated by this channel in vivo. However, various alternative approaches have been employed in more recent studies to determine the nature of VDCC currents gated by Cav2.3 in neurones. Injection of Cav2.3 antisense oligonucleotides into
cerebellar granule cells reduced native R-type currents (Piedras-Renteria and Tsien, 1998). Additionally, a recently discovered pharmacologically specific peptide toxin, SNX-482, which was isolated from the venom of the tarantula *Hysterocrates gigas*, selectively blocks Cav2.3 channels stably expressed in mammalian cell lines with an IC$_{50}$ of 15-30nM. When applied to acutely dissociated rat neurones, although a potent inhibitor of R-type current in neurohypophyseal nerve endings, it was ineffective at inhibiting R-type currents of cell body preparations of cerebellar granule cells, retinal ganglion cells or hippocampal pyramidal cells (Newcomb *et al.*, 1998). This suggests that the somal R-type currents of central neurones are unlikely to be generated by channels containing a Cav2.3 subunit, whilst it was likely to be present in synaptic R-type channels. Reports from another laboratory suggest that R-type currents from cerebellar granule cells have multiple SNX-482 sensitive and resistant components, although Cav2.3 antisense treatment was effective in abolishing both currents (Tottene *et al.*, 2000). Finally, in an attempt to clarify these ambiguities, generation of a Cav2.3 knockout mouse has demonstrated the R-type currents are indeed heterogeneous and that Cav2.3 only encodes a portion of R-type current which varies in a tissue dependent manner (Wilson *et al.*, 2000). Moreover, an unexpected finding was that the reduction in the magnitude of R-type current recorded from cerebellar granule neurones in the Cav2.3 knockout mice was much smaller than the reduction in R-type current recorded from the granule neurones from the Cav2.1 knockout (Jun *et al.*, 1999), highlighting an as yet undetermined link between Cav2.1 and non-Cav2.3 R-type channels, or that native Cav2.3 currents are sensitive to block by DHPs and $\omega$-AgalIVA as had been previously demonstrated *in vitro* (Stephens *et al.*, 1997). This latter observation casts doubt on the accuracy of the original classification of R-type current (Randall and Tsien, 1995; Randall *et al.*, 1993), because the DHPs and $\omega$-AgalIVA appear to be less specific than originally reported.

1.2.2.1.3 **Low voltage activated T-type $\alpha_1$ subunits (Cav3.1-Cav3.3)**

One of the salient discoveries in the field of VDCC research was the elucidation of the molecular structure and properties of a family of proteins that gate the LVA T-type current. Rhythmic firing in cardiac pacemaker cells and neuronal tissues is reliant on T-
type activity and T-type malfunction can contribute to epileptic seizures (Huguenard, 1996). In much the same way that the characterisation of the HVA channels lead to greater understanding of the processes involved in neurotransmission and Ca\(^{2+}\) activated second messenger pathways, it is hoped that the cloning and characterisation of the molecules that control T-type current will help deconstruct native T-type currents and develop therapeutic strategies in the treatment of epilepsies and arrhythmias (Huguenard, 1996; Shorofsky and Balke, 2001).

Perez-Reyes and colleagues identified the first member of a T-type VDCC family by probing the Genbank database using a text-based search of the terms “calcium” and “channel” to search for sequences with homology to the conserved regions of VDCC \(\alpha_1\) subunits, which were then compared by the basic sequence alignment tool (BLAST) (Altschul et al., 1990; Perez-Reyes et al., 1998). They concentrated in particular on the expressed sequence tag (EST) division, a collection of partial cDNA fragments cloned from normalised cDNA libraries (Soares et al., 1994). This approach recognised a candidate EST that contained sequence that encoded a putative voltage-dependent ion channel, shared low sequence identity with the carp Cav1.1 subunit (P23316) and another putative channel in C. elegans genomic sequence (C.e. C54). The completely sequenced EST (AF029228) was then used to generate a probe to screen rat brain and human heart cDNA libraries. Overlapping cDNAs from the rat brain library were assembled to generate a complete Cav3.1 (\(\alpha_{1C}\)) sequence (Perez-Reyes et al., 1998). Lower stringency screening of the human cardiac library identified a separate but related sequence, Cav3.2 (\(\alpha_{1H}\)) (Cribbs et al., 1998). The newly identified Cav3.1 sequence was used to re-probe a rat brain cDNA library to search for other related sequences. This identified a third putative Ca\(^{2+}\) channel gene, Cav3.3 (\(\alpha_{1I}\)) and the rat brain isoform of Cav3.2 (Lee et al., 1999d). Subsequent articles detailed the identification and cloning of multiple human and rat brain Cav3 splice variants (Cribbs et al., 2000; Cribbs et al., 2001; McRory et al., 2001; Mittman et al., 1999a; Mittman et al., 1999b; Satin and Cribbs, 2000). Structural predictions for the Cav3 family indicated they conformed to the conserved cation channel four transmembrane domain model, each domain containing an S4 voltage sensing segment, P-loop, and conserved residues that form the cation selectivity filter (Lee et al., 1999d; McRory et al., 2001; Mittman et al., 1999a; Mittman et al., 1999b; Perez-Reyes, 1998). Although highly
similar in the transmembrane regions, they share little similarity in the intracellular loops linking each domain and also lack the domains present in the HVA channels involved in binding β subunits and Ca^{2+} (Pragnell et al., 1994; Walker and De Waard, 1998). PCR, in situ hybridisation and immunohistochemical analysis suggested Cav3.1, Cav3.2 and Cav3.3 are ubiquitously expressed in the CNS (Craig et al., 1999; Kase et al., 1999; Talley et al., 1999) but a recent investigation has localised Cav3.3 expression more selectively to the striatum (McRory et al., 2001). Cav3.1 and Cav3.2 have also been localised to non-neuronal tissues. Transcripts of Cav3.1 have been detected in rat insulin secreting cells, kidney, and neonatal heart (Zhuang et al., 2000). Cav3.2 has been detected in heart, kidney, liver, pancreas and skeletal muscle (Cribbs et al., 1998).

Functional expression of Cav3 channels in various expression systems in the absence of any auxiliary subunits produced currents that activated slowly at potentials near threshold, but stronger depolarisations increased both rate of activation and inactivation (Cribbs et al., 1998; Lee et al., 1999b; McRory et al., 2001; Monteil et al., 2000a; Monteil et al., 2000b; Perez-Reyes et al., 1998; Williams et al., 1999). This was reminiscent of the signature pattern of native T-type currents (Randall and Tsien, 1997). The recombinant Cav3 currents also displayed slow, voltage-dependent deactivation and the single channel conductance was equivalent to that of native T-type currents (~8 pS). The scorpion toxin kurtoxin specifically inhibits Cav3.1 and Cav3.2 currents by modifying channel gating, shifting the threshold for activation to more positive potentials (Chuang et al., 1998). These channels are also modulated by divalent cations. Cd^{2+} blocks the pores of all three isoforms with similar potency (Lacinová et al., 2000) whilst Ni^{2+} modulated the gating properties of the channels, inhibiting them with a decreasing order of potency Cav3.2 >> Cav3.3 > Cav3.1. (Lee et al., 1999c). The concentrations required to block Cav3.1 and Cav3.3 are sufficient to also affect HVA channels. The biophysical properties of heterologous Cav3.1 channels are apparently not modulated by co-expression of the auxiliary β or α2δ subunits (Dolphin et al., 1999b; Lacinová et al., 1999); however, interactions may occur to facilitate trafficking or stabilisation of functional Cav3.1 at the plasma membrane (Dolphin et al., 1999b).
Chapter 1

Introduction

The biophysical and pharmacological properties clearly defined the Cav3 family of proteins and their splice variants as major determinants of the diversity of T-type currents recorded in vivo.
### Chapter 1: Introduction

#### Table 1.2: The cloned VDCC α1 subunits and most widely studied alternate splice forms, presented together with their primary tissue distribution, biophysical properties and pharmacological antagonists.

This table was compiled using data collected from references cited in the main body of text for the α1 subunit section.

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Gene name</th>
<th>Human chromosomal location</th>
<th>Splice variant</th>
<th>Primary tissues</th>
<th>Channel type</th>
<th>Activation Threshold</th>
<th>Pharmacology</th>
</tr>
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<tbody>
<tr>
<td>CaV1.1</td>
<td>CACNA1S</td>
<td>1q31-32</td>
<td>CaV1.1a, CaV1.1b</td>
<td>Sk. Muscle</td>
<td>High</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>CaV1.2a</td>
<td>Heart</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CaV1.2b</td>
<td>Smooth muscle</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CaV1.2c</td>
<td>Brain, heart, pituitary, adrenal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CaV1.2</td>
<td>CACNA1C</td>
<td>12p13.3</td>
<td>CaV1.3a</td>
<td>Brain, pancreas, kidney, ovary, cochlea</td>
<td>Intermediate</td>
<td>DHPs, PAAs, BTZs</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CaV1.3b</td>
<td>Brain</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>CaV1.3c</td>
<td>Brain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CaV1.3d</td>
<td>Brain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CaV1.3</td>
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<td>3p14.3</td>
<td>CaV1.4a</td>
<td>Retina</td>
<td>Intermediate</td>
<td></td>
<td></td>
</tr>
<tr>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>CaV1.4</td>
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<td>Xp11.23</td>
<td>CaV1.5a</td>
<td>Brain, cochlea, pituitary</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>CaV1.5b</td>
<td>Brain, cochlea, reti, heart, pituitary</td>
<td>Intermediate</td>
<td></td>
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<tr>
<td>CaV1.5</td>
<td>CACNA1G</td>
<td>17q22</td>
<td>CaV1.6a</td>
<td>Brain, nervous system</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td>CaV1.6b</td>
<td>Foetal cardiac myocytes</td>
<td></td>
<td></td>
<td>Kurtoxin (&lt;350nM)</td>
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<td>16p13.3</td>
<td>CaV1.7a</td>
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<td></td>
<td></td>
<td>Low</td>
</tr>
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<td>CaV1.8a</td>
<td>Brain</td>
<td>T</td>
<td></td>
<td>Low</td>
</tr>
</tbody>
</table>

Table 1.2 - The cloned VDCC α1 subunits and most widely studied alternate splice forms, presented together with their primary tissue distribution, biophysical properties and pharmacological antagonists.
1.2.2.2 Structural and functional properties integral for VDCC function.

1.2.2.2.1 The channel pore and selectivity filter

Under normal physiological ionic conditions the VDCCs are able to be > 1000 fold more selective for Ca^{2+} over Na^{+} and K^{+} despite being in the presence of much higher concentrations of the monovalent cations (Tsien et al., 1987). The molecular structure of their pore allows them perform this intricate feat.

It has been widely accepted that the structural determinants that define the permeation characteristics of voltage-dependent cation channels are localized within the four “P-loop” motifs (also termed the SS1-SS2 region) of all the voltage dependent ion channels. This region dips into and back out of the plasma membrane (Yellen et al., 1991) as it connects the transmembrane spanning segments S5 and S6 of each of the four homologous repeat domains of the α1 subunit (Albright et al., 2000). The majority of research into the molecular architecture of the voltage dependent cation channel pore has been performed on K^{+} channel subunits because their simple tetrameric arrangement in the plasma membrane lends itself more readily to mutational and biophysical analysis (Armstrong and Hille, 1998; Hille, 1992). Furthermore, understanding if the molecular structure of voltage dependent cation channel pores has recently been revolutionised by the reporting of the crystal structure of the inward rectifier like KcsA K^{+} channel from the bacterium Streptomyces lividans (Doyle et al., 1998). The pore of the channel is approximately 45 Å in length and if travelling from the external to the internal mouths, its shape resembles a distorted hourglass figure (Figure 1.5). The outer part of the P-loop of the four identical K^{+} channel subunits form the funnel shaped outer vestibule that at its narrowest point contains the “selectivity filter” (Doyle et al., 1998; Lu and Miller, 1995; MacKinnon et al., 1990; MacKinnon and Miller, 1989). A highly conserved “signature sequence region”, lies at the heart of the selectivity filter. The conserved sequence Thr-X-X-Thr-X-Gly-Tyr-Gly found in at least 50 cloned K^{+}-selective channels contains three threonines, whose -OH groups have the potential to complex and select K^{+} ions. However, only the hydroxyl group of the first of these threonines is necessary for a selective channel (Heginbotham et al., 1994), and it is the carbonyl backbone rather than the amino acid side-chains of the Gly-Tyr-Gly motif.
arranged in a hairpin loop which serves to complex a $K^+$ ion (Doyle et al., 1998). This
structure discriminates between the potentially permeant ions on the basis of the energy
of stripping a hydrated ion down to its crystal radius (Hille, 1973). This high affinity
site is flanked by two low affinity $K^+$ interaction sites, one each in the wider outer and
inner vestibules, which allows high flux of ions via sequential energy steps rather than
by mutual repulsion the cations in a narrow pore (Kiss et al., 1998). The S5 and S6
segments (or the helices which are connected by the P-loop) shape the latter region of
the pore inner vestibule and the channel to the cytoplasm, which is lined by
hydrophobic amino acids that present a relatively inert surface to the cations diffusing
through the rest of the pore (Choi et al., 1993; Doyle et al., 1998; Holmgren et al.,
1997; Kirsch et al., 1993; Lopez et al., 1994).

This model for channel structure is thought to be applicable to most voltage dependent
cation channels. The structure of the VDCC pore is predicted to permit permeation by
similar mechanisms but utilizes different methods of ion selection. $Ca^{2+}$ channels are
highly selective for $Ca^{2+}$ in its presence, but become non-selectively permeable to
monovalent cations in its absence (McCleskey and Almers, 1985). The flow of
monovalents can be blocked by the application of µM concentrations of $Ca^{2+}$ and $Ca^{2+}$-
flux restored at mM $Ca^{2+}$ concentrations (Almers and McCleskey, 1984; Hess and
Tsien, 1984). This implies that VDCC do not exclude monovalent cations using a
"selectivity filter" but utilize a competitive, mutually exclusive binding mechanism.
Akin to the $K^+$ channels, the primary loci of ion selectivity in VDCCs appear to reside
within the P-loop motifs of each of the four transmembrane domains of the $Ca^{2+}$ channel
$\alpha_1$ subunit (Tang et al., 1993b; Williamson and Sather, 1999; Yang et al., 1993a). Each
of the P-loops contains a conspicuous glutamate residue that is conserved among all
types of HVA VDCCs, creating a structure at the narrowest point of the pore designated the EEEE locus (Heinemann et al., 1992; Kim et al., 1993; Tang et al., 1993a). Single and double mutations in this locus severely impair Ca\textsuperscript{2+} permeation (Cibulsky and Sather, 2000; Ellinor et al., 1995; Tang et al., 1993b; Yang et al., 1993b), and it is eliminated by quadruple alanine (AAAA), glutamine (QQQQ), or aspartate (DDDD) substitutions (Ellinor et al., 1995). Furthermore, insertion of these glutamates at corresponding positions into a voltage-dependent Na\textsuperscript{+} channel confers Ca\textsuperscript{2+} selectivity (Heinemann et al., 1992). The EEEE locus therefore forms a single high affinity Ca\textsuperscript{2+} binding locus in the pore of HVA VDCCs (Cibulsky and Sather, 2000), but unlike the K\textsuperscript{+} channels it thought that it is the carboxylate bearing side chains of the glutamate residues in an asymmetrical arrangement, and not the carbonyl backbones which project into the lumen to form the ion binding locus (Varadi et al., 1999; Wu et al., 2000).

The permeation and selectivity properties of the LVA VDCCs differ somewhat from the HVA channels. The Ba\textsuperscript{2+} conductance of most HVA channels is approximately twice as large as the Ca\textsuperscript{2+} conductance (Mangoni et al., 1997; McDonald et al., 1994; Wakamori et al., 1998), whereas Ca\textsuperscript{2+} conductance in LVA channels is the same or larger than that of Ba\textsuperscript{2+} (Carbone and Lux, 1987; Fox et al., 1987a; Huguenard, 1996; Nilius et al., 1985). A putative EF binding motif (da Silva and Reinach, 1991) has been discovered in the pore region of domain III of the Cav2.2 \(\alpha_1\) subunit, which is conserved in all HVA \(\alpha_1\) subunits. This motif has been hypothesised to underlie the difference in Ca\textsuperscript{2+} and Ba\textsuperscript{2+} permeability in these channels (Feng et al., 2001b). Furthermore, under certain conditions in solutions where the [Ca\textsuperscript{2+}] + [Ba\textsuperscript{2+}] is held constant, the total HVA VDCC current can be smaller in the mixture than when measured in a solution of Ca\textsuperscript{2+} or Ba\textsuperscript{2+} alone. Thus the Ba\textsuperscript{2+} conductance of HVA channels is susceptible to block by Ca\textsuperscript{2+} ions due to a higher binding affinity for Ca\textsuperscript{2+}. This phenomenon is termed the anomalous mole fraction effect (AMFE) but does not occur in LVA channels (Almers and McCleskey, 1984; Byerly et al., 1985; Campbell et al., 1988; Friel and Tsien, 1989; Hess and Tsien, 1984; Yue and Marban, 1990). HVA channels are also more sensitive to block by Cd\textsuperscript{2+} than LVA channels (Huguenard, 1996; McDonald et al., 1994).

The residues of the high affinity locus from domains III and IV of the LVA VDCC \(\alpha_1\) subunits are aspartate rather than glutamate, resulting in the motif EEDD (Cribbs et al.,
Permeation properties of T-type VDCCs critically depend upon the presence of the aspartate residues in this locus although their replacement by glutamates in a Cav3.1 subunit does not completely confer HVA permeation properties upon the channel (Talavera et al., 2001). In particular, the transfer of a single amino acid D1487E (Domain III) in the Cav3.1 subunit is sufficient to induce Ba$^{2+}$ over Ca$^{2+}$ permeability, AMFE, and high Cd$^{2+}$ sensitivity to the T-type channel, and cause a positive shift in the voltage dependence of channel activation. However, mutating the EEDD locus of LVA channels to EEEE alone is obviously insufficient to confer all the aspects of HVA permeation onto a T-type channel. Other differences that may account for this are; lysine and asparagine residues lie close to the EEDD locus of Cav3.1, whilst phenylalanines and alanines are found in the equivalent positions in all the HVA channels (Yang et al., 1999), the putative EF hand motif located outside of the EEEE locus of HVA channels is absent from LVA channel $\alpha_1$ subunits (Feng et al., 2001b) and the backbone structures of EEEE and EEDD have different spatial arrangements of their negative charges in the pore (Talavera et al., 2001). Interestingly, the permeation properties of the R-type Cav2.3 channel conform to neither HVA nor LVA parameters but tend to be more like those of LVA VDCCs, exhibiting larger Ca$^{2+}$ or Sr$^{2+}$ whole cell currents compared to Ba$^{2+}$ (Bourinet et al., 1996). The molecular features underlying this discrepancy are still to be determined as Cav2.3 possesses the EEEE loci and the domain III EF hand motif conserved in all HVA $\alpha_1$ subunits, however, it does lack a conserved aspartate at residue 264 in domain I. The loss of the negatively charged side chain at this region may alter the spatial organisation of the adjacent residues and hence affect the pore permeation properties (Wu et al., 2000).
Models predicting how Ca\(^{2+}\) permeates through the VDCCs revolve around the high affinity EEEE/EEDD locus, which is flanked on either side by low affinity sites. As described previously for the K\(^+\) channel, multiple Ca\(^{2+}\) ions are predicted to permeate in single file, and one recent theory suggested the low affinity sites either side of the EEEE/EEDD locus provide steps of potential energy that speed the exit of Ca\(^{2+}\) from the high affinity site (Dang and McCleskey, 1998; Yue and Marban, 1990). The steps could be provided by weak binding in the non-selective vestibules, by specific protein structures in a long pore, or by stepwise re-hydration of a permeating ion (Dang and McCleskey, 1998). However, current opinion maintains earlier theories that repulsive forces between the permeating cations are necessary to drive their movement through the pore (Almers and McCleskey, 1984; Corry et al., 2001; Hess and Tsien, 1984).

<table>
<thead>
<tr>
<th>Motif I</th>
<th>Motif II</th>
<th>Motif III</th>
<th>Motif IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca(_{1.2})</td>
<td>TMGGWT</td>
<td>TGDDWN</td>
<td>TFGGWP</td>
</tr>
<tr>
<td>Ca(_{1.3})</td>
<td>TMGGWT</td>
<td>TGDDWN</td>
<td>TFGGWP</td>
</tr>
<tr>
<td>Ca(_{1.1})</td>
<td>TMGGWT</td>
<td>TGDDWN</td>
<td>TFGGWP</td>
</tr>
<tr>
<td>Ca(_{2.1})</td>
<td>TMGGWT</td>
<td>TGDDWN</td>
<td>TFGGWP</td>
</tr>
<tr>
<td>Ca(_{2.2})</td>
<td>TMGGWT</td>
<td>TGDDWN</td>
<td>TFGGWP</td>
</tr>
<tr>
<td>Ca(_{2.3})</td>
<td>TMGGWT</td>
<td>TGDDWN</td>
<td>TFGGWP</td>
</tr>
<tr>
<td>Ca(_{3.2})</td>
<td>TLAGWV</td>
<td>TGDDWN</td>
<td>SHGWV</td>
</tr>
<tr>
<td>Ca(_{3.1})</td>
<td>TLAGWV</td>
<td>TGDDWN</td>
<td>SHGWV</td>
</tr>
</tbody>
</table>

Figure 1.6 - Sequence of the P-loop SS2 segment in HVA and LVA VDCCs in all four conserved motifs.

Amino acids are marked by the one letter nomenclature; the constituents of the selectivity filter are highlighted in grey. LVA channels contain an EEDD motif rather than EEEE, which probably accounts for the differences in ion selectivity between LVA and HVA channels. Reproduced and modified from Varadi et al. (1999).

Figure 1.7 - Properties that facilitate cation permeation through the pore

Two mechanisms by which the channel stabilises a cation in the middle of the membrane. First, a large aqueous cavity stabilises an ion (green) in the otherwise hydrophobic membrane interior. Second, oriented helices point their partial negative charge (carboxyl end, red) towards the cavity where a cation is located. Reproduced and modified from Doyle et al. (1998).
1.2.2.2 Activation and the voltage sensor

Activation, or the process by which the channel opens, is controlled by important structures within the pore-forming α_1 subunit. Examination of the amino acid sequence of the cloned Cav_1.1 subunit predicted the protein formed a voltage dependent channel (Tanabe et al., 1987). The S4 segment of each conserved transmembrane domain contained positively charged residues every third or fourth amino acid along its α-helix. The presence of such a motif had already been hypothesised to be critical in bestowing voltage sensitive properties upon Na^+ and K^+ channels (Noda et al., 1982; Papazian et al., 1987), therefore this segment was predicted to serve the same purpose in VDCCs. It was predicted that depolarisation caused an outward movement of the S4 segments, which leads to channel opening (Catterall, 1988). This event can be observed by recording the tiny “gating-currents” that result from the movement of the positively charges residues the transmembrane electric field (Aggarwal and MacKinnon, 1996). The neutralisation of charged residues in the S4 segment reduced the number of gating charges (Aggarwal and MacKinnon, 1996). Alternatively, in separate investigations, sequential substitution of the positive residues of S4 for cysteines or histidines revealed the segment moves through the membrane in response to depolarisation, presenting external charges to the outer surface and burying inner charges in the membrane (Yang et al., 1996; Yellen, 1998). There is also strong evidence that the S4 helix rotates during depolarisation to present the charged residues to the extracellular compartment of the pore (Cha et al., 1999; Glauner et al., 1999). It has been hypothesised that the outward movement and rotation of the S4 in response to voltage changes pulls or twists the short linkers connecting it to neighbouring S3 and S5 segments and these segments transmit this movement to the S6 pore lining segments, opening the activation gate at their base. Alternatively, S4 and S6 are intimately intertwined and one segment cannot move without compensatory rearrangements of the other (Horn, 2000).

1.2.2.3 Inactivation

Inactivation is an important regulatory mechanism for tightly controlling the amount of Ca^{2+} entering a cell during an action potential. As a result, neurosecretion is more
precisely controlled, even suppressed during periods of short-term synaptic depression (Forsythe et al., 1998), and cytosolic Ca\(^{2+}\) concentration can be prevented from reaching cytotoxic levels. Inactivation occurs when the channel moves from the open activated state to an inactivated impermeant state, as opposed to a resting closed state. Unlike resting channels that are readily available for opening upon arrival of an action potential, channels that have progressed to an inactivated state will no longer gate the flow of ions on the arrival of new a depolarising impulse, and will have to return to a closed state before it is able to activate once again in response to a voltage change (Figure 1.8). Three different modes of inactivation have been reported for VDCCs: Fast (Zhang et al., 1994), slow (Sokolov et al., 2000) and Ca\(^{2+}\)-dependent (Peterson et al., 1999). Each type of inactivation is intrinsically controlled by the structural properties of the \(\alpha_1\) subunit and its interactions with the auxiliary subunits. The contribution of one of four auxiliary \(\beta\) subunits to this process is covered later in the section describing \(\beta\) subunit function (Section 1.2.2.3 page 72).

Figure 1.8 - Ca\(^{2+}\) channel state transitions during an action potential

Ca\(^{2+}\) channels reside in different conformational populations: closed (resting) states, activated (open) states and several inactivated states. Channel activation occurs rapidly in response to prolonged depolarisations or following trains of stimuli. Progression to inactivated states can be fast or slow and voltage or calcium dependent. The fast and slow voltage dependent inactivated states are closely interrelated. Transitions to inactivation may occur from the open as well as from the resting state. Negative shifts of the membrane potential promote transitions of open and/or inactivated channels to the resting state. Diagram modified from Hering et al. (2000).

1.2.2.2.3.1 Voltage dependent

Unlike the voltage-dependent Na\(^+\) and K\(^+\) channels that inactivate rapidly due to block of the pore by a particle or cytoplasmic loop that is part of the same channel (Hoshi et al., 1990; Vassilev et al., 1988), inactivation of VDCCs is much less understood and for a long time was not believed to involve blocking of the pore by another part of the channel. During a prolonged depolarisation or a rapid train of stimuli, channels can proceed rapidly or slowly to inactivation and moreover channels can move to the slow
inactivating state from the fast inactivating state (Sokolov et al., 2000). However, they do not necessarily have to pass through the open state, a phenomenon that is represented in plots of activation and steady state inactivation curves, which barely overlap. In fact, recent reports suggest inactivation occurs more readily in channels in a "partially activated" closed state; an intermediate state where the voltage sensor is in the activated position, but the channel is not open and from which it can progress directly to an inactivated state (Patil et al., 1998).

The fast and slow components of inactivation can be measured by fitting a double exponential to recordings of decaying whole cell currents (See Chapters 5 and 6). However, it is not uncommon for inactivating currents to only present a single component that can be fitted by a single exponential. The time constants calculated from the fitting of these curves (τ_{inactivation}) are often referred to as τ_1 or τ_{fast}, and τ_2 or τ_{slow} and are measured in the order of milliseconds. The τ_{inactivation} and its voltage dependence is a defining characteristic for each particular channel type.

Mutations of the α_i subunit, either deliberately introduced by the researcher (Herlitze et al., 1997; Stotz et al., 2000; Zhang et al., 1994) or those associated with disease states related with altered VDCC inactivation (e.g. Familial Hemiplegic Migraine, FHM - (Kraus et al., 1998)), coupled with the different inactivation properties of splice variants of the same VDCC α_i subunit (Bourinet et al., 1999), have helped to elucidate some regions or residues of the α_i subunit that control channel inactivation. Transplantation of IS6 between rabbit Cav2.1 and marine ray Cav2.3 (doe-1) transferred the inactivation properties of the donor channel to the acceptor (Zhang et al., 1994). However, because these channels normally demonstrate moderate to fast inactivation and are closely phylogenetically related (Ertel et al., 2000), it was highly probable that in these experiments other regions were also contributing to inactivation. Indeed, point mutations in the pore lining regions of IIS6, IIIS6 and IVS6 (Hering et al., 2000; Kraus et al., 1998), the S5-S6 linker of domains II, III and IV (Hans et al., 1999; Kraus et al., 1998), and in the I-II linker (Bourinet et al., 1999; Herlitze et al., 1997) of the α_i2.1 subunit all influence its rate and voltage-dependence of inactivation. More recent investigations have determined that all four transmembrane domains are involved controlling the voltage dependence of activation process, and domains II, III, and IV the
rate, but domains II and III playing the principal role in both processes (Spaetgens and Zamponi, 1999). Moreover, concurrent substitution of the domain I-II linker with IIS6 and IIIS6 segments of Cav1.2 into Cav2.3 removed inactivation from the acceptor subunit (Stotz et al., 2000). The findings reported in this latter article lead the authors to suggest that the fast component of Ca\textsuperscript{2+} channel inactivation may after all be mediated by block of the pore by an inactivation particle formed in the I-II linker, similar to that proposed for the domain III-IV linker of voltage-dependent sodium channels (Stotz et al., 2000). This hypothesis is supported by the earlier findings of Cens et al. (Cens et al., 1999), who accelerated the rate in voltage-dependent inactivation of a Cav2.1 channel by concomitantly over-expressing peptides corresponding to its I-II linker. Although this effect is possibly a function of interfering with \( \beta \) subunit interaction, point mutation or alternative splicing of the I-II linker of Cav1.2 or Cav2.1 respectively, both support a role of the cytosolic loop in fast voltage-dependent inactivation (Bourinet et al., 1999; Herlitze et al., 1997). The molecular nature of a putative hinged particle, and whether it is involved in inactivation of all VDCC types remains to be determined.

The molecular determinants of slow voltage-dependent inactivation are much more of an enigma. Association of the \( \beta_{2a} \) subunit (Section 1.2.2.3) appears to increase the probability that channels will inactivate slowly (Sokolov et al., 2000), and binding of use-dependent VDCC antagonists (e.g. PAAs) induce a slow rate of recovery from inactivation (Hering et al., 1997; Sokolov et al., 1999). Indeed, it would appear that there is a synergism between the residues or regions involved in use-dependent block by organic molecules and those involved in inactivation. Furthermore, under certain conditions, channels enter into the slowly inactivating conformation from the open state more willingly than they do from the fast inactivating conformation (Sokolov et al., 2000), indicating that slow inactivation is not merely a progression from fast inactivation. Further research is required to fully understand this component of the inactivation process; however, it is possible that it may occur via similar mechanism as the pore distortion process proposed for the C-type inactivation on voltage-dependent K\textsuperscript{+} channels (Hering et al., 2000; Liu et al., 1996).
1.2.2.2.3.2 **Ca$$^{2+}$$-dependent**

In addition to voltage dependent inactivation, the L-type VDCCs display Ca$$^{2+}$$-dependent inactivation. Originally identified in the Ca$$^{2+}$$ currents from axotomised Aplysia neurones (Chad et al., 1984; Chad and Eckert, 1984), this process has been most extensively characterised in the L-type VDCC currents conducted by Cav1.2 in the heart but is also reported to occur in some splice variants of the Cav2.1 (Bourinet et al., 1999; Lee et al., 1999a). During long depolarising stimuli, Ca$$^{2+}$$-dependent inactivation underlies the rapid component of Cav1.2/Cav2.1 inactivation, with increases in [Ca$$^{2+}$$]$_i$ resulting in currents decaying to baseline in milliseconds (Lee et al., 1985; Nilius and Benndorf, 1986). Ca$$^{2+}$$-dependent inactivation is therefore the primary determinant of current duration in depolarised cardiac cells and provides a critical autoregulatory means for controlling [Ca$$^{2+}$$]$_i$ under physiological and pathological conditions. The molecular mechanism underlying this process is independent of the participation of regulatory enzymes or processes requiring the hydrolysis of ATP (Haack and Rosenberg, 1994) and is caused by the direct interaction of Ca$$^{2+}$$ and calmodulin with the cytoplasmic C-terminal domain of Cav1.2 or Cav2.1 (Lee et al., 2000; Lee et al., 1999a; Qin et al., 1999; Soldatov et al., 1997; Zuhlke et al., 1998). Ca$$^{2+}$$-dependent inactivation is a cooperative process involving the binding of Ca$$^{2+}$$ to calmodulin and the association of the Ca$$^{2+}$$/calmodulin complex with a putative IQ calmodulin-binding motif (Lee et al., 2000; Lee et al., 1999a; Soldatov et al., 1997; Zuhlke and Reuter, 1998). Splice variants not possessing this critical motif lack Ca$$^{2+}$$-dependent inactivation. Additionally, both mutations that preclude calmodulin binding to Cav$\alpha_1$ subunits and calmodulin mutants that cannot bind Ca$$^{2+}$$ also prevent Ca$$^{2+}$$-dependent inactivation (Lee et al., 1999a; Peterson et al., 1999; Qin et al., 1999; Zuhlke et al., 1999). These studies indicate that calmodulin is the Ca$$^{2+}$$ sensor for channel regulation.
and imply that in its Ca\(^{2+}\) free state (apicalmodulin) it is likely to be constitutively
bound at a saturable site that provides preferential access to the IQ region on association
with Ca\(^{2+}\) (DeMaria et al., 2001; Jurado et al., 1999; Pitt et al., 2001), ready to bind
Ca\(^{2+}\) and initiate a conformational change that inactivates the VDCC (DeMaria et al.,
2001; Peterson et al., 1999; Zuhlke et al., 1999). A recent investigation proved this
hypothesis correct, definitively demonstrating VDCC-calmodulin pre-association with
Cav1.2, Cav2.1, and Cav2.3 \(\alpha_1\) subunits (Erickson et al., 2001), the latter result being
surprising because to date no Ca\(^{2+}\) modulation of R-type current has been demonstrated.

On the other hand, VDCCs are also subject to Ca\(^{2+}\)-dependent facilitation (Gurney et
al., 1989; Lee et al., 1999a; McCarron et al., 1992). The same calmodulin molecule
constitutively bound to the \(\alpha_1\) subunit C-terminus can also activate the phosphorylation
of the channel by Ca\(^{2+}\)/calmodulin-regulated protein kinase II (CaMKII) (Anderson et
al., 1994; McCarron et al., 1992; Xiao et al., 1994; Yuan and Bers, 1994; Zuhlke et al.,
1999), causing facilitation by increasing single Ca\(^{2+}\) channel open probability and
lengthening open times (Dzhura et al., 2000). It has been shown that the bifurcation of
calmodulin properties arises from divergent signalling capabilities of calmodulin lobes.
Ca\(^{2+}\) binding to the N-terminal lobe selectively initiates channel inactivation, whereas
Ca\(^{2+}\) sensing by the C-terminal lobe induces facilitation (DeMaria et al., 2001).

### 1.2.2.4 Agonist/antagonist binding sites

#### 1.2.2.4.1 A multisubsite domain in L-type channels

The major organic L-type antagonists DHPs, PAAs and BTZs, are able to influence one
another's binding to VDCCs in a non-competitive manner, suggesting L-type channels
are allosterically modulated by these three classes of molecule (Glossmann et al., 1983).
This allosteric model was extended with the realisation that binding of DHPs and PAAs
was also dependent upon the extracellular divalent cation concentration, indicating an
allosteric coupling between Ca\(^{2+}\) binding sites and drug receptor regions of the channels
(Glossmann et al., 1985a; Knaus et al., 1992). Purification and subsequent cloning of
the high-affinity sites of DHP binding from skeletal muscle identified that the $\alpha_1$ subunit of L-type VDCCs contains the required DHP, PAA and BTZ binding-domains (Curtis and Catterall, 1984; Glossmann et al., 1987; Naito et al., 1989; Striessnig et al., 1990a; Striessnig et al., 1990b; Tanabe et al., 1987). Photo-affinity labelling and antibody peptide mapping localised the drug-binding regions close to the pore of the $\alpha_1$ subunit (Nakayama et al., 1991; Striessnig et al., 1990a; Striessnig et al., 1991). The amino acids of the discontinuous domain IIIS5-S6 region and domain IVS6 segment were hypothesised to be arranged in close proximity at the extracellular surface of the channel pore to form the high affinity drug binding sites. Indeed transfer of these two regions to the DHP insensitive Cav2.1 channel conferred full DHP sensitivity onto the channel (Grabner et al., 1996). This "gain of function" approach was refined to pinpoint the amino acids responsible for DHP interaction by specifically mutating particular residues of domain IIIS5 of the Cav2.1 (BI-2) channel to that of the L-type channel. Eight critical residues from the cardiac Cav1.2 ($\alpha_1$) (M67515) supported DHP sensitivity in the neuronal Cav2.1, however high-affinity DHP binding was only conferred by the presence of an additional methionine in IIIS6 (He et al., 1997; Mitterdorfer et al., 1996b; Sinnegger et al., 1997). Further mutational analysis confirmed these findings and identified additional residues that have been identified in the formation of the DHP binding pocket, IIIS6 and in IVS6 (Hockerman et al., 1997b; Peterson et al., 1997; Peterson et al., 1996; Schuster et al., 1996). Four of these residues are conserved in non-L-type channels, but their presence is apparently insufficient to confer high sensitivity to DHPs.

Four residues of the Cav1.2 IVS6 segment contribute to high affinity PAA binding (Hering et al., 1996; Hockerman et al., 1995) with the aid of four further residues from IIIS6 conserved in both L- and non-L-type channels (Hockerman et al., 1997a) and two glutamate residues from the $Ca^{2+}$ selectivity filter (Mitterdorfer et al., 1995). Interestingly some of these residues also contribute to BTZ sensitivity, implying some overlap in the binding domains of these reagents or even identical sites (Hering et al., 1996; Hockerman et al., 2000). There is also overlap with some residues mapped as part of the DHP binding site demonstrating the close proximity of each domain required for allosteric function. However, other residues in IVS5 have also been implicated in the use dependent block of L-type channels by PAAs and BTZs but not DHPs (Motoike
et al., 1999). This motif may provide an auxiliary binding site or a fulcrum for movement of the pore helices within the membrane allowing use-dependent blockade.

The allosteric relationship between Ca\(^{2+}\) and DHPs was demonstrated in experiments incubating brain and cardiac VDCC with EDTA, which converted the DHP binding domain to a low affinity state (Glossmann et al., 1985b; Mitterdorfer et al., 1995), demonstrating that divalent cations were a prerequisite for high-affinity DHP binding. One model suggested VDCC channel modulation by DHPs was accomplished by improving Ca\(^{2+}\) coordination at the selectivity filter and thus promoting an inactivated or blocked state (Mitterdorfer et al., 1995). Furthermore, electrophysiological studies have determined that DHPs preferentially bind to L-type VDCCs when they are an inactivated state (Bean, 1984; Sanguinetti and Kass, 1984b), during which a single Ca\(^{2+}\) is predicted to bind with high affinity site at the selectivity filter and block the channel. Therefore, association of a Ca\(^{2+}\) ion at the selectivity filter will stabilise DHP binding, and DHP will co-operatively stabilise Ca\(^{2+}\) interaction in the channel. Whether this effect is caused by direct interaction of the divalent cation with the channel antagonist (Zhorov and Ananthanarayanan, 1996; Zhorov and Ananthanarayanan, 1997) or a conformational change that presents the residues associated with DHP binding in a more favourable configuration, or both, remains to be determined. The association of \(\beta\) subunits with the \(\alpha_i\) has also been implicated in improving DHP binding, causing a conformational change in the pore-forming subunit that further stabilises the interaction of Ca\(^{2+}\) at the selectivity filter and promotes high-affinity DHP binding (Castellano et al., 1993a; Lacerda et al., 1991; Mitterdorfer et al., 1994).

Current thinking now proposes that the pore region of L-type channels contains a “multisubsite domain” that functions as a focus for DHP, PAA and BTZ binding (Table 1.3 and Figure 1.10). Critical residues within this domain differentially accommodate the three chemical classes of VDCC modulators, but some overlap in their affinities for these reagents can result in one class of drug affecting the potency of another to modulate gating of L-type currents (Mitterdorfer et al., 1998).
### Table 1.3 and Figure 1.10 – A multisubsite domain in the pore of L-type VDCC is critical in the binding of DHPs, PAAs and BTZs.

<table>
<thead>
<tr>
<th>Location</th>
<th>DHP</th>
<th>PAA</th>
<th>BTZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>III5</td>
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<td>Y1152, I1153, F1165, V1166</td>
<td>I1150, M1160</td>
</tr>
<tr>
<td>III6</td>
<td>Y1463, M1464, I1471, I1472</td>
<td>Y1463, M1464, A1467, I1470</td>
<td>I1460</td>
</tr>
<tr>
<td>IVS6</td>
<td>Y1463, M1464, I1471, I1472</td>
<td>Y1463, M1464, A1467, I1470</td>
<td>I1460</td>
</tr>
</tbody>
</table>

Table 1.3) Amino acids written in the single letter code grouped according to their involvement in binding each of the L-type channel modulators and their location in each transmembrane segment of domains III and IV. Numbering is according to the CavL2,splice variant (M67515). Figure 1.10) Schematic drawing of the “multisubsite domain”. The colour of the sphere marking binding residues denotes the its involvement with each drug: Green – DHPs, Blue – PAAs, Yellow – DHPs & PAAs, Orange – PAAs and BTZs and Magenta – All three classes.

### 1.2.2.4.2 ω-conotoxin and ω-agatoxin binding sites

Understanding of the sites of the neuronal non-L-type α1 subunits involved in binding the peptide ω-conotoxins and ω-agatoxins is less than that for the DHP binding site of the L-type channels. It is generally thought that these toxins occlude the pore, binding to a region that has been termed the “macrosite” (Olivera et al., 1994). This motif is hypothesized to contain the necessary residues to associate ω-toxins, arranged as multiple “microsites” within its structure, whose sequences vary between the channel types, creating the selectivity between different toxins and α1 subunits (Olivera et al., 1994). Although this has not been proved, research into the binding of the N-type antagonist ω-conotoxin GVIA to the mouth of the Cav2.2 subunit is determining the structures that participate in toxin block. Mutations of residues in the P-loop between S5 and the glutamate that forms part of the selectivity filter in domains II and III causes a significant slowing of the onset of toxin block (Ellinor et al., 1994). Specific mutations in the domain III P-loop identified individual residues essential for toxin block, the most prominent being Glu$^{1335}$ (Ellinor et al., 1994). The adjacent region in the P-loop is predicted to adopt a helix-turn-helix conformation and form the putative EF-hand motif at the mouth of Cav2.2 that is proposed to be involved in the Ca$^{2+}$
permeation process (Doughty et al., 1998b; Feng et al., 2001b). Mutation of a pair of glycines within this motif, Gly$^{1326}$ and Gly$^{1332}$ showed that residues outside the $\omega$-ctx GVIA binding region identified by Ellinor et al. (Ellinor et al., 1994) can also participate in toxin binding (Feng et al., 2001a). In fact, one of the substitutions, Gly$^{1326}$Pro, is sufficient to convert the binding of $\omega$-ctx GVIA and $\omega$-ctx MVIIA, which are considered irreversible in wild type Cav2.2 channels, to be fully reversible in less than 10 minutes (Feng et al., 2001a). Within the toxin structure, Tyr$^{13}$ is essential for the activity of $\omega$-ctx MVIIA and $\omega$-ctx GVIA (Kim et al., 1995b), and within the Cav2.2 Gly$^{1335}$ is essential for toxin binding (Ellinor et al., 1994). Molecular modelling has determined that the distance between the proposed EF-hand motif and Glu$^{1335}$ is equivalent to that between the N-terminal amino group and Tyr$^{13}$ of the $\omega$-ctx GVIA peptide. The N-terminal amino group of $\omega$-ctx GVIA associates with the EF-hand, while Tyr$^{13}$ interacts with Glu$^{1335}$ (Doughty et al., 1998b). The same molecular models predicted L-type channels are not inhibited by $\omega$-ctx GVIA because the Glu$^{1335}$ is orientated in the opposite orientation within the P-loop and the putative $\omega$-ctx GVIA binding site is crowded with tyrosines not present in the Cav2.2 subunit (Doughty et al., 1998b). Moreover, although Cav2.2 and Cav2.3 share considerable homology within the predicted pore EF-hand motif Cav2.3 contains the differences Glu1332Asn and Gln1337Lys, which are sufficient to confer toxin resistance. Presumably similar interactions occur between the pore region of Cav2.1 channels and $\omega$-AgaIVA, but differences in the disulphide bridge architecture of the toxin and alternative residues at key positions of the Cav2.1 P-loop confer selectivity over other toxins.

### 1.2.2.2.5 G-protein modulation of VDCCs

In native neurones, modulation of VDCC current by the neurotransmitters GABA or noradrenaline, was demonstrated to be a result of G-protein mediated signal transduction (Holz et al., 1986; Scott and Dolphin, 1986). Seven transmembrane neurotransmitter receptors mediate synaptic transmission by activating pertussis toxin-sensitive heterotrimeric G-proteins that interact with N- and P/Q-type VDCCs inhibiting Ca$^{2+}$ activation (Currie and Fox, 1997; Dolphin, 1998; Zamponi and Snutch, 1998). Association of a G-protein with a VDCC causes a positive shift in the voltage-
dependence of activation, pushing the channel from a "willing" gating mode, characterised by rapid activation during depolarisations within the physiological range, to a "reluctant" state, typified by slow activation at more positive voltages, usually beyond the normal physiological range (Bean, 1989). This results in a partial inhibition that can be relieved by applying a strong depolarising prepulse, which facilitates dissociation of the G-protein from the VDCC (Dolphin, 1995; Elmslie et al., 1990; Ikeda, 1992).

The work of Ikeda (Ikeda, 1996) and Herlitze et al. (Herlitze et al., 1996) determined that it was the $G_{\beta y}$ moiety, not the $G_{\alpha}$, of the G-protein heterotrimer that mediated this inhibitory effect. Site-directed mutagenesis, and construction of channel chimeras determined that $G_{\beta y}$ associates with the $\alpha_1$ subunit at two separate sites within the domain I-II linker (De Waard et al., 1997; Page et al., 1997; Zamponi et al., 1997), a 38 amino acid region in the C-terminus (Qin et al., 1997), and the amino acids 44-55 in the N-terminus (Canti et al., 1999; Page et al., 1998; Zhang et al., 1996). The identification of a putative consensus binding motif for $G_{\beta y}$ subunits (QXXER) located in the domain I-II linker within the region determined to bind the VDCC $\beta$ subunit ($\alpha_1$ interaction domain - AID) (Chen et al., 1995; De Waard et al., 1997; Pragnell et al., 1994; Walker and De Waard, 1998; Zamponi et al., 1997), indicated that this shared binding region possibly underlies the functional antagonism between the $\beta$ subunit and $G_{\beta y}$, observed during $G_{\beta y}$ mediated inhibition. However, Qin et al. proposed that a different $\beta$ subunit/$G_{\beta y}$ binding site in the C-terminus determined this behaviour (Qin et al., 1997). Indeed, Zhang and colleagues had already established that the I-II linker was not the primary determinant of $G_{\beta y}$ mediated inhibition, rather that G-protein modulation depended on determinants within domain I and/or the C-terminus (Zhang et al., 1996). It was subsequently determined that the presence of amino acids 44-55 of the $\alpha_1$ subunit N-terminus of non-L type (Cav2.1, Cav2.2, Cav2.3) was actually the primary determinant for G-protein modulation (Canti et al., 1999; Page et al., 1998). The first of these investigations identified Cav2.3 in rat brain expressed a longer N-terminus than the previously described Cav2.3 rbEI (Soong et al., 1993). Named Cav2.3long, when co-expressed with dopamine (D2) receptors in Xenopus oocytes or $G_{\beta_1 y_2}$ subunit in COS-7 cells, unlike the rbEI clone, a slowing of activation and reduction in current amplitude characteristic of G-protein modulation was observed (Page et al., 1998). Moreover,
addition of the N-terminal 55 amino acids of Cav2.2 onto the N-terminal of Cav2.3 rbEII also resulted in G-protein modulation whereas deletion of the same 55 amino acids from Cav2.2 prevented G-protein modulation (Page et al., 1998). Site directed mutagenesis later determined that S48, R52, and R54 within an 11 amino acid sequence of the Cav2.2 N-terminus were essential for G-protein modulation (Canti et al., 1999). Therefore, it was proposed the N-terminus of the neuronal Cav2 family of channels is the primary contributor to a multicomponent site involved in Gpy binding and/or in subsequent modulation of channel gating, with accessory contributions from the sites in the I-II linker and C-terminus (Dolphin et al., 1999a).

Figure 1.11 - Prepulse facilitation of Cav2.2 currents modulated by co-expressed G||f. Cav2.2/β2/α2δ1 co-expressed with G-protein Gβγ subunits. The cell was held at -100mV, and voltage stepped according to the protocol shown, with two 50ms steps P1 and P2 to -40, -30 and -20mV, separated by an 80ms, +120mV depolarising prepulse. The amplitude of the currents in P2 after the prepulse are much larger than in P1 and their rate of activation is faster at all potentials. Figure is taken from Dolphin (1998).

1.2.2.2.6 VDCC phosphorylation

Activated Gβγ subunits inhibit VDCCs via a membrane-delineated pathway, however the Gα subunits can also modulate Ca^{2+} current via a non-membrane delineated pathway. Activation of the Gα subunit can stimulate the activity of second messenger cascades controlled by cytosolic concentrations of cyclic AMP (cAMP) or diacylglycerol (DAG), which can activate PKA or PKC respectively. It is well established that phosphorylation enhances Ca^{2+} currents in cardiac myocytes (Bean et al., 1984; Cachelin et al., 1983), neurones (Gray and Johnston, 1987; Mogul et al., 1993) or cells expressing cloned VDCCs (Bourinet et al., 1994; Bourinet et al., 1992; Fournier et al., 1993). The first example of ion channel regulation through second messenger pathways was the β-adrenergic stimulation of Ca^{2+} current in cardiac myocytes (Reuter and Scholz, 1977). In the presence of β-adrenergic agonist, the
VDCC single channel conductance did not change and neither did the number of functional channels seen within a membrane patch (Bean et al., 1984; Cachelin et al., 1983; Reuter et al., 1982). Facilitation of Ca\(^{2+}\) current occurred in the presence of noradrenaline due to an increase in the probability of channel opening and mean channel open time (Bean et al., 1984; Yue et al., 1990). Stimulation of the β-adrenoceptor catalysed G\(_{\alpha}\)-GTP formation, which activated adenylate cyclase and the resulting increase in intracellular cAMP concentration stimulated PKA to phosphorylate the cardiac VDCCs. This shifted them from a "null" mode of gating, in which they have a low probability of opening and can only be stimulated by unphysiological, very positive membrane potentials, to a "high P" mode in which the gating occurs within the physiological range (Bean et al., 1984). Similar mechanisms of Ca\(^{2+}\) current facilitation can be observed in bovine chromaffin cells following activation of dopamine D1-receptors (Artalejo et al., 1990). The most prevalent VDCC current modulated by cAMP dependent pathways is the L-type; however, both N- and T-type currents can be inhibited following PKA phosphorylation (Brown and Seabrook, 1995; Pfeiffer-Linn and Lasater, 1993) and adenosine stimulation of an A\(_{2B}\) receptor has been found to potentiate P-type current (Mogul et al., 1993). DAG activated PKC can enhance L-type currents in cardiac or smooth muscle (Lacerda et al., 1988; Schuhmann and Groschner, 1994) and neuronal tissue (Yang and Tsien, 1993b). Ca\(^{2+}\) current facilitation by PKC, like PKA, is a result of increasing the open probability and prolonging the open times of VDCCs (Yang and Tsien, 1993b). N-type currents can be both augmented (Yang and Tsien, 1993a) or inhibited (Cox and Dunlap, 1992) following PKC phosphorylation.

The molecular determinants of PKA- and PKC-mediated VDCC modulation are still relatively undetermined. The α\(_1\) subunits are probably the principal targets for phosphorylation as they contain multiple sites for phosphorylation by PKA, PKC and several other protein kinases not discussed in this brief review (Cribbs et al., 1998; Gerstin et al., 1998; Lee et al., 1999d; Mori et al., 1991; Perez-Reyes et al., 1998; Soong et al., 1993; Strom et al., 1998; Takahashi et al., 1987; Tanabe et al., 1988; Williams et al., 1992a; Williams et al., 1992b; Yoshida et al., 1992b), although the VDCC β subunit also possesses sites for phosphorylation by PKA (Curtis and Catterall, 1985; Gao et al., 1997a; Haase et al., 1996). Phosphorylation by a particular kinase may even depend upon the subunit composition of the channel at that given moment.
(Puri et al., 1997). The presence of a particular serine, Ser1928, in the C-terminus of full length Ca\textsubscript{v}1.2 is important in conferring the functional effects of PKA phosphorylation onto the channel, its absence causing a marked reduction in current facilitation by PKA (De Jongh et al., 1996; Gao et al., 1997b). However, the effect of phosphorylation of many heterologously expressed VDCCs by PKA is poor, falling well short of the 2-4 fold increases in current amplitude in native cells, indicating that a constitutive component of the phosphorylation pathway is missing in these cells. In point of fact, the co-transfection of A-kinase anchoring proteins (AKAPs) (Gray et al., 1998) with full-length skeletal muscle or cardiac L-type channels containing the critical C-terminal Ser1928 residue has been shown to be a necessary determinant of PKA modulation \textit{in vitro} (Fraser et al., 1998; Gao et al., 1997a; Gray et al., 1997; Johnson et al., 1997). However, of the 79 kDa and 15/18 kDa isoforms (AKAP-79 and AKAP-15/18 respectively) expressed in these heterologous expression systems, only AKAP-15/18 has shown \textit{in vivo}, association with cardiac L-type channels where it localises PKA at the plasma membrane to compartmentalise the kinase near its putative sites of action (Fraser and Scott, 1999; Gray et al., 1997). However, the inclusion of AKAP-15/18 in transient transfections of HEK-293 cells with the cardiac L-type Ca\textsuperscript{2+} channel only stimulated cAMP responsive currents by 35%, which is still markedly lower than the PKA potentiated L-type currents in native cells (Fraser et al., 1998).

Modulation of the L-type Ca\textsubscript{v}1.2 channels by PKC is dependent upon the presence of two threonines at positions 27 and 31 of the N-terminus (McHugh et al., 2000). These residues are present in the cardiac splice variant (Mikami et al., 1989) but not in the brain isoform (Snutch et al., 1991), indicating the N-terminal sequence is a target for tissue specific regulation of Ca\textsubscript{v}1.2. Several neurotransmitters acting through PKC can reverse the G-protein mediated inhibition of N- or P/Q-type channels (Swartz, 1993; Swartz et al., 1993). Phosphorylation of residues just downstream of the G\textsubscript{\beta\gamma} binding site within the domain I-II linker of the Ca\textsubscript{v}2.2 subunit lessens the degree of current inhibition (Zamponi et al., 1997).
1.2.2.2.7 Association of presynaptic VDCC with the SNARE complex

N- and P/Q-type channels are intrinsically involved in mediating synaptic transmission at fast synapses. The N-type channels have been implicated more particularly in peripheral neurotransmission whilst control by P/Q-type channels is more prevalent in central synapses (Castillo et al., 1994; Olivera et al., 1994; Wheeler et al., 1994). Biochemical and immunochemical experiments have demonstrated a tight association of both syntaxin and synaptotagmin with both Cav2.1 and Cav2.2 α1 subunits (Bennett et al., 1992; El Far et al., 1993; Leveque et al., 1994; Yoshida et al., 1992a). The II-III loops of both subunits purify syntaxin 1A and SNAP-25 in vitro, specifically interacting with one of two possible adjacent regions (Sheng et al., 1996; Sheng et al., 1997; Sheng et al., 1994). These sites were collectively designated the “synprint” region, abbreviating synaptic protein interaction. Interaction of this region in Cav2.2 with syntaxin 1A, SNAP-25 or the synaptic core complex is dependent on the cytosolic Ca\(^{2+}\) concentration (Sheng et al., 1996). This interaction occurs in the same Ca\(^{2+}\) concentration range as the threshold [Ca\(^{2+}\)], for fast neurotransmitter release, suggesting a role for the N-type channels in the docking and fusion of neurotransmitter vesicles. However, the synprint region is subject to alternative splicing in the Cav2.1 subunit, resulting in some splice variants being unable to bind syntaxin (Sheng et al., 1994; Starr et al., 1991). Moreover, association of SNARE proteins with Cav2.1 is predominantly Ca\(^{2+}\)-independent, indicating a different regulatory role in neurotransmitter release for some splice variants of P/Q-type channels. Experimental evidence suggests that Ca\(^{2+}\) binding to synaptotagmin is part of the signal that initiates neurotransmitter release (Brose et al., 1992; Davletov and Sudhof, 1993; Geppert et al., 1994; Perin et al., 1990) and coupled to the fact that binding of the synprint region by syntaxin and synaptotagmin is competitive (Sheng et al., 1997), fluctuations in Ca\(^{2+}\) concentration exerts a temporal-spatial control over the interaction of synaptic VDCCs with the with the synaptic core complex by modulating their affinities for binding one another (Catterall, 1998; Catterall, 1999; Meir et al., 1999).
Figure 1.12 – Regions of the VDCC $\alpha_1$ subunit of functional importance in channel activation, inactivation and interaction with cytosolic proteins.

Schematic diagram of the VDCC $\alpha_1$ subunit inserted into the plasma membrane, arranged as four conserved domains (I-IV) of six transmembrane spanning segments S1-S6. The pore of the channel is formed from the S5-S6 P-loop (red) that contains the structural determinants for Ca$^{2+}$ ion selectivity and the putative EF hand motif (green) is located in the III P-loop towards the outer mouth of the channel. The voltage sensors lie within the S4 segment of each transmembrane domain with positive charges every two or three residues along their helices (red with “+” annotation). Regions determined to control inactivation properties are labelled in yellow with red outlining. In particular S6 of each domain and the I-II loop have been implicated in fast voltage-dependent inactivation, although numerous other residues in the P-loop and voltage sensors have been potentially implicated in this process. Purple highlighting in the C-terminus denotes the Ca$^{2+}$/calmodulin binding domain (CBD), an alternatively spliced region in the L-type and some Cav2 channels that contains an IQ and/or EF hand domain implicated in the process of Ca$^{2+}$ dependent inactivation. Regions of interaction of the $\alpha_1$ subunit with the $\beta$ subunit are labelled in magenta. The N- and C-terminal sites can be $\beta$ isoform selective in Cav2.1 and Cav2.3 and a $\beta$ subunit can potentially associate with the $\alpha_1$-interaction domain (AID) plus one or other of the N- or C-termine. Yellow boxed regions in the I-II loop, N- and C-termini mark regions of potential interaction with the G$_{\beta\gamma}$ subunits of activated G-proteins. These regions are all within or in close proximity to $\beta$ subunit binding sites. When the channel is folded in its correct conformation these sites are probably all in close proximity with one another and form a multi-component site for binding G$_{\beta\gamma}$ proteins. The primary contributor to this multi-component of Cav2 channels site is the G$_{\beta\gamma}$ binding site in the N-terminus. The synprint site on the domain I-II linker shown in grey stripes is implicated in the binding of the SNARE complex proteins involved in neurosecretion.

1.2.2.3 The $\beta$ subunit

The auxiliary $\beta$ subunit forms part of the functional multimeric neuronal VDCC protein. Four VDCC $\beta$ subunit genes $\beta_1$-$\beta_4$ and their alternative splice-variants are expressed differentially in multiple tissue types (Castellano et al., 1993a; Castellano et al., 1993b; Hullin et al., 1992; Perez-Reyes et al., 1992; Pragnell et al., 1991). Importantly, two homologues of the $\beta_3$ subunit were identified in Xenopus oocytes, and therefore their
influence must always be considered when analysing the data from VDCCs expressed transiently in this system (Tareilus et al., 1997). Originally identified as part of the purified DHP receptor from skeletal muscle (Curtis and Catterall, 1984), cloning of the primary sequence of the ~50kDa VDCC β subunit predicted it to be a hydrophilic species lacking putative transmembrane domains or glycosylation sites (Ruth et al., 1989). This indicated that it was an entirely intracellular species and the fact that it separated as part of the DHPR complex in SDS-PAGE gels under non-reducing conditions (Curtis and Catterall, 1984) indicated that the β subunit was associated with the α1 through non-covalent protein-protein interactions.

A major β subunit-binding site has been described on the intracellular loop connecting domains I and II of all the HVA α1 subunits (Pragnell et al., 1994). Located 23 amino acids downstream of the transmembrane domain I, the α1-interaction domain (AID) is an 18 amino acid sequence, in which half the residues are conserved amongst all HVA α1 subunits (Figure 1.12; Figure 1.13). The 10th, 13th and 14th residues of the AID are critical for the binding of the subunit (De Waard et al., 1996), their mutation to alternative amino acids precluding all β subunit modulatory effects (Walker and De Waard, 1998). This is also demonstrated in the Cav3 T-type family of α1 subunits that do not possess an AID, a structural feature that correlates with the autonomous properties of these subunits that apparently lack biophysical modulation by β subunits and do not require its co-expression to drive the expression of classical T-type currents (Dolphin et al., 1999b; Perez-Reyes, 1998). The complementary region of the β subunit that binds to the AID is designated the β-interaction domain (BID)(De Waard et al., 1994; De Waard et al., 1995). This 30 amino acid sequence has been co-expressed alone with α1 subunits and is sufficient to cause the hyperpolarizing shift in voltage dependence of channel activation characteristic of β subunit co-expression (De Waard et al., 1994). Secondary sites of interaction are probably required to drive other typical β subunit effects including, large current stimulation or shifts in the voltage dependence and kinetics of inactivation. Lower affinity β subunit interaction sites have been described on the carboxyl termini of Cav2.3 (Qin et al., 1997; Tareilus et al., 1997) and Cav2.1 (Walker et al., 1999)(Figure 1.12). Unlike the I-II loop AID-BID interaction, the Cav2.1 N- and C-termini sites are β subunit selective (Walker et al., 1999).
Furthermore, binding to these sites is separate from relations with the I-II loop and it has been shown that the $\alpha_\text{I}$ subunit can bind to both the I-II loop and one secondary site (either the N- or C- terminal site, but not both) (Walker et al., 1999). The presence of multiple and differential combinations of $\alpha_\text{I}$-$\beta$ interaction sites probably underlies many of the subtle nuances of a functional VDCC complex.

Figure 1.13 - Structure of the $\alpha_\text{I}$-interaction domain (AID) and a functional map of the $\beta$ subunit.

a) Top - Structure and location of the consensus AID sequence of all HVA $\alpha_\text{I}$ subunits 23 amino acids downstream of domain 1 S6 and 55-86 residues upstream from domain II S1. Centre - Individual AID sequences of the L-type and non-L-type neuronal Ca$\text{v}$ proteins with respective consensus sequences. Conserved residues are labelled in bold. Bottom - critical motifs for interaction with G$\text{p}$ subunits (light grey highlight) and the $\beta$ subunit (dark grey highlight) or both (black). b) Domain structure of a VDCC $\beta$ subunit with the two conserved domains, D2 and D4, highlighted in boxed regions with their sequence identity to consensus $\beta$ sequence displayed. The location of BID in the N-terminal region of D4 is marked and the consensus BID sequence magnified above. Residues important in binding the $\alpha_\text{I}$ subunit are highlighted in grey. Below, Region 1 represents the BID, functionally involved in shifts of voltage-dependence of activation and small current increases; Region 2 - modulates inactivation kinetics; region three modulates kinetics and voltage dependence of inactivation. Figure adapted from Walker and De Waard (1998) & Birnbaumer et al. (1998).
Chapter 1

The overall secondary conformation of the β subunit remains largely undetermined. Structural predictions from the primary sequence of the skeletal muscle β₁ subunit indicate that it possesses six or seven α-helices and several β sheets (Ruth et al., 1989). However, the spatial arrangement of these structures when the subunit is in its native environment is unknown. Nevertheless, based upon the sequence conservation between β isoforms, a simplistic model of five domains D1-D5 has been formulated. Between all subunits, the N- and C- termini (D1 and D5) display the largest degree of sequence divergence (2% and 3% identity respectively), the central alternative splice region D3 being only slightly more conserved (11%). The interconnecting 130 amino acid D2 and 150 amino acid D4 domains are the most conserved between all isoforms (65% and 78% respectively) (Birnbaumer et al., 1998; Walker and De Waard, 1998), the latter domain containing the conserved BID within its N-terminal sequence (De Waard et al., 1994) (Figure 1.13). Examination of these domains in the β₁b subunit revealed the N-terminus D1 region resembled a Post synaptic-Disc-Zona occludens 1 (PDZ) domain, D2 was a putative SH3 (src homology 3) domain and domain D4 bore a resemblance to the membrane-associated guanylate kinase protein family (MAGUKs) (Hanlon et al., 1999). It follows that SH3 and guanylate kinase domains are possibly also present in the β₂, β₃ and β₄ subunits because they are located in the conserved D2 and D4 regions. Furthermore, it is established that many MAGUKs also contain SH3 and PDZ domains and are involved in clustering ion channels (Kim et al., 1995a) indicating a possible link between the β subunit and this family of proteins.

1.2.2.3.1.1 Post-translational modification

Palmitoylation

Although the β subunits are hydrophilic, when expressed in HEK293 or COS-7 mammalian cells, immunocytochemistry has often localised the β₁b and β₂a subunits to the plasma membrane even in the absence of a co-expressed α₁ subunit (Brice et al., 1997; Chien et al., 1995). The β₂a subunit is unique amongst the β isoforms because it undergoes a post-translational modification process termed palmitoylation. This occurs because its N-terminus contains a unique Cys-Cys doublet in positions 3 and 4 found in no other β subunit (Chien et al., 1996), which are modified by covalent attachment of
Chapter 1

Introduction

16-carbon fatty acid thioester groups. Although, hypothesised to facilitate insertion of the β2a into the plasma membrane, mutants where Cys 3 and/or Cys 4 were substituted for alanine still localised to membrane particulate fractions and were still able to target functional channel complexes to the plasma membrane (Chien et al., 1996). Mutagenesis experiments determined that in the β1b subunit (which is not palmitoylated), clusters of positively charged, acidic residues in the C-terminus are most likely responsible for the membrane clustering behaviour (Bogdanov et al., 2000). β2a also contains clusters of positively charged residues, indicating that these motifs rather than palmitoylation probably control plasma membrane localisation (Birnbaumer et al., 1998). The functional consequences of palmitoylation of the β2a subunit are in fact to preclude the voltage dependent prepulse facilitation of VDCC currents (Cens et al., 1996) and to cause a positive shift in the voltage dependence of inactivation of Cav2.3 (Olcese et al., 1994). Mutation of Cys3 and Cys4 to alanine or substitution of the N-terminal β2a sequence for that of β3 restores prepulse facilitation to Cav1.2 channels expressed in Xenopus oocytes (Birnbaumer et al., 1998; Cens et al., 1996).

Phosphorylation

As stated previously, the L-type VDCCs represent the best-studied ion-channels modulated by protein kinases. The β1a subunit of skeletal muscle possesses both PKA and PKC phosphorylation sites, however experimental evidence has determined that the primary substrate for PKA or PKC mediated current stimulation resulting from activation is the Cav1.1 protein rather than the β subunit (Gutierrez et al., 1994; Mundina-Weilenmann et al., 1991). Nevertheless, phosphorylation of the Cav1.1 subunit can be dependent on the β isoforms with which it is co-expressed (Puri et al., 1997). β-adrenergic stimulation of cardiac L-type channels may be primarily dependent upon phosphorylation of the cardiac β2a subunit by PKA (Haase et al., 1993). However evidence to refute this finding was produced by Gao et al. (1997a), who determined that phosphorylation of β2a appears to be independent of AKAP's and not sufficient to drive PKA mediated stimulation of cardiac L-type currents.
1.2.2.3.2 Functional properties

Functionally, the presence of VDCC β subunits in expression system studies has revealed a repertoire of effects on the major α₁ subunit. These are summarised below.

1.2.2.3.2.1 β subunit is obligatory for surface expression and correct functional assembly of VDCCs

The β subunit is thought to act as a chaperone to traffic VDCCs to the cell membrane. Several groups have investigated this property. To determine the role of the β₁ subunit in channel activity and excitation-contraction coupling, Gregg et al. (1996) used gene targeting to inactivate the β₁ subunit in mice. These mice died at birth from asphyxia resulting from a lack of excitation-contraction coupling. Immunohistochemistry of cultured myotubes showed that not only was the β₁ subunit absent, but the amount of Cav1.1 in the membrane was also undetectable. Immunocytochemistry in Cav1.1 null myotubes reveal that β₁a expression is normal and appropriately localised. Gregg et al. therefore concluded that the β₁ subunit plays an important role in both the transport and insertion of the Cav1.1 subunit into the membrane and the targeting of the muscle DHPR complex to its appropriate functional location (Gregg et al., 1996). Supporting evidence for the chaperoning role of β subunits came from several other sources; the rescue of excitation-contraction coupling in β₁ null myotubes following restoration of the β₁ subunit expression (Beurg et al., 1997); the knockout of endogenous Xenopus oocyte β subunits resulting in inhibition of transient VDCC expression (Tareilus et al., 1997); co-expression of Cav2.1 with β₁b, β₂a, or β₃ resulted in channel targeting to the membrane of COS-7 cells (Brice et al., 1997) and moreover differential β subunit association controlled the sub-cellular localisation of the VDCC complex (Brice and Dolphin, 1999); a point mutation in the AID on the α₁ I-II linker prevented β subunit mediated chaperoning of Cav1.2 to the cell membrane (Gerster et al., 1999); the α₁ subunit I-II linker possesses an endoplasmic reticulum retention signal that is masked on interaction with a β subunit, facilitating insertion of the channel into the plasma membrane (Bichet et al., 2000). Although Cav3
T-type channels possess no classical β subunit interaction domains, Dolphin et al. reported that β_{1b} produced an increase of functional expression of Cav3.1, either in the absence or the presence of heterologously expressed α2δ, whereas the other β subunits had much smaller effects (Dolphin et al., 1999b), though co-expression of a β subunit was not obligatory for channel function.

The chaperoning properties of the β subunit are, however, independent of its allosteric modulation of the α_1 subunit, which influences the activation and inactivation properties of the channel (Gerster et al., 1999; Yamaguchi et al., 1998).

1.2.2.3.2.2 Setting of proper kinetics of activation and voltage dependence of activation.

The currents expressed by Cav1.1 subunits transfected alone in L-cells were extremely abnormal and only resembled native channels upon co-expression of the β subunit (Lacerda et al., 1991; Varadi et al., 1991). Co-expression of the β_{1α} subunit with the cardiac Cav1.2 channel consistently enhanced expressed currents, accelerated activation kinetics and shifted the voltage-dependence of activation to hyperpolarized potentials (Singer et al., 1991; Wei et al., 1991). These properties are reproduced in the Cav2 family of channels (Canti et al., 2000; De Waard and Campbell, 1995; Olcese et al., 1996; Stephens et al., 2000). β subunits facilitate the opening of the channels by improving the coupling between channel opening and charge movement during a depolarisation (Neely et al., 1993). This alteration manifests as an increase in the steepness of the conductance-voltage relationship, by increasing the open probability of channels and hence a left-shift of the half maximal value for voltage dependent activation (Kamp et al., 1996; Olcese et al., 1996).

1.2.2.3.2.3 Inactivation

Whilst α_1 subunits contain the innate determinants of voltage-dependent inactivation (Cens et al., 1999; Herlitze et al., 1997; Spaetgens and Zamponi, 1999; Zhang et al., 1994), association with β subunit isoforms dictates their overall inactivation rate (De Waard and Campbell, 1995; Olcese et al., 1994). In general, the differential association
of a HVA $\alpha_1$ subunit with a $\beta$ subunit influences the rate of voltage-dependent inactivation in the following order (fastest to slowest) $\beta_3 > \beta_{1b} \approx \beta_4 >> \beta_{2a}$ (De Waard and Campbell, 1995; Patil et al., 1998; Stea et al., 1994). The properties of the $\beta_{2a}$ are in fact the opposite of the other three $\beta$ subunits, shifting the voltage dependence of inactivation more positive rather than more negative, and significantly slowing the inactivation kinetics (Olcese et al., 1994; Qin et al., 1996; Wyatt et al., 1998). This property is controlled by the differential splicing of the N-terminus (Olcese et al., 1994), which as previously described is unique in the $\beta_{2a}$ subunit, with additional contributions from the long form of the D3 region of the subunit (Qin et al., 1996)(Figure 1.13 B- Region 3).

1.2.2.3.2.4 Prepulse facilitation

As previously described, the $\beta$ subunit and the $G_{\beta\gamma}$ subunit appear to share common interaction motifs on the $\alpha_1$ subunit of members of the $Cav2$ family and that the $\beta$ subunit reduces the magnitude of G-protein inhibition by enhancing the rate of dissociation of the $G_{\beta\gamma}$ from the $\alpha_1$ subunit during a depolarising prepulse (Roche et al., 1995; Roche and Treistman, 1998). This theory is supported by experimental evidence where the $\beta$ subunits were depleted by injection of antisense nucleotides into dorsal root ganglion cells, resulting in increased $G_{\beta\gamma}$ mediated inhibition (Campbell et al., 1995). However, subsequent findings reported that although varying the concentration of $\beta$ subunit alters G-protein modulation of VDCC calcium currents (Canti et al., 2001), when $\beta$ subunit expression is completely absent, voltage-dependent facilitation of currents by prepulse is lost because the $\beta$ subunit is no longer present to facilitate the removal of bound $G_{\beta\gamma}$ subunits (Meir et al., 2000).

L-type channels also experience voltage-dependent facilitation, but although it is independent of G-protein involvement, research suggests that co-expression of the auxiliary $\beta$ subunit is necessary (Bourinet et al., 1994; Kamp et al., 2000).
1.2.2.4 The $\alpha_2\delta$ subunit

Affinity purification, or immunoprecipitation of the HVA $\alpha_1$ subunits from skeletal muscle, heart and brain revealed that the auxiliary $\alpha 2\delta$ subunits are an integral part of the VDCC complex in all of these tissues (Cooper et al., 1987; Curtis and Catterall, 1984; Hamilton et al., 1989; Tokumaru et al., 1992; Witcher et al., 1993). Each $\alpha 2\delta$ isoform is the product of a single gene (Barclay and Rees, 2000; Ellis et al., 1988; Klugbauer et al., 1999) that encodes a polypeptide that is post-translationally cleaved into the disulphide linked $\alpha 2$ and $\delta$ proteins (De Jongh et al., 1990; Jay et al., 1991; Takahashi et al., 1987). The $\alpha 2$ subunit its highly glycosylated, approximately 30 kDa of its molecular weight being lost after incubation in deglycosylation reagents (Jay et al., 1991). This indicated that the majority of the $\alpha 2$ protein was extracellular, and topographical mapping with $\alpha 2$ specific antibodies later determined that the entire $\alpha 2$ moiety was located extracellularly (Brickley et al., 1995; Gurnett et al., 1996; Wiser et al., 1996). The $\delta$ portion of the subunit contains a single transmembrane segment that is orientated in the plasma membrane with its N-terminal located extracellularly and serves to anchor the $\alpha 2$ subunit to the surface of the cell (Brickley et al., 1995; Gurnett et al., 1996; Jay et al., 1991; Wiser et al., 1996). Co-immunoprecipitation experiments demonstrated that in the absence of $\delta$ subunits, $\alpha 2$ subunits do not assemble with $\alpha 1$ subunits. Moreover, the transmembrane and cytoplasmic sequences in $\delta$ can be substituted with those of an unrelated protein without any effect on the association between the $\alpha 2\delta$ and $\alpha 1$ proteins (Gurnett et al., 1997).

Three $\alpha 2\delta$ genes have been identified to date. $\alpha 2\delta_1$, originally cloned from skeletal muscle (Ellis et al., 1988) is universally expressed as one of up to five alternatively spliced forms ($\alpha 2\delta_{1a-c}$), depending on tissue and species (Angelotti and Hofmann, 1996; Brust et al., 1993; Kim et al., 1992; Williams et al., 1992b). The $\alpha 2\delta_2$ subunit, of which four splice variants have been identified (Gao et al., 2000a; Hobom et al., 2000), displays 56% identity with the $\alpha 2\delta_1$ subunit and is expressed in human heart, pancreas skeletal muscle, kidney, liver, placenta, testis, and brain (Gao et al., 2000a; Klugbauer et al., 1999a). Differential distribution of the mouse $\alpha 2\delta_2$ subunit varies slightly from human, in that it is predominantly in brain with lower levels in kidney and testis (Barclay et al., 2000).
and the highest level of neuronal expression is in the cerebellum, where it is the principal isoform expressed in Purkinje cell bodies (Barclay et al., 2001; Hobom et al., 2000). The $\alpha_2\delta_3$ subunit is only 30% identical to $\alpha_2\delta_1$ and expressed exclusively in brain in the mouse, particularly localised to the caudate putamen, hippocampus, enthorinal complex, cortex, thalamic nuclei (Klugbauer et al., 1999a; Marais et al., 2001). However two human splice variants of the $\alpha_2\delta_3$ subunit have been amplified from multiple foetal tissue types as well as adult kidney (Hanke et al., 2001).

Regardless of the $\alpha_2\delta$ isoform or the expression system in which studies are performed the general consensus of all research is that the principal function of $\alpha_2\delta$ subunits is to upregulate HVA VDCC current density, increasing whole cell current amplitude for a particular magnitude of depolarisation step (Bangalore et al., 1996; Barclay et al., 2001; Brodbeck et al., 2002; De Waard and Campbell, 1995; Gao et al., 2000a; Hobom et al., 2000; Klugbauer et al., 1999a; Mikami et al., 1989; Mori et al., 1991; Shistik et al., 1995; Williams et al., 1992b). This effect can also be measured as an increase in the number of DHP (Felix et al., 1997; Williams et al., 1992b), or conotoxin binding sites (Brust et al., 1993; Williams et al., 1992a), but is most cases is dependent upon or significantly enhanced by the co-expression of the $\beta$ subunit (Brust et al., 1993; De Waard and Campbell, 1995; Singer et al., 1991). Mechanism by which $\alpha_2\delta$ stimulates current is unknown but evidence suggests that it may facilitate or stabilise plasma membrane incorporation of the $\text{Ca}^{2+}$ channel complex. Other biophysical consequences of $\alpha_2\delta$ expression are relatively minor, and usually an extension of the properties of the $\beta$ subunit; these include the acceleration of the rate of channel activation and inactivation and shifting of the voltage dependence of both parameters in a hyperpolarizing direction (De Waard and Campbell, 1995; Felix, 1999; Klugbauer et al., 1999a; Singer et al., 1991; Tomlinson et al., 1993).

Apparently, it is the interaction of the glycosylated extracellular $\alpha_2$ subunit with the $\alpha_1$ subunit that underlies these functional properties. Deletion of the $\delta$ subunit transmembrane anchor results in a loss of current stimulation (Wiser et al., 1996) whilst, as previously mentioned, replacing the $\delta$ subunit with an unrelated transmembrane segment does not disrupt the functional properties of an $\alpha_2\delta$ (Gurnett et al., 1997),
although this finding is contested by Felix et al. who reported that the δ subunit is responsible for some of the modulations of gating properties (Felix et al., 1997). Nevertheless, deglycosylation abolishes the current stimulation attributed to the α2δ subunit, possibly because glycosylation increases channel stability at the plasma membrane (Gurnett et al., 1996).

Although, the α1 subunit is the principal target for the majority of pharmacological reagents modulating VDCC function, the α2δ1 has been implicated as the in-vivo target for the anti-epileptic drug gabapentin (a structural analogue if the inhibitory neurotransmitter GABA) (Gee et al., 1996). However, another study using a different purification procedure showed that there might be an additional gabapentin binding protein in brain that was detected with a polyclonal α2 antibody (Brown et al., 1998). It was subsequently determined that gabapentin binds α2δ1 and α2δ2, but not α2δ3 (Marais et al., 2001). The interaction of α2 and δ subunits is important in gabapentin binding as neither subunit binds the drug when expressed alone (Wang et al., 1999). The mechanism of action of gabapentin is unclear, and data for its effects on VDCCs is inconsistent, with some groups failing to detect significant modulation of Ca\(^{2+}\) current at therapeutic concentrations (10μM) (Rock et al., 1993; Schumacher et al., 1998), whilst other report fast and moderate voltage-independent inhibition of whole cell calcium currents in pyramidal neocortical cells (Fink et al., 2000; Stefani et al., 1998), and a subsequent reduction in neurotransmitter release (Fink et al., 2000). It is possible that the actions of this drug are very subtle, only targeting a specific subpopulation of channels or are dependent upon the experimental procedures employed in each investigation (Alden and Garcia, 2001).
### Table 1.4 -Summary of the VDCC auxiliary subunit genes, chromosomal location, splice-variants and primary tissue distribution

All the information within this table is compiled from references cited in the appropriate sections in main body text.
1.2.3 The VDCC γ subunit

Much work has concentrated on the characterisation of functional properties of the α, β, and α2δ subunits (Birnbaumer et al., 1998; Catterall, 2000; Dolphin, 1998; Ertel et al., 2000; Jones, 1998; Perez-Reyes, 1998). However, research concerning the role of the γ subunit has not been as extensive.

1.2.3.1 The skeletal muscle γi subunit.

The γi subunit was identified as a 32 kDa constituent of the DHPR complex purified from skeletal muscle (Curtis and Catterall, 1984; Flockerzi et al., 1986; Sharp and Campbell, 1989; Takahashi and Catterall, 1987). However, purification of VDCCs from other tissues including, cardiac tissue and brain, revealed that this subunit did not co-purify with these complexes (Chang and Hosey, 1988; McEnery et al., 1991; Witcher et al., 1993). For many years, only a single, four-exon gene (CACNLG1/CACNG1) localised on human chromosome 17q24 and exclusively expressed in skeletal muscle, was reported to encode a VDCC γ subunit, the γi (Bosse et al., 1990; Eberst et al., 1997; Jay et al., 1990; Powers et al., 1993; Wissenbach et al., 1998). Analysis of the subunit’s primary sequence (222 amino acids in human and rabbit, 223 amino acids in rat and mouse) predicted the polypeptide possessed four α-helical transmembrane spanning segments, and was inserted into the plasma membrane with its N- and C- termini localised in the cytoplasm. The first extracellular loop possesses two conserved sites for N-linked glycosylation (Bause, 1983) and a single residue in the intracellular C-terminus is potentially phosphorylated by casein kinase II (Pinna, 1990). No alternatively spliced variants have been reported, although a 500 base pair cDNA containing the open reading frame for a 68 amino acid protein was consistently amplified by reverse transcriptase polymerase chain reaction (RT-PCR) together with the murine skeletal muscle γi subunit cDNA (Wissenbach et al., 1998). This protein, designated γi by the authors, represented the first 24 bases of exon 1 of the γi subunit at which point an alternative splice site was utilised to omit the majority of exon 1 and splice in a sequence completely unrelated to γi or any other known protein. γi had no functional effect upon a transiently expressed CaV1.2 channel and has not been reported in any
other species, indicating that it was possibly an artefact of the RT-PCR in that particular investigation (Wissenbach et al., 1998).

Because of complications expressing Cav1.1, in vitro studies characterising the functional properties of the γt subunit were limited in their findings. In Xenopus oocytes, the voltage dependence of steady state inactivation of a Cav1.2 channel was shifted more hyperpolarized by ~30mV and peak amplitudes were increased slightly (Singer et al., 1991; Wei et al., 1991). However this was only observed upon co-expression of a β, α2δ or both auxiliary subunits with the γt and Cav1.2. To determine the role of γt in its native environment, gene targeting was used to establish a mouse model, in which γt expression is eliminated (Ahern et al., 2001; Freise et al., 2000). In contrast to the experiments with recombinant Cav1.2 in oocytes, recordings of Ca\(^{2+}\) currents of skeletal myotubes in 10mM Ca\(^{2+}\) from mice lacking the γt subunit, suggest that its role is to limit calcium entry through these channels (L-type VDCC G_{max} 21-25% larger in γt knockout myotubes), increase the rate at which the channels inactivate, and shift the half-maximal potential for the voltage dependence of inactivation 8.6-13.5 mV closer to the resting potential (Ahern et al., 2001; Freise et al., 2000). The increase in Ca\(^{2+}\) current recorded in the knockout mice was suggested to be due to changes in the open probability rather than an alteration in single channel conductance in the absence of γt (Freise et al., 2000). Together, these results indicate that the inclusion of γt in the DHPR complex decreases the Ca\(^{2+}\) entry during stimulation of skeletal muscle by reducing the number of channels available for activation.
Figure 1.14 - Functional properties of the skeletal muscle γ subunit in vivo.

The generation of γ knockout mice facilitated the accurate characterisation of the functional properties of the subunit by comparing the myotube Ca\(^{2+}\) currents recorded from knockout (-/-) and wild type mice (+/+). a) The average current-voltage relationship of -/- and +/- prepared from litter-matched mice revealed that peak current amplitudes are significantly larger in -/- mice at test potentials from -20mV to +40mV (Freise et al., 2000). b) Steady state inactivation of VDCC currents in -/- mice occurs at more depolarised potentials (+13.5mV) than +/- mice (Ahern et al., 2001). c) The kinetics of inactivation are significantly slower in -/- mice compared to +/- . Pulses 1, 2 and 3 correspond to 2.5 ms depolarisations to -70mV, +30mV and +60mV respectively (Ahern et al., 2001).

1.2.3.2 Ca\(^{2+}\) channelopathies: Insights into channel structure and function from nature's mistakes

Certain epilepsies, episodic ataxias, migraine headache, and night-blindness can all be classed as “calcium channelopathies”, because mapping of their genetic loci has determined that each is linked to the mutation of a calcium channel gene. Elucidation of the particular gene mutated in each syndrome has facilitated the discovery of several novel ion channel subunits, and furthered our understanding of the functional properties of others that had been previously discovered. Channelopathies can directly alter the functional properties of an ion channel by mutating the gene encoding the primary pore-forming subunits, or alternatively indirectly alter channel properties via mutations in its auxiliary subunits. Of the many diseases investigated, several human conditions including hypokalaemic periodic paralysis (HypoPP) (Elbaz et al., 1995; Jurkat-Rott et
al., 1994; Ptacek et al., 1994), episodic ataxia type-2 (EA-2) (Kullmann et al., 2001; Ophoff et al., 1996), X-linked congenital stationary night blindness (Bech-Hansen et al., 1998) and juvenile myoclonic epilepsy (Escayg et al., 2000) are linked to mutations of known α1 and auxiliary VDCC subunits. The mutations linked to characterised VDCC channelopathy phenotypes in both humans and mice are summarised in Table 1.5.

Table 1.5 – Human Disorders arising from VDCC channelopathies

The table on the following page summarises the mutations underlying several human syndromes caused by VDCC channelopathies. The affected gene, resulting translation effect and disease phenotype are all listed. References for table 1.5 are listed below.

<table>
<thead>
<tr>
<th>Disorder/ Mutant name</th>
<th>Gene (Gene product)</th>
<th>Common mutations*</th>
<th>Phenotype</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypokalaemic periodic paralysis 1 (HypoPP1)</td>
<td>CACNA1S (Cav1.1)</td>
<td>R1239H (IVS4), R1239G (IVS4), R528H (IIS4)</td>
<td>Episodic muscle weakness and periodic paralysis. Attacks aborted by exercise or administration of potassium. Attacks precipitated by insulin or glucose administration</td>
<td>1, 2</td>
</tr>
<tr>
<td>Malignant hyperthermia susceptibility 5 (MHS 5)</td>
<td>CACNA1S (Cav1.1)</td>
<td>R1086H (III-IV loop)</td>
<td>Potentially lethal in susceptible individuals on exposure to commonly used inhalation anaesthetics and depolarising muscle relaxants. Crises reflect the consequences of disturbed skeletal muscle calcium homeostasis</td>
<td>3, 4</td>
</tr>
<tr>
<td>MHS 3</td>
<td>CACNA2D1 (a28)</td>
<td>?</td>
<td>Precipitated by general anaesthesia. Hypertonicity of voluntary muscles, hyperthermia, myopathy. Rhabdomyolysis may follow severe exercise in hot conditions, neuroleptic drugs, alcohol, or infections</td>
<td>5</td>
</tr>
<tr>
<td>X-linked congenital stationary night blindness 2 (CSNB2)</td>
<td>CACNA1F (Cav1.4)</td>
<td>20 mutations 70% truncation 30% aa subst. No clustering of changes</td>
<td>Congenital stationary night blindness Decreased visual acuity Loss of night vision</td>
<td>6, 7, 8</td>
</tr>
<tr>
<td>Episodic ataxia type 2 (EA-2)</td>
<td>CACNA1A (Cav2.1)</td>
<td>Early stop (IIS1), (IIS2-S3), (IVS1) Splice error (IIS2), G293R (IIS5-S6).</td>
<td>Ataxia triggered by emotional or physical stress, last up to several hours, and associated with truncal instability, interictal nystagmus, vertigo, double vision, migraine and vomiting</td>
<td>9, 10, 11, 12</td>
</tr>
<tr>
<td>Familial hemiplegic migraine (FHM)</td>
<td>CACNA1A (Cav2.1)</td>
<td>R192Q (IS4), T666M (IIS5-S6), V714A (IIS6), D715E (IIS6), I1811L (IVS6)</td>
<td>Hemiplegic migraine, drowsiness, confusion, aura may consist of: Hemianoptic blurring of vision, unilateral paresthesias, numbness, dysphasia, retinal degeneration, nystagmus, persistent cerebellar dysfunction, and coma.</td>
<td>9, 13, 14, 15</td>
</tr>
<tr>
<td>Spinocerebellar ataxia type 6 (SCA6)</td>
<td>CACNA1A (Cav2.1)</td>
<td>CAGn expansion = polyglutamine in C-termini</td>
<td>Disease is insidious with gradual development of balance and coordination difficulties (spinocerebellar ataxia). Cerebellar atrophy with predominant loss of the Purkinje cell</td>
<td>16, 17, 18</td>
</tr>
<tr>
<td>Absence epilepsy, episodic ataxia and progressive, disabling fixed cerebellar syndrome</td>
<td>CACNA1A (Cav2.1)</td>
<td>Early stop (IVS6)</td>
<td>Nocturnal generalised tonic-clonic seizures in early years, absence epilepsy, gait unsteadiness, dysarthria and double vision.</td>
<td>19</td>
</tr>
<tr>
<td>Juvenile myoclonic epilepsy 1 (EJM1)</td>
<td>CACNA4B (B4)</td>
<td>R482X early stop codon (38 aa deletion)</td>
<td>Morning myoclonic jerks, morning generalised tonic-clonic seizures, absence seizures, 4-6 Hz polyspike EEG</td>
<td>20</td>
</tr>
<tr>
<td>Epilepsy, generalized idiopathic episodic ataxia included</td>
<td>CACNA4B (B4)</td>
<td>C104F missense mutation</td>
<td>Idiopathic generalized epilepsy with rare juvenile atypical prolonged absences and occasional generalised tonic-clonic seizures</td>
<td>20</td>
</tr>
</tbody>
</table>
1.2.3.3 Channelopathies causing absence epilepsy in mice are linked to mutations in VDCC subunits.

Hereditary factors play an important role in the aetiology of common idiopathic human epilepsies, including absence epilepsy. Absence epilepsy is a generalised, non-convulsive seizure disorder, occurring mainly in children. Seizures are typically brief (<30s), occur spontaneously, and can be recorded by electro encephalogram (EEG) as 3Hz cortical spike wave discharges (SWD). Simultaneous to the generation of the SWD, the patient displays behavioural arrest and impaired awareness (Noebels and Tharp, 1995). Unfortunately, the heterogeneous nature of many human epilepsies makes mapping the principal defect to a single genetic locus almost impossible. Nevertheless, in recent years, our understanding of the primary causes of absence epilepsy have been significantly improved with the elucidation of the underlying genetic defects of rodent models displaying SWD and abnormal behaviour analogous to human absence epilepsy. Although unsurprising that mutations in voltage-dependent ion channels are associated with epilepsy, the correlation between VDCC mutants and absence epilepsy models is striking. Four of the six distinct mouse models of inherited absence epilepsy, tottering (tg), lethargic (lh), ducky (du), and stargazer (stg) have been associated with VDCC defects (Barclay et al., 2001; Burgess et al., 1997; Fletcher et al., 1996; Letts et al., 1998). Absence epilepsy mouse models not attributed to VDCC mutation are slow wave epilepsy (swe) and mocha (mh), in which the ubiquitous Na+/H+ exchanger gene Slc9a1 (Cox et al., 1997), and the adaptor-related protein δ subunit gene Ap3d (Kantheti et al., 1998) are mutated respectively. Although the causal mechanisms of absence epilepsy emerging from a VDCC channelopathy are poorly understood, it is tempting to assume that tg, lh, du and stg are the result of a VDCC malfunction directly altering the properties of the channel itself and/or disrupting the one of the many second messenger pathways or protein-protein interactions with which VDCCs are inextricably linked. However, of more interest to this body of work is that mapping of the disease loci in these mice identified the murine isoforms and/or the chromosomal location of the previously known Cav2.1 (tg), β4 (lh) and α2δ2 (du) subunits (Barclay et al., 2001; Burgess et al., 1997; Fletcher et al., 1996), but in particular, identified a previously unknown putative VDCC auxiliary subunit, the neuronal γ2 (stg) (Letts et al., 1998).
<table>
<thead>
<tr>
<th>Mutation name</th>
<th>Gene / subunit</th>
<th>Nucleotide mutation</th>
<th>Translation effect</th>
<th>(Ca^{2+}) channel effect</th>
<th>Phenotype</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tottering (tg)</td>
<td>Cacnala / Ca(_{v2.1})</td>
<td>Missense C1802</td>
<td>P601L (IIIS5-S6)</td>
<td>Pore lining region</td>
<td>40% ↓ Purkinje cell P-type current density</td>
<td>Absence epilepsy, NA LCH of CNS, Motor seizures, Ataxia</td>
</tr>
<tr>
<td>Leaner (tg(^{os}))</td>
<td>Cacnala / Ca(_{v2.1})</td>
<td>98nt insertion @ 5901/2 or Deletion of nt5763-5901</td>
<td>Out of frame readthrough → novel C-terminus</td>
<td>65% ↓ ↓ Purkinje cell P-type current density</td>
<td>3 × ↓ (P_g) P-type channels</td>
<td>Absence seizures, aberrant tyrosine hydroxylase gene expression, no motor seizures, severely ataxic, progressive cerebellar degeneration</td>
</tr>
<tr>
<td>Rolling-Nagoya (tg(^{os}))</td>
<td>Cacnala / Ca(_{v2.1})</td>
<td>Missense C3784T</td>
<td>R1262G (IIIS4)</td>
<td>↓ current density, ↓ in slope factor and +8 mV shift of Purkinje cell P-type I-V curve</td>
<td>Unknown</td>
<td>More severe ataxia than (tg). No motor seizures</td>
</tr>
<tr>
<td>Rocker (tg(^{AK}))</td>
<td>Cacnala / Ca(_{v2.1})</td>
<td>Missense C3929A</td>
<td>T1310K (IIIS5-S6)</td>
<td>Unknown</td>
<td>Unknown</td>
<td>More severe ataxia than (tg). No motor seizures</td>
</tr>
<tr>
<td>Lethargic</td>
<td>Cacnb4 / (\beta_4)</td>
<td>4 nt insertion into a splice donor site → exon skipping,</td>
<td>Translational frameshift, and protein truncation with loss of BID</td>
<td>VDCC currents (\text{in vivo}) indistinguishable from (wt) because of (\beta) subunit reshuffling</td>
<td>Absence epilepsy, ataxia, episodic dyskinesia and generalised spike-wave epilepsy</td>
<td>9, 10, 11</td>
</tr>
<tr>
<td>Ducky</td>
<td>Cacna2d2 / (\alpha2\delta_2)</td>
<td>Genomic rearrangement</td>
<td>Truncated protein of only exons 1-3</td>
<td>↓ P-type current density</td>
<td>Ataxia, absence epilepsy, paroxysmal dyskinesia</td>
<td>12, 13</td>
</tr>
<tr>
<td>Stargazer (stg)</td>
<td>Cacng2 / (\gamma_2)</td>
<td>Etn insertion into intron 2</td>
<td>Premature transcriptional termination – null mutation</td>
<td>-7mV shift in P/Q-type steady state inactivation (\text{in vitro}). Hypothesised ↑ (Ca^{2+}) entry stg brain.</td>
<td>Head-tossing, ataxic gait, prolonged spike-wave discharges associated with absence epilepsy</td>
<td>14, 15, 16, 17, 18, 19, 20</td>
</tr>
<tr>
<td>Wagglver (stg(^{sus}))</td>
<td>Cacng2 / (\gamma_2)</td>
<td>Possible Etn insertion into intron 1</td>
<td>Reduction of (wt) mRNA</td>
<td>Unknown</td>
<td>Severely ataxic but head toss less frequently than (stg), but have a more pronounced side-to-side head motion</td>
<td>20</td>
</tr>
</tbody>
</table>

Table 1.6 – Mutant mouse models of absence epilepsy: Mutations and physiological consequences
Table 1.6 – (From previous page) Mutant mouse models of absence epilepsy: Mutations and physiological consequences

Abbreviations: NA LCH of CNS – noadrenergic locus ceruleus hyperinnervation of the central nervous system; nt – nucleotide; I-V – current-voltage; wt – wild type; Etn – Early transposon. Single letter amino acid code is used to describe mutations. Data in brackets in the translation effect column refers to α subunit conserved domains I-IV and transmembrane segments S1-S6. References used to create table:


Multiple alleles of each mutant mouse have been identified and a summary of the phenotypes and genotypes of these absence epileptic mice is given in Table 1.6. However, the following passages describe in more detail the research surrounding the stargazer mouse and the discovery that a mutation of a putative VDCC γ subunit was responsible for its absence epilepsy phenotype.

1.2.3.4 The stargazer mouse (stg)

1.2.3.4.1 Aetiology

The stargazer mouse (stg) arose spontaneously at the Jackson Laboratory on the A/J inbred mouse line (Noebels et al., 1990). The phenotype of this spontaneous epileptic mutant mouse is characterised by an ataxia, head tossing and recurrent spike-wave seizures characteristic of absence epilepsy (Noebels et al., 1990; Qiao et al., 1996). Both stg and the allelic waggler (wag) (Sweet et al., 1991) mutants display a severe
impairment in the acquisition of classical eye blink conditioning in adulthood, indicating a cerebellar dysfunction (Bao et al., 1998; Qiao et al., 1998). Despite having apparently normal gross cerebellar morphology, high power examination of stg cerebellum reveals an increased number of external granule cells at postnatal day 15 (P15) and the presence of immature looking granule cells in the adult (Qiao et al., 1996). Other immature features in the molecular layer of adult stg cerebellar cortex when compared to age matched wild type mice include the presence of desmosoid plaques, concentric profiles of parallel fibres and a significantly lower number of synapses where the presynaptic termini are smaller and contain fewer synaptic vesicles (Qiao, 2001). Ataxia first becomes manifest in stg mice around postnatal day 14 (P14) and has been attributed to the coincident and specific near-total reduction in brain derived neurotrophic factor (BDNF) mRNA (Qiao et al., 1996) and resultant protein expression (Qiao et al., 1998) in the cerebellar granule cells (GCs). Although expression levels of the BDNF receptor TrkB remain normal in the stg mouse, the ligand induced tyrosine phosphorylation of several TrkB-activated signal transduction molecules is reduced (Qiao et al., 1998). More recent investigations have highlighted that excitatory post-synaptic currents (EPSCs) at mossy fibre (MF) to GC synapses are devoid of their fast component mediated by AMPA (alpha-amino-3-hydroxyl-5-methyl-4-isoxazolepropionate) glutamate receptors whilst retaining the slow NMDA (N-methyl-D-aspartate) receptor mediated component. There is no deficit in glutamate release from the mossy fibre terminals, and AMPA receptor mediated EPSCs in CA1 pyramidal cells remain normal in the stg brain. This indicates an impairment of AMPA receptor function specifically localised to the stg GCs, although mRNA levels of four GluR subunit genes are comparable to the wild type. Impaired AMPA receptor function is however not a result of the reduced BDNF-TrkB signalling as EPSCs at the MF-GC synapses of BDNF knockout mice retain their AMPA receptor component (Hashimoto et al., 1999).

Figure 1.15 – The stargazer mouse
Stargazer displays a characteristic head tossing and an ataxic gait.
1.2.3.4.2 Identification of a putative neuronal VDCC $\gamma$ subunit ($\gamma_2$) in stargazer mice

Efforts to elucidate the $stg$ genotype resulted in the construction of a fine genetic map of the $stg$ region on mouse chromosome 15 (Letts et al., 1997). Subsequent research determined that an early transposon (BTn) was inserted into the second intron of a four-exon gene in this region, resulting in its premature transcriptional termination. The non-mutated gene was named stargazin, and analysis of the predicted stargazin polypeptide suggested that it was a 323 amino acid, 36kDa, tetra-spanning transmembrane protein (Figure 1.16A). Regardless of having only weak homology with the $\gamma_1$ subunit (25% identity or 38% similarity), the authors proposed that the brain-specific stargazin protein represented the first example of a neuronal VDCC $\gamma$ subunit on the basis of the similarity of its polypeptide secondary structure its genomic architecture ($cacngl$ and stargazin both possess very large first introns within a gene whose coding sequence is assembled from four exons). Stargazin was therefore designated the VDCC $\gamma_2$ subunit and its gene renamed $cacng2$. In situ hybridisation determined that $\gamma_2$ is expressed throughout the brain, particularly in the cerebellum, and to a lesser extent in hippocampus, cerebral cortex, thalamus and olfactory bulb. Additionally, a specific polyclonal antibody detected that its expression was particularly enriched in synaptic plasma membranes. This antibody also detected two bands in SDS-PAGE that were 35 kDa and 38 kDa in size, which were proposed to represent non-glycosylated and glycosylated forms respectively (Letts et al., 1998).

Electrophysiology ascertained that the primary influence of transiently expressed $\gamma_2$ subunit was to cause a 7mV, hyperpolarizing shift in the voltage dependence of steady state inactivation of in $Ca_{\text{v2.1}}/\beta_1d/\alpha2\delta_1$ VDCC currents (Letts et al., 1998). Both the neuronal stargazin protein and the skeletal muscle $\gamma_1$ subunit had therefore been shown to exert inhibitory effects upon VDCC currents (Ahern et al., 2001; Freise et al., 2000; Letts et al., 1998). The consequent hypothesis stated that the mutation resulting in the effective absence of stargazin/$\gamma_2$ expression would lead to inappropriate Ca$^{2+}$ entry and functional changes in cortical neurones, contributing to the pronounced seizure phenotype observed in $stg$ mice (Di Pasquale et al., 1997; Letts et al., 1998).
Figure 1.16 — Properties of stargazin that identify it as a candidate neuronal VDCC γ subunit

a) Secondary structure of the VDCC γ (grey line) and putative neuronal γ2 subunits (black line) predicted by the prediction program TM Pred (Hofmann and Stoffel, 1993). Positive scores show hydrophobic regions predicted to form transmembrane spanning segments whilst negative scores denote hydrophilic residues. The transmembrane profiles of γ1 and γ2 are extremely similar apart from γ2 being approximately 100 amino acids longer in the C-terminus. b) Average steady-state inactivation curves for Cav2.1/βα2δ1 channels stably expressed in BHK cells alone, with a green fluorescent protein (GFP) marker, or GFP and the γ2 subunit. A 7mV hyperpolarizing shift is recorded on co-expression of the γ2 subunit. Figures reproduced and modified from Letts et al. (1998).

1.3 Stargazer foretells a constellation of γ subunits: Aims of the investigation

Despite many years of evidence to the contrary, the work of Letts et al. (1998) provided the first indication that neuronal VDCC γ subunits exist, and possibly form part of functional channels in vivo. The aim of this thesis project was to identify, clone and characterise the functional properties of a family of human stargazin-like genes, starting with the human orthologue of mouse stargazin (γ2). The identification of the human γ2 orthologue and any other homologues was performed using “in silico” technologies to search for fragments of human nucleotide and protein sequence similar to the mouse cacng2 gene or γ2 protein sequence. The Genbank/EMBL databases presented multiple genomic and expressed nucleotide “hits” that were assembled into clusters of similar overlapping sequences that represented the nucleotide sequences of putative VDCC γ subunits. Subsequently, more traditional laboratory molecular biology techniques were employed to clone the cDNAs for each predicted γ subunit and analysis of their predicted primary structure identified regions of conservation with other known proteins.
The differential expression profile of each putative \( \gamma \) subunit was probed by northern blot in both multiple human tissues and multiple brain regions. Specific polyclonal antibodies were generated against regions of lowest shared homology between the putative \( \gamma \) subunits and used in immunohistochemistry experiments to develop a more detailed protein distribution data for these species in human cerebellum and hippocampus. Whether alone, or in combination with functional VDCC complexes transiently expressed in COS-7 cells, these same antibodies also determined the subcellular localisation of the \( \gamma \) subunits by immunocytochemistry combined with confocal scanning laser microscopy. This investigation served a dual purpose in ensuring the \( \gamma \) cDNAs expressed \textit{in vitro} before embarking upon the characterisation of their influence upon VDCC biophysics. This was determined by two-electrode voltage clamp of \textit{Xenopus} oocytes expressing functional VDCC complexes in the absence or presence of one of the newly identified \( \gamma \) subunits. Their influence upon whole cell \( \text{Ba}^{2+} \) current, the voltage dependence of activation and inactivation, and the inactivation kinetics were investigated.

The combined molecular biology, expression and functional evidence amassed for the proteins identified and cloned on the basis of their similarity to stargazin, resulted in their classification into two distinct subfamilies of human \textit{stargazin}-like genes on the basis of their differing level of homology to stargazin and their vastly contrasting functional properties when expressed \textit{in vitro} with VDCCs.

### 1.4 Epilogue to introduction

During the years in which this thesis project has proceeded, the identification, cloning and characterisation of proteins related to stargazin, has become a highly dynamic and controversial field of study. In addition to my own work, many laboratories have also sought novel homologues of the putative neuronal mouse VDCC \( \gamma_2 \) subunit and to characterise their functional properties. The findings of these laboratories were reported in the following publications (Black and Lennon, 1999; Burgess \textit{et al.}, 1999b; Burgess \textit{et al.}, 2001; Chen \textit{et al.}, 2000; Chu \textit{et al.}, 2001; Green \textit{et al.}, 2001; Kang \textit{et al.}, 2001; Klugbauer \textit{et al.}, 2000; Roussel \textit{et al.}, 2001; Sharp \textit{et al.}, 2001). Reference to these articles will be made at the appropriate times when discussing my own findings in the
ensuing chapters, and their bearing upon any experiments performed subsequent to their publication explained.
Chapter 2  

Materials & Methods

2

Materials & Methods
2.1 Cloning on a computer

2.1.1 An introduction to “in silico” cloning

Until the late 1990’s, the conventional approach to identifying the orthologues (the same genes but in a different species) and paralogues (related genes in the same species) of a particular gene was by hybridisation screening cDNA libraries or bacterial or yeast artificial chromosome (BAC or YAC respectively) Southern blots to detect colonies or restriction fragments containing sequence complimentary to the gene of interest. Alternatively, “shotgun” polymerase chain reaction (PCR) experiments using degenerate oligonucleotide primers and low annealing temperatures have been used to amplify homologues or splice variants of different genes. Whilst successful in identifying a plethora of genes from many species, these techniques are labour intensive, time consuming and success depends upon the researcher optimising each experimental variable, particularly when attempting to clone a gene expressed at low levels or with poor homology to the probes/primers being used.

The more modern approach to identifying novel genes is by applying the relatively new bioinformatics techniques. Bioinformatics is the science of developing computer databases and algorithms for the purpose of speeding up and enhancing biological research. An integration of mathematical, statistical and computer methods are applied to analyse biological, biochemical and biophysical data. Bioinformaticians who apply their skills in the field of molecular biology, can use computer software to query the sequence of a known gene, cDNA (complementary DNA) fragment, protein or polypeptide against the wealth of DNA and protein sequences stored in the databases of the International Nucleotide Sequence Database Collaboration (comprised of the DNA DataBank of Japan (DDBJ), the European Molecular Biology Laboratory (EMBL), and GenBank at the National Center for Biotechnology Information (NCBI)), and predict the sequences of entire novel genes or proteins from the fragments of DNA or protein shown to have homology to the query sequence, all without performing a single experiment in a laboratory. The phrase “in silico” cloning has been coined to describe this technique.
Chapter 2 Materials & Methods

Naturally, conventional molecular biology techniques are still required to physically clone cDNAs and genes, but in silico cloning facilitates this process by providing a full or partial sequence of a predicted gene that can be used as a template to design primers that will specifically amplify the intended target in a PCR reaction. Alternatively, the BAC, YAC, or cDNA library clone in which a sequence of interest has been determined to reside can be ordered from the relevant source for use in subsequent experiments.

A prime example of the successful application of in silico cloning in neuroscience was the cloning of the previously elusive Cav3 family of T-type VDCC $\alpha$ subunits (Perez-Reyes, 1998). In a similar fashion, this investigation utilised in silico techniques to identify a family of putative human neuronal VDCC $\gamma$ subunits, a group of proteins that like the T-type $\alpha$ subunits had escaped being cloned by conventional molecular biological approaches since the discovery of the skeletal-muscle $\gamma_1$ subunit many years before (Bosse et al., 1990; Powers et al., 1993).

2.1.2 In silico cloning of a family of human VDCC $\gamma$ subunits

2.1.2.1 Identification of human ESTs related to the mouse cacng2 by in silico analysis

The GenBank and EMBL databases were searched for sequences possessing homology to the full-length sequence of the mouse cacng2 gene and $\gamma_2$ protein (AF077739) (Letts et al., 1998) using the BLASTn and tBLASTn alignment programs (Altschul et al., 1990). BLAST (Basic Local Alignment Search Tool) is a set of similarity search programs designed to explore all of the available sequence databases regardless of whether the query is protein or DNA. BLASTn directly compares a nucleotide query sequence against a nucleotide sequence database, whilst tBLASTn compares a protein query sequence against a nucleotide sequence database dynamically translated in all reading frames (Altschul et al., 1990). Multiple human expressed sequence tags (ESTs) (Boguski et al., 1993; Soares et al., 1994) and genomic sequence from both databases with identities to cacng2 $\geq$ 40% were clustered to identify overlapping identical sequence belonging to the same transcript using the GCG package (Wisconsin Package Version 9.0, Genetics Computer Group), and a program developed by GlaxoSmithKline.
Research and Development Bioinformatics group, termed ESTBlast (Gill et al., 1997). Multiple alignments of in silico nucleotide sequences or conceptually translated proteins were generated using the Clustal W algorithm (Omiaga 1.1, Oxford Molecular Group, Oxford, UK).

2.1.2.2 Identification and analysis of a human genomic sequence related to CACNG7

The in silico-derived 487bp EST cluster subsequently determined to be part of the CACNG7 nucleotide sequence was used as a query sequence using the tBLASTx program (compares the six-frame translations of a nucleotide query sequence against the six-frame translations of a nucleotide sequence database) to identify related human genes in the genomic sequence division of Genbank. BACs identified to contain sequence with homology to the query were analysed with the gene prediction program Genscan (Burge and Karlin, 1997) and each predicted gene was submitted to the tBLASTn program to identify which of these genes was related to the CACNG7 sequence.

2.1.2.3 Identification and analysis of mouse genomic sequences related to CACNG5 and CACNG7

The nucleotide and protein sequences of putative human γ5 and γ7 were compared to the high throughput genomic sequence (HTGS) subset of Genbank using BLASTn and tBLASTn respectively with default parameters. Genscan identified putative genomic structure of the murine orthologues within the BAC clones in which they had been identified. The complete in silico genes were assembled from these data.

2.2 Standard molecular biology procedures

2.2.1 cDNA sources and synthesis

Human brain total RNA was purchased from Invitrogen (Paisley, UK) or mouse cerebellar total RNA purified from the cerebella of 31 day old mice using the RNeasy
kit (Qiagen, Crawley, UK), were used to generate cDNA using the Superscript Pre-amplification System (Invitrogen) primed with random hexamers (section 2.2.1.2). 5' or 3' rapid amplification of cDNA ends (RACE) experiments were performed using Marathon Ready total human brain cDNA (BD Biosciences Clontech, Basingstoke, UK – section 2.2.1.2.3) or cDNA generated from human brain total RNA using the SMART PCR cDNA Synthesis Kit (BD Biosciences Clontech – section 2.2.1.2.4).

Mouse cerebellum total RNA was prepared from freshly obtained tissue or tissue that had been snap frozen in liquid nitrogen and stored at -70°C. Mice were killed by cervical dislocation and 25-50mg of dissected tissue placed in an Eppendorf tube and homogenised in 350ml of the RLT buffer (proprietary buffer containing guanidinium thiocyanate, 25-50%) from the RNeasy kit. The tissue lysate was centrifuged for 3 min at maximum speed in a microcentrifuge. The supernatant was carefully transferred to a new microcentrifuge tube by pipetting. 350 μl of 70% ethanol was added to the cleared lysate and mixed. The sample, including any precipitate that may have formed was applied to an RNeasy mini column placed in a 2 ml collection tube and centrifuged for 15 s at 8000 × g. The flow-through was discarded and 700μl buffer RW1 (proprietary buffer containing 2.5-10% guanidinium thiocyanate and 2.5-10% ethanol) added to the RNeasy column. The tube was centrifuged for 15s at 8000 × g to wash the column. Flow-through was discarded. The RNeasy column was transferred into a new 2 ml collection tube, 500μl buffer RPE added onto the RNeasy column and centrifuged for 15 s at 8000 × g to wash the column. This was repeated with another 500μl RPE buffer and spun for 2 min to dry the column. To elute the RNA, 30–50 μl RNase-free water was pipetted directly onto the RNeasy silica-gel membrane. The tube was centrifuged for 1 min at 8000 × g. RNA was stored at -70°C until ready for use.

2.2.1.1 Concentration and purity of extracted mRNA

Using a spectrophotometer the quantity and purity of mRNA extracted from the cells was determined. A 5μl sample of the mRNA was added to 595μl of DEPC treated water (using 5μl of sodium dodecyl sulphate in 595μl of DEPC treated water as a negative control blank). From this dilute sample absorbance readings were taken at 260 nm and 280 nm wavelengths. At 260 nm an optical density (OD$_{260}$) of 1 is equivalent to 40
\( \mu g/ml \), therefore allowing calculation of the concentration of mRNA in the sample. The ratio of \( OD_{260}/OD_{280} \) defines the sample purity: a ratio <1.8 is indicative of protein impurities, whilst a ratio >2.0 suggests sample degradation. Only samples giving \( OD_{260}/OD_{280} \) ratios between 1.8-2.0 were used.

### 2.2.1.2 First strand cDNA synthesis from Total RNA

The first strand cDNA synthesis reaction was catalysed by Superscript II RNase H reverse transcriptase (RT) (Invitrogen). RNase H activity degrades mRNA during the first strand reaction and is present in most naturally occurring RTs, however Superscript II RT has been engineered from M-MLV (Moloney murine leukaemia virus) RT (Gerard, 1978; Kotewicz et al., 1988) to remove the RNase H activity. This modification improves the enzymes ability to copy long RNA compared to M-MLV RT, whilst retaining the full DNA polymerase activity. Frequently, RNA preparations contain small amounts of genomic DNA that may be subsequently amplified along with the target cDNA. In all reverse transcriptions a without RT control was performed to determine whether amplified fragments were of genomic of cDNA origin. The positive control was reverse transcription of a control human placental total RNA, and PCR amplification of a known product from the resulting cDNA.

#### 2.2.1.2.1 Priming with Random hexamers

Priming with random hexamers is the most non-specific method of synthesising first strand cDNA. As their name implies, these primers are collections of six base oligonucleotides of multiple possible combinations of the four deoxyribonucleotide triphosphates (dNTPs - dATP, dTTP, dGTP and dCTP; 10mM dNTP mix, Invitrogen). All RNAs in a population are templates for reverse transcription in this method, and specific PCR primers confer the needed specificity during subsequent PCR reactions. To maximise the size of cDNA synthesised using random hexamers, the ratio of primers to RNA needs to be optimised for each preparation. Lowering the proportion of hexamers to template may reduce the cDNA yield but increases the number of full-length transcripts.
2.2.1.2.2 First strand synthesis reaction conditions

<table>
<thead>
<tr>
<th>Component</th>
<th>Sample</th>
<th>No RT control</th>
<th>Control RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total RNA 1μg/μl</td>
<td>1μl</td>
<td>1μl</td>
<td>-</td>
</tr>
<tr>
<td>Control RNA (50ng/μl)</td>
<td>-</td>
<td>-</td>
<td>1μl</td>
</tr>
<tr>
<td>Random hexamers (50ng/μl)</td>
<td>1μl</td>
<td>1μl</td>
<td>1μl</td>
</tr>
<tr>
<td>DEPC-treated water</td>
<td>10μl</td>
<td>10μl</td>
<td>10μl</td>
</tr>
</tbody>
</table>

RNA/primer mixtures were prepared in sterile tubes as outlined in Table 2.1. Samples were incubated at 70°C for 10 min and then placed on ice for at least 1 min. The reaction mixture was prepared by adding each component in the order displayed in Table 2.2.

<table>
<thead>
<tr>
<th>Component</th>
<th>Each reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 X PCR Buffer</td>
<td>2μl</td>
</tr>
<tr>
<td>25mM MgCl₂</td>
<td>2μl</td>
</tr>
<tr>
<td>10mM dNTP mix</td>
<td>1μl</td>
</tr>
<tr>
<td>0.1M DTT</td>
<td>2μl</td>
</tr>
</tbody>
</table>

For n samples, plus one no RT control and one control RNA reaction, reaction mix for n + 3 reactions was prepared. 7μl of reaction mixture was added to each RNA/primer mixture, mixed gently, collected by brief centrifugation and incubated at 25°C for 5 min, after which 200 units of RT was added to each tube except the no RT control (substitute with dH₂O). Tubes were incubated at 25°C for a further 10 min before being transferred to a thermostatically controlled water-bath or programmable thermocycler holding a constant temperature of 42°C for 50 min. Reactions were terminated by incubating tubes at 70°C for 15 min then chilled on ice. Following brief centrifugation, 2 units of RNase H was added to each tube, which were incubated at 37°C for 20 min to digest parent RNA strands.

2.2.1.2.3 Marathon ready cDNA

Each tube of Marathon Ready total human brain cDNA (BD Biosciences Clontech) is essentially an uncloned library of double stranded (ds) cDNA to which the suppliers have ligated a specific adaptor sequence to each end. Gene specific primers (GSPs) and primers complementary to the adaptor sequence can be used in 5’ or 3’ RACE. The
Chapter 2
Materials & Methods

specificity of marathon race reactions is greatly enhanced by the absence of an extension from the GSP during the first RACE cycle. The presence of an amine group on the Marathon cDNA adaptor blocks the extension of the 3' end of the adaptor-ligated ds cDNAs, and thus prevents formation of an adaptor primer 1 (AP1) site in the general population of cDNAs, and facilitates its inclusion only in the cDNA of interest during the first RACE cycle Figure 2.1.

![Diagram of Marathon RACE reactions](image)

**Figure 2.1 – The templates and primers used in Marathon RACE reactions**

cDNA synthesis and adaptor ligation creates a population of cDNAs with the depicted structure. RACE PCR using AP1 and a GSP followed by second nested race using AP2 and a nested GSP (NGSP) amplifies the region of interest. Figure reproduced from Marathon Ready cDNA user manual.

2.2.1.2.4 **SMART cDNA synthesis**

The SMART (Switching Mechanism At 5' end of RNA Transcript) RACE cDNA amplification kit (BD Biosciences Clontech) provides a mechanism for generating full-length cDNAs in reverse transcription reactions. When certain M-MLV RT variants reach the end of an RNA template, they exhibit a terminal transferase activity that adds 3-5 residues (predominantly deoxycytosine (dC)) to the 3' end of the first-strand cDNA. This activity is harnessed by the SMART oligonucleotide (5'-AAGCAGTGGTAAC-AACGCAGAGTACGCGGG-3'), whose terminal stretch of deoxyguanosine (dG) bases can anneal to the dC-rich cDNA tail and serve as an extended template for RT. After RT switches templates from the mRNA molecule to the SMART oligonucleotide, a complete cDNA copy of the original RNA is synthesised with the additional SMART
sequence at the end. Since the dC tailing activity is typically most efficient if the enzyme has reached the end of the mRNA template, the SMART sequence is usually added only to complete first strand cDNAs. Therefore the use of high quality mRNA template will result in the formation of a set of cDNAs that has maximum amount of 5' sequence. The SMART sequence can then be used as the 5' primer target in 5' RACE PCR protocols (Figure 2.2).

![Figure 2.2- Mechanism of SMART cDNA synthesis](image)

First-strand synthesis is primed using a modified oligo(dT) primer. After RT reaches the end of the mRNA template, its terminal transferase adds several dC residues. The SMART oligonucleotide anneals to the tail of the cDNA and serves as an extended template for RT.

### 2.2.1.2.4.1 SMART first strand cDNA synthesis protocol

RNA/primer mixtures were prepared in sterile tubes as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Sample</th>
<th>No RT control</th>
<th>Control RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total RNA (1 μg/μl)</td>
<td>3 μl</td>
<td>1 μl</td>
<td>-</td>
</tr>
<tr>
<td>Control RNA (50 ng/μl)</td>
<td>-</td>
<td>-</td>
<td>1 μl</td>
</tr>
<tr>
<td>Oligo (dT) (0.5 μg/μl)</td>
<td>1 μl</td>
<td>1 μl</td>
<td>1 μl</td>
</tr>
<tr>
<td>SMART oligonucleotide</td>
<td>1 μl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEPC-treated water</td>
<td>5 μl</td>
<td>10 μl</td>
<td>10 μl</td>
</tr>
</tbody>
</table>

Table 2.3 - RNA/primer mixtures for SMART 1st strand cDNA synthesis

From this point, the standard RT protocol as described for random hexamer primed cDNA was followed including the same reaction mixtures described in Table 2.2. A successful reverse transcription was checked by a control RACE experiment using the 5' SMART universal primer mix and a Transferrin receptor (TFR) GSP that should result in the amplification of a 2.6-kilobase (kb) band.
2.2.2 **Polymerase Chain Reaction (PCR)**

The polymerase chain reaction (PCR) results in the selective amplification of a particular portion of chosen DNA molecule (Frohman *et al.*, 1988). Any region can be chosen, so long as the sequences at the borders are known. These border regions provide the sequence to which two short oligonucleotide primers hybridise, one to each strand of the double helix, delineating the region to be amplified. A thermostable DNA polymerase 1 enzyme extends the primers towards each other in a reaction cycle of three steps:

1) **Denaturation** – heating to 94-98°C denatures DNA double helix presenting bases for hybridisation with primers.

2) **Primer annealing** – cooling the temperature to 50-55°C allows primers to anneal to their targets on template sequence. The higher the annealing temperature the more specific is the primer-template association. The optimum annealing temperature depends upon the primer melting temperature ($T_m$), the temperature at which a correctly base paired primer-template hybrid "melts", which is dictated by primer length and nucleotide content. $T_m$ can be calculated by the simple formula:

$$T_m = (4 \times [G + C]) + (2 \times [A + T]) \degree C$$

If annealing temperature is too high, primers and template remain dissociated. If annealing temperature is too low primer mismatch occurs generating false positives.

3) **Extension** – Usually occurs at 72°C, the approximate optimum temperature at which thermostable polymerases extend the primers towards one another by adding dNTPs from the reaction mixture to their 3’- ends.

The region of DNA encompassed by the primers is exponentially amplified by repetition of these cycles.

Different thermostable polymerase 1 molecules have been isolated that can be utilised for different purposes in PCR reactions. Each thermophilic polymerase has unique characteristics, such as pH and salt optima that affect the efficacy of the PCR protocol.
Chapter 2  
Materials & Methods

The enzymes are usually sold in unit quantities. One unit (1U) is defined as the amount of enzyme required to catalyse the incorporation of 10nmol of dNTP into acid-insoluble material in 30 minutes at 74°C.

2.2.2.1 Taq polymerase

*Taq* DNA Polymerase (Chien *et al.*, 1976) is a thermostable enzyme isolated from thermophilic bacterium *Thermus aquaticus*. This unmodified enzyme replicates DNA at 74°C and exhibits a half-life of 40 minutes at 95°C (Chien *et al.*, 1976). The enzyme catalyzes the polymerization of nucleotides into duplex DNA in the 5'→3' direction in the presence of magnesium. However, *Taq* has a relatively high error rate (one error ~ every 125,000 nucleotides) since it does not have a 3'→5' exonuclease proofreading function. The most common mutations caused by *Taq* are AT to GC transitions (Keohavong and Thilly, 1989) or it can generate deletion mutations if the template DNA has the potential to form secondary structures (Cariello *et al.*, 1991). However, the error rate of *Taq* polymerase can be greatly reduced by modifying the reaction conditions (Ling *et al.*, 1991). If used to amplify a novel DNA fragment, sequencing of products from at least three repeats of the same PCR experiment is required to confirm correct sequence. *Taq* polymerase reaction mixtures and PCR protocols are outlined in Table 2.4 and Table 2.5 respectively.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount per reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNA template (25ng/µl)</td>
<td>1-2.5 µl</td>
</tr>
<tr>
<td>Gene Specific Primer 1 (10pmol/µl)</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>Gene Specific Primer 2 (10pmol/µl)</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>10 × Thermophilic buffer *</td>
<td>5µl</td>
</tr>
<tr>
<td>MgCl₂ (25mM)</td>
<td>2.5 µl</td>
</tr>
<tr>
<td><em>Taq</em> polymerase</td>
<td>1µl (2.5 U)</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>0.5 µM</td>
</tr>
<tr>
<td>Sterile deionised H₂O (dH₂O)</td>
<td>To 50µl</td>
</tr>
</tbody>
</table>

Table 2.4 - Standard *Taq* polymerase reaction mix for a 50µl PCR reaction

* Stock 10 × Thermophilic Reaction Buffer without MgCl₂: 500mM KCl, 100mM Tris-HCl (pH 9.0 at 25°C) and 1.0% Triton X-100. If reaction mixtures differed the altered parameter will be stated in the text.
**Chapter 2**

**Materials & Methods**

<table>
<thead>
<tr>
<th>Protocol</th>
<th><em>Taq 30</em></th>
<th><em>Taq 60</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Temp</strong></td>
<td><strong>Time</strong></td>
<td><strong>Cycles</strong></td>
</tr>
<tr>
<td>Denature</td>
<td>95°C</td>
<td>1 min</td>
</tr>
<tr>
<td>Denature</td>
<td>95°C</td>
<td>30 s</td>
</tr>
<tr>
<td>Anneal</td>
<td>50°C</td>
<td>30 s</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>30 s</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>10 min</td>
</tr>
<tr>
<td>Cool</td>
<td>Hold @ 4°C</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 2.5 - *Taq* polymerase protocols

Referred to as *Taq30* and *Taq60* from this point onwards, these were the standard PCR parameters programmed into thermocycler for reactions employing *Taq* polymerase.

*Taq* polymerase possesses a nontemplate-dependent terminal transferase activity that adds a single deoxyadenosine (dA) to the 3’ ends of PCR products. These 3’ A overhangs can be exploited to insert the fragment into linearised vectors containing single deoxythymidine (dT) residue overhangs. This activity can also be exploited to add single 3’ A overhangs onto the ends of blunt ended PCR products or restriction fragments, by incubating the cDNA fragment with *Taq* at 72°C for 15 minutes in buffer containing dATPs.

### 2.2.2.2 *Pfu* and *Pfu Turbo* polymerases

*Pfu* is a high fidelity polymerase identified in *Pyrococcus furiosus*, which is very thermostable (Stratagene, La Jolla, CA). It can tolerate temperatures exceeding 95°C, enabling it to PCR amplify GC-rich targets and has a very low error rate (~ once every 767,000 nucleotides) because it possesses a 3’→5’ exonuclease proofreading function. However, *Pfu* is much slower than *Taq*, requiring a minimum extension time of 1.5 - 2 minutes/kb of amplified template and it generates blunt ended PCR products.

*Pfu Turbo* polymerase is merely an enhanced version of *Pfu* polymerase, sold as a blend of cloned *Pfu* and a novel thermostable factor added by the suppliers (Stratagene) that enhances PCR product yields without altering DNA replication fidelity. The error rate of *Pfu Turbo* is the same as for *Pfu* and significantly lower than that of *Taq*. The main advantage of using this enzyme over *Pfu* is that its enhanced performance allows the use of shorter extension times, which dramatically shortens a PCR protocol if amplifying a large target. Moreover, *Pfu* and *Pfu Turbo* both have minimal activity at 40-50°C resulting in fewer mispaired primer-extension reactions than occur with *Taq*. 

108
Chapter 2  Materials & Methods

Pfu and Pfu Turbo reaction mixes and PCR protocols are detailed in Table 2.6 and Table 2.7 respectively. Where stated, some amplification reactions additionally contained 20% DMSO in the reaction mix. This served to reduce the energy requirements to melt G-C rich regions and disrupt any mRNA secondary structure when the amplification of a particular target proved difficult using standard parameters.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount per reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNA template (25ng/μl)</td>
<td>1-2.5 μl</td>
</tr>
<tr>
<td>Gene Specific Primer 1 (10pmol/μl)</td>
<td>2.5 μl</td>
</tr>
<tr>
<td>Gene Specific Primer 2 (10pmol/μl)</td>
<td>2.5 μl</td>
</tr>
<tr>
<td>10 × Pfu Thermophilic buffer *</td>
<td>5μl</td>
</tr>
<tr>
<td>Pfu/ Pfu turbo polymerase</td>
<td>1 μl (2.5 U)</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>0.5 μM</td>
</tr>
<tr>
<td>Sterile deionised H2O (dH2O)</td>
<td>To 50μl</td>
</tr>
</tbody>
</table>

Table 2.6 - Standard Pfu and Pfu Turbo polymerase reaction mix for a 50μl PCR reaction

* Stock 10 × Pfu Thermophilic Reaction Buffer: 100mM KCl, 20mM MgSO4, 100mM (NH4)2SO4, 200mM Tris-HCl (pH 8.8 at 25°C), 1.0% Triton X-100 and 1mg/ml nuclease free BSA. If reaction mixtures differed the altered parameter will be stated in the text.

Table 2.7 - Pfu and Pfu Turbo polymerase protocols

These were the standard PCR parameters programmed into thermocycler for reactions employing Pfu and Pfu Turbo polymerases.

<table>
<thead>
<tr>
<th>Protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pfu</td>
</tr>
<tr>
<td>Temp</td>
</tr>
<tr>
<td>Denature</td>
</tr>
<tr>
<td>Denature</td>
</tr>
<tr>
<td>Anneal</td>
</tr>
<tr>
<td>Extension</td>
</tr>
<tr>
<td>Extension</td>
</tr>
<tr>
<td>Cool</td>
</tr>
</tbody>
</table>

2.2.2.3 "Touchdown" PCR

Touchdown PCR involves decreasing the annealing temperature every second cycle to a 'touchdown' annealing temperature, which is then used for 10 or so cycles. It was originally intended to simplify the complicated process of determining optimal annealing temperatures. Annealing takes place at approximately 15°C above the calculated Tm. During the following cycles, the annealing temperature is gradually reduced by 1°C until it has reached a level of approximately 5°C below Tm. The concept is that any differences in Tm between correct and incorrect annealing gives a 2-fold difference in product amount per cycle (4-fold per °C). This allows a critical concentration of the correct product to build, therefore increasing the ratio between correct and incorrect products (Don et al., 1991).
Splice-overlap PCR is a method of amplifying a single cDNA fragment from two or more cDNAs with overlapping sequence. This technique was used in this investigation to assemble complete cDNAs of putative γ subunits where restriction digest and ligation was not feasible because of a lack of unique restriction sites. PCR parameters are much the same as for a normal PCR, however, two or more template cDNAs are included in the mix with a GSP designed to the 5' and 3' extremities of the intended final cDNA fragment. If we consider the simplest case scenario with two template fragments, the GSPs anneal to their specific targets and extend each fragment during the first cycle. However if sufficient overlap between the target sequences exists, they also prime one another resulting in their extension in the 5' → 3' direction. This generates a template for the GSPs in the second round of PCR from which the intended final cDNA fragment can be amplified Figure 2.3.

Figure 2.3 – The splice overlap PCR principle
Top) The two cDNA template fragments are primed in the PCR reaction by a pair of gene specific primers (GSP1 & GSP2), one designed to a specific region of either fragment. The regions of sequence overlap between the two template strands also act as primers and during the first round of PCR (middle) the templates are extended as well as each individual short fragment. Bottom) The next PCR cycle begins to amplify the intended cDNA following creation of the extended templates to which GSP1 and GSP2 can anneal. This fragment is exponentially amplified because GSPs are able to extend sense and antisense strands in each cycle whereas amplification of the short original templates is linear, only amplifying the complementary strands to each template, resulting in greatly enhanced levels of “spliced” product over amplified templates.
2.2.3 DNA isolation and purification

2.2.3.1 Agarose gel electrophoresis

The number and size of the cDNA fragments produced by a PCR reaction or restriction digest (section 2.2.5.1) can be determined by agarose gel electrophoresis. An appropriate amount of agarose was weighed and then dissolved in 1 x TAE buffer (0.04M Tris acetate, 0.001M EDTA (pH 8)). The agarose concentration was chosen depending on the size of DNA to be separated Table 2.8.

<table>
<thead>
<tr>
<th>Agarose</th>
<th>Size of DNA fragments separated</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5%</td>
<td>1 - 30 kb</td>
</tr>
<tr>
<td>0.7%</td>
<td>0.8 - 12 kb</td>
</tr>
<tr>
<td>1.0%</td>
<td>0.5 - 10 kb</td>
</tr>
<tr>
<td>1.2%</td>
<td>0.4 - 7 kb</td>
</tr>
<tr>
<td>1.5%</td>
<td>0.2 - 3 kb</td>
</tr>
<tr>
<td>2.0%</td>
<td>0.05 - 2 kb</td>
</tr>
</tbody>
</table>

The gels were cast in agarose gel electrophoresis apparatus (Flowgen, Ashby de la Zouche, UK) and the electrophoresis tank filled with 1 x TAE prior to gel loading. Loading buffer (final concentration: sucrose 40% (v/v), bromophenol blue 0.25%, EDTA 0.1M (pH 8), SDS 0.5% (w/v)) was mixed with the sample before loading and the appropriate markers were loaded in adjacent lanes. Gels were routinely run at 90-110 V. DNA bands were visualised by staining the gel in a 1μg/ml ethidium bromide (EtBr) in TAE solution for 10-15 min and placed on an ultraviolet light box. If the DNA was required for further cloning the bands were excised from the gel with a sterile scalpel and purified. Agarose gels were photographed using a Polaroid camera or a Gel Doc imaging system (Bio-Rad, Hercules, CA, USA).

2.2.3.1.1 Purification of cDNA from agarose gels

DNA was purified from agarose gels using the Qiaex II kit (Qiagen, Crawley, UK). Gel slices excised from electrophoresis gels were weighed and dissolved in three volumes (e.g. 300μl to 100mg of gel) of buffer QX1 (a high salt agarose solubilisation buffer that does not contain NaI). 10μl of Qiaex II silica beads was added to the tube (for up to 2μg DNA) and the mixture waswarmed to 50°C to melt the agarose and allow the silica
beads to adsorb the DNA in the high salt conditions. Following a 10min incubation, the mixture was centrifuged for 30s and the supernatant drawn off. The pellet was washed with 500μl QX1 and re-centrifuged. The pellet was washed twice in 500μl buffer PE (ethanol, TrisCl, EDTA, and NaCl). The pellet was allowed to air dry for 15 minutes then the DNA was eluted from the glass beads by re-suspending in the required amount of double distilled sterile water and pelleting the beads by centrifugation. For fragments <4kb the suspension was incubated for 5 min at room temperature before centrifuging. For fragments of 4-10kb incubation was for 5min at 50°C, and fragments >10kb, 10 min at 50°C.

2.2.3.1.2 Purification of cDNA from solutions

For fragments of 100bp-4kb three volumes of QX1 were added to the solution. For fragments less than 100bp 6 volumes were added and for fragments larger than 4kb three volumes of QX1 plus two volumes of dH2O were added. 10μl of Qiaex II silica beads were added per 5μg of DNA, the solution mixed and incubated at room temperature with frequent vortexing. The pellet was washed twice in 500μl PE buffer and DNA eluted as described for agarose gels.

2.2.4 Cloning cDNA

2.2.4.1 pCR-TOPO

PCR products were usually cloned in to the cloning vectors pCRII-TOPO, pCR2.1-TOPO or pCR4Blunt-TOPO (Invitrogen, Figure 2.4) following amplification and purification. These vectors exploit the ligation activity of topoisomerase I by providing a linearised vector that is “activated” by the association of topoisomerase molecules to either end. Ligation of the vector with a PCR product containing 3‘ A overhangs (use pCRII-TOPO or pCR2.1-TOPO) or blunt products (use pCR4Blunt-TOPO), occurred spontaneously within 5 minutes at room temperature. The cloning reactions were transformed directly into chemically competent E.coli and replicated. This facilitated rapid identification of PCR products because purification of the plasmids from the transformed E.coli yielded large amounts of template that contained T7, T3, Sp6, M13 and M13R common primer sites, which could be used to sequence the insert without requiring primers specific to the insert. The site of insert ligation was in the middle of
a multiple cloning site (MCS) of each vector that could be used to subclone the fragment into other vectors if desired.

Transformed *E.coli* were selected for ampicillin or kanamycin resistance and these vectors also allowed blue/white screening of positive clones because they contain the *LacZα* gene that is interrupted by the MCS. When transformed *E.coli* were grown in the presence of X-gal substrate (40μl of 40 mg/ml stock per dish) positive clones produced white colonies but vector without insert resulted in blue colonies. Whilst usually sufficient for colony selection, blue/white screening was sometimes ambiguous, with some colonies being light blue. pCR4Blunt-TOPO circumvented this problem because it contains a *LacZα-ccdB* gene fusion, which is lethal to the *E.coli* if not interrupted by an insert. Therefore any colonies that survive almost certainly contain plasmid with the PCR product inserted at the MCS.
Materials & Methods

pCR® II-TOPO (a) was used to clone fragments amplified by Taq polymerase, the overhanging dT residues ligate with overhanging dA residues on the PCR fragment. pCR2.1-TOPO differs from pCRII-TOPO as it lacks the Sp6 promoter site. PCR4Blunt-TOPO (b) was used to clone Pfu polymerase blunt ended PCR products. Maps reproduced from http://www.invitrogen.com.

Figure 2.4
Invitrogen pCR-TOPO vector maps and multiple cloning sites

pCRII-TOPO (a) was used to clone fragments amplified by Taq polymerase, the overhanging dT residues ligate with overhanging dA residues on the PCR fragment. pCR2.1-TOPO differs from pCRII-TOPO as it lacks the Sp6 promoter site. PCR4Blunt-TOPO (b) was used to clone Pfu polymerase blunt ended PCR products. Maps reproduced from http://www.invitrogen.com.
2.2.4.2 Transformation of competent cells

DNA was produced by overexpression in chemically competent Top 10 (Invitrogen) or DH5α (Gibco) E.coli strains. DNA was introduced into the cells by heat-shock transformation and reproduced episomally. 50μl of competent cell suspension was placed into chilled 1.5ml tubes and incubated with 10-100ng of plasmid. The tubes were left on ice for 30 min to allow the DNA to adhere to the bacterial cell wall. Top 10 bacteria were then heat-shocked by placing in a hot water-bath at 42°C for 2 min, whilst DH5α bacteria were heat-shocked at 37°C for 40 seconds. This allows the DNA to enter the cells. Pre-warmed Luria-Bertani (LB) media (1.0% tryptone, 0.5% yeast extract, 1.0% NaCl, 20 mM glucose, pH 7.0) (200 μl for Top 10F; 995 μl for DH5α cells) was added to each tube and they were shaken horizontally at 37°C for 30min - 1h to allow antibiotic resistance to develop. The cell suspension was spread onto an autoclaved a 15g/l agar (in LB) plate containing the antibiotic (ampicillin 50 μg/ml unless otherwise stated). Plates were incubated overnight in a 37°C incubator to allow colonies to grow.

2.2.4.3 Bacterial culture

2.2.4.3.1 Small-scale

Small-scale cultures were grown for purification of plasmid cDNA on the miniprep scale or as starter cultures for large-scale cultures. A single colony selected from an agar plate was picked using a sterile inoculation loop and cultured overnight (for miniprep) or for 6-8 hours (starter culture) in 4ml of LB (containing 100μg/ml ampicillin) at 37°C whilst being shaken in a rotary incubator.

2.2.4.3.2 Large-scale

Large-scale cultures were grown for purification of plasmid cDNA on the maxiprep scale. 200μl of bacterial culture from a small-scale preparation was added to 200-250ml LB (containing 100μg/ml ampicillin) and cultures overnight at 37° C whilst being shaken in a rotary incubator.
2.2.4.4  Plasmid purification

2.2.4.4.1  Miniprep

Miniprep plasmid DNA purifications were performed to check correct insertion of ligations into the chosen vector by restriction digest. For this purpose the Qiagen spin miniprep kit was employed. This kit is based upon the alkaline lysis of bacterial cells (Birnboim and Doly, 1979) followed by adsorption of DNA onto silica in the presence of high salt. RNA, proteins, and low-molecular-weight impurities are removed by a medium-salt wash. Plasmid DNA is eluted in a low-salt buffer, and then concentrated and desalted by isopropanol precipitation. The buffers supplied with kit are summarised in Table 2.9. 1.5 ml of small-scale culture was centrifuged at 12,000 × g to pellet the E.coli. Supernatant was removed and the cells re-suspended in 250 µl of cell suspension buffer P1, containing RNase A. 250 µl cell lysis solution P2 was added to the homogenous cell suspension and the tubes were incubated at room temperature for 5 min. This allowed sodium dodecyl sulphate (SDS) to denature the bacterial proteins, NaOH to denature the chromosomal and plasmid DNA and RNase A to digest the RNA. 350 µl high-salt neutralisation buffer N3 was then added to each tube, which was capped and inverted 4-5 times to mix the contents. Vortexing was avoided at this step to prevent shearing of genomic DNA. The suspension was centrifuged 12,000 × g for 10 min at room temperature and the supernatant was transferred to a spin-cartridge placed in a wash tube. The spin-cartridge/wash tube assembly was centrifuged at 12,000 × g for 1 min at room temperature and the flow-through was discarded. The spin-cartridge was then washed with 700 µl of wash buffer PE, and centrifuged at 12,000 × g for 1 min at room temperature. The spin-cartridge was then placed in a 1.5 ml recovery tube and 75 µl of warm TE buffer was added directly to the centre of the spin-cartridge. After an incubation period of 1 min the spin-cartridge/recovery tube was centrifuged at 12,000 × g for 1 min in order to collect the eluted DNA.
Chapter 2  Materials & Methods

Buffer Composition

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>Cell Suspension Buffer (stored at 4°C) 50 mM Tris-HCl (pH 8) 10 mM EDTA, RNase A 100 μg/ml</td>
</tr>
<tr>
<td>P2</td>
<td>Cell Lysis Solution 200 mM NaOH, 1% SDS (w/v)</td>
</tr>
<tr>
<td>N3</td>
<td>Neutralisation Buffer Proprietary high salt buffer to create dehydrating conditions required for DNA to bind silica in miniprep columns. Contains 25-50% guanidinium chloride and 10-25% acetic acid</td>
</tr>
<tr>
<td>P3</td>
<td>Neutralisation Buffer 3.0 M Potassium Acetate pH 5.5, Guanidine hydrochloride</td>
</tr>
<tr>
<td>PE</td>
<td>Wash Buffer Proprietary Ethanol based wash buffer</td>
</tr>
<tr>
<td>QBT</td>
<td>Equilibration buffer 750 mM NaCl, 50 mM MOPS pH 7.0, 15%, isopropanol, 0.15% Triton X-100</td>
</tr>
<tr>
<td>QC</td>
<td>Wash Buffer 1.0 M NaCl, 50 mM MOPS pH 7.0, 15% isopropanol</td>
</tr>
<tr>
<td>QF</td>
<td>Elution buffer 1.25 M NaCl, 50 mM Tris Cl pH 8.5, 15% isopropanol</td>
</tr>
</tbody>
</table>

Table 2.9 - Plasmid purification kit buffer compositions

2.2.4.4.2 Maxi-prep

The Qiagen maxiprep kits were used in large-scale plasmid purifications. These kits are based upon the alkaline-lysis technique followed by binding of plasmid DNA to an anion-exchange resin under appropriate low-salt and pH conditions. All the necessary buffers are essentially as described in Table 2.9. 200-250 ml of large-scale bacterial culture was centrifuged at 6,000 × g for 15 min at 4°C to pellet the bacteria and the supernatant was removed. The cell pellet was re-suspended in 10 ml of Buffer P1. 10 ml of buffer P2 was then added to lyse the bacteria. 10 ml of chilled buffer P3 was added to neutralise and terminate these reactions. The suspension was centrifuged immediately at 20,000 × g for 30 min at 4°C then the supernatant was re-centrifuged for a further 15 min to remove any remaining particles. The supernatant was poured onto an equilibrated anion-exchange column (15 ml QBT) that binds double and single stranded DNA, and RNA and allowed to drain by gravity flow. The nucleic acids were then washed with 60 ml of Wash Buffer QC. The plasmids were eluted from the column by adding 15 ml elution buffer QF. 10.5 ml isopropanol was added to the eluate and the mixture was centrifuged at 15,000 × g for 30 min at 4°C in order to precipitate and pellet the DNA. The supernatant was discarded and the DNA pellet was washed with 5 ml 70% ethanol and re-centrifuged at 15,000 × g for 10 min at room temperature. The
pellet was air-dried and dissolved in the appropriate amount of water. The DNA concentration was checked by using a Beckman spectrophotometer and diluted to a working concentration of 1μg/μl.

2.2.5 Subcloning cDNA into expression vectors

2.2.5.1 Restriction Digest and ligation

2.2.5.1.1 Restriction digest

Restriction enzymes were purchased from New England Biolabs (Hatfield, UK), Promega or GibcoBRL (now part of Invitrogen). All digests in this investigation were to completion and digested DNA in preparation for subcloning, and to perform test digests to establish if ligations had been successful. Usually DNA was digested in excess enzyme at 37°C for at least 1hr. However this could be varied according to the specific enzymes being used. The total volume of enzyme and buffer used did not exceed 10% of the total volume of the reaction.

2.2.5.1.2 Ligation

Purified restriction fragments were ligated into the appropriately digested accepting vectors using 10% T4 DNA ligase and 10% ligase buffer (Gibco BRL). Reactions were incubated overnight at 14-16°C. A vector-only ligation was always performed as a negative-control. Ratios of insert to vector were varied from equimolar to 3:1 insert : vector to find optimal ligation conditions. Reaction volumes usually did not exceed 10μl and 2μl of completed reaction was transformed into supercompetent E. coli.

2.2.5.2 pMT2

The mammalian expression vector pMT2 carries eukaryotic regulatory regions derived from several sources. These include the origin of DNA replication containing T-antigen binding sites, early gene enhancer and polyadenylation signal from the Simian virus 40.
(SV40), and the adenovirus major late promoter, 21bp direct repeats that are recognised by cellular transcription factors (tripartite leader) and VA\textsubscript{I} gene. Expression of the T-antigen by the cell into which the plasmid is transfected increases plasmid copy number and therefore provides more templates that become transcriptionally activated over the course of transfection. The adenovirus tripartite leader and VA\textsubscript{I} gene serve to increase the efficiency of translation of the foreign gene inserted into the plasmid. pMT2 also possesses the murine dihydrofolate reductase (DHFR) gene at the 3’ end of the transcription unit. This is inefficiently translated, but serves to enhance the stability of mRNA transcribed from the plasmid, but can also be used as a marker of successful transient transfection. pMT2 was the expression vector of choice in this investigation because, the principal mammalian cell line used for transient expression in our laboratory is the COS-7 cell line that constitutively expresses the T-antigen (section 2.5.3.1.1). Moreover, cDNAs cloned into pMT2 are also efficiently expressed by Xenopus oocytes following intranuclear injection with the plasmid (Swick et al., 1992). The MCS of pMT2 has been modified in our laboratory to facilitate the easy cloning of many cDNAs for expression in COS-7 cells and oocytes. This linker is inserted at the EcoR\textsubscript{I} site of pMT2 in either the forward or reverse orientation to make the constructs pMT2LF or pMT2LR respectively (see Table 2.10 for linker restriction sites). All new constructs made in this investigation were subcloned into the pMT2LR version.

<table>
<thead>
<tr>
<th>Vector</th>
<th>Polylinker</th>
</tr>
</thead>
</table>

Table 2.10 – The pMT2LR multiple cloning site.

2.2.5.3 pcDNA3.1- myc/his

Fusion proteins of the novel γ subunits with C-terminal myc/his epitope were expressed by subcloning their cDNAs minus the stop codon, in frame into the multiple cloning site of the mammalian expression vector pcDNA3.1- myc/his (Invitrogen). These fusion proteins were used in immunocytochemistry investigations to co-localise the γ subunits in COS-7 cells with other transiently expressed proteins. This vector is part of the
pcDNA3 family of vectors (Mizushima and Nagata, 1990) that contain the powerful cytomegalovirus promoter, SV40 poly-adenylation region, and fl origin of replication used to transiently express many different proteins in multiple mammalian cell lines. Three different versions of the vector are supplied when purchased, named A, B and C that allow easy insertion of the chosen cDNA in frame with the myc/his C-terminal tag. Furthermore, the MCS is orientated in a + or – orientation to increase the versatility of the vector. Figure 2.5 displays the generic map of pcDNA3.1-myc/his. The vector version and restriction sites used to subclone the γ subunits are described in section 2.4.1.2.

Figure 2.5 – The pcDNA3.1/mvc-His vector map

The vector map displays key regulatory elements for mammalian expression and antibiotic resistance cassettes for bacterial and mammalian selection if required. The restriction site within the MCS of version A of both the – and + variations of the plasmid are displayed above the map. Differences from the illustration include:

In the – version
* There are two Apa I sites in version A only.

** There are two Xba I sites in version B only.

In the + version
* There is a unique BstE II site, but no Xba I or Apa I sites in version C.

** There is a unique Sac II site between the Apa I site and the Sfu I site in version B only.

2.2.6 Automated DNA sequencing

Sequencing reactions were essentially a modification of the Sanger-Coulson method (Sanger et al., 1977). A linear PCR is set up with template DNA and a GSP plus each of the four dNTPs. Also added to the PCR reaction tube is a modified dideoxynucleotide (ddNTP), which can be incorporated into a growing polynucleotide
strand just as efficiently as any of the dNTPs, but its incorporation blocks further strand synthesis. This is because ddNTPs lack the –OH group at the 3’ position of the sugar component that is needed to attach to the next sugar molecule in the chain. Four reactions per primer/template combination are set up, one tube containing ddATP, ddTTP, ddCTP and ddGTP which produce a mixture of length of amplified DNA strands because they are in competition with the normal dATP, dTTP, dCTP or dGTP. Upon completion of the PCR, samples are run in separate lanes on a high-resolution agarose gel.

The ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Mix (PE Biosystems) was used for fluorescence-based automated sequencing. The Ready reaction Mix in this kit contains the dye terminators (fluorescent labels), deoxynucleoside triphosphates (dNTPs), AmpliTaq DNA Polymerase, magnesium chloride and buffer. The dye terminators are labelled with high-sensitivity fluorescence dyes. The dye structures contain a fluorescein donor-dye coupled to a rhodamine acceptor dye. The excitation maximum of each dye label corresponds to the fluorescein donor, and the emission spectrum corresponds to the rhodamine acceptor. The argon-ion laser in the DNA sequencing instrument efficiently excites the donor dye. Because the emission spectrum of the rhodamine acceptor of each of the four dNTPs is distinct, each base termination in the DNA sequence can be identified. The reactions are run adjacent to one another on a high-resolution agarose gel and the fluorescence data is collected automatically by the DNA sequencing instrument. Sequences were analysed using the Lasergene computer suite (DNA Star, Madison, WI, USA), or Chromas v1.45 sequence analysis software (Freeware, Technelysium Pty Ltd, Helensvale, Australia).

2.3 Isolation and cloning of human γ subunit cDNAs

2.3.1 Isolation and cloning of the CACNG2 cDNA

Human CACNG2 cDNA was amplified from human brain cDNA in Taq 30 PCR reactions containing the GSPs FM13, 5’-GCGGCCGCACCATGGGGCTGTTTGATC-3’ and FM39, 5’-GCTAGCCTCGAGTTAGTGGGAATGAAGAA-3’. FM13 corresponds to the start of the open reading frame (ORF) of the CACNG2 in silico
sequence, with a 5’extension of a Not I restriction site and partial Kozak sequence for initiation of translation in vertebrates (ACC) (Kozak, 1987). Most eukaryotic mRNAs contain a short Kozak recognition sequence (consensus (GCC) GCCRCCATGG, where R is a purine (A or G) and the start codon is underlined) that greatly facilitates the initial binding of mRNA to the small subunit of the ribosome. Its inclusion in the 5’untranslated region (5’UTR) can therefore improve the expression of a cloned cDNA as it acts as an initiating signal for translation. FM39 corresponds to the complementary sequence of the last 22 nucleotides of the ORF to the stop codon, with a 5’extension of Xho I and Nhe I restriction enzyme sites.

Amplified fragments were purified from agarose gels using the Qiaex II kit, cloned into pCR2.1-TOPO vector. Plasmid was transformed into TOP 10 supercompetent E.coli and grown over night on agar plates. Positive colonies were confirmed by EcoRI restriction digest of purified plasmid from overnight miniprep cultures of the white colonies from the cloning plate. Selected clones were sequenced on both strands using T7 (5’-TAATACGACTCAGATATGG-3’) and M13R (5’-CAGGAAACAGCTATGAC-3’) universal primers and gene specific primers in an automated dye terminator sequencer. Clones containing the consensus cDNA sequence of CACNG2 were cultured on the maxiprep scale, purified plasmid diluted to a working dilution of 1µg/ml, and stored at -20°C until required. Glycerol stocks of these clones were made by snap freezing 750µl of miniprep culture with glycerol added to a final concentration of 25% (750µl 50% glycerol) in liquid nitrogen. These were stored at -70°C. From this point forth, all newly identified clones were prepared and stored in an identical fashion.

2.3.2 Isolation and cloning of the CACNG3 cDNA

The same procedures as for CACNG2 were followed, but using the primer pair FM62, 5’-GCGGCGCCACCATGAGGTGTGACAGAGGTA-3’ and FM2, 5’-GCTAGCCTCGAGTTCAGACGGGTGTTG-3’. These primers correspond to the 5’ coding, and 3’ complimentary sequence the ORF of the CACNG3 cDNA sequence respectively, and contain the same three restriction enzyme sites and partial Kozak sequence (Kozak, 1987) in their 5’ extensions as the CACNG2 primers.
2.3.3 Isolation and cloning of the CACNG4 cDNA

The partial sequence of the CACNG4 cDNA predicted by in silico data was amplified from human brain cDNA by Taq 30 PCR reactions containing the GSPs FM20, 5'-CTTCCAGGGATCTATAAAGGG-3' corresponding to nucleotides 1-21 of in silico prediction coding strand, and FM21, 5'-CACAGGGGTCGTCGTCGGTTCAGCA-3', complimentary to nucleotides 748-774 of the in silico prediction. The resulting band was purified from an agarose gel using the Qiaex II protocol (Qiagen), cloned in to the pCR2.1-TOPO vector (Invitrogen), and sequenced in an automated sequencer using the universal primers T7 and M13 Reverse and GSPs.

2.3.3.1 SMART amplification of missing 5'-end

The 5'-sequence was determined by SMART 5' RACE. The first round of PCR reactions contained 25ng SMART synthesised total brain cDNA, the SMART PCR primer, 5'-AAGCAGTGGTAACAACGCAAGT-3', and 10pmol of one of a set of nested primers FM32, FM33 or FM34 (Table 2.11) designed to the complementary strand of the 5'-end of the partial CACNG4 sequence. Three separate reactions plus controls, one for each of the GSPs were set up. Reaction conditions otherwise followed a Taq60 protocol.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>CACNG4 (777bp) Complementary region</th>
</tr>
</thead>
<tbody>
<tr>
<td>FM32</td>
<td>5'-AGTGATTGGATCCGGAAAGCAG-3'</td>
<td>nt 24-43</td>
</tr>
<tr>
<td>FM33</td>
<td>5'-GAGGAGGTACTCCGAGCTGT-3'</td>
<td>nt 71-90</td>
</tr>
<tr>
<td>FM34</td>
<td>5'-TCGTAGTCTATTGTCCGTCGTTG-3'</td>
<td>nt 46-65</td>
</tr>
</tbody>
</table>

Table 2.11 – 5' RACE primers for CACNG4

The second round of PCR reactions contained 2µl of reaction product from the initial PCR and a primer nested to the SMART PCR primer (FM57, 5'-AAGCAGTGGTAACAACGCAAGTACG-3'). The second primer of the pair in each tube was one of either FM32 or FM34. To the products of the first FM33 primed reaction was added 10pmol FM32 or FM34. To the products of the FM34 primed reaction was added 10pmol FM32. The remainder of the reaction mixture consisted of a standard Taq reaction mix (Table 2.4). A Taq PCR protocol was followed. Amplified
fragments were purified from an agarose gel, cloned into pCR 2.1-TOPO and sequenced (see Figure 2.6 for diagram of full procedure).

**Figure 2.6 – 5'RACE to amplify unknown portion of CACNG4 from SMART human brain cDNA**

The SMART tagged cDNA generated by reverse transcription with the SMART oligonucleotide was used as a template to amplify the missing 5' region of CACNG4. The primers FM32-34 were designed to the 5' region of the incomplete CACNG4 so that FM34 and 32 were nested to FM33. A first round of PCR using an oligonucleotide complementary to the SMART primer region at the 5' region of the cDNA and one of primers FM32-34 generated 3 products which were then isolated and used in a second round of nested PCR. The products of these PCRs were then cloned into sequencing vectors and sequenced. The sequences of the PCR product were checked for identity with the partial CACNG4 and other sequence in the Genbank database in BLASTn and tBLASTn alignments. One of the resulting clones yielded a sequence with 60 nt overlap with the known CACNG4 sequence, a putative in frame start codon and 203 nt of 100% identity with human chromosome 17 BAC clone AC005988. Further analysis of these BAC clone revealed sequence 5' of the start codon which had good homology to that of the Kozak consensus sequence (Kozak, 1987) for initiation of vertebrate translation and identified an upstream out of frame stop codon. This clone’s sequence was assembled with the incomplete CACNG4 sequence and used to design primers to amplify the whole protein.

### 2.3.3.2 Amplification of complete CACNG4 cDNA

Following a *Taq* 30 protocol, the cDNA of the complete CACNG4 ORF was amplified from 25ng of human brain cDNA with the GSPs FM43, 5'-GCGGCCGCACCATGGTGCGATGCGACCGCG-3' and FM44, 5'-GCTAGCTCGAGTCACACAGGGGTCGTCCGTC-3', corresponding to nucleotides 1-19 of the predicted complete CACNG4 coding strand 5' end (including a 5' extension of a *Not* I restriction site and partial Kozak sequence (Kozak, 1987)) and complimentary sequence to nucleotides 965-984 of the predicted complete CACNG4 ORF (including the stop codon TGA and two added restriction enzyme recognition sites for *Nhe* I and *Xho* I), respectively.
Chapter 2  Materials & Methods

The amplified fragment was purified from an agarose gel, cloned into pCR2.1-TOPO and sequenced using T7, M13 reverse and gene specific primers.

2.3.4 Isolation and cloning of the CACNG7 subunit cDNA

2.3.4.1 Cloning human CACNG7 cDNA

2.3.4.1.1 Amplification of CACNG7 487 bp cDNA predicted by \textit{in silico} cloning

Following a Taq 30 protocol, the CACNG7, 487 bp fragment predicted by \textit{in silico} cloning was amplified from 25 ng human brain cDNA using 25 pmol each of primer pair FM49, 5'-'CGGGAGAAGGTGCTGTGCT-3' corresponding to the first 20 nucleotides of the coding strand and FM50, 5'-'TCATTGATGGACACGTCG-3' complementary to nucleotides 468-487 of coding strand. The amplified band was purified from an agarose gel, cloned into pCR2.1-TOPO and sequenced.

2.3.4.1.2 Elucidation of unknown 5' and 3' sequence by Marathon RACE

The unknown 5' and 3' CACNG7 sequence was determined by 5' and 3' RACE using Marathon Ready total human brain cDNA (section 2.2.1.2.3). Sets of three nested primers were designed to each end of the known cDNA sequence (Table 2.12).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>CACNG7 (487bp)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>FM51</td>
<td>5'-CGTAGGAAGAGGCTGACCAT-3'</td>
<td>Nt 36-55 (cs)</td>
<td>5' RACE</td>
</tr>
<tr>
<td>FM52</td>
<td>5'-GTGGCCGATGTTGGATGA-3'</td>
<td>Nt 71-90 (cs)</td>
<td>5' RACE</td>
</tr>
<tr>
<td>FM53</td>
<td>5'-GAAGAGATGCAGAGCAGA-3'</td>
<td>Nt 119-138 (cs)</td>
<td>5' RACE</td>
</tr>
<tr>
<td>FM54</td>
<td>5'-CTCCAGCGACGTCCTC-3'</td>
<td>Nt 461-480</td>
<td>3' RACE</td>
</tr>
<tr>
<td>FM55</td>
<td>5'-TTCACGCAGCTGCTGGACT-3'</td>
<td>Nt 384-403</td>
<td>3' RACE</td>
</tr>
<tr>
<td>FM56</td>
<td>5'-ATCTCCAGCATCAAAGCAGA-3'</td>
<td>Nt 181-200</td>
<td>3' RACE</td>
</tr>
</tbody>
</table>

Table 2.12 – Marathon RACE primers for CACNG7

(cs) complementary strand.

Each initial 5' RACE CACNG7 PCR experiment contained a standard Taq reaction mix (Table 2.4), 0.5 ng of the Marathon Ready total brain cDNA template, and 10 pmol Adaptor Primer 1 (AP1), 5'-'CCATCCTAATACGACTCAGATATAGGTC-3'. Four samples (and their controls) were set up by adding 10 pmol of one of the GSPs FM50-
FM53 to the reaction mix. A Taq 30 protocol was performed. The majority of the RACE product was visualised on an agarose gel, keeping back a small volume for use in nested PCR. Six new samples and appropriate positive and negative controls were set up for this second step with the same reaction mix as the previous experiment, but without AP1 or the Marathon Ready cDNA. In place of these were 2μl of first experiment RACE product, 10pmol Adapter Primer 2 (AP2), 5'-ACTCACTATAGGGCTCGAGCGGC-3', and 10pmol of an appropriate nested primer. To the tubes containing the product of the FM50 RACE, was added FM51, FM52 or FM53. To the tubes containing the product of the FM53 RACE, was added FM51 or FM52. To the product of the FM52 RACE was added FM51. The Taq 30 reactions were performed in the thermocycler, except the annealing temperature was raised to 55°C to increase the specificity of amplification. Samples of each tube were run out on an agarose gel, and the remainder of the RACE products were kept for use in a second nested PCR. Conditions were identical to the previous PCR set using AP2 and 10pmol of appropriate GSP, except 5μl of the nested RACE product served as the template. The FM50+FM53 product was primed with either FM51 or FM52, the FM50+FM52 product with FM51, and the FM53+FM52 product with FM51. The bands from this final PCR set were isolated using the Qiaex II kit, cloned into pCR2.1-TOPO and sequenced using the T7 and M13R universal primers.

Each 50μl sample in the initial 3' RACE CACNG7 PCR experiment contained the standard Taq reaction mix and 0.5ng of the Marathon Ready total brain cDNA template, as used in the 5' RACE experiment. Three different tubes primed with 10pmol AP1 and 10pmol of FM54, FM55 or FM56, were cycled according to the Taq 30 protocol. Part of the sample was run on an agarose gel, and distinct bands were purified. 2μl of each remaining sample was then used as the template for nested PCRs. FM56 RACE product was primed with 10pmol AP2 and 10pmol of either FM55 or FM54. The FM55 RACE product was primed with 10pmol FM54 and 10pmol AP2. 30 PCR cycles were performed as in the first experiment, but with an annealing temperature of 55°C. The majority of each sample was run on a high percentage agarose gel and bands in the expected size range were purified. Together with the bands
extracted from the first PCR set, these were cloned into pCR2.1-TOPO vector and sequenced using the T7 and M13 Reverse primers.

### 2.3.4.1.3 Assembly of a full CACNG7 ORF cDNA

The complete CACNG7 cDNA was assembled from two of the generated RACE clones and the original 487 bp cDNA by a splice overlap PCR protocol using a primer pair specific for the predicted ORF. A 330bp fragment of CACNG7 was primed from 5ng of CloneG4_5_23 by 25pmol of FM85, 5’-GCGGCCGCCACCATGAGTCACTGCAGCAGCCG-3’, corresponding to nucleotides 1-20 of coding strand of the predicted full length CACNG7 (including a 5’ extension of a Not I restriction enzyme recognition site and a partial Kozak sequence (Kozak, 1987)) and 25pmol FM51, which now corresponded to the complementary sequence to nucleotides 313-331 of the predicted complete ORF. 25pmol each of primer pair FM49 and FM50, now corresponding to nucleotides 278-296, and the complimentary sequence to nucleotides 744-763 of coding strand, respectively, amplified the central portion of the CACNG7 ORF from 5ng of CloneG4_02. The final 169bp portion of the ORF was amplified from 5ng of CloneG4_242_05 using primers FM55, now corresponding to nucleotides 660-679 of the predicted ORF, and FM84, 5’-GCTAGCCTCGAGTCAGCAGGGCGAGGTGAGAGA-3’, complementary to nucleotides 809-828 of coding sequence (including the TGA stop codon and a 5’ extension of Nhe I and Xho I restriction enzyme recognition sites). In all cases the Pfu turbo reaction mix and protocol was used (Table 2.6 & Table 2.7). The bands were purified from an agarose gel using the Qiaex II protocol.

The complete CACNG7 sequence was amplified in a single splice overlap PCR reaction containing approximately 50ng of each of the 300bp, 487bp and 169bp PCR products, primed with FM84 and FM85 in a Pfu Turbo reaction mix. Following a Pfu turbo PCR program, the band was purified from an agarose gel, cloned into pCR2.1-TOPO and sequenced using T7, M13 reverse and gene specific primers.
2.3.4.2 Cloning mouse cacng7 cDNA

The complete coding sequence was found within chromosome 19 BAC AC079557 and used to design gene-specific primers FM 144, 5' -ATGAGTCACTGCAGCAGCCG-3' and FM 145, 5' - TCAGCACGGCGAAGTGGAGA-3'. The complete ORF was amplified in a single *Pfu* PCR reaction from 20 ng mouse cerebellar cDNA and cloned into pCR4-TOPO for sequencing with GSPs, T7 and M13R primers.

2.4 Subcloning into expression vectors

2.4.1.1 pMT2

γ subunit cDNAs were subcloned into the multiple cloning site of PMT2LR using the *Not* I and *Spe* I sites. γ subunit cDNAs were cut from the TOPO cloning vector into which they had been initially cloned using the *Not* I restriction site introduced by the 5' PCR primer extensions and the *Nhe* I site introduced by the 3' PCR primer extensions. These steps were performed in two separate restriction digests because of incompatibility of restriction enzyme buffers. Restriction fragments were purified by the Qiaex II kit solution technique following the first cut. Following the second cut the samples were run on agarose gels and the desired fragments purified by the Qiaex II gel purification process. Ligations were performed overnight with T4 DNA ligase at 14°C with appropriate controls.

2.4.1.2 pcDNA3.1 myc/his

Human γ2-myc/his fusion was constructed by amplifying the CACNG2 ORF in a *Pfu* Turbo PCR minus the stop codon but including *Not* I + Kozak 5' extension and *Hind* III 3' extension and cloning the fragment into pCR2.1-TOPO. The fragment was then subcloned in frame into pcDNA3.1 myc/his B (-) using the *Not* I and *Hind* III restriction sites. Successful subcloning and correct in-frame insertion was confirmed by restriction digest and sequencing across the insert sites. The same procedure was followed for the human γ4-myc/his fusion. Human γ3-myc/his fusion was created by inserting the
CACNG3 ORF minus Stop codon but with Not I + Kozak 5’ extension and Hind III 3’ extension into pcDNA3.1 myc/his A (-).

Human γ7-myc/his was constructed by amplifying the complete ORF minus the stop codon from the pMT2-γ7 clone and inserting the fragment in frame into the multiple cloning site of pCDNA3.1 myc/his A (+) using the Not I and Apa I restriction sites.

2.5 Expression Analysis

2.5.1 Northern Blot

2.5.1.1 Human blots

Human 12-lane multiple tissue blots and brain II, III and IV blots were purchased from BD Biosciences Clontech.

2.5.1.2 COS-7 and Xenopus oocyte blot

2.5.1.2.1 RNA purification

RNA from COS-7 cells and Xenopus oocytes was isolated using the RNAzol B Phenol-chloroform extraction method (GibcoBRL). COS-7 cells were cultured to confluence in a T75 tissue culture flask (see section 2.5.3.1.1), washed twice with sterile, cold PBS and 4ml of RNAzol B reagent added to the flask. Cells were washed from flask and 1ml placed into four sterile 1.5 ml tubes. Approximately 25 stage V and VI oocytes were placed in a 1.5ml tube and any ND96 buffer (see section 2.6.1) drawn off. 1ml of RNAzol B was added to each tube. Cells were homogenised using a glass-teflon homogeniser. 200μl of chloroform was added per 2ml of homogenate and tubes were shaken vigorously for 15 seconds and then placed on ice for 5 min. The suspension was centrifuged at 12,000 × g at 4°C for 15 min. The upper aqueous phase was drawn off and placed in a fresh tube. An equal volume of isopropanol was added and samples were stored at 4°C for 15 min. Samples were centrifuged at 12,000 × g at 4°C for 15 min. The RNA formed a white yellow pellet at the base of the tube. Optional washes were performed until the pellet was almost completely white (especially required for
Chapter 2  Materials & Methods

Oocyte RNA). These proceeded as follows: The pellet was re-suspended in 500μl dH₂O, and 50μl NaAcetate and 500μl Phenol:Chloroform:isoamylalcohol (25:24:1) added. The tube was vortexed and centrifuged at 12,000 × g at 4°C for 5 min. The aqueous phase was removed, 1ml absolute ethanol added and the samples stored at -20°C for 30 min. Samples were spun at 12,000 × g at 4°C for 15 min. Ethanol was removed and the tubes spun for a further 2 min. Excess ethanol was drawn off and the pellet air-dried for 5 min. Pellet was re-suspended in 50μl DEPC dH₂O.

2.5.1.2.2 Blot creation

A denaturing agarose gel was cast (see Table 2.13 for constituents).

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH₂O</td>
<td>152.25 ml</td>
</tr>
<tr>
<td>Agarose</td>
<td>2.45 g</td>
</tr>
<tr>
<td>10 × MOPS</td>
<td>17.5 ml</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>5.25 ml</td>
</tr>
</tbody>
</table>

Table 2.13 – Constituents of a 1xMOPS denaturing agarose gel

Agarose and dH₂O was warmed until the agarose has dissolved, then allowed to cool to approximately 60°C. The MOPS and formaldehyde was added at this point and the gel allowed to set.

RNA samples were prepared as in Table 2.14.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA</td>
<td>20 μg</td>
</tr>
<tr>
<td>10 × MOPS</td>
<td>5 μl</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>8.75 μl</td>
</tr>
<tr>
<td>Deionised formamide</td>
<td>2.5 μl</td>
</tr>
<tr>
<td>dH₂O</td>
<td>To 50 μl</td>
</tr>
</tbody>
</table>

Table 2.14 – RNA sample preparation for denaturing gel

Samples were heated to 55°C and placed on ice and 0.5μl EtBr and 5μl gel dye added.

Samples were loaded onto the gel and run overnight at 20mV (in 1 × MOPS running buffer). The gel was then washed in 1 × TAE for 30 min and a nitrocellulose filter cut to the size of the gel, in 1 × TAE for 10 min. RNA was transferred onto the filter membrane by overnight electroblotting (Figure 2.7) run at 0.7 - 0.8 mA in 1 × TAE. RNA was cross-linked to the membrane using an ultraviolet stratalinker (Stratagene) set on auto cross-link (120 000 μjoules / cm²). Blots were stored in an air-tight bag at -20°C until ready for use.
Figure 2.7 – Apparatus for transfer of RNA from denaturing gel to nitrocellulose membrane using an electroblotter

Blot performed overnight in 1 x TAE at 0.7-0.8mA. + and − denote anode and cathode respectively.

2.5.1.3 Probe design and synthesis

2.5.1.3.1 cDNA probes

The γ subunit northern probe templates were synthesised from 100ng of plasmid containing the appropriate complete γ subunit cDNA. Pfu Turbo parameters were employed to amplify fragments using the primer pairs detailed in Table 2.15, and the products of the PCR reactions purified by agarose gel electrophoresis and gel extraction. The quantity of probe synthesised was estimated by running a sample of the purified probe template on a high percentage agarose gel against a low mass DNA ladder (GibcoBRL).

<table>
<thead>
<tr>
<th>Probe</th>
<th>Primer</th>
<th>Strand</th>
<th>Primer Sequence</th>
<th>γ subunit cDNA location</th>
<th>Probe size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y2</td>
<td>FM24</td>
<td>Coding</td>
<td>5'-CATGTTTATCGACCGGC-3' 5'-TGTACATGGAGATCCCG-3'</td>
<td>nt 597-814</td>
<td>218nt</td>
</tr>
<tr>
<td></td>
<td>FM42</td>
<td>Complement</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y3</td>
<td>FM26</td>
<td>Coding</td>
<td>5'-CAGAATTGTAGGGTGGTT-3' 5'-GGTGACATCGAGATCGTAC-3'</td>
<td>nt 560-792</td>
<td>233nt</td>
</tr>
<tr>
<td></td>
<td>FM27</td>
<td>Complement</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y4</td>
<td>FM28</td>
<td>Coding</td>
<td>5'-GGGCGTTGCCTGGGTGGAAA-3' 5'-GGGGCTTGCATCTTCAG-3'</td>
<td>nt 594-804</td>
<td>211nt</td>
</tr>
<tr>
<td></td>
<td>FM29</td>
<td>Complement</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y7</td>
<td>FM120</td>
<td>Coding</td>
<td>5'-GGCGTGATGGTCCGTGAC-3' 5'-TCATTTGGATGCACGTAC-3'</td>
<td>nt 577-763</td>
<td>187nt</td>
</tr>
<tr>
<td>Burgess</td>
<td>FM142</td>
<td>Coding</td>
<td>5'-CGGTGCACACTGACTGTGGG-3' 5'-ATGACCTCGTGGTGATGC-3'</td>
<td>nt 80-482</td>
<td>403nt</td>
</tr>
<tr>
<td></td>
<td>FM143</td>
<td>Complement</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.15 - γ subunit northern probe primers

Stripable [α-32P] radio-labelled γ subunit cDNA probes were assembled according to the Strip-EZ DNA Probe Synthesis and Removal Kit (Ambion, Abingdon, UK). 25ng
linearised template was placed in a tube, made up to 9µl with TE buffer and denatured by heating at 95-100°C in a hot block for 5 min. The sample was snap frozen on dry ice, thawed, microcentrifuged and placed on ice. To the denatured DNA was added: 2.5µl 10 × decamer solution, 5µl 5 × buffer –dATP/cCTP, 2.5µl 10 × dCTP, 5.0µl [α-32P] dATP (3000 Ci/mmol (370MBq/ml), Amersham, AA0004), 1.0 µl exonuclease-free Klenow fragment. Contents were gently mixed and incubated at 37°C for 30 minutes. The reaction was terminated by adding 1µl 0.5M EDTA. Unincorporated nucleotides were removed by passing the synthesis reaction through a Chromaspin-10 column (BD Biosciences Clontech). This removed >90% of products <4bp and dNTPs, and recovers >50% 10bp, >70% 15bp and >90% of >30bp fragments. Once storage buffer had been spun out of the column and a semi-dry-column matrix established, the sample was applied to the centre of the gel bed’s flat surface. The column was placed in a 1.5 ml tube and spun at 700 x g for 5 min. The purified sample was collected in the tube.

27µl of the purified probe was transferred to a fresh tube and diluted ten-fold in 10mM EDTA (243 µl). The diluted probe was incubated at 90°C for 10 min. The probe was transferred directly to a container containing a pre-hybridised blot (see section 2.5.1.4).

2.5.1.3.2 Oligonucleotide probes

A 49mer oligonucleotide probe FM98, 5’-GCTGGGTGTTCATGGGCATCACATAT-TCTATGGTGAAGCAACGCCCCCG-3’ was designed to the complementary sequence of nt 349-397 of the complete CACNG5 in silico prediction, a region that did not cross any exon-intron boundaries. 10pmol of the probe was labelled with [γ-32P] dATP according to the KinaseMax 5’ End-Labeling Kit (Ambion) forward reaction protocol. The T4 polynucleotide kinase catalyses the transfer of the γ phosphate from [γ-32P] dATP to the 5’ –OH of the oligonucleotide. 10 pmol of the FM98 oligonucleotide was incubated with 21 pmol [γ-32P] dATP (7000, Ci/mmol, 150mCi/ml), 2µl 5 × forward buffer and 1µl T4 polynucleotide kinase (10 U/µl) in a final volume of 10 µl at 37°C for 30 min and then the reaction halted by a 2 min 95°C incubation.
2.5.1.4 Hybridisation and washing

2.5.1.4.1 Blot pre-hybridisation

Before every hybridisation blots were pre-hybridised for 1h in ExpressHyb solution (BD Bioscience Clontech).

2.5.1.4.2 Hybridisation

Blots hybridised with cDNA radio-labelled probes were incubated overnight at 65°C in ExpressHyb solution. Blots hybridised with oligonucleotide radio-labelled probes were incubated overnight at 37°C in ExpressHyb solution.

2.5.1.4.3 Washes

High stringency washes for cDNA probe hybridised blots were performed as follows: ExpressHyb and probe was poured off from container and blots were washed briefly in solution 1 (Table 2.16) at 65°C. Wash solution was poured off and the blot washed again in solution 1 for 10 min at 65°C. Blots were transferred to a tray containing solution 2 at 65°C and washed whilst shaking slowly on a rotary shaker for 10 min. Blots were removed, emissions checked and re-washed in solution 2 (Table 2.16) until a suitable reading obtained (just above background).

<table>
<thead>
<tr>
<th>Solution 1</th>
<th>Solution 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 × SSC</td>
<td>0.1 × SSC</td>
</tr>
<tr>
<td>0.05 % SDS</td>
<td>0.1 % SDS</td>
</tr>
</tbody>
</table>

Table 2.16 – Northern blot wash solutions

SSC = Saline Sodium Citrate, SDS = Sodium dodecylsulphate. Solutions made fresh in 1 l volumes in dH₂O.

Low stringency washing of oligonucleotide probe hybridised blots were performed as follows: Blots were washed for 30-40 min in solution 1 at 37°C with changes of the solution every 10 min. Blots were then washed in solution 2 at room temperature for 40 min with continuous shaking and one change of solution after 20 min.
2.5.1.5 Exposure, developing and image processing

Blots were wrapped in cling-film and placed down on X-OMAT blue film (Kodak) in an auto-cassette. The cassette was stored at -80°C for 1-4 days, or until a suitable exposure was obtained. Films were developed and fixed using and automatic X-OMAT processor (Kodak) or similar device. Developed films were scanned into a PC and images processed using the Adobe Photoshop versions 4.0 & 5.5 software (Adobe Systems, London, UK). In all circumstances, no attempt was made to quantify the intensity of probe signal, although relative transcript detection levels could be compared on the same blots.

2.5.2 Antibodies

2.5.2.1 Design

Multiple sequence alignments and BLAST searches to compare all the putative VDCC γ subunits with one another and other known proteins in the genetic databases were compared to find the regions of lowest homology between all the subunits that are not present in any other known proteins. Peptides for each predicted γ subunit that matched these criteria and had good predicted antigenicity were designed according to the sequences in Table 2.17.

<table>
<thead>
<tr>
<th>Antibody target</th>
<th>Epitope</th>
<th>γ subunit amino-acid position</th>
</tr>
</thead>
<tbody>
<tr>
<td>γ2</td>
<td>TARATDYLQASAITRIPS</td>
<td>211-228</td>
</tr>
<tr>
<td>γ3</td>
<td>FHNSTPKEFKESLHNNPAN</td>
<td>291-309</td>
</tr>
<tr>
<td>γ4</td>
<td>VHDFQQQDLKEGFHVSMLN</td>
<td>303-321</td>
</tr>
<tr>
<td>γEXT</td>
<td>CVASEYFLEPEINLVTEN</td>
<td>72-89</td>
</tr>
<tr>
<td>γ7</td>
<td>YPPAIKYPDHLHIS</td>
<td>258-271</td>
</tr>
</tbody>
</table>

Table 2.17 – Sequences of peptides designed to raise rabbit polyclonal anti γ subunit antibodies

All antibodies were designed to have 100% cross reactivity with both human and mouse γ subunits. The corresponding amino acid position of the antigenic epitope to the human γ sequence is displayed in the third column. γEXT is the antibody raised to a sequence in the first extracellular loop of the γ7 protein. γ7 antibody corresponds to a C-terminal motif in the same protein.
2.5.2.2 Peptide Synthesis

The peptides were synthesised by standard solid-phase techniques at Severn Biotech (Kidderminster, UK). Each was coupled to the carrier protein tuberculin PPD using sulpho-SMCC (Pierce, Tattenhall, UK) via a Cys residue added at the N-terminus during synthesis.

2.5.2.3 Antibody production and affinity purification

To raise rabbit polyclonal anti-\( \gamma \) antibodies, the resulting conjugates were used to immunise BCG-sensitised Dutch rabbits at monthly intervals (Lachmann et al., 1986). The immune response was monitored by indirect enzyme-linked immunosorbent assay (ELISA) with free peptide-coated microtitre plates. Immunoglobulins from the terminal bleeds were purified using immobilised peptide antigen columns (Sulpho-link, Pierce).

2.5.2.3.1 Column Construction

2mg of peptide was weighed and dissolved in 1ml of dimethylsulphoxide (DMSO, Sigma). A polypropylene column (Pierce No.29924) was assembled then 4ml of Sulpho-link coupling gel was added to the column and allowed to drain (50:50 solution: gel therefore final column volume is 2ml). The remaining gel was equilibrated with 6-10 column volumes of 50mM Tris, 5mM EDTA-Na, pH 8.5. The 1ml of dissolved peptide was added to the column and incubated overnight on a spiramixer at room temperature. The following morning, the top cap was removed and the contents allowed to drain. 2ml of a 50mM cysteine solution (in 50mM Tris, 5mM EDTA-Na, pH 8.5) was added to the column and mixed for 15min, then a further 30min at room temperature without mixing. The column was washed with 4ml 1M NaCl (2 column volumes). The column was then pre-cycled with 4ml 0.1M glycine, pH 2.5 elution buffer and re-equilibrated with PBS. When not in use, columns were stored in PBS containing 0.005% thimerosal and kept at 4°C.
Materials & Methods

2.5.2.3.2 Antibody affinity purification

During each stage of purification, all effluent from the column was kept to check correct antibody binding or elution depending upon the purification stage. Terminal bleed antiserum (2ml) was diluted to 10ml in PBS. With a sterile Pasteur pipette, 2mls at a time of the diluted serum was slowly added to the column to allow antibodies (Abs) to bind. Serum was then chased through with 4ml of PBS, and washed with a further 4ml PBS. To elute the Abs, 9ml of 0.1M glycine pH 2.5 was added to the columns, and elutate collected in a 15ml falcon tube containing 1ml Tris buffer, pH 8.5. 4ml of PBS re-equilibrated the column following elution.

Purified antibodies were dialysed against PBS in two oversight steps using the Pierce Slide-A-lyzer. Ab concentration was estimated on a spectrophotometer at an absorption wavelength of 280nm (a reading of 1.4 = 1mg/ml), or for more accurate measurements, determined by the BCA protein assay (Pierce).

2.5.2.4 Antibody specificity

Each γ subunit Ab was checked for specificity for its target by immunocytochemistry (see section 2.5.3 for methods and Chapter 4 for results). COS-7 cells transfected only by a single γ subunit cDNA were checked for positive staining following incubation with the correct affinity purified Ab. Moreover, COS-7 cells transfected with γ subunit cDNAs other than the intended target for the antibody were checked for positive staining. For example, cells transfected with γ3, γ4 and γ7 subunits were shown not to display positive staining if incubated with an anti-γ2 Ab. Control slides were also checked for cross-reaction with untransfected COS-7 cells and other VDCC subunits used in the investigation. Finally, positive staining of target protein was abolished following overnight pre-incubation (at 4°C) of the primary antibody with a 10× molar excess of the peptide against which it was raised.
Chapter 2  

Materials & Methods

2.5.3 Immunocytochemistry

2.5.3.1 Cell culture

2.5.3.1.1 COS-7 flask culture

COS-7 cells are derived from African Green Monkey kidney fibroblast cells and are used as a transient expression system. These cells constitutively express the SV40 T antigen and contain the cellular factors required to drive replication of SV40 origin-containing plasmids e.g. pMT2 (see section 2.2.5.2). Cells were obtained from ECACCs (European Collection of Animal Cell Cultures, Salisbury, UK). The cells were maintained in DMEM (Dulbecco's Modified Eagle Medium) containing penicillin/streptomycin (P/S) and 10% new born calf serum (NBCS) and grown to 100% confluency in 75cm² (T75) vented flasks (Falcon). To passage the cells, the medium was removed from the flasks and the cells were washed twice with cold PBS (10 mM phosphate buffer, 2.7 mM potassium, 137 mM NaCl; made from tablets (Sigma) dissolved in 1 l dH₂O). The cells were dissociated enzymatically by incubation in 1 ml of trypsin containing Mg²⁺ and Ca²⁺ (0.5 mM in Pucks saline solution) for 5-10 min at 37°C. The trypsin was inactivated by addition of the required amount of medium and 1 ml of the resultant suspension was added to new flasks containing 9 ml of fresh culture medium.

2.5.3.1.2 Plating for cytochemistry on coverslips

Cells were grown on sterile glass coverslips pre-treated with tissue culture grade poly-L-lysine (15 mg/ml, Sigma). This allowed cells to adhere firmly to the coverslips. Excess poly-L-lysine was removed, slides washed in sterile dH₂O and air dried in a tissue culture hood. A single coverslip was placed in a 3.5 cm Petri dish and stored at 4°C until ready for use.

In early immunocytochemistry experiments (Chapter 4), cells that had already been transfected 2-3 days previously and grown in 3.5 cm Petri dishes were washed twice with sterile PBS, dissociated from the dish by incubation a non-enzymatic cell dissociation solution (CDS, Sigma) at 37°C for 5 min and diluted in an appropriate
volume of fresh culture media. Cells were then re-plated onto coverslips 2-3 h prior to fixation to allow cells to settle and adhere to the coverslip. However, during the course of the investigation, it was thought that re-plating the cells so soon before fixation may could possibly result in inappropriate observations regarding cellular morphology. Therefore in later experiments (Chapter 6), cells were plated onto coverslips at lower dilutions, but 2-3 h prior to transfection. The cells were then grown cultured for a further 2-3 days whilst transient protein expression occurred and were fixed in situ in their normal morphological condition.

2.5.3.2 Transfections

2.5.3.2.1 Geneporter transfections

Lipophilic transfection involves absorption of the DNA coated with a cationic lipid. The GenePorter reagent (Gene Therapy Systems Inc.) was used for lipophilic transfection of COS-7 cells. The reagent is a formulation of the neutral lipid dioleoyl phosphatidylethanolamine (DOPE) and a proprietary cationic lipid, DC-cholesterol. Cells were plated onto coverslips on the day of transfection so that they were approximately 30% confluent. This ensured that the cells were still growing in monolayers four days later. The DNA and GenePorter reagent were diluted separately in 500 µl of serum-free medium. The amount of DNA used was 2 µg (for subunit cDNA ratios see section 2.5.3.2.2), for which the recommended volume of the GenePorter reagent is 10 µl. The diluted DNA was added to the diluted GenePorter reagent, vortexed, and incubated at room temperature for 20-60 min. Once the cells had adhered to the coverslips (normally after 2 hours), the culture medium from the cells was removed and the DNA- GenePorter mixture was added. The cells were then incubated at 37°C. Three to five hours following transfection, 1ml of medium containing 20% NBCS was added. The cells were incubated for 2-3 days at 37°C, and the medium (10% NBCS calf serum) was changed as necessary.
Chapter 2  

Materials & Methods

2.5.3.2.2 cDNA mixes

Unless otherwise stated, the α₁, β, α₂δ and γ subunits were mixed and transfected in a 1:1:1:1 ratio by DNA weight. In the absence of any subunit cDNAs from the mix, blank pMT2 vector was substituted. The exception to this rule was when the Kv3.1b subunit was expressed with γ₁-myc/his. In these transfections the cDNA ratio was 1:1 by DNA weight, and in the absence of γ₁-myc/his cDNA, blank pcDNA3.1 was substituted.

2.5.3.2.3 Non-γ subunit cDNAs used

The following cDNAs were used: rabbit Cav2.2 (D14157), N-terminal GFP tagged rabbit Cav2.2 (Raghib et al., 2001a), rabbit Cav2.2 Δ3’UTR, rabbit Cav2.1 (X57689), rat brain Cav1.2 (isoform CII, M67515), rat β₁b (X61394, except R417S, V435, V449A, W492R, V511A, a gift from Dr. T. P. Snutch), mouse α₂δ₂ (AF247139, common brain splice variant), mut-3b Green Fluorescent Protein (GFP, M62653, except S72A and S65G, a gift from Dr. T. E. Hughes), rat Kv3.1b (M68880, a gift from Dr. L. Kaczmarek). The 3’ untranslated region (UTR) of rabbit Cav2.2 was removed to generate Cav2.2 Δ3’UTR, by introducing an Spe 1 site immediately after the stop codon by PCR and subcloning the Cav2.2 back into the vector. All cDNAs were subcloned into expression vector pMT2 (section 2.2.5.2 & (Swick et al., 1992)) with the exception of Kv3.1b that was in pRC-CMV.

2.5.3.3 Fixing and staining

The transfected COS-7 cells were washed with Tris Buffered Saline (TBS; 154 mM NaCl and 20 mM Tris, pH 7.4) (2 × 15 min) 2-3 days post transfection. Cells were fixed with 4% paraformaldehyde (Sigma) in TBS solution for 15 min at room temperature in order to cross-link and immobilise proteins in the cells. To allow Ab penetration into the cells, the cells were permeabilised with 0.02% Triton X-100 (BDH) (2 × 8 min) in TBS; this step was omitted if permeabilisation was not required. The cells were then incubated with blocking solution (20% goat serum (Sigma), 4% bovine albumin serum (Sigma) and 0.1% DL-lysine (Sigma) in TBS (4 × 5 min) to block non-specific binding
sites prior to primary antibody application. Cells were incubated overnight with the primary Ab at 4°C. All Abs were diluted in a solution comprising 10% goat serum, 2% bovine serum albumin (BSA) and 0.05% DL-lysine in TBS. Cells were washed again with the blocking solution and incubated with the secondary Ab for 2h at 4°C. For experiments using the rabbit-polyclonal primary Abs, the secondary antibody was goat anti-rabbit IgG biotin (Table 2.18 for dilutions). For experiments using the mouse-monoclonal primary Abs (anti-myc Ab and anti-GFP Ab), the secondary antibody was goat anti-mouse IgG biotin (Table 2.18 for dilutions). Following incubation with the secondary antibody, the cells were washed again with the blocking solution (4 × 5 min) then incubated with the fluorophore for 1hr at room temperature. The fluorophores routinely used were either streptavidin Texas-Red (Molecular Probes; 2 μg/ml) or streptavidin fluorescein isothiocyanate (FITC) (Sigma; 2 μg/ml) at a dilution of 3.33μg/ml (Table 2.18). Excess fluorophore was removed by washing with TBS (5 × 5 min). The samples were then mounted on microscope slides in an anti-fade agent (Vectorshield, Vector, CA, USA) and sealed with nail varnish. All solutions were applied to the coverslips at a volume of 200 μl.

2.5.3.3.1 Organelle Markers

In some experiments, the plasma membrane and nucleus were labelled to delineate their localisation more precisely (Table 2.19). All organelle markers were applied after application of the Texas-Red or FITC fluorophores, and the cells were washed with TBS (5 × 5 min).

2.5.3.3.1.1 Plasma Membrane

Oregon-Green conjugated phalloidin (Molecular Probes, Oregon, USA) was used to delineate the plasma membrane. Phalloidin is a phallotoxin isolated from the mushroom *Amanita phalloides*. The peptide binds selectively to F-actin, a component of the actin cytoskeleton underlying the plasma membrane. Phalloidin is cell-impermeant, and therefore was used in conjunction with permeabilised cells. Each coverslip was treated with 1:50 dilution of the marker (from supplied stock) and incubated for 20-40 min at room temperature.
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Target sequence origin</th>
<th>Host species</th>
<th>Type</th>
<th>Working dilution</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>γ2</td>
<td>Human or mouse</td>
<td>Rabbit</td>
<td>Polyclonal; Affinity purified</td>
<td>0.2 µg/ml (1:1000)</td>
<td>F. J. Moss</td>
</tr>
<tr>
<td>γ3</td>
<td>Human or mouse</td>
<td>Rabbit</td>
<td>Polyclonal; Affinity purified</td>
<td>0.2 µg/ml (1:650)</td>
<td>F. J. Moss</td>
</tr>
<tr>
<td>γ4</td>
<td>Human or mouse</td>
<td>Rabbit</td>
<td>Polyclonal; Affinity purified</td>
<td>0.2 µg/ml (1:1000)</td>
<td>F. J. Moss</td>
</tr>
<tr>
<td>γTEXT</td>
<td>Human or mouse</td>
<td>Rabbit</td>
<td>Polyclonal; Affinity purified</td>
<td>1.2 µg/ml (1:250)</td>
<td>F. J. Moss</td>
</tr>
<tr>
<td>γ7</td>
<td>Human or mouse</td>
<td>Rabbit</td>
<td>Polyclonal; Affinity purified</td>
<td>0.8 µg/ml (1:500)</td>
<td>F. J. Moss</td>
</tr>
<tr>
<td>Anti-myc</td>
<td>Mouse</td>
<td>Mouse</td>
<td>Monoclonal; Affinity purified</td>
<td>4 µg/ml (1:500)</td>
<td>Santa-Cruz (Autogen-Bioclear in UK)</td>
</tr>
<tr>
<td>Anti-GFP</td>
<td>GFP</td>
<td>Mouse</td>
<td>Monoclonal; Affinity purified</td>
<td>20 µg/ml (1:50)</td>
<td>BD Biosciences Clontech</td>
</tr>
<tr>
<td>Kv3.1b</td>
<td>Rat</td>
<td>Rabbit</td>
<td>Polyclonal; Affinity purified</td>
<td>1.5 µg/ml (1:200)</td>
<td>Alomone</td>
</tr>
<tr>
<td>Biotin conjugated Goat anti-rabbit 2° Ab</td>
<td>Rabbit</td>
<td>Goat</td>
<td>Polyclonal; Affinity purified</td>
<td>5 µg/ml (1:200)</td>
<td>Sigma</td>
</tr>
<tr>
<td>Biotin conjugated Goat anti-mouse 2° Ab</td>
<td>Mouse</td>
<td>Goat</td>
<td>Polyclonal; Affinity purified</td>
<td>10 µg/ml (1:200)</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td>Texas-Red conjugated Goat anti-rabbit 2° Ab</td>
<td>Rabbit</td>
<td>Goat</td>
<td>Polyclonal; Affinity purified</td>
<td>2 µg/ml (1:300)</td>
<td>Molecular Probes</td>
</tr>
</tbody>
</table>

Table 2.18 - Primary and secondary Abs used in immunocytochemistry experiments

The table details the target, target species, host in which Abs were raised, type of Ab, working dilution and source or supplier of the Ab. Secondary antibodies are shaded in grey.

2.5.3.3.1.2 Nucleus

Nuclear staining was obtained by incubating cells with 4',6-diamidino-2-phenylindole (DAPI, Molecular Probes). DAPI emits blue fluorescence upon binding to the minor groove of double-stranded DNA. It is a plasma membrane permeable dye and can be used with unpermeabilised cells. Cells were incubated with DAPI (diluted in TBS to 300 nM) for 1 min at room temperature.
Chapter 2 Materials & Methods

<table>
<thead>
<tr>
<th>Organelle Marker</th>
<th>Organelle</th>
<th>Exc/Emi (nm)</th>
<th>Concentration</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAPI</td>
<td>Nucleus</td>
<td>358/461</td>
<td>300nM</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td>Texas-Red Phalloidin</td>
<td>Sub-plasma membrane</td>
<td>591/608</td>
<td>6.6 μM</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td>Oregon-Green Phalloidin</td>
<td>Sub-plasma membrane</td>
<td>496/520</td>
<td>6.6 μM</td>
<td>Molecular Probes</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fluorophore</th>
<th>Conjugated to</th>
<th>Exc/Emi (nm)</th>
<th>Concentration</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescin isothyocyanate</td>
<td>Streptavidin</td>
<td>494/518</td>
<td>2 μg/ml</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td>Texas Red</td>
<td>Streptavidin</td>
<td>595/615</td>
<td>2 μg/ml</td>
<td>Molecular Probes</td>
</tr>
</tbody>
</table>

Table 2.19 – Organelle markers and streptavidin-conjugated fluorophores used in immunocytochemistry experiments

Exc/Emi = Excitation/Emission wavelengths of fluorophores.

2.5.4 Confocal laser scanning microscopy

The confocal laser scanning microscope (CLSM) was used to examine fixed samples labelled with fluorescently labelled antibodies or cellular markers.

The key feature of confocal microscopy is that only a small region of specimen is illuminated by the laser beam at any one time, eliminating “out-of-focus” interference resulting from light above and below the plane of focus. In order to produce an image of a whole sample in focus, the beam must scan across the specimen. A laser beam passes into a light microscope and is reflected into a scanning unit which generates x and y movement of the point of illumination. The beam is focused on the specimen by the objective lens, and any reflected light (e.g. from fluorescent markers) passes back through the objective lens and scanning unit into the detecting apparatus (Figure 2.8). Photomultiplier tubes (PMTs) detect the reflected or fluorescent light from the specimen and relay a compound image of the specimen to a computer screen. Furthermore, alterations in the z axis by adjustment of the objective lens of the microscope allowing series of images of different sections of the specimen to be produced. In this investigation CLSM images were captured using a Leica TCS NT microscope, and all images were taken at an optical section thickness of 0.5μm.

Confocal images were post-processed using Adobe Photoshop versions 4.0 & 5.5 software (Adobe Systems, London, UK).
Figure 2.8 - Diagram of the Illumination Path in a Confocal Microscope

The laser light enters from the right side (blue). Two irises define the optical path. The excitation filter selects a laser wavelength. The beam is expanded in a telescope and filtered using a pinhole. The dichroic mirror deflects the beam towards a scan unit consisting of two independent rotatable mirrors (x and y scanners). A scan lens focuses the beam inside the microscope. Light emitted from the sample (red & green) retraces the path of the illuminating beam through the first dichroic until it reaches the second dichroic mirror where it is split and different wavelengths of light are focused into the pinholes in front of photomultiplier I (PMT) or PMT2.

2.5.5 Human Brain Immunohistochemistry

2.5.5.1 Tissue sources

Access to human brain tissue blocks, which were already fixed and embedded in wax was kindly provided by the Immunohistochemistry Group at the GlaxoSmithKline
Materials & Methods

Medicines Research Centre, Stevenage, UK. The human hippocampus and cerebellum tissue samples came from two elderly male patients of 76 and 80 years of age, whose causes of death were listed as metastatic carcinoma and left ventricular failure respectively.

2.5.5.2 Tissue sectioning

7μm sections were cut on a Microm HM3555S microtome, floated onto 42°C warm water bath and lifted onto Vectashield (BDH) coated microscope slides. Slides were place in a 37°C warm air blower overnight.

2.5.5.3 Tissue processing and staining

Slides were brought to water by serial immersions for 2 min in Xylene (×2), 100% industrial methylated spirits (IMS)(×2), 90% IMS, 70% IMS and then rinsed in running tap water and finally left to stand in distilled water. The limits of the tissue on the slide were marked with a Pap Pen (Daido Sangyo Co Ltd., Japan). To lower the threshold of antigen detection, tissue slices were processed according to the microwave antigen retrieval process (McKee et al., 1993; Shi et al., 1991). This removes cross-linking between antigens and fixative reagents. Sections were immersed in 0.01M citrate buffer pH6 (HDS supplies, Aylesbury, UK) and heated at 100°C for 5min in a molecular pathology microwave (BioRad). Sections were left to stand for 5min, boiled for a further 5min at 100°C and left to stand for 2min. Endogenous peroxidase activity was blocked by incubation with 3% H₂O₂ (Sigma) in water for 20min. Slides were then washed in running tap water for 5min then placed in distilled water for 5 min and finally washed for a further 5min in running distilled water. Non-specific binding of Abs was blocked by incubation with 5% milk powder, diluted in PBS and 0.1% Triton X-100 for 30min. Sections were incubated overnight with primary antibody diluted with 3% goat serum and 0.1% Triton X-100 at 4°C in a moist chamber (see Table 2.20 for primary Ab dilutions). Slides were then washed 3 × 5min in PBS and the secondary biotinylated rabbit link antibody applied (1:20 dilution, Biogenex, Wokingham, UK) and incubated for 20min at room temperature. Slides were then washed 3 × 5min in PBS and incubated
for 20min with Horse Radish Peroxidase (HRP) - Strepaavidin-conjugated antibody (Biogenex, prediluted (1/20)). Slides were then washed 3 × 5min in PBS. 3,3',5,5' Diaminobenzidine Tetrahydrochloride (DAB) solution was made up according to Vector kit SK-4100, and incubated with sections for 2-10 min. The reaction was stopped by plunging slides into distilled water. Slides were dehydrated by immersing for 2min each in a series of 70% IMS, 90% IMS, 2 × 100 % IMS and 2 × Xylene. Slides were mounted in Xylene and cover-slips attached using an automated coverslipping machine.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Conc μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>γ2</td>
<td>1:200</td>
<td>0.85 μg/ml</td>
</tr>
<tr>
<td>γ3</td>
<td>1:100</td>
<td>1.3 μg/ml</td>
</tr>
<tr>
<td>γ4</td>
<td>1:200</td>
<td>0.85 μg/ml</td>
</tr>
</tbody>
</table>

Table 2.20 — Working dilutions for primary Abs in immunohistochemistry experiments

2.5.5.4 Control Procedures

The following controls were performed in all immunohistochemistry experiments. Sections in which PBS was substituted for the primary antibody were run in every experiment. Additionally, sections were incubated with primary Ab that had been pre-absorbed with 100μM of specific synthesis peptide overnight at 4°C prior to the experiment.

2.5.5.5 Microscopy and image analysis

Slides were analysed using a Zeiss Axioplan Optical Microscope (Carl Zeiss Ltd., Welwyn Garden City, UK). All sections were viewed under bright-field illumination using ×2.5, ×10, ×20, or ×40 objectives and a stabilised light source. For each tissue section, both The Central Nervous System (Brodal, 1998), and The Human Brain: an introduction to its functional anatomy (Nolte, 2001) were used for reference, together with corresponding counterstained sections to locate and define nuclear groups. A JVC KY-F55B television camera attached to the microscope and a PC running AcQuis image capture software (Syncroscopy, Cambridge, UK) was used to obtain colour images of each section. Images were processed using Adobe Photoshop versions 4.0 &
Chapter 2

Materials & Methods

5.5 software (Adobe Systems, London, UK). No attempt was made to quantify the intensity of tissue staining in any section.

2.5.6 Mouse cerebellum immunocytochemistry

2.5.6.1 Paraformaldehyde perfusion and cryo-sectioning of mouse cerebellum

P21-24 mice were either anaesthetised by asphyxiation with CO₂ and perfused transcardially via the ascending aorta, initially with 5ml of a 100mM sodium phosphate buffer (PBS, pH 7.2) and then with 15ml 4% paraformaldehyde (Agar Aids, Stanstead, UK) in PBS. The brains were immediately removed from the cranium and postfixed for 2 hours at 4°C with fresh 4% paraformaldehyde in PBS and then incubated over-night in PBS with 30% sucrose and 0.02% sodium azide at 4°C for cryoprotection. The brains were then frozen in isopentane, previously pre-cooled with liquid nitrogen and 15μm parasagittal sections were cut with a cryostat (CM 1900, Leica). The sections were transferred to poly-L-lysine-coated microscope slides (BDH, Merck Ltd, Lutterworth, UK), left to dry then used immediately.

2.5.6.2 Staining of cerebellar sections

Sections were circled with a Pap Pen, and incubated with 2% Triton X-100 in PBS for 2 7.5 min steps to permeabilise the tissue. Non-specific binding was prevented by performing 3 × 5 min incubations in 3% goat serum (in PBS with 0.1% Triton X-100). The primary Abs were appropriately diluted (2μg/ml) in a solution comprising 10% goat serum, 2% bovine serum albumin (BSA) and 0.05% DL-lysine in PBS and incubated overnight at 4°C. The following day sections were washed twice with 200μl PBS for 15 minutes before incubation with biotinylated goat anti-rabbit Ab (5 μg/ml) as the secondary antibody (2h at 4°C). Following a second washing step with two changes of PBS, the sections were incubated with a Texas-red-coupled streptavidin conjugate (2μg/ml), for one hour at room temperature and in the dark. Sections were washed four times with PBS, then 22x50mm coverslips mounted using Vectashield and sealed with nail polish. Slides were kept in the dark at 4°C until examination by confocal microscopy.
2.6 Xenopus oocyte two-electrode voltage clamp (TEVC)

2.6.1 Preparation of oocytes

Adult *Xenopus laevis* females were anaesthetised by immersion in 0.2% tricaine (Sigma) then killed by decapitation and pithing. Oocytes were surgically removed and defolliculated by treatment with 2 mg/ml collagenase type Ia (Sigma) in a Ca^{2+}-free ND96 saline containing (in mM): 96 NaCl, 2 KCl, 1 MgCl₂, 5 Hepes, pH adjusted to 7.4 with NaOH for two hours at 21°C. Plasmid cDNAs for the different α₁ subunits, plus auxiliary β α2δ and γ subunits were mixed in a ratio of 1:1:1:1 (unless otherwise stated using cDNAs as detailed in section 2.5.3.2.3), and ~10 nl was injected into the nuclei of stage V and VI oocytes using a Drummond microinjector following centrifugation of oocytes at 1200 rpm for 14 min. Injected oocytes were incubated at 18°C for 3-5 days in ND96 saline (as above plus 1.8 mM CaCl₂) supplemented with 100 µg/ml penicillin-100 U/ml streptomycin (Life Technologies).

2.6.2 Two-electrode voltage clamp

In the two-electrode voltage clamp (TEVC) electrophysiology configuration, the membrane potential is held constant (i.e. "clamped") while the current flowing through the membrane is measured. As long as channel gating is determined by transmembrane voltage alone, voltage clamp offers control over the key variable that determines the opening and closing of voltage-dependent ion channels. Furthermore, clamping the voltage eliminates the capacitive current, except for a brief time following a step to a new voltage (recorded as transient spikes at the beginning and end of a voltage step) and the currents that flow are proportional only to the membrane conductance, (i.e. to the number of open channels). Figure 2.9 shows a schematic diagram of the TEVC clamp circuit. The membrane voltage (V_m) is recorded by a unity-gain buffer preamplifier (A1), which receives inputs from the voltage-recording electrode (VE) and the bath Ag/AgCl pellet (Clark Electromedical Instruments, Reading, UK) and agar bridge reference electrode (RE). This electrode grounds the perfusion bath in which the cell lies connecting to an earthing socket located on the VE pipette holding headstage, completing the circuit. To prevent Ag⁺ ions leaching into the perfusion chamber the
Ag/AgCl pellet was isolated from the chamber by encasing it in an agar/salt bridge. The salt bridge was formed of a modified Gilson pipette tip pipette filled with 3% (w/v) agar (Sigma) made in 1M KCl, allowing electrical continuity between the pellet and perfusion chamber whilst maintaining physical isolation. The amplifier A1 records differentially between VE and the RE to give output \( V_m \). This output is compared to the command potential (\( V_{cmd} \)) in a high-gain differential clamping amplifier (A2). The output of the clamping amplifier is proportional to the difference (\( \varepsilon \)) between \( V_m \) and \( V_{cmd} \) and the voltage at its output forces current to flow through the current-passing microelectrode (IE) into the cell. The polarity of the gain in the clamping amplifier is such that the current in IE reduces \( \varepsilon \). A third differential amplifier (A3) measures the voltage drop across the current setting resistor, \( R_0 \). This voltage drop (\( V_i \)) is proportional to the total current (\( I \)) flowing through \( R_0 \) and the output signal, \( V_i \), is a good representation of the membrane current (\( I_m \)).

![Schematic of the two-electrode voltage clamp circuit](https://example.com/schematic.png)

**Figure 2.9 – Schematic of the two-electrode voltage clamp circuit**

See body text for description of circuit function. Key to symbols: A1 = differential preamplifier, A2 = clamping amplifier, A3 = voltage drop differential amplifier to record membrane current (\( I_m \)), VE = voltage recording electrode, IE = current injecting electrode, RE = bath reference electrode. \( V_m \) = membrane voltage, \( V_{cmd} \) = command voltage, \( R_0 \) = current setting resistor, \( I \) = total current.
Chapter 2

Materials & Methods

Whole-cell recordings from oocytes were performed in the two-electrode voltage clamp configuration under continuous gravity-fed superfusion (~1ml/min) of a chloride-free solution containing (in mM): 5 Ba(OH)$_2$, 80 TEA-OH, 2 CsOH, 5 Hepes (pH 7.4 with methanesulfonic acid). The cells were injected systematically with 30-40 nl of a 100 mM solution of K$_3$-1,2-bis(aminophenoxy)ethane-$N,N,N',N'$-tetra-acetic acid (BAPTA) to suppress endogenous Ca$^{2+}$-activated Cl$^-$ currents. Recording microelectrodes, were pulled from thick walled borosilicate glass capillary tubing with the following dimensions: 1.5 mm outer diameter, 1.0 mm bore diameter and with an internal 0.1 mm fibre (Plowden and Thompson, UK). The TEVC pipettes were pulled using a P-87 Flaming/Brown microelectrode puller (Sutter Instrument Company, Novato, CA, USA). Recording electrodes contained 3M KCl and had resistances of 0.3-2 MΩ. The holding potential was -100 mV. All the experiments were performed at room temperature (20-24°C).

2.6.3 Data analysis and presentation

The currents were amplified and low-pass filtered at 1 KHz (to prevent extraneous low frequency noise) by means of a Geneclamp 500 amplifier (Axon Instruments, Burlingame, CA, USA), digitised through a Digidata 1200 interface (Axon Instruments) and stored on a PC using data acquisition software pClamp6.02 (Axon Instruments). Additional analyses including calculation of means, S.E.M, significance (paired or unpaired Student's $t$-tests, where applicable) and curve fitting were calculated using Microcal Origin 5.0 (Microcal Software Inc., MA, USA). Where mean values are presented they are shown as mean ± sem (with $n$ depicting the number of cells or experiments from which the mean was calculated). Statistical significance was defined as $p<0.05$ (*) and $p<0.01$ (**).

2.6.3.1 Equations

Curve fitting analysis was performed where appropriate using the fitting function of Microcal Origin (Microcal Software Inc.). The following three equations were used:
2.6.3.1.1 Current-voltage (I-V) relation

Current-voltage (I-V) relation curves were fitted with a combined Boltzmann and linear fit function:

\[ I = G_{\text{max}}(V-V_{\text{rev}})/(1+\exp(-(V-V_{0.5\text{activ}})/k)) \]

where \( I \) is the whole cell current (\( \mu \)A), \( G_{\text{max}} \) is the maximum slope conductance, \( V_{0.5\text{activ}} \) is voltage of the mid-point of activation, \( V_{\text{rev}} \) is the reversal potential and \( k \) is the slope factor for activation. This function provides a practical and simplified form of curve fitting approximating the \( V_{\text{rev}} \).

2.6.3.1.2 Steady-state inactivation

Steady-state inactivation data were fitted with a Boltzmann function of the form:

\[ I/I_{\max}=1/(1+\exp((V-V_{50\text{inact}})/k)) \]

where \( I/I_{\max} \) is the normalised peak current, \( V_{50\text{inact}} \) is the voltage for the mid-point of inactivation, \( V \) is the conditioning voltage potential and \( k \) is the slope factor for inactivation.

2.6.3.1.3 Inactivation time-course

\( I_{\text{Ba}} \) inactivation time-courses evoked by a 4.75s depolarising pulse to +10mV were best fitted with a sum of a two-exponential decay.

\[ I = I_0 + A_{\text{fast}}e^{-(t-t_0)/\tau_{\text{fast}}} + A_{\text{slow}}e^{-(t-t_0)/\tau_{\text{slow}}} \]

Where \( I \) is the current at time \( t \), and \( t_0 \) is the time at the commencement of decay and \( I_0 \) is the current at \( t_0 \). \( \tau_{\text{fast}} \) represents the time constant for the fast component of inactivation, and \( \tau_{\text{slow}} \) is the time constant for the slow component. \( A_{\text{fast}} \) represents the proportion of the fast component of inactivation, and \( A_{\text{slow}} \) represents the proportion of the slow part of inactivation.
3

Identification and cloning of human stargazin-related genes
3.1 Introduction

Stargazin is the product of the gene mutated in the epileptic mouse, Stargazer (cacng2). Despite poor sequence homology to the voltage dependent calcium channel (VDCC) \( \gamma_1 \) subunit of skeletal muscle (25%), early investigations hypothesised it to be the first example of a neuronal VDCC \( \gamma \) subunit on the basis of the similarity of its predicted protein secondary structure to the \( \gamma_1 \) subunit and its ability to cause a small but statistically significant shift in the half maximal voltage for steady state inactivation \( (V_{50\text{inact}}) \) of a P/Q-type \( \text{CaV}2.1/\beta_1d/\alpha2\delta_1 \) VDCC complex expressed in a mammalian cell line (Letts et al., 1998).

Organisms utilise genetic heterogeneity of calcium channel subunits as an important mechanism for generating functional diversity in different cell types. Ten genes have been reported to encode \( \text{CaV-} \alpha_1 \) subunits, four encode \( \beta \) subunits, and three \( \alpha2\delta \) genes have been identified (Catterall, 2000), but with only two genes reported to encode \( \gamma \) subunits, this class of subunit was relatively under-represented. Furthermore, because the \( \gamma_1 \) and \( \gamma_2 \) subunits are expressed in only skeletal muscle (Jay et al., 1990; Powers et al., 1993; Wissenbach et al., 1998) and neuronal tissue respectively (Letts et al., 1998), and not in any other tissues in which VDCCs are highly expressed, it was hypothesised that other \( \gamma \) subunit genes remained to be discovered.

This chapter describes the identification and cloning of a family of human stargazin-related genes that encode putative VDCC \( \gamma \) subunits. Previous attempts to identify further \( \gamma \) subunits using conventional molecular biology techniques had proved unsuccessful (Eberst et al., 1997). The cloning of \( \text{cacng2} \) from mouse brain highlighted that this was probably because the homology between \( \text{cacnlg} \) (\( \text{cacng1} \)) and \( \text{cacng2} \) was too low for nucleic acid hybridisation, or degenerate RT-PCR methods to succeed. In this investigation, the approach of searching genome and expressed sequence tag (EST) databases using text based similarity search algorithms was utilised to identify human genes related to \( \text{cacng2} \). In recent years, this "in silico" methodology has proved successful in identifying many novel genes, with the
notable example of the identification of the genes that encode the T-type VDCC $\text{Cav}_3.1$ (Perez-Reyes et al., 1998), $\text{Cav}_3.2$ (Cribbs et al., 1998) and $\text{Cav}_3.3$ (Lee et al., 1999d) subunits.

Following the identification of genomic and EST sequences identified in silico with significant homology to stargazin, the coding sequences of a family of putative human VDCC $\gamma$ subunits were subsequently cloned using conventional molecular biology techniques and the primary and secondary structure of the resulting proteins determined.


3.2 Results

3.2.1 Identification and virtual cloning of a family of human stargazin-related genes by electronic database mining

The submission of the mouse cacng2 sequence as a query into the tBLASTn alignment program identified regions of human genomic sequence and multiple ESTs derived from different tissue sources with identities greater than 40% to cacng2. These were grouped into four clusters according to sequence overlap and their relative identities to cacng2. For each cluster, the tissue source was assigned according to the annotations in the EST database (Table 3.1).

A 972 bp cluster contained the entire ORF of a human gene related to cacng2 (969bp) and the predicted protein encoded by this ORF displayed >99% identity to the putative mouse γ2 subunit but only 27% identity with the human γ1 subunit (Table 3.1 & Figure 3.1). This indicated that it represented the human stargazin orthologue and it was accordingly designated CACNG2 encoding a putative human γ2 subunit. As reported previously by Letts and colleagues (Letts et al., 1998), the identification of exon 1, 3 and 4 of the human orthologue CACNG2 in partially sequenced regions of chromosome 22 (accession no. Z83733 and AL022313) allowed the gene to be localised to human chromosome {Homo sapiens chromosome - Hsa} 22q12-13. However, following completion of the draft of the human genome (Birney et al., 2001; Jasny and Kennedy, 2001; McPherson et al., 2001; Pennisi, 2001; Venter et al., 2001; Wolfsberg et al., 2001) submission of the predicted CACNG2 ORF to the newly established NCBI Genome BLAST and Map View search facility (www.ncbi.nih.gov/Entrez/map_search) more specifically localised the gene to Hsa 22q13.1 (Figure 3.2).

Another 1065 bp cluster contained a 948 bp predicted ORF that would encode a 315 amino acid protein with 74% identity to stargazin (Table 3.1 & Figure 3.1). Searches of the NCBI protein databases with this amino acid sequence identified an identical 35 kDa predicted protein (AAC15246) annotated as an "unknown gene product" from Hsa 16p12-13.1 (AC004125). Genscan analysis of bacterial artificial
chromosome (BAC) AC004125 predicted the intron-exon structure of the gene encoding this unknown gene product to be extremely similar to those of both human and mouse γ2 genes. It was therefore concluded that this gene was a homologue of CACNG2 and the corresponding in silico cluster identified in this investigation was named CACNG3 encoding a predicted human γ3 subunit. More recent searches of the human genome map have now localised CACNG3 to Hsa 16p12.2 (Table 3.1 & Figure 3.3).

The remaining 777 bp and 487 bp clusters were shorter than the mouse stargazin open reading frame (969bp) and possessed no obvious start codon indicating they were unlikely to represent full-length stargazin-related genes. The longer of these sequences had a predicted translation product (reading-frame 1) with 59% identity to exons 2, 3 and 4 of cacng2, but was apparently lacking a first exon. Nevertheless, it was designated CACNG4, which encoded a partial predicted human γ4 subunit (Figure 3.1). Interestingly, this partial gene was located on Hsa 17q24, the same region in which the human γ1 subunit gene resides (Iles et al., 1993). The predicted amino acid product of the 487 bp cluster only displayed 26% identity to cacng2 over 158 amino acids and was thought to be part of a more distantly related gene. All further work arising from the discovery of this short, low homology stargazin-like cluster is described in detail in Chapter 6.

<table>
<thead>
<tr>
<th>Cluster ID</th>
<th>Tissue Distribution</th>
<th>Mapping</th>
<th>% homology to stargazin</th>
<th>In silico Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>CACNG2</td>
<td>Neuronal</td>
<td>22q13.1</td>
<td>&gt;99%</td>
<td>Complete ORF</td>
</tr>
<tr>
<td>CACNG3</td>
<td>Neuronal / Retina</td>
<td>16p12.2</td>
<td>74%</td>
<td>Complete ORF</td>
</tr>
<tr>
<td>CACNG4</td>
<td>Neuronal</td>
<td>17q24.2</td>
<td>59%</td>
<td>Partial ORF</td>
</tr>
<tr>
<td>487 bp cluster</td>
<td>Neuronal</td>
<td>?</td>
<td>26% over 158 aa</td>
<td>Partial ORF</td>
</tr>
</tbody>
</table>

Table 3.1– Summary of human genes identified in silico by their homology to murine cacng2.

Tissue distribution for each cluster was predicted according to sources of the ESTs from which they were assembled. Each cluster was mapped to a human chromosome according to homology with sequence in the genomic database. (aa = amino acid)
Figure 3.1 - A line up of the predicted protein sequences of human genes identified in silico by their homology to cacin2.

The products of the predicted genes CACNG2, CACNG3 and CACNG4, γ2, γ3 and γ4 respectively were lined up against the protein sequence of the human γ1 subunit. Subunits shaded in dark grey have 100% identity to the consensus sequence and those in light grey have 75% identity. The intron-exon boundaries are marked by solid dots (•) above the residue whose codon is interrupted by the adjacent intron. Note the γ4 predicted protein is incomplete, lacking sequence equivalent to the first exon of its homologues.
### Table 3.2 - Chromosomal location of CACNG2

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Location</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCM5</td>
<td>22q13.1</td>
<td>minichromosome maintenance deficient (S. cerevisiae) 5 (cell division cycle 46)</td>
</tr>
<tr>
<td>DEXRAS1</td>
<td>22q13.1</td>
<td>similar to mouse Ras, dexamethasone-induced 1</td>
</tr>
<tr>
<td>CACNG2</td>
<td>22q13.1</td>
<td>calcium channel, voltage-dependent, gamma subunit 2</td>
</tr>
<tr>
<td>RAYL</td>
<td>22q13.1</td>
<td>putative GTP-binding protein similar to RAY/RAB1C</td>
</tr>
<tr>
<td>MPST</td>
<td>22q13.1</td>
<td>mercaptoypyruvate sulfurtransferase</td>
</tr>
<tr>
<td>KIAA1904</td>
<td>22q13.1</td>
<td>KIAA1904 protein</td>
</tr>
<tr>
<td>LGALS2</td>
<td>22q13.1</td>
<td>lectin, galactoside-binding, soluble, 2 (galectin 2)</td>
</tr>
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<td>SH3BP1</td>
<td>22q13.1</td>
<td>SH3-domain binding protein 1</td>
</tr>
<tr>
<td>GALR3</td>
<td>22q13.1</td>
<td>galanin receptor 3</td>
</tr>
<tr>
<td>POLR2F</td>
<td>22q13.1</td>
<td>polymerase (RNA) II (DNA directed) polypeptide F</td>
</tr>
<tr>
<td>PRKCAHP</td>
<td>22q13.1</td>
<td>protein kinase C, alpha binding protein</td>
</tr>
<tr>
<td>KIAA1660</td>
<td>22q12</td>
<td>KIAA1660 protein</td>
</tr>
<tr>
<td>KDELR3</td>
<td>22q13.1</td>
<td>KDELR (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 3</td>
</tr>
<tr>
<td>C22orf2</td>
<td>22q12</td>
<td>chromosome 22 open reading frame 2</td>
</tr>
<tr>
<td>KIAA0668</td>
<td>22q13.1</td>
<td>KIAA0668 protein</td>
</tr>
<tr>
<td>NPTXR</td>
<td>22q13.1</td>
<td>neuronal pentraxin receptor</td>
</tr>
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<td>RPL3</td>
<td>22q13</td>
<td>ribosomal protein L3</td>
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<td>TAB1</td>
<td>22q13.1</td>
<td>transforming growth factor beta-activated kinase-binding protein 1</td>
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<td>HSU79252</td>
<td>22q13</td>
<td>hypothetical protein</td>
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<tr>
<td>KIAA1093</td>
<td>22q13.1</td>
<td>KIAA1093 protein</td>
</tr>
</tbody>
</table>

**Figure 3.2 - Chromosomal location of CACNG2**

An ideogram of human chromosome 22 annotated with the position of CACNG2 at position q13.1 (red line). To the right, the region surrounding this location, highlighted in grey on the ideogram is magnified with a diagram and table of genes surrounding the putative γ subunit sequence. Columns of table are from left to right, gene symbol, location, and full name.
Chapters
Clonins stargazin-related genes

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Location</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMG1</td>
<td>16p12.3</td>
<td>PI-3-kinase-related kinase SMG-1</td>
</tr>
<tr>
<td>ARL6IP</td>
<td>16p12-p11.2</td>
<td>ADP-ribosylation factor-like 6 interacting protein</td>
</tr>
<tr>
<td>LOC51760</td>
<td>16p12.3</td>
<td>B/K protein</td>
</tr>
<tr>
<td>FLJ13593</td>
<td>16p12.3</td>
<td>hypothetical protein FLJ13593</td>
</tr>
<tr>
<td>MIR16</td>
<td>16p12-p11.2</td>
<td>membrane interacting protein of RGS16</td>
</tr>
<tr>
<td>KIAA0419</td>
<td>16p12.3</td>
<td>KIAA0419 gene product</td>
</tr>
<tr>
<td>LOC57020</td>
<td>16p12.3</td>
<td>hypothetical protein</td>
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<tr>
<td>HS39850</td>
<td>16p12.3</td>
<td>elongation factor-2 kinase</td>
</tr>
<tr>
<td>KIAA1203</td>
<td>16p12.3</td>
<td>KIAA1203 protein</td>
</tr>
<tr>
<td>SCN1H</td>
<td>16p12.2-p12.1</td>
<td>sodium channel, nonvoltage-gated 1, beta (Liddle syndrome)</td>
</tr>
<tr>
<td>GGA2</td>
<td>16p12</td>
<td>Golgi-associated, gamma-adaptin ear containing, ARF-binding protein 2</td>
</tr>
<tr>
<td>UBPH</td>
<td>16p12</td>
<td>similar to ubiquitin binding protein</td>
</tr>
<tr>
<td>PLK</td>
<td>16p12.3</td>
<td>polo (Drosophila)-like kinase</td>
</tr>
<tr>
<td>LOC63928</td>
<td>16p12.3</td>
<td>hepatocellular carcinoma antigen gene 520</td>
</tr>
<tr>
<td>PRKCB1</td>
<td>16p11.2</td>
<td>protein kinase C, beta 1</td>
</tr>
<tr>
<td>CACNG3</td>
<td>16p12-p13.1</td>
<td>calcium channel, voltage-dependent, gamma subunit 3</td>
</tr>
<tr>
<td>KIAA1460</td>
<td>16p12.2</td>
<td>KIAA1460 protein</td>
</tr>
<tr>
<td>RKST1</td>
<td>16pter-p11</td>
<td>putative sodium-coupled cotransporter RKST1</td>
</tr>
<tr>
<td>AQP8</td>
<td>16p12</td>
<td>aquaporin 8</td>
</tr>
<tr>
<td>HS3ST4</td>
<td>16p11.2</td>
<td>heparan sulfate (glucosamine) 3-O-sulfotransferase 4</td>
</tr>
</tbody>
</table>

Figure 3.3 – Chromosomal location of CACNG3
An ideogram of human chromosome 16 annotated with the position of CACNG3 at position p12.2 (red line). To the right, the region surrounding this location, highlighted in grey on the ideogram, is magnified with a diagram and table of genes surrounding the putative γ subunit sequence. Columns of table are from left to right, gene symbol, location, and full name.
3.2.2 Amplification and cloning of cDNAs encoding novel human stargazin like proteins

The *in silico* predicted sequences were used to design primer pairs that amplified 972bp and 948bp cDNA's encoding the ORF's of *CACNG2* and *CACNG3*, respectively, from human brain total RNA by RT-PCR. Translation of the sequences predicted a putative 323 amino acid, 36kD \( \gamma_2 \) protein and a 315 amino acid, 35.5kD \( \gamma_3 \) protein. The \( \gamma_2 \) subunit displayed >99% identity to the mouse *cacng2* and 75% identity with its sub-family member \( \gamma_3 \). Identity with the skeletal muscle \( \gamma \) was much lower at approximately 25% for both \( \gamma_2 \) and \( \gamma_3 \) subunits (Figure 3.4).

Using the same total brain RNA source as used to amplify *CACNG2* and *CACNG3* ORFs, the partial sequence of the *CACNG4* ORF predicted by *in silico* data was cloned. Sequence analysis of multiple clones from different PCR reactions confirmed the computer predictions with the exception of nucleotides 93-96 to be CATC (*TNNN in silico* – Figure 3.5). More interestingly, the cloned sequences did not posses a 6bp run, TCAGGC present at nucleotides 234-240 of the 777 bp *in silico* sequence. In the cDNA, the first T of this sequence was a C and the remaining 5nt were absent, eliminating a frame shift from the prediction (Figure 3.5).

The absent 5' sequence predicted to correspond to exon 1 of *CACNG4* was isolated by nested PCR from human total brain first strand cDNA, synthesised from purified human whole brain total RNA using the SMART PCR cDNA Synthesis Kit. One of the many products of these reactions included a band of approximately 340 bp, amplified by the SMART oligonucleotide with primers FM33 then FM34 that was subsequently cloned and sequenced. Amongst the multiple clones that were analysed, one particular colony (designated CloneG8_5_28) contained sequence with 60nt overlap with the known *CACNG4* sequence, a putative in-frame start codon with the rest of the *CACNG4* ORF, and 203 nucleotides of 100% identity to nucleotides 163453-163655 of clone AC005988 derived from Hsa 17. Further analysis of this BAC clone revealed an 18 nucleotide sequence, ATGGTGCGATCGGACCGC that ran immediately 5' to the region of 100% identity to the cloned sequences and contained another in-frame ATG start codon that was preceded by 9 nucleotides.
Cloning stargazin-related genes

GCGCCCACCATGG (initiation codon is underlined), that displayed good homology to the consensus Kozak sequence (Kozak, 1987). Protein synthesis is regulated by the sequence and structure of the 5' untranslated region (UTR) of the mRNA transcript. Most eukaryotic mRNAs contain a short recognition sequence that greatly facilitates the initial binding of mRNA to the small subunit of the ribosome. This consensus Kozak sequence for initiation of translation in vertebrates is (GCC) GCCRCCCATGG, where R is a purine (A or G), and the initiation codon is underlined (Kozak, 1987). The GCG and the ESTBlast programs were used to assemble this short sequence with that of clone G8_5_28 and the previously cloned 771nt sequence. The resulting prediction was used to design the oligonucleotide primers that would amplify the complete human CACNG4 ORF from total human brain RNA by RT-PCR. The complete 984nt CACNG4 ORF predicted a protein of 327 amino acids (MW 36.6kDa). This protein exhibits 59%, 56% and 23% identity with human γ2, γ3, and γ1 subunits, respectively (Figure 3.4). Submission of the complete CACNG4 ORF to the NCBI Genome BLAST located the gene to human chromosome 17q24.2 (Table 3.1 & Figure 3.6).
Chapter 3

Cloning stargazin-related genes

Figure 3.4 – Multiple alignments of the predicted protein sequences of human γ1.4 subunits

Sequences were aligned with the Clustal W multiple alignment program with default values. Dark grey and light grey areas highlight regions of 100% and 75% identity between all subunits respectively. Two cysteine residues highly conserved amongst all γ subunits are highlighted in bold white typeface. The exon/intron boundaries are marked by solid dots above the residue whose codon is interrupted by the adjacent intron. Underlined regions as predicted by the TMpred program denote the transmembrane (TM 1-4) segments. Dotted lines and bold font denote potential N-glycosylation sites and residues in lower case mark residues potentially phosphorylated by PKA, PKC, casein kinase II or tyrosine kinase. Boxed residues in the C-termini mark residues with homology to the PDZ-domain binding motif present in NMDA receptors and voltage-dependent potassium channels.
Figure 3.5 - CACNG4 ORF cDNA sequence compared to the partial in silico prediction.

The cDNA sequence of the complete CACNG4 ORF was amplified from human total brain RNA and its sequence compared with the partial in silico prediction. The majority of missing first exon of CACNG4 was identified by 5'RACE (underlined region) which itself identified an Hsa 17 BAC containing identical sequence and with a small additional 5' fragment containing the translation “start” codon (bold residues). Regions of the in silico prediction found to be different in the cDNA clone are highlighted in grey. Large triangles above sequence mark the consensus exon boundaries. Only sequence corresponding to exons 1, 2 and part of exon 3 are displayed, as all nucleotides are identical beyond this region.
### Table 3.6 - Chromosomal location of CACNG1 and CACNG4

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Location</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCN4A</td>
<td>17q23-q25.3</td>
<td>Sodium channel, voltage-gated, type IV, alpha polypeptide</td>
</tr>
<tr>
<td>LOC92340</td>
<td>17q24.2</td>
<td>Similar to putative</td>
</tr>
<tr>
<td>ICAM2</td>
<td>17q23-q25</td>
<td>Intercellular adhesion molecule 2</td>
</tr>
<tr>
<td>LOC124644</td>
<td>17q24.2</td>
<td>Similar to TCAM-1 (H. sapiens)</td>
</tr>
<tr>
<td>HT008</td>
<td>17q24.2</td>
<td>Uncharacterized hypothalamus protein HT008</td>
</tr>
<tr>
<td>LOC124643</td>
<td>17q24.2</td>
<td>Similar to ribosomal protein L31, 60S ribosomal protein L31 (H. sapiens)</td>
</tr>
<tr>
<td>FALZ</td>
<td>17q24</td>
<td>Fetal Alzheimer antigen</td>
</tr>
<tr>
<td>LOC124638</td>
<td>17q24.2</td>
<td>Similar to 60S RIBOSOMAL PROTEIN L17 (L23) (H. sapiens)</td>
</tr>
<tr>
<td>RDG660</td>
<td>17q24.2</td>
<td>Retinal degeneration B beta</td>
</tr>
<tr>
<td>PSMD12</td>
<td>17q24.2</td>
<td>Proteasome (prosome, macropain) 26S subunit, non-ATPase, 12</td>
</tr>
<tr>
<td>LOC124659</td>
<td>17q24.2</td>
<td>Similar to keratin, type I cytoskeletal 18 (cytokeratin 18) (k18) (ck 18) (h. sapiens)</td>
</tr>
<tr>
<td>LOC124658</td>
<td>17q24.2</td>
<td>Hypothetical gene supported by XM_074734</td>
</tr>
<tr>
<td>CACNG1</td>
<td>17q24.2</td>
<td>Calcium channel, voltage-dependent, gamma subunit 1</td>
</tr>
<tr>
<td>CACNG4</td>
<td>17q24.2</td>
<td>Calcium channel, voltage-dependent, gamma subunit 4</td>
</tr>
<tr>
<td>PRKCA</td>
<td>17q22-q23.2</td>
<td>Protein kinase C, alpha</td>
</tr>
<tr>
<td>APOH1</td>
<td>17q23-qter</td>
<td>Apolipoprotein H (beta-2-glycoprotein I)</td>
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<td>17q24.3</td>
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**Figure 3.6 – Chromosomal location of CACNG1 and CACNG4**

An ideogram of human chromosome 17 annotated with the position of CACNG1 and CACNG4 at position q24.2 (red line). To the right, the region surrounding this location, highlighted in grey on the ideogram, is magnified with a diagram and table of genes surrounding the putative γ subunit sequences. Columns of table are from left to right, gene symbol, location, and full name.
According to the TMPred program (Hofmann and Stoffel, 1993) the hydrophobicity profiles predicting the transmembrane topology of the deduced γ2, γ3 and γ4 subunits were almost identical (Figure 3.7). Each subunit comprises four transmembrane spanning segments, with the amino- and carboxy-termini localised intracellularly, agreeing with those postulated for skeletal muscle γ1 (Bosse et al., 1990; Eberst et al., 1997; Jay et al., 1990), and mouse brain γ2 (Letts et al., 1998). These plots also provide a good graphical representation of the much longer intracellular C-termini of the neuronal γ2, γ3 and γ4 subunits compared to the skeletal muscle γ1.

**Figure 3.7 – Predicted transmembrane topology of the VDCC γ subunits**

Comparison of the transmembrane architecture of the human skeletal muscle γ1 subunit with the predicted neuronal γ2, γ3 and γ4 subunits. Positive TMPred (Hofmann and Stoffel, 1993) scores highlight the four transmembrane spanning segments (TM 1-4). The program also predicts that the most favourable orientation of the subunit in the plasma membrane is with the N- and C-termini on the intracellular side. Evidence to support this orientation includes the lack of N-terminal signal sequences, location of the predicted N-glycosylation sites on the extracellular side and the majority of the predicted phosphorylation sites in intracellular domains. The plots for each subunit are remarkably similar and form a large part of the evidence that suggests γ2, γ3 and γ4 are VDCC γ subunits despite having substantially larger C-termini than the γ1 subunit.
3.3 Discussion

3.3.1 Identification and cloning of human putative VDCC $\gamma_2$, $\gamma_3$ and $\gamma_4$ subunits

The cDNA sequences of three novel human stargazin-like proteins have been identified and cloned from the brain. CACNG2, CACNG3 and CACNG4 encode proteins that form a specific subfamily of putative human neuronal VDCC $\gamma$ subunits that according to the sources of the ESTs used to assemble their predicted sequences, are expressed primarily in neuronal tissue. The amino acid sequences of the resulting $\gamma_2$, $\gamma_3$ and $\gamma_4$ proteins exhibit 99%, 74% and 59% identity with stargazin but only ~18% identity in comparison with the human $\gamma_1$ subunit (Powers et al., 1993). The identities of the human $\gamma_3$ and $\gamma_4$ subunits to $\gamma_2$ are however 84% and 64% respectively. These findings agree with those of two other laboratories that have published the human CACNG2, CACNG3 and CACNG4 sequences during the course of this investigation (Black and Lennon, 1999; Burgess et al., 1999b).

The approximate chromosomal locations of the CACNG2, CACNG3 and CACNG4 genes were initially determined from the annotations of the BACs containing $\gamma$ subunit sequence to be Hsa 22q12-13, 16p12-13.1 and 17q24 respectively. However, following the recent completion of the draft human genome, these were more specifically located to Hsa 22q13.1, 16p12.2 and 17q24.2. A mutation in the mouse cacng2 results in the stargazer or waggler phenotypes, which show an ataxia and suffer from spike-wave seizures analogous to absence epilepsies in humans (Letts et al., 1998; Noebels et al., 1990). Mutations of ion channel subunits or channelopathies have also been associated with several seizure disorders in humans (Barchi, 1998; Biervert et al., 1998; Steinlein et al., 1995; Wallace et al., 1998). Furthermore, mutations in VDCC $\alpha_1$ subunits have been linked to human familial hemiplegic migraine, episodic ataxia and congenital stationary night blindness (Bech-Hansen et al., 1998; Brandt and Strupp, 1997; Ophoff et al., 1996). Consequently, the Online Mendelian Inheritance in Man (OMIM) database was searched for linkage between any of the human CACNG genes identified in this study and neurological disorders in man. The location of CACNG3 yielded a possible link
to a human seizure disorder, with benign familial infantile convulsions 2 (BFIC2) mapping to Hsa 16p12-q12. Similar to benign familial neonatal convulsions (BFNC) that is caused by mutations in the $K^+$ channel subunit genes $KCNQ2$ that maps to Hsa 20q (EBN1) (Singh et al., 1998) and $KCNQ3$ on Hsa 8q (EBN2)(Charlier et al., 1998), or benign familial infantile convulsions 1 (BFIC1) that maps to Hsa 19q but whose gene remains unidentified (Guipponi et al., 1997), BFIC2 is possibly allelic to infantile convulsions and choreoathetosis (ICCA) (Caraballo et al., 2001; Lee et al., 1998), but the identity of the affected gene remains to be determined. More precise mapping of the BFIC2 locus or screening for mutations in $CACNG3$ from patients suffering from the disorder could confirm or eliminate the $\gamma_2$ subunit as a candidate for mutation in this disorder. As more neurological disorders are mapped in the future, the possible involvement of $CACNG2$, $CACNG3$ and $CACNG4$ should be monitored.

### 3.3.2 Predicted structure and motifs of potential functional significance

The $\gamma_2$, $\gamma_3$ and $\gamma_4$ proteins are all predicted to possess a very similar four-transmembrane secondary structure to the VDCC $\gamma$ subunit cloned from skeletal muscle but possess C-termini that are approximately 100 amino acids longer. Within these longer C-termini reside multiple sites for post-translational modification. The $\gamma_2$ subunit possesses six potential phosphorylation sites, three protein kinase C sites ($PKC$; residues 211, 228, 240 and 290), one cAMP site (residue 318) and one caesin kinase II site (residue 290 - Figure 3.4). The $\gamma_3$ subunit is predicted to possess ten potential sites for phosphorylation. PKC has the potential to act at three sites (residues 236, 282, 295), cAMP dependent kinase at four sites (PKA; residues 217, 232, 233 and 310) and caesin kinase II at three sites (211, 282, and 295), which like the $\gamma_2$ subunit are all in the carboxy-terminal chain (Figure 3.4). $\gamma_4$ also has the potential to be highly phosphorylated in the C-terminal sequence at residues 244 and 325 by PKA, residue 218 by caesin kinase II and residues 218, 236 and 247 and one other site at residue 132 between TM II and III by tyrosine kinase (Figure 3.4). Another post-translational modification conserved amongst all the $\gamma$ subunit is a site for N-linked glycosylation in the first putative extracellular loop between
transmembrane segments I and II. This modification occurs at residue 43 of $\gamma_1$, residue 48 of both $\gamma_2$ and $\gamma_3$, and residue 42 of $\gamma_4$. A second N-linked glycosylation site is also predicted at residue 79 of $\gamma_1$. As for other VDCC subunits, both phosphorylation and glycosylation sites are of possible functional significance in the normal function of the subunit *in vivo* (Burgess and Norman, 1988; Chien and Hosey, 1998; Gao et al., 1997a; Gurnett et al., 1996; Gutierrez et al., 1994; Henning et al., 1996; Lu et al., 1995; Nunoki et al., 1989; Shistik et al., 1998). The location of these predicted sites for post-translational modification help to predict the orientation of the $\gamma$ subunits in the plasma membrane. The strongly favoured prediction according to the TM Pred program (Hofmann and Stoffel, 1993) is for the N- and C-termini to be located on the intracellular side, which favours phosphorylation at all the residues previously mentioned and locates the putative N-linked glycosylation sites in what becomes the first extracellular loop.

Closer analysis of the newly identified neuronal $\gamma$ subunits with other proteins in the database revealed that they form part of a super-family of tetra-spanning membrane proteins. Although overall sequence identity between many of these proteins is low, they contain a region of conserved domain architecture highlighted by the consensus sequence in Figure 3.8. This group of proteins includes peripheral membrane protein 22 (PMP22 – also called paroxisomal membrane/myelin protein) (Snipes et al., 1992; Taylor et al., 1995), epithelial membrane proteins (EMP1-3) (Chen et al., 1997; Taylor and Suter, 1996), lens intrinsic membrane proteins (MP20) (Kumar et al., 1993) and the claudin tight junction (TJ) forming subunits (Furuse et al., 1998a; Morita et al., 1999), which have been implicated in the regulation of cell development (Magyar et al., 1996; Patel et al., 1992; Zoidl et al., 1995) and/or are involved in intercellular connections and cell adhesion events (D'Urso et al., 1999; Furuse et al., 1998b; Gow et al., 1999). The transmembrane regions of these proteins contain conserved motifs, however, the first extracellular loop of almost all members of the group also contains a conserved GLWxxC motif.
Chapter 3

Cloning stargazin-related genes

250  260

**Figure 3.8 - Regions of the γ2, γ3 and γ4 subunits exhibit structural similarity to a group of integral membrane proteins.**

A sequence alignment of tetra-spanning membrane proteins, epithelial membrane protein-1 (EMP-1_B4B), EMP-1 squamous cell-specific protein CL-20 (EMP-1_CL20), Peripheral myelin protein 22 (PMP-22), lens fibre membrane intrinsic protein (MP20) and tight junction forming subunits, Claudins 1, 7 and 10, with the VDCC γ2, γ3, and γ4 subunits. The conserved domain architecture is highlighted by the consensus sequence. Putative transmembrane regions are underlined. Residues exhibiting a conservation score >2.5 bits with consensus sequence are highlighted in magenta.

The cysteine residue of this motif has the potential to form a disulphide bond with a second conserved cysteine approximately ten amino acids downstream (Figure 3.8).

The functional importance this highly conserved region in any of these species is unknown but it is possible that they may be involved in the formation interfacial "kissing points" characteristic of TJ (Tsukita and Furuse, 1999). However, the mechanism underlying such interactions remains to be clarified. Nevertheless, this apparent link between the putative γ2, γ3 and γ4 subunits and TJ molecules such as the Claudins may implicate them in cell adhesion events at neuronal synapses where VDCCs are abundant (Doughty et al., 1998a; Westenbroek et al., 1998).

Evidence that stargazin and its orthologues are possibly involved in the molecular architecture of specialised cellular regions strengthened by the presence of another conserved motif in the novel γ subunits. The final four amino acids of the C-terminal of all three putative human neuronal γ subunits are identical. This sequence, TTPV (Figure 3.4), is strikingly similar to the {T/S}-X-V motif in the C-terminal of N-methyl-D-aspartic acid (NMDA) receptor subunits (Kornau et al., 1995) and voltage-dependent K+ channels (Kv) (Kim et al., 1995a) that has been implicated in the binding of post synaptic density 95 (PSD-95), a prominent cytoskeletal protein of post-synaptic densities that belongs to the family of proteins known as membrane...
Chapters

Cloning stargazin-related genes

associated guanylate kinases (MAGUKs) (Cho et al., 1992; Kistner et al., 1993). The motif associates to another conserved sequence in PSD-95, the PDZ domain. This is a conserved structural element of 80 to 100 amino acids that was originally identified in PSD-95, the Drosophila tumour suppressor discs-large (Woods and Bryant, 1991), and the tight-junction protein Zona occludens-1 (ZO-1) (Morais Cabral et al., 1996; Willott et al., 1993). The presence of multiple PDZ domains in these scaffold proteins can facilitate the assembly of multi-protein signalling complexes (Fanning and Anderson, 1999), or clustering of structural elements as has been shown by ZO-1, ZO-2, and ZO-3, which contain three PDZ domains (PDZ1 to -3), and are concentrated at TJs in epithelial cells via an interaction with the C-terminal tyrosine and valine residues of the distant relatives of the γ subunits, the claudins (Itoh et al., 1999).

There is a reasonable likelihood that the putative neuronal γ2, γ3 and γ4 subunits are trafficked and clustered via such interactions, and they may also underlie any incorporation into a VDCC complex or integrate the VDCC into a larger signalling network. In mice expressing a mutated PSD-95 it has been shown that synaptic morphology is unaffected and the NMDA receptor is able to traffic to the postsynaptic density independently of PSD-95. However, the mutation compromises the link between the NMDA receptor and downstream signalling mechanisms resulting in dramatically impaired spatial learning and enhanced long-term potentiation (Migaud et al., 1998). Interestingly both stargazer and waggler mice, display a severely impaired eye-blink conditioning response, indicating adversely affected cerebellar function and behavioural learning (Bao et al., 1998; Qiao et al., 1998). It is possible that similarly to the PSD-95 mutant mice, the mutations in the γ2 subunit underlying the stargazer and/or waggler phenotypes disrupt the interaction of the subunit with synaptic MAGUKs or other PDZ domain proteins impairing downstream signalling pathways in cerebellar granule cells involved in learning processes. Further investigation is required to prove this hypothesis.

The nature of the interaction between γ subunits and VDCCs remains undetermined. The γ1 subunit can be purified as part of the DHPR complex of skeletal muscle (Flockerzi et al., 1986; Jay et al., 1990), although it cannot be via a PDZ interaction because the required domain is absent from this subunit, and is primarily believed to
be a transmembrane domain interaction (Catterall, 1998). Nevertheless, the potential for the PDZ-interaction domains of the neuronal isoforms with VDCC subunits, either by direct or indirect interactions remains. The neuronal \( \text{Cav}2.1 \) and \( \text{Cav}2.2 \) subunits are both recognised by PDZ domain proteins (Maximov et al., 1999) and the auxiliary \( \beta_{1b} \) subunit contains a PDZ domain like region within its N-terminus (Hanlon et al., 1999) which gives rise to the possibility of an as yet undetermined interaction between these subunits and a \( \gamma \) at the post-synaptic density.

Subsequent chapters investigate the differential tissue distribution of this newly identified family of human neuronal proteins and investigates the hypothesised link with VDCCs by recording their influence when co-expressed \textit{in vitro} with heterologous channels.
In vitro expression and differential tissue distribution of the putative γ₂, γ₃ and γ₄ subunits
In vitro expression and tissue distribution

4.1 Introduction

Chapter 3 described the identification and cloning of the cDNAs of a family of putative human neuronal VDCC γ subunits, whose functional properties would need to be investigated by expressing them in heterologous expression systems. However, before this could commence, I needed to determine whether the cloned subunits would successfully express in vitro, and if so, with which VDCC subunits to co-express them in future functional characterisation experiments. To this end, the sub-cellular distribution of the \( \gamma_2, \gamma_3, \) and \( \gamma_4 \) subunits when expressed transiently in COS-7 cells was determined. This required the generation of specific polyclonal antibodies raised against each γ subunit. Once generated, the antibodies were used in immunocytochemistry experiments to detect expression of each γ subunit in COS-7 cells transfected with \( \gamma_2, \gamma_3, \) or \( \gamma_4 \) alone or in combination with a VDCC complex. This served the additional purposes of checking the specificity of the newly raised antibodies and determined whether COS-7 cells endogenously express γ subunits. *Xenopus* oocytes and COS-7 cells were also examined for endogenous γ subunit mRNAs by northern blot.

Having determined that the cloned γ subunits express in vitro and that the cell types in which they were being expressed did not possess endogenous γ subunits, northern blot analysis established the gross tissue distribution of each of the putative human γ subunits. These results were extended by immunohistochemical examination of the differential expression of each subunit in human hippocampus and cerebellum. The γ specific antibodies were used in these studies and the expression profiles generated for the \( \gamma_2, \gamma_3 \) and \( \gamma_4 \) subunits when compared with previously published expression data for the other VDCC subunits allowed me to speculate with which VDCC combinations the γ subunits were likely to associate in vivo. These conclusions would determine the subunit combinations with which I would express the γ subunits in electrophysiological characterisation experiments (Chapter 5) providing an insight into the possible modulatory function of each neuronal γ subunit in vivo.
4.2 Results

4.2.1 Transient expression of cloned γ subunit cDNAs in COS-7 cells

COS-7 cells were transiently transfected with the pMT2 vector containing the cDNAs of the human γ2, γ3, and γ4 subunits. Their expression was detected 2-3 days after transfection with a polyclonal rabbit anti γ2, γ3, or γ4 antibody (Ab) labelled with biotinylated anti-rabbit IgG and a Texas Red-streptavidin conjugated fluorophore. When expressed alone, all three γ subunits expressed well, and localised largely to the plasma membrane of the cells. The staining pattern resembled a continuous red halo in cells transfected with the γ2 or γ4 subunit, but the γ3 immuno-reactivity (IR) presented as clusters of intense staining in the membrane (Figure 4.1). The specificity of the Abs was confirmed by incubating non-transfected cells with each primary Ab, which in each instance showed no IR (Figure 4.1 non). Similar results were obtained when cells expressing one of the γ isoforms were incubated with Abs directed against the other two (data not shown). This result confirmed that the Abs did not cross-react with any species except the one with which they were intended to bind. Cells transfected with a γ subunit but which were not permeabilised after fixation exhibited no IR (Figure 4.1np). The anti-γ subunit Abs were designed to epitopes in the C-terminal region after transmembrane segment 4 of each subunit. This indicated that γ2, γ3, and γ4 subunits are orientated in the plasma membrane with their N- and C-termini located in the intracellular side.

The γ subunits were then transfected with VDCC subunits Cav2.1 and β4 to check that their expression was not disrupted by the co-expression of other subunits and that the antibodies did not cross-react with these VDCC proteins. Although at this stage of the investigation, the particular tissue distributions of each human γ isoform had not been determined, the Cav2.1 and β4 subunits were chosen because these were the prevalent VDCC subunits of their respective classes expressed in cerebellum (Ludwig et al., 1997; Volsen et al., 1995; Volsen et al., 1997), the tissue in which mouse γ2 had been shown to be predominantly expressed (Letts et al., 1998). The expression of the γ2, γ3, and γ4 subunits remained localised to the plasma membrane when co-expressed with these subunits, and the anti-γ subunit antibodies did not cross-react with the Cav2.1 or
β₄ subunits (Figure 4.2). However, although all three subunits expressed well throughout the plasma membrane, the IR pattern for γ₂ and γ₄ was clustered like that observed for the γ₃ subunit when expressed alone, rather than displaying the continuous red “halo” that had been observed when γ₂ or γ₄ were expressed alone.

Figure 4.1 – Transient expression and subcellular localisation of cloned neuronal γ subunits in COS-7 cells.

Cells were transfected with each subunit according to the annotation of each row. **non** = non-transfected cells. The transfections of non-permeablised (np) cells are labelled above each panel. Primary antibody specific for each subunit was incubated with the permeablised transfected cells and labelled with Texas Red conjugated fluorophore (red). The cell nuclei were labelled with the UV fluorescent dye, DAPI (blue). The γ₂, γ₃, and γ₄ subunits all localise to the plasma membrane when expressed alone. Un-transfected COS-7 cells incubated with each antibody show no cross-reaction with endogenous proteins. None of the anti-γ antibodies detect expressed protein if the cells are not permeablised. Scale bars in all panels represent 10μm.
Figure 4.2 – Co-expression of neuronal $\gamma$ subunits with VDCC Ca\(_{2.1}\) and $\beta_4$ subunits in COS-7 cells.

Cells were transfected with subunit combinations as annotated to the left of each row. Primary antibody specific for each subunit was incubated with the permeabilised transfected cells and labelled with Texas Red conjugated fluorophore (red). The cell nuclei were labelled with the UV fluorescent dye, DAPI (blue). Membrane localisation of the $\gamma$ subunits is not altered by co-expression of a Ca\(_{2.1}/\beta_4\) VDCC complex but the incidence of spots of intense staining is more prevalent than when the $\gamma$ subunits were expressed alone (examples shown by white arrows). This is particularly the case for the $\gamma_2$ and $\gamma_4$ subunits (see Figure 4.1) and could indicate clustering with VDCC complexes. The bottom row reveals that the $\gamma$ subunit antibodies do not cross-react with the Ca\(_{2.1}\) or $\beta_4$ subunits. Scale bars represent 10μm in all panels.

### 4.2.2 Probing for endogenous $\gamma$ subunit mRNAs in Xenopus oocytes and COS-7 cells

The previous set of experiments determined that the $\gamma$ subunit cDNAs cloned in Chapter 3 expressed in vitro when inserted into the pMT2 expression vector. They also established that no detectable $\gamma_2$, $\gamma_3$ or $\gamma_4$ subunit protein was endogenously expressed in COS-7 cells. The electrophysiological characterisation of $\gamma$ subunit properties is described in Chapter 5 and was performed in Xenopus oocytes. However, before those investigations commenced Xenopus oocytes were examined for the expression of endogenous $\gamma$ subunit mRNAs and the absence of $\gamma$ subunits in COS-7 cells was confirmed by northern blot analysis. A northern blot was constructed with lanes containing the total RNA from COS-7 cells or Xenopus oocytes. Northern probes
Chapter 4  

In vitro expression and tissue distribution

approximately 200 nt in length specific to each γ subunit were amplified from the human γ subunit cDNAs. These were then labelled with α-^32^P-dATP in probe synthesis reactions and all three probes were mixed and incubated with the blot, which was subsequently washed at low stringencies. Development of the blot revealed that no RNAs of the expected size for γ subunits (between 2 kb and 7 kb) from *Xenopus* oocytes or COS-7 cells hybridised to the γ subunit probes, indicating that they were probably not endogenously expressed in these cell types (Figure 4.3). Only bands corresponding to the non-specific signal emitted by the 16S and 23S ribosomal RNAs were detected in each cell type. Only repetition of these experiments using purified mRNA will abolish these sources of noise, which could possibly have masked low-level γ subunit signals from these cells, and conclusively determine whether γ subunit message is transcribed in *Xenopus* oocytes or COS-7 cells.

![Figure 4.3 - Northern blot to probe for endogenous γ2, γ3 and γ4 subunit RNAs in Xenopus oocytes and COS-7 cells.](image)

A mixture of γ subunit specific probes failed to specifically hybridise to any bands in northern blots containing total RNA run from *Xenopus* oocytes and COS-7 cells. The two signals seen in each lane represent the ribosomal 23S and 16S RNA or their equivalents from each cell type. Because washing was performed at low stringency, non-specific hybridisation to these species occurred. The positions of the RNA size markers are marked to the left of the blot in kilobases (kb). This blot is representative of three experiments.

### 4.2.3 Northern Blot analysis of mRNA distribution

The tissue distribution of the novel γ2, γ3 and γ4 mRNAs was analysed by northern blot. A human multiple-tissue northern blot (Figure 4.4a) and two brain region blots (Figure 4.4b and c) were hybridised with specific cDNA probes generated from the cDNA clones identified in the previous chapter. The results of these northern blots would provide information about the tissues in which the sub-cellular distribution of these...
putative γ subunits could be further investigated and affect the decision concerning the other VDCC subunits to be used in the co-expression studies to characterise their functional properties in vitro.

4.2.3.1 γ₂

A cDNA probe corresponding to nucleotides 597-814 (218nt) of the CACNG2 ORF cDNA was amplified from one of the γ₂ cDNA clones generated in Chapter 3. This region is contained entirely within the fourth exon of the gene, not crossing any intron-exon boundaries and had the lowest observed homology with the CACNG3 and CACNG4 sequences. The CACNG2 specific probe detected an mRNA of approximately 7kb and a smaller 3kb band. Interestingly, these signals were almost identical to those produced in a mouse multiple-tissue blot probed with a murine γ₂ cDNA probe (Letts et al., 1998). That investigation also detected a major transcript of approximately 7kb and a minor 3kb transcript specifically localised in brain. This indicated the message of the γ₂ subunit is the same or very similar size in both species. Of the multiple human tissues probed, the transcript was only present in brain (Figure 4.4a). However, probing of the brain region blots determined that the γ₂ mRNA is particularly abundant in cerebellum, cerebral cortex, occipital lobe, frontal lobe and temporal lobe, hippocampus and thalamus (Figure 4.4b & c). The transcripts were detected at slightly lower levels in medulla, putamen, amygdala and substantia nigra whilst weak signals were detected in caudate nucleus, but were apparently absent from corpus callosum and the sub-thalamic nucleus and spinal cord.

4.2.3.2 γ₃

A 233nt cDNA probe corresponding to nucleotides 560-792 of the CACNG3 cDNA determined that the expression of a single 2.0-2.1kb γ₃ transcript was much more selective than that for the γ₂. Like the γ₂ subunit, the γ₃ transcript was exclusively localised to the brain, but was only detected in cerebral cortex, including occipital lobe, frontal lobe, and temporal lobe, the putamen, caudate nucleus, amygdala and hippocampus. Expression of a CACNG3 mRNA was notable in its absence from all of the other regions probed (Figure 4.4).
4.2.3.3 \( \gamma_4 \)

The \( \gamma_4 \), 211nt specific cDNA probe, corresponding to nucleotides 594-804 of the CACNG4 ORF detected an mRNA of approximately 4kb that was expressed exclusively in brain (Figure 4.4). Investigation of particular brain regions determined that the mRNA was expressed in the majority of tissues probed. The signal was most prevalent in the putamen and caudate nucleus, which together are often collectively termed the striatum, one of the structures that make up the basal ganglia. Apart from the \( \beta \)-actin control, the \( \gamma_4 \) probe was the only one to hybridise to a transcript in spinal cord, albeit faintly.
Figure 4.4 - Northern blot analysis of the expression of the mRNA transcripts of the $\gamma_2$, $\gamma_3$, and $\gamma_4$ subunits.

Human multiple-tissue northern blots (a) probed specifically for the $\gamma_2$, $\gamma_3$, and $\gamma_4$ subunits reveal that all three species are exclusively expressed in the brain. Probing the brain region blots (b & c) determined that the $\gamma_2$ and $\gamma_4$ subunits are almost ubiquitously expressed, but at differential levels in the same tissues, whereas the $\gamma_3$ subunit is more specifically localised to cerebral cortex, amygdala, caudate nucleus and hippocampus. Size markers are from a RNA ladder provided on the blot by Clontech and the $\beta$-actin control probe results are displayed in the bottom panels.
4.2.4 Immunohistochemical analysis of neuronal $\gamma$ subunits

The results of the northern blots provided data about the regional distribution of the mRNA transcripts for the $\gamma_2$, $\gamma_3$, and $\gamma_4$ subunits. However, they did not provide information about protein expression or the cell types within these regions that actually express each $\gamma$ isoform. To this end, I performed immunohistochemical analysis of the expression of the $\gamma_2$, $\gamma_3$, and $\gamma_4$ subunits in human cerebellum and hippocampus. The reasons for choosing to study $\gamma$ subunit expression in these tissues were as follows. The stargazer and waggler mice display a loss of the fast component of EPSC at mossy fibre to cerebellar granule cell synapses (Chen et al., 1999; Hashimoto et al., 1999), plus reduced synaptic transmission at parallel fibre Purkinje cell synapses in waggler (Chen et al., 1999). However, the synaptic transmission to CA1 pyramidal cells (Schaffer collateral projection) in stargazer was not altered (Hashimoto et al., 1999). In situ hybridisation studies determined that murine $\gamma_2$ is normally expressed at its highest levels in the cerebellum and at slightly lower levels in hippocampus (Letts et al., 1998) but is severely reduced in stargazer brains. This would suggest that $\gamma_2$ is the major and possibly the only neuronal $\gamma$ subunit in the cerebellum, whereas other $\gamma$'s could be present at sufficient levels to compensate for the loss of $\gamma_2$ in hippocampus. It was therefore of great interest to see if expression of the human $\gamma$ subunits identified in this work paralleled those of mouse and if they were differentially expressed in the different cell types of each tissue type. Furthermore, the distribution of other VDCC subunits has been investigated in the greatest detail in these two brain regions.

4.2.4.1 Anatomy of the cerebellum

The cerebellum coordinates muscular activity and controls the force, direction and extent of voluntary movements. It is also important as a centre for body equilibrium and in the maintenance of posture. It receives afferent sensory input from the eyes, ears, cutaneous sensory receptors and proprioceptors. However, in spite of its large sensory input no conscious sensations are perceived in the cerebellum. Its efferent connections are to motor control centres in the brainstem, some of which in turn are linked to the cerebral cortex. Despite containing more than half the total number of neurones in the
CNS, the structural organisation of the cerebellum is remarkably simple in comparison to other structures such as the cerebral cortex or hippocampus. The cerebellar cortex consists of three layers. The outermost is the molecular layer, which contains mainly dendrites and axons from cells in the deeper layers and only a few cell bodies. Immediately below the molecular layer lies a monolayer of Purkinje cell bodies and below the Purkinje cells lies the granule cell layer, packed with cerebellar granule cells, the most abundant neurones in the CNS. Deep within the cerebellum, lie the cerebellar nuclei. The cells in these regions act as a relay station for outgoing information from the cerebellar cortex. Outlined below is a brief description of the principal cell types of the cerebellum and their relative locations are displayed in Figure 4.5.

### 4.2.4.1.1 Purkinje cells

Purkinje cells are the biggest neurones in the cerebellar cortex and among the biggest neurones in the body. They have vast, extensively branched dendritic arbours, which are unusual in that they are confined to a single plane, forming an espalier orientated perpendicular to the long axis of the folia. Their axons, which are the only output from the cerebellar cortex, generally pass to the deep cerebellar nuclei. The Purkinje cells are GABAergic, and inhibit their target neurones.
4.2.4.1.2 Granule cells

The granule cells are glutamatergic, excitatory neurones that have their cell bodies in the granule cell layer. The axons of the granule cells ascend through the Purkinje cell layer into the molecular layer in which they bifurcate at right angles into two branches running parallel with the surface of the cortex. These branches are called parallel fibres, run in the direction of the long axis of the folia and form numerous synapses with the Purkinje cell dendrites, which are at right angles to the parallel fibres. This arrangement ensures that each parallel fibre forms synapses with many Purkinje cells. At the same time each Purkinje cell integrates the activity of a large number of granule cells.

4.2.4.1.3 Inhibitory inter-neurones

Three types of inhibitory, GABAergic inter-neurones are also located in the cerebellar cortex. The basket cells are located in the molecular layer and receive input from the parallel fibres. Each projects to many Purkinje cells enveloping the cell soma and axon hillock of the cell they innervate in a “basket”. The stellate cells are similar to basket cells, are similarly confined to the molecular layer and terminate on the shafts of the Purkinje cell dendrites. The Golgi cells are located in the granule layer. Their dendrites project into the molecular layer and receive input from the parallel fibres. Their cell bodies receive input from collaterals from the incoming mossy fibres (see below) and Purkinje cells, whilst their axons project to the dendrites of the granule cells.

The cells of the cerebellar cortex receive afferent input from multiple sources and output the processed information via a particular efferent pathway. These inputs and output are outlined below.

4.2.4.1.4 Afferent connections – Mossy and climbing fibres

Apart from the climbing fibres (see below) the mossy fibres constitute the majority of the cerebellar input and conduct signals relatively rapidly and end in the granule cell
Chapter 4

In vitro expression and tissue distribution

layer, establishing synapses with the granule cell dendrites. One mossy fibre branches extensively and contacts a large number of granule cells, each of which contacts many Purkinje cells. Thus each mossy fibre influences many Purkinje cells, but the excitatory effect on each is weak, so that many mossy fibres must be active together to provide sufficient excitation (via the parallel fibres) to fire a Purkinje cell.

Figure 4.6 – Schematic of cerebellum circuitry

Black denotes inhibitory neurones and nerve endings, white denotes excitatory. B, basket cell; P, Purkinje cell; s, stellate cell; G, Golgi cell; g, granule cell; nc, nuclear cell; cf, climbing fibre; mf, mossy fibre; pf, parallel fibre. Arrows indicate direction of impulses.

The climbing fibres end very differently from the mossy fibres. The fibres ascend directly to the molecular layer from the inferior olive and divide into several branches, each entwining themselves around the primary dendrites of a Purkinje cell. As they climb, they form numerous synapses with the dendrites. Each Purkinje cell receives branches from only one climbing fibre. However, the branches of a climbing fibre are required to innervate many Purkinje cells because there are many fewer olivary cells from which the fibres originate than target Purkinje cells. Thus in contrast to mossy fibre connections whose multiple connections must give a coordinated high frequency input to stimulate a Purkinje cell, a single action potential in a climbing fibre elicits a burst of action potentials in the Purkinje cell it contacts. As a result, the firing frequency of the climbing fibres is very low under natural conditions.
4.2.4.1.5 Efferent pathways of the cerebellum

The axons of the Purkinje cells terminate onto the cerebellar nuclei. The neurones of these nuclei forward the information to the various target regions in the rest of the brain via the brainstem and thalamus. The cerebellar nuclei are located deep in the white matter of the cerebellum and can be split into four different nuclei, the fastgial nucleus, the small globose and emboliform nuclei and the large, folded dentate nucleus. Generally impulses from different parts of the cerebellum are kept separated within the nuclei and are forwarded to other brain regions. However because there are a greater number of Purkinje neurones than nuclear cells, there is marked convergence of corticonuclear connections in these regions. In addition the inhibitory connections from Purkinje cell axons, the nuclei also receive excitatory inputs from mossy and climbing fibre collaterals. The neurones of the deep cerebellar nuclei are excitatory, and fire at high frequency. The entire cerebellar circuitry is therefore tailored to modulate or control the timing of the excitatory output from these cells.

4.2.4.2 Immunohistochemistry of the putative neuronal \( \gamma \) subunits in the cerebellum

The specificity of each antibody had been previously confirmed by immunocytochemistry on COS-7 cell transiently expressing the human \( \gamma \) subunit cDNAs alone or in combination with other VDCC subunits (Section 4.2.1). Two negative controls were always performed for each experiment. These were usually the absence of primary Ab and pre-absorption of the primary Ab with a 10\( \times \) molar excess of synthesis peptide. No positive immuno-reactivity (IR) was observed in all cases and example images of peptide pre-absorption sections are shown in all appropriate figures.

4.2.4.3 The localisation of the \( \gamma_2 \) subunit in human cerebellum

Figure 4.7, panel a displays a macroscopic image of cerebellum displaying \( \gamma_2 \)-IR in the cerebellar folia (f) and lower level in the dentate cerebellar nucleus (dcn). White matter (wm) is not stained. Panel b displays a peptide pre-absorption control of the \( \gamma_2 \) Ab,
which resulted in greatly reduced IR. Panels c and d display medium (×20) high power
(×40) magnification of the molecular layer (M) and a small part of the adjacent granule
cell layer (g) of a folium of the cerebellum. Moderate γ2 staining is seen in the
molecular layer. This is most probably a consequence of γ2 expression in the dendrites
(d) of cerebellar Purkinje neurones (p). Cell bodies in the molecular layer that stain
positively for the γ2 subunit are small inter-neurones. These are most likely stellate or
basket cells (s). Certainly IR for the γ2 subunit in the soma of Purkinje cells is strong
and this spreads out up the dendrites, but is not as intense following the first few
bifurcations. In panel e, which displays a high magnification image of the granule cell
layer, the granule cells themselves (g) are moderately stained for the γ2 subunit.
However, assessment of IR in this cell type is difficult because the majority of the cell
mass is comprised of the nucleus. The strongest IR in this region is actually in the inter­
neurones (i). Panel f displays a ×10 magnification image of peptide pre-absorption
control of the γ2 Ab. Panel g displays the dentate cerebellar nucleus, which gives a
strong signal in the cell bodies of this nucleus with only weak to moderate staining in
the surrounding neuropil. The blue stain marks cell nuclei as a result of the treatment of
this particular slide with a haematoxylin counter-stain. Panel h displays the DCN in the
peptide pre-absorption section. Note light brown deposits in nucleus cell bodies. This
is lipofuscin (l), a yellow-brown fatty hydrocarbon pigment that accumulates in certain
animal tissue upon aging. Lipofuscin deposits result from accumulation of residual
bodies, which are lysosomes that have absorbed the worn-out, non-digestible parts of
the cell. Scale bars in panels a and b are 5mm. Scale bars in panel c-h are 25μm.
Figure 4.7 - \( \gamma_2 \) subunit immuno-reactivity in human cerebellum

Scale bars in panels a and b are 5mm. Scale bars in panels c-h are 25µm
Chapter 4  
In vitro expression and tissue distribution

4.2.4.4 The localisation of the $\gamma_3$ subunit in human cerebellum

The cerebellum shows poor IR when incubated with the $\gamma_3$ Ab. Figure 4.8, panel a displays a (×20) magnified cerebellar folia with the central white matter (wm) granule cell layer (g) and molecular layer (m) labelled. Weak IR is observed in Purkinje cell bodies and the inter-neurones of the granule cell layer. Staining of the molecular layer neuropil and the granule cells is comparable to the peptide pre-absorption control in panel b. One could deduce that the $\gamma_3$ subunit is poorly represented, but not absent from the cerebellum. Nevertheless, an alternative interpretation of the anti-$\gamma_3$ Ab IR pattern in cerebellum is that the Ab cross-reacts with an epitope present in an undiscovered protein that is expressed in human cerebellum. This is a circumstance for which there is no experimental control. This second interpretation also concurs with the results of the northern blots, which determined that $CACNG3$ mRNA was absent from human cerebellum.

Figure 4.8 - $\gamma_3$ subunit immuno-reactivity in human cerebellar folia.

Panel a shows low level IR in the cerebellar folia when incubated with the anti-$\gamma_3$ Ab. The central white matter (wm) granule cell layer (g) and molecular layer (m) are all displayed. Weak IR is observed in Purkinje cell bodies and the inter-neurones of the granule cell layer. Staining of the molecular layer neuropil and the granule cells is comparable to the peptide pre-absorption control in panel b. Scale bars represent 25µm.
4.2.4.5 The localisation of the $\gamma_4$ subunit in human cerebellum

Figure 4.9 - $\gamma_4$ subunit immuno-reactivity in human cerebellum

Scale bars in panels a and b are 5mm. Scale bars in panel c-g and i are 25µm. Scale bar in panel h represents 50µm.
In vitro expression and tissue distribution

Figure 4.9, panel a displays a macroscopic image of cerebellum displaying $\gamma_4$-IR in the cerebellar folia (f) and high IR in the dentate cerebellar nucleus (dcn). White matter (wm) is very lightly stained. Panel b displays a peptide pre-absorption control of the $\gamma_4$ Ab, which resulted in greatly reduced IR. Panel c displays a $\times 10$ magnification image of a cerebellar folium. The molecular layer (m) is light to moderately stained as is the adjacent granule cell layer (g), but this layer is punctuated by many moderately stained inter-neurones. However, most striking of all are the strongly stained Purkinje cells (p). Their somata and dendrites are both strongly stained. In panel d, a higher magnification image of the same section shows that the high level of IR extends well into the Purkinje cell dendrites (d) and with slightly weaker IR in the surrounding neuropil, the majority of which consists of the Purkinje cell dendritic arbours, indicating that $\gamma_4$ expresses well throughout this cell type. Cell bodies in the molecular layer that stain positively for the $\gamma_4$ subunit are the stellate and basket cell inter-neurones (i), which are displayed at high power magnification in panel e. A high power image of the granule cell layer in panel f shows that the granule cells themselves (g) are lightly stained for the $\gamma_4$ subunit. The strongest IR in this region is actually in the inter-neurones (i). Panel g displays the dentate cerebellar nucleus, which gives a strong $\gamma_4$-IR signal in the perisomatic neuropil with only weak staining in the nucleus cell bodies. Interestingly, this staining pattern is almost the complete inverse of the $\gamma_2$-IR in the same region. Panel h, displays a folium from a peptide pre-absorbed Ab incubated section. Panel i displays the DCN in the peptide pre-absorption section.

4.2.4.6 The localisation of the $\gamma_2$, $\gamma_3$, and $\gamma_4$ subunit in mouse cerebellum

For comparative purposes, the expression of the $\gamma$ subunits in mouse cerebellum was investigated using fluorescence immunocytochemistry. This extended the mRNA distribution studies of mouse brain (Klugbauer et al., 2000; Letts et al., 1998) by also elucidating the protein expression levels in mouse cerebellum. The peptides used to generate the antibodies raised against human sequence were shown to be 100% identical to the same regions of the mouse $\gamma_2$ subunit identified by Letts et al. (1998) and the $\gamma_3$ and $\gamma_4$ subunits identified from mouse brain by Klugbauer et al. (2000). The $\gamma_2$ subunit was expressed most strongly in the Purkinje cell bodies of mouse cerebellum, and this
Figure 4.10 – Distribution of the neuronal $\gamma_2$, $\gamma_3$ and $\gamma_4$ subunits in mouse cerebellum

The $\gamma_2$ subunit expressed most strongly in the Purkinje cell bodies (p), with lower levels in the molecular (m) and granule cell layers (g). Low power magnification images of cerebella stained with the $\gamma_3$ Ab show that the subunit is absent or very poorly expressed. High power images (b) show some low level fluorescence but this could not be specifically assigned to a cell type and could be non-specific staining. The $\gamma_4$ subunit is expressed in Purkinje cell bodies and the molecular layer, but is absent from the granule cells. The negative control was to incubate slices without primary Ab. These slices showed markedly reduced fluorescence. Scale bars in row a represent 50µm. Scale bars in row b represent 15µm.
extended out at lower levels into the molecular layer. The granule cell layer was only lightly stained in comparison. The $\gamma_3$ subunit was not detected in the murine cerebellum, which echoed the findings from the human cerebellum and the recently reported mRNA distribution (Klugbauer et al., 2000). The $\gamma_4$ subunit was expressed in Purkinje cell bodies and throughout the molecular layer, but at lower levels than the $\gamma_2$ subunit. The $\gamma_4$ subunit is apparently absent from the granule cell layer. This is of great functional significance when considering the stargazer mutation. Effective absence of $\gamma_2$ subunit in the stargazer mouse and lack of $\gamma_3$ or $\gamma_4$ in granule cells could suggest that part of the pleiotropic phenotype of the mutant mouse arises because there are no other $\gamma$ subunits expressed in these cells that can compensate for the loss of the $\gamma_2$ subunit.

4.2.4.7 Outline of the anatomy of the hippocampus

For the purposes of this chapter the term hippocampal formation encapsulates three structures, the dentate gyrus, Ammon’s horn (hippocampus proper), and the subiculum. The structural organisation of the hippocampal formation is outlined below and schematically represented in Figure 4.11.

Figure 4.11 – Schematic of a coronal section of the hippocampal formation

**CA1-CA4**, fields of the cornu Ammonis (Ammon’s Horn); 1, alveus; 2, stratum oriens; 3, pyramidal layer; 3′, stratum lucidum; 4, stratum radiatum; 5, stratum lacunosum; 6, stratum moleculare; 7, vestigial hippocampal sulcus (7′, residual cavity). **Dentate Gyrus**; 8, stratum moleculare; 9, stratum granulosum; 10, polymorphic layer; 11, fimbria; 12, margo denticulatus; 13, choroidal fissure; 14, fused hippocampal fissure; 15, subiculum. Figure reproduced from (Duvernoy, 1998).
4.2.4.7.1 Dentate gyrus

The dentate gyrus forms a C-shaped structure, which is separated from the CA1 region of Ammon's horn by the fused hippocampal fissure. The principal cell type populating the gyrus is the dentate granule cell. These cells typically send unipolar dendritic processes into the overlying molecular layer. However in humans and other primates it has been observed that dentate granule cells also project basal dendritic trees into the polymorphic layer. Although controversy has surrounded whether to classify the polymorphic layer as part of the dentate gyrus or hippocampus, connectional studies determined that all the cells of the layer give rise to projections only to the dentate gyrus and not the hippocampus.

4.2.4.7.2 Hippocampus

Whilst the hippocampus has effectively one cell layer, the pyramidal cell layer, the plexiform layers above and below are also classified into distinct regions. The limiting surface layer is formed by the axons of the pyramidal cells and is termed the alveus. Between the alveus and the pyramidal cell layer is the stratum oriens, occupied mainly by the basal dendrites of the pyramidal cells, although also containing some other cell types. The region superficial to the pyramidal layer is subdivided into multiple laminae, the stratum lucidum, stratum radiatum and stratum lacunosum-moleculare. The mossy fibres from the dentate granule cells travel within the stratum lucidum, a lamina only present adjacent to the CA3 region of Ammon's horn because pyramidal cells of the CA1 and CA2 do not receive inputs from mossy fibres. The remaining plexiform regions are mainly constituted by the stratum radiatum that contains the CA3 to CA1 associational fibre connections known as the Schaffer Collaterals. These dendritic processes ultimately terminate in a tuft of thin branches on the distal apical dendrites of the hippocampal pyramidal cells contained in the stratum lacunosum-moleculare.

Ammon's horn or the hippocampus proper can be divided into three regions, designated CA1, CA2 and CA3. A fourth region, that in this study has been labelled CA4, is the subject of classification contention. It describes the region of the hippocampus that doubles back upon itself and is encapsulated by the dentate gyrus and although it may
just represent an extension of the CA3 region I have labelled it CA4 in diagrams and figures for ease of description. The CA3 extends away from this region and contains the CA3 pyramidal neurones, the largest in the hippocampus. The major hallmark of the CA3 region is the afferent mossy fibres that project from the dentate granule cells onto the CA3 pyramidal dendrites. As previously mentioned, the two sets of processes meet at the region just above the pyramidal cell layer termed the stratum lucidum. The border between the CA3 and the adjacent CA2 is not distinct due to overlap of the pyramidal cell type from each region. However, CA2 has the most compact pyramidal layer and can be identified by the lack of mossy fibre afferents. It does however receive strong input from the hypothalamus. The boundary of the CA2 and the CA1 regions can be placed as the point at which the pyramidal layer starts to broaden. The CA1 contains multiple cell types, but the pyramidal cells are by far the most abundant. They are arranged as two sub-layers that become progressively more difficult to differentiate as one approaches the subiculum, where the cells are much less densely packed and more loosely organised.

4.2.4.7.3  Subiculum

The subiculum, receives and integrates information from the perirhinal, entorhinal and prefrontal cortices as well as the presubiculum. It is also the principal target of CA1 pyramidal cell axons and represents the final relay in a synaptic loop between the entorhinal cortex, dentate gyrus and hippocampus. However the precise organisation of this region and its connections are poorly understood.

4.2.4.8  Immunohistochemistry of the putative neuronal \( \gamma \) subunits in the hippocampus

Immunohistochemistry revealed differential expression patterns of the \( \gamma_2, \gamma_3 \) and \( \gamma_4 \) subunits in the different regions and cell types of human hippocampus. At least two negative controls were always performed for each experiment. These were usually the absence of primary Ab and pre-absorption of the primary Ab with a 10× molar excess of synthesis peptide. Otherwise a third control of using control rabbit IgG (Sigma) to the same dilution as the \( \gamma \) specific primary was applied. No positive IR was observed in all
Chapter 4  

**In vitro expression and tissue distribution**

cases and example images of peptide pre-absorption sections are shown in all appropriate figures.

The observations regarding the staining by each γ specific Ab are explained in the figure legends for this section. Abbreviations used to label figures were as follows:

- `a`: alveus  
- `CA1`: CA1 field of hippocampus  
- `CA3`: CA3 field of hippocampus  
- `cf`: Choroidal fissure  
- `f`: fimbria  
- `GL`: Granule layer of dentate gyrus  
- `I`: Stratum lucidum of the hippocampus  
- `l-m`: Stratum lacunosum-moleculare of the hippocampus  
- `ML`: Molecular layer of the dentate gyrus  
- `o`: Stratum oriens of the hippocampus  
- `p`: Pyramidal layer of the hippocampus  
- `PL`: Polymorphic layer of the dentate gyrus  
- `r`: Stratum radiatum of the hippocampus  
- `s`: Subiculum

### 4.2.4.9 The localisation of the γ2 subunit in human hippocampus

Figure 4.12a, b and c display no primary Ab, pre-immune IgG and peptide pre-absorption incubated negative control sections, all of which show no or greatly reduced γ2 subunit IR. Figure 4.12d displays generalised staining in the cell layers and neuropil throughout the hippocampus when incubated with the γ2 Ab. The dotted line represents the border between the CA4 region of the hippocampus and the polymorphic layer of the dentate gyrus. Figure 4.13-Figure 4.15 investigate the γ2 IR in each hippocampal region in more detail, presenting images of sections at progressively increasing magnifications.
Figure 4.12 – Macroscopic view of human hippocampus immuno-reactivity with the \( \gamma_2 \) subunit specific antibody
Chapter 4  

In vitro expression and tissue distribution

Figure 4.13 - low power magnification of γ2 Ab staining of the hippocampus

a) The alveus, stratum oriens most adjacent the pyramidal layer, and the pyramidal layer all stain moderately for γ2 subunit. The stratum radiatum and lacunosum-moleculare stain much more weakly. The dentate gyrus molecular layer stains moderately for γ2 and the granule cells strongly. γ2 staining is weak or absent in the polymorphic layer. This suggests that the γ2 subunit may be present in the soma and apical dendrites of dentate granule cells at higher levels than in the basal dendrites. b) The alveus and CA2 and 3 pyramidal layers stain well for the γ2 subunit. The apparently higher level of γ2 staining in the CA2 and CA3 is probably due to a higher density of pyramidal cell soma in these regions compared to the CA1 region (Mouritzen Dam, 1979). It is however apparent that there is only a very weak staining of the strata oriens and radiatum in the CA2/3 regions; yet, moderate IR is detected in the stratum lucidum. Weak staining is visible in the stratum lacunosum-moleculare.

c & d) Synthesis peptide pre-absorption controls of a serial section from the same hippocampus. Scale bar = 250μm.
Chapter 4

In vitro expression and tissue distribution

Figure 4.14 – 10 × magnification of hippocampal regions stained with the γ2 Ab.

a) The staining of the part of the CA1 hippocampal region towards the subiculum is generally moderate in both the somata and perisomatic neuropil of the pyramidal layer. b) Where the CA1 region approaches the CA2, the perisomatic staining remains moderate but the somatic staining becomes more intense. c) Weak γ2-IR is apparent in the strata oriens and radiatum, moderate to strong staining in the alveus and stronger staining in the soma of the pyramidal layer with moderate perisomatic staining. d) Moderate staining of dentate gyrus molecular layer and dense IR in the granule cells. The polymorphic layer in weakly stained but distinct fibres are stained which course across this region. Staining of the CA4/Hilus cells is seen in the corner of the image. Scale bar = 50 μm.
Figure 4.15 – High power images (×40) of cellular $\gamma_2$ staining in the hippocampal regions.

The cells of the CA1 region are only lightly stained with a similar light staining of the surrounding neuropil. The staining is moderate in the cell bodies and neuropil of the CA2. Staining of the soma of CA3 hippocampal neurones is slightly more intense and results in greater definition of the cells. The surround in neuropil is moderately stained. The granule neurones of the dentate gyrus (D) are stained similarly to the CA3 cell bodies with weak to moderate staining of the surrounding neuropil. Scale bar = 25 $\mu$m.
4.2.4.10 The localisation of the $\gamma_3$ subunit in human hippocampus

Figure 4.16 – Specific staining of human hippocampus with an anti $\gamma_3$ subunit antibody.

Panels a and b display no IR when the primary antibody was the pre-immune sera or $\gamma_3$ Ab pre-absorbed with excess synthesis peptide respectively. Panel c shows strong $\gamma_3$-IR is apparent throughout the pyramidal layer of the CA1, CA2 and CA3 regions of Ammon's horn. This staining is strongest in the CA3 and becomes progressively weaker through the CA2 and CA1 and into the subiculum. This is most likely to be caused by increased cell densities in the CA2/3 regions rather than more intense staining of pyramidal neurones. IR is absent from the alveus, stratum oriens, stratum radiatum and lacunosum-moleculare. Weak staining appears in the CA4 region (separated from polymorphic layer of dentate gyrus by dotted line). The granule layer of the dentate gyrus is distinctly labelled whereas the molecular and polymorphic layers display weak to no IR. The staining of the somal regions suggests that $\gamma_3$ is probably principally localised in hippocampal cell bodies rather than neuronal processes which course through the poorly stained neuropil. Scale bars = 5mm.
synthesis peptide. Scale bars = 250μm.

**Figure 4.17** – Low power magnification images of γ3 Ab immuno-reactivity in human hippocampus.

In panel a, the pyramidal cells of the CA1 region of Ammon’s horn stain intensely for the γ3 subunit. More moderate immuno-reactivity (IR) is observed in the perisomatic neuropil. Weaker staining is observed in the stratum oriens and weak IR in stratum radiatum. The alveus and lacunonsum-molecular are in general, absent of IR. In the dentate gyrus it is only the granule cell layer that stains for γ3. IR is very weak to absent from the molecular and polymorphic layers. In panel b, even more intense staining of the pyramidal cell layers of the CA2 and CA3 compared to the CA1 region is observed. The perisomatic staining in these regions is only moderate. The somal staining of pyramidal cells continues into the CA4 region, where the surrounding neuropil is devoid of IR. The strata oriens and radiatum of the CA2/3 regions are not stained by the γ3 subunit Ab. Panels c and d display the absence of γ3 IR from the adjacent hippocampal section that was incubated with the γ3 Ab pre-absorbed with an excess of its
The wide band of pyramidal neurones of the subiculum (S) stain densely in their soma for the $\gamma_3$ subunit, but immunoreactivity is absent from the surrounding neuropil. A high power image of the subiculum cells (S-HP) confirms this observation. The pyramidal cells of the CA1 region of Ammon’s horn have strong somal staining for the $\gamma_3$ subunit with much lower perisomatic IR. Somal staining for $\gamma_3$ is dense in the CA3 pyramidal cells, but perisomatic staining is not much greater than in the CA1, indicating the dense staining seen in the central low power image is probably due to more tightly packed cells in the CA3 layer. Granule cells of the dentate gyrus (DG) are stained moderately to strongly by the $\gamma_3$ Ab. The IR is mainly in the soma of these cells, but can also be seen in the early branches of the granule cells dendritic trees, which extend into the molecular layer (ML), which is lightly stained. Unless otherwise labeled, scale bars represent 25 $\mu$m.
4.2.4.11 The localisation of the $\gamma_4$ subunit in human hippocampus

In panel a, the dotted line in the dentate gyrus indicates the border between the polymorphic layer and the CA3 and CA4 regions of the hippocampus. At the macroscopic level, $\gamma_4$ is seen throughout the cell bodies in the pyramidal cells of the CA1, CA2 and CA3 regions of Ammon's horn. The staining is apparently more intense in the CA2/3 region but this most likely to be due to increased cell density in these regions compared to the CA1. Weak to moderate staining is observed throughout the neuropil. In the dentate gyrus, the granule cell layer appears to stain slightly more strongly than the adjacent molecular or polymorphic regions. Panel b displays the adjacent section incubated with $\gamma_4$ antibody pre-absorbed with its immunising peptide. The IR is greatly reduced in this section. Scale bars represent 5mm.
Figure 4.20 - \( \gamma_4 \) immuno-reactivity in pyramidal cells and dentate gyrus

In panel a, the strongest staining is observed in the pyramidal cell layers and in the granule layer of the dentate gyrus, weak staining is observed in the neuropil surrounding these layers. Panel b is a high power magnification of pyramidal cells from the CA3 region, showing strong staining in the soma that extends into the cellular processes. The perisomatic neuropil is stained at low levels. Panel c displays cells from the CA4 region, and demonstrates that like the CA3 pyramidal cells, cell soma stain well for \( \gamma_4 \) subunit, whilst the perisomatic neuropil staining is comparable to that of the CA3 indicating that it is the higher density of cells in the CA3 that accounts for the apparently denser staining of CA3 in the macroscopic sections. Panel d shows that although positively stained, the granule neurones of the dentate gyrus do no stain as strongly for \( \gamma_4 \) as do pyramidal cells. Scale bar represents in panel a 250\( \mu \)m, in panel b-d, 25\( \mu \)m.
4.3 Discussion

4.3.1 In vitro expression of neuronal γ subunits and antibody specificity

The transient expression of the newly identified and cloned γ2, γ3 and γ4 subunits cDNAs in COS-7 cells served four purposes.

1. Determined that the human γ subunit cDNA clones generated in Chapter 3 express in vitro.
2. Demonstrated the specificity of three anti-γ subunit antibodies, one generated to detect each of the three isoforms.
3. Revealed that the putative γ2, γ3 and γ4 subunits all localise to the plasma membrane of COS-7 cells when expressed alone or in combination with other VDCC subunits.
4. COS-7 cells do not endogenously express γ2, γ3 or γ4 subunit protein.

It was therefore established that as predicted from analysis of the predicted secondary structure of each subunit, that they are indeed transmembrane proteins. Furthermore, they must be orientated with their N- and C-termini on the cytoplasmic side of the membrane, because the anti-γ antibodies, all of which were designed to epitopes in the C-terminal sequence after the fourth transmembrane segment failed to stain COS-7 cells that had not been permeablised.

In addition to the antibodies not detecting γ subunit protein in untransfected COS-7 cells, a northern blot of total RNA from COS-7 cells and Xenopus oocytes determined that neither cell type possesses γ subunit mRNAs. It was critical to establish that γ subunits were absent from these cell types for any data from functional characterisation experiments in these cell types to be interpretable.
4.3.2 **Northern blot distribution**

Northern blot experiments determined that the mRNAs for the $\gamma_2$, $\gamma_3$ and $\gamma_4$ subunits are exclusively expressed in the brain. The $\gamma_2$ and $\gamma_4$ transcripts expressed in most of the brain regions probed and mainly in the same tissues; however, in certain regions (e.g. the cerebellum) $\gamma_2$ is probably the predominant species whereas in the basal ganglia regions of putamen and caudate nucleus $\gamma_4$ is probably the most highly represented transcript. The $\gamma_3$ subunit is much more selectively expressed in the cortex, striatum, amygdala and hippocampus, but most notably is absent from the cerebellum and thalamus. Defects in these two regions have been implicated as underlying ataxias and epilepsies respectively, and interestingly the $\gamma_2$ subunit is most prevalent in both. One could therefore hypothesise that should a human condition be discovered which, like stargazer, is a result of loss of $\gamma_2$ expression, its phenotype may also present as ataxia and epileptic seizures because there is no $\gamma_3$ and little $\gamma_4$ in the cerebellum and thalamus to compensate for the loss of $\gamma_2$ (also see Table 4.1).

An additional observation was that these neuronal $\gamma$ subunits are differentially expressed in some of the nuclei that comprise the basal ganglia, a region believed to be involved in the planning and programming of movement, or more broadly in the processes by which abstract thought is converted into voluntary action. The putamen has a heterogeneous neuronal $\gamma$ population with $\gamma_4$ possibly the most prevalent. The caudate nucleus also expresses transcript for all three subunits; however, the signal detected with the $\gamma_2$ probe was extremely weak. On the other hand, $\gamma_2$ was the most prevalent species detected in substantia nigra, with faint detection of $\gamma_4$ and no $\gamma_3$ signal. No signals for any of the $\gamma$ transcripts were detected in the sub-thalamic nucleus. The differential distribution of these subunits may allude to subtly different properties for each of the subunits in these nuclei, but without definitive cellular or sub-cellular localisation and functional electrophysiological characterisation of the properties of these subunits from the native cells of each nucleus, few conclusions can be drawn about the functional significance of this differential $\gamma$ subunit distribution in basal ganglia.
The γ2 specific probe detected two mRNAs indicating potential splice variation of this subunit. The probe was designed to be complementary to a sequence entirely within exon 4. The existence of two transcripts could therefore possibly result from alternatively splicing of exons 1-3. However, this is an unlikely explanation because of the magnitude of the size difference between the two transcripts, and as Letts et al. (1998) suggested for the mouse transcripts, is more probably due to the 7 kb species possessing a long 3' un-translated region. Additionally, the expression pattern does not fit with differential expression of alternative splice variants of the same gene, because the 3kb transcript is detected in all the same brain regions as the 7 kb mRNA, albeit at much lower levels. However, one can observe that the message sizes for γ2 mRNA are the same for both human and mouse, indicating a high degree of inter-species conservation (Letts et al., 1998). A recent study that identified mouse γ3 and γ4 subunits (Klugbauer et al., 2000), showed by northern blot that they too are exclusively localised to the brain and have messages of similar (in the case of γ3) or identical size (in the case of γ4), revealing that these two species are also highly conserved between humans and mice, even in un-translated regions.

The results of the northern blots from this study were compared with data from other groups regarding the expression of these novel γ subunits published during the course of my own investigations that were generated by in situ hybridisation and western blot (Klugbauer et al., 2000; Sharp et al., 2001). The mRNA levels of each subunit in mouse brain generally agreed with my human northern blots (Klugbauer et al., 2000). Protein expression in mouse brain determined by western blot performed in a study subsequent to my own work by Sharp et al. (2001) compared the expression of the γ2, γ3 and γ4 isoforms in normal and stargazer mouse. Western blot analysis determined that in normal mice, γ2 expression is strongest in cerebellum, followed by cortex, medium levels in midbrain, striatum, hippocampus, and thalamus and weak in pons and brainstem. γ3 was detected strongly only in midbrain, striatum and cortex with low levels in hippocampus and thalamus, but these bands were masked somewhat by the broad γ2 bands produced on the same blot by a γ2+γ3 detecting antibody. γ4 expression was detected at its strongest in cortex with medium detection in midbrain, striatum, hippocampus and weak detection in thalamus, pons, brainstem and cerebellum.
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Table 4.1 - Differential distribution of YDCC subunits in the brain.
From previous page Table 4.1 - Differential distribution of VDCC subunits in the brain

Data was compiled from several sources and although not quantitative, represents a qualitative evaluation of the expression levels of neuronal VDCC subunits. Labelling is scored as follows, +++ = strong, ++ = moderate, + = weak, and - = no labelling. Fields labelled with more than one staining intensity e.g. -/+ are areas where immuno-reactivity varies within the one region. Tissue sources are as follows, h = human, r = rat, m = mouse. Techniques include i.s. = in situ hybridisation, n = northern blot, w = western blot, ar = autoradiographic labelling by radioactive conotoxin. The references consulted to compile the table were:


F. J. Moss Results from this thesis chapter.

These combined data were also compared with brain region distribution studies of the other neuronal VDCC subunits and compiled in Table 4.1. The highly differentiated regional expression of the VDCC genes in brain is without doubt of great significance in controlling the plethora of specialised for neuronal functions in each region. Nevertheless, without specific immuno-purification of VDCC complexes from particular tissues and the identification of each particular component, these data can only be used as a guide to predict the molecular composition of the VDCC channels expressing in each cell type.

4.3.3 Expression of γ subunits in cerebellum and hippocampus

The major limiting factor in the immunohistochemistry experiments was reproducibility. The ability to control variables when preparing rodent samples, in terms of age, gender, agonal state and post mortem delay, serves to limit protein degradation and ensure the quality of the tissue. Such controls are not possible for
human specimens for obvious reasons. The only control that could effectively be applied was to use tissue from patients who had no history of neurological illness that could affect the distribution of ion channel subunits. The tissue in this investigation came from two elderly male patients of 76 and 80 years of age, whose causes of death were listed as metastatic carcinoma and left ventricular failure respectively. Although both patients were of the same sex, similar age and neither patient had any obvious neurological disorder, temporal changes in expression profiles of each γ subunit might be observed when comparing the results obtained in this study with tissue samples from a child, adolescent or adult of 30-40 years of age. Furthermore, during of the patients’ lifetimes, they will have experienced different medical histories and been subject to different drug treatments. Additionally, tissues from elderly subjects present another potential source of error, in that a yellow-brown fatty hydrocarbon pigment, lipofuscin accumulates in the more permanent cells like neurones. This was observed in particular sections, especially in the dentate cerebellar nuclei cell bodies (Figure 4.7h). The observation of low Horse Radish Peroxidase-conjugated IR should therefore always be checked with pre-absorbed control sections to confirm that it is not in fact lipofuscin accumulation. In spite of this, little variation in staining was observed between sections of the same tissue from either patient, and the data correlated with the gross expression profiles generated by northern blots. Nevertheless, all the immunohistochemistry results can only be considered to be preliminary due to the low number of patient replicates, and narrow sample of ages from which tissue was obtained.

Despite these shortfalls this study presents data regarding the distribution of putative human neuronal VDCC subunits in two extremely important brain regions. Apart from a few investigations, the majority of our knowledge of VDCC distribution arises from studies in rats and mice. Whilst obviously of great value, all anatomical, physiological and pharmacological research is ultimately targeted at understanding of human systems and the elucidation of human γ subunit distribution may be of therapeutic benefit should any neurological syndrome ever be attributed to loss or mutation of one of these proteins in human brain.
### Chapter 4

In vitro expression and tissue distribution

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Table 4.2 - The relative expression levels of VDCC subunits expressed in cerebellum

Data was compiled from several sources and although not quantitative, represents a qualitative evaluation of the expression levels of neuronal VDCC subunits. Labelling is scored as follows: +++ = strong, ++ = moderate, + = weak, and - no labelling. Fields labelled with more than one staining intensity e.g. /+ are areas where immunoreactivity varies within the one region. Fields containing two expression strengths separated by “or”, result from conflicting reports from separate publications. +++ denotes increasing labelling in sequential sections from anterior to posterior cerebellum. ? = IR is possibly the result of cross-reaction with an undiscovered protein. Where a field is blank, no data was available. ML = molecular layer, GL = granule cell layer, DCN = deep cerebellar nuclei. Un-shaded columns represent data from human tissue, light-grey columns represent data from mouse tissue, and dark-grey columns represents data from rat tissue. Where possible data is a measure of immuno-reactivity following incubation with specific antibodies, however the following exceptions apply. The Ca3 family information is mainly from in situ hybridisation (Craig et al., 1999; Talley et al., 1999), but some Ca3.1 info also includes immuno-reactivity of protein (Craig et al., 1999). α2δ and α2δ3 data is from in situ investigations whereas α2δ3; data is a combination of in situ and protein immuno-reactivity expression data. In addition to the data presented in this thesis chapter, references consulted to compile this table include: (Barclay et al., 2001; Brodbeck et al., 2002; Chung et al., 2000; Craig et al., 1999; Kase et al., 1999; Klugbauer et al., 1999a; Ludwig et al., 1997; Talley et al., 1999; Volsen et al., 1995; Volsen et al., 1997).
This staining however, may not be true $\gamma_3$ IR. It is likely that the $\gamma_3$ Ab cross-reacted with an unknown protein in human cerebellum; a possibility supported by the failure to detect $CACNG3$ mRNA on northern blots.
4.3.3.1 The distribution of the putative VDCC \(\gamma\) subunits in cerebellum

The expression profile for the human neuronal \(\gamma\) subunits is summarised in Table 4.2, which also compares the expression of the \(\gamma\) subunits with expression data for other VDCC subunits in cerebellum. Where possible, human protein expression data is used. Nonetheless, for some subunits, in particular the more recently discovered Cav3 T-type \(\alpha_1\) subunits and the \(\alpha_2\delta_2\) and \(\alpha_2\delta_3\) subunits, data from rat or mouse brain had to be used. The \(\gamma_2\) and \(\gamma_4\) subunits are both expressed in human and mouse cerebellum, with \(\gamma_2\) being the predominant species. However, in Purkinje cells, the \(\gamma_2\) subunit was localised more to the cell bodies and proximal dendrites, whereas the \(\gamma_4\) expression extended throughout the cell well into the dendritic trees. When compared to other VDCC expressed in Purkinje cells the \(\gamma_4\) IR most closely resembled Cav2.3 and \(\beta_3\) subunit expression patterns, whilst \(\gamma_2\) expression more closely resembled the generalised IR observed for Cav2.1 and \(\beta_2\) (Volsen et al., 1995; Volsen et al., 1997). In spite of this, the \(\beta_4\) subunit is the prevalent subunit throughout the cerebellum and has the potential to associate with both the \(\gamma_2\) or \(\gamma_4\) subunits via a VDCC complex (Volsen et al., 1997).

Interestingly, of the newly identified T-type Cav3 \(\alpha_1\) subunits, Cav3.1 expresses extremely well throughout Purkinje cells (Craig et al., 1999; Talley et al., 1999). To date, no direct interactions of the Cav3.1 subunit and any VDCC auxiliary subunit has been demonstrated. Nevertheless, it is tempting to hypothesise that one reason that neuronal \(\gamma\) subunits were not identified in early experiments immuno- or affinity-purifying neuronal VDCCs was because they associate with T-type channels, the molecular identity of which until recently remained undetermined (Perez-Reyes, 1999). However, much work remains to be performed before any bold statements can be made about such putative interactions.

The \(\gamma_3\) subunit protein was detected at very low levels in human cerebellum despite no message being detected in northern blots. The positive IR to the \(\gamma_3\) Ab was shown to mainly be in the inter-neurones of the cerebellum, but low levels were also detected in Purkinje cell bodies. Investigation of mouse brain \(\gamma_3\) protein expression in this study determined that is was not present mouse cerebellum. This finding agreed with the findings of Klugbauer et al. (2000) who by in situ hybridisation produced similar
findings for γ₂ mRNA. Mouse cerebellum immunocytochemistry also determined that as reported in other investigations the γ₂ is expressed strongly throughout the cerebellum. However, γ₄ expression was restricted to the molecular layer and Purkinje cell bodies. Staining in the granule cell layer was extremely weak or absent. This finding added weight to the suggestion arising from the northern blot studies, that the γ₂ subunit is the only γ isoform expressed in cerebellar granule neurones and its loss in the stargazer mice gives rise to the ataxic phenotype because other γ isoforms are not present to substitute for the missing γ₂. Whether the loss of γ₂ subunit in human brain would result in a similar phenotype will remain open to debate until and unless such a condition is discovered, but there does appear to be some γ₄ expression in human granule cells indicating that, providing the functional properties of γ₂ and γ₄ are similar, any ataxic phenotype resulting from a γ₂ mutation in humans may not be as strong due to compensatory substitution of the γ₂ subunit for γ₄ subunit in a similar manner to which β₁,₂ subunits compensate for β₄ subunit loss in lethargic mice (Burgess et al., 1999a).

Staining by the γ₂ and γ₄ subunits in the dentate cerebellar nucleus (DCN) was strikingly different. With a few exceptions, the Purkinje cells only route out of the cerebellar cortex is via the DCN, and very few project out of the cerebellum itself. The DCN serve as a relay for the Purkinje cell output and forward the signals to the rest of the central nervous system via the thalamus, a region in which the γ₂ subunit predominates. However, this is not their sole function, as they receive inputs from collateral branches of mossy fibres and climbing fibres that carry excitatory inputs to the granule and Purkinje neurones. Much of the perisomatic neuropil surrounding the DCN somata consists of afferents from the Purkinje cells, and it is interesting to note that this neuropil is stained particularly strongly for γ₄ whilst γ₂ showed much lower IR. The DCN cell bodies stained strongly for γ₂ but were devoid of γ₄ staining. A clear differential distribution is therefore observed, and it is reasonable to assume that the γ₄ subunit is pre-synaptically localised in the Purkinje cell afferents to the DCN whilst the γ₂ subunit localises at the post-synaptic regions the DCN cell bodies. Indeed this observation holds with the finding that γ₄ expressed well throughout Purkinje cell processes. The γ₄ subunit also appears to be more concentrated in GABAergic neurones of the cerebellum more than the excitatory glutamatergic cell types. In addition to showing strong IR throughout
Purkinje cells, $\gamma_4$ is detected in the stellate and basket cells of the molecular layer and also the Golgi (type II) inter-neurones of the granule layer. Whilst $\gamma_2$ is also detected in all these cell types, the expression of $\gamma_4$ is definitely lower in the excitatory granule cells than is that of $\gamma_2$ subunit, and is absent from the DCN somata.

### 4.3.3.2 The distribution of the putative VDCC $\gamma$ subunit in hippocampus

This study has determined that $\gamma_2$, $\gamma_3$ and $\gamma_4$ subunits show differential but overlapping expression patterns in the human hippocampus and dentate gyrus. As was the case for the cerebellum, the expression patterns of $\gamma_2$ and $\gamma_4$ subunits more closely resemble one another than that of $\gamma_3$, although $\gamma_3$ is more closely related to $\gamma_2$ than $\gamma_4$. The $\gamma_2$, and $\gamma_4$ subunits are expressed throughout the hippocampus and dentate gyrus, although there are variations in the staining of cell bodies, dendrites and neuropil in the different regions. The $\gamma_3$ subunit localises more specifically to the somatal regions and IR is similarly intense in the neuronal cell bodies of the hippocampal structure. This possibly indicates that $\gamma_2$ and $\gamma_4$ are involved in synaptic modulation of neurotransmission, whereas the $\gamma_3$ subunit is solely involved in regulation of VDCC or other functions in the cell soma. Indeed, only the $\gamma_2$ subunit is apparently present in the CA3 stratum lucidum, indicating a potential presynaptic distribution. This could be more effectively determined in future investigations by staining adjacent slices with an anti-calbindin antibody. Calbindin IR is intense in mossy fibre presynaptic terminals in the stratum lucidum of CA3 (Seress et al., 1993) so would therefore confirm if the $\gamma_2$ subunit was localised similarly. If this is the case $\gamma_2$ could associate with Cav2.2 or Cav2.3, which have shown intense IR in the stratum lucidum of human CA3 (Day et al., 1996). The expression profiles of the novel $\gamma$ subunits in hippocampus are summarised in Table 4.3 and compared with expression data previously published for other neuronal VDCC subunits. The potential diversity of channel combinations that could be predicted in each cell type is overwhelming and without more functional data, few conclusions can be draw to the functional significance of $\gamma$ subunit distribution in hippocampus. Table 4.3 does however provide a useful reference when interpreting VDCC currents recorded from hippocampal slices, or when attempting to simulate VDCC currents from hippocampal cells during in vitro expression investigations.
### Chapter 4

**In vitro expression and tissue distribution**

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Table 4.3 - The relative expression levels of VDCC subunits expressed in hippocampus

Data was complied from several sources and although not quantitative, represents a qualitative evaluation of the expression levels of neuronal VDCC subunits. Labelling is scored as follows, +++ = strong, ++ = moderate, + = weak, and no labelling, d > s = dendrites greater than somata, d = dendrites only. Fields labelled with more than one staining intensity e.g. -/+ are areas where immuno-reactivity varies within the one region. Where a field is blank, no data was available. DG = dentate gyrus; DGL = dentate granule layer; DML = dentate molecular layer; PoL = polymorphic layer; pCA3 = proximal CA3; dCA3 = distal CA3; SL = stratum lucidum; SO = Stratum oriens; SR = stratum radiatum; SUB = subiculum. (np) in the subiculum neuropil refers to non-pyramidal cell staining. Proximal CA3 is the region abutting the polymorphic region and the distal CA3 is adjacent to the CA2. Un-shaded columns represent data from human tissue; light-grey columns represent data from mouse tissue and dark-grey columns represents data from rat brain. Where possible data is a measure of immuno-reactivity following incubation with specific antibodies, however the following exceptions apply. The Ca\textsubscript{v}3 family information is mainly from in situ hybridisation (Craig et al., 1999; Talley et al., 1999), but some Ca\textsubscript{v}3.1 info also immuno-reactivity of protein (Craig et al., 1999). α\textsubscript{2}δ\textsubscript{1}, α\textsubscript{2}δ\textsubscript{2}, and α\textsubscript{2}δ\textsubscript{3} data is from in situ investigations. In addition to the data presented in this thesis chapter, references consulted to compile this table include: (Barclay et al., 2001; Craig et al., 1999; Craig et al., 1998; Day et al., 1996; Day et al., 1998; Klugbauer et al., 1999a; Talley et al., 1999).
4.4 Future work

4.4.1 In vitro expression of γ subunits

This chapter has presented data which shows that the cloned human neuronal γ₂, γ₃ and γ₄ subunits all expressed well in COS-7 cells and are localised to the plasma membrane. Future investigations could extend these findings in several ways:

1) Determining whether γ subunits are expressed at the plasma membrane of *Xenopus* oocytes by fixing, sectioning and staining oocytes injected with the γ subunits alone or in combination with other VDCC subunits.

2) The membrane distribution of the γ subunits could be investigated further by co-staining transfected cells with the fluorescently conjugated phalloidin markers (Molecular probes, Oregon, USA). These markers delineate the plasma membrane by staining the actin cytoskeleton just beneath its surface.

3) Co-immunoprecipitation of VDCC subunits or other proteins from transfected mammalian cells or from native tissue with the neuronal γ subunits would determine binding partners with which the γ subunits associate both in vitro and in vivo.

4.4.2 Differential tissue distribution

An in depth analysis of the brain mRNA and protein levels of each γ isoform should be continued. *In situ* hybridisation using the same cDNA probes generated for the northern blots in this study would determine the differential mRNA levels in each brain region, whilst more immunohistochemistry on sections from more patients and different brain regions will help to confirm the data presented in this chapter, and increase our knowledge of the distribution of these γ isoforms in each brain region. In future investigations, alternate sections should also be stained with cellular markers or counter-stains to aid in the identification of particular structures. For example, staining the hippocampus with in a Timm's silver sulphide solution, or incubating with an anti-calbindin antibody highlights the mossy fibres, enabling the researcher to visualise
regions of synaptic connectivity in the tissue. Comparisons of the distribution of each γ subunit isoform between human and rodent species may also highlight some inter-species distribution variations that once the functional properties of these proteins are fully understood may facilitate the understanding of phenotypic variations in neurological syndromes between humans and mice.
The functional properties of a family of human stargazin-like genes when co-expressed with neuronal voltage-dependent calcium channels
Chapter 5

5.1 Introduction

In the previous chapters I described the identification and cloning of a family of human neuronal proteins related to the mouse stargazin (γ2) protein and discovered their differential tissue distributions. This chapter investigates the influence of these human stargazin-like proteins upon the electrophysiological properties of VDCCs heterologously expressed in *Xenopus leavis* oocytes. The aim of the experiments in this section was to examine whether there was any biophysical evidence for a role of the human γ2, and γ4 subunits as part of a human neuronal VDCC complex.

At the time this work commenced, only the original study describing the identification and functional characterisation the mouse γ2 subunit (stargazin) had been published (Lett's et al., 1998). The principal electrophysiological finding was that co-expression of the mouse γ2 in a BHK (baby hamster kidney) cell line stably expressing Cav2.1/β1a/α2δ1 VDCCs shifted the half maximal voltage of steady-state inactivation (V50inact) by −7 mV. The logic behind the use of this un-physiological channel complex was presumably that expression of the Cav2.1 subunit would produce a P/Q-type current, although the β1a is a skeletal muscle isoform. Several studies have shown that Cav2.1 is strongly expressed in the cerebellum (Stea et al., 1994; Tanaka et al., 1995; Westenbroek et al., 1995) and gates the P/Q-type VDCC current that is predominant in this tissue (Randall and Tsien, 1995; Zhang et al., 1993), which is also where the expression of the γ2 subunit is strongest (Lett's et al., 1998). However, the skeletal muscle β1a and the ubiquitous α2δ1 auxiliary subunits with which the Cav2.1 was co-expressed in this cell line could not be considered wholly appropriate considering that the group was characterising the exclusively neuronal γ2 subunit. The fact that the γ2 subunit is principally expressed in the cerebellum of both humans and mice, as shown by the results of distribution studies in Chapter 4, and the work of other laboratories (Klugbauer et al., 2000; Letts et al., 1998), meant that I chose to concentrate on expressing a calcium channel that would more closely resemble the predominant type found in cerebellar Purkinje cells, and to investigate the properties of only the γ2 and γ4 subunits that are expressed in the cerebellum. The γ3 subunit was not further characterised in this study because it is apparently absent from human and mouse.
cerebellum. Furthermore, I aimed to determine whether any effects of $\gamma_2$ or $\gamma_4$ subunit co-expression with a VDCC were dependent upon the inclusion of an $\alpha2\delta$ subunit as part of the channel complex.
Chapter 5

5.2 Results

5.2.1 Selection of a suitable VDCC complex with which to characterise the human \( \gamma_2 \) and \( \gamma_4 \) subunits

Although the \( \gamma_1 \) subunit is not expressed in cardiac muscle, early studies investigated its influence of upon the biophysical properties of the DHP-sensitive voltage-dependent cardiac L-type channels (Cav1.2) expressed in *Xenopus* oocytes (Singer *et al.*, 1991; Wei *et al.*, 1991) and mammalian HEK293 cells (Eberst *et al.*, 1997; Lerche *et al.*, 1996). These all reported a pronounced hyperpolarizing shift in the membrane potential for half maximal steady state inactivation \( (V_{50\text{inact}}) \) upon co-expression of \( \gamma_1 \) subunit, whilst comparison of the highly variable effects of the \( \gamma_1 \) subunit upon other voltage-dependent properties of the channel was difficult, mainly because different subunit combinations and \( \text{Ba}^{2+} \) concentrations that were used in each investigation. Ideally, it would have been of more physiological relevance to perform these studies using the skeletal muscle Cav1.1 subunit, but the technology to reliably express the Cav1.1 subunit in *Xenopus* oocytes or mammalian cell lines does not exist, and \( \gamma_1 \) knockout mice have only recently been generated (Ahern *et al.*, 2001; Freise *et al.*, 2000). Given the structural similarity between \( \gamma_2 \) and \( \gamma_1 \), Letts *et al.* (1998) investigated the biophysical influence of their putative \( \gamma_2 \) subunit upon a BHK cell line stably expressing Cav2.1/\( \beta_{1\alpha}/\alpha2\delta_1 \) VDCCs. This cell-line was intended to produce a P/Q-type \( \text{Ca}^{2+} \) current bestowed by the Cav2.1 subunit, but the auxiliary \( \beta_{1\alpha} \), and \( \alpha2\delta_1 \) subunits are not specifically neuronal and in particular, not selectively expressed in the cerebellum where the \( \gamma_2 \) subunit predominates. Therefore, their investigation into the \( \gamma_2 \) subunit's functional properties, like those into the functional properties of the \( \gamma_1 \), was performed using a \( \text{Ca}^{2+} \) channel complex that is not likely to exist in vivo.

The tissue distribution evidence of Chapter 4 showed the human \( \gamma_2 \) and \( \gamma_4 \) subunits are both highly expressed in the cerebellum. For this investigation, I therefore chose to characterise the properties of the human \( \gamma_2 \) and \( \gamma_4 \) subunits with VDCC subunits that predominate in the Purkinje neurones of the cerebellum. Several distribution and localisation studies have shown the principal pore-forming subunit expressed in the Purkinje cell to be Cav2.1 (Craig *et al.*, 1998; Stea *et al.*, 1994; Tanaka *et al.*, 1995;
Chapter 5  \(\gamma_2\) & \(\gamma_3\) VDCC electrophysiology

Volsen et al., 1995; Westenbroek et al., 1995). I chose to express the rabbit B1_II Ca\(_{v}2.1\) subunit (X57689) (Mori et al., 1991) as it is a \(\Delta\)NP isoform. This splice variant does not contain an asparagine and proline at positions 1605 and 1606 in the domain IV extracellular linker separating transmembrane segments S3 and S4. Such splice variants of the Ca\(_{v}2.1\) subunit have been reported to express more P-type than Q-type currents (Bourinet et al., 1999). In all cases I expressed this Ca\(_{v}2.1\) together with the \(\beta_4\) which is the predominant \(\beta\) subunit expressed in the Purkinje cell soma (Volsen et al., 1997) and has been shown in a previous study to be the \(\beta\) subunit that most strongly modulates the activation and inactivation properties of the rabbit B1_II Ca\(_{v}2.1\) clone when expressed in *Xenopus* oocytes (De Waard and Campbell, 1995).

It has been reported previously that any alterations in Ca\(_{v}1.2\) channel properties attributable to the \(\gamma_1\) subunit are dependent upon the co-expression of a \(\beta\) and/or a \(\alpha 2\delta\) subunit (Singer et al., 1991; Wei et al., 1991). The presence or absence of an \(\alpha 2\delta\) subunit may therefore also have a critical bearing on any effects that may be attributable to addition of a putative neuronal \(\gamma\) subunit. Recent studies have reported the identification and cloning of another two \(\alpha 2\delta\) subunits, \(\alpha 2\delta_2\) and \(\alpha 2\delta_3\) (Gao et al., 2000a; Klugbauer et al., 1999a). The genes encoding the \(\alpha 2\delta_1\), \(\alpha 2\delta_2\), and \(\alpha 2\delta_3\) subunits show generally distinct patterns of expression in the cerebellum (Barclay et al., 2001; Hobom et al., 2000; Klugbauer et al., 1999a). However, the \(\alpha 2\delta_2\) subunit has been reported to be most strongly expressed \(\alpha 2\delta\) subunit in cerebellar Purkinje cells (Barclay et al., 2001; Hobom et al., 2000) and therefore was included in the VDCC complexes used to investigate the neuronal \(\gamma\) subunit properties. The dependence of modulation by any neuronal \(\gamma\) subunit upon the co-expression of an \(\alpha 2\delta\) subunit was also investigated by omitting the \(\alpha 2\delta_2\) subunit from the cDNA mixes for some experiments.

5.2.2 Influence of the \(\alpha 2\delta_2\) subunit upon the biophysical properties of Ca\(_{v}2.1/\beta_4\) currents expressed in *Xenopus* oocytes

In initial experiments, the biophysical properties of the Ca\(_{v}2.1/\beta_4\) VDCCs expressed in *Xenopus* oocytes, and the influence of the \(\alpha 2\delta_2\) subunit on this complex in the absence of any \(\gamma\) subunits were determined.
5.2.2.1 Activation

Figure 5.1 shows the peak I-V relationship with representative traces and Table 5.1 the results of the modified Boltzmann fitting to determine values for the parameters defining the I-V relationship of Cav$_{2.1}$/\beta_4 currents expressed in *Xenopus* oocytes alone and in the presence of the \(\alpha 2\delta_2\) auxiliary subunit. Cav$_{2.1}$/\beta_4 cDNAs injected in oocytes produced functional HVA VDCCs that activated at -20mV, had peak current amplitude of -0.25\(\mu\)A at +10mV and reversed at approximately +50mV. Co-expression of the \(\alpha 2\delta_2\) subunit caused a 2.5 fold increase in the peak current amplitude at +10mV and a corresponding 2.4 fold increase in the maximum conductance, \(G_{\text{max}}\). These data indicate Cav$_{2.1}$/\beta_4 current was up-regulated by the \(\alpha 2\delta_2\) subunit without altering the voltage dependence of activation.

![Figure 5.1](image)

**Figure 5.1 - Influence of the \(\alpha 2\delta_2\) subunit upon activation of a Cav$_{2.1}$/\beta_4 VDCC expressed in *Xenopus* oocytes.**

Whole-cell currents recorded from *Xenopus* oocytes by TEVC in 10mM Ba$^{2+}$ (holding potential -100mV; +10mV increments). 

a) The peak current-voltage relationships for Cav$_{2.1}$/\beta_4 (n=16) and Cav$_{2.1}$/\beta_4/\(\alpha 2\delta_2$$^3$$^5$$^7$$^9$ (n=25) displays a doubling in the maximal conductance and peak current amplitude at 0mV and +10mV upon co-expression of the \(\alpha 2\delta_2\) subunit. The mean I-V relationship was fitted with a combined Boltzmann and linear fit, as described in Materials and methods (2.6.3.1.1). b) The I-V relationship normalised to peak current amplitude at +10mV shows that there are no shifts in the \(V_{50}\) of activation as a result of co-expression of the \(\alpha 2\delta_2\) subunit. c) Representative traces of currents recorded from a single oocyte from each injection set and the activation protocol waveform.
Chapter 5  

7) VDCC electrophysiology

Channel | Activation | Inactivation | n
---|---|---|---
| $V_{0.5\text{activ.}}$ (mV) | $k_{\text{activ.}}$ | $G_{\text{Max}}$ (µS) | Peak (+10mV)

| Ca$\nu2.1/\beta_4$ | -3.76 ± 0.71 | 4.60 ± 0.43 | 7.04 ± 0.48 | -0.25 ± 0.05 | 16 |
| Ca$\nu2.1/\beta_4/\alpha2\delta_2$ | -5.30 ± 0.86 | 4.05 ± 0.19 | 17.0 ± 3.22* | -0.63 ± 0.13* | 25 |

Table 5.1 - Characteristics of $I_{Na}$ via Ca$\nu2.1/\beta_4$ channels alone or co-expressed with $\alpha2\delta_2$ subunits in *Xenopus* oocytes.

To analyse the voltage-dependent activation, current-voltage (I-V) relationships (Figure 5.1) were fitted with a modified Boltzmann equation $I = G_{\text{max}}(V-V_{\text{rev}})/(1+\exp(- (V-V_{0.5\text{activ.}})/k))$ where $I$ was the current, $G_{\text{max}}$ was the maximum conductance, $V$ was the test pulse potential and $V_{\text{rev}}$ was the measured reversal potential. $V_{0.5\text{activ.}}$ is the midpoint voltage for current activation and $k_{\text{activ.}}$ is the slope factor. Data are expressed as mean ± S.E.M of the number of replicates, $n$. According to an unpaired Student’s $t$-test, * marks significance values $P < 0.05$ in pairs differing by the presence of an $\alpha2\delta_2$ subunit.

5.2.2.2 Inactivation

The voltage-dependence and kinetics of Ca$\nu2.1/\beta_4$ current inactivation in *Xenopus* oocytes alone, or when co-expressed with an $\alpha2\delta_2$ subunit were then investigated. The effect of co-expression an $\alpha2\delta_2$ subunit upon the half-maximal voltage of current inactivation ($V_{50\text{inact}}$) of Ca$\nu2.1/\beta_4$ currents was indistinguishable from the controls lacking a $\alpha2\delta_2$ subunit (Figure 5.2). The $\alpha2\delta_2$ subunit did however cause a slight decrease in the slope factor ($k_{\text{inact}}$), but this proved to be statistically non-significant according to Students $t$-test (Table 5.2). The presence of $\alpha2\delta_2$ ($n=28$) slowed the time course of Ca$\nu2.1/\beta_4$ ($n=15$) current inactivation at the membrane potential of +10mV by reducing the proportion of the fast time constant, $\tau_{\text{fast}}$ correspondingly increasing and the proportion of the slow time constant, $\tau_{\text{slow}}$.

| Channel | Activation | Inactivation | n
---|---|---|---
| $V_{50\text{inact}}$ (mV) | $k_{\text{inact}}$

| Ca$\nu2.1/\beta_4$ | -32.4 ± 1.09 | 8.43 ± 0.61 | 22 |
| Ca$\nu2.1/\beta_4/\alpha2\delta_2$ | -32.5 ± 1.28 | 6.92 ± 0.29 | 26 |

Table 5.2 - Parameters of the voltage-dependence of steady state inactivation of $I_{Na}$ via Ca$\nu2.1/\beta_4$ channels alone or co-expressed with the $\alpha2\delta_2$ subunit in *Xenopus* oocytes.

Steady state inactivation data from Figure 5.2 were fitted with a single Boltzmann equation of the form $I/I_{\text{max}} = (A_1 - A_2) / (1 + \exp(- (V-V_{50\text{inact}})/k_{\text{inact}})) + A_2$ where $I_{\text{max}}$ is the maximal current, $V_{50\text{inact}}$ is the half-maximal voltage for current inactivation, $k_{\text{inact}}$ is the slope factor, and $A_1$ and $A_2$ represent the proportion of inactivating and non-inactivating current, respectively. Values are expressed as mean ± S.E.M of the number of replicates, $n$. Statistical significance was calculated according to an unpaired Student’s $t$-test.

224
Figure 5.2 - Effects of the α2δ2 subunit upon steady state inactivation properties of Cav2.1/β4
VDCCs expressed in Xenopus oocytes.

Calcium channel currents recorded in 10mM Ba2+ from Xenopus oocytes injected with Cav2.1/β4 or
Cav2.1/β4/α2δ2. a) Steady state inactivation data of Cav2.1/β4 or Cav2.1/β4/α2δ2 currents (n = 22 and 26
respectively) normalised to the maximum current I_max and fitted with a single Boltzmann equation as
described in materials and methods (2.6.3.1.2). b) Waveform of protocol for data acquisition by stepping
from -100mV holding potential to the conditioning potential for 25 seconds before measuring the current
at test potential of 0mV for 100ms. Unless otherwise stated, this protocol applied to all steady-state
inactivation recordings.

Figure 5.3 - The influence of the α2δ2 subunit on the time course of Cav2.1/β4 current inactivation
expressed in Xenopus oocytes.

a) The time course of the currents during a 4.75 second depolarising pulse to +10mV with the zero level
indicated by the horizontal line. Currents shown are the mean time course from each group scaled to the
same amplitude. Individual measurements were fitted to the sum of two exponentials (white line).
Results of fitting procedures are displayed graphically in panels b) the time constants for the fast (τ_fast)
and slow (τ_slow) components of current inactivation and c) the proportion of the fast (A_fast) and slow
(A_slow) components of current inactivation. The number of replicates (n) for Cav2.1/β4 and
Cav2.1/β4/α2δ2 are 15 and 28 respectively. According to an unpaired Student’s t-test, * marks
significance values P < 0.05.
5.2.3 The effects of γ2 and γ4 subunits upon the biophysical properties of Ca_{v}2.1/β4 currents ± the α2δ2 subunit, expressed in *Xenopus* oocytes.

Having characterised the properties of the α2δ2 subunit when co-expressed with the Ca_{v}2.1/β4 Ca^{2+} channel in *Xenopus* oocytes, I investigated the biophysical consequences of co-expression of the γ2 and γ4 subunits and whether their influence was dependent upon the co-expression of the α2δ2 subunit.

5.2.3.1 Activation

Figure 5.4 and Figure 5.5 show the I-V relations of I_{Ba} via Ca_{v}2.1/β4 and Ca_{v}2.1/β4/α2δ2 in the absence and presence of the γ2 or γ4 subunits. The data defining the I-V relationship in each circumstance was generated by fitting the curves with a combined Boltzmann and linear fit (see methods 2.6.3.1.1). Neither the γ2 nor the γ4 subunit caused a significant shift in the V_{0.5activ} of Ca_{v}2.1/β4. The k_{activ}, G_{max}, and peak current amplitude at +10mV were also very similar to controls on co-expression of either of the γ subunits in the absence the α2δ2 subunit. When the α2δ2 subunit was added to the Ca^{2+} channel complex, co-expression of either γ2 or γ4 caused no significant changes in the I-V relationship. Its is important to note is that the increases in G_{max} and peak current amplitude attributable to the co-expression of an α2δ2 subunit with Ca_{v}2.1/β4 were unaffected by the co-expression of either the γ2 or the γ4 subunit.
Chapter 5  

\( \gamma_2 \) & \( \gamma_4 \) VDCC electrophysiology

Figure 5.4 - Influence of the \( \gamma_2 \) and \( \gamma_4 \) subunits on the I-V relationship of \( Ca_{2.1}/\beta_4 \) VDCCs.

Whole-cell currents recorded from \textit{Xenopus} oocytes by TEVC in 10mM Ba\(^{2+}\) (holding potential -100mV; +10mV increments; 100ms depolarisation – see inset for step protocol waveform). The peak current-voltage relationships for \( Ca_{2.1}/\beta_4 \) (n=16), \( Ca_{2.1}/\beta_4/\gamma_2 \) (n=14) and \( Ca_{2.1}/\beta_4/\gamma_4 \) (n=11) show no significant differences in any parameters (Table 5.3). The right-hand panels display representative traces recorded from a single oocyte injected with each subunit combination investigated. For clarity, only traces from -50 to +10mV are displayed.

Figure 5.5 - Influence of the \( \gamma_2 \) and \( \gamma_4 \) subunits on the I-V relationship of \( Ca_{2.1}/\beta_4/\alpha2\delta_2 \) VDCCs.

Whole-cell currents recorded from \textit{Xenopus} oocytes by TEVC in 10mM Ba\(^{2+}\) (holding potential -100mV; +10mV increments; 100ms depolarisation – see inset for step protocol waveform). The peak current-voltage relationships for \( Ca_{2.1}/\beta_4/\alpha2\delta_2 \) (n=43), \( Ca_{2.1}/\beta_4/\alpha2\delta_2/\gamma_2 \) (n=34) and \( Ca_{2.1}/\beta_4/\alpha2\delta_2/\gamma_4 \) (n=20) show the only significant difference in any of the measured parameters was an increase in the value of the \( k_{act} \) by 10.7% on co-expression of the \( \gamma_4 \) subunit (Table 5.3). The right-hand panels display representative traces recorded from a single oocyte injected with each subunit combination investigated. For clarity only traces from -50 to +10mV are displayed.
Chapter 5

\[ \gamma_2 \text{ and } \gamma_4 \text{ VDCC electrophysiology} \]

### Table 5.3 - Characteristics of I_{na} via Cav2.1/β4 or Cav2.1/β2/α2δ2 channels alone or co-expressed with γ2 or γ4 subunits in Xenopus oocytes.

To analyse the voltage-dependent activation, current-voltage (I-V) relationships (Figure 5.4 and Figure 5.5) were fitted with a modified Boltzmann equation (see methods 2.6.3.1.1). One-way analysis of variance (ANOVA) and post hoc Tukey-Kramer “Honestly Significantly Different” (HSD) tests determined no statistically significant changes in any pairs of data differing by the presence of a γ subunit (\( P < 0.05 \) was considered significant).

<table>
<thead>
<tr>
<th>Channel</th>
<th>Activation</th>
<th>( n )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{Cav2.1/β4} )</td>
<td>( V_{0.5\text{activ.(mV)}} )</td>
<td>16</td>
</tr>
<tr>
<td>&amp; ( k_{\text{activ.}} )</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>&amp; ( G_{\text{max}} (\mu S) )</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>&amp; ( \text{Peak at } +10\text{mV} )</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>&amp; ( \text{(μA)} )</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>&amp; ( \text{Peak at } +10\text{mV} )</td>
<td>20</td>
<td></td>
</tr>
</tbody>
</table>

5.2.3.2 Steady-state inactivation

When the mouse γ2 subunit (stargazin) was initially cloned and characterised, the principal biophysical consequence of its co-expression with a VDCC containing the Cav2.1 α subunit was a small hyperpolarizing shift in the half-maximal voltage of steady-state inactivation (Letts et al., 1998), a result subsequently repeated by another laboratory who also observed a similar effect upon co-expression of the γ4 subunit using the same core VDCC complex combination previously used by Letts et al. (Klugbauer et al., 2000; Letts et al., 1998). In my investigation, I could not repeat the findings of either group when co-expressing the human γ2 or γ4 subunits with Cav2.1/β2/α2δ2 VDCC in Xenopus oocytes. For further analysis, the control data for Cav2.1/β2/α2δ2/γ2 and Cav2.1/β2/α2δ2/γ4 injections were separated because recordings made to compare the effects of γ2 and γ4 were made on different dates, and hence were only compared to control injections recorded on the same days. The γ4 experiments were performed during September and October 2000, and the γ2 experiments during January and February 2001. Averaged steady state inactivation curves for the co-expression of the γ2 subunit with Cav2.1/β2/α2δ2 Ca\(^{2+}\) channels show that the subunit caused a very small and statistically non-significant hyperpolarizing shift in \( V_{50\text{inact}} \). Co-expression of the
The $\gamma_4$ subunit produced a hyperpolarising shift in the $V_{50\text{inact}}$ of the Cav2.1/$\beta_4/\alpha 2\delta_2$ inactivation curve by 3.2mV, but this shift was also non-significant according to an unpaired Student’s t-test. Both the $\gamma_2$ and $\gamma_4$ subunits caused almost no alteration in the value of $k_{\text{inact}}$.

![Figure 5.6](image)

**Figure 5.6 - Influence of the $\gamma_2$ and $\gamma_4$ subunits upon the steady state inactivation properties of Cav2.1/$\beta_4/\alpha 2\delta_2$ channels expressed in Xenopus oocytes.**

Mean steady state inactivation data of $I_{\text{Ba}}$ recorded in 10mM Ba$^{2+}$ from Xenopus oocytes injected with a) Cav2.1/$\beta_4/\alpha 2\delta_2$ (n=18) or Cav2.1/$\beta_4/\alpha 2\delta_2/\gamma_2$ (n=18) and b) Cav2.1/$\beta_4/\alpha 2\delta_2$ (n=20), or Cav2.1/$\beta_4/\alpha 2\delta_2/\gamma_4$ (n=20) normalised to the maximum current $I_{\text{max}}$ and fitted with a single Boltzmann function (see methods 2.6.3.1.2). Co-expression of the $\gamma_2$ subunit produced data almost identical to the control. The $\gamma_4$ subunit caused a small but statistically non-significant hyperpolarising shift in the $V_{50\text{inact}}$. The numerical data for parameters defining the steady-state inactivation relationship are displayed in Table 5.4.

<table>
<thead>
<tr>
<th>Channel</th>
<th>Inactivation</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$V_{50\text{inact}}$ (mV)</td>
<td>$k_{\text{inact}}$</td>
</tr>
<tr>
<td>Cav2.1/$\beta_4/\alpha 2\delta_2$</td>
<td>-31.52 ± 1.58</td>
<td>7.04 ± 0.19</td>
</tr>
<tr>
<td>Cav2.1/$\beta_4/\alpha 2\delta_2/\gamma_2$</td>
<td>-33.44 ± 1.49</td>
<td>7.10 ± 0.23</td>
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<tr>
<td>Cav2.1/$\beta_4/\alpha 2\delta_2/\gamma_4$</td>
<td>-31.4 ± 0.89</td>
<td>7.04 ± 0.28</td>
</tr>
<tr>
<td>Cav2.1/$\beta_4/\alpha 2\delta_2/\gamma_4$</td>
<td>-34.6 ± 1.43</td>
<td>6.61 ± 0.28</td>
</tr>
</tbody>
</table>

**Table 5.4 - Influence of the $\gamma_2$ and $\gamma_4$ subunits upon the steady state inactivation properties of Cav2.1/$\beta_4/\alpha 2\delta_2$ channels.**

Steady state inactivation data from Figure 5.6 were fitted with a single Boltzmann function (see methods 2.6.3.1.2). Values are expressed as mean ± S.E.M of the number of replicates, n. None of the shifts in any of the parameters measured that occurred on co-expression of $\gamma_2$ or $\gamma_4$ proved to be statistically significant according to an un-paired Student’s t-test.

The $\gamma_2$ and $\gamma_4$ subunits did not affect the steady-state inactivation properties of a Cav2.1/$\beta_4$ VDCC expressed with the $\alpha 2\delta_2$ subunit. Similarly, the $\gamma_4$ subunit did not significantly affect the steady state inactivation of a Cav2.1/$\beta_4$ channel in the absence of an $\alpha 2\delta$ subunit. However, when the Cav2.1/$\beta_4$ VDCCs were expressed in the absence of
an α2δ subunit, the γ2 subunit caused an obvious −4.9mV shift in the $V_{\text{shift}}$ which proved statistically significant ($p<0.05$) according to a Students $t$-test and was similar to the shifts reported in the initial characterisation of stargazin (Letts et al., 1998) and subsequent study by Klugbauer et al. (Klugbauer et al., 2000). However, in this instance the means of two test samples were compared to a single control, suggesting analysis of variance (ANOVA) was probably a more appropriate test of significance. ANOVA and post hoc Tukey-Kramer HSD calculations determined differences in either the $V_{\text{shift}}$ of Cav2.1/β4 and Cav2.1/β4/γ2 or Cav2.1/β4 and Cav2.1/β4/γ4 currents to be non-significant.
5.2.3.3 Inactivation kinetics

During a sustained 4.75s membrane depolarisation to +10mV, the current amplitude of Cav2.1/β4 containing VDCC expressed with in the presence or absence of the α2δ2 subunit and γ2 or γ4 subunits, decayed as a result of inactivation of the channel. The time course of inactivation for all subunit combinations expressed consisted of two kinetic components. The first inactivating component followed a fast time course, \( \tau_{\text{fast}} \) whereas the second component followed a slow time course, \( \tau_{\text{slow}} \). The proportion of the inactivated current constituted by \( \tau_{\text{fast}} \) was termed \( A_{\text{fast}} \) and the proportion of the inactivated current constituted by \( \tau_{\text{slow}} \) was termed \( A_{\text{slow}} \). Both \( A_{\text{fast}} \) and \( A_{\text{slow}} \) were expressed as a percentage of the total current, \( A_{\text{fast}} + A_{\text{slow}} + C \), where \( C \) represents the proportion of the total current which is not inactivated.

Co-expression of the γ2 or γ4 subunit with Cav2.1/β4/α2δ2 caused no significant alterations in the inactivation kinetics. Figure 5.8 with Table 5.6 and display all the data recorded for each condition. The γ2 and γ4 subunit also did not alter the inactivation kinetics when co-expressed with Cav2.1/β4 in the absence of an α2δ subunit (Figure 5.9).
Figure 5.8 - The influence of co-expression of the $\gamma_2$ and $\gamma_4$ subunit on the inactivation kinetics of Cav$_{2.1}/\beta_4/\alpha_2\delta_2$ VDCCs expressed in Xenopus oocytes.

The time course of the currents during a 4.75 second depolarising pulse to $+10$ mV of Cav$_{2.1}/\beta_4/\alpha_2\delta_2$ co-expressed with a) $\gamma_2$ and b) $\gamma_4$ with the zero level indicated by the horizontal line. Currents shown are the mean time course from each group scaled to the same amplitude. Individual measurements were fitted to the sum of two exponentials (white lines). Results of fitting procedures are displayed in Table 5.6. No statistically significant changes were measured in any of the parameters of inactivation kinetics on co-expression of either $\gamma$ subunit.

<table>
<thead>
<tr>
<th>Channel</th>
<th>Inactivation kinetics</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\tau_{\text{fast}}$ (ms)</td>
<td>$\tau_{\text{slow}}$ (s)</td>
</tr>
<tr>
<td>Ctrl</td>
<td>$261.5 \pm 17.4$</td>
<td>$1.15 \pm 0.06$</td>
</tr>
<tr>
<td>$+\gamma_2$</td>
<td>$291.9 \pm 13.7$</td>
<td>$1.08 \pm 0.08$</td>
</tr>
<tr>
<td>Ctrl</td>
<td>$257.7 \pm 17.0$</td>
<td>$1.19 \pm 0.06$</td>
</tr>
<tr>
<td>$+\gamma_4$</td>
<td>$243.6 \pm 19.6$</td>
<td>$1.18 \pm 0.06$</td>
</tr>
</tbody>
</table>

Table 5.6 - Data to compare the influence of co-expression of the $\gamma_2$ or $\gamma_4$ subunit on the inactivation kinetics of Cav$_{2.1}/\beta_4/\alpha_2\delta_2$ VDCCs.

Small changes in Cav$_{2.1}/\beta_4/\alpha_2\delta_2$ (Ctrl) inactivation kinetics are seen upon co-expression of a) $\gamma_2$ and b) $\gamma_4$, none of which are statistically significant ($t$-test, $P < 0.05$ considered significant). Data are expressed as mean ± S.E.M. of the number of replicates, n.
Figure 5.9 - The influence of co-expression of the γ2 and γ4 subunit on the inactivation kinetics of Cav2.1/β4 VDCCs expressed in Xenopus oocytes.

The time course of the currents during a 4.75 second depolarising pulse to +10mV of Cav2.1/β4 co-expressed with a) γ2 and b) γ4 with the zero level indicated by the horizontal line. Currents shown are the mean time course from each group scaled to the same amplitude. The two test conditions expressing a γ subunit were plotted on separate graphs with the same control data for clarity. Individual measurements were fitted to the sum of two exponentials (white line). Results of fitting procedures are displayed in Table 5.7.

<table>
<thead>
<tr>
<th>Channel</th>
<th>Inactivation kinetics</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctrl</td>
<td>τ_{fast} (ms)</td>
<td>τ_{slow} (s)</td>
</tr>
<tr>
<td>γ2</td>
<td>233.4 ± 12.1</td>
<td>1.34 ± 0.09</td>
</tr>
<tr>
<td>γ4</td>
<td>257.2 ± 14.0</td>
<td>1.17 ± 0.09</td>
</tr>
<tr>
<td>γ4</td>
<td>279.0 ± 20.1</td>
<td>1.28 ± 0.15</td>
</tr>
</tbody>
</table>

Table 5.7 - Data to compare the influence of co-expression of the γ2 or γ4 subunit on the inactivation kinetics of Cav2.1/β4 VDCCs.

The time constants for the fast (τ_{fast}) and slow (τ_{slow}) components of current inactivation and the proportion of the fast (A_{fast}) and slow (A_{slow}) components of current inactivation were calculated from the fits in Figure 5.9. ANOVA and post hoc Tukey-Kramer HSD tests determined no statistically significant changes in any pairs of data differing by the presence of a γ subunit (confidence level set at 95%). Data are expressed as mean ± S.E.M of the number of replicates, n.
5.3 Discussion

5.3.1 Selection of a suitable channel complex with which to characterise the novel neuronal VDCC γ subunits

Many articles have been published in the last decade, characterising the functional properties of each of the VDCC auxiliary subunits as they were identified and cloned. These have provided researchers with invaluable insights into the roles of each of these subunits in fine-tuning the functioning of the pore forming and current gating Cav α1 subunits. Early investigations elucidated the crude properties of the β, α2δ and γ subunits in modulating L- and P/Q-type VDCCs heterologously expressed in Xenopus oocytes (Hullin et al., 1992; Mikami et al., 1989; Mori et al., 1991; Singer et al., 1991). It has only been in more recent years, following the discovery of the superabundance of VDCC subunit genes and their splice variants, that the appreciation of the potential molecular diversity of Ca\(^{2+}\) channels and their differential tissue distributions, has made it possible to study the particular role of each auxiliary subunit in channel complexes that resemble those in which they may be expressed in vivo (Birnbaumer et al., 1998; Catterall, 2000; Jones, 1998). Furthermore, the generation of VDCC subunit knockout mice, and the characterisation of epileptic mice with spontaneously arising mutations in particular VDCC subunits has greatly enhanced our understanding of the normal roles of these subunits in vivo (Ahern et al., 2001; Barclay et al., 2001; Freise et al., 2000; Freise et al., 1999; Puranam and McNamara, 1999). However, the generation of knockout mice is not always a successful technique because the process is both expensive, labour intensive and often results in the creation of a lethal phenotype or one in which alternative compensatory mechanisms have occurred. Therefore heterologous expression systems are still the method of choice for functionally characterising novel subunits. It is however imperative that in such experiments, every effort is made to express the subject of investigation with partner subunits with which it may interact in its native surroundings.

The aim of the present investigation was therefore to characterise the functional properties of the human neuronal γ\(_2\) and γ\(_4\) subunits with other VDCC subunits that are expressed at high levels in the same tissues where the γ subunits predominate. Tissue
distribution studies had shown that the $\gamma_2$, $\gamma_3$ and $\gamma_4$ subunit were exclusively localised to the human brain and differentially distributed in the many brain regions (Chapter 4). The distribution of the neuronal $\gamma$ subunits in cerebellum was of particular interest because the effective absence of $\gamma_2$ in mouse brain was of functional significance in the stargazer mouse. Both the $\gamma_2$ and $\gamma_4$ subunits are expressed in the cerebellum according to northern blot, and immunostaining was particularly strong in Purkinje cells. The $\gamma_3$ subunit is absent from or expressed at very low levels in cerebellum (Chapter 4 and (Klugbauer et al., 2000)). The stargazer phenotype is also very similar to those of other mice that have mutations in the predominant Purkinje cell subunits, $\text{Ca}_v 2.1$ (Fletcher et al., 1996), $\beta_4$ (Burgess et al., 1997) and $\alpha 2\delta_2$ (Barclay et al., 2001) subunits, resulting in cerebellar ataxia and spike-wave seizures. The subunit combination of choice to study $\gamma_2$ and $\gamma_4$ properties was therefore the rabbit BI-2 $\text{Ca}_v 2.1$ splice form that produces a more P- than Q- type current (Bourinet et al., 1999; Mori et al., 1991) together with $\beta_4$ and $\alpha 2\delta_2$.

Early investigations into the functional properties of the skeletal muscle $\gamma_1$ subunit showed that its effects are dependent upon the co-expression of a $\beta$ and/or $\alpha 2\delta$ subunit (Singer et al., 1991; Wei et al., 1991). At that time only a single gene encoding the $\alpha 2\delta_1$ subunit had been identified. More recent studies have identified two further $\alpha 2\delta$ genes, both of which are expressed neuronally (Klugbauer et al., 1999a), but it is the $\alpha 2\delta_2$ subunit that predominates in the cerebellum (Barclay et al., 2001; Hobom et al., 2000). $\text{Ca}_v 2.1/\beta_4$ channels were therefore expressed in Xenopus oocytes in the absence of any $\alpha 2\delta$ subunit or with the neuronal $\alpha 2\delta_2$ subunit, and compared with data published from other laboratories investigating the neuronal $\gamma$ isoforms (Klugbauer et al., 2000; Letts et al., 1998) to examine the possibility that $\gamma_2$ and $\gamma_4$ require the co-expression of an $\alpha 2\delta$ subunit in order to modulate VDCC currents.

5.3.2 The $\alpha 2\delta_2$ increases maximum conductance of $\text{Ca}_v 2.1/\beta_4$ channel expressed in Xenopus oocytes

Before investigating the biophysical influence of the $\gamma_2$ and $\gamma_4$ subunits, the functional effects of co-expression $\alpha 2\delta_2$ with the $\text{Ca}_v 2.1/\beta_4$ VDCC in Xenopus oocytes were
determined. The $\alpha_2\delta_2$ subunit significantly increased peak current amplitude and $G_{\text{max}}$ compared to the Cav2.1/\beta_4, but did not significantly alter steady-state inactivation properties. These findings reinforced the findings of similar experiments performed in our laboratory that both rat and rabbit Cav2.1 co-expressed with $\beta_4$ are strongly enhanced by $\alpha_2\delta_2$ in both Xenopus oocytes and mammalian COS-7 expression systems (Barclay et al., 2001; Brodbeck et al., 2002). In vitro studies by other laboratories have shown that $\alpha_2\delta_1$, $\alpha_2\delta_2$ and $\alpha_2\delta_3$ subunits can all act to increase maximal conductance of various Cav-$\alpha_1/\beta$ subunit combinations (Dolphin et al., 1999b; Gao et al., 2000a; Hobom et al., 2000; Klugbauer et al., 1999a; Walker and De Waard, 1998). However, it has been reported that when associated with the $\beta$ subunit, the $\alpha_2\delta$ subunits can also induce shifts in the voltage dependence of activation of Cav1.2, Cav2.1, and Cav2.3 in various expression systems (Felix et al., 1997; Hobom et al., 2000; Qin et al., 1998; Singer et al., 1991). Differences between the present results and those of previous studies may highlight a dependence on the specific combination of Cav $\alpha_1$ and $\beta$ subunits expressed.

The kinetics of inactivation of Cav2.1/\beta_4 channels were slightly but significantly altered by $\alpha_2\delta_2$ subunit co-expression. The $\alpha_2\delta_2$ subunit slowed both the $\tau_{\text{fast}}$ and $\tau_{\text{slow}}$ time constants but not significantly. However, it did cause a significant reduction in the proportion of the fast time constant, $A_{\text{fast}}$ and a corresponding increase in the proportion of the slow inactivating time constant, $A_{\text{slow}}$. The overall effect was that at any given point after peak activation, whole cell current of oocytes expressing the channels containing an $\alpha_2\delta_2$ subunit would be slightly less inactivated that those not expressing an $\alpha_2\delta_2$ subunit. In the few studies investigating the $\alpha_2\delta_2$ subunit, effects on inactivation kinetics have in general been slight and usually non-significant (Brodbeck et al., 2002; Gao et al., 2000a; Klugbauer et al., 1999a). Nevertheless, all effects on inactivation kinetics in this and in previous investigations are minor and like other biophysical parameters, are likely to be dependent upon specific subunit combinations expressed and the expression systems used.
Chapter 5  γ2 & γ4 VDCC electrophysiology

5.3.3 Activation properties of Cav2.1/β4 channels are unaltered by the γ2 and γ4 subunits in the presence or absence of α2δ2 subunits

The co-expression of the γ2 or the γ4 subunits with Cav2.1/β4 VDCCs in the absence or presence of α2δ2 subunits, did not significantly affect any of the activation properties. These results suggest that neither the human γ2 nor the γ4 subunit strongly modulate the I-V relationship of Cav2.1/β4 VDCCs expressed in the absence or presence of an α2δ2 subunit.

5.3.4 Modulation of VDCC inactivation by γ2 or γ4 subunits

Both γ2 and γ4 failed to shift the voltage-dependence of steady-state inactivation of a complete Cav2.1/β4/α2δ2 VDCC complex. However, when the α2δ2 subunit cDNA was omitted from the expressed complex, the γ2 subunit shifted the voltage-dependence of steady-state inactivation 4.9mV in the hyperpolarising direction, which proved significant (P < 0.05) according to an unpaired Student’s t-test. γ4 did not alter the steady-state inactivation of a Cav2.1/β4 VDCC. This result highlighted either a possible differential effect of γ2 and γ4 when expressed with the Cav2.1/β4 VDCC in the absence of an α2δ2 subunit, or a potential type I error in statistical significance. Firstly, let us consider the biological reasons for this difference. Although oocytes express an endogenous α2δ1 subunit (Singer-Lahat et al., 1992), the extent to which it is incorporated in the exogenously expressed channels is probably minor. In previous investigations, oocytes showed very low levels of endogenous calcium channel activity that did not interfere with the expression of exogenous Cav1.2, which was enhanced significantly by co-expression of exogenous α2δ1 (Singer et al., 1991). It is therefore unlikely that a preferential α2δ1/γ2 association with Cav2.1/β4 underlies the shift in V_{50\text{inact}} recorded in the “absence” of an α2δ subunit. Rather, co-expression of an α2δ2 subunit with the γ2 subunit somehow masks or prevents the modulation by γ2 subunit. However, the difference observed in the V_{50\text{inact}} on co-expression of the γ2 subunit with Cav2.1/β4 VDCCs more likely to be the result of a type I error in the statistical analysis. The t-test tells us if the variation between two groups is "significant", but if all the data
in this chapter for VDCCs expressing a γ subunit were compared to data from control VDCCs not expressing a γ subunit this would entail forty separate t-tests. With the alpha level for significance set at P = 0.05, it is not surprising that amongst the forty samples at least one result gave a P < 0.05. To determine whether the significant difference in this instance was due to a type I error, leading to the rejection of the null hypothesis (H₀) when it is in fact true, ANOVA of the data was performed where appropriate. ANOVA compared the variance of the whole population compared to the changes in one data set to give one P for the H₀, which simply indicated if there was a difference between two or more of the group means, but it did not reveal between which groups the difference occurred. Therefore, a post hoc Tukey-Kramer HSD test (allows analysis of unequal group sizes) performed a pairwise comparison of the means of each group to see where any significant differences occurred. ANOVA and Tukey-Kramer HSD tests determined that differences in the V₅₀inact and k₅₀inact between the control group and those expressing a γ₂ or a γ₄ subunit did not exist, and therefore the significant difference calculated by a Student’s t-test was probably the result of a type I error.

Although minor variations were observed in the parameters defining the inactivation kinetics of Caᵥ₂.1/β₄ currents in the absence or presence of an α₂δ₂ subunit, neither the γ₂ nor γ₄ subunit caused significant modulation.

5.3.5 Comparison of data with results from other laboratories investigating the influence of putative neuronal γ subunit on VDCC electrophysiology

5.3.5.1 Activation

The apparent inability to modulate the activation properties of HVA VDCC complexes in the present study concurs with the of findings from other laboratories that have characterised the stargazin-like group of neuronal γ subunits with other calcium channel complexes performed before, during, and since the acquisition of my data (Klugbauer et al., 2000; Letts et al., 1998). However, minor hyperpolarising shifts by γ₂, γ₃ or γ₄ (Rousset et al., 2001) or alternatively a small depolarising shift caused by γ₂ in the
voltage dependence of activation of P/Q-type VDCCs (Klugbauer et al., 2000) have also been reported. Rousset et al. (2001) also reported that the effects of γ2 and γ4 subunits on activation voltage-dependence were differentially dependent upon the co-expression of the β2 or β4 subunit. Additionally, the γ3 subunit only had significant effects in the absence of a β subunit (Rousset et al., 2001). The variation between the findings of the different laboratories are probably in part due to the different subunit combinations expressed and charge carriers used (Ca2+ or Ba2+). However, in probably the most salient publication to date regarding the functional role of the γ2 subunit (discussed in more detail below), Chen et al., (2000) recorded the whole cell Ca2+ currents from stargazer and wild type cerebellar granule cells. They reported that absence of the γ2 subunit did not alter the I-V relationship of the native whole cell Ca2+ current. They did not use pharmacological agents to break down the whole cell Ca2+ current into its component parts, which had this been performed, may have highlighted subtle changes particular to P/Q-, N-, R-, or L-type currents present in this cell type (Pearson et al., 1995; Randall and Tsien, 1995). Although it is also possible that another γ isoform could functionally substitute for the γ2 subunit, this is unlikely in mouse granule cells, because distribution studies have shown that the other γ isoforms are probably not expressed in this cell type (Chapter 4 & Klugbauer et al., 2000). Nevertheless, the importance of this result lies in the finding that absence of the γ2 subunit did not result in changes in VDCC biophysics in the same manner that omission of the γ1 subunit in knockout mice altered skeletal muscle VDCC properties (Ahern et al., 2001; Freise et al., 2000). This indicated that even if incorporated as part of the VDCC complex, γ2 did not modulate HVA VDCC activation properties.

Even so, the more recent electrophysiological analyses of one laboratory showed that γ2 co-expression caused a significant decrease in the current amplitude of both Cav2.1 and Cav2.2 Ca2+ channels co-expressed with the β3 and α2δ1 subunits (Kang et al., 2001). Interestingly, the effects of the γ2 subunit on current amplitude were dependent on the co-expression of the α2δ1 subunit in a manner similar to that which had been previously reported for the skeletal muscle γ1 subunit (Singer et al., 1991; Wei et al., 1991), albeit that the γ1 subunit potentiated Ca2+ current in those in vitro studies. Moreover, the reduction of peak Ca2+ current amplitude by γ2 co-expression bore a similarity to the
Chapter 5 γ2 & γ4 VDCC electrophysiology

reduction in Ca\(^{2+}\) entry into skeletal muscle myotubes expressing the γ₁ subunit compared to myotubes of γ₁ knockout mice (Ahern et al., 2001; Freise et al., 2000). However, it remains an exception to the rule, and the majority of research, including the observations reported herein suggest that that γ₂ and γ₄ subunits do not significantly modulate the activation properties of both native and heterologous VDCCs (Chen et al., 2000; Green et al., 2001; Klugbauer et al., 2000; Letts et al., 1998; Rousset et al., 2001).

5.3.5.2 Inactivation

Letts et al. (1998) reported that the murine γ₂ subunit caused a -7mV shift in the \(V_{50inact}\) of the Cav₂.1/β₁₈/α2δ₂ channel. This was the only significant modulation of Ca\(^{2+}\) current in that investigation and formed part of the evidence implicating stargazin (γ₂) as a neuronal γ subunit of native VDCCs. Similar small shifts with the same subunit combination gating Ca\(^{2+}\) current (instead of Ba\(^{2+}\)) expressed in HEK-293 cells, were reported by a different laboratory for both γ₂ and γ₄ subunits, although the magnitude of the hyperpolarisation was only 3-5mV (Klugbauer et al., 2000). However, when the skeletal muscle β₁₈ subunit was replaced by a neuronal β₂₆ subunit, the hyperpolarising shifts were only observed when Ca\(^{2+}\) was used as the charge carrier. When Ba\(^{2+}\) was used as the charge carrier significant depolarising shifts in \(V_{50inact}\) were caused by γ₂ subunit co-expression (Klugbauer et al., 2000). A separate investigation using Ba\(^{2+}\) as the charge carrier reported that γ₂ and γ₃ subunits hyperpolarised the \(V_{50inact}\) of Cav₂.1/α2δ₁ (no β) channels by 3-5mV, γ₂ and γ₄ shifted the \(V_{50inact}\) of Cav₂.1/β₂₆/α2δ₁ channels more hyperpolarised by 4-5mV, whilst γ₂ shifted the \(V_{50inact}\) of Cav₂.1/β₂₆/α2δ₁ 4mV in the depolarising direction (Rousset et al., 2001). Therefore, when Cav₂.1 or Cav₁.2 are co-expressed with β₂₆, γ₂ induces positive shifts in the voltage dependence of steady-state inactivation, whilst co-expression of γ₂, γ₃ or γ₄ with other β subunits and Cav₂.1 usually results in small or non-significant hyperpolarisation of \(V_{50inact}\). In this chapter, I reported no significant alterations in any of the parameters of steady state inactivation of the recombinant neuronal type Cav₂.1/β₄ VDCC regardless of α2δ₂ subunit co-expression. This agreed more closely with the findings of Chen et al. (2000) and Kang et al. (2001). The first of these two studies recorded the steady-state inactivation of VDCC current recorded from the cerebellar granule neurones of
stargazer mice and observed no alterations in any of the measured inactivation parameters compared to wild type cells (Chen et al., 2000). Kang et al. (2001) recorded from Xenopus oocytes expressing recombinant Cav2.2 or Cav2.1 with $\beta_3$ and $\alpha 2\delta_1$ and observed no shifts in the voltage dependence of inactivation and only a small change in the slope factor of $\text{Cav2.1}/\beta_3/\alpha 2\delta_1$ channel inactivation when co-expressed with the $\gamma_2$ subunit. It would therefore appear that modulation of VDCC steady-state inactivation by the neuronal $\gamma_{2,4}$ subunits is only minor if it occurs at all.

The inactivation kinetics of Cav2.1 or Cav2.2 expressed in Xenopus oocytes have been reported to be unaffected by the co-expression of a $\gamma_2$ subunit (Kang et al., 2001). Conversely, it has been reported that both $\tau_{\text{slow}}$ and $\tau_{\text{fast}}$ are reduced on co-expression of $\gamma_2$, $\gamma_3$ or $\gamma_4$ subunits (Klugbauer et al., 2000; Rousset et al., 2001), with in some instances a decrease in the proportion of inactivating current represented by $A_{\text{slow}}$ (Rousset et al., 2001). The latter study also reported that when $\gamma$ subunits were co-expressed an unexpected subpopulation of currents with much slower inactivation arose that were dependent on the co-expression of a $\beta$ subunit. No such populations were observed in my recordings and further investigation is required to understand the physiological mechanisms and consequences of the unexpected slow inactivation effects if they are determined to be a consistent observation on $\gamma$ subunit co-expression. The studies by Letts et al. (1998) and Chen et al. (2000) did not investigate the influence of the $\gamma$ subunits on inactivation kinetics.

Overall, the reported modulation of HVA VDCCs by the $\gamma_2$, $\gamma_3$ and $\gamma_4$ subunits is minor or non-existent. Even when co-expressed with LVA Cav3.1 or Cav3.3 subunits modulations of channel properties are very small (Green et al., 2001; Klugbauer et al., 2000). Furthermore, some of the multitude of small but "significant" differences reported by different laboratories could also be attributed to type I errors arising from multiple $t$-tests on different groups of data that shared a common control (Klugbauer et al., 2000; Rousset et al., 2001). ANOVA may have been more appropriate in these circumstances, but the variation of subunit combinations expressed, expression systems used, and divalent cation concentrations in the recording solutions used by the different laboratories should still be considered when comparing all the published data. The electrophysiological data therefore cannot be used as a strong argument to warrant the
considering the γ subunits as an integral part of HVA or T-type channels because modulation of current properties not reproducible between different studies. Nevertheless, biochemical evidence for the association of the γ₂ and γ₃ subunits with the N-type Cav2.2 subunit both in vitro and in brain tissue has been generated (Kang et al., 2001; Sharp et al., 2001). It is quite possible that the γ subunits play another role in VDCC function (e.g. trafficking and assembly) that does not interfere with channel biophysical properties.

5.3.6 γ₂, γ₃ and γ₄ are important chaperones in the trafficking of AMPA receptor subunits.

As discussed previously, Chen et al. (2000) reported that the absence of a γ₂ subunit in the stargazer mouse did not affect the activation or inactivation properties if VDCC currents recorded from native neurones. Furthermore, at the time of publication, evidence for the inclusion of these neuronal γ isoforms as part of a VDCC complex is not convincing (Klugbauer et al., 2000; Letts et al., 1998). Chen et al. (2000) were the first group to use biochemical techniques to show a physical interaction of γ₂ with any other cellular protein. The specific proteins with which the γ₂ subunit could be co-immunoprecipitated were the GluR AMPA receptor subunits and the MAGUKs, PSD-95 and SAP-97. The conclusions of the investigation proposed a two-step model in which γ₂ first conveys AMPA receptors to the plasma membrane, and then sweeps them laterally into post-synaptic sites. An association of γ₂ with GluR subunits, probably via transmembrane region interaction, is involved in the insertion of the AMPA receptor into the plasma membrane. The PDZ-interaction motif in the C-terminal of the γ₂ subunit interacts with the PSD-95 protein, and is necessary for the correct trafficking of the AMPA/γ₂ complex to the post-synaptic density. If γ₂ is unable to dock with PSD-95 in any way, targeting of the AMPA receptor to the post-synaptic density is lost although the AMPA receptor is still inserted into the plasma-membrane. Recent experiments from the same laboratory have shown that γ₃ and γ₄ both behave similarly (Chen et al., 2001). Transfection of the stargazin family members into the cerebellar granule cells of stargazer mice is able to rescue the EPSCs attributed to aberrant AMPA receptor expression in these cells. Previous investigations have shown that the GluR message
and protein are both present in stargazer brains (Hashimoto et al., 1999), therefore transfection of a neuronal γ subunit promotes the insertion and trafficking of AMPA receptors to their correct location in these cells, although it is not essential for functional AMPA receptor expression, as has been demonstrated in recombinant expression systems.

### 5.3.7 Are the γ₂, γ₃ and γ₄ proteins VDCC subunits?

Evidence for substantial modulation of VDCC current by the stargazin-related family of proteins is inconsistent and the work of Chen and colleagues (Chen et al., 2000; Chen et al., 2001) highlighted that the major role of the stargazin-like family of proteins is probably to control trafficking of the AMPA receptor to its correct location in the synapse. Additionally, electron-microscopy evidence suggests that the γ subunits are strongly localised to the post-synaptic density (Sharp et al., 2001). However this work has not eliminated the interaction of γ₂, γ₃ and γ₄ with other ion channels, although the I-V relationship of Kv3.1b channels transiently expressed in Xenopus oocytes is not altered by the co-expression of the γ₂ subunit (A.C. Dolphin, personal communication). The intricacies of the γ₂ interaction with the GluR subunits and PSD-95 remain to be determined. For example, once at the synapse does the γ₂ subunit remain associated with the AMPA receptor, or is it released to interact with other species? The co-immunoprecipitation of the γ₂ and γ₃ subunits with a Cav2.2 subunit (Kang et al., 2001; Sharp et al., 2001) highlights that the γ subunits are able to interact with VDCCs. However, whether this interaction is a direct association between Cavα₁ protein and γ subunits, or an interaction via an intermediate protein (e.g. PSD-95) remains to be determined. The γ subunits may play a role in anchoring VDCCs at the post-synaptic density, in the vicinity of other ion channels such as the AMPA receptor, therefore mediating clustering of the two channel types (or other ion channels) in a large post-synaptic complex via interaction with PSD-95 (Kim et al., 1995a). A test for such an association would be detection of both an AMPA receptor and a VDCC α₁ subunit in the same fraction of native neurone lysate immunoprecipitated with a γ subunit antibody. Furthermore, generation of γ subunit chimeras or yeast-two-hybrid baits
could determine the specific domains of the \( \gamma \) subunit involved in interaction with AMPA receptors and VDCC \( \alpha_1 \) subunits, and whether these domains overlap.

It would however seem that the classification of these proteins as VDCC \( \gamma \) subunits may have been somewhat premature, and only a great deal more investigation will determine all the roles of this family of proteins and result in a more appropriate nomenclature. Until such a time, the current nomenclature will suffice whilst bearing in mind that the proteins are probably not integral for normal neuronal VDCCs function.
Identification, cloning and functional characterisation of a pair of low-homology stargazin-related proteins
6.1 Introduction

The $\gamma_2$, $\gamma_3$, and $\gamma_4$ subunits form a subfamily exclusively localized to the central nervous system (CNS) (Klugbauer et al., 2000; Letts et al., 1998) whose expression and functional influence upon VDCCs has been investigated in chapters 4 and 5 and in several studies by other groups (Kang et al., 2001; Klugbauer et al., 2000; Letts et al., 1998; Rousset et al., 2001). The $\gamma_5$ and $\gamma_7$ subunits (Burgess et al., 1999b; Burgess et al., 2001), however, are predicted to represent another sub-family of stargazin-related proteins, with extremely low sequence identity to $\gamma_1$ and approximately 25% identity to $\gamma_2$. These putative subunits, like other members of the $\gamma$ subunit family, are predicted to be proteins with four transmembrane segments and intracellular N- and C-termini, encoded by a gene assembled from four exons. However, assembly of the full-length $\gamma_5$ and $\gamma_7$ cDNAs has not been described and there are no functional data for either of these $\gamma$ subunits.

This chapter reports the identification, cloning and functional characterisation of a novel protein that represents the complete $\gamma_7$ subunit. The first four exons of the gene encoding this protein are identical to those encoding the predicted $\gamma_7$ subunit described by Burgess et al. (2001), but the transcription and translation of a final fifth exon results in the protein having a very different and much longer C-terminus. Results show that the co-expression of the five-exon $\gamma_7$ subunit almost abolishes the functional expression and markedly suppresses the level of Cav2.2 subunit protein. Additionally, a second protein that retains high sequence similarity to $\gamma_7$ has been identified. Designated the $\gamma_5$ subunit, like its homologue, it is predicted to be encoded by a five-exon gene, the first four of which also encode the first 190 amino acids of the $\gamma_5$ subunit predicted by Burgess et al. (1999b).
6.2 Results

6.2.1 The identification and cloning of a pair of putative $\gamma$ subunits with low homology to stargazin.

Chapter 3 described the identification of 37 ESTs from different tissue sources resulting from the submission of the full-length mouse stargazin sequence (Letts et al., 1998) as a query sequence to probe the genetic databases. Amongst the resulting clusters, a short 487 bp sequence, assembled from three ESTs (from superior temporal cortex tissue, right temporal lobe tissue and a hNT2 cell line, which was derived from a human teratocarcinoma that exhibited properties characteristic of a committed neuronal precursor at an early stage of development) but no genomic sequence, represented an incomplete ORF that displayed 50% nucleotide identity and 26% amino acid identity with the central region of stargazin. It possessed no obvious start or stop codons, which indicated it was unlikely to represent full-length stargazin-related gene (Figure 6.1).

The 487 bp fragment identified by in silico cloning was amplified from human whole brain cDNA as predicted by the tissue sources of the ESTs from which it had been assembled (Figure 6.2). Sequence analysis of multiple clones from different PCR reactions confirmed the computer predictions. Gene-specific primers to the extremities of this 487bp fragment were used in 5' and 3' RACE experiments to generate two bands that contained the missing parts of a complete ORF. A 330 bp fragment contained the missing 5' sequence, possessing an in-frame start ATG codon (Figure 6.3a) and a 400 bp band contained the missing 3' sequence possessing an in-frame stop codon (Figure 6.3b). The portions of these fragments containing ORF sequence were subsequently amplified and assembled in a single "splice overlap" PCR reaction with the previously identified 487 bp sequence to obtain a full-length 828bp stargazin-like nucleotide sequence (Figure 6.4).
Chapter 6  Low homology stargazin-related proteins

Figure 6.1 - A 487bp in-silico sequence displayed 26% identity to stargazin.
A gapped protein sequence alignment of the mouse stargazin sequence with the predicted 161amino acid translation product of a 487bp nucleotide cluster generated from ESTs with homology to stargazin. Identical residues are highlighted in grey.

Figure 6.2 - The 487bp in-silico prediction of a partial stargazin-like gene was amplified from human brain total RNA by RT-PCR.
Lanes 1 and 2 contain a ~500bp band amplified using primers specific for the 487 bp sequence prediction. Lane 3 is the no cDNA template negative control reaction, and lane 4 is the positive control, amplifying the CACNG3 sequence (948 bp). Arrows to the left of the gel highlight important size markers of the 1kb DNA ladder.
Chapter 6 Low homology stargazin-related proteins

Figure 6.3 - 5' and 3' RACE on human brain cDNA generated bands containing sequence predicted to complete the ORF of a low-homology stargazin-like gene.

a) 5'RACE and b) 3' RACE generated a 330bp and ~400bp bands respectively which when sequenced contained regions with overlap with the previously cloned 487bp sequence and contained putative start and stop codons for an ORF. For both panels, lane 1 is the reaction sample and lane 2 the no cDNA template negative control.

Figure 6.4 - Splice overlap PCR generated an 828bp complete ORF

a) Diagram of template cDNAs and primers used in a single splice overlap PCR reaction to generate b) an 828bp band in lane 1 and no false positives in the negative control (lane 2).
When this full-length sequence was used to search the human high throughput genomic sequences (HTGS) using the BLASTn algorithm, a single bacterial artificial chromosome (BAC), clone AC008440, derived from human chromosome 19 was identified. Analysis of this BAC using the gene prediction program Genscan (Burge and Karlin, 1997) predicts the 828 bp ORF is assembled from five exons and encodes a 275 amino acid protein.

When compared to all of the previously published γ subunits this 828 bp cDNA clone exhibited 100% identity to the predicted human γ7 subunit over the first four exons (Burgess et al., 2001). However the sequence of the cDNA clone described here diverges from the published γ7 due to the presence of the fifth exon (Figure 6.9). This difference indicated that either I had identified a potential splice variant of the γ7 or alternatively, the complete CACNG7 gene and that the previously published gene sequence was incomplete. To date, there is no evidence for the existence of the predicted four-exon γ7 cDNA being cloned. I attempted to amplify this isoform from human brain cDNA using primers designed to specifically amplify the predicted ORF. However, none of the resulting bands (~750 bp, ~650 bp, ~200 bp) were of the correct size (603 nt) or contained the γ7 sequence. However, this does not eliminate the possibility that the γ7 exists, because the five-exon variant identified in this study was assembled from RT-PCR fragments and never amplified in a single reaction from human brain mRNA.

BLAST searches of mouse HTGS identified the mouse orthologue of the five-exon γ7 within BAC AC079557. Confirmation that this variant is expressed in mouse was obtained by amplifying the complete cDNA in a single Pfu RT-PCR reaction (containing 20% DMSO) from mouse cerebellar total RNA (Figure 6.5). The mouse orthologue possesses 70% identity to the equivalent human γ7 at the nucleic acid level and, remarkably, 100% identity at the protein level (Figure 6.6). Further analysis of BAC AC079557 failed to identify the orthologue of the predicted four-exon human γ7. An in-frame stop codon in the same or similar position to that of the human prediction could not be found in the murine genomic sequence, and hence GSPs could not be designed to specifically amplify an ORF for this species.
BLASTn searches and Genscan analysis of human chromosome 17 BAC AC005988 identified a gene encoding a second 275 amino acid protein sequence with which the human y7 cDNA clone had sequence identity of greater than 60%. This gene also possesses five exons and exhibits 70.5% amino acid identity compared to the human y7 protein sequence and 27% identity compared to y2. It exhibits 100% identity to the predicted human y5 subunit (Burgess et al., 1999b) over its first four exons, but like the y7 subunit, the additional fifth exon encodes an alternative C-terminus. Like the y7, this predicted protein either represents an alternative splice variant of the previously predicted y5 or the product of the complete CACNG5 gene. The five-exon mouse orthologue was subsequently identified on mouse chromosome 16 BAC AC079424. This in silico murine y5, exhibited 89.5% identity at the nucleotide level and 97% identity at the protein level to the human sequence. Very recent experiments performed with Dr. Yuri Bogdanov from our laboratory, have successfully amplified this isoform from mouse brain cDNA using the very sensitive touchdown PCR technique. Figure 6.7 displays an 828bp band amplified with primers specific to the five-exon mouse y5 sequence predicted from BAC AC079424. Sequencing of this product showed that it retained 90% nucleotide identity and 97% amino acid identity with the human y5 (Figure 6.8). This is the first strong evidence that the complete mRNA for y5 is transcribed in any tissue. However, whether it is translated in mouse brain or any other tissues remains undetermined. It would therefore appear that I have identified both the complete human CACNG5 and mouse cacng5 genes that encode the y5 subunit, and if transcribed, the previously predicted four-exon human y5 possibly represents an alternative splice from of the five-exon y5 subunit.
Figure 6.6 – Nucleotide alignment of five-exon human and mouse γ7 nucleotide sequences. The mouse γ7 nucleotide sequence possesses 70% identity to its human orthologue with all differences being silent resulting in identical human and mouse γ7 protein sequences. Identical nucleotides are highlighted in grey.
Figure 6.7 - 828bp mouse γ5 band can be amplified in a single PCR reaction from mouse brain cDNA.

Samples of PCR reactions containing: lane 1, mouse γ5 gene specific primers; lane 2, no cDNA negative control of lane 1. An 828bp band is specifically amplified from whole mouse brain cDNA.

Figure 6.8 - Amino acid alignment of human and mouse γ5 sequences.

The in silico nucleotide sequence of the mouse γ5 was used to design primers to amplify the complete mouse γ5 ORF from brain cDNA. Mouse γ5 subunit exhibits 90% nucleotide and 97% amino acid identity to the human γ5. Identical residues are highlighted in grey.

Hydropathy plots predict that, like all the stargazin related proteins, γ7 and γ5 have four transmembrane spanning helices with intracellular N- and C-termini (Figure 6.10). With helix prediction parameters set between 17 and 33 amino acids, the program TMpred (Hofmann and Stoffel, 1993) also predicted the final transmembrane spanning segments of γ7 and γ5 to have 28 and 25 amino acids respectively, 6 and 8 amino acids longer than their equivalents in the four-exon γ7 or γ5 predictions. Additionally, the γ7 protein identified in this study has a much more substantial cytosolic C-terminus than the published γ7 in which only four intracellular amino acids are predicted after the final transmembrane segment.

The sequences encoding the entire human γ5 and γ7 and mouse γ5 and γ7 subunits have been deposited in the Genbank/EMBL/DDBJ databases under the accession numbers AF458898, AF458897, AF458900 and AF458899 respectively.
Figure 6.9 - Protein sequences of a family of low homology stargazin related genes.

Alignment of the five exon $\gamma_5$ and $\gamma_7$ subunits with the previously identified four exon $\gamma_5$ and $\gamma_7$ subunits using the Clustal W algorithm (Thompson et al., 1994). Dotted lines indicate consensus N-glycosylation sites and solid triangles (▲) beneath residues mark consensus sites for phosphorylation by cAMP- and cGMP-dependent protein kinase, protein kinase C, casein kinase II, or tyrosine kinase. The exon/intron boundaries are marked by solid dots (•) above the residue whose codon is interrupted by the adjacent intron. Note that the sequence identity between five exon $\gamma_5$ and $\gamma_7$ is 80% conserved within the additional fifth exon, whereas the four-exon $\gamma_5$ and $\gamma_7$ subunits differ greatly in sequence identities and length of the C-terminus. The two large open triangles (▲) designate a pair of cysteine residues that are conserved amongst the putative VDCC $\gamma$ subunits and may be involved in the formation of a disulfide bridge. The transmembrane helices, as predicted by the TMpred program (Hofmann and Stoffel, 1993), are indicated by solid underlining. The residues highlighted in bold in the C-terminal of five-exon $\gamma_7$ identify the epitope for the anti-$\gamma_7$ antibody.
Chapter 6  
Low homology stargazin-related proteins

Figure 6.10 – Hydropathy plots comparing five-exon human $\gamma_7$ and $\gamma_5$ to the previously published predicted four-exon human $\gamma_7$ and $\gamma_5$ amino acid sequences.

Hydropathy plots of the four- and five-exon $\gamma_7$ and $\gamma_5$ subunits predicted by the TMpred program (Hofmann and Stoffel, 1993) with the length of the hydrophobic part of the transmembrane helix restricted between 17 and 33 amino acids. Amino acid position is shown on the x-axis, and positive TMpred scores indicate putative membrane spanning regions. All are predicted to have four transmembrane spanning helices with intracellular N- and C-terminals but the length of the final transmembrane segment varies between four- and five-exon versions of each protein. These TM predictions also strongly favour the N- and C-terminals to be located on the intracellular side of the plasma membrane.
6.2.2 Tissue distribution

6.2.2.1 γ7 mRNA distribution

The tissue distribution of the novel γ7 mRNA was analysed by northern blot. A human multiple-tissue northern blot (Figure 6.11a) and two brain region blots (Figure 6.11b & c) hybridised with a probe corresponding to nucleotides 576-763 of the γ7 ORF. This region was chosen because it contains the least identity when compared to other γ subunits (53% to human γ2), and is unique to γ7. Thus, this probe will not detect the predicted four-exon γ7 should this isoform be expressed. This γ7-specific probe reveals two transcripts of approximately 2.4 kilobases (kb) and 3.0 kb both of which are expressed only in brain. Both of these transcripts are expressed in all brain regions probed, although the shorter transcript is expressed at greater levels in several areas including cerebellum, amygdala, hippocampus and thalamus (Figure 6.11). These blots were stripped and re-probed using a probe designed by Burgess et. al. (Burgess et al., 2001) for RT-PCR expression analysis to detect four-exon γ7. This probe, corresponding to nucleotides 80-482 of the γ7 ORF would detect both the predicted four-exon γ7 and full-length γ7 transcripts. No additional transcripts were seen on any of the blots with this probe and it gave precisely the same expression profile as the full-length γ7 specific probe, even the same differential expression of the short and long transcripts seen in cerebellum, amygdala, hippocampus and thalamus (Figure 6.11).

6.2.2.2 γ5 mRNA distribution

At the time this experiment was performed, no ESTs that possessed any γ5 sequence were present in the genetic databases. To determine a potential source of RNA from which I could attempt to amplify the human gene by RT-PCR, a 49mer oligonucleotide probe complementary to nucleotides 349-397 of the in silico γ5 sequence was labelled with [γ-32P] dATP and used to probe a human multiple tissue northern blot at room temperature and low salt concentration. A message of approximately 7.0kb was seen to be strong in skeletal muscle and placenta and somewhat weaker in heart (Figure 6.12). Other smaller messages were also labelled, the most prevalent of which was a present in
all tissues probed and was ~1.9 kb in size. In some tissues it appeared as a doublet, with a smaller message at ~1.7 kb, which in skeletal muscle gave a significantly stronger signal. Because of the low temperatures used for hybridisation and the small size of the probe, which of these signals is genuine is hard to determine. Nevertheless, whichever signal should prove to be genuine, it would appear that γ5 is the first example of a stargazin homologue that can be expressed in non-neuronal tissue. Although the blot in Figure 6.12 suggests only extremely weak expression of γ5 in brain, if at all, a γ5 cDNA has recently been amplified from mouse brain (see above). Nevertheless, this was achieved using a highly sensitive PCR technique and does not necessarily follow that the protein is translated in this tissue. Future experiments will utilise this clone to generate a much more specific cDNA probe generic to both human and mouse sequences that can be used to identify the true expression profile of the γ5 mRNA in both species.
Figure 6.11 – Northern blot analysis of the tissue distribution of the γ7 subunit.

(a) Multiple tissue northern blots probed specifically for the full-length, five-exon γ7 subunit show two mRNA species of approximately 2.4kb and 3.0kb that are specifically localized to human brain. Multiple brain region blots (b and c) show the γ7 subunit is however expressed in all the individual brain regions probed. A second probe that detected both predicted four-exon γ7, if expressed, and cloned five-exon γ7 subunits produced an identical expression profile to the five-exon γ7 specific probe. The bottom section of each panel displays the mRNA detected by the control β-actin probe for each blot.
Figure 6.12 – Northern blot analysis of the mRNA distribution of the human $\gamma_6$ subunit.

A human multiple tissue northern blot probed with a 49mer oligonucleotide probe complementary to nt 349-397 of the human $\gamma_6$ in silico prediction, hybridised at low temperature and washed at low salt concentration. Signals ~7.0kb in size occur in heart, skeletal muscle and placenta and smaller ~1.9 kb and ~1.7 kb signals occur in all tissues probed with the 1.7 kb signal in skeletal muscle being particularly strong. These results suggest the $\gamma_6$ may be the first example of a gene identified by its homology to stargazin that is expressed in non-neuronal tissues.
6.2.3 Influence of the γ7 subunit on heterologous expression of VDCCs

6.2.3.1 Electrophysiology

Having investigated the tissue distribution of the γ7 subunit in human brain, I next investigated the effect of expression of this protein on Ba\(^{2+}\) currents recorded from neuronal VDCCs expressed in *Xenopus* oocytes or COS-7 cells. Figure 6.13a shows recordings made in 5mM Ba\(^{2+}\) from *Xenopus* oocytes expressing Cav2.2 either with or without co-expression of γ7. Co-expression of an α2δ subunit was omitted in these experiments to avoid recording any potential α2δ dependent influence that may possibly occur, as had been reported by another laboratory for the γ2 subunit (Kang et al., 2001). The maximum conductance (Gmax), determined from fitting a modified Boltzmann function (see methods) to the current-voltage (I-V) plots, was dramatically and significantly reduced when the human γ7 subunit was co-expressed compared to oocytes where it was not (1.5 ± 0.3 μS (n = 24, p < 0.001) versus 12.7 ± 2.3 μS (n = 26)), with a corresponding 92.6% reduction in peak current amplitude at 0mV from -0.5 ± 0.1 μA to -0.037 ± 0.006 μA (p <0.001, Table 6.1). The half-maximal value for the voltage dependence of activation (V0.5activ) was also shifted from -7.3 ± 0.8 mV in Cav2.2 (n = 26) to -3.9 ± 0.9 mV upon co-expression of γ7 (n = 24, p < 0.01). To examine whether the residual current represents the endogenous current in *Xenopus* oocytes (Lacerda et al., 1994), a comparison between currents recorded from oocytes transfected with only the auxiliary β1b and those additionally expressing γ7 was made. However we observed that these were also reduced from -28.1 ± 9.6 nA (n=10) to -8.3 ± 5.3 nA (n=9) upon co-expression of γ7. We next investigated the effects of γ7 on other Cav-α1 subtypes. Figure 6.13b and Figure 6.13c show representative traces and I-V plots which indicate that the γ7 subunit significantly reduced the Gmax of Cav2.1 and Cav1.2 VDCCs. However, the effect of γ7 on these channels is not as striking as that seen with Cav2.2 + γ7. The Gmax in cells expressing Cav2.1 was 5.8 ± 1.0 μS (n = 17), and was reduced to 3.0 ± 0.5 μS in the additional presence of γ7 (n = 19, p < 0.01, Table 6.1). Cav1.2 currents showed a Gmax of 5.9 ± 1.4 μS (n = 10) whereas in the additional presence of γ7 it was −2.8 ± 0.6 μS (n = 14, p < 0.05, Table 6.1).
Figure 6.13 - Peak current amplitude of neuronal voltage dependent calcium channels is reduced upon co-expression of the \( \gamma_7 \) subunit.

Peak current-voltage relationships and individual representative traces for (a) \( \text{Ca}_v^{2.2} \) (\( \bullet \), \( n=26 \)), \( \text{Ca}_v^{2.2+\gamma_7} \) (\( \bigcirc \), \( n=24 \)), \( \text{Ca}_v^{2.1} \) (\( \square \), \( n=17 \)), (b) \( \text{Ca}_v^{2.1+\gamma_7} \) (\( \bigcirc \), \( n=19 \)), (c) \( \text{Ca}_v^{1.2} \) (\( \Delta \), \( n=10 \)) and \( \text{Ca}_v^{1.2+\gamma_7} \) (\( \Delta \), \( n=15 \)) expressed in Xenopus oocytes with the \( \beta_{1b} \) auxiliary subunit were determined by measuring peak \( \text{Ba}^{2+} \) current amplitudes recorded during 100ms test pulses between \(-70\) and \(+40\) mV (holding potential \(-100\) mV; \(+10\) mV increments; \([\text{Ba}^{2+}]\) for \( \text{Ca}_v^{2.2} \) recordings \(5\) mM; \([\text{Ba}^{2+}]\) for \( \text{Ca}_v^{2.1} \) and \( \text{Ca}_v^{1.2} \) recordings \(10\) mM).
Chapter 6  
Low homology stargazin-related proteins

| Channel | [Ba^{2+}] (mM) | Activation | | | | n |
|---------|----------------|------------|-----------------|---------------|------|
| Cav2.2  | 5              | -7.34 ± 0.79 | 4.34 ± 0.24 | 12.7 ± 2.29 | -0.50 ± 0.10 | 26 |
| Cav2.2 + γ7 | 5          | -3.88 ± 0.92** | 4.66 ± 0.40 | 1.50 ± 0.27*** | -0.04 ± 0.01*** | 24 |
| Cav2.1  | 10             | -0.63 ± 0.73  | 5.03 ± 0.24  | 5.76 ± 0.95  | -0.17 ± 0.02  | 17 |
| Cav2.1 + γ7 | 10        | 0.92 ± 0.68   | 4.71 ± 0.41  | 2.98 ± 0.46** | -0.09 ± 0.02** | 19 |
| Cav1.2  | 10             | -0.68 ± 2.08  | 6.80 ± 0.31  | 5.88 ± 1.40  | -0.17 ± 0.04  | 10 |
| Cav1.2 + γ7 | 10        | 1.75 ± 0.70   | 5.68 ± 0.59  | 2.84 ± 0.57* | -0.10 ± 0.02  | 14 |

Table 6.1- Influence of the γ subunit on the activation properties of VDCC expressed in *Xenopus* oocytes determined from the I-V plots. Data are expressed as mean ± S.E.M of the number of replicates, n. * marks significance values P < 0.05, ** marks P < 0.01, and *** P < 0.001 according to an un-paired student’s t-test. All transfections contained the β subunit.

In an additional set of experiments the I investigated whether the reduction in Cav2.2 current amplitude on co-expression γ subunit was influence by the addition of an α2δ2 subunit to the VDCC complex. These recordings were performed in 10mM Ba^{2+} in an attempt to improve the resolution of currents recorded in the presence of γ. However, even in the presence of an α2δ2 subunit, Gmax was dramatically and significantly reduced in oocytes expressing Cav2.2/β1β/α2δ2/γ compared to control oocytes expressing Cav2.2/β1β/α2δ2 (4.9 ± 2.1 μS (n = 5, p < 0.05) versus 41.5 ± 8.6 μS (n = 11)), with a corresponding 95.2 % reduction in peak current amplitude at 0mV from -1.9 ± 0.5 μA to -0.08 ± 0.03 μA (p <0.05) (Figure 6.14). The half-maximal value for the voltage dependence of activation (V_{0.5activ}) was also shifted from -6.5 ± 1.6 mV in oocytes expressing Cav2.2/β1β/α2δ2 (n = 11) to -0.2 ± 2.7 mV upon co-expression of γ (n = 5, p = 0.05). This depolarising shift was similar in size to the shift observed in recordings made in the absence of an α2δ subunit. The value of the slope factor (kactiv) of the activation curve was also increased from 3.9 ± 0.5 in Cav2.2/β1β/α2δ2 expressing oocytes (n = 11), to 5.7 ± 0.4 (p < 0.05) in the additional presence of the γ subunit.
Figure 6.14 - Peak current amplitude of Cav2.2/β1b/α2δ2 voltage dependent calcium channels is reduced upon co-expression of the γ7 subunit.

a) Representative traces of Cav2.2/β1b/α2δ2 and Cav2.2/β1b/α2δ2/γ7 currents during a 100ms depolarisation to 0mV from a holding potential of −100mV, recorded in 10mM Ba2+. b) The current-voltage relationship for oocytes expressing Cav2.2/β1b/α2δ2 (●) or Cav2.2/β1b/α2δ2/γ7 (○) determined by measuring peak Ba2+ current amplitudes recorded during 100ms test pulses between −70 and +40 mV (holding potential −100mV; +10mV increments).

The influence of γ7 co-expression on voltage and time-dependent inactivation properties of each channel combination was also investigated. The additional presence of the γ7 subunit did not significantly alter the V50inact for any channel combination investigated and neither did it alter k_inact for Cav2.1 or Cav1.2 co-expressed with β1b (Figure 6.15, Table 6.2). The only significant difference was in the value of k_inact for Cav2.2 that increased almost 2-fold when co-expressed with γ7 as shown by the shallow dependence of the steady-state inactivation curve on voltage in the presence of γ7 (P < 0.01; Figure 6.15, Table 6.2). However, this result should be viewed with some caution. As the representative traces in Figure 6.15a right hand panels display, currents recorded from Cav2.2 + γ7 expressing oocytes were extremely small, therefore when analysing the data the signal to noise ratio was much greater than in control cells and near the limit of sensitivity for the TEVC set-up. Furthermore, the peak current amplitude of Cav2.2 + γ7 expressing oocytes is very near that of the endogenous current of oocytes that is enhanced by the expression of only the β1b subunit without a heterologous Cav-α1 subunit. However, as stated earlier, this enhanced endogenous current is itself reduced upon co-expression of the γ7 subunit with the β1b subunit.
Figure 6.15 – Properties of the γ7 subunit upon the steady state inactivation properties of VDCCs expressed in *Xenopus* oocytes.

Averaged steady-state inactivation curves for a) Cav2.2, b) Cav2.1 and c) Cav1.2 co-expressed in *Xenopus* oocytes with the β1b subunit ± the γ7 subunit. Peak current amplitudes were normalised to the peak reached during each step of the protocol (see methods). Data expressed as mean ± S.E.M. The right hand panels of part (a) display representative recordings from oocytes expressing Cav2.2 in the absence and presence of the γ7 subunit. The numerical data for parameters defining the steady-state inactivation relationship are displayed in Table 6.2.

<table>
<thead>
<tr>
<th>Channel</th>
<th>Inactivation</th>
<th>n</th>
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<tbody>
<tr>
<td></td>
<td>V_{50inact} (mV)</td>
<td>k_{inact}</td>
</tr>
<tr>
<td>Cav2.2</td>
<td>-55.28 ± 0.96</td>
<td>8.22 ± 0.29</td>
</tr>
<tr>
<td>Cav2.2 + γ7</td>
<td>-52.51 ± 1.56</td>
<td>15.96 ± 2.90**</td>
</tr>
<tr>
<td>Cav2.1</td>
<td>-29.22 ± 1.30</td>
<td>8.66 ± 0.92</td>
</tr>
<tr>
<td>Cav2.1 + γ7</td>
<td>-23.49 ± 4.58</td>
<td>9.97 ± 1.48</td>
</tr>
<tr>
<td>Cav1.2</td>
<td>-14.76 ± 3.66</td>
<td>14.49 ± 1.17</td>
</tr>
<tr>
<td>Cav1.2 + γ7</td>
<td>-23.51 ± 3.82</td>
<td>11.78 ± 2.65</td>
</tr>
</tbody>
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Table 6.2 – Average steady-state inactivation data from recordings displayed in Figure 6.15.

Steady state inactivation plots from Figure 6.15 were fitted with a single Boltzmann equation. V_{50inact} is the half maximal voltage for current inactivation, and k_{inact} is the slope factor. Values are expressed and mean ± S.E.M. of the number of replicates, n. ** marks P < 0.01.
In the presence of the $\gamma_7$ subunit, the very small $\text{Cav}_2.2 \ 	ext{Ba}^{2+}$ currents also inactivated rapidly due to a significantly reduced $\tau_{\text{fast}}$ (Figure 6.16a, Table 6.3). The portion of the current inactivating with the slow time constant was also significantly increased (Table 6.3). This occurred because the fraction of total current that did not inactivate in the $\text{Cav}_2.2$ expressing oocytes was reduced to almost zero in the presence of the $\gamma_7$ subunit. However, as for the steady-state inactivation data for these subunit combinations, the importance of these results is questionable due to the small size of $\text{Cav}_2.2 + \gamma_7$ currents. The inactivation kinetics of oocytes expressing $\text{Cav}_2.1$ or $\text{Cav}_1.2$ VDCCs were not significantly altered by the co-expression of the $\gamma_7$ subunit (Figure 6.16b & c, Table 6.3).

Further experiments performed in collaboration with P. Viard, a postdoctoral member of our laboratory, investigated the possibility that the striking reduction in $\text{Cav}_2.2$ current when co-expressed with the $\gamma_7$ subunit was peculiar to the *Xenopus* oocyte expression system (Chapter 6A). For comparison with immunocytochemistry data (section 6.2.4), an N-terminal green fluorescent protein tagged $\text{Cav}_2.2$ construct (GFP-$\text{Cav}_2.2$) previously shown to have no significant differences in its biophysical properties compared to the non-tagged channel (Raghib *et al.*, 2001b) was transfected. These experiments confirmed the almost total abolition of whole-cell $\text{Ba}^{2+}$ current in mammalian COS-7 cells transfected with GFP-$\text{Cav}_2.2/\beta_{1b}/\gamma_7$, compared to GFP-$\text{Cav}_2.2/\beta_{1b}$ controls. Furthermore, unlike in a recent publication that reported the partial inhibitory influence of the $\gamma_2$ subunit upon $\text{Cav}_2.2$ currents to be dependent upon the co-expression of the $\alpha_2\delta$ subunit as part of the VDCC complex (Kang *et al.*, 2001), and as demonstrated above in *Xenopus* oocytes (Figure 6.14), it was demonstrated that the influence of the $\gamma_7$ subunit upon $\text{Cav}_2.2/\beta_{1b}$ currents was independent of co-expression of an $\alpha_2\delta$ subunit (Chapter 6A). Together with the data generated in *Xenopus* oocytes, the near complete abolition of $\text{Cav}_2.2$ current seen in COS-7 cells upon co-expression of the $\gamma_7$ subunit, suggests it may be principally affecting the expression of these channels with only a minor influence on their biophysical properties.
The influence of the $\gamma_7$ subunit on the inactivation of a) Cav2.2, b) Cav2.1 and c) Cav1.2 currents; Currents shown are the mean time course from each group. Individual measurements were fitted with the sum of two exponentials (white line). $\beta_{1b}$ subunits were present in all transfections, and recordings were made in 5mM Ba$^{2+}$. Results of these procedures are displayed in Table 6.3.

### Table 6.3 - Modulation of inactivation kinetics by the $\gamma_7$ subunit

Data expressed as mean ± S.E.M of the number of replicates, n. ** and *** mark significant difference from the control P < 0.01 and P < 0.001 respectively. $\beta_{1b}$ subunit was present in all transfections.

<table>
<thead>
<tr>
<th>Channel</th>
<th>Inactivation</th>
<th>$\tau_{\text{fast}}$ (ms)</th>
<th>$\tau_{\text{slow}}$ (s)</th>
<th>$A_{\text{fast}}$ %</th>
<th>$A_{\text{slow}}$ %</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cav2.2</td>
<td></td>
<td>348.7 ± 32.7</td>
<td>2.047 ± 0.214</td>
<td>56.13 ± 1.65</td>
<td>41.24 ± 1.93</td>
<td>27</td>
</tr>
<tr>
<td>Cav2.2 + $\gamma_7$</td>
<td></td>
<td>190.4 ± 12.2***</td>
<td>1.682 ± 0.119</td>
<td>52.54 ± 2.09</td>
<td>49.68 ± 2.13**</td>
<td>17</td>
</tr>
<tr>
<td>Cav2.1</td>
<td></td>
<td>163.6 ± 7.8</td>
<td>0.952 ± 0.103</td>
<td>55.80 ± 3.27</td>
<td>44.20 ± 3.27</td>
<td>15</td>
</tr>
<tr>
<td>Cav2.1 + $\gamma_7$</td>
<td></td>
<td>166.5 ± 9.2</td>
<td>1.149 ± 0.118</td>
<td>62.11 ± 2.34</td>
<td>37.89 ± 2.33</td>
<td>15</td>
</tr>
<tr>
<td>Cav1.2</td>
<td></td>
<td>218.6 ± 26.7</td>
<td>2.280 ± 0.696</td>
<td>45.10 ± 5.09</td>
<td>38.60 ± 7.83</td>
<td>7</td>
</tr>
<tr>
<td>Cav1.2 + $\gamma_7$</td>
<td></td>
<td>154.3 ± 15.5</td>
<td>1.305 ± 0.155</td>
<td>56.75 ± 6.11</td>
<td>32.60 ± 3.37</td>
<td>7</td>
</tr>
</tbody>
</table>
6.2.4 Immunocytochemical analysis of the effects of $\gamma_7$

The sub-cellular distribution of the expressed $\gamma_7$ subunit and its effects upon the expression of Cav2.2 VDCCs were determined using immunocytochemistry and confocal laser scanning microscopy. When the $\gamma_7$ subunit alone was transiently transfected into COS-7 cells, expression of $\gamma_7$ protein was detected using a specific $\gamma_7$ Ab raised against an internal C-terminal epitope (Table 2.17, Chapter2). $\gamma_7$ expression was observed throughout the cytoplasm with a non-uniform distribution, possibly associated with intracellular organelles and also in the region of the plasma membrane in some cells (delineated by co-localisation with Oregon Green-phalloidin - Figure 6.17). However, non-permeabilised cells transfected with the $\gamma_7$ subunit and incubated with a specific antibody raised against an epitope in the first “extracellular” loop of the $\gamma_7$ subunit ($\gamma_{7\text{EXT}}$ Ab, Table 2.17, Chapter2) mainly positively stained very small, unhealthy looking cells, which were probably dead or dying at the time of fixation, and therefore had compromised plasma membranes (Figure 6.18). Staining of permeabilised $\gamma_7$ transfected COS-7 cells with the same $\gamma_{7\text{EXT}}$ Ab produced staining pattern the same as observed with the $\gamma_7$ Ab in large healthy cells, indicating when expressed alone, the $\gamma_7$ subunit was primarily located intracellularly with little insertion into the plasma membrane of healthy cells.

![Figure 6.17 - Sub-cellular distribution of the $\gamma_7$ subunit transiently expressed in COS-7 cells](image)

a) COS-7 cells were transfected with the $\gamma_7$ subunit alone and stained with (from left to right) Oregon Green phallolidin (Phal) to delineate the plasma membrane, Texas Red labelled anti-$\gamma_7$ subunit antibody ($\gamma_7$ Ab), DAPI nuclear stain, and in the final panel all images were merged. b) Magnification of region marked by square in merged image of panel (a). White arrows in $\gamma_7$ Ab panel highlight $\gamma_7$ subunit staining concentrated in the region of the plasma membrane. Scale bars in all panels represent 10$\mu$m.
Figure 6.18 – Detection of the subcellular distribution of the γ7 subunit using an antibody directed to the loop linking transmembrane segments 1 and 2

COS-7 cells were transfected with the γ7 subunit alone. Some cells were left non-permeabilised and others were permeabilised. When incubated with the γ7EXT Ab, non-permeabilised cells that stained positively for γ7 were all extremely small and looked unhealthy (non-perm, a). Some larger, healthier cells showed some patchy staining the membrane (non-perm, b), but most remained unstained. Untransfected cells (non-perm, c) showed no positive immunoreactivity for γ7. However, permeabilised cells stained positively throughout the cell when incubated with the γ7EXT Ab (perm, a & b). Untransfected permeabilised cells displayed no positive immunoreactivity (perm, c). Scale bars in all panels represent 10 μm.

The events underlying the almost complete abolition of Cav2.2 current when co-expressed with the γ7 subunit were investigated in the following experiments. Figure 6.19 shows the typical fluorescence of cells transiently transfected with GFP-Cav2.2/β1b and shows that the anti-γ7 subunit antibody does not cross-react with any of the transfected VDCC subunits. Figure 6.20 shows cells where the γ7 subunit has also been co-transfected. A striking reduction in the fluorescence of GFP-Cav2.2 was observed upon co-expression of the γ7 subunit, whilst the γ7 levels and subcellular distribution remained comparable to those in cells transfected with the γ7 subunit alone.

In transient transfections, co-transfected subunits are not always co-expressed in every cell. I therefore sought to quantify the extent of the reduction in observed GFP-Cav2.2 fluorescence caused by γ7 subunit co-expression. This was measured by counting the number of GFP-positive cells in both the GFP-Cav2.2/β1b and GFP-Cav2.2/β1b/γ7 transfected cultures. The mean percentage of the total cells per 16 mm² field of view (FOV) with GFP-Cav2.2 fluorescence was reduced from 16.4 ± 3.4 % (n = 12 FOV from 3 transfections) in GFP-Cav2.2/β1b transfected cells to 3.3 ± 0.7 % (n = 11 FOV from 3 transfections, P < 0.01) in cells additionally transfected with the γ7 subunit (Figure 6.21). The same histogram also shows that almost all cells that exhibited GFP-Cav2.2 fluorescence in the Cav2.2/β1b/γ7 transfections, were also labelled for γ7 (3.0 ± 0.7 %),
implying that there is some residual GFP-Cav2.2 fluorescence in some cells expressing
the γ7 subunit as seen in Figure 6.21c. It was also observed that the percentage of cells
expressing γ7 in the Cav2.2/β1b/γ7 transfections (3.0 ± 0.7%) was much lower than the
percentage of cells expressing another auxiliary subunit, β1b, in the GFP-Cav2.2/β1b
transfections (16.4 ± 3.4%), although Cav2.2 is also entirely co-localized with β1b
(Figure 6.21b).
Figure 6.19 – Expression of N-terminal GFP tagged Ca\textsubscript{v}2.2 subunit in COS-7 cells

Rows a-e show five example COS-7 cells transiently transfected with a GFP-Ca\textsubscript{v}2.2 cDNA. Columns from left to right display GFP-Ca\textsubscript{v}2.2 fluorescence, Texas Red labelled anti-\gamma\textsubscript{7} antibody staining, DAPI nuclear staining, and merged images of each channel. Scale bars in every panel represent 10\micron. All cells were co-transfected with \beta_{1b}. 

270
Figure 6.20 – Co-expression of the γ7 subunit almost abolishes GFP-Ca\textsubscript{v}2.2 fluorescence when expressed transiently in COS-7 cells.

Rows a-e show five examples of COS-7 cells transiently transfected with a GFP-Ca\textsubscript{v}2.2 + γ7 cDNA. Columns from left to right display GFP-Ca\textsubscript{v}2.2 fluorescence, Texas red-labelled anti-γ7 antibody staining, DAPI nuclear staining, and merged images of each channel. Scale bars in every panel represent 10μm. All cells were co-transfected with β1b.
Figure 6.21 – The effect of co-expression of $\gamma_7$ upon the expression of GFP-Cav2.2 in COS-7 cells.

(a) Histogram of mean percentage of total cells per field of view (± S.E.M.) expressing GFP-Cav2.2 or Texas red labelled $\beta_{1b}$ or $\gamma_7$, with the subunit combinations stated. * marks $P < 0.01$ according to an unpaired students t-test. (b) Representative image of GFP-Cav2.2/$\beta_{1b}$ transfected cells labelled with Texas red labelled anti-$\beta_{1b}$ antibody. (c) GFP-Cav2.2/$\beta_{1b}/\gamma_7$ transfected cells labelled with Texas red labelled anti-$\gamma_7$ antibody. Staining is labelled in panel and scale bars represent 1mm.
The effect of the $\gamma_i$ could be due to reduced synthesis, accelerated degradation or prevention of the correct folding of the complete GFP-$\operatorname{Cav}_{2.2}$ protein or just the GFP tag. However, western blot experiments performed in collaboration with Dr A. Davies from our laboratory showed that when the $\gamma_i$ subunit was co-expressed with either the GFP-$\operatorname{Cav}_{2.2}$ or untagged $\operatorname{Cav}_{2.2}$ the intensity of the $\operatorname{Cav}_{2.2}$ band was greatly reduced, but expression of the protein was not completely abolished (Chapter 6A). These results were obtained both in the absence and presence of auxiliary $\beta_{1b}$ subunit. These data extended the findings from confocal imaging and electrophysiology experiments by revealing that the $\gamma_i$ subunit suppresses the expression of GFP-$\operatorname{Cav}_{2.2}$ protein rather than interfering with the correct conformational folding or trafficking of the GFP-labelled $\operatorname{Cav}_{2.2}$ or untagged $\operatorname{Cav}_{2.2}$ that could result in reduced fluorescence or recorded current densities.

The almost complete abolition of $\operatorname{Cav}_{2.2}$ current in *Xenopus* oocytes and COS-7 cells (6.2.3.1-Electrophysiology and Chapter 6A respectively) and GFP-$\operatorname{Cav}_{2.2}$ fluorescence in COS-7 cells resulting from co-expression of the $\gamma_i$ subunit, meant that in subsequent experiments the possibility that the $\gamma_i$ subunit was down-regulating the expression of all transiently expressed proteins was investigated.

The first of these studies investigated if the $\operatorname{Cav}_{2.2}$ subunit was the only part of the calcium channel complex whose expression was adversely affected by co-expression of the $\gamma_i$ subunit. The $\operatorname{Cav}_{\beta}$ subunit plays a major role in facilitating the incorporation of $\operatorname{Cav}_{\alpha_i}$ subunits into the plasma membrane and as a result enhances expressed VDCC currents compared to currents from $\alpha_i$ subunit alone (Berrow *et al.*, 1997; Brice *et al.*, 1997; Brice and Dolphin, 1999; Walker and De Waard, 1998). Reduced expression, more rapid degradation or incorrect folding of the $\beta_{1b}$ subunit on co-expression of the $\gamma_i$ could all contribute to the reduced $\operatorname{Cav}_{2.2}$ currents reported above. COS-7 cells were transfected with a $\operatorname{Cav}_{2.2}/\beta_{1b}$-GFP complex with, or without the $\gamma_i$ subunit. The typical fluorescence of the GFP-tagged $\beta_{1b}$ subunit is displayed in Figure 6.22 a-c. The GFP-$\beta_{1b}$ fluorescence appeared to be reduced, but in most cases not abolished on co-expression of the $\gamma_i$ subunit (Figure 6.22 d-f). However, Dr A Davies from our laboratory has subsequently performed western blot analysis of COS-7 cells expressing
the GFP-Cav2.2/β1b complexes with the γ7 subunit and shown that the levels of β1b subunit expressed in such transfections is only slightly, reduced compared to the controls not expressing γ7 (Chapter 6A).

Next, the possibility that the γ7 subunit could alter the expression of the putative VDCC γ2 subunit was examined. As shown in previous chapters, the γ2 subunit is localised to the plasma membrane when expressed alone in COS-7 cells. Figure 6.23a shows that this expression is not altered when the γ2 subunit is tagged with C-terminal myc/his motif. When co-expressed in equal proportions with the γ7 subunit, the expression of the γ2-myc/his is not reduced (Figure 6.23b-e). However its sub-cellular distribution is markedly changed, with little plasma membrane localisation, and the majority of the γ2 staining occurring within the cytoplasmic regions. Expression of the γ7 subunit remained typical of all previously examined cells expressing the subunit. This result suggests that whilst the γ7 subunit is not reducing the amount of γ2 subunit expressed, it is retaining it within organelles within the cytoplasm.
Figure 6.22 - $\text{Ca}_{\text{v}}{\gamma_7}$-GFP fluorescence is reduced in the majority of cells co-expressing $\gamma_7$.

COS-7 cells transiently expressing a-c) $\text{Ca}_{\text{v}}2.2/\beta_{1\text{b}}$-GFP and d-e) $\text{Ca}_{\text{v}}2.2/\beta_{1\text{b}}$-GFP/$\gamma_7$. Columns from left to right display $\beta_{1\text{b}}$-GFP fluorescence, Texas Red labelled anti-$\gamma_7$ antibody staining, DAPI nuclear staining, and merged images of each channel. Scale bars in every panel represent 10 μm.
Figure 6.23 - The γ_7 subunit does not reduce staining for γ_2-myc/his but alters its subcellular distribution

UT) Untransfected COS-7 cells, a) γ_2-myc/his transfected cells, and b-e) cells transfected with both the γ_2-myc/his subunit and the γ_7 subunit labelled in columns 1-4 from left to right show γ_2-myc/his labelled with anti-myc antibody and FITC, Texas Red staining for the γ_7 subunit, DAPI nuclear staining and the merged image of all channels. The scale bar in all panels represents 10 μm.
Chapter 6

Low homology stargazin-related proteins

The effect of γ upon the Ba\(^{2+}\) currents through Cav2.2 VDCCs expressed in Xenopus oocytes and its influence on the fluorescence of GFP-Cav2.2 expressed in COS-7 cells was so striking, we investigated the possibility that it down-regulates other heterologously expressed ion channels by co-expressing γ with the Shaw-like voltage-dependent potassium channel, Kv3.1b. Figure 6.24 a-c displays the typical expression of a γ-myc/his fusion protein expressed alone in a COS-7 cell. The expression of this construct is unaltered compared to that of the untagged γ (Figure 6.17). Figure 6.24 d-f shows the expression of Kv3.1b when transfected alone. The distribution and the expression level of Kv3.1b were unaltered by the co-expression of the γ-myc/his fusion protein (Figure 6.25). These findings have been extended by recording the currents from Xenopus oocytes co-expressing Kv3.1b and γ. These experiments showed that the γ subunit had no effect on the peak current amplitude of heterologously expressed Kv3.1b channels (Chapter 6A) and confirms that the γ subunit does not adversely affect the expression of the Kv3.1b subunit in either amphibian or mammalian expression systems.

In all the previous experiments, the Cav2.2, β\(_{1b}\), and γ subunits were transfected in a 1:1:1 ratio by cDNA weight. In this ratio, approximately twice as many copies of expression vector containing the γ ORF (pMT2-γ ~6200bp) would be included in the transfection mix compared to pMT2-GFP-Cav2.2 (~12000bp). It was therefore a possibility that the γ subunit could be affecting the expression of GFP-Cav2.2 by saturating the transcription and/or translation machinery of the cells in which it was expressed, which could result in the reduced the expression of the larger Cav-α\(_{1}\) subunit. GFP-Cav2.2/β\(_{1b}\) complexes cDNAs were mixed in a 10:10:10 ratio and transfected.
Figure 6.24: Subcellular distribution of the $\gamma_{7}$-myc/his and $K_{v}3.1b$ subunits transiently expressed alone in COS-7 cells

Cells were transfected with a-c) $\gamma_{7}$-myc/his alone or d-f) $K_{v}3.1b$ subunit alone. Columns 1-4 from left to right show $\gamma_{7}$-myc/his labelled with anti-myc antibody and FITC, Texas Red staining for the $K_{v}3.1b$ subunit; DAPI nuclear staining and the merged image of all channels. The scale bar in all panels represents 20 µm.
Figure 6.25 – Staining of the Kv3.1b subunit is not reduced by the co-expression of the γ-myc/his subunit

Cells were transfected with a-d) both the γ-myc/his subunit and the Kv3.1b subunit. Columns 1-4 from left to right show γ-myc/his labelled with anti-myc antibody and FITC; Texas Red staining for the Kv3.1b subunit; DAPI nuclear staining and the merged image of all channels. The scale bar in all panels represents 20μm.
Chapter 6  

Low homology stargazin-related proteins

into COS-7 cells with different dilutions of the γ7 subunit. The proportion of γ7 subunit included in transfections was 10, 5, 2.5, 1 and 0 parts with the difference in cDNA mass being made up by blank pMT2 expression vector. As a control, the experiment was repeated simultaneously using the γ2 subunit instead of γ7. Figure 6.26 shows representative images of a cell from each transfection condition with γ7. The suppression of GFP-Cav2.2 expression is clearly dependent on the amount of γ7 with which it is co-transfected. At the maximum concentration of γ7, GFP-Cav2.2 fluorescence is completely abolished in agreement with previous experiments. Very low levels of fluorescence localised to what is probably the trans-Golgi network, begin to be seen when the amount of γ7 cDNA was halved. Fluorescence levels of GFP-Cav2.2 continue to increase as the amount of γ7 included in the transfections is progressively reduced. However, in the control experiments expressing the γ2 subunit (Figure 6.27), GFP-Cav2.2 fluorescence was also almost completely abolished at the maximum concentration of co-transfected γ2 subunit. This returned steadily as the amount of γ2 cDNA in each transfection was reduced, but was comparatively brighter at each of these dilutions, compared to γ7 transfected COS-7 cells. This result was unexpected, because previous chapters have reported that co-expression of the γ2 subunit with the Cav2.1 subunit does not reduce peak current amplitudes when expressed in Xenopus oocytes.
Chapter 6  Low homology stargazin-related proteins

Figure 6.26 - Reduction in GFP-Ca\textsubscript{v}2.2 fluorescence is dependent upon the concentration of γ7 subunit cDNA with which it is co-transfected

The effect of varying the proportion of the γ7 subunit cDNA in transfection mixes upon the GFP-Ca\textsubscript{v}2.2 fluorescence was investigated. UT marks untransfected cells. COS-7 cells were transfected with Ca\textsubscript{v}2.2/β\textsubscript{II}γ7 subunits in the ratio 10:10:X μg plasmid cDNA where X is represented by the figure to the left of each set of panels. The cDNA levels were kept constant in all transfections by the addition of an appropriate amount of the empty pMT2 plasmid to the cDNA mixes. Panels display from left to right GFP-Ca\textsubscript{v}2.2 fluorescence, staining with the anti-γ7 subunit antibody, DAPI nuclear labelling and the merged image of the red, green and blue channels. Scale bar in all pictures represents 10 μm.
Chapter 6  Low homology stargazin-related proteins

Figure 6.27 - Effects of the concentration of $\gamma_2$ subunit cDNA in the transfection mix upon GFP-Cav2.2 fluorescence when it is co-transfected in COS-7 cells

The effect of varying the proportion of the $\gamma_2$ subunit cDNA in transfection mixes upon the GFP-Cav2.2 fluorescence was investigated. COS-7 cells were transfected with Cav2.2/$\beta_2$/$\gamma_2$ subunits in the ratio 10:10:X µg plasmid cDNA where X is represented by the figure to the left of each set of panels. The cDNA levels were kept constant in all transfections by the addition of an appropriate amount of the empty pMT2 plasmid to the cDNA mixes. Panels display from left to right GFP-Cav2.2 fluorescence, staining with the anti-$\gamma_2$ subunit antibody, DAPI nuclear labelling and the merged image of the red, green and blue channels. Scale bar in all pictures represents 10 µm
6.3 Discussion

6.3.1 Identification and cloning of splice variants of putative human $\gamma$ subunits.

We have described the identification of two genes that encode putative VDCC subunits $\gamma_1$ and $\gamma_5$, by their sequence and structural homology to the mouse stargazin gene ($cacng2$), and have cloned and expressed the cDNA for both human and mouse $\gamma_1$. The $\gamma_1$ subunit is predicted to be a 275 amino acid, 31 kDa protein possessing four transmembrane spanning domains with intracellular N- and C-termini. A physical clone of human $\gamma_5$ is still to be expressed, but the in silico sequence predicts a 275 amino acid 31 kDa protein with identical transmembrane topology to $\gamma_1$. The transcription of the $\gamma_5$ has, however, been confirmed by the cloning of a complete cDNA encoding the $\gamma_5$ from mouse brain. The overall size and structure of $\gamma_1$ and $\gamma_5$ is much closer to that predicted for the skeletal muscle $\gamma_1$ subunit than for the neuronal $\gamma_2$, $\gamma_3$, and $\gamma_4$ subunits. However their sequence identity with the $\gamma_1$ subunit is only approximately 21%, and only slightly better for the human $\gamma_2$ subunit at approximately 25% identity, with higher degrees of conservation occurring in the predicted transmembrane regions (up to 36%). It must also be noted that the C-termini of the $\gamma_1$ and $\gamma_5$ subunits differ from those of $\gamma_2$, $\gamma_3$, and $\gamma_4$ subunits, as they are much shorter and lack the classical consensus motif for interaction with PDZ-domains (T[T/S]-X-V) (Kornau et al., 1997; Tomita et al., 2001), although their final four residues ([T/S]S-P-C) do have some similarity. This C-terminal motif of $\gamma_2$ (also present in $\gamma_3$ and $\gamma_4$ subunits) has been shown to play a crucial role in trafficking AMPA (alpha-amino-3-hydroxyl-5-methyl-4-isoxazolepropionate) receptor subunits to their correct location in the post-synaptic density (Chen et al., 2000). This structural difference suggests that $\gamma_1$ and $\gamma_5$ may have a different function to the $\gamma_2$, $\gamma_3$ and $\gamma_4$ subunits.

The $\gamma_1$ and $\gamma_5$ subunits are, however, 70.5% identical to one another and, unlike all other putative $\gamma$ subunits, are assembled from five exons, the first four of which are identical to exons 1-4 of the genes encoding the previously published predicted four-exon $\gamma_1$ and $\gamma_5$ subunits (Burgess et al., 1999b; Burgess et al., 2001). Sequence analysis of the BAC
clones in which these genes were found suggest that if they are expressed, the four-exon γ; and γ5 subunits would result from an apparent intron retention event at the exon 4-intron 4 boundary of the γ; and γ5 genes (Figure 6.28). Intron retention is a rarely reported mode of alternative splicing in vertebrates (Barber et al., 1999; Goodison et al., 1998; Hampson et al., 1989; Hampson and Rottman, 1987; Jung et al., 2001) and has been found to occur in another ion channel auxiliary subunit. The β1a subunit of the voltage dependent sodium channel is encoded by the alternative splicing of a retained third intron within the β1 gene that includes an in-frame termination codon (Kazen-Gillespie et al., 2000). Inspection of the sequence of intron 4 of the human γ; and γ5 genes reveals that in-frame stop codons are found respectively, 25 nt and 319 nt downstream of the predicted exon 4-intron 4 splice site. If translated, the intron-retained variant mRNA would encode a four-exon γ; subunit having 190 N-terminal amino acids identical to γ; subunit followed by 8 C-terminal amino acids encoded by the first 24 bases of intron 4 before the stop codon. Similarly the intron-retained γ5 human mRNA would encode 190 N-terminal amino acids which are identical to γ5 subunit but the C-terminal 106 amino acids would be encoded by the entirety of intron 4, resulting in a C-terminal γ5 sequence which has extremely low sequence homology to the other γ subunits. In contrast, the C-terminal sequences after the final transmembrane segments of the five-exon γ5 and γ; are of equal length and retain 80% identity with one another.

The sequence of the corresponding intron 4-retained mouse orthologues of human γ; and γ5 can be predicted by analysis of the relevant mouse BACs. However, the stop
codons within the fourth intron of each mouse gene are not in the same or even similar positions to their human equivalents, and their C-terminal regions do not retain homology to their human counterparts. In contrast, the in silico prediction and cDNA clone of five-exon mouse \( \gamma_5 \) sequence exhibit 90% identity to the human \( \gamma_5 \) at the nucleic acid level and 97% identity at the protein level.

The presence of particular elements within terminal exons has been reported to be critical in regulating the correct splicing of other genes in which intron retention has been well characterised (Dirksen et al., 1994; Hampson and Rottman, 1987). Further research is required to discover if intron retained \( \gamma_5 \) and \( \gamma_7 \) subunits are expressed in vivo, if their expression is species and tissue-dependent, and if any motifs in exon 5 are of functional significance in regulating their expression. If intron retained \( \gamma_5 \) or \( \gamma_7 \) were shown to be expressed they would be the first examples of alternatively spliced \( \gamma \) subunit variants.

The \( \gamma_5 \) and \( \gamma_7 \) subunits represent a distinct subdivision of the \( \gamma \) subunit family of proteins identified by structural and sequence homology to stargazin. In addition to the lower sequence homology to the neuronal \( \gamma_2 \), \( \gamma_3 \) and \( \gamma_4 \) subunits, the identification of these gene products, assembled from five exons rather than four, as in the case of stargazin and its closer homologues, is evidence for further evolutionary diversion from a common ancestor.

### 6.3.2 Tissue distribution of the \( \gamma_7 \) subunit

Northern blot analysis of the \( \gamma_7 \) mRNA revealed it to be expressed exclusively in neuronal tissue. Two probes were used in different hybridisations; one specific for \( \gamma_7 \) and the other, used in RT-PCR based expression analysis in a previous study (Burgess et al., 2001), would detect mRNAs for both \( \gamma_7 \) and the putative four-exon \( \gamma_7 \). Both probes hybridised to \( \sim 2.4 \) kb and \( \sim 3 \) kb mRNAs, with identical expression patterns in all tissues in which they are expressed. Presumably these bands represent transcripts that are either alternatively spliced or are differently terminated but clearly they cannot represent different transcripts for four- or five-exon \( \gamma_7 \). Northern blotting with a four-
exon $\gamma_7$ specific probe would be required to determine if a distinct $\gamma_7$ mRNA species exists, but this would appear unlikely given the data presented here.

$\gamma_7$ transcripts were expressed in all the brain regions probed, but were especially abundant in cerebral cortex, with high expression in occipital, frontal and temporal lobes. $\gamma_7$ mRNA was also expressed at high levels in the amygdala, hippocampus, substantia nigra, thalamus and cerebellum. Interestingly, differential expression of the two transcripts was observed within some tissues. A much stronger signal for the smaller 2.4 kb mRNA is most apparent within the cerebellum, but is also demonstrated in amygdala, hippocampus and thalamus.

\subsection*{6.3.3 Tissue distribution of the $\gamma_5$ subunit}

A preliminary northern blot using a small oligonucleotide probe suggested that the human $\gamma_5$ mRNA is found predominantly in non-neuronal tissue with possible low levels of expression in brain. 7.0 kb signals were seen in skeletal muscle, placenta and heart, and smaller 1.9 kb and 1.7 kb signals arose in all of the tissues probed. The low specificity of this result does not greatly further our knowledge of the tissue distribution of the $\gamma_5$ subunit.

The PCR technique used to amplify the $\gamma_5$ cDNA from mouse brain was much more sensitive than the northern blot detection technique, and possibly amplified the sequence from a residual $\gamma_5$ mRNA from brain that one cannot automatically assume is translated. It is also possible that the $\gamma_5$ mRNA originated from other non-neuronal cell types present in total brain (e.g. blood vessels). This clone will however allow the generation of a $\gamma_5$ specific northern or \textit{in situ} hybridisation probe that will elucidate the correct tissue distribution of the protein in both human and mouse in future experiments.
6.3.4 Co-expression of the $\gamma_7$ subunit almost totally abolishes heterologous $\text{Ca}_{\text{v}}2.2$ currents by reducing protein expression.

Co-expression of the $\gamma_7$ subunit almost completely abolished $\text{Ba}^{2+}$ current through N-type $\text{Ca}_{\text{v}}2.2$ VDCCs expressed in both *Xenopus* oocyte and COS-7 mammalian expression systems. It also significantly reduced maximum $\text{Ba}^{2+}$ conductance through $\text{Ca}_{\text{v}}2.1$ and $\text{Ca}_{\text{v}}1.2$ VDCCs, but the effects were not so pronounced. In contrast to the effects of the neuronal $\gamma_2$ subunit, which has been reported in a previous study to inhibit N-type $\text{Ca}_{\text{v}}2.2/\beta_3/\alpha 2\delta_1$ currents by about 37% (Kang *et al.*, 2001), the influence of the $\gamma_7$ subunit is not dependent upon co-expression of an $\alpha 2\delta$ subunit as part of the $\text{Ca}^{2+}$ channel complex. Some minor effects upon channel inactivation were also recorded. The significance of these data is, however, open to question given the tiny amplitudes of the currents from which the data was obtained.

Several mechanisms could underlie the suppression of VDCC currents by the $\gamma_7$ subunit including (1) interference with the functioning of the channel at the plasma membrane, (2) inhibition of the delivery of the channel to the plasma membrane, (3) prevention of the correct folding of the pore-forming subunit, (4) more rapid protein degradation, and (5) suppression of channel protein synthesis.

Confocal imaging of COS-7 cells transiently expressing N-terminally tagged GFP-$\text{Ca}_{\text{v}}2.2$ $\text{Ca}^{2+}$ channel complexes revealed that co-expression of the $\gamma_7$ protein, which itself is highly expressed throughout the cell, including the plasma membrane, resulted in an almost complete loss of GFP fluorescence. This suggested reduced synthesis or stability of GFP-$\text{Ca}_{\text{v}}2.2$, or interference of the $\gamma_7$ subunit with the correct folding of the GFP-tagged protein. Western blot analysis of the levels of both GFP-$\text{Ca}_{\text{v}}2.2$ and untagged $\text{Ca}_{\text{v}}2.2$ revealed that co-expression of $\gamma_7$ results in a much reduced steady state level of $\text{Ca}_{\text{v}}2.2$ (Chapter 6A). This is likely to be due to a reduction in the level of $\text{Ca}_{\text{v}}2.2$ protein synthesized when $\gamma_7$ is co-expressed because no smaller molecular weight products of degradation were detected either by the GFP-Ab or the $\text{Ca}_{\text{v}}2.2$-II-III linker Ab.
These results raised the possibility that the γ1 was causing a non-specific inhibition of translation. However, we have shown that the influence of γ1 does not cause a generalised down-regulation of all heterologously expressed ion channels, because the I-V relationship for the voltage-dependent potassium channel Kv3.1b expressed in *Xenopus* oocytes, was unaffected by co-expression of the γ1 subunit (Chapter 6A). Furthermore, immunocytochemistry experiments confirmed that the expression of Kv3.1b in COS-7 cells was unaltered by the co-expression of the γ1 subunit. Moreover, despite some reduction in the fluorescence of β1b-GFP when expressed as part of a Cav2.2/β1b-GFP complex with γ1, western blot analysis has shown there to be little effect of the γ1 protein on expression of the VDCC β1b subunit (Chapter 6A). Additionally, the expression levels of the similarly sized and structured γ2 subunit were not lowered upon co-expression of equal levels of γ1. However, the normal plasma membrane localisation of the γ2 was lost, suggesting that co-expression of the γ1 subunit resulted in the retention of the γ2 subunit in the intracellular membranes. This could be consequence of a direct interaction between the two proteins, or be a result of congestion of the cellular trafficking machinery following over expression of the γ1 subunit. By whichever process this event occurs, the ability of the γ1 protein to quell the propensity of the γ2 subunit to traffic itself and possibly other proteins that would normally be dependent on γ2 for their trafficking to the plasma membrane may be of functional significance if both proteins are found to be expressed in the same cell types (Chen et al., 2000). On the other hand, the retention of γ2 within intracellular organelles seen in this heterologous expression system may be prevented *in vivo* due to the interaction of other proteins that are absent from the transfections, which would normally interact with either γ species in their native environment.

These results display a striking resemblance to the dominant-negative synthesis suppression of the Cav2.2 subunit induced by the co-expression of truncated Cav2.2 constructs (Raghib et al., 2001b). In that investigation it was proposed that the translation of full-length Cav2.2 was halted when Domain I or Domain I-II of Cav2.2 interacted with the initially synthesized transmembrane α-helices of the nascent full-length Cav2.2. It may also be possible here, that while the Cav2.2 is in a nascent state and only associated with the endoplasmic reticulum (ER) via ionic interactions rather
than inserted into the lipid bi-layer (Borel and Simon, 1996), certain critical residues of the \( \text{Cav}_{2.2} \) may be exposed to inappropriate interaction with the much shorter \( \gamma_3 \) subunit resulting in the premature termination of \( \text{Cav}_{2.2} \) synthesis. In a transient heterologous expression system, \( \gamma_3 \) will be synthesized more rapidly than the \( \text{Cav}_{2.2} \), given that its ORF is only approximately one eighth of the size of the pore-forming subunit, therefore creating an excess of molecules capable of inhibiting \( \text{Cav}_{2.2} \) synthesis. This could explain the almost complete abolition of recorded \( \text{Cav}_{2.2} \) currents expressed in COS-7 and *Xenopus* oocytes. It is nevertheless also possible \( \gamma_3 \) induces rapid and complete targeting of \( \text{Cav}_{2.2} \) to a degradation pathway. Additional experiments incubating cells expressing GFP-\( \text{Cav}_{2.2} \) and \( \gamma_3 \) with ALLN/lactacystin should be performed to determine whether inhibition of proteasome activity causes a recovery the GFP-\( \text{Cav}_{2.2} \) fluorescence (Kagan et al., 2000; Lee and Goldberg, 1998).

Further evidence produced in collaboration with another member of our laboratory, Dr. F. Bertaso, indicates that the influence of \( \gamma_3 \) expression may be upon newly synthesized \( \text{Cav}_{2.2} \) protein. Expression of the \( \gamma_3 \) subunit in sympathetic neurons from the SCG, in which at least 80% of the whole cell peak \( \text{Ca}^{2+} \) current is carried through N-type \( \text{Ca}^{2+} \) channels (Delmas et al., 2000; Plummer et al., 1989), did not significantly alter endogenous current densities (Chapter 6A). The \( \gamma_3 \) subunit therefore does not affect the function of pre-existing VDCC protein. Future experiments will allow a longer duration between transfection with the \( \gamma_3 \) subunit and recording of currents to permit channel turnover mechanisms to have a significant bearing upon the recorded current densities and to discover if the presence of the \( \gamma_3 \) subunit interferes with normal channel recycling. Additionally, one could investigate whether \( \gamma_3 \) can suppress the increase in N-type VDCC current density in pheochromocytoma (PC12) cells, stimulated as cells are differentiated upon culture in the presence of nerve growth factor (NGF) (Greene and Tischler, 1976; Plummer et al., 1989). In un-differentiated PC12 cells, whole cell HVA VDCC current is prominently L-type with a few cells expressing a very small N-type component (Plummer et al., 1989). Upon differentiation, whole cell currents increase approximately 5-fold, consisting of 20% L-type, 40% N-type and 40% currents resistant to conotoxin or dihydropyridine block. Un-differentiated PC12 cells could be transfected with \( \gamma_3 \) and the components of whole cell calcium current determined in both
un-differentiated and differentiated PC12 cells to discover whether γ7 is able to suppress the NGF stimulated expression of the endogenous Cav2.2 subunit.

It would therefore appear that the influence of γ7 on VDCC expression and function is via a mechanism unlike that reported previously for the other neuronal γ subunits identified by their homology to stargazin (Chen et al., 2000; Kang et al., 2001; Klugbauer et al., 2000; Letts et al., 1998). It may act by similar mechanisms to the truncated Cav2.2 constructs to reduce the expression of Cav2.2 and other VDCC α1 subunits. Nevertheless, in the present study, inhibition of expression by the γ7 subunit is apparently specific for VDCC subunits, particularly Cav2.2, because co-expression with Kv3.1b did not adversely affect the expression of the potassium channel. Deletion and mutation analysis of the γ7 subunit will be necessary to establish the critical regions involved in down-regulation of VDCC expression.

Preliminary immunocytochemistry investigations may suggest that the suppression of Cav2.2 expression is dependent upon the amount of γ7 protein expressed in the same cell. Although a similar, albeit slightly less powerful effect was seen upon the co-expression of the different amounts of the γ2 subunit with GFP-Cav2.2, it is possible that this set of experiments suggests that the GFP tag may itself be adversely effected by the over-expression of a small membrane proteins such as γ7 or γ2. Other experiments co-expressing the γ2 or with Cav2.1 (Chapter 5) have shown no reduction in peak current amplitude of recorded currents, which would suggest that expression or trafficking of the Cav-α1 subunit is not adversely effected. Another laboratory has reported a 37% reduction in Cav2.2 current amplitude when co-expressed with γ2 (Kang et al., 2001), but still, this is not of sufficient magnitude to explain the loss of GFP-Cav2.2 fluorescence seen in Figure 6.27. Furthermore, levels of β1b-GFP fluorescence were apparently reduced upon co-expression of the γ7 protein. This must also be caused by disruption of the correct folding of the GFP tag. Evidence against a reduction in the levels of expressed β subunit includes the fact that the properties of Cav2.2, Cav2.1 or Cav1.2 currents in the presence of γ7 did not mimic those of previous investigations in Xenopus oocytes and COS-7 cells expressing these channels without β subunits, where both the $V_{50}$ of activation and steady-state inactivation were markedly depolarised.
Low homology stargazin-related proteins (Canti et al., 2000; De Waard and Campbell, 1995; Meir et al., 2000; Singer et al., 1991; Stephens et al., 2000). Furthermore, subsequent western blot analysis has shown that the level of expressed β1b protein in Cav2.2/β1b transfections is not reduced on co-expression of the γ7 (Chapter 6A). Additional western blot experiments and electrophysiology recordings are needed to discover how dependent the suppression of Cav2.2 expression is upon γ7 concentration.

Whether γ7 is actually a subunit of a functional plasma membrane calcium channel complex appears unlikely, at least for Cav2.2. However, the low homology to the stargazin-like γ subunits, the presence of a fifth exon, the lack of classical PDZ-interaction motif and unique functional properties of γ7 imply that it represents a distinct subdivision of the family of proteins identified by their structural and sequence homology to stargazin.

6.4 Direction of future research to determine the true identity and functional properties of the γ7 and γ5 subunits

Several proposals for future experiments have already been suggested in the above discussion. However additional investigations are outlined below that would expand our understanding of the expression and functional properties of this novel sub-family of proteins.

6.4.1 Cloning the human γ5 subunit and determining functional properties

To date, attempts to amplify and clone the complete or partial human γ5 coding sequence by RT-PCR have not produced a clone. A northern blot experiment using an oligonucleotide probe suggested that the γ5 subunit might be a non-neuronal species (section 6.2.2.2). However published research has suggested low levels of the predicted γ5 isoform to be expressed in human brain in addition to kidney, spleen, thymus, prostate, testis, foetal brain and spinal cord (Burgess et al., 2001). This study identified several candidate tissues as sources of mRNA from which the four- and/or five-exon γ5
could be amplified in future experiments, but is not a definitive expression profile for either of subunits, as the sensitivity of the RT-PCR methodology used was extremely high. The same investigation managed to amplify the human \( \gamma_1 \) subunit from 16 out of the 24 tissues probed, when it is widely accepted that this subunit is only expressed in skeletal muscle \textit{in vivo} \cite{Powers1993}. Only when the template was diluted 1:1500 was the target specifically amplified from skeletal muscle \cite{Burgess2001}. Similar dilutions of the template were not performed in experiments to amplify part of the \textit{CACNG5} gene, so more accurate distribution data for this subunit was not generated. Should it remain difficult to clone the human \( \gamma_5 \) subunit from mRNA by RT-PCR, alternative strategies could be pursued, for example, assembly of the coding sequence from the genomic material within BAC AC005988 by PCR and/or restriction digests and ligations. Alternatively, the \( \gamma_5 \) cDNA from mouse brain could serve as a more suitable northern blot or \textit{in situ} hybridisation probe than the oligonucleotide previously used, to discover whether the \( \gamma_5 \) subunit is expressed in humans and in which tissues. Otherwise the same mouse \( \gamma_5 \) cDNA could be used to probe a human cDNA library to find whole or partial \( \gamma_5 \) cDNAs.

The functional characterisation of the properties of the \( \gamma_5 \) subunit may already proceed by expressing the mouse clone with functional VDCC complexes in repeats of all the experiments performed with the \( \gamma_7 \) subunit. This will determine if the \( \gamma_5 \) behaves in the same manner as \( \gamma_7 \) in transient expression systems. It will also be of interest to discover if expression of both \( \gamma_5 \) and \( \gamma_7 \) with Cav2.2 still results in a marked suppression of expression of the Cav-\( \alpha_1 \) subunit.

### 6.4.2 Distribution of the \( \gamma_7 \) and \( \gamma_5 \) subunits

Northern blot analysis has determined the regions of the human brain in which the \( \gamma_7 \) subunit is expressed. More detailed investigations of the cell types and brain regions in which the protein is predicted to be expressed should be performed by immunohistochemistry and \textit{in situ} hybridisation. The anti-\( \gamma_7 \) antibody will detect both human and mouse proteins as they possess 100\% identity with one another. A \( \gamma_7 \) specific probe for \textit{in situ} hybridisation can be easily amplified from any of the \( \gamma_7 \)
cDNAs generated for previous work, although the cDNA northern probe already used in previous investigations would be suitable. More detailed knowledge of the cell types in which this protein is expressed will help to determine its true function.

The recent cloning of the mouse γ5 cDNA has provided a template from which northern blot and \textit{in situ} probes may be generated. These can be used to determine the correct distribution of the both human and mouse tissues in which the γ5 mRNA is transcribed. It will be particularly interesting if the γ5 and γ7 co-localise in any regions or cell types. Generation of a γ5 specific antibody will also help to determine the tissues in which γ5 protein is expressed by immunohistochemistry or western blot.

\subsection*{6.4.3 Binding partners for γ7 and γ5}

With which other proteins do the γ7 and γ5 interact \textit{in vivo}? Although a striking influence of the γ7 upon Cav2.2 expression is seen \textit{in vitro}, whether any interaction with VDCC subunits occurs in the native surroundings of the γ7 must be determined, although the results obtained here suggest that they interact at an early stage in Cav2.2 synthesis. Co-immunoprecipitation of Cav-α1 subunits with the γ7 from mouse brain would demonstrate whether interactions occurred with calcium channels \textit{in vivo}. However, a negative result would not mean that they do not interact. A more sensitive technique to determine potential binding partners of the novel γ7 and γ5 is the yeast-two-hybrid assay (Fields and Song, 1989). Suitable GAL-4 binding domain baits could be generated from the intracellular C-terminus of each subunit and used to probe human and mouse activation domain cDNA libraries. Once any false positives have been eliminated, the identity of the remaining candidates can be determined by isolation and sequencing of the cDNAs. These binding partners should subsequently be cloned and the interaction confirmed by co-immunoprecipitation with the γ7 or γ5 from native tissue and/or \textit{in vitro} expression systems. It will be of great interest to determine whether γ5 and γ7 directly interact with one another, any of the other stargazin related proteins, or other related tetra-spanning membrane proteins.
6.4.4 Functional importance of critical residues and motifs of the \( \gamma_5 \) and \( \gamma_7 \) proteins

Figure 6.9 highlights residues of \( \gamma_5 \) and \( \gamma_7 \) that are predicted to be modified by N-linked glycosylation and phosphorylation. Some or all of these putative post-translational modifications are likely to play a role in modifying the functional properties of the proteins. Site-directed mutagenesis of the critical serine, threonine or tyrosine at each predicted phosphorylation site will determine the role of phosphorylation at each of these intracellular regions when the subunits are expressed alone or co-expressed with other proteins. The role of glycosylation at the predicted sites in the extracellular loops of \( \gamma_5 \) and \( \gamma_7 \) can also be determined by incubating transiently expressed protein with different concentrations of the de-glycoylation reagents, N-glycosidase F (Chu, 1986; Tarentino et al., 1985) and \( \beta \)-galactosidase (Hamos et al., 1987).

All proteins related to stargazin possess a pair of conserved cysteines in their first extracellular loop (residues 62 and 72 of \( \gamma_5 \) and \( \gamma_7 \)). These are predicted to form disulphide bridges that are possibly of structural and functional significance. Treating cells transfected with either subunit with biotinylated methanethiosulfonate (MTS) can determine whether a disulphide bridge is present between these residues. Biotinylated-MTS only associates with free sulphhydryl groups, and therefore will only detect the cysteines of the \( \gamma \) subunits if they are not involved in forming disulphide linkages (Figure 6.29). Therefore, if the predicted disulphide bond is present in \( \gamma_5 \) or \( \gamma_7 \) in their native form, they will not be detected by biotinylated-MTS. Additional evidence for the normal existence of a disulphide bond can be generated by mutating either one of the cysteines to an alanine, which should result in the detection of \( \gamma_5 \) or \( \gamma_7 \) by biotinylated-MTS. Moreover, the signal should no longer be detected if both cysteines are mutated to alanines. Immunoprecipitation and western blot would be the method of choice for specifically detecting biotinylated-MTS labelled \( \gamma \) subunits with HRP-conjugated streptavidin and enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech, Little Chalfont, UK). Once it has been established if and how disulphide bonds are formed between Cys 62 and 72, their functional significance can be determined in electrophysiology and immunocytochemistry experiments by expressing wild type and each Cys/Ala mutation of the \( \gamma \) subunits with Cav-\( \alpha \)1 subunits.
Figure 6.29 – Association of biotinylated methanethiosulfonate (MTS) to free sulphhydril groups of proteins

Reaction scheme of how free cysteines are labelled by reaction with biotinylated MTS where the biotin is represented by the R group. The reaction cannot occur if a disulphide bond already exists between a pair of cysteines.

6.4.5 Identifying the mechanism underlying the suppression of \( \text{Ca}_v2.2 \) expression

This investigation has already established that expression of the \( \gamma \) greatly reduces the expression of \( \text{Ca}_v2.2 \) protein in transient transfections. Future research will determine if there is also a reduction in \( \text{Ca}_v2.2 \) mRNA, and if so, whether it is a primary event or a result of synthesis inhibition. Additionally, our laboratory has generated truncations of \( \text{Ca}_v2.2 \) that have been used in a previous investigation (Raghib et al., 2001b) which will be useful in elucidating where \( \gamma \) interacts with \( \text{Ca}_v2.2 \), how it causes suppression of synthesis and determine at which stage of \( \text{Ca}_v2.2 \) synthesis the suppression occurs. It would be of particular interest to ascertain whether the synthesis of the \( \text{Ca}_v2.2 \) Dom I-II or Dom III-IV constructs are reduced upon co-expression of \( \gamma \) in COS-7 cells. Preliminary experiments could be performed by immunocytochemistry using GFP tagged \( \text{Ca}_v2.2 \) domain constructs. However, more conclusive results would be obtained by western and northern blot analysis of the protein and mRNA levels.

6.4.6 Cloning of \( \gamma_5 \) and \( \gamma_7 \) and investigation of the predicted intron retention events underlying their potential expression

It is imperative that future investigations ascertain whether the predicted \( \gamma_5 \) and \( \gamma_7 \) subunits are actually expressed \textit{in vivo}. RNase protection analysis is the most suitable technique to achieve this goal. BAC clones AC008440 and AC005988 contain the complete and ordered genes encoding \( \gamma_7 \) and \( \gamma_5 \) respectively and can be used to generate probes for RNase protection analysis of the four- or five exon \( \gamma_5 \) or \( \gamma_7 \). Fragments
Chapter 6  Low homology stargazin-related proteins

corresponding to exon 4 plus intron 4 and part of exon 5 of each gene could be excised by restriction digest or amplified from the relevant BAC and subcloned into a vector containing a T3, T7 or SP6 RNA polymerase promoter/binding site to generate pRPA-γ7 or pRPA-γ5 plasmids. Synthesis of labeled cRNA probe is achieved by linearising pRPA-γ7 or pRPA-γ5 plasmids with a single cutter restriction enzyme, ethanol precipitating the product, and incubating the re-suspended DNA with an in-vitro transcription kit mixture (e.g. Maxiscript, Ambion) and radiolabelled [α-32P] UTP/CTP.

The RPA-γ7 probe can be hybridized to human brain total or poly-(A) selected RNA. The RPA-γ5 probe should be hybridized with RNA from a number of different tissues unless more accurate distribution data for the four- or five-exon γ5 has been generated. If expressed, the terminal nucleotides of four-exon γ7 or γ5 corresponding to intron 4 of the 5 exon gene will be fully protected by their respective probes resulting in bands 564 nt and 528 nt in size (Figure 6.30). Five-exon γ7 and γ5 will not be fully protected and produce bands of 143 nt and 147 nt respectively (Figure 6.30).

Analysis of BACs AC079424 and AC079557 suggests that four-exon γ7 or γ5 will not be expressed in mouse because there are no stop codons in the same or even similar positions to their human equivalents. However, intron retention may still occur resulting in more alternative splice variants of the five-exon γ7 and γ5 subunits. This can be determined using a similar RNase protection experiments as described above for the human subunits but using probes corresponding to exon 4 plus intron 4 of the mouse γ7 and γ5 genes.
Chapter 6  Low homology stargazin-related proteins

Figure 6.30  RNase protection strategy to determine splicing or retention of intron 4 of the genes CACNG5 and CACNG7.

a) Probe RPA-γ7 generated by PCR from BAC AC008440 corresponds the sequences of exon 4 plus intron 4 and the first 98 nucleotides of exon 5 of gene CACNG7. If expressed the four- and five-exon γ7 variants will be resolved as 564 nt and 142 nt protected fragments respectively in RNase protection experiments. b) Probe RPA-γ5 generated by PCR from BAC AC005988 corresponds the sequences of exon 4 plus intron 4 and the first 34 nucleotides of exon 5 of gene CACNG5. If expressed the four- and five-exon γ5 variants will be resolved as 528 nt and 147 nt protected fragments respectively.

If it is found that four-exon human γ5 and γ7 are expressed in vivo, a set of experiments designed to discover the motifs influencing the alternative processing of the five-exon genes that encode γ5 and γ7 are outlined below.

The entire gene for each subunit, including the 5' and 3' untranslated regions and polyadenylation signal could be subcloned from BAC clones AC008440 and AC005988 into an appropriate plasmid for expression in mammalian COS-7, CHO or tsA201 cells. Once transfected into cells, the relative expression of four- and five-exon γ5 and γ7 can be determined by using RPA-γ7 and RPA-γ5 probes in RNase protection assays to determine the relative expression of correctly spliced and intron-retained isoforms.

A gene in which intron retention has been well characterised is bovine growth hormone. The fraction of bovine growth hormone transcripts that retain intron D can be increased to more than 95% upon deletion of a critical CTTCCGGAAG element within the flanking exon 5 (Hampson and Rottman, 1987). Further research identified the GGAAG portion of this palindrome as the motif responsible for regulating the gene’s correct splicing.
Homologous sequences also occur within the exon flanking the retained intron of a PAX3 transcription factor splice variant (Barber et al., 1999). Such a sequence cannot be found in exon 4 of either CACNG5 or CACNG7, however the CTTCC portion of the palindrome is present 73 nt downstream from the consensus splice site of exon 4 of CACNG7. Absence of the GGAAG portion of the sequence could possibly favour expression of the four-exon γ in preference to correctly spliced γ (Dirksen et al., 1994). However, this may also be dependent upon particular motifs present in intron 4 (Hampson et al., 1989). Additionally, the CTTCC motif bears a close resemblance to the CUUCGG sequence that is often associated with particularly stable RNA stem-loop structures (Tuerk et al., 1988). Currently there is no evidence that this motif is associated with stem-loop structures in the putative γ subunits, or whether it plays a role in stabilisation of pre-mRNA secondary structure, resulting in intron retention.

The generation and expression of clones with deletions of different regions in exon 5 of CACNG7 and CACNG5 using appropriate restriction strategies will determine if any particular motifs are required to be present in order to promote the correct splicing of these genes. Furthermore they will be able to determine whether the proximity of any identified motifs to intron 4 alters the efficiency of intron removal (Hampson et al., 1989; Reed and Maniatis, 1986). Once again, RNase protection analysis will determine the proportion of correctly spliced to intron-retained isoform expressed by each deletion clone.
Supplementary data for Chapter 6 regarding the functional characterisation of the $\gamma_7$ subunit
6.5 Introduction

Additional experiments were performed in close collaboration with colleagues from my laboratory in order to more completely characterise the functional properties of the γ subunit when co-expressed with the Cav2.2 VDCC subunit. These data have been included together with my own data from chapter 6 as part of a publication currently in press at EMBO Journal, (Vol. 21, No. 7, April 2nd 2002) entitled “The novel product of a five-exon stargazin-related gene abolishes Cav2.2 calcium channel expression”. At the time of submission of this thesis, the article will not have been published. Furthermore, because the data generated was not entirely my own work, it was inappropriate to include the results as part of chapter 6. I have therefore presented it in this appendix to chapter 6 in order to extend my own findings regarding the functional characterisation of the novel γ subunit.

6.6 Conjoint experiments

6.6.1 Influence of the γ subunit upon the heterologous expression of Kv3.1b in Xenopus oocytes
(A. C. Dolphin & F. J. Moss)

The effect of γ upon the Ba^{2+} currents through Cav2.2 VDCCs expressed in Xenopus oocytes was so striking, we investigated the possibility that it down-regulates other heterologously expressed ion channels by co-expressing it with the Shaw-like voltage-dependent potassium channel, Kv3.1b.

6.6.1.1 Methods

Xenopus oocytes. Xenopus oocytes were prepared, injected and utilized for electrophysiology as previously described in Chapter 2, with the following exceptions. cDNAs injected were rat Kv3.1b (M68880) in pRC-CMV expression vector (a gift of Dr. L. Kaczmarek -Yale University School of Medicine), and human γ (AF458897) in pMT2. Kv3.1b currents were recorded 3 days after intranuclear injection of oocytes with 9nl of cDNA together with an equal amount of γ in pMT2, or water for the control. The external recording medium contained (in mM): NaCl 96; HEPES 5; KCl 2; MgCl2 1; CaCl2 1.8, (pH 7.4).
6.6.1.2 Results

Figure 6.30 shows representative traces and the peak I-V relationship of Kv3.1b currents expressed in Xenopus oocytes alone and co-expressed with the γ subunit, which indicate that the γ subunit had no effect on the peak current amplitude of heterologously expressed Kv3.1b channels.

![Graph showing peak I-V relationship for Kv3.1b and Kv3.1b + γ](image)

Figure 6.31 - Co-expression of the γ subunit with Kv3.1b in Xenopus oocytes does not modulate expressed peak current amplitudes.

Peak I-V relationship for Kv3.1b (■, n = 10), and Kv3.1b + γ (□, n = 10) and current traces for Kv3.1b (top left panel), Kv3.1b + γ (bottom left panel). Holding potential was -100 mV, and steps were between -30 and +40 mV for 100 ms. The scale bar refers to both panels.

6.6.2 Influence of the γ subunit upon Cav2.2 VDCC currents expressed in mammalian COS-7 cells (P. Viard and F. J. Moss)

To determine whether the effects of γ were peculiar to the Xenopus oocyte expression system we examined its effect on Cav2.2 current in the mammalian cell line, COS-7. Additionally, in a recent report it was stated that inhibition of Cav2.2 currents by the γ subunit was dependent upon co-expression of the αδ subunit (Kang et al., 2001). To investigate if the same is true of the much more robust suppressive effect of γ, recordings were made from cells in the absence of co-transfected αδ subunit.
6.6.2.1 Methods

cDNAs. The following cDNAs were used: N-terminal GFP tagged rabbit Cav2.2 (Raghib et al., 2001b), rat β₁₆ (X61394, except R417S, V435A, V449A, W492R, V511A, a gift from Prof. T. P. Snutch), mouse α₂δ₂ (AF247139), human γ₁ (AF458897) and mut-3b Green Fluorescent Protein (GFP, M62653, except S72A and S65G, a gift from Prof. T. E. Hughes). All cDNAs were subcloned into expression vector pMT2 (Swick et al., 1992).

Cell culture. Monkey COS-7 cells were cultured as previously described in Chapter 2. Transfection was performed with Geneporter transfection reagent (Gene Therapy Systems, San Diego, CA). Cells were plated onto coverslips 2-3 h. prior to transfection. The DNA and Geneporter reagent (2 µg and 10 µl respectively) were each diluted in 500 µl of serum-free medium, mixed, and applied to the cells. The α₁, β, α₂δ and γ subunits were mixed and transfected in a 1:1:1:1 ratio by DNA weight. In the absence of α₂δ₂ and/or γ₁ subunit, blank pMT2 vector was transfected. After 3.5 h., 1 ml of medium containing 20% serum was added to the cells, which were then incubated at 37°C for 3 days. Prior to electrophysiological recording cells were re-plated using a non-enzymatic cell dissociation solution (Sigma, Dorset, UK) and maintained at 27°C for between 2 and 6 h.

Patch-clamp electrophysiology. Recordings were made from fluorescent COS-7 cells expressing GFP-Cav2.2. Because GFP-Cav2.2 was only weakly fluorescent, free GFP (ratio 0.1) was included in the transfection to identify transfected cells from which recordings were made. Borosilicate glass electrodes of resistance 2-5 MΩ were filled with a solution containing (mM): cesium aspartate 140; EGTA 5; MgCl₂ 2; CaCl₂ 0.1; K₂ATP 2; and Hepes 10; pH 7.2. The external solution containing (mM): TEA-Br 160; KCl 3; NaHCO₃ 1; MgCl₂ 1; Hepes 10; glucose 4; and BaCl₂ 1 or 10 as stated; pH 7.4. 1 mM Ba²⁺ was used as a charge carrier except in the presence of γ₁ subunit where 10 mM Ba²⁺ was also used. Whole-cell currents were recorded using the whole-cell patch technique, with an Axopatch-200A amplifier. Currents were elicited by 200 ms step depolarisations from a holding potential of -80 mV. Data were filtered at 1 kHz and
analyzed using pCLAMP 6 and Origin 5.0 software. Current records are shown following leak and capacitance current subtraction (P/4 protocol). Experiments were performed at room temperature. Data are expressed as mean ± S.E.M. Statistical analysis was performed using paired or unpaired Student's t test, as appropriate.

6.6.2.2 Results

These experiments confirmed the almost total abolition of whole-cell Ba\textsuperscript{2+} current in cells transfected with GFP-Cav\textsubscript{2.2}/\beta\textsubscript{1b}/\alpha\delta\gamma, compared to GFP-Cav\textsubscript{2.2}/\beta\textsubscript{1b}/\alpha\delta\gamma controls (Fig. 1.2a, upper traces). In 1mM Ba\textsuperscript{2+}, mean current density of cells expressing GFP-Cav\textsubscript{2.2}/\beta\textsubscript{1b}/\alpha\delta\gamma was 13.5 ± 4.3 pA/pF at 0mV (n=20) (Figure 6.32), but even when extracellular [Ba\textsuperscript{2+}] was increased to 10mM, currents from GFP-Cav\textsubscript{2.2}/\beta\textsubscript{1b}/\alpha\delta\gamma transfected COS-7 cells remained extremely small (0.23 ± 0.08 pA/pF at 0mV, n=20, p < 0.01)(Figure 6.32a, lower trace and Figure 6.32b).

The histograms in Figure 6.32b show that the influence of the \gamma subunit upon Cav\textsubscript{2.2}/\beta\textsubscript{1b} currents was independent of co-expression of an \alpha\delta subunit (Cav\textsubscript{2.2}/\beta\textsubscript{1b}, 14.9 ± 2.7 pA/pF at 0mV in 1mM Ba\textsuperscript{2+}, n = 16; Cav\textsubscript{2.2}/\beta\textsubscript{1b}/\gamma, 0.02 ± 0.03 pA/pF at 0mV in 10mM Ba\textsuperscript{2+}, n = 18, p < 0.01). Recordings were made from all cells where the whole cell configuration was achieved with a high-resistance seal, including those without currents. The mean data therefore included all cells examined.
Chapter 6A

Chapter 6 supplementary data

Figure 6.32 - Heterologously expressed VDCC current density is reduced by γ7 in COS-7 cells.

GFP-Cav2.2/β1s cDNAs were transiently transfected into COS-7 cells with or without α2δ2 and γ7 subunits. (a) Example traces elicited by a 200ms step depolarization to +10mV from a holding potential of −80 mV in the presence of 1mM Ba2+ (upper panel). In the presence of γ7, extracellular Ba2+ solution was increased to 10 mM (lower panel). (b) Histogram of mean current densities measured at +10mV in 1mM Ba2+ for controls and 10mM Ba2+ in the presence of γ7. Co-expression of γ7 abolished currents both in presence and absence of α2δ2 subunits. The number of experiments (n) for each condition is given in parentheses above the columns and data from all cells tested is included (** P < 0.01, Student’s t-test).

6.6.3 Influence of the γ7 subunit on endogenous Ca2+ currents recorded from cultured sympathetic neurones (F. Bertaso and F. J. Moss)

We investigated the electrophysiological consequence of the acute expression of γ7 upon the endogenous VDCC currents from sympathetic neurones of the rat superior cervical ganglion (SCG). These neurones express 80-90% N-type and 10% L-type current (Delmas et al., 2000; Plummer et al., 1989) and provided a vehicle in which we could determine if the γ7 subunit could exert its influence upon pre-existing Ca2+ channels.

6.6.3.1 Methods

cDNAs. mut-3b Green Fluorescent Protein (GFP, M62653, except S72A and S65G, a gift from Prof T. E. Hughes) and human γ7 (AF458897), both of which were subcloned into the pMT2 expression vector(Swick et al., 1992).

SCG sympathetic neurone preparation, transfection, and electrophysiology. Sympathetic neurones were isolated from 17 day-old Sprague-Dawley rats killed by CO2 inhalation, following a modified procedure described by Delmas et al. (Delmas et
al., 1998), in which collagenase type IA (500 U.mL⁻¹, Sigma) and trypsin type XI (3000 U.mL⁻¹, Sigma) were used. Aliquots of the cell suspension were immediately transferred to the electroporation cuvettes together with 10 μg of each of the cDNAs to be transfected. After electroporation (Morales et al., 2000) (250 volts, 900 μF, EasyjecT Plus, EquiBio, Ashford, UK) the cells were resuspended in pre-warmed growth medium, plated on poly-L-lysine (Sigma) coated Petri dishes and incubated at 37°C, 5% CO₂, for 36-48 h before use for immunocytochemistry or electrophysiological experiments. Currents were elicited in the whole-cell patch-clamp configuration from a holding potential of -80 mV by a 200 ms ramp from -40 to +40 mV. Otherwise all other recording conditions were as described above for COS-7 cells with the exception of the addition of 0.5 μM tetrodotoxin to the extracellular solution.

**Immunocytochemistry.** SCG neurons were fixed and permeabilised for immunocytochemistry essentially as previously described (Brice et al., 1997). Primary antibody, affinity purified anti-γ₇ was used at 0.8 μg/ml and secondary goat anti-rabbit (Sigma, Dorset, UK) antibody was applied at and 5 μg/ml. Texas red-conjugated streptavidin was applied at 3.33 μg/ml. The nuclear dye 4',6-diamidino-2-phenylindole (DAPI, 300 nM, Molecular Probes) was also used to visualize the nucleus. Cells were examined on a confocal scanning laser microscope (Leica TCS SP, Milton Keynes, UK). All images were scanned sequentially to eliminate cross-talk and photomultiplier settings kept constant in each experiment.

**6.6.3.2 Results**

Whole-cell patch-clamp recordings, performed 36-48h post isolation and transfection from control sympathetic neurons transfected with GFP marker, give a mean Iₒₙ current density at 0mV of -13.6 ± 2.6 pA/pF (n=6). However, this was not significantly altered in neurons transfected with GFP and the γ₇ subunit (-15.2 ± 3.5 pA/pF, n=6) (Figure 6.33a and Figure 6.33b). Expression of γ₇ in these experiments was verified by immunocytochemistry, which also showed that γ₇ was not detectable endogenously in SCG sympathetic neurones or in the associated glial cells present in the culture preparation (Figure 6.34). It would therefore appear that the γ₇ subunit is unable to
acutely influence the properties of pre-existing VDCCs in neurons. Interestingly, the morphology of the sympathetic neurones changes drastically when expressing the $\gamma_7$ subunit. They have more numerous but shorter and less ordered neurites than the control transfected neurones. This may suggest a role for the $\gamma_7$ subunit in controlling development of cellular morphology like its distant relations, the claudin subunits of tight junction, or the myelin membrane proteins PLP and PMP-22.
Figure 6.33 - The endogenous VDCC current from sympathetic neurons is unaltered by \( \gamma_7 \) expression.

a) Example traces from sympathetic neurons transiently transfected with GFP or GFP and \( \gamma_7 \) subunit, elicited by a 100ms step depolarization to +10mV from a holding potential of -80mV in medium containing 10mM Ba\(^{2+}\). b) Histogram of mean current densities measured from sympathetic neurons at +10mV. The number of experiments (n) for each condition is given in parentheses above the columns.

Figure 6.34 - Acute expression of the \( \gamma_7 \) subunit in sympathetic neurones from the SCG.

a) Control sympathetic neurone transfected with GFP alone. b & c) sympathetic neurones transfected with GFP and \( \gamma_7 \) subunit. From left to right, the panels in column 1 show GFP fluorescence, column 2 shows Texas Red staining for the \( \gamma_7 \) subunit, column 3 displays the blue DAPI staining of the nucleus, and the panels in column 4 show the merged images. The scale bar in panel a represents 20 \( \mu m \) and in panels b and c, 10 \( \mu m \).
6.6.4 Effect of co-expression of the \( \gamma \) subunit on the Ca\(_{\text{v}2.2} \) protein levels
(A. Davies and F. J. Moss)

Previous experiments had quantified the number of green fluorescent GFP-Ca\(_{\text{v}2.2}/\beta_{1b} \) transfected cells in the presence or absence of the \( \gamma \) subunit. This gave an indication of the scale of the reduction of GFP-Ca\(_{\text{v}2.2} \) fluorescence when co-expressed with \( \gamma \), but did not provide any suggestion of the mechanism underlying the phenomenon. Detection of the Ca\(_{\text{v}2.2} \) or \( \beta_{1b} \) subunits by western blot aimed to elucidate whether prevention of the correct folding of the pore-forming subunit, more rapid protein degradation, or suppression of VDCC subunit synthesis could explain the previous immunocytochemistry results.

6.6.4.1 Methods

cDNAs. N-terminal GFP tagged rabbit Ca\(_{\text{v}2.2} \) (Raghib \textit{et al.}, 2001b), rabbit Ca\(_{\text{v}2.2}\) \( \Delta 3' \)UTR, rat \( \beta_{1b} \) (X61394, except R417S, V435A, V449A, W492R, V511A, a gift from Prof. T. P. Snutch). The 3' untranslated region (UTR) of rabbit Ca\(_{\text{v}2.2} \) was removed to generate Ca\(_{\text{v}2.2} \) \( \Delta 3' \)UTR (D14157), by introducing an \( \text{Spe I} \) site immediately after the stop codon by PCR and subcloning the Ca\(_{\text{v}2.2} \) back into the vector. All cDNAs were subcloned into expression vector pMT2 (Swick \textit{et al.}, 1992).

COS-7 cells were cultured and transfected as described in Chapter 2, then re-suspended in hypotonic buffer (10 mM Tris, pH 7.4), containing protease inhibitors (Complete EDTA-free, Roche Diagnostics, Lewes, U.K.) and 2 mM EDTA. Aliquots were taken for assay of total lysate protein (BCA, Perbio Science, Chester, UK), and the remainder of each sample was then solubilised in (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer containing 2% SDS. The samples were sonicated briefly (3 x 5 s. on ice) and then centrifuged (10,000 x \( g \), 15 min., 4°C) to remove any insoluble material. Samples (50 \( \mu \)g total protein/lane in Figure 6.35a and 10 \( \mu \)g total protein/lane in Figure 6.35b) were separated by SDS-PAGE using 4-20 % gradient gels and then transferred electrophoretically to polyvinylidene fluoride membranes. The membranes were blocked with 3% BSA for 5 h at 55°C and then incubated overnight at 20°C with a 1:1000 dilution of either an anti-peptide Ab raised in rabbits against residues 846-861.
within the II-III loop of rabbit brain Cav2.2 (Raghib et al., 2001b), or against an Ab raised against whole rat β1b subunit. Secondary Ab (a 1:1000 dilution of goat anti-rabbit IgG-horseradish peroxidase conjugate) was added and the membranes incubated for 1 h. Following extensive washing, bound Abs were detected using enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech, Little Chalfont, UK).

6.6.4.2 Results

GFP-Cav2.2 or untagged Cav2.2 expressed alone in COS-7-cells are detectable by western blot using an antibody (Ab) against the II-III loop of Cav2.2 (band at ~240kDa). When the γ7 subunit was co-expressed with either the GFP-Cav2.2 or untagged Cav2.2 the intensity of the Cav2.2 band was greatly reduced, but expression of the protein was not completely abolished (Figure 6.35a). No smaller molecular weight bands were observed that might represent degradation products of the GFP-Cav2.2 or untagged Cav2.2 or partially synthesized protein. Similar results were obtained both in the absence and presence of auxiliary β1b subunit, and also using the anti-GFP antibody (data not shown). Additionally, the co-expression of γ7 or γ7-myc/his with the Cav2.2/β1b did not reduce the expression of β1b subunit (Figure 6.35b). These data extend the findings from confocal imaging and electrophysiology experiments by revealing that the γ7 subunit suppresses the expression of GFP-Cav2.2 protein rather than interfering with the correct conformational folding or trafficking of the GFP-labeled or untagged protein that could result in reduced fluorescence or recorded current densities.
Figure 6.35 - The effect of co-expression of γ7 subunit on the expression of CaV2.2 in COS-7 cells.

(a) Western blotting and immunodetection using anti-rabbit brain CaV2.2 II-III loop Ab. Lane 1, GFP-CaV2.2; lane 2, GFP-CaV2.2 and γ7; lane 3, CaV2.2 Δ3'UTR; and lane 4, CaV2.2 Δ3'UTR and γ7. Positions of molecular weight markers are shown to the left. Blots are representative of three similar experiments. (b) Western blotting and immunodetection with anti β1b Ab. Lane 1, untransfected COS-7 cells; lane 2, GFP-CaV2.2/β1b; lane 3, GFP-CaV2.2/β1b/γ7; lane 4, GFP-CaV2.2/β1b/γ7--myc/his.
Chapter 7

General Discussion

7

General Discussion
7.1 Identification and cloning of proteins related to stargazin

Using a combination of *in silico* and conventional molecular biology techniques, I have identified and cloned the cDNAs of five members of a family of human proteins according to their homology to the mouse stargazin/γ2 protein. The first of these was the human orthologue of stargazin (Letts *et al*., 1998), the sequence of which was used to identify a further four human paralogues. These proteins were designated γ2, γ3, γ4, γ5 and γ7, due to their homology with stargazin, the first example of a putative neuronal VDCC γ subunit and are encoded by the genes CACNG2, CACNG3, CACNG4, CACNG5 and CACNG7 respectively. Co-incident work from other laboratories has extended the number of members of this family to eight (γ1,8) that have been identified in human, mouse, rat and chick (Black and Lennon, 1999; Burgess *et al*., 1999b; Burgess *et al*., 2001; Chu *et al*., 2001; Kious *et al*., 2002; Klugbauer *et al*., 2000; Letts *et al*., 1998). In a recent study, it was suggested that a ninth putative γ protein and its orthologues, originally designated mouse γ5 (AJ272046) by Klugbauer *et al*. (2000), should not be considered a member of the γ-subunit family of proteins because its primary sequence and its intron/exon gene structure bore little resemblance identity with other family members (approximately 8% protein sequence identity to γ1, and 19% to γ2). It was therefore re-classified “protein distantly related (pr) to the γ subunit family” (Chu *et al*., 2001).

The γ1,8 proteins share a conserved predicted tetra-spanning transmembrane topology, with the N- and C-termini located on the cytoplasmic side of the plasma membrane, a GLWxxC motif in the first extracellular loop linking transmembrane segments 1 and 2, and a pair of cysteine residues in the same extracellular loop. This conserved domain architecture includes the γ subunit family as a subdivision of a super-family of tetra-spanning proteins including peripheral membrane protein PMP22, lens intrinsic membrane proteins (MP20) and the claudin tight-junction forming proteins (PMP22/EMP/MP20/Claudin super-family). Although overall sequence identity between many of these proteins is low, they all contain a region of conserved domain architecture highlighted by the GLWxxC consensus sequence and pair of conserved cysteines (Figure 3.4, Figure 3.8 and Figure 6.9). The functional importance of
possessing this domain architecture is unknown but many proteins that do are known to be involved in cell adhesion events or regulation of cell development. Interestingly, a very recent investigation reported a possible developmental role for the \( \gamma_4 \) subunit, determining that its expression was co-incident with the onset of neuronal differentiation in chick cranial and dorsal root ganglia (Kious et al., 2002).

Analysis of the sequence similarity and chromosomal locations of all eight putative \( \gamma \) subunits reveals their phylogenetic relationship to one another (Figure 7.1). In all cases a higher degree of sequence conservation is observed between \( \gamma \) subunit orthologues than paralogues. This indicates that all the \( \gamma \) subunit isoforms diverged prior to the separation of primate, rodent and possibly even avian orders (Chu et al., 2001). Furthermore the sequence identity between paralogous \( \gamma \) subunits has identified that they can be subdivided into four groups: \( \gamma_2 \) and \( \gamma_3 \) that share 75% identity, \( \gamma_4 \) and \( \gamma_8 \) that share 50% identity, \( \gamma_5 \) and \( \gamma_7 \) that share 70% identity and \( \gamma_1 \) and \( \gamma_6 \) that share 33% identity (but 62% similarity). Identities between groups range from as low as 16% between \( \gamma_8 \) and \( \gamma_6 \), and as high as 50% between \( \gamma_4 \) and \( \gamma_2 \).

![Figure 7.1 - Phylogenetic relationship of human \( \gamma \) subunits](image)

A phylogram of full-length \( \gamma \) subunit protein sequences was constructed by aligning sequences using the CLUSTALX 1.8 program (Thompson et al., 1997) and the neighbour-joining method (Saitou and Nei, 1987). Two members of the distantly related claudin family, claudin 4 and claudin 10 were included as the outgroup to which the tree was rooted. The rectangular cladogram comparing the \( \gamma \) subunit and claudin sequences was generated from the ClustalX output using the TREEVIEW software (Page, 1996).

However, the \( \gamma_2 \), \( \gamma_3 \), \( \gamma_4 \) and \( \gamma_8 \) subunits are more closely related to one another than to the \( \gamma_5 \) and \( \gamma_7 \) or \( \gamma_1 \) and \( \gamma_6 \) subunit branches. These subunits show the closest homology to the mouse protein stargazin and have longer C-termini than the other \( \gamma \) subunits that contain the TTPV consensus motif for interaction with PDZ-domain containing proteins.
General Discussion

(Kornau et al., 1997). This domain has been shown to be critical in mediating the correct trafficking of AMPA receptor subunits to the post-synaptic density through association of the $\gamma_2$, $\gamma_3$, or $\gamma_4$ subunits with the PDZ-domain containing cytoskeletal scaffold protein, PSD-95 (Chen et al., 2000; Chen et al., 2001). $\gamma_5$ and $\gamma_7$ possess an alternative S/TSPC motif in their equivalent region, which although not a classical PDZ-domain binding region, shows close similarity with the TTPV motif. This could be a previously unknown example of a PDZ-domain binding region or is a consensus motif for a different kind of protein-protein interaction. $\gamma_1$ and $\gamma_6$ are approximately 100 or 50 amino acids shorter than the $\gamma_2$ or $\gamma_7$ subunits respectively and do not possess any known protein-protein interaction motifs. These differences in the C-termini of members of each branch of the phylogenetic tree are an indication of possible functional variation between each subdivision of the $\gamma$ subunit family. In addition to sequence variation, a second indication of evolutionary divergence came in the form of different intron/exon gene structure. Unlike all of the other putative $\gamma$ subunits, the $\gamma_5$ and $\gamma_7$ subunits contained five rather than four exons. The identification and cloning of human and mouse $\gamma_5$ and $\gamma_7$ orthologues detailed in Chapter 6 of this study highlighted an additional splice site within the sequence encoding the fourth transmembrane segment. Another laboratory had predicted that these two genes contained only four exons (Burgess et al., 1999b; Burgess et al., 2001). However, amplification of these four exon predictions from human or mouse brain proved impossible (Chapter 6) and the size and sequence homology of their C-terminals bore little resemblance to any of the other known $\gamma$ subunits. It would appear that these four-exon $\gamma_5$ and $\gamma_7$ subunits were predicted as a result of overlooking this fourth splice site, resulting in their predicted C-terminals being encoded by all or part of intron 4 respectively. A more recent extensive genomic analysis of the $\gamma$ subunit family confirmed my own findings (Chu et al., 2001) identifying and amplifying five-exon human, mouse and rat $\gamma_5$ and $\gamma_7$ subunits.

Mapping of the chromosomal locations of each of the human $\gamma$ subunits revealed that $\gamma_2$ is located on Hsa 22q13.1 and $\gamma_3$ on Hsa 16p12-p13.1, whilst $\gamma_1$, $\gamma_4$ and $\gamma_5$ are clustered in close proximity on Hsa 17q24.2 and $\gamma_6$, $\gamma_7$ and $\gamma_8$ are clustered on Hsa 19q13.4. The work of two separate laboratories has deduced that diversity and clustering of $\gamma$ genes arose from tandem duplication that generates paralogues that remain in close proximity

314
on the same chromosome, and chromosome duplication, which results in the simultaneous duplication of many genes on a daughter chromosome (Burgess et al., 1999b; Burgess et al., 2001; Chu et al., 2001). Subsequent gene deletions or unequal crossing-over events led to the arrangement of the eight γ genes on four different human chromosomes (Figure 7.2). Burgess et al. (1999b & 2001) actually used these evolutionary mechanisms to predict the location of new γ family members using the locations of γ1 and γ2 or the closely linked protein kinase C β genes as a starting point (Burgess et al., 1999b; Burgess et al., 2001).

Figure 7.2 – Evolutionary replication of γ subunit genes by tandem or chromosomal duplication

A model of gene duplication based on the phylogenetic analysis and genomic mapping of mammalian γ subunits is consistent with their evolution from a single ancestral gene. Two possible evolutionary pathways (A and B) could have led to the chromosomal distribution of γ subunit genes identified to date. Pathway A contains four evolutionary steps. Following a single round of tandem gene duplication (A1) and chromosome duplication (A2), an unequal crossing-over event (A3) leads to a gene cluster with three paralogous γ genes and an additional single γ gene on another chromosome. A final chromosome duplication event (A4) follows to generate a second homologous gene cluster as well as a second single homologous γ gene. Pathway B contains five steps with two rounds of tandem duplications (B1 and B2) resulting in a gene cluster with three ancestral γ subunit genes. The first round of chromosome duplication (B3) generates another homologous gene cluster. The deletion of two genes from one of the gene clusters (B4) is followed by another round of chromosome duplication (B5). Each pathway results in the chromosomal location reported for human and mouse γ subunits. Reproduced from Chu et al. (2001)

Determination of the chromosomal locations of each γ subunit gene also allowed their comparison with the genetic linkage of neurological disorders that have been mapped and submitted to the OMIM database. Of the five genes identified in this investigation only the location of CACNG3 mapped to a region identified to harbour a disease locus. Benign familial infantile convulsions 2 (BFIC2) is possibly allelic to infantile convulsions and choreoathetosis (ICCA) (Caraballo et al., 2001; Lee et al., 1998), and maps to Hsa 16p12-q12. This is a large region that encompasses the loci of many
genes, including CACNG3, and more precise mapping is required to determine the underlying genetic cause of this disorder.

7.2 Tissue distribution and subcellular localisation

Northern blot analysis determined that the mRNA encoding human \( \gamma_2, \gamma_3, \gamma_4 \) and \( \gamma_7 \) are all exclusively expressed in the CNS. There is little scope for splice variation, and those subunits for which multiple transcripts were detected (\( \gamma_2 \) and \( \gamma_7 \)) probably express mRNAs with different length untranslated regions rather than alternative exons in the coding sequence. Furthermore, the same investigations could not detect additional transcripts that could possibly encode the four-exon versions of the \( \gamma_5 \) and \( \gamma_7 \) subunits. Probing blots of the different brain regions revealed that each \( \gamma \) isoform was differentially expressed throughout the brain, with some subunits being particularly localised to certain tissues. The \( \gamma_2, \gamma_4 \) and \( \gamma_7 \) subunits were expressed in the majority of the regions probed. The \( \gamma_2 \) mRNA is most abundant in cerebellum, and present at slightly lower levels in cerebral cortex, hippocampus and thalamus. The \( \gamma_4 \) transcript was detected at a similar level in most brain regions, but particularly strongly in the striatum. The \( \gamma_7 \) subunit was expressed most strongly in the cerebral cortex and cerebellum, but was well represented in all of the regions probed. The two transcripts were present at different levels in certain tissues, the shorter \( \sim 2.4 \)kb message more prevalent in cerebellum, amygdala, hippocampus and thalamus. Both sizes of mRNA were also detected in the spinal cord and corpus callosum, two tissues that contain significant amounts of white matter. It is therefore a possibility that \( \gamma_7 \) is expressed in glial or Schwann cells. Future in situ hybridisation and immunohistochemistry studies should determine the particular cell types in which this subunit is expressed in each brain region. The \( \gamma_3 \) mRNA was only expressed in cerebral cortex, the putamen, caudate nucleus, amygdala and hippocampus. No transcript was detected in any of the other regions on the blots indicating a lower extent of \( \gamma \) subunit heterogeneity in several tissues including the cerebellum and thalamus. These expression profiles agreed with mouse brain in situ hybridisation studies from other laboratories for the \( \gamma_{2,4} \) subunits (Klugbauer et al., 2000; Letts et al., 1998), but expression studies by RT-PCR have suggested that \( \gamma \) subunits shown to be exclusively neuronal in this study can also be detected in non-neuronal tissues (Burgess et al., 2001; Chu et al., 2001). In particular,
that $\gamma_4$ could also be detected in lung, prostate, atrium and aorta (Burgess et al., 2001; Chu et al., 2001), whilst $\gamma_7$ was detected in cardiac tissue, lung and testis (Chu et al., 2001). In one of these investigations, the $\gamma_1$ subunit, previously only identified in skeletal muscle (Jay et al., 1990; Wissenbach et al., 1998), was also detected at low levels in brain (Burgess et al., 2001). This highlighted the extreme sensitivity of modern PCR techniques and the findings of these investigations should be validated by further in situ hybridisation experiments in these non-neuronal tissues to determine the spatial distribution of the $\gamma_4$ and $\gamma_7$ mRNAs and elucidate whether the genes really are transcribed outside the CNS.

At the time that the sequence of the human $\gamma_5$ subunit was first determined by mining the genetic databases, no EST's containing any CACNG5 sequence had been identified. In order to identify a tissue source from which the cDNA could be amplified, a northern blot using an oligonucleotide probe complementary to a 49-nucleotide region of the predicted gene was performed in this study to determine its tissue localisation. Although multiple transcripts were detected with this probe, none were detected in the brain. Therefore $\gamma_5$ was possibly the first example of a protein identified by its homology to stargazin that was expressed outside the CNS. However, later experiments showed that the $\gamma_5$ cDNA could be amplified from adult mouse brain, and has been detected specifically in human foetal brain and adult rat brain in two separate laboratories (Burgess et al., 1999b; Burgess et al., 2001; Chu et al., 2001). This is not definitive proof that the protein is expressed in CNS neurones as residual amounts of $\gamma_5$ mRNA could be present in brain that may not be translated or alternatively the template may have originated from non-neuronal tissues in the brain (e.g. blood vessels). Further research is required to determine the true distribution of this subunit, but this will be an easier task now that the complete cDNA has been amplified.

The distribution of the $\gamma_2$, $\gamma_3$ and $\gamma_4$ subunits in cerebellum and hippocampus was investigated in more detail by immunohistochemistry. Purkinje cells of the human cerebellum predominantly stain for the $\gamma_2$ subunit in the cell bodies and proximal dendrites, whereas the $\gamma_4$ expression extended throughout the cell well into the dendritic arbours. When compared to other VDCC expressed in Purkinje cells the $\gamma_4$
immunoreactivity most closely resembled Cav2.3 and β3 subunit expression patterns, whilst γ2 expression more closely resembled the generalised immunoreactivity observed for Cav2.1 and β2 (Volsen et al., 1995; Volsen et al., 1997). However there was plenty of overlap with other VDCC subunit expression patterns, particularly the β4 subunit that is the most prevalent β throughout the cerebellum (Volsen et al., 1997). The γ2 and γ4 subunits also have similar distribution to Cav3.1 in cerebellum, especially in Purkinje cells. However, strong biochemical evidence for any member of the Cav3 family of subunits to interact with other VDCC subunits is required before much can be interpreted from the coincident distribution of the γ subunits with Cav3.1. The γ3 subunit protein was detected at very low levels in human cerebellum despite the fact that no message was detected in northern blots, but it was apparently completely absent from mouse cerebellum. The low level of staining that was observed for γ3 in human cerebellum appeared to be predominantly in the interneurones of the molecular and granule cell layers. γ subunit immunoreactivity also determined that in mouse cerebellum, as reported in other investigations the γ2 is expressed strongly throughout the cerebellum (Klugbauer et al., 2000; Letts et al., 1998; Sharp et al., 2001). However, γ4 expression was restricted to the molecular layer and Purkinje cell bodies. Staining in the granule cell layer was extremely weak or absent. This finding added weight to the suggestion that the γ2 subunit is the only γ isoform expressed in cerebellar granule neurones and its loss in the stargazer mice gives rise to the ataxic phenotype because other γ isoforms are not present to substitute for the missing γ2. Away from the cerebellar cortex, γ2 was shown to be expressed well in the cell bodies of the deep cerebellar dentate nucleus neurones, but present only a low levels in the surrounding neuropil, which consists of afferents from the Purkinje neurones and some collaterals from mossy and climbing fibres. Expression of the γ4 subunit in the dentate nuclei was concentrated in the neuropil and was very low in the nuclei cell bodies. This clear differential distribution highlights a possible pre-synaptic role for γ4 and post-synaptic role for γ2 in this structure.

The γ2, γ3 and γ4 subunits show differential but overlapping expression patterns in the human hippocampus and dentate gyrus. Expression patterns of γ2 and γ4 subunits more closely resemble one another than that of γ3, expressed throughout the hippocampus and
dentate gyrus, but with varying intensity in the staining of cell bodies, dendrites and neuropil in the different regions. The $\gamma_3$ subunit localises more specifically to the regions containing high densities of cell bodies, i.e. the strata pyramidale of the hippocampus and the granule cell layer of the dentate gyrus. This suggested possible dendritic and axonal roles for the $\gamma_2$ and $\gamma_4$ subunits whilst $\gamma_3$ function is probably confined to the cell soma.

Although subcellular distribution of the $\gamma$ subunits in these tissue samples could only be hypothesised from the observed staining patterns, transient expression of $\gamma_2$, $\gamma_3$ and $\gamma_4$ subunits in COS-7 cell determined that all were preferentially located in the plasma membrane when expressed alone or in conjunction with a VDCC complex. However, the $\gamma_7$ subunit was expressed throughout the cell but appears to be poorly, if at all inserted in to the plasma membrane.

7.3 Functional properties of the neuronal $\gamma$ subunits

Modulation by the $\gamma_2$ or the $\gamma_4$ subunits of Ba$^{2+}$ currents via recombinant Cav2.1/$\beta_4$ VDCCs with or without an $\alpha 2\delta$ subunit was only very slight. The hyperpolarising shifts in the voltage dependence of steady-state inactivation reported by other laboratories could not be reproduced (Klugbauer et al., 2000; Letts et al., 1998; Rousset et al., 2001) with the exception of when the $\gamma_2$ subunit was co-expressed with the Cav2.1/$\beta_4$ channel in the absence of an $\alpha 2\delta$ subunit. It is therefore possible that inclusion of an $\alpha 2\delta$ subunit as part of the VDCC complex somehow masks or prevents the modulation by the $\gamma_2$ subunit. Additionally, it has been reported that the absence of the $\gamma_2$ subunit in the stargazer mouse does not significantly alter activation or inactivation properties of whole cell VDCC currents recorded from cerebellar granule cells (Chen et al., 2000). The variations between the findings of this report and other laboratories are probably in part due to the different subunit combinations expressed and/or the charge carriers used (Ca$^{2+}$ or Ba$^{2+}$), but in all instances the modulation of VDCC (no matter the constituent subunits) is small and the affected parameters of activation or inactivation vary. However, one laboratory has reported that $\gamma_2$ co-expression caused a significant decrease in the current amplitude of both Cav2.1 and Cav2.2 Ca$^{2+}$ channels (Kang et al.,
Chapter 7

General Discussion

2001). Interestingly, the effects of the \( \gamma_2 \) subunit on current amplitude were dependent on the co-expression of the \( \alpha_2 \delta_1 \) subunit in a manner similar to that which had been previously reported for the skeletal muscle \( \gamma_1 \) subunit (Singer et al., 1991; Wei et al., 1991). Moreover, the reduction of peak \( \text{Ca}^{2+} \) current magnitude by \( \gamma_2 \) co-expression bore a similarity to the reduction in \( \text{Ca}^{2+} \) entry into skeletal muscle myotubes expressing the \( \gamma_1 \) subunit compared to myotubes of \( \gamma_1 \) knockout mice (Ahern et al., 2001; Freise et al., 2000). However the same investigation showed no significant changes in the steady-state inactivation properties that were one of the main pieces of evidence that led to the original classification of \( \gamma_2 \) as a VDCC subunit (Letts et al., 1998). Nevertheless, it has been shown that the \( \gamma_2 \) subunits co-sediments and co-immunoprecipitates with Cav2.2 VDCC subunits in vivo (Kang et al., 2001; Sharp et al., 2001). Whether this is a direct interaction or a result of the two proteins being linked via another VDCC subunit or cytoskeletal protein remains to be determined.

With much research concerning stargazin and its family members centering on their putative role as VDCC auxiliary subunits, Chen and colleagues reported that \( \gamma_2 \), and the closely related \( \gamma_3 \) and \( \gamma_4 \) subunits, are able to rescue cell surface expression of AMPA receptor subunits in the cerebellar granule cells of stargazer mice (Chen et al., 2000; Chen et al., 2001). They proposed that a major role of these proteins was to traffic the AMPA receptor to its correct somatic location in a two-step process. Firstly interaction between the GluR AMPA receptor subunits and \( \gamma_2 \) promotes the insertion of the AMPA receptor into the plasma membrane, then an interaction of the C-terminal PDZ-interaction motif of \( \gamma_2 \) with PSD-95 facilitated the correct trafficking of the whole complex to the post-synaptic density, where the AMPA receptor gates the fast component of glutamate activated post-synaptic current. Interestingly, this behaviour is selective for AMPA receptor subunits over kainate or NMDA receptor subunits (Chen et al., 2001). How association with VDCC subunits fits into this process remains to be determined, but it is possible that \( \gamma_2 \) and its close homologues may participate in the trafficking and/or membrane anchoring of many synaptic proteins. The high density of stargazin-like proteins in the synaptic regions (Sharp et al., 2001) could also play a role in maintaining the structural integrity of the synapse. The possession of the PMP-22/EMP/MP20/Claudin motif in the first extracellular loop between transmembrane segments 1 and 2 may permit the stargazin-like subunits to behave similarly to the
claudin subunits that form tight junctions in epithelial tissues. Juxtaposed γ subunits on the exofacial surfaces either side of the synapse may form kissing points that maintain the contact between the two synaptic boutons (Tomita et al., 2001).

Analysis of the gene structure and sequence of the γ5 and γ7 subunits suggested that they possibly have an alternative functional role compared to other members of the family of proteins identified by their homology to stargazin. Work in this investigation concentrated upon the properties of recombinant γ7 when transiently expressed in different expression systems. The first indication that γ7 behaved differently from the γ2, γ3 or γ4 subunits came when immunocytochemistry revealed that when the γ7 was transfected into COS-7 cells it expressed throughout the cytoplasm, but did not show clear plasma membrane insertion. However, when co-expressed with a VDCC Cav2.2 subunit gating N-type currents in COS-7 cells or Xenopus oocytes the results were astounding and completely unlike anything previously reported for a putative ion-channel regulatory protein. The γ7 subunit almost completely abolished the expression of Cav2.2 N-type VDCC current in Xenopus oocytes and COS-7 cells and also significantly reduced the maximum Ba^{2+} conductance in the non N-type Cav2.1 and Cav1.2 channels, but the effects were not so pronounced. In contrast to the previously reported reduction of Cav2.2 current by the γ2 subunit (Kang et al., 2001), the effects of the γ7 subunit were not dependent on the co-expression of an α2δ subunit. Immunocytochemistry and western blot determined that co-expression of the γ7 subunit resulted in almost the complete loss of Cav2.2 expression, which was likely to be due to a reduction in Cav2.2 synthesis rather than degradation of the α1 subunit because no smaller molecular weight products were detected with the anti-Cav2.2 antibody. However, γ7 could induce rapid and complete targeting of the Cav2.2 subunit to a degradation pathway precluding detection of Cav2.2 fragments. Nevertheless, reduction of ion channel expression was not a non-specific effect because the γ7 fails to reduce the expression or alter cellular localisation of the potassium channel Kv3.1b in Xenopus oocytes and COS-7 cells. Although a mechanism for this process of reducing VDCC Cav2.2 expression remains to be determined, it would appear that the influence of the γ7 subunit was upon newly synthesised protein, because it failed to reduce N-type currents endogenously expressed by sympathetic neurones of the superior cervical ganglion.
Overall, the functional properties of γ7 identified in this body of work are unlike anything previously reported for any of the proteins with homology to stargazin, but its effects are apparently specific for reducing VDCC subunit expression, particularly Cav2.2. That γ7 is actually part of a functional plasma membrane VDCC appears unlikely, at least for Cav2.2. This is reinforced by the absence of significant endogenous γ7 in sympathetic neurones, where the current is predominantly N-type. Future work will examine in which cell types γ7 is expressed endogenously in the brain and whether its endogenous function is to down regulate Cav2.2 expression.

### 7.4 Proposed Future Research

1) Mutation/deletion and yeast-two hybrid studies to determine protein-protein interactions for each of the subunits identified in this investigation. In particular these should be performed for the γ7 and γ5 subunits for which a true in vivo function remains to be determined. Mutational analysis will also help determine some of the roles of conserved regions elsewhere in the γ subunits, i.e. the pair of conserved cysteines in the first extracellular loop and the PMP22/EMP/MP20/Claudinlike motifs.

2) Co-immunoprecipitation of VDCC subunits or other proteins from transfected mammalian cells or from native tissue with the neuronal γ subunits would determine binding partners with which the γ subunits associate both in vitro and in vivo. Does each γ subunit preferentially associate with a particular VDCC complex or AMPA receptor complex?

3) Confirming the subcellular distribution of γ subunits when expressed in Xenopus oocytes by fixing, sectioning and staining oocytes injected with the γ subunits alone or in combination with other VDCC subunits.

4) The membrane distribution of the γ2, γ3 and γ4 subunits could be confirmed by co-staining transfected COS-7 cells with the fluorescently conjugated phalloidin.
markers (Molecular probes, Oregon, USA). These markers delineate the plasma membrane by staining the actin cytoskeleton just beneath its surface.

5) More detailed investigation of brain mRNA and protein distribution for each γ subunit in rodent and human, especially γ7 and γ5. In situ hybridisation and immunohistochemistry may also highlight regions where the mRNA is transcribed but protein is not translated. Furthermore, they could determine whether the reduction in expression of Cav2.2 by γ7 is an artefact of the in vitro expression systems used in this study or whether it also occurs in vivo by ascertaining if Cav2.2 and γ7 are endogenously expressed in the same cells at both the protein and mRNA levels. Alternatively the same goal could be achieved by recording the endogenous VDCC currents from cells in which γ7 is shown to be endogenously expressed.

6) Northern blot for cells transiently expressing Cav2.2 and γ7 will determine if reduction in Cav2.2 expression is solely at the protein level or is a function of reduced Cav2.2 mRNA.

7) RNAse protection assays could be used to confirm that the four exon γ7 and γ5 subunits are not expressed in vivo.

8) Immunocytochemistry could be employed to ascertain whether co-expression of a MAGUK influence the expression pattern of γ7, or a γ7/VDCC complex in the same way that co expression of PSD95 with γ2 and the GluR4 subunit facilitated the trafficking of AMPA receptors to the post-synaptic density (Chen et al., 2000).

7.5 Concluding remarks

The members of a family of tetra-spanning transmembrane proteins identified and cloned in this study, together with those identified by other laboratories are diverse in both their sequence homologies and potential in vivo functions. Whether, as originally proposed they form part of functional VDCCs in vivo remains an open question.
Chapter 7

General Discussion

Biophysical evidence for modulation of VDCC current by any member of the \( \gamma \) family is inconsistent, but biochemical research has shown a physical interaction between the \( \gamma_2 \) subunit and Cav2.2. However, certain members of the family with the closest homology to the stargazin protein are certainly involved in the trafficking of AMPA receptors to their correct location in the cell. The role of the \( \gamma_? \) subunit and its close homologue \( \gamma_5 \) is somewhat of an enigma. The reduction of Cav2.2 expression on co-transfection of \( \gamma_7 \) in heterologous expression systems is striking in both its magnitude and selectivity. A great deal of further research is required to determine if its endogenous function is the same.
Acknowledgements

I have been very fortunate to work with two highly professional and welcoming groups during the course of my Ph. D. studies. The close support and attention I have received from my co-workers at University College London and GlaxoSmithKline has been excellent. Firstly I wish to thank my co-supervisors, Annette Dolphin (UCL) and Jeff Clare (GlaxoSmithKline, Stevenage, UK) who have expertly guided me through this project, patiently listened to all my queries and concerns no matter how trivial, and enthusiastically supported my efforts in the pursuit of both this investigation and my future career aspirations.

Thank you to every member of Annette Dolphin's laboratory, both past and present, with which I have had the pleasure to work with these last few years. My gratitude is extended to Carles Canti (whose patience knows no bounds) for teaching me *Xenopus* oocyte preparation and two-electrode voltage clamp electrophysiology, to Ayesha Raghib for teaching me the immunocytochemistry and confocal laser scanning microscopy techniques with the help of Darren Clements from the UCL confocal unit, and to Tony Davies for his resolute western blotting efforts and biochemistry advice for which Nick Berrow and Adrian Butcher also deserve thanks. Jens Brodbeck, Jo-Maree Courtney, Manuela Nieto-Rostro, Karen Page, Wendy Pratt and Jack Wratten, all deserve thanks for their technical assistance and it was a great pleasure to supervise Alex Graham who provided sterling assistance during his summer studentship in the laboratory. Special thanks are reserved for Federica Bertaso and Patricia Viard who graciously allowed me to invade their part of the laboratory during my final months in the lab, guided me through some of the mysteries of analysis of electrophysiology data, and who worked so hard to produce the patch-clamp results in my paper. I only hope that in return I at least provided some entertainment even if I was unable to improve the quality of the English spoken in that room.

I also wish to thank everyone with whom I have worked in the Ion Channels Group at GSK, Stevenage. In particular Stephen Burbidge, who sacrificed much of his own valuable time to introduce me to the delights of molecular biology, and Andy Powell
**Acknowledgements**

who has acted as a sounding board for many of my ideas and organised the arrival of many of the materials I required to coincide with my frequent visits to the laboratory in Stevenage. My gratitude is also extended to Chris Plumpton, whose time and efforts were fundamental in the generation of γ subunit specific antibodies, Mary Plumpton, Phillippe Sanseau and Daniel Crowther who performed the initial bioinformatics searches that gave birth to the whole project and also reviewed much of the bioinformatics presented in this thesis, and Mick Hurle and Margaret Flint who allowed me to invade their immunohistochemistry laboratory, provided access to a source of human tissue and taught me all the necessary histochemistry techniques.

Outside of the laboratory special thanks are reserved for “The Usual Suspects” for restoring sanity on a regular basis, through healthy debate over a pint of cold Guinness (or is that Kilkenny?). I look forward to regular reunions in the Californian sunshine. I also salute the “Easy Riders”. If I ever decide to give up the riches and trappings of science, I’ll always have a career in Formula 1 to fall back upon. Lastly, but by no means least, thank you to Jenny Davies for providing great friendship and cheer, no matter how far I put her nose out of joint! Coffee-time crosswords and the state of my shins will never quite be the same!
Publications and meeting abstracts

Journal articles


Conference posters


Glossary of terms

Ab antibody
ADP adenosine diphosphate
A_{fast} amplitude of the fast component of current inactivation
AID \(\alpha_1\)-subunit interaction domain
AKAP A-kinase anchoring protein
AKAP-15/18 15/18 kDa A-kinase anchoring protein
AKAP-79 79 kDa A-kinase anchoring protein
AMFE anomalous mole fraction effect
AMPA alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate
ANOVA analysis of variance
AP adaptor primer
A_{slow} amplitude of the slow component of current inactivation
ATP adenosine triphosphate
Ba^{2+} barium ion
BAC bacterial artificial chromosome
BAPTA \(K_{3-1,2}\)-bis(aminophenoxy)ethane-\(N,N,N',N'\)-tetra-acetic acid
BDNF brain derived neurotrophic factor
BGH bovine growth hormone
BHK baby hamster kidney
BID \(\beta\)-subunit interaction domain
BLAST Basic Local Alignment Search Tool
BLASTn BLAST search directly comparing nucleotide against nucleotide
\(t\)BLASTn BLAST search comparing protein query sequence to nucleotide sequence
\(t\)BLASTx BLAST search comparing the six-frame translations of a nucleotide query sequence against the six-frame translations of a nucleotide sequence database
bp base pair
BTZ benzothiazepine
\(\beta_{xo}\) \textit{Xenopus} VDCC \(\beta\) subunit
C-terminus Carboxyl terminus of a protein
cDNA complementary DNA
### Glossary of terms

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
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<tbody>
<tr>
<td>cAMP</td>
<td>cyclic 3', 5'-adenosine monophosphate</td>
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<tr>
<td>cGMP</td>
<td>cyclic 3', 5'-guanosine monophosphate</td>
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<tr>
<td>Ca$^{2+}$</td>
<td>calcium ion</td>
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<td>[Ca$^{2+}$]$_i$</td>
<td>intracellular free calcium concentration</td>
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<td>CaMKII</td>
<td>Ca$^{2+}$/calmodulin-regulated protein kinase II</td>
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<td>Ca$_V$-α$_1$</td>
<td>voltage-dependent calcium channel alpha 1 subunit</td>
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<tr>
<td>Ca$_V$-β</td>
<td>voltage-dependent calcium channel beta subunit</td>
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<td>Cd$^{2+}$</td>
<td>cadmium ion</td>
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<td>CHO</td>
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<td>CLSM</td>
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<td>dideoxynucleotide</td>
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<td>dideoxthymidine triphosphate</td>
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<td>ddCTP</td>
<td>dideoxycytidine triphosphate</td>
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<td>DAG</td>
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<td>DAPI</td>
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<td>DCN</td>
<td>dentate cerebellar nucleus</td>
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<td>DDBJ</td>
<td>DNA DataBank of Japan</td>
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<td>Term</td>
<td>Definition</td>
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<tr>
<td>DEPC</td>
<td>diethyl-pyrocarbonate</td>
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<td>DHFR</td>
<td>dihydrofolate reductase</td>
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<td>dihydroxypidine</td>
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<td>DHPR</td>
<td>dihydroxypidine receptor</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
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<tr>
<td>DNA</td>
<td>deoxyribose nucleic acid</td>
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<tr>
<td>ΔNP</td>
<td>Cav2.1 splice variant lacking asparagine and proline residues at positions 1605 and 1606</td>
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<tr>
<td>Dom X</td>
<td>transmembrane domain X</td>
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<tr>
<td>DOPE</td>
<td>dioleoyl phosphatidylethanolamine</td>
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<td>dud</td>
<td>ducky absence epilepsy mouse</td>
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<td>EA-2</td>
<td>episodic ataxia type-2</td>
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<tr>
<td>ECC</td>
<td>excitation-contraction coupling</td>
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<td>ECL</td>
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<td>EEG</td>
<td>electroencephalogram</td>
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<td>EMBL</td>
<td>European Molecular Biology Laboratory</td>
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<tr>
<td>EPSC</td>
<td>excitatory post-synaptic current</td>
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<td>expressed sequence tag</td>
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<td>EtBr</td>
<td>ethidium bromide</td>
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<tr>
<td>ETn</td>
<td>early transposon</td>
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<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<td>FHM</td>
<td>familial hemiplegic migraine</td>
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<td>G-protein</td>
<td>GTP-binding protein</td>
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<td>G_{max}</td>
<td>maximum conductance</td>
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<td>GABA</td>
<td>γ-amino butyric acid</td>
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<td>GCs</td>
<td>cerebellar granule cells</td>
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<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>GSP</td>
<td>gene specific primer</td>
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<td>H_{0}</td>
<td>null hypothesis</td>
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<td>HRP</td>
<td>horse radish peroxidase</td>
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<td>Hsa</td>
<td>Homo sapiens chromosome</td>
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<tr>
<td>HSD</td>
<td>honestly significant difference</td>
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<tr>
<td>HTGS</td>
<td>high throughput genomic sequence</td>
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Glossary of terms

HVA  high voltage-activated
HypoPP  hypokalaemic periodic paralysis
I  current
IE  current passing electrode
I_{max}  maximum current
I-V  current-voltage
I-II loop  intracellular loop linking domain I and II of VDCC or voltage dependent Na^+ channels
IMS  industrial methylated spirits
in silico  computer based predictions or simulations of biological data
in vitro  in a heterologous environment
in vivo  in an endogenous environment
IR  immunoreactivity
K^+  potassium ion
k_{activ}  the slope factor of the activation curve
kb  kilobase
kDa  kilodalton
k_{inact}  the slope factor of steady-state inactivation
K_V  voltage-dependent potassium channel subunit
LB  Luria-Bertani media
LEMS  Lambert-Eaton myasthenic syndrome
lh  lethargic absence epilepsy mouse
LVA  Low voltage-activated
MAGUK  membrane-associated guanylate kinase
MCS  multiple cloning site
MF  mossy fibre
mh  mocha absence epilepsy mouse
M-MLV  Moloney murine leukaemia virus
mRNA  messenger RNA
MTS  methanethiosulfonate
MW  molecular weight
myc/his  c-myc / 6 histidine fusion protein antigen epitope
Na^+  sodium ion
<table>
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<tr>
<td>NBCS</td>
<td>New born calf serum</td>
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<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
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<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
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<tr>
<td>NGSP</td>
<td>Nested gene specific primer</td>
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<tr>
<td>Ni&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Nickel ion</td>
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<tr>
<td>NGF</td>
<td>N-Methyl-D-aspartate</td>
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<td>nt</td>
<td>Nucleotide</td>
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<td>N-terminus</td>
<td>Amino terminus of a protein</td>
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<td>oligo</td>
<td>Oligonucleotide</td>
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<td>Open reading frame</td>
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<td>Phenylalkylamine</td>
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<td>Phosphate Buffered Saline</td>
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<td>Polymerase chain reaction</td>
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<td>Pheochromocytoma cells</td>
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<td>Post synaptic-Disc-Zona occludens I</td>
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<td>Pfu</td>
<td><em>Pyrococcus furiosus</em> DNA Polymerase</td>
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<td>PKA</td>
<td>cAMP dependent protein kinase / Protein kinase A</td>
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<tr>
<td>PKC</td>
<td>DAG dependent protein kinase / Protein kinase C</td>
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<td>P-loop</td>
<td>Pore loop</td>
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<td>PMT</td>
<td>Photomultiplier tube</td>
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<tr>
<td>P/S</td>
<td>Penicillin/streptomycin</td>
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<tr>
<td>PSD-95</td>
<td>Post-synaptic density protein of 95kDa</td>
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<tr>
<td>RACE</td>
<td>Rapid amplification of cDNA ends</td>
</tr>
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<td>RE</td>
<td>Reference electrode</td>
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<tr>
<td>RNA</td>
<td>Ribose nucleic acid</td>
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<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
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<td>rRNA</td>
<td>Ribosomal RNA</td>
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<td>RNase</td>
<td>Ribonuclease</td>
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<td>Ribonuclease protection assay</td>
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<td>RT</td>
<td>Reverse transcriptase</td>
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<td>RyR</td>
<td>Ryanodine receptor</td>
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<td>SCG</td>
<td>Superior cervical ganglion</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
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### Glossary of terms

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
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<tr>
<td>SDS-PAGE</td>
<td>sodium dodecylsulphate-polyacrylamide gel electrophoresis</td>
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<tr>
<td>S.E.M.</td>
<td>standard error of the mean</td>
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<tr>
<td>SMART</td>
<td>Switching Mechanism At 5' end of RNA Transcript</td>
</tr>
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<td>SNAP-25</td>
<td>synaptosome-associated protein of 25 kDa</td>
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<td>SNARE</td>
<td>soluble NSF attachment protein receptor</td>
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<td>SR</td>
<td>sarcoplasmic reticulum</td>
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<td>ss</td>
<td>single-stranded</td>
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<td>SSI</td>
<td>steady-state inactivation</td>
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<td>stg</td>
<td>stargazer absence epilepsy mouse</td>
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<td>stargazin</td>
<td>the gene mutated in the stargazer mouse</td>
</tr>
<tr>
<td>stargazin</td>
<td>the protein mutated in the stargazer mouse</td>
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<td>SWD</td>
<td>spike wave discharges</td>
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<tr>
<td>swe</td>
<td>slow wave epilepsy</td>
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<td>Synprint</td>
<td>synaptic protein interaction site</td>
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<td>75cm$^2$ vented flask</td>
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<td>Taq</td>
<td><em>Thermus aquaticus</em> DNA Polymerase</td>
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<td>TAE</td>
<td>Tris acetate EDTA</td>
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<td>TBS</td>
<td>Tris Buffered Saline</td>
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<td>TEVC</td>
<td>two-electrode voltage clamp</td>
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<td>$\tau_{fast}$</td>
<td>time constant for the fast component of current inactivation</td>
</tr>
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<td>TFR</td>
<td>Transferrin receptor</td>
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<td>tottering absence epilepsy mouse</td>
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<td>TJ</td>
<td>tight junction</td>
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<td>transmembrane segment</td>
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<td>melting temperature</td>
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<td>tRNA</td>
<td>total RNA</td>
</tr>
<tr>
<td>TrkB</td>
<td>BDNF receptor</td>
</tr>
<tr>
<td>$\tau_{slow}$</td>
<td>time constant for the slow component of current inactivation</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>V</td>
<td>voltage</td>
</tr>
<tr>
<td>$V_{0.5activ}$</td>
<td>the half-maximal value for the voltage dependence of activation</td>
</tr>
<tr>
<td>$V_{50inact}$</td>
<td>the half-maximal voltage for current inactivation</td>
</tr>
<tr>
<td>$V_{cmd}$</td>
<td>command potential</td>
</tr>
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333
### Glossary of terms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>VDCC</td>
<td>voltage dependent calcium channel</td>
</tr>
<tr>
<td>VE</td>
<td>voltage recording electrode</td>
</tr>
<tr>
<td>Vm</td>
<td>membrane voltage</td>
</tr>
<tr>
<td>V_{rev}</td>
<td>current reversal potential</td>
</tr>
<tr>
<td>ω-AgaIVA</td>
<td>ω-agatoxin IVA</td>
</tr>
<tr>
<td>ω-ctx GVIA</td>
<td>ω-conotoxin GVIA</td>
</tr>
<tr>
<td>ω-ctx MVIIA</td>
<td>ω-conotoxin MVIIA</td>
</tr>
<tr>
<td>ω-ctx MVIIC</td>
<td>ω-conotoxin MVIIC</td>
</tr>
<tr>
<td>wag</td>
<td>waggler absence epilepsy mouse</td>
</tr>
<tr>
<td>wt</td>
<td>wild-type</td>
</tr>
<tr>
<td>YAC</td>
<td>yeast artificial chromosome</td>
</tr>
<tr>
<td>ZO</td>
<td>zona occludens</td>
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<tr>
<td>+/-</td>
<td>wild-type</td>
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<tr>
<td>-/-</td>
<td>knockout</td>
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<tr>
<td>-/+</td>
<td>heterozygote</td>
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