The glycine transporter GlyT2: mutations, interactors and diseases

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ABSTRACT

Hyperekplexia or startle disease (OMIM: 149400) is caused by defects in mammalian glycinergic neurotransmission, resulting in a complex motor disorder characterised by neonatal hypertonia and an exaggerated startle reflex. This affects newborn children and is characterised by noise or touch-induced seizures which result in muscle stiffness and breath-holding episodes that can lead to brain damage and/or sudden infant death. Hyperekplexia is caused by mutations in the glycine receptor α1 and β subunit genes. However, a significant proportion of hyperekplexia patients do not have mutations in these genes. Mutations in the presynaptic glycine transporter 2 (GlyT2) gene, SLC6A5, are a recently discovered cause of hyperekplexia (Rees et al., 2006). This work aims to study the functional consequences of novel SLC6A5 mutations, to characterise new GlyT2 interacting proteins as candidates for genetic studies in hyperekplexia, and to determine whether any animal disorders linked to glycinergic dysfunction might have defects in SLC6A5. The functional consequences of hyperekplexia mutations in SLC6A5 were assessed using [3H]-glycine uptake assays and immunocytochemistry and revealed defective glycine uptake compared to controls, often resulting from aberrant trafficking of the transporter to the cell membrane. Using the GAL4 yeast two-hybrid (YTH) system, seven new proteins potentially involved in the localisation and trafficking of GlyT2 were identified, of which the cytoskeletal protein FMNL2 appears to be the most promising candidate for genetic analysis in hyperekplexia. Lastly, a new example of GlyT2 dysfunction in animals was characterised: congenital muscular dystonia 2 (CMD2) in Belgian blue cattle. Whole-genome screening and DNA sequencing of candidate genes revealed a missense mutation in SLC6A5, resulting in a L270P substitution in the third membrane-spanning domain of GlyT2. Functional studies showed that this alteration abolishes [3H]-glycine uptake in recombinant systems. Identification of this mutation had an immediate translation into breeding practice, allowing marker assisted selection against CMD2 by avoiding ‘at risk’ matings.
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This thesis describes research conducted in the School of Pharmacy, University of London between October 2005 and August 2008 under the supervision of Professor Robert J. Harvey. I certify that the research described is original and that any parts of the work that have been conducted by collaboration are clearly indicated, I also certify that I have written all the text herein and have clearly indicated by suitable citation any part of this dissertation that has already appeared in a publication.

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Eloisa Carta
CONTENTS
List of figures ..............................................................................................................12
List of tables ...............................................................................................................15

1. INTRODUCTION .............................................................................16
   1.1. Hyperekplexia ........................................................................................17
   1.2. The glycinergic inhibitory synapse ................................................................18
   1.3. Animal defects in GlyR dysfunction ...................................................20
   1.4. Postsynaptic causes of human hyperekplexia ...............................23
   1.5. Glycine transporters: essential regulators of neurotransmission .................................................................................27
       1.5.1. The SLC6 transporter superfamily ...................................................27
       1.5.2. Characterisation and differential expression of the glycine transporters GlyT1 and GlyT2 ........................................................28
       1.5.3. Function of glycine transporters at inhibitory synapses .................30
   1.6. Glycine transporters mouse knockouts as models for human disease .....................................................................................................31
       1.6.1. GlyT1 knockout mice reveal an essential role for GlyT1 in terminating glycinergic neurotransmission .....................................31
       1.6.2. GlyT1 knockout mice: a new animal model of glycine encephalopathy ........................................................................32
       1.6.3. GlyT1 knockout mice: studies on excitatory NMDARs .....................33
       1.6.4. GlyT2 knockout mice: a new model for human hyperekplexia ......34
   1.7. Presynaptic components of human hyperekplexia .......................35
   1.8. Thesis aims ............................................................................................37

2. METHODS ........................................................................................39
   2.1. Bacterial methodology ........................................................................ 40
       2.1.1. Materials.........................................................................................40
       2.1.2. Bacteria culture ............................................................................41
       2.1.3. Preparation of competent E. coli cells ...........................................................................................................40
       2.1.4. Transformation of competent E. coli cells ...................................41
       2.1.5. Small-scale preparation of plasmid DNA (minipreps) .................41
       2.1.6. Large-scale preparation of plasmid DNA (maxipreps) ..................42
2.2. DNA methodology .................................................................43
   2.2.1. Materials ........................................................................43
   2.2.2. Plasmid construct generation ......................................44
   2.2.3. Polymerase Chain Reaction (PCR) ...............................44
   2.2.4. Oligonucleotide primers ................................................47
   2.2.5. Agarose gel electrophoresis .........................................48
   2.2.6. Agarose gel purification of DNA fragments .................48
   2.2.7. Restriction enzyme digestion ........................................49
   2.2.8. Phenol-Chloroform extraction ......................................49
   2.2.9. Ligation of DNA inserts into plasmid vectors ..............50
   2.2.10. Restriction enzyme digestion to remove recircularised vector 50
   2.2.11. DNA sequencing ..........................................................50
   2.2.12. Rapid Amplification of cDNA Ends (RACE) ...............51
   2.2.13. TOPO cloning ...............................................................55
   2.2.14. Site-directed mutagenesis ............................................55

2.3. Yeast methodology .................................................................57
   2.3.1. Materials ........................................................................57
   2.3.2. Maintenance of yeast ....................................................57
   2.3.3. Yeast recovery and growth ............................................58
   2.3.4. Transformation of competent yeast cells .......................58
   2.3.5. The LacZ freeze-fracture assay ....................................59
   2.3.6. YTH library screening ..................................................60
   2.3.7. Analysis of putative positive clones from the library screen ......62

2.4. Cell culture methodology .......................................................63
   2.4.1. Materials ........................................................................63
   2.4.2. The cell culture laboratory and equipment ....................64
   2.4.3. Preparation of cell culture medium .................................65
   2.4.4. Thawing of cryopreserved cells ....................................66
   2.4.5. Maintaining of HEK293 cells in culture ..........................66
   2.4.6. Cryopreservation of cells ...............................................66
   2.4.7. Transfection of cells with plasmid DNA ..........................66
   2.4.8. Immunocytochemistry ....................................................66
   2.4.9. Confocal microscopy ......................................................67
   2.4.10. [H]-glycine uptake assays .............................................67
3. NEW MUTATIONS IN GLYT2 GENE (SLC6A5) IN INDIVIDUALS WITH HYPEREKPLEXIA

3.1. Identification of new hyperekplexia mutations in the human GlyT2 gene (SLC6A5)

3.2. Study aims

3.3. Identification of novel N- and C-terminal exons in the GlyT2 gene using 5' and 3' rapid amplification of cDNA ends (RACE)

3.3.1. Alternative splicing of human GlyT2 transcripts

3.3.2. 5' RACE reveals two novel GlyT2 N-termini

3.3.3. 3' RACE reveals an additional C-terminal GlyT2 isoform

3.4. Further characterisation of the novel isoform GlyT2-NFS

3.4.1. Cloning of pRc/CMV-hGlyT2-NFS

3.4.2. Functional activity of the hGlyT2 variants

3.5. Functional characterisation of new mutations in SLC6A5

3.5.1. Generation of pRc/CMV-hGlyT2 and pRK5myc-hGlyT2 mutants

3.5.2. Subcellular localisation of new hGlyT2 hyperekplexia mutants

3.5.3. Functional characterisation of hGlyT2 mutants

3.6. Discussion

3.6.1. Identification of new hyperekplexia mutations and splicing patterns in the human GlyT2 gene

3.6.2. GlyT2-NFS: a new C-terminal GlyT2 variant

3.6.3. New GlyT2 hyperekplexia mutations affect subcellular localisation and/or functionality of the transporter

3.6.4. W151X, A275T and R439X do not exert dominant-negative effects

4. IDENTIFICATION OF GLYT2 INTERACTING PROTEINS WITH THE YTH TECHNIQUE

4.1. GlyT2 interacting proteins are presynaptic candidates for genetic analysis in hyperekplexia

4.2. Characterising new GlyT2 interacting proteins using the YTH system

4.2.1. The basis of the YTH system

4.2.2. Yeast reporter strains

4.2.3. The LacZ and HIS3 reporter genes
4.3 Study aims

4.4 Identification of non autoactivating, functional GlyT2 baits
   4.4.1. pYTH16-GlyT2N bait and pACT2-ULIP6 construct testing
   4.4.2. pYTH16-GlyT2C bait and pACT2-syntenin-1 construct testing

4.5 cDNA library screening with pYTH16-GlyT2C bait
   4.5.1. Choice and amplification of a cDNA library
   4.5.2. Library transformation
   4.5.3. Selection of positive clones and analysis of plasmid DNAs
   4.5.4. Eliminating false positives

4.6. The PDZ binding motif in GlyT2C is necessary and sufficient for interactions with diverse PDZ domain containing proteins

4.7. Selectivity of newly identified GlyT2 interactors for GlyT1 versus GlyT2

4.8. Discussion
   4.8.1. Syntrophin-a1
   4.8.2. LNX1
   4.8.3. MAGI1
   4.8.4. SAP97
   4.8.5. SAP102
   4.8.6. PICK-1
   4.8.7. FMNL2

4.9. The specific interaction of GlyT1 and GlyT2 with diverse PDZ domain containing proteins is mediated via C-terminal type I and type III motifs

5. PRIORITISING NEW GLYT2 INTERACTING PROTEINS FOR GENETIC ANALYSIS IN HYPEREKPLEXIA
   5.1. Biological relevance of the novel GlyT2 interacting proteins
   5.2. Study aims
   5.3. Bioinformatic study of novel GlyT2 interacting proteins: gene locations, knockout mouse models and associated human diseases
   5.4. FMNL2 gene structure, alternative splicing and cDNA cloning
   5.4.1. Generation of a FLAG-tagged FMNL2 construct
5.5. Investigating possible co-localisation of GlyT2 and FMNL2 in mammalian cells using immunofluorescence and confocal microscopy .................................................. 126

5.6. Functional consequences of co-expression of GlyT1 or GlyT2 with PICK-1 and FMNL2 using [3H]-glycine uptake assays ........................................ 128

5.7. Discussion .................................................................................................. 134
  5.7.1. Functional effect of PICK-1 on GlyT-mediated uptake .................... 135
  5.7.2. FMNL2: a new candidate gene for genetic screening in hyperekplexia? .............................................................................. 136

6. GLYT2 DYSFUNCTION IN CONGENITAL MUSCULAR DYSTONIA TYPE 2 .................................................. 138

6.1. Congenital Muscular Dystonia type 2 (CMD2) .................................. 139

6.2. Study aims .............................................................................................. 141

6.3. Prediction of the bovine GlyT2 gene, cDNA and protein sequences and identification of a SLC6A5 mutation in CMD2 ........................................ 141

6.4. Amplification and TOPO cloning of SLC6A5 exons 4 and 5 from bovine genomic DNA ................................................................. 143

6.5. Characterisation of the functional consequences of the bovine CMD2 mutation .................................................................................. 144
  6.5.1. Subcellular localisation of the hGlyT2 L269P mutant ................... 145
  6.5.2. [3H]-glycine uptake is impaired in the L269P mutant .................... 146

6.6. Discussion .............................................................................................. 147
  6.6.1. A missense mutation in SLC6A5 causes CMD2 ......................... 147

7. GENERAL DISCUSSION .............................................................................. 148

7.1. GlyR versus GlyT2 mutations in human hyperekplexia ................... 149

7.2. Identification of new hyperekplexia mutations and exons in the human GlyT2 gene .................................................................................. 149

7.3. Identification of GlyT2 interactors reveals a new candidate gene for hyperekplexia .................................................................................. 151

7.4. Potential role for PICK-1 in GlyT1 function at NMDAR ................. 152

7.5. GlyT2 dysfunction in animals .............................................................. 153
List of figures

Figure 1.1. The glycineric inhibitory synapse .................................................. 19
Figure 1.2. Mutation spectrum in GlyR α1 and β subunits in hyperekplexia .... 25
Figure 1.3. Distribution of GlyT1 and GlyT2 immunoreactivity in mouse E18 embryos .............................................................................. 29
Figure 1.4. Model of GlyT2 topology and hyperekplexia mutations ............... 36
Figure 1.5. A model of GlyT2 and the hyperekplexia N509 mutation affecting Na⁺ affinity ................................................................. 38
Figure 2.1. Schematic representation of the 3' RACE protocol ......................... 52
Figure 2.2. Schematic representation of the 5' RACE protocol ......................... 52
Figure 2.3. Schematic representation of the LacZ assay .................................. 60
Figure 3.1. Amino acid sequence of human GlyT2 indicating the relative positions of new SLC6A5 hyperekplexia mutations ................................. 71
Figure 3.2. Agarose gel showing 5' RACE PCR products (adult spinal cord) .......... 73
Figure 3.3. Plasmid map of pCR-Blunt II-TOPO ........................................ 74
Figure 3.4. Genomic sequence showing mapping of the 5' RACE cDNAs onto the corresponding region of SLC6A5 ................................. 75
Figure 3.5. Agarose gel showing 3' RACE PCR products (embryonic spinal cord) ................................................................. 76
Figure 3.6. cDNA sequences for human GlyT2-TQC and GlyT2-NFS variants .............................................................................. 77
Figure 3.7. Transport activity of hGlyT2-TQC and hGlyT2-NFS isoforms ....... 79
Figure 3.8. Plasmid maps of pRc/CMV-hGlyT2 and pRK5myc-hGlyT2 .......... 80
Figure 3.9. Subcellular localisation of myc-hGlyT2 and hyperekplexia mutants .............................................................................. 81
Figure 3.10. Transport activity of hGlyT2 and hyperekplexia mutants ........... 83
Figure 3.11. Kinetic properties of hGlyT2 and A275T mutant .......................... 84
Figure 3.12. Schematic representation of MLPA technique ............................ 88
Figure 4.1. The YTH system ............................................................................. 92
Figure 4.2. Genotype of yeast reporter strains L40, Y190 and AH109 .............. 93
Figure 4.3. Map of the bait construct pYTH16-GlyT2N and the prey construct pACT2-ULIP6 ................................................................. 95
Figure 4.4. Maps of the bait construct pYTH16-GlyT2C and the prey construct pACT2-ULIP6 ................................................................. 97
Figure 4.5. LacZ freeze-fracture assay on SD/-Leu/-Trp plates (left panels, filters 1-4) and growth on SD/-Leu/-Trp/-His + 3-AT plates (right panels, filters 5-8) ................................................................. 98
Figure 4.6. A flowchart depicting the steps involved in a YTH library screen ....... 99
Figure 4.7. Map of the pACT2-cDNA library plasmid ..................................... 100
Figure 4.8. Restriction enzyme analysis of miniprep DNA derived from HIS+ colonies ................................. 102
Figure 4.9. Sequence analysis of clone 1.1 ................................................................. 102
Figure 4.10. Sequence analysis of clones 1.2, 5.4, 5.5, 9.4, 9.6, 11.3, 12.1, 12.5 .................................................... 103
Figure 4.11. Sequence analysis of clones 2.1 and 14.2 ................................................... 103
Figure 4.12. Sequence analysis of clones 3.2, 3.11, 3.12, 4.3 and 4.4 ......................... 104
Figure 4.13. Sequence analysis of the clone 5.2 .......................................................... 104
Figure 4.14. Sequence analysis of clones 7.1, 11.1 and 12.2 .................................................. 105
Figure 4.15. Sequence analysis of the clone 14.3 ...................................................... 105
Figure 4.16. Sequence analysis of clones 1.5 and 3.10 ..................................................... 106
Figure 4.17. Sequence analysis of clones 5.3, 6.1, 8.2, 9.2, 10.4 and 11.4 .................... 106
Figure 4.18. Sequence analysis of clones 3.1, 3.3, 3.5, 5.1, 7.2, 10.3, 14.1 and 15.1 ...................................................... 106
Figure 4.19. Verification of bait-prey interaction for GlyT2 interactors ............................... 109
Figure 4.20. Representation of the domain structures of new GlyT2 interacting proteins .................................................. 110
Figure 4.21. GlyT2-PDZ domain interactions are dependent on the C-terminal class III PDZ binding motif .......................................................... 111
Figure 4.22. Plasmid map of pGBK7-GlyT1C .............................................................. 112
Figure 4.23. Interaction of syntrophin-α1, but not other GlyT2 interacting proteins, with a GlyT1 bait .............................................................. 113
Figure 5.1. Potential alternative splicing and functional domains of FMNL2 ......................... 125
Figure 5.2. Plasmid map of pRK5FLAG-FMN2 .......................................................... 126
Figure 5.3. Subcellular localisation of FMNL2 and GlyT2 expressed separately in HEK293 cells .............................................................. 127
Figure 5.4. Subcellular localisation of FMNL2 and GlyT2 co-expressed in HEK293 cells .............................................................. 128
Figure 5.5. Plasmid map of pEGFP-PICK-1 ............................................................. 129
Figure 5.6. Functional consequences of PICK-1 co-expression on GlyT1 and GlyT2-mediated transport activity ...................................................... 130
Figure 5.7. Effect of PICK-1 wild-type and mutant on the kinetics of GlyT1-mediated glycine transport .............................................................. 131
Figure 5.8. Prediction of phosphorylation sites in GlyT1 and GlyT2 baits in yeast and mammalian cells .............................................................. 133
Figure 5.9. Functional consequences of FMNL2 on GlyT1 and GlyT2 mediated transport activity .............................................................. 134
Figure 6.1. CMD2 in Belgian blue cattle ................................................................. 140
Figure 6.2. Alignment of the human and bovine GlyT2 proteins ...................................... 142
Figure 6.3. Confirmation of the T809C mutation in CMD2 and carrier animals .................. 143
Figure 6.4. DNA sequencing chromatograms showing the CMD2 mutation .................. 144
Figure 6.5. Sequence alignment of the GlyT2 TM3 domain in vertebrate species................................................................................................144
Figure 6.6. Subcellular localisation of hGlyT2 and L169P mutant.................145
Figure 6.7. Lack of [3H]-glycine uptake for hGlyT2 mutants L269P(1) and L269P(2).............................................................................................146

Appendix 1: Predicted bovine GlyT2 gene sequence............................................154
Appendix 2: Predicted bovine GlyT2 cDNA sequence...........................................166
List of tables

Table 1.1. Genetic defects in mouse, bovine and zebrafish GlyR genes ...............23
Table 1.2. Human postsynaptic defects in glycinergic transmission ..................23
Table 1.3. Hyperekplexia mutations in GLRA1 and GLRB ..............................26
Table 1.4. Mouse presynaptic and glial defects in glycinergic transmission ......35
Table 1.5. Hyperekplexia mutations in SLC6A5 ..............................................36
Table 2.1. Oligonucleotide primers used for PCR amplification ......................47
Table 2.2. Sequencing primers ........................................................................51
Table 2.3. 3' RACE primers ...........................................................................53
Table 2.4. 5' RACE primers ...........................................................................54
Table 2.5. Human and bovine GlyT2 mutagenesis primers ...............................56
Table 2.6. PCR conditions for site-directed mutagenesis ................................56
Table 2.7. YTH bait and prey vectors ................................................................58
Table 3.1. New hyperekplexia mutations in SLC6A5 .......................................71
Table 3.2. SNPs detected in SLC6A5 .................................................................88
Table 4.1. Pilot transformations of Y190 with pYTH16-GlyT2N and
pACT2-ULIP6 ............................................................................................96
Table 4.2. Pilot transformations of Y190 with pYTH16-GlyT2C and
pACT2-syntenin-1 .....................................................................................97
Table 4.3. Small scale transformation strategy to eliminate false positives ......107
Table 4.4. GlyT2-interacting proteins identified in the GlyT2C YTH screen ..108
Table 5.1. Novel GlyT2 interactors: mouse models of dysfunction .................123
Table 5.2. Novel GlyT2 interactors: human diseases .......................................124
1. INTRODUCTION
1.1. Hyperekplexia

Hyperekplexia or startle disease is a rare neurological disorder, first described by Kirstein and Silfverskiold in 1958 as an “emotionally precipitated drop seizure” (Kirstein and Silfverskiold, 1958) and can appear in utero or soon after birth. Newborns affected by hyperekplexia present with an excessive startle response to unexpected acoustic, visual or tactile stimuli, hyperreflexia and hypertonia (Zhou et al., 2002). Startle reactions are followed by a short period of generalised stiffness, during which voluntary movements are impossible, but without loss of consciousness (Gaitatzis et al., 2004). Early manifestations include abnormal intrauterine movements, nocturnal myoclonus and hernias and tonic spasms can lead to apnoea episodes, bradycardia and associated brain damage or sudden death (Nigro and Lim, 1992). Affected children might refuse to move around because of fear of frequent falls (Praveen et al., 2001). Hypertonia diminishes spontaneously during the first year of life, probably due to the maturation of a compensatory mechanism, such as GABAergic neurotransmission. However, in a minority of cases, the exaggerated startle reflex may persist into adulthood causing individuals to become wheelchair-bound to avoid injuries from sudden falls (Zhou et al., 2002). The intelligence of most hyperekplexia patients is normal although approximately 18% of individuals affected present mild neurological deficiency (Andrew and Owen, 1997), perhaps as a result of neonatal anoxia. The aetiology of hyperekplexia is unclear; the excessive startle reflex is probably caused by a normal but exaggerated startle response, due to either an increased reticular neuronal excitability in the brain stem and spinal cord or to lack of cortical inhibition. Hyperekplexia is a rare disease with an unknown prevalence. The disease is usually familial and mostly autosomal dominant, but recessive cases of hyperekplexia are also known which may explain some ‘sporadic’ cases. However, others can be associated with brainstem pathologies or trauma (Gaitatzis et al., 2004). Recognition of hyperekplexia in the neonatal period is essential to prevent life-threatening events (Sharma et al., 2006). Diagnosis is based on the evaluation of the startle response by nose tapping and should be included in the routine examination of all newborns. Unlike most neurological disorders, hyperekplexia is highly treatable. A simple intervention called the Vigevano manoeuvre (flexing of the head and limbs towards the trunk) dissipates and counteracts the effects of acute hypertonia and breath-holding spells (Vigevano et al., 1989). Pharmacological therapy is also
effective: for example clonazepam, a benzodiazepine that positively modulates GABA\textsubscript{A} receptor-mediated neurotransmission is presumed to compensate for impaired glycineric transmission (Zhou \textit{et al.}, 2002). High doses (0.1-0.2 mg/kg/day) of clonazepam result in a dramatic reduction of startle responses and associated breath-holding episodes without development of tolerance. However, as expected with benzodiazepines, drowsiness and sedation are often reported as side effects (Ryan \textit{et al.}, 1992; Tijssen \textit{et al.}, 1997) making childhood life difficult (e.g. attendance at nursery and school). Several other antiepileptic drugs have been tried as therapeutic agents, such as vigabatrin, valproic acid and phenytoin. Both vigabatrin and valproic acid inhibit the GABA metabolising enzyme GABA-transaminase (Gidal \textit{et al.}, 1999; Landmark, 2007). Phenytoin inhibits competitively the Na\textsuperscript{+} dependent high affinity synaptosomal transport (Wong and Teo, 1986). Although all these drugs aim to enhance the GABA levels in the brain, they have not always consistently controlled the hypertonia and/or abnormal startle response (Praveen \textit{et al.}, 2001).

1.2. The glycineric inhibitory synapse

Glycine is the main inhibitory neurotransmitter in the caudal region of the central nervous system. Glycine levels are highest in the medulla oblongata, pons and spinal cord, where inhibitory glycine receptors (GlyRs) are prominently expressed (Betz and Laube, 2006). In the spinal cord and brain stem, glycineric inhibitory interneurones control motor rhythm generation, coordination of spinal reflexes and processing of sensory signals (Legendre, 2001). CNS glycine derives from both metabolic precursors and uptake and is synthesised \textit{de novo} by catalysis of serine by serine-hydroxymethyltransferase (Harvey \textit{et al.}, 2008a). At glycineric synapses (Figure 1.1), glycine is released from presynaptic terminals by Ca\textsuperscript{2+}-triggered exocytosis of synaptic vesicles. Glycine then diffuses across the synaptic cleft and binds to postsynaptic GlyRs, opening an intrinsic anion channel. The resulting influx of Cl\textsuperscript{-} ions into the postsynaptic cytoplasm leads to hyperpolarisation and inhibition of the postsynaptic neurone (Eulenburg \textit{et al.}, 2005). Glycineric transmission is terminated by the closure of individual GlyRs and by the removal of glycine into surrounding glial cells via GlyT1. The presynaptic terminal is refilled with glycine by GlyT2-mediated uptake, which provides glycine for the refilling of presynaptic vesicles via the vesicular inhibitory amino acid transporter (VIAAT; Eulenburg \textit{et al.}, 2005; Betz \textit{et al.}, 2006). Accessory proteins such as ULIP6 and syntenin-1 regulate steady-state
Figure 1.1. The glycinergic inhibitory synapse. Postsynaptic GlyRs are composed of α (red) and β (blue) subunits in a 2α3β stoichiometry and are clustered opposite presynaptic terminals releasing glycine by gephyrin and the RhoGEF collybistin (Figure modified from Harvey et al., 2008a). The glial glycine transporter GlyT1 removes glycine from the synaptic cleft, thereby terminating neurotransmission. The neuronal glycine transporter GlyT2, which binds ULIP6 and syntenin-1, is essential for glycine re-uptake into the presynaptic terminal, providing glycine for refilling of synaptic vesicles by the vesicular transporter VIAAT/VGAT. Glycine is also synthesised by a de novo pathway involving serine-hydroxymethyltransferase (SHMT).

levels of GlyT2 at the plasma membrane. The binding of glycine to GlyRs is antagonised by the plant alkaloid strychnine, a highly selective antagonist, widely used for distinguishing glycinergic from GABAergic inhibition and important tool for the study of GlyRs (Betz and Laube, 2006). Postsynaptic GlyRs belong to the cysteine-loop ligand-gated ion channel family, which includes nicotinic acetylcholine receptors, GABA<sub>A/C</sub> receptors and serotonin type 3 receptors (Lynch, 2004). Four inhibitory GlyR isoforms have been identified in mammals containing the α1, α2, α3 or α4 subunits in combination with the β subunit in a 2α3β stoichiometry (Grenningloh et al., 1987; Harvey et al., 2004b). Each GlyR subunit has a large N-terminal extracellular domain, four membrane-spanning domains (M1-M4 connected by intracellular or extracellular loops) and a short extracellular C-terminus. The M2 region lines the Cl⁻ ion channel, and the M2/M3 linker region presents charged residues involved in signal transduction (Betz and Laube, 2006). The different GlyR isoforms have different biological roles, reflected in their temporal and spatial patterns of expression (Harvey et al., 2004b). GlyRs containing the α1 subunit predominate in adult brain stem and spinal cord, whilst the α2 subunit is generally considered to
represent an embryonic/neonatal isoform involved in glycinergic synapse formation (Malosio et al., 1991; Sato et al., 1992). During embryonic development, due to the high intracellular Cl⁻ concentration of nerve cells, activation of GlyRs results in Cl⁻ efflux and depolarisation. Hyperpolarising α2 subunit-containing GlyRs trigger intracellular Ca²⁺ influx, allowing the correct formation of inhibitory glycinergic synapses, and play a role in the neurogenesis in neocortex and retina (Kirsch and Betz, 1998; Flint et al., 1998; Young and Cepko, 2004). By contrast, GlyRs containing the α3 subunit gene are located in the lamina II of the spinal cord dorsal horn and have a crucial role in the processing of inflammatory pain stimuli (Harvey et al., 2004b). Lastly, the GlyR α4 subunit is found in the retina, the autonomic nervous system and the developing male genital ridge (Harvey et al., 2000; Heinze et al., 2007). Curiously, the structural GlyR β subunit is found at all developmental stages and is widely expressed in the brain, even in regions that are not thought to express GlyRs (Malosio et al., 1991). The GlyR β subunit is crucial for the correct synaptic localisation of GlyRs: the large intracellular loop of the β subunit binds to the scaffolding protein gephyrin, which in turn is translocated to the cell membrane by the GDP-GTP exchange factor callybistin (Harvey et al., 2004a).

1.3. Animal defects in GlyR dysfunction

Comparative studies using mouse, bovine and zebrafish models of inhibitory glycinergic dysfunction (Figure 1.1; Table 1.1) have traditionally served as leads for genetic analysis of the orthologous genes in human hyperekplexia (Harvey et al., 2008b). For example, five mouse models have been found that are caused by mutations in the mouse GlyRs α1 and β subunit genes (Legendre, 2001; Harvey et al., 2008b). Spastic and spasmodic mice are phenotypically similar: they appear normal at birth but around two weeks of age develop a severe neuromuscular disorder characterised by an exaggerated startle response, rapid tremor, rigidity and inability to right. The appearance of this phenotype is consistent with the timing of the developmental switch in GlyR subunit composition from α2- to α1-subunit containing receptors in spinal cord (Buckwalter et al., 1994). In the homozygous spastic mouse (spa) this phenotype is determined by the insertion of a 7.1 kb LINE-1 element into the intron 5 of the mouse GlyR β subunit gene (Glrβ). This element leads to aberrant splicing of the β subunit pre-mRNA inducing skipping of exons 4 and/or 5 and results
in a reduced level of Glrb mRNA. However, a small amount of Glrb transcript remains intact, preventing this model from being lethal (Kingsmore et al., 1994; Mülhardt et al., 1994). By contrast, homozygous spasmodic mice (spd) harbour a missense mutation (A52S) in the N-terminus of the GlyR α1 subunit gene (Glrα1). This mutation results in a reduced agonist sensitivity of GlyRs without affecting receptor expression (Ryan et al., 1994; Saul et al., 1994). More severe symptoms occur in the lethal mutants oscillator (Buckwalter et al., 1994; Kling et al., 1997), cincinatti (Holland et al., 2006) and Nmf11 (Traka et al., 2006): mice begin to exhibit rapid, violent trembling at around two weeks of age, which increases in severity daily. At three weeks of age they display prolonged periods of rapid tremor, producing extreme rigour and stiffness that is normally fatal. These lethal phenotypes are caused by different mutations in each case. In oscillator, a microdeletion of seven nucleotides in exon 8 of the Glrα1 results in a frameshift at the end of the third putative transmembrane domain, resulting in a truncated α1 subunit. Western blot and [3H]-strychnine binding experiments showed that the spinal cord of homozygous oscillator mice is completely devoid of the α1 subunit (Buckwalter et al., 1994; Kling et al., 1997). Cincinatti harbours a duplication of Glrα1 exon 5, again resulting in a frameshift and a truncated GlyR α1 subunit (Holland et al., 2006). Nmf11, an ethynitrosourea (ENU)-induced mutant mouse, has a missense mutation in Glrα1, resulting in a N46K substitution in the GlyR α1 subunit extracellular domain. However, the functional effects of the Nmf11 N46K mutation are still unclear, since GlyR α1 subunit mRNA and protein levels are normal and the mutant GlyR localises correctly to synaptic sites (Traka et al., 2006). Genetic defects in GlyR genes have also been described in Poll Hereford cattle (Gundlach et al., 1988; Pierce et al., 2001) and the zebrafish bandoneon (beo) mutant (Hirata et al., 2005) and are suspected in familial reflex myoclonus in labrador retrievers (Fox et al., 1984) and inherited myoclonus in Peruvian Paso horses (Gundlach et al., 1993).
<table>
<thead>
<tr>
<th>Gene $^1$</th>
<th>Protein</th>
<th>Species</th>
<th>Mutant</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlrA1 (11qB1.3)</td>
<td>GlyR α1</td>
<td>Mouse</td>
<td>spasmodic</td>
<td>A52S missense mutation: reduced agonist affinity</td>
<td>[Ryan et al., 1994]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>oscillator</td>
<td>Exon 8 microdeletion: frameshift/protein truncation</td>
<td>[Buckwalter et al., 1994; Kling et al., 1997]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Nmfl</td>
<td>N46K missense mutation: consequences unknown</td>
<td>[Traka et al., 2006]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cincinatti</td>
<td>Exon 5 duplication, frameshift/protein truncation</td>
<td>[Holland et al., 2006]</td>
</tr>
<tr>
<td>GLRA1 (Chr 7)</td>
<td>GlyR α1</td>
<td>Cow</td>
<td>ICM</td>
<td>Y24X nonsense mutation in Poll Hereford cattle: protein truncation. Hyperesthesia, spontaneous and startle-evoked myoclonic jerks of musculature. Death owing to failure to stand and feed</td>
<td>[Gundlach et al., 1988; Pierce et al., 2001]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Exaggerated startle response, muscle rigidity, tremor, impaired righting reflex, neonatal lethality (~P21) in oscillator, Nmfl I and cincinatti</td>
<td></td>
</tr>
<tr>
<td>Glrb (3qE3)</td>
<td>GlyR β</td>
<td>Mouse</td>
<td>Spastic</td>
<td>LINE 1 element insertion: aberrant mRNA splicing, exaggerated startle, resembles spasmodic</td>
<td>[Kingsmore et al., 1994; Mülhardt et al., 1994]</td>
</tr>
<tr>
<td>Glrb2 (Chr 14)</td>
<td>GlyR β2</td>
<td>Zebrafish*</td>
<td>bandoneon</td>
<td>Missense or nonsense mutations D78X, L255R, R275H result in aberrant simultaneous trunk muscle contractions in response to tactile stimuli</td>
<td>[Hirata et al., 2005]</td>
</tr>
</tbody>
</table>

Table 1.1. Genetic defects in mouse, bovine and zebrafish GlyR genes. $^1$Chromosomal locations were determined using the genome browser at http://genome.ucsc.edu/. $^2$Note that, unusually, there are two GlyR β subunit genes in zebrafish, GlrB1 and GlrB2. Amino acid numbering shown for the zebrafish GlyR β2 subunit mutations is based on the mature polypeptide after signal peptide cleavage predicted with the Signal P programme (http://www.cbs.dtu.dk/services/SignalP/).
1.4. Postsynaptic causes of human hyperekplexia

Hyperekplexia is caused by a defective glycinergic transmission in reflex circuits of the spinal cord and brain stem, resulting in an increased level of excitability of motor neurones (Lynch, 2004). Hyperekplexia-associated mutations present autosomal dominant and recessive inheritance (the last mostly transmitted as compound heterozygous or resulting from consanguineous marriages). As found in animal models, mutations in the GlyR α1 subunit gene (GLRA1) are the most common postsynaptic cause of hyperekplexia (Table 1.2; Figure 1.2). Rare mutations have also been found in the GlyR β subunit gene (GLRB) and those encoding the clustering proteins gephyrin (GPHN) and collybistin (ARHGEF9).

<table>
<thead>
<tr>
<th>Gene Location</th>
<th>Protein</th>
<th>OMIM</th>
<th>Associated human disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLRA1 5q33.1</td>
<td>GlyR α1</td>
<td>138491</td>
<td>Hyperekplexia</td>
</tr>
<tr>
<td>GLRA2 Xp22.2</td>
<td>GlyR α2</td>
<td>305990</td>
<td>Unknown</td>
</tr>
<tr>
<td>GLRA3 4q34.1</td>
<td>GlyR α3</td>
<td>600421</td>
<td>Unknown</td>
</tr>
<tr>
<td>GLRA4 Xq22.2</td>
<td>GlyR α4</td>
<td>-</td>
<td>Pseudogene in humans</td>
</tr>
<tr>
<td>GLRB 4q32.1</td>
<td>GlyR β</td>
<td>138492</td>
<td>Hyperekplexia</td>
</tr>
<tr>
<td>GPHN 14q23.3</td>
<td>Gephyrin</td>
<td>603930</td>
<td>MOCO deficiency, hyperekplexia, leukaemia</td>
</tr>
<tr>
<td>ARHGEF9 Xq11.1</td>
<td>Collybistin</td>
<td>300429</td>
<td>X-linked mental retardation, additional features can include seizures, increased anxiety, hyperactivity, facial dysmorphisms, sensitivity to thermal pain and hyperekplexia</td>
</tr>
</tbody>
</table>

Table 1.2. Human postsynaptic defects in glycinergic transmission. Chromosomal locations were determined using the human genome browser at http://genome.ucsc.edu/. Online Mendelian Inheritance in Man (OMIM) identifiers and associated human diseases were obtained from http://www.ncbi.nlm.nih.gov/omim/.

Mutations in GLRA1 were discovered following extensive genetic mapping studies by Shiang et al., (1993), who identified missense mutations in GLRA1 in four families with autosomal dominant hyperekplexia. These mutations resulted in the substitution of the highly conserved charged residue arginine with uncharged amino acids leucine (R271L) or glutamine (R271Q) and cause reduced glycine affinity and Cl⁻ conductance (Langosch et al., 1994). Since this initial study at least 28 independent mutations have been discovered in GLRA1 (Figure 1.2; Figure 1.3; Harvey et al., 2008b) and mutation R271 is recurrent and the most common cause of this disorder. Many other dominant missense mutations in GLRA1 have been identified, including Q266H, located within the pore-forming TM2 domain and K276E and Y279C, which lie in the TM2-TM3 extracellular loop. These mutations affect the duration of channel
opening. P250T, located at the intracellular border with M2, causes an enhanced
desensitisation rate (Saul et al., 1999). The first recessive form of hyperekplexia was
described in a patient with consanguineous parents who presented homozygosity for
the missense mutation I244N which causes a reduced glycine current magnitude and
glycine sensitivity (Rees et al., 1994). Other recessive forms of hyperekplexia are
caused by large deletions, nonsense mutations, frameshift or splice site errors (e.g.
Brune et al., 1996; Rees et al., 2001; Figure 1.2; Table 1.3). Rare mutations have also
been identified in genes encoding other key components of the glycinergic synapse,
such as the GlyR β subunit, gephyrin and collybistin. Compound heterozygous
mutations in the GlyR β subunit gene (GLRB) were found in one individual with a
transient hyperekplexia phenotype (Rees et al., 2002). These were a missense
mutation (G229D) that results in a reduced sensitivity to agonist mediated activation
of heteromeric αβ GlyRs, and a splice site mutation (IVS5+5G>A) resulting in the
omission of GLRB exon 5. A heterozygous missense mutation (N10Y) in the gephyrin
gene (GPHN) was found in a young male infant with neonatal hypertonia and
excessive startle response. However, this mutation did not affect GlyR β-gephyrin
interactions nor collybistin-induced clustering of gephyrin, so the functional
significance of this mutation is unclear (Rees et al., 2003). Deletions in GPHN are
also associated with molybdenum co-factor (MOCO) deficiency (Reiss et al., 2001),
and GPHN-MLL (mixed gene leukaemia) gene products have been detected in acute
monoblastic leukaemia (Eguchi et al., 2004). Lastly, a missense mutation (G55A) in
the collybistin gene (ARHGEF9) was identified in a patient with symptoms of both
hyperekplexia and epilepsy. This mutation disrupted collybistin-mediated gephyrin
clustering in cultered neurones and the clinical phenotype was probably consequence
of the loss of gephyrin and major GABA_A and GlyR subtypes from inhibitory
synapses (Harvey et al., 2004a). Chromosomal rearrangements in ARHGEF9 result in
a spectrum of clinical symptoms with mental retardation as a common feature (Marco
et al., 2008; Kalscheuer et al., 2008). For these reasons, mutations in gephyrin and
collybistin are unlikely to represent major causes of hyperekplexia. In addition, no
pathogenic mutations have been identified in other GlyR α subunit genes (Rees et al.,
2006) consistent with the different spatial and temporal patterns of expression and
specialised functions of GlyRs containing the α2 and α3 subunits (Young and Cepko,
2004; Harvey et al., 2004b). GLRA4 has been excluded from analysis as it is a
pseudogene in humans, due to the presence of a premature stop codon in exon 9 (Simon et al., 2004).

Figure 1.2. Mutation spectrum in GlyR α and β subunits in hyperekplexia. This schematic diagram depicts the suggested four-membrane spanning domain topology of GlyR α and GlyR β subunits and relative positions of amino acid alterations caused by recessive (blue circles) and dominant (red circles) mutations found in human hyperekplexia (Figure modified from Harvey et al., 2008b). Note that the vast majority of alterations affect the M1-M2 domains of the GlyR α subunit. Alterations found in glycineergic disorders in animals are also indicated (purple circles). X denotes a stop codon. Numbered boxes indicate potential membrane-spanning domains.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Alteration</th>
<th>Location</th>
<th>Mode</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLRA1</td>
<td>ΔExons1-7</td>
<td>N-terminus-M2</td>
<td>AR</td>
<td>Large deletion, protein truncation</td>
</tr>
<tr>
<td>GLRA1</td>
<td>W68C* R316X*</td>
<td>N-terminus M3-M4 loop</td>
<td>CH</td>
<td>Missense, no functional data; Nonsense, protein truncation</td>
</tr>
<tr>
<td>GLRA1</td>
<td>R72H</td>
<td>N-terminus</td>
<td>AR</td>
<td>Missense, <em>no functional data</em></td>
</tr>
<tr>
<td>GLRA1</td>
<td>ΔC (601-605) M147V</td>
<td>N-terminus N-terminus</td>
<td>CH</td>
<td>Deletion, frameshift, protein truncation; Missense, <em>no functional effect detected</em></td>
</tr>
<tr>
<td>GLRA1</td>
<td>Y202X</td>
<td>N-terminus</td>
<td>AR</td>
<td>Nonsense, protein truncation</td>
</tr>
<tr>
<td>GLRA1</td>
<td>R218Q</td>
<td>N-terminus</td>
<td>AD†</td>
<td>Missense, defective GlyR trafficking, reduced agonist sensitivity</td>
</tr>
<tr>
<td>GLRA1</td>
<td>Y228C</td>
<td>M1</td>
<td>AR</td>
<td>Missense, <em>no functional data</em></td>
</tr>
<tr>
<td>GLRA1</td>
<td>S231R</td>
<td>M1</td>
<td>AR</td>
<td>Missense, defective GlyR trafficking</td>
</tr>
<tr>
<td>GLRA1</td>
<td>W239C</td>
<td>M1</td>
<td>AD</td>
<td>Missense, <em>no functional data</em></td>
</tr>
<tr>
<td>GLRA1</td>
<td>I244N</td>
<td>M1</td>
<td>AR</td>
<td>Missense, defective GlyR assembly</td>
</tr>
<tr>
<td>GLRA1</td>
<td>P250T</td>
<td>M1-M2 loop</td>
<td>AD</td>
<td>Missense, impaired signal transduction and altered desensitisation</td>
</tr>
<tr>
<td>GLRA1</td>
<td>R252H R392H</td>
<td>M1-M2 loop M3-M4 loop</td>
<td>CH</td>
<td>Missense, defective GlyR trafficking</td>
</tr>
<tr>
<td>GLRA1</td>
<td>V260M</td>
<td>M2</td>
<td>AD</td>
<td>Missense, impaired signal transduction</td>
</tr>
<tr>
<td>GLRA1</td>
<td>Q266H</td>
<td>M2</td>
<td>AD</td>
<td>Missense, impaired signal transduction</td>
</tr>
<tr>
<td>GLRA1</td>
<td>S267N</td>
<td>M2</td>
<td>AD</td>
<td>Missense, impaired signal transduction and reduced alcohol sensitivity</td>
</tr>
<tr>
<td>GLRA1</td>
<td>S270T</td>
<td>M2</td>
<td>AD</td>
<td>Missense, <em>no functional data</em></td>
</tr>
<tr>
<td>GLRA1</td>
<td>R271Q</td>
<td>M2-M3 loop</td>
<td>AD</td>
<td>Missense, impaired signal transduction</td>
</tr>
<tr>
<td>GLRA1</td>
<td>R271L</td>
<td>M2-M3 loop</td>
<td>AD†</td>
<td>Missense, impaired signal transduction, reduced potency and efficacy of agonists</td>
</tr>
<tr>
<td>GLRA1</td>
<td>K276E</td>
<td>M2-M3 loop</td>
<td>AD</td>
<td>Missense, impaired signal transduction</td>
</tr>
<tr>
<td>GLRA1</td>
<td>K276Q*</td>
<td>M2-M3 loop</td>
<td>AD†</td>
<td>Missense, no functional data</td>
</tr>
<tr>
<td>GLRA1</td>
<td>Y279C</td>
<td>M2-M3 loop</td>
<td>AD</td>
<td>Missense, impaired signal transduction</td>
</tr>
<tr>
<td>GLRA1</td>
<td>Y279S</td>
<td>M2-M3 loop</td>
<td>AD</td>
<td>Missense, <em>no functional data</em></td>
</tr>
<tr>
<td>GLRA1</td>
<td>Y279X</td>
<td>M2-M3 loop</td>
<td>AR</td>
<td>Nonsense, protein truncation</td>
</tr>
<tr>
<td>GLRA1</td>
<td>S296X</td>
<td>M3</td>
<td>AD†</td>
<td>Nonsense, protein truncation, dominant-negative effects on GlyR expression</td>
</tr>
<tr>
<td>GLRA1</td>
<td>G342S</td>
<td>M3-M4 loop</td>
<td>AD</td>
<td>Missense, <em>no functional effect detected</em></td>
</tr>
<tr>
<td>GLRB</td>
<td>G229D IVS5+5G-A</td>
<td>N-terminus TM3-TM4</td>
<td>CH</td>
<td>Decreased agonist sensitivity; Splice-site mutation</td>
</tr>
</tbody>
</table>

Table 1.3. Hyperekplexia mutations in GLRA1 and GLRB. *In a minority of cases (denoted by an asterisk), GlyR α1 subunit amino acid numbering was indicated using the start methionine as +1. The correct residues in the mature GlyR α1 subunit are identified in this table. *The N-terminus indicates the large N-terminal extracellular domain, while M1-M4 denotes the four membrane spanning domains of GlyR subunits. Loops are sequences linking the membrane-spanning domains. X denotes a stop codon. *AR: autosomal recessive; AD: autosomal dominant; CH: compound heterozygosity; †: de novo mutation.
1.5. Glycine transporters: essential regulators of neurotransmission

Curiously, despite fairly comprehensive genetic studies of postsynaptic receptors and clustering proteins, many hyperekplexia patients do not appear to harbour mutations in GLRA1, GLRB, GPHN or ARHGEF9. However, since hyperekplexia is caused by defective glycinergic transmission, it was considered possible that mutations might be found in other proteins involved in the formation, maintenance and function of glycinergic synapses. This resulted in a shift of attention towards presynaptic mechanisms of disease (Rees et al., 2006; Harvey et al., 2008a) and in particular to glycine transporters.

1.5.1. The SLC6 transporter superfamily

Glycine transporters (GlyTs) belong to the Na⁺/Cl⁻ dependent neurotransmitter transporter family (SLC6 gene family) which includes transporters for GABA, biogenic amines (dopamine, noradrenaline, adrenaline and serotonin), for the amino acid proline, and for the osmolytes taurine, betaine and creatine. Orphan transporters with unknown substrates also belong to this family (Bröer, 2006). These proteins play a central role in the termination of synaptic transmission, as they retrieve released neurotransmitter into presynaptic terminals or surrounding glial cells, thus limiting the spread and duration of synaptic excitability (Hahn and Blakely, 2002). SLC6 transporters are related in sequence and share a common topology of 12 membrane-spanning domains connected by six extracellular and five intracellular loops and have intracellular N- and C- termini harbouring potential phosphorylation sites. A large extracellular loop (EL2) between transmembrane domains 3 and 4 may contain N-glycosylation sites important for the efficient insertion of the transporters in the plasma membrane (Olivares et al., 1995). The sequence identity of those proteins suggests a similar mechanism of transport (Nelson, 1998) and indeed uptake is an active process energetically coupled to the transmembrane Na⁺ electrochemical gradient maintained by a Na⁺/K⁺-ATPase. Extracellular binding of Na⁺, Cl⁻ and the substrate determines a conformational change that permits the translocation from the outward to the inward side of the membrane (Aragon and López-Corcuera, 2003). Following the release of bound substrate and ions, the 'empty' transporter returns to the original conformation (Eulenburg et al., 2005). Genetic variation in neurotransmitter transporter genes underlies several human disorders and pathologies.
For example, a loss-of-function missense mutation with dominant-negative effect has been reported in the noradrenaline transporter (NET), resulting in orthostatic intolerance in heterozygous patients (Shannon et al., 2000). By contrast, a gain-of-function mutation in the serotonin transporter (SERT) results in a severe form of autism/obsessive-compulsive disorder (Kilic et al., 2003). Polymorphisms identified in the 5'-flanking promoter of SERT might also contribute to depression, Alzheimer's disease and alcohol dependence. Polymorphisms in the 3' untranslated region of the dopamine transporter gene (DAT) might also be associated with attention deficit hyperactivity disorder (Hahn and Blakely, 2002).

1.5.2. Characterisation and differential expression of the glycine transporters GlyT1 and GlyT2

Two distinct GlyTs were initially characterised based on their sequence similarity with the GABA transporter GAT1, the first neurotransmitter transporter cloned from the purified neuronal protein (Guastella et al., 1990) in combination with functional assays (Guastella et al., 1992; Liu et al., 1993). The human GlyT1 gene (SLC6A9) is located on human chromosome 1p34.1, while the human GlyT2 gene (SLC6A5) is found on chromosome 11p15.1. GlyTs share 50% amino acid sequence identity but differ in term of localisation, function, uptake capability and pharmacology (Eulenburg et al., 2005). Both GlyTs exist in variants created by alternative splicing: GlyT1 exists in five isoforms, resulting by combination of N-and C-terminal exons (Adams et al., 1995; Borowsky and Hoffman, 1998; Hanley et al., 2000), whilst two N-terminal variants have been reported for GlyT2 (Ebihara et al., 2004). GlyT2 is a larger protein than GlyT1 due to an extended N-terminus of 200 amino acids, a unique characteristic of GlyT2 compared with the other SLC6 proteins (Liu et al., 1993). Although other SLC6 transporters appear to dimerise, independent studies suggested at first that GlyTs were monomers (López-Corcuera et al., 1993; Horiuchi et al., 2001). However, more recent studies based on cysteine-mediated cross-linking and FRET microscopy approaches suggested that GlyTs oligomerise via an interface that is conserved between SLC6 family members (Bartholomäus et al., 2008). The distribution of GlyTs has been studied using in situ hybridisation and immunocytochemistry using antibodies raised against specific epitopes for GlyT1 and GlyT2. This analysis revealed differential expression of the two transporters at both regional and cellular levels. Both GlyT1 and GlyT2 are expressed in caudal regions of
the CNS, supporting a clear involvement of the two proteins in glycinergic inhibitory transmission (Aragón and López-Corcuera, 2003). GlyT2 expression is restricted to spinal cord, brain stem and cerebellum, with the highest transporter levels found in the dorsal and ventral horn of the spinal cord, the auditory system and in the nuclei of the cranial nerves. In general, GlyT2 expression correlates well with the distribution of GlyRs, making it a reliable marker of glycinergic neurones (Jursky and Nelson, 1995; Poyatos et al., 1997). However, GlyT1 is also expressed in areas that are supposedly devoid of strychnine-sensitive GlyRs, such as neocortex, hippocampus and olfactory bulb, where it is associated with N-methyl-D-aspartate (NMDA) receptors (NMDAR) which mediate glutamatergic transmission (Zafra et al., 1995a; Zafra et al., 1995b). Furthermore, GlyT1 is also present in non-neuronal tissues such as liver and pancreas (Jursky and Nelson, 1996; Figure 1.3). At the cellular level immunocytochemical studies have indicated a glial distribution for GlyT1 and a neuronal localisation for GlyT2. However, exceptions to this rule include the retina, where GlyT1 is found in the amacrine cells of the inner nuclear layer, and the cerebellum, where GlyT2 is localised both in presynaptic terminals and in glial cells (Zafra et al., 1995a; Zafra et al., 1995b).

**Figure 1.3. Distribution of GlyT1 and GlyT2 immunoreactivity in mouse E18 embryos.** GlyT2 expression is restricted to caudal regions of CNS, such as spinal cord (Sc) and brain stem; GlyT1 is also expressed in higher brain regions and in peripheral organs as liver (L) and pancreas (P). Reproduced with permission from Jursky and Nelson (1996).
1.5.3. Function of glycine transporters at inhibitory synapses

GlyT1 and GlyT2 both contribute to the control of the extracellular glycine concentration at glycinergic synapses, where they display complementary roles. GlyT1, present in the plasma membrane of glial cells surrounding synapses, terminates inhibitory transmission by removing glycine from the synaptic cleft. By contrast, GlyT2, localised in the presynaptic membrane of the incoming glycinergic axon, recycles glycine back into the presynaptic terminal, thus facilitating the refilling of synaptic vesicles by VIAAT (Eulenburg et al., 2005; Supplisson and Roux, 2002). Because GlyT2 is localised at some distance from the glycine-releasing active zones, a role for the transporter in glycine clearance from the synapse has been excluded (Spike et al., 1997; Roux and Supplisson, 2000). López-Corcuera et al., (1998) compared the functional properties of GlyT1 and GlyT2 through biochemical and electrophysiological methods in a stable expression system (HEK293 cells). Considering the substrate-transport mechanism, both the transporters catalyze the symport of glycine with Na\(^+\) and Cl\(^-\), but GlyT2 shows a higher Na\(^+\) coupling than GlyT1. Using electrophysiological and radio-tracing techniques on Xenopus oocytes, Roux and Supplisson (2000) confirmed that the two transporters exhibit different Na\(^+\) affinities and stoichiometries: 2 Na\(^+\)/1 Cl\(^-\) glycine for GlyT1 and 3 Na\(^+\)/1 Cl\(^-\) glycine for GlyT2. Under physiological conditions, glycine concentrations at both glycinergic and glutamatergic synaptic clefts is 0.1-0.2 \(\mu\)M. However, a considerably higher presynaptic glycine concentration is required to allow an efficient refilling of synaptic vesicles, due to the low affinity of VIAAT for GABA and glycine (Roux and Supplisson, 2000). GlyT2 has a higher driving force that allows 'uphill' glycine transport in order to maintain a concentration of 20-40 mM in the synaptic terminal. By contrast its lower Na\(^+\) stoichiometry allows GlyT1 to work close to equilibrium, and to operate in a reverse uptake mode, releasing glycine from the astrocyte to the extracellular space, depending on the ionic or electrical environment (Aragón and López-Corcuera, 2003). GlyT1 and GlyT2 are quite distinct in terms of pharmacology. The inhibition of GlyT1 by the selective and potent antagonist sarcosine results in an enhancement in the NMDAR mediated transmission by increasing the availability of synaptic glycine (Martina et al., 2004). Positive clinical results obtained in individuals treated with sarcosine suggest that GlyT1 inhibitors may have therapeutic potential as a novel class of antipsychotic drugs (Tsai et al.,
By contrast, GlyT2 is selectively inhibited by the tricyclic antidepressant amoxapine, which is 10-fold more potent on GlyT2 than GlyT1, and prevents binding of both glycine and Cl⁻ to the transporter (Núñez et al., 2000). Several selective glycine uptake inhibitors have been developed, but their effects await complete characterisation (Isaac et al., 2001; Caulfield et al., 2001).

1.6. Glycine transporters mouse knockouts as models for human disease

The generation of knockout mice for the GlyTs genes *SLC6A9* and *SLC6A5* has greatly improved our understanding of the function of GlyT1 and GlyT2 *in vivo* (Aragón and López-Corcuera, 2005; Harvey et al., 2008a; Betz et al., 2006; Table 1.4).

1.6.1. GlyT1 knockout mice reveal an essential role for GlyT1 in terminating glycinergic transmission

Gomeza et al., (2003a) inactivated the GlyT1 gene by homologous recombination, deleting exon 3 which encodes the second membrane-spanning domain of GlyT1. Homozygous GlyT1 knockout mice appear anatomically normal but soon after birth exhibit severe motor deficits characterised by lethargy, hypotonia and hyporesponsivity. GlyT1 knockouts die 6-14 hours after birth, most likely from respiratory failure, combined with wasting and dehydration caused by an inability to feed. In support of this theory, electrophysiological recordings from GlyT1 knockout brainstem slices showed a depressed and irregular respiratory rhythm that normalised after application of GlyR antagonist strychnine. Recordings performed in hypoglossal motoneurons displayed an increased Cl⁻ conductance, consistent with a tonic activation of GlyRs by elevated glycine concentrations, and prolonged decay times for spontaneous inhibitory postsynaptic currents. Those experiments excluded a significant role for excitatory NMDARs in respiratory rhythm generation and indicated that the respiratory failure seen in this model was due to a high level of extracellular glycine and consequent sustained activation of GlyRs (Gomeza et al., 2003a). Potentiation of glycinergic transmission was also seen in the zebrafish mutant *shocked*, where a missense mutation causing a G81D substitution in the second transmembrane domain of GlyT1 was responsible for motor deficits such as reduced spontaneous coiling of the trunk, diminished escape responses when touched and
absence of swimming. Since the application of strychnine restored the normal phenotype in both models, GlyT1 apparently has an essential role in terminating glycinergetic transmission, removing glycine from the synaptic cleft into neighbouring glial cells (Cui et al., 2005; Harvey et al., 2008a).

1.6.2. GlyT1 knockout mice: a new animal model of glycine encephalopathy

Interestingly, hyperglycinergic GlyT1 knockout mice also have motosensory deficits that resemble symptoms of glycine encephalopathy (or non-ketotic hyperglycinemia) in humans. This disease is typified by early neonatal onset, with convulsive seizures, coma and respiratory distress developing within a few days after birth. One-third of patients die within the first year of life, many in the neonatal period, often from respiratory impairment. Those who survive can live for many years, but suffer varying degrees of mental retardation, having a developmental age of 3-6 months, as well as intractable seizures (Applegarth and Toone, 2006; Sakata et al., 2001; Conter et al., 2006). However, this disease is typically associated with defects in the mitochondrial glycine cleavage system, which degrades excess extracellular glycine (Applegarth and Toone, 2006). Confirmation of a diagnosis of glycine encephalopathy requires: i) detection of raised cerebrospinal fluid (CSF) and plasma glycine concentrations and exclusion of organic acid disease by urinary acid assays; ii) a biochemical assay revealing a disrupted glycine cleavage enzyme complex in liver and iii) identification of mutations in one of the four genes of the mitochondrial glycine cleavage system (GCS), encoding the P-protein (a pyridoxyl-dependent glycine decarboxylase, GLDC), the H-protein (a lipoic acid-containing hydrogen carrier protein), the T protein (a tetrahydrofolate-dependent aminomethyltransferase) and the L protein (lipoamide dehydrogenase). Mutations in the P-, H- and T-proteins have been identified in glycine encephalopathy. More than 80% of patients have a defect in the P-protein gene (GLDC, 9p24.1), while 15% of patients have defects in the T-protein gene (AMT, 3p21.31). Defects in the H-protein gene (GCSH, 16q23.2) are extremely rare. However, there are also reports of individuals with glycine encephalopathy who have normal GCS activity in liver and brain, but defective CNS glycine transport in post-mortem tissue (Applegarth and Toone, 2006; Mayor et al., 1984, 1985). Hence, mutations in the human GlyT1 gene could underlie glycine encephalopathy in these cases, since the loss of glial GlyT1 uptake would be expected to raise CSF glycine,
while loss of GlyT1 from peripheral sites, including the liver (Figure 1.3), could also lead to raised serum glycine. The activity of the isolated glycine cleavage system would not be affected, since this complex is assayed in a cell-free lysate isolated from liver biopsy, and thus would be independent of GlyT1 function. However it has not yet been demonstrated that GlyT1 knockout mice present the main features for a mouse model of glycine encephalopathy, such as raised CSF and serum glycine in presence of normal mitochondrial glycine cleavage enzyme system activity (Applegarth and Toone, 2006; Harvey et al., 2008a).

1.6.3. GlyT1 knockout mice: studies on excitatory NMDARs

The role of GlyT1 in the control of glycine levels at central glutamatergic synapses is now well established (López-Corcuera et al., 2001). Glycine is a neuromodulator of excitatory NMDAR activity, acting as an obligatory co-agonist with glutamate to activate these receptors (Johnson and Ascher, 1987). NMDARs are hetero-oligomers formed by the association of NR1 subunits, that contain the glycine binding site, and NR2 subunits, that control the affinity of the co-agonist site (Danysz and Parsons, 1998). However, reservations have been raised about the in vivo role of glycine, since the concentration of glycine in CSF is in the micromolar range (5-10 μM), whereas the affinity of glycine for the glycine modulatory site of the NMDAR ranges from 0.1 to 3 μM depending on the NR2 subtype. Under these conditions, the NMDAR co-agonist site would always be saturated and therefore of no physiological or pharmacological relevance. Moreover recent evidence suggests that glycine and endogenous D-serine compete for the same binding site (Mothet et al., 2000; Panatier et al., 2006). However, Zafra et al., (1995a) localised GlyT1 expression on astroglia cells in close proximity to NMDARs and more recently showed that GlyT1 is expressed at pre- and postsynaptic aspects of glutamatergic synapses, especially in the neocortex and hippocampus (Cubelos et al., 2005b). The close spatial association of GlyT1 and glutamatergic synapses supports a role for GlyT1 in the maintenance of glycine under subsaturating levels in the synaptic cleft (Supplisson and Bergman, 1997). This has been demonstrated in hippocampal slices (Bergeron et al., 1998), brain stem hypoglossal motoneurons (Berger et al., 1998; Lim et al., 2004), prefrontal cortex slices (Chen et al., 2003) and in a co-expression model in Xenopus oocytes (Supplisson and Bergman, 1997). Moreover, because GlyT1 is capable of both glycine import and export, under particular physiological conditions GlyT1 might enable
glycine release from astrocytes, so enhancing NMDAR responses. The understanding of the role of GlyT1 in the modulation of NMDARs is complicated by the neonatal lethality of homozygous GlyT1 knockout mice. However, experiments using heterozygous GlyT1 mice and tissue-specific GlyT1 knockouts have been more illuminating (Harvey et al., 2008a). Heterozygous mice carrying only one functional GlyT1 allele appear normal at birth, but show a 50% reduction in $[^3H]$-glycine uptake in brain homogenates compared to wild-type littermates (Gomeza et al., 2003a; Tsai et al., 2004b). Notably, heterozygous mice present an enhanced NMDA/AMPA receptor activity ratio, suggesting that increased glycine levels lead to major functional changes at glutamatergic synapses (Tsai et al., 2004b; Gabernet et al., 2005; Martina et al., 2005). Furthermore, heterozygous mice are less sensitive to an amphetamine disruption of prepulse inhibition (PPI). PPI occurs when a low-intensity stimulus (prepulse) precedes a startle stimulus, reducing the startle response, and is linked to sensorimotor gating deficits seen in schizophrenia (Tsai et al., 2004b). Lastly GlyT1 heterozygotes present better spatial memory retention, while selective disruption of GlyT1 in forebrain neurones result in an enhanced associative and objects recognition memory (Tsai et al., 2004b; Yee et al., 2006). Taken together, these studies suggest that reduced expression of GlyT1 enhances memory retention and protects against disruptions of sensory gating, supporting the view that GlyT1 may represent a target for novel cognition-enhancing and antipsychotic drugs (Harvey et al., 2008a).

1.6.4. GlyT2 knockout mice: a new model for human hyperekplexia

Gomeza et al., (2003b) also generated a mouse line where the GlyT2 gene (Slc6a5) has been inactivated via deletion of exon 4 via homologous recombination. GlyT2 knockout mice gained weight slowly and died prematurely at the end of the second postnatal week, displaying a complex neurological phenotype characterised by spasticity, rigid muscle tone, strong spontaneous tremor, hind feet clasping and a severely impaired righting reflex. Death was probably caused by inability to feed, desiccation and continued convulsions. Electrophysiological recordings of glycine-mediated activity in brain stem slices and spinal cord cultures from GlyT2 knockout mice showed a striking reduction in the amplitude of glycinergetic spontaneous and miniature inhibitory currents. However, there was no obvious change in the
expression or clustering of postsynaptic GlyRs. These experiments highlighted the crucial role of GlyT2 in replenishing the presynaptic pool of glycine needed for efficient neurotransmitter loading into synaptic vesicles. Interestingly, Gomeza et al., (2003b) suggested that the phenotype of GlyT2 knockout mice resembled human hyperekplexia. While this is clearly not the case, the GlyT2 knockout phenotype does resemble several mutants of the mouse GlyR α1 subunit gene (Glra1), in particular oscillator, Nmf11 and cincinnati (Buckwalter et al., 1994; Kling et al., 1997; Traka et al., 2006; Holland et al., 2006).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>Animal</th>
<th>Mutant</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slc6a9</td>
<td>GlyT1</td>
<td>Mouse</td>
<td>Knockout</td>
<td>Motor deficits, lethargy, hypotonia, hyporesponsivity, postnatal death (P0) due to respiratory failure [Gomeza et al., 2003a]</td>
</tr>
<tr>
<td>Slc6a9</td>
<td>GlyT1</td>
<td>Zebrafish</td>
<td>shocked</td>
<td>G81D missense mutation, spontaneous trunk coiling, diminished escape responses, absence of swimming [Cui et al., 2005]</td>
</tr>
<tr>
<td>Slc6a5</td>
<td>GlyT2</td>
<td>Mouse</td>
<td>Knockout</td>
<td>Increased muscle tone, spontaneous tremor, severely impaired righting reflex, neonatal death (~P14) [Gomeza et al., 2003b]</td>
</tr>
</tbody>
</table>

Table 1.4. Mouse presynaptic and glial defects in glycinergic transmission.

1Chromosomal locations were determined using the human genome browser at http://genome.ucsc.edu/

1.7. Presynaptic components of human hyperekplexia

Given the phenotypic similarities of mouse models of GlyR α1 and GlyT2 deficiency, Rees et al., (2006) assessed whether mutations in the human GlyT2 gene (SLC6A5) could cause hyperekplexia. The researchers scanned all 16 coding exons of SLC6A5 by denaturing high-performance liquid chromatography (dHPLC) in an international cohort of 83 individuals affected by sporadic and familial hyperekplexia devoid of mutations in GLRA1, GLRB, GPHN and ARHGEF9. Direct sequencing of aberrant dHPLC profiles revealed a mosaic of missense and nonsense mutations in the GlyT2 gene, and additional regions of common single nucleotide polymorphisms (SNPs; Figure 1.4, Table 1.5).
Figure 1.4. Model of GlyT2 topology and hyperekplexia mutations. This diagram depicts the suggested twelve-membrane spanning domain topology of GlyT2 and relative positions of amino acid alterations caused by recessive (blue circles) and dominant (red circle) mutations found in human hyperekplexia (Rees et al., 2006; Figure modified from Harvey et al., 2008b). The alteration found in CMD2 in Belgian blue cattle (see chapter 6) is also indicated by a purple circle. X stands for stop codon. The positions of glycine and two of the three Na⁺ ions are depicted by a large yellow triangle and inverted blue triangles respectively. Numbered boxes represent potential membrane-spanning domains.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Alteration</th>
<th>Location</th>
<th>Mode</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLC6A5</td>
<td>Y377X</td>
<td>TM3-TM4 loop</td>
<td>CH</td>
<td>Nonsense/protein truncation</td>
</tr>
<tr>
<td></td>
<td>V432F+ fs97</td>
<td>TM5</td>
<td></td>
<td>Frameshift/protein truncation</td>
</tr>
<tr>
<td>SLC6A5</td>
<td>Y491C</td>
<td>TM6-TM7 loop</td>
<td>CH</td>
<td>Missense, functionally inert</td>
</tr>
<tr>
<td></td>
<td>Q630X</td>
<td>TM10</td>
<td></td>
<td>In-frame stop codon/protein truncation</td>
</tr>
<tr>
<td>SLC6A5</td>
<td>P108L+fs25</td>
<td>N-terminus TM6</td>
<td>CH</td>
<td>Frameshift/protein truncation</td>
</tr>
<tr>
<td></td>
<td>W482R</td>
<td>TM6</td>
<td></td>
<td>Missense, glycine binding site</td>
</tr>
<tr>
<td>SLC6A5</td>
<td>L306V</td>
<td>TM3-TM4 loop</td>
<td>CH</td>
<td>Missense, functionally insert with N509S</td>
</tr>
<tr>
<td></td>
<td>N509S</td>
<td>TM7</td>
<td></td>
<td>Missense, Na⁺ binding site</td>
</tr>
<tr>
<td>SLC6A5</td>
<td>Y377X</td>
<td>TM3-TM4 loop</td>
<td>AD</td>
<td>Nonsense/protein truncation</td>
</tr>
<tr>
<td>SLC6A5</td>
<td>T425M</td>
<td>TM5</td>
<td>AR</td>
<td>Missense, functionally inert</td>
</tr>
<tr>
<td>SLC6A5</td>
<td>S510R</td>
<td>TM7</td>
<td>AD</td>
<td>Missense, forms large intracellular aggregates, dominant-negative mutation</td>
</tr>
</tbody>
</table>

Table 1.5. Hyperekplexia mutations in SLC6A5. ¹TM1-TM12 denote the twelve membrane spanning domains of GlyT2. X denotes a stop codon. Loops are sequences linking the membrane-spanning domains. ²AR: autosomal recessive; AD: autosomal dominant; CH: compound heterozygosity.

In the majority of cases GlyT2 mutations were inherited as compound heterozygotes, indicating that this gene is mostly associated with recessive hyperekplexia, although one dominant mutation (S510R) was also found. Individuals with mutations in SLC6A5 displayed classical symptoms of hyperekplexia, but also prolonged spasms and life-threatening apnoea and breath-holding attacks, which often improved after the first year of life. The functional relevance of these mutations was assessed by cell-
surface localisation and $[^3H]$-glycine uptake assays in HEK293 cells transfected with EGFP-tagged wild-type and mutant GlyT2 plasmids. GlyT2 mutations resulted in a defective subcellular GlyT2 localisation or decreased glycine uptake or both, affecting the correct functioning of the transporter (Table 1.5). The crystal structure of the leucine transporter (LeuT), a bacterial homologue of the mammalian SLC6 family (Yamashita et al., 2005), helped understanding the precise molecular mechanisms underlying SLC6A5 missense mutations. Alignment of the Aquifex aeolicus LeuT with GlyT2 allowed the identification of residues potentially involved in coordinating glycine and Na$^+$ binding. For example, mutations W482R (TM6) and N509S (TM7) were predicted to affect the binding to GlyT2 of glycine and Na$^+$ respectively (Figures 1.4 and 1.5). Electrophysiological studies in Xenopus oocytes confirmed these predictions. The Y377X nonsense mutation was subsequently found in a multigenerational family, together with two additional variants A89E and G767R, which are caused by common SNPs in SLC6A5 (Eulenburg et al., 2006). These studies demonstrated that SLC6A5 is a second major hyperekplexia gene and should have equal priority to GLRA1 in genetic screening. Interestingly, there are several marked differences between mouse knockouts for GlyT2 and individuals with mutations in SLC6A5. In mice, muscle stiffness and tremor are spontaneous, whereas in humans they are induced by acoustic or tactile stimuli. Furthermore, SLC6A5 mutations are not lethal in humans and in most cases symptoms resolved within the first year of life following intensive postnatal and infant care.

1.8. Thesis aims

Following the initial identification of mutations in SLC6A5, in collaboration with Professor Mark Rees (Institute of Life Sciences, Swansea University) several new GlyT2 mutations have been identified in individuals from the UK, Spain, Holland, France and Italy. One main aim of my work was to characterise the functional consequences of these novel GlyT2 variants to assess whether they affect $[^3H]$-glycine transport and subcellular location. Since in some individuals only one defective allele was identified, I also studied alternative splicing of the human GlyT2 gene in order to make sure that I had analysed all coding exons. Furthermore, since the correct synaptic localisation of GlyT2 is vital, I decided to characterise GlyT2 interacting proteins using the YTH system. As well as revealing mechanisms underlying the
trafficking and regulation of the transporter, I intended to prioritise GlyT2 interacting proteins for genetic analysis in remaining hyperekplexia patients devoid of defects in known causative genes. Lastly, I undertook a retrospective analysis of glycinergic dysfunction in animal models, in order to determine whether there were any ‘unresolved’ cases that might be caused by GlyT2 rather than GlyR deficits. This resulted in the characterisation of a GlyT2 defect in CMD2, a recessive disorder that affects Belgian blue cattle (Charlier et al., 2008a).

Figure 1.5. A model of GlyT2 and the hyperekplexia N509S mutation affecting Na⁺ affinity. A: model of the hGlyT2 dimer (Figure modified from Harvey et al., 2008b) showing coloured transmembrane helices (ribbons). For each monomer, glycine, as well as two (out of three) sodium ions, are shown in CPK representation, in yellow and small blue spheres respectively. Note that the TM3-TM4 extracellular loop (EL2) was not modelled due to an insertion of residues 331-389 relative to LeuTAA. View of the sodium ion Na1 binding-site in the native N509 (B) and mutant S509 (C) structures. Na1, Na2, and residues from TM1, TM6 and TM7, as well as the bound glycine are shown. N509, which is located in TM7, is able to interact with Na1 whilst S509 is too distant (3.4Å) to co-ordinate Na1. All models were visualised using the molecular graphics program Chimera (http://www.cgl.ucsf.edu/chimera/).
2. METHODS
2.1. Bacterial methodology

2.1.1. Materials

TOP10 chemically competent *E. coli* cells were purchased from Invitrogen Ltd (Paisley, UK). Luria-Bertani (LB) medium, LB Agar, calcium chloride dehydrate, glycerol and magnesium chloride were purchased from Sigma-Aldrich Company LTD (Dorset, UK). HiSpeed Plasmid Maxi kit, QuickLyse Miniprep kit and Elution Buffer were purchased from Qiagen Ltd (West Sussex, UK). Ampicillin was purchased from Calbiochem (Nottingham UK). Kanamycin was purchased from Merck (Dorset, UK).

2.1.2. Bacteria culture

LB medium and LB agar medium were used for routine bacteria culture. *LB medium*: 1% (w/v) peptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl; *LB Agar medium*: LB medium supplemented with 1.5% (w/v) agar. ‘In-house’ competent cells were prepared from TOP10 *E. coli* after restreaking onto LB agar plates which were inverted and incubated at 37°C overnight. LB liquid cultures were grown at 37°C overnight with shaking. For plasmid selection, LB cultures were supplemented with the antibiotics at final concentration of 100 μg/ml (ampicillin) or 50 μg/ml (kanamycin).

2.1.3. Preparation of competent *E. coli* cells

Solutions required: 80 mM CaCl₂/ 50 mM MgCl₂; 0.1 mM CaCl₂; 50% (v/v) glycerol. Competent cells were prepared using a modified calcium chloride protocol as described by Cohen *et al.* (1972). A sterile loop was used to streak chemically competent TOP10 cells onto an LB agar plate that was then incubated at 37°C overnight. The following day a colony was picked from the plate and used to inoculate 2 ml of LB medium. This culture was grown overnight with vigorous shaking at 37°C. The following day, the overnight culture was used to inoculate 200 ml of pre-warmed LB medium. This overnight culture was then grown at 37°C with shaking and the optical density at 600 nm (OD₆₀₀) was determined every 20 minutes after the first 2 hours of incubation in order to monitor culture growth. When an OD₆₀₀ of 0.95 was reached, the culture was poured into pre-chilled 50 ml falcon tubes and subjected to centrifugation at 4,000 g for 5 minutes at 4°C. The supernatants were carefully removed and the pellets were then resuspended in 10 ml of ice-cold
CaCl₂/MgCl₂ solution. After incubation on ice for 10 minutes, the cells were pelleted by centrifugation at 3,000 g for 3 minutes at 4°C. This spin/wash/store on ice step was repeated twice, after which the cells were resuspended in 5 ml of ice-cold CaCl₂ solution and then mixed with 5 ml of 50% glycerol. The cells were then dispensed into 550 µl aliquots in prechilled microcentrifuge tubes and frozen immediately in liquid nitrogen. The tubes were stored at -80°C.

2.1.4. Transformation of competent E. coli cells

Bacteria were transformed using a protocol based on work of Mandel and Higa (1970) and Cohen et al. (1972) who showed that bacteria treated with ice-cold CaCl₂ and then subjected to a brief heat-shock could take up plasmid DNA. A tube containing frozen competent cells was gently thawed on ice. For each DNA to be transformed, 100 µl of competent cells were pipetted into a clean microcentrifuge tube and 10 ng of plasmid DNA or 5 µl ligation mix was added to the cells. The cells were incubated on ice for 30 minutes, heat-shocked for 30 seconds at 42°C and placed on ice for 5 minutes. A 300 µl volume of LB medium was added and the tube was shaken at 37°C for 1 hour. During this step the cells were able to express the antibiotic resistance gene encoded by the plasmid. After one hour the cells were spread using a sterile glass spreader on agar plates containing the appropriate antibiotic. The plates were then inverted and incubated overnight at 37°C.

2.1.5. Small-scale preparation of plasmid DNA (minipreps)

Solutions required: Lysis Buffer: 200 mM NaOH, 1% (w/v) SDS, RNase A, Lysozyme; Wash Buffer: 1 M NaCl, 50 mM MOPS (pH 7.0), 15% (v/v) isopropanol; Elution Buffer: 10 mM Tris-HCl (pH 8.5). DNA minipreps were obtained according to the QuickLyse Miniprep procedure (Qiagen). This is based on the alkaline lysis of bacteria followed by the absorption of DNA onto a silica membrane and elution of DNA. Briefly, single colonies from the transformed agar plates were picked using a pipette tip and used to inoculate 2 ml of LB medium supplemented with the appropriate antibiotic in a 15 ml falcon tube. After incubation of the culture at 37°C overnight with vigorous shaking, bacterial cells were pelleted by centrifugation at 16,000 g for 1 minute. The supernatant was discarded and the pellet was resuspended in 400 µl pre-chilled lysis buffer containing lysozyme and RNase. After 3 minutes incubation, the lysate was transferred to a spin column and centrifuged at 16,000 g for
1 minute. The column-attached DNA was washed with an isopropanol-based solution in order to remove any cellular debris. Two centrifugation steps at 16,000 g of 1 minute each were performed to remove the residual washing buffer and dry the column. The column was placed in a fresh microcentrifuge tube and a 50 µl low-salt volume of elution buffer (Buffer EB; Qiagen) was added to the matrix to elute the DNA by centrifugation at 16,000 for 1 minute. The yield was determined using a UV spectrophotometer and varied from 0.2 to 0.6 µg/µl. Miniprep DNAs were stored at -20°C.

2.1.6. Large-scale preparation of plasmid DNA (maxipreps)

Solutions required: Buffer P1 (resuspension buffer): 50 mM Tris-Cl (pH 8.0), 10 mM EDTA, 100 µg/ml RNase A; Buffer P2 (lysis buffer): 200 mM NaOH, 1% (w/v) SDS; Buffer P3 (neutralisation buffer): 3.0 M potassium acetate (pH 5.5); Buffer QBT (equilibration buffer): 750 mM NaCl, 50 mM MOPS (pH 7.0), 15% (v/v) isopropanol, 0.15 % Triton X-100; Buffer QC (wash buffer): 1.0 M NaCl, 50 mM MOPS (pH 7.0), 15% (v/v) isopropanol; Buffer QF (elution buffer): 1.25 M NaCl, 50 mM Tris-HCl (pH 8.5), 15% (v/v) isopropanol; Elution Buffer (EB): 10 mM Tris-HCl (pH 8.5). Maxipreps of plasmid DNA were carried out according to the HiSpeed Plasmid Purification procedure (Qiagen), which is based on a modified alkaline lysis method followed by the binding of plasmid DNA to a resin in low-salt and pH conditions. A single bacteria colony was picked from the agar plate and resuspended in 2 ml of LB medium supplemented with the appropriate antibiotic in a 15 ml falcon tube. The tube was incubated for approximately 8 hours at 37°C with vigorous shaking. A 200 µl volume of the bacteria culture was transferred into a sterile flask containing 200 ml of LB medium and the appropriate antibiotic. The culture was grown for 12-16 hours at 37°C with vigorous shaking. The bacterial cells were harvested by centrifugation at 4,000 g for 15 min at 4°C. After removing the supernatant, the pellet was resuspended completely in 10 ml pre-chilled buffer P1. Then 10 ml of lysis buffer P2 were added and the suspension was mixed by inverting six times and incubated at room temperature for 5 minutes. Finally, 10 ml of pre-chilled buffer P3 were added to the lysate, the suspension was poured into the barrel of the Qiafilter Cartridge and incubated at room temperature for 10 minutes. During this incubation step, a HiSpeed Maxi Tip was equilibrated by applying 10 ml buffer QBT, allowing the column to empty by gravity flow. After removing the cap from the Qiafilter outlet nozzle, the
cell lysate was filtered into the previously equilibrated HiSpeed Tip and allowed to enter the resin by gravity flow. After washing the Tip with 60 ml Buffer QC, the DNA was eluted with 15 ml Buffer QF and the eluate was collected in a clean 50 ml falcon tube. DNA was precipitated adding 10.5 ml isopropanol to the eluate and the mix was incubated at room temperature for 5 minutes. During the incubation a Qiaprecipitator Maxi Module was attached to a 30 ml syringe. The eluate/isopropanol mixture was filtered through the Qiaprecipitator. DNA was washed adding 2 ml 70% (v/v) ethanol to the syringe. After drying the membrane, the Qiaprecipitator was attached to a 5 ml syringe and 0.5 ml EB buffer was added to the syringe. The DNA was eluted into a fresh 1.5 ml microcentrifuge tube. The eluate was transferred again into the syringe and the elution step was repeated in order to maximise the amount of DNA solubilised and recovered from the Qiaprecipitator. The DNA concentration was determined by spectrophotometry at 260 nm and varied between 0.5 and 4 µg/µl. Maxiprep DNAs were stored at -20°C.

2.2. DNA methodology

2.2.1. Materials

Agarose, DNA molecular weight marker, Zero Blunt TOPO PCR cloning kit, GeneRacer Kit, Accuprime Pfx DNA polymerase, 25:24:1 (v/v) phenol:chloroform:isoamyl alcohol, SyBRSafe DNA gel stain and Safe Imager Transilluminator were purchased from Invitrogen Ltd (Paisley, UK). Poly(A)+ RNA (hippocampus and spinal cord) was purchased from Clontech (Berks, UK). Acetic acid, bromophenol blue, ethylenediaminetetraacetic acid disodium salt (EDTA), glycogen from oyster (type 2), sodium acetate anhydrous, sucrose and tris(hydroxymethyl)methylamine (Tris-base) were purchased from Sigma-Aldrich Company Ltd (Dorset, UK). Sephaglas Bandprep kit was purchased from Amersham Biosciences (Bucks, UK). Ethanol was purchased from BDH Laboratory Supplies (Poole, UK). Elution buffer EB was purchased from Qiagen Ltd (West Sussex, UK). PfuTurbo master mix was purchased from Stratagene (Cambridge, UK). Restriction enzymes were purchased from New England Biolabs (Ipswich, UK). T4 DNA ligase was purchased from Roche Diagnostic Ltd (West Sussex, UK). HiSpeed Plasmid Maxi kit and QIAprep Spin Miniprep kits were purchased from Qiagen Ltd (West Sussex, UK).
2.2.2. Plasmid construct generation

The majority of expression constructs were generated by (i) performing a PCR amplification using oligonucleotide primers where restriction enzyme recognition sites had been incorporated into the 5' end of the primer; (ii) agarose gel electrophoresis of the PCR product and subsequent DNA gel purification; (iii) restriction enzyme digestion of the gel-purified product and cloning vector; (iv) phenol/chloroform extraction of the digested DNA; (v) ligation of the purified PCR product and cloning vector, catalysed by T4 ligase; (vi) transformation of E. coli with the ligation product and (vii) dideoxy sequencing of the recombinant clones to confirm a full-length insert and an encoded in-frame fusion protein. Cloning procedures are detailed in the following sections.

2.2.3. Polymerase Chain Reaction (PCR)

Human hippocampal cDNA (Clontech), human spinal cord cDNA (Clontech) or previously constructed plasmids were used as templates for PCR amplification. For all PCR reactions the components were pipetted into 0.2 ml microcentrifuge tubes, mixed by tapping and briefly centrifuged before loading on the Hybaid PCR Express thermal cycler. Stratagene PfuTurbo Hotstart PCR master mix is a 2 × formulation of PfuTurbo Hotstart PCR DNA polymerase, PCR buffer, magnesium and dNTPs. This formulation was used to amplify fragments longer than 1 kb. Invitrogen Accuprime Pfx DNA polymerase has a proofreading 3' to 5' exonuclease activity that provides higher fidelity than Pfu and was also used to amplify large DNA targets. The error rate of the two enzymes, expressed as mutation frequency per base pair per duplication, is respectively of $1.3 \times 10^{-6}$ for Pfu Turbo and $3 \times 10^{-7}$ for Accuprime Pfx. The four types of PCR reactions are tabulated below.

<table>
<thead>
<tr>
<th>PCR reaction 1: Accuprime Pfx DNA polymerase PCR, cDNA template</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PCR component</strong></td>
</tr>
<tr>
<td>cDNA</td>
</tr>
<tr>
<td>Primer, forward</td>
</tr>
<tr>
<td>Primer, reverse</td>
</tr>
<tr>
<td>Pfx mastermix</td>
</tr>
</tbody>
</table>
PCR reaction 2: Accuprime *Pfx* DNA polymerase PCR, plasmid DNA template

<table>
<thead>
<tr>
<th>PCR component</th>
<th>Stock concentration</th>
<th>Volume added to a 25 µl reaction</th>
<th>Working concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid DNA</td>
<td>50 ng/µl</td>
<td>1</td>
<td>2 ng/µl</td>
</tr>
<tr>
<td>Primer, forward</td>
<td>10 pmol/µl</td>
<td>1</td>
<td>0.4 pmol/µl</td>
</tr>
<tr>
<td>Primer, reverse</td>
<td>10 pmol/µl</td>
<td>1</td>
<td>0.4 pmol/µl</td>
</tr>
<tr>
<td><em>Pfx</em> mastermix</td>
<td>$2.2 \times 10^4$ U/µl</td>
<td>22</td>
<td>$1.9 \times 10^2$ U/µl</td>
</tr>
</tbody>
</table>

PCR reaction 3: *PfuTurbo* master mix PCR, cDNA template

<table>
<thead>
<tr>
<th>PCR component</th>
<th>Stock concentration</th>
<th>Volume added to a 50 µl reaction</th>
<th>Working concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNA</td>
<td>0.2-0.5 µg/µl</td>
<td>0.5</td>
<td>2-5 ng/µl</td>
</tr>
<tr>
<td>Primer, forward</td>
<td>10 pmol/µl</td>
<td>1</td>
<td>0.2 pmol/µl</td>
</tr>
<tr>
<td>Primer, reverse</td>
<td>10 pmol/µl</td>
<td>1</td>
<td>0.2 pmol/µl</td>
</tr>
<tr>
<td><em>PfuTurbo</em> master mix</td>
<td>$2 \times$</td>
<td>25</td>
<td>$1 \times$</td>
</tr>
<tr>
<td>dH₂O</td>
<td></td>
<td>22</td>
<td></td>
</tr>
</tbody>
</table>

PCR reaction 4: *PfuTurbo* master mix PCR, plasmid DNA template

<table>
<thead>
<tr>
<th>PCR component</th>
<th>Stock concentration</th>
<th>Volume added to a 50 µl reaction</th>
<th>Working concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid DNA</td>
<td>50 ng/µl</td>
<td>1</td>
<td>1 ng/µl</td>
</tr>
<tr>
<td>Primer, forward</td>
<td>10 pmol/µl</td>
<td>1</td>
<td>0.2 pmol/µl</td>
</tr>
<tr>
<td>Primer, reverse</td>
<td>10 pmol/µl</td>
<td>1</td>
<td>0.2 pmol/µl</td>
</tr>
<tr>
<td><em>PfuTurbo</em> master mix</td>
<td>$2 \times$</td>
<td>25</td>
<td>$1 \times$</td>
</tr>
<tr>
<td>dH₂O</td>
<td></td>
<td>22</td>
<td></td>
</tr>
</tbody>
</table>

Template DNA used for PCR amplification

Human brain cDNA (Clontech), plasmid DNAs harbouring cDNA clones purchased from RZPD (German Resource Centre for Genome Research) and bovine genomic DNA were used as template DNA for PCR amplifications.

PCR amplification conditions

PCR conditions used for the generation of each construct are detailed below. PCR amplifications involved an initial 94°C denaturation (2 minutes) followed by up to 35 cycles of denaturing at 94°C (1 minute), annealing at 55, 60 or 65°C (1 minute) and extension at 68 or 72°C. The extension time was dependent on the length of the fragment to amplify and on the enzyme used. Details of each PCR reaction type are provided below.
**pYTH16-GlyT2N:** a partial cDNA encoding the human GlyT2 N-terminus was amplified from human adult hippocampal cDNA using the primers hGlyT2NSalI and hGlyT2NEcoRI (PCR reaction type 1) and 35 cycles of 94°C for 1 minute, 60°C for 1 minute and 68°C for 2 minutes.

**pYTH16-GlyT2C:** a partial cDNA encoding the human GlyT2 C-terminus was amplified from human adult hippocampal cDNA using the primers hGlyT2CSalI and hGlyT2CEcoRI (PCR reaction type 1) and 35 cycles of 94°C for 1 minute, 60°C for 1 minute and 68°C for 1 minute.

**pYTH16-GlyT2CdelTQC:** a partial cDNA encoding the human GlyT2 C-terminus, but lacking the C-terminal PDZ domain binding site (TQC) was amplified from human adult hippocampal cDNA using the primers hGlyT2CSalI and hGlyT2CEcoRI-TQC and 35 cycles of 94°C for 1 minute, 60°C for 1 minute and 68°C for 1 minute (PCR reaction type 1).

**pACT2-ULIP6:** the complete coding region of human ULIP6 was amplified from human adult hippocampal cDNA using the primers ULIP6-EcoRI and ULIP6-XhoI and 30 cycles of 94°C for 1 minute, 60°C for 1 minute and 72°C for 5 minutes (PCR reaction type 3).

**pACT2-Syntenin-1:** the complete coding region of human syntenin-1 was amplified from cDNA clone (NM_005625) from RZPD using the primers SDB1-BamHI and SDB-XhoI and 30 cycles of 94°C for 1 minute, 60°C for 1 minute and 72°C for 3 minutes (PCR reaction type 3).

**pcDNA3-hGlyT2:** a full-length GlyT2 cDNA was amplified from human hippocampal cDNA (Clontech) using the primers hGlyT2A-1 and hGlyT2A-2 and 30 cycles of 94°C for 1 minute, 60°C for 1 minute and 72°C for 6 minutes (PCR reaction type 3). The insert from this clone was subcloned into three additional vectors: pEGFP-C1 (Clontech, resulting in pEGFP-hGlyT2), pRK5myc (resulting in pRK5myc-hGlyT2) and pRc/CMV (Invitrogen, resulting in pRc/CMV-hGlyT2).

**TOPO-bGlyT2Ex4+5:** a genomic DNA fragment containing SLC6A5 exons 4 and 5 were amplified from bovine genomic DNAs (CMD2 homozygote and heterozygote
samples) using the primers bGlyT2Ex4+5A and bGlyT2Ex4+5B and 25 cycles of 94°C for 1 minute, 60°C for 1 minute and 68°C for 2 minutes (PCR reaction type 1).

**pRe/CMV-GlyT2-B:** a partial cDNA encoding the C-terminus of GlyT2-B was amplified from human adult spinal cord first-strand cDNA. The first stage (PCR reaction type 1) was performed using the primers hGlyT2CNaeI3 and hGlyT2CXbaI3 and 25 cycles of 94°C for 1 minute, 60°C for 1 minute and 68°C for 2 minutes. The second stage, or nested PCR, followed the same conditions using hGlyT2CEcoNI and hGlyT2CXbaI1 primers.

**pRK5FLAG-FMN2:** the complete FMNL2 coding region was amplified from KIAA1902 cDNA (obtained from RZPD) using the primers FMNL2-XmaI and FMNL2-XbaI and 25 cycles of 94°C for 1 minute, 60°C for 1 minute and 72°C for 6 minutes (PCR reaction type 3).

### 2.2.4. Oligonucleotide primers

Table 2.1 contains the sequences of the oligonucleotide primers used for PCR amplifications.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5' to 3')</th>
<th>Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>hGlyT2NSalI</td>
<td>TGTGTCGACCATTGGAATTGGCAGTGCTCCCC</td>
<td>SalI</td>
</tr>
<tr>
<td>hGlyT2EcoRI</td>
<td>GGAGAATTCTCAGTCAGTCAGTTTGCTGGACC</td>
<td>EcoRI</td>
</tr>
<tr>
<td>hGlyT2CsalI</td>
<td>GGTGTCGACAATAACTGATCTGGGCCC</td>
<td>SalI</td>
</tr>
<tr>
<td>hGlyT2EcoRI</td>
<td>ACCGAATTCTCAGACTGATCTGCCCAGTTCC</td>
<td>EcoRI</td>
</tr>
<tr>
<td>hGlyT2EcOrITQC</td>
<td>CTAGAATTCTCAGGAGTTGCCAATCCTT</td>
<td>EcoRI</td>
</tr>
<tr>
<td>hGlyT2A-1</td>
<td>TGGGATGCCACATGGAATTGGCAGTGCTGGACC</td>
<td>SalI</td>
</tr>
<tr>
<td>hGlyT2A-2</td>
<td>ACCCTCGAGCTAGCTGAGTCAGTGGCCCAGTTTC</td>
<td>XhoI</td>
</tr>
<tr>
<td>hGlyT2CNaeI3</td>
<td>TTTGCGGCGCTCGCTACATCTCTTCGGTTATAC</td>
<td>NaeI</td>
</tr>
<tr>
<td>hGlyT2XbaI3</td>
<td>AATTCTAAGCTCAGTCTGATGTTTCAGCAGTGGG</td>
<td>XbaI</td>
</tr>
<tr>
<td>hGlyT2ECNaeI</td>
<td>CCGGAAGCTTAAACCAGGCTGGCCTCTCC</td>
<td>EcoRI</td>
</tr>
<tr>
<td>hGlyT2CXMbaI</td>
<td>AATTCTAAGGCAATGCGCTCTTCCTACTATCTGCAGTCGCTG</td>
<td>XbaI</td>
</tr>
<tr>
<td>FMNL2-XmaI</td>
<td>CGCCCGGCGGAACTGGCAGGCGGAGCACCAGCTGAGGAT</td>
<td>XmaI</td>
</tr>
<tr>
<td>FMNL2-XbaI</td>
<td>GCCCTCAGACAGGCTACATTGTTATTTCCGGACC</td>
<td>XbaI</td>
</tr>
<tr>
<td>ULIP6-EcoRI</td>
<td>CCGGAATTCTAATGCTCTGCTGAGCTGGAACC</td>
<td>EcoRI</td>
</tr>
<tr>
<td>ULIP6-XhoI</td>
<td>GCACTCGAGTACGACGCTGGCAGTTACC</td>
<td>XhoI</td>
</tr>
<tr>
<td>SDB1-BamHI</td>
<td>ATCCGATCACATTGCTCTCTCTCTCTCTCTCTCTCTCT</td>
<td>BamHI</td>
</tr>
<tr>
<td>SDB1-XhoI</td>
<td>CGCCGATGTTACGACGCTGGCAGTTACC</td>
<td>XhoI</td>
</tr>
<tr>
<td>bGlyT2Ex4+5A</td>
<td>CCCCAGCATCTAGATATAGAGGCC</td>
<td></td>
</tr>
<tr>
<td>bGlyT2Ex4+5B</td>
<td>GCTTGGGGCTCTGTGAGCTCC</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.1. Oligonucleotide primers used for PCR amplification.** The restriction enzyme recognition site incorporated into the different primers for cloning purposes are indicated by underlining.
2.2.5. Agarose gel electrophoresis

Solutions required: *Tris-acetate EDTA (TAE) buffer*: 40 mM Tris-base, 20 mM acetic acid, 1 mM EDTA (pH 8.0); *Loading buffer*: 0.25% bromophenol blue, 40% (w/v) sucrose in distilled water. DNA fragments were visualised and separated using agarose gels containing SYBR Safe™ DNA gel stain. SYBR Safe™ DNA gel stain provides sensitive DNA and RNA detection with substantially reduced mutagenicity, making it safer to use than ethidium bromide. To pour a gel, 1 g of agarose powder was dissolved in 100 ml 1 x TAE buffer and heated in a microwave oven until completely melted. After cooling the solution a volume of 10 μl of SYBR Safe™ DNA gel stain was added to facilitate the visualisation of DNA after electrophoresis. The solution was poured in a casting tray containing a sample comb and allowed to solidify at room temperature for 15-30 minutes. The gel was then placed in an electrophoresis chamber filled with TAE buffer and the comb was removed. Samples were mixed with 3 μl loading buffer then loaded into the wells against a 1 μg 1 Kb DNA ladder (Invitrogen). Electrophoresis was performed at 90 V for 30 minutes and fragments of interest were visualised using the Safe Imager Transilluminator or photographed using transmitted UV light on an InGenius gel doc system (Syngene).

2.2.6. Agarose gel purification of DNA fragments

Solutions required: *Wash buffer*: 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.1 mM NaCl solution; *Elution Buffer*: 10 mM Tris-HCl (pH 8.5), 1 mM EDTA. DNA was purified from the agarose gel using the Sephaglas BandPrep Kit (Amersham Biosciences). Agarose slices containing the DNA band of interest were cut out from the gel using a sterile scalpel and transferred to fresh 1.5 ml microcentrifuge tubes. For slices weighing less than 250 mg, a volume of 250 μl gel solubiliser was added to the tube. For slices weighing more than 250 mg, an additional 1 μl gel solubiliser for each mg of agarose was added. The tube was vortexed and incubated at 60°C with shaking until the slice was dissolved. The Sephaglas BP, a glass matrix, was resuspended by vortexing the container and 5 μl of the suspension were added to the dissolved slice. The mixture was incubated for 5 minutes at room temperature, mixing gently every minute to resuspend the Sephaglas. The tube was subjected to centrifugation at 16,000 g for 1 minute and the supernatant was discarded taking care not to disturb the Sephaglas pellet. The centrifugation step was repeated to remove
any residual liquid. Next, 80 µl of wash buffer were added to the tube and the Sephaglas pellet was resuspended by pipetting up and down several times. The tube was centrifuged for 1 minute at 16,000 g and the supernatant removed. This step was repeated twice for a total of three washes. The tube was subjected to a further centrifugation at 16,000 g for 1 minute to remove any residual liquid and let air dry for 5 minutes at room temperature. The DNA was eluted from the Sephaglas pellet by adding 25 µl elution buffer and incubating at 37°C for 10 minutes with vigorous shaking. The tube was subjected to centrifugation at 16,000 g for 1 minute and the supernatant was carefully removed and placed into a clean 1.5 ml microcentrifuge tube, taking care not to disturb the glass pellet. The eluted DNA was stored at -20°C.

2.2.7. Restriction enzyme digestion

Gel purified PCR product restriction enzyme digestions
The gel purified DNA (25 µl) was mixed with 3 µl 10 × enzyme buffer and 1 µl of each enzyme (New England Biolabs: concentration 10 units/µl). The reaction was mixed, briefly centrifuged and incubated at 37°C for 1 hour.

Plasmid DNA restriction enzyme digestions
Plasmid DNA (2 µl at 0.5 µg/µl) was mixed with 2 µl 10 × enzyme buffer, 1 µl of each restriction enzyme and 14 µl dH2O to a final volume of 20 µl. The reaction was mixed, briefly centrifuged and incubated at 37°C for 1 hour.

2.2.8. Phenol-Chloroform extraction

This procedure was carried out to inactivate and remove restriction enzymes and buffer components before proceeding to the ligation reaction. The volume of the restriction enzyme digestion was increased to 100 µl with dH2O. In a fume hood a volume of 100 µl phenol: chloroform: isoamyl alcohol (25:24:1) was added to this mix and the tube content was mixed by tapping until an emulsion formed. The tube was then subjected to centrifugation at 16,000 g for 15 minutes. The upper aqueous phase was transferred to a clean 1.5 ml microcentrifuge tube containing 250 µl of 96% (v/v) ethanol, 10 µl 3 M sodium acetate (pH 4.8) and 1 µl oyster glycogen (1 µg). The contents were mixed thoroughly and the tube was incubated on dry ice for 15 minutes. The DNA was recovered by centrifugation at 16,000 g for 15 minutes and the supernatant was discarded. The pellet was washed with 250 µl 80% (v/v) ethanol and centrifuged at 16,000 rpm for 1 minute. The supernatant was removed, the tube was
subjected to a further centrifugation step at 16,000 g and any residual ethanol was removed. The pellet was air dried for 5 minutes at room temperature, dissolved in an appropriate volume of elution buffer EB and incubated at 37°C for 10 minutes with shaking. In case of purification of vector DNA for subsequent ligation, the pellet was resuspended in 50 μl of buffer EB for a final vector concentration of 50 ng/μl. When purifying DNA fragments to be inserted into a vector the pellet was resuspended in 10 μl of buffer EB.

2.2.9. Ligation of DNA inserts into plasmid vectors

Solutions required: *Ligation buffer*: 66 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 1 mM dithiothreitol, 1 mM ATP. After carrying out a restriction enzyme digestion and phenol-chloroform extraction on both the DNA insert and the plasmid vector, a volume of 7 μl DNA insert was added to 1 μl vector (50 ng/μl), 1 μl 10 x ligation buffer and 1 μl bacteriophage T4 DNA ligase (1 U/μl) in a 1.5 ml microcentrifuge tube. The reaction was mixed, briefly centrifuged and incubated at 4°C for 12-16 hours.

2.2.10. Restriction enzyme digestion to remove recircularised vector

To increase the number of colonies of interest, a post-ligation digest step was sometimes performed using a restriction enzyme that would cut religated vector but not a plasmid harbouring an insert. For this strategy to work: i) a unique restriction enzyme recognition site must be present in the vector multiple cloning site between the two restriction enzyme sites used for cloning and ii) this restriction enzyme must not cut the chosen insert DNA. Digesting the ligation product with this enzyme linearises religated vectors, but not those carrying inserts. When using this strategy, 8 μl of ligation reaction was mixed with 1 μl restriction enzyme (10 U) and 1 μl of 10 x restriction enzyme buffer in a 1.5 ml microcentrifuge tube. The reaction was mixed and incubated at 37°C for 1 hour.

2.2.11. DNA sequencing

Plasmid DNA samples were sent to the Sequencing Service, School of Life Science, University of Dundee, Scotland (http://www.dnaseq.co.uk/) for DNA sequencing. DNA sequencing was performed using Applied Biosystems BigDye V3.1 chemistry
on an Applied Biosystems 3730 Automated Capillary DNA Sequencer. Template was supplied at a concentration of 200-300 ng in a 15 µl volume per reaction. Sequencing primers were provided at a concentration of 3.2 pmol per reaction. DNA templates and primers were diluted in dH2O. Sequences of oligonucleotide primers used for sequencing reactions are shown in Table 2.2. The results were downloaded and analyzed with Sequencer software (GeneCodes Corporation) version 4.1.2.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Template</th>
<th>Direction</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>T7</td>
<td>pCR4Blunt-TOPO</td>
<td>F</td>
<td>TAATACGACTCACTATATAGG</td>
</tr>
<tr>
<td>T3</td>
<td>pCR4Blunt-TOPO</td>
<td>F</td>
<td>AATTAACCTCTACTAAGGG</td>
</tr>
<tr>
<td>M13F</td>
<td>pCR4Blunt-TOPO</td>
<td>F</td>
<td>GCAAACAGCTATGACCATG</td>
</tr>
<tr>
<td>M13R</td>
<td>pCR4Blunt-TOPO</td>
<td>F</td>
<td>GGAACAGCTATGACCATG</td>
</tr>
<tr>
<td>pACT2 F</td>
<td>pACT2</td>
<td>F</td>
<td>AATACCACCTACAAATGGATGGATAT</td>
</tr>
<tr>
<td>pYTH16 F</td>
<td>pYTH16</td>
<td>F</td>
<td>CAGGAGAGCTCTACACATGAGG</td>
</tr>
<tr>
<td>pACT2 R</td>
<td>pACT2</td>
<td>R</td>
<td>GAGGTACATGGCCAGATG</td>
</tr>
<tr>
<td>SP6</td>
<td>pRK5MYC</td>
<td>F</td>
<td>ATTTAGGTGACACTATAG</td>
</tr>
<tr>
<td>SP6 Rev</td>
<td>pRK5FLAG</td>
<td>R</td>
<td>GACAAACCACAACACTAGAATGC</td>
</tr>
<tr>
<td>GlyT2S1</td>
<td>GlyT2 cDNA</td>
<td>F</td>
<td>GAACGTGAGTGGGCCA</td>
</tr>
<tr>
<td>GlyT2S2</td>
<td>GlyT2 cDNA</td>
<td>F</td>
<td>TGCCGCTGGTGGGCCG</td>
</tr>
<tr>
<td>GlyT2S3</td>
<td>GlyT2 cDNA</td>
<td>F</td>
<td>TGCGGGATTTGGATG</td>
</tr>
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<td>GlyT2S4</td>
<td>GlyT2 cDNA</td>
<td>F</td>
<td>TCAACATGGAGATG</td>
</tr>
<tr>
<td>GlyT2S5</td>
<td>GlyT2 cDNA</td>
<td>F</td>
<td>GTGAGATGATGAGATG</td>
</tr>
</tbody>
</table>

Table 2.2. Sequencing primers. The sequence, orientation (5'-3') and template used for each primer are shown. F: forward. R: reverse.

2.2.12. Rapid Amplification of cDNA Ends (RACE)

The GeneRacer kit (Invitrogen) was used to amplify cDNAs encoding alternative N- and C- termini for GlyT2. Two general RACE strategies exist: one amplifies 3' ends of transcripts (3' RACE) whereas the other captures 5' ends of mRNAs (5' RACE). Briefly, in 3' RACE mRNA was reverse-transcribed using a modified oligo dT primer with a known priming site (anchor primer 1) at the 5' end. This first-strand cDNA was then amplified using a forward gene specific primer (GSP1) identical to a known sequence in SLC6A5 together with anchor primer 1 (AP1). To increase the specificity of the PCR, a second stage nested PCR was performed using an additional gene specific primer (GSP2) and anchor primer 2 (AP2), using the product of the first stage PCR as template (Figure 2.1).
In 5' RACE, after dephosphorylation and decapping of the mRNA, a RNA oligo was ligated to the 5' end of the mRNA in order to provide a known priming site for GeneRacer PCR primers. The ligated mRNA was reverse transcribed into first-strand cDNA using random primers. The first-strand cDNA was amplified using a gene specific primer complementary to a known sequence in \textit{SLC6A5} and an anchor primer homologous to part of the RNA oligo. A second stage of PCR was carried out using nested primers as described above, with the product of the first stage PCR as template (Figure 2.2).

**Figure 2.1. Schematic representation of the 3' RACE protocol.** GSP1: gene specific primer 1; GSP2: gene specific primer 2; AP1: anchor primer 1; AP2: anchor primer 2

**Figure 2.2. Schematic representation of the 5' RACE protocol.** GSP1: gene specific primer 1; GSP2: gene specific primer 2; AP1: anchor primer 1; AP2: anchor primer 2.
3' RACE

Human poly(A)+ RNA from different brain regions (adult spinal cord, embryonic spinal cord, pons, medulla oblongata, cerebellum and hippocampus) from Clontech was reverse transcribed into cDNA. In a clean 1.5 ml microcentrifuge tube, 0.5 μg of poly(A)+ RNA were added to 10.5 μl DEPC-treated dH2O and 1 μl of Oligo dT GeneRacer primer and incubated at 65°C for 5 minutes. The tube was then chilled on ice and centrifuged briefly. A mix containing 4 μl of first strand buffer, 1 μl dNTP mix (10 mM each dNTP), 1 μl 0.1 M DTT, 1 μl RNaseOUT RNase inhibitor (40 U/μl) and 1 μl Superscript III reverse transcriptase was added to the RNA/primer mixture and the reaction was incubated at 50°C for 1 hour. The first stage of PCR was performed using 1 μl of the resulting cDNA, 1.5 μl of each primer (GeneRacer 3' Primer and the gene specific primer hGlyT2 3' RACE 1) at 10 pmol/μl with 21 μl Accuprime Pfx MasterMix. The amplification conditions for the first-stage PCR were 30 cycles of 94°C for 1 minute, 65°C for 1 minute and 68°C for 3 minutes. For the second stage (nested PCR), the first-stage PCR product was diluted 1:50 in dH2O and 1 μl of this dilution was mixed with 1.5 μl of each primer (GeneRacer 3' Nested Primer and the gene specific primer hGlyT2 3' RACE 2) at 10 pmol/μl with 21 μl Accuprime Pfx MasterMix. The amplification conditions were the same as above.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>GeneRacer 3' Primer</td>
<td>GCTGTCAACGATAACGCTAGCTAAG</td>
</tr>
<tr>
<td>hGlyT2 3' RACE 1</td>
<td>ACATCTTTCTGGAAAGTCTGCGGC</td>
</tr>
<tr>
<td>GeneRacer 3' Nested Primer</td>
<td>CGCTAGCTAACGCCATGAGCTTTG</td>
</tr>
<tr>
<td>hGlyT2 3' RACE 2</td>
<td>CTAGTGACCTGGTCCATGCTGGGTG</td>
</tr>
</tbody>
</table>

Table 2.3. 3'RACE primers. The sequence and orientation (5'-3') for each primer is shown.

5' RACE

Poly(A)+ RNA (adult spinal cord, embryonic spinal cord, pons, medulla oblongata, cerebellum and hippocampus) was treated with calf intestinal phosphatase (CIP) in order to remove 5' phosphate. In a clean microcentrifuge tube 1 μl RNA (~1-2 μg) was mixed with 1 μl 10 x CIP buffer, 1 μl RNaseOUT (40 U/μl), 1 μl CIP (10 U/μl) and 6 μl dH2O. This reaction was incubated at 50°C for 1 hour. The dephosphorylated RNA was extracted with phenol/chloroform and ethanol precipitated as described in Section 2.2.8 and the RNA pellet was resuspended in 7 μl H2O. In order to remove the 5' CAP structure from full-length mRNA, dephosphorylated RNA was treated with
tobacco acid pyrophosphatase (TAP). In a clean microcentrifuge tube 7 µl RNA were mixed with 1 µl 10 × TAP buffer, 1 µl RNaseOUT (40 U/µl) and 1 µl TAP (10 U/µl). After mixing the reaction was incubated at 37°C for 1 hour. The tube was then centrifuged briefly and placed on ice. The RNA was extracted with phenol/chloroform and ethanol precipitated as described in Section 2.2.8 and the RNA pellet was resuspended in 7 µl H₂O. After precipitation the GeneRacer RNA oligonucleotide was ligated to the 5' end of the decapped RNA in order to provide a known priming site for GeneRacer primer 1. In this reaction, 7 µl dephosphorylated, decapped RNA was mixed with 0.25 µg lyophilised GeneRacer RNA Oligo and the reaction was incubated at 65°C for 5 minutes then chilled on ice. Next, 1 µl 10 × Ligase Buffer, 1 µl 10 mM ATP, 1 µl RNaseOUT (40 U/µl) and 1 µl T4 RNA ligase (5 U/µl) were added to the tube. The reaction was incubated at 37°C for 1 hour, extracted with phenol/chloroform, ethanol precipitated as described in Section 2.2.8 and the RNA pellet was resuspended in 10 µl dH₂O. This 5' end ligated mRNA was reverse transcribed into cDNA. In a clean microcentrifuge tube, 10 µl ligated mRNA were mixed with 1 µl random primers and 1 µl dH₂O. The reaction was incubated at 65°C for 5 minutes then chilled on ice. A mix containing 4 µl RT buffer, 1 µl dNTP mix (10 mM each dNTP), 1 µl 0.1 M DTT, 1 µl RNaseOUT RNase inhibitor (40 U/µl) and 1 µl Superscript III reverse transcriptase was added to the RNA/primer mixture and the reaction was incubated at 50°C for 1 hour. The first stage of PCR was performed using 1 µl of the resulting cDNA, 1.5 µl of each primer (GeneRacer 5’ Primer and the gene specific primer hGlyT2 5’ RACE 1) at 10 pmol/µl with 21 µl Accuprime Pfx MasterMix. The amplification conditions for the first-stage PCR were 30 cycles of 94°C for 1 minute, 65°C for 1 minute and 68°C for 3 minutes. A 1:50 dilution of the first-stage PCR product was used as template for the nested PCR which was carried out using 1.5 µl of the primers (GeneRacer 5’ Nested Primer and the gene specific primer hGlyT2 5’ RACE 2) with 21 µl Accuprime Pfx MasterMix. The amplification conditions were the same used for the first round of PCR (Table 2.4).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GeneRacer 5’ Primer</td>
<td>CGACTGGAGCAGCAAGGACACTGA</td>
</tr>
<tr>
<td>hGlyT2 5’ RACE 1</td>
<td>AGGGAAACCTCCAGACATTGCCCCAG</td>
</tr>
<tr>
<td>GeneRacer 5’ Nested Primer</td>
<td>GGACACTGACATGGACAGGAGGAGTA</td>
</tr>
<tr>
<td>hGlyT2 5’ RACE 2</td>
<td>CAGGTCGACGTCCAGTTTGCTGGACCAGTT</td>
</tr>
</tbody>
</table>

Table 2.4. 5’ RACE primers. The sequence and direction used for each primer is shown.
2.2.13. TOPO cloning

RACE PCR products were cloned using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen). This method provides a highly efficient and fast cloning strategy based on the enzyme topoisomerase I which functions both as a restriction enzyme and as a ligase. Its biological role is to cleave and rejoin DNA during replication. *Vaccinia* virus topoisomerase I specifically recognises the pentameric sequence 5' -CCCTT-3' and forms a covalent bond with the phosphate group attached to the 3' thymidine. It cleaves one DNA strand enabling the DNA to unwind. The enzyme then relegates the ends of the cleaved strand and releases itself from the DNA. To harness the religating activity of topoisomerase, TOPO vectors are provided linearised with topoisomerase I covalently bound to each 3' phosphate. This enables the vectors to readily ligate DNA sequences with compatible ends. The ligation is complete in 5 minutes at room temperature. In a TOPO cloning reaction, 4 µl of the gel-purified blunt-ended PCR product were mixed with 1 µl salt solution and 1 µl TOPO vector in a clean 1.5 ml microcentrifuge tube. The reaction was incubated for 5 minutes at room temperature, placed on ice and then immediately transformed into *E. coli* competent cells as described in Section 2.1.4.

2.2.14. Site-directed mutagenesis

Mutations were introduced into GlyT2 expression constructs using the Quikchange site-directed mutagenesis method (Stratagene), a rapid procedure that uses a supercoiled double-stranded DNA vector with an insert of interest and two synthetic oligonucleotide primers harbouring the desired mutation. The two primers, each complementary to opposite strands of the vector, are extended during temperature cycling by *Pfu* DNA polymerase. Subsequently, the PCR reaction is digested using the restriction enzyme *DpnI*, which recognises and cuts methylated DNA from *E. coli*. Since only the non-mutated template DNA is methylated, the new PCR products containing the desired mutation are resistant to digestion and can be transformed into competent *E. coli*, which re-circularise the plasmid. Mutations were introduced into pRK5myc-hGlyT2 or pRc/CMV-hGlyT2 plasmids using the *PfuUltra* Hotstart PCR master mix (Stratagene) and specific primer pairs for each mutation (Table 2.5). For each mutagenesis reaction, PCR components were added to a 0.2 ml microcentrifuge tube as described in Table 2.6. After mixing, the tube was briefly centrifuged and
placed in a Hybaid PCR Express thermal cycler. The PCR reaction was carried out using the following conditions: an initial denaturation at 95°C for 1 minute was followed by 18 cycles of denaturing at 95°C (1 minute), annealing at 60°C (1 minute) and extension at 72°C (16 minutes).

<table>
<thead>
<tr>
<th>GlyT2 mutant</th>
<th>Mutagenesis primer sequence (5'–3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>W151X</td>
<td>CCTGTTGTGGCGTATTAGACCATAGGC</td>
</tr>
<tr>
<td></td>
<td>GCTCATGTTTGCTGACCACACAGG</td>
</tr>
<tr>
<td>L198R+fs123</td>
<td>CTGGTCCAGCAAAGCTTCTACTGGCG</td>
</tr>
<tr>
<td></td>
<td>GACAGGATAGTGGCTTTGTGGGACACAG</td>
</tr>
<tr>
<td>L237P</td>
<td>TACCCTGATGACCCCGGTCGTGGGA</td>
</tr>
<tr>
<td></td>
<td>TCCAGCACGACGCCCCAATCACTCAGAG</td>
</tr>
<tr>
<td>P243T</td>
<td>CTGGTCCAAGCAAAGCTTCTACTGGCG</td>
</tr>
<tr>
<td></td>
<td>CAAAGAAGATGTTTAAATCCGACAGG</td>
</tr>
<tr>
<td>L269P</td>
<td>GCCATCCCAGCTCAAGCGCGTGTCGCG</td>
</tr>
<tr>
<td></td>
<td>GCCACAGCTTGTGGGAGCTGGGATGCC</td>
</tr>
<tr>
<td>A275T</td>
<td>GCCGTGGCAGCAGACATGCGTACCAC</td>
</tr>
<tr>
<td></td>
<td>GTGATCGACAGCTGAGCTCCACAGCC</td>
</tr>
<tr>
<td>R439X</td>
<td>ATCTCTCTCCTACTCGGGAGTACACCCTG</td>
</tr>
<tr>
<td></td>
<td>CAGGGTGACTCCCTCGATGGAGGAGAT</td>
</tr>
<tr>
<td>I665K+fs1</td>
<td>ATCTTGTTTCAAGAAGGATGATGATTG</td>
</tr>
<tr>
<td></td>
<td>CAATCATCATCTCTTTAGAAACACAAAT</td>
</tr>
<tr>
<td>L269P</td>
<td>GCCATCCCAGCTCAAGCGCGTGTCGCG</td>
</tr>
<tr>
<td></td>
<td>GCCACAGCTTGTGGGAGCTGGGATGCC</td>
</tr>
</tbody>
</table>

Table 2.5. Human and bovine GlyT2 mutagenesis primers. The forward primer is shown above the reverse primer for each mutation. The sequence underlined indicates the mutated codon or site of the deletion.

<table>
<thead>
<tr>
<th>PCR component</th>
<th>Stock concentration</th>
<th>Volume added to a 50 µl reaction (µl)</th>
<th>Working concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid DNA</td>
<td>50 ng/µl</td>
<td>1</td>
<td>1 ng/µl</td>
</tr>
<tr>
<td>Mutagenesis</td>
<td>10 pmol/µl</td>
<td>1</td>
<td>0.25 pmol/µl</td>
</tr>
<tr>
<td>primer, forward</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mutagenesis</td>
<td>10 pmol/µl</td>
<td>1</td>
<td>0.25 pmol/µl</td>
</tr>
<tr>
<td>primer, reverse</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PfuUltra Hotstart PCR master mix</td>
<td>2 x</td>
<td>25</td>
<td>1 x</td>
</tr>
<tr>
<td>dH₂O</td>
<td></td>
<td>22</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.6. PCR conditions for site-directed mutagenesis.

**DpnI digestion and electrophoresis of the mutagenesis PCR product**
The PCR products were digested with *DpnI* in order to eliminate methylated template DNA and select for newly synthesised DNA containing the desired mutation. 1 µl *DpnI* (New England Biolabs; 20 U/µl) was added to the 50 µl mutagenesis PCR product, mixed thoroughly and incubated for 1 hour at 37°C. Part of the *DpnI* digested
PCR product (10 µl) was then loaded on a 1% (w/v) agarose gel and subjected to electrophoresis at 90 V for 30 minutes. Detection of a linear DNA fragment with a size consistent with vector plus insert would indicate the presence of mutated DNA, since all non-mutated DNA was removed by digestion with DpnI. Finally, 10 µl from each reaction were transformed into *E. coli* competent cells as described in Section 2.1.4 and miniprep DNAs were made for analysis as described in Section 2.1.5. The full coding region of all constructs was sequenced to confirm the incorporation of the desired mutation and to ensure that no unwanted changes had been incorporated via PCR errors.

### 2.3. Yeast methodology

#### 2.3.1. Materials

A human foetal brain Matchmaker cDNA library, yeast dropout (DO) supplements, minimal synthetic dropout (SD) base and yeast extract peptone dextrose (YPD) medium were purchased from Clontech (Berks, UK). Adenine, 3-amino-1,2,4-triazole (3-AT), dimethylformamide (DMF), dimethyl sulphoxide (DMSO), Herring sperm DNA, lithium acetate, lyticase from *Arthrobacter luteus*, magnesium sulphate heptahydrate, polyethylene glycol (PEG) molecular weight 3350, β-mercaptoethanol and tris(hydroxymethyl)methylamine-ethylendiamine-tetraacetic acid disodium salt (Tris-EDTA) were purchased from Sigma-Aldrich Company Ltd (Dorset, UK). Disodium hydrogen orthophosphate dehydrate, potassium chloride, sodium dihydrogen orthophosphate dehydrate and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) were purchased from Qiagen Ltd (West Sussex, UK).

**YTH vectors and yeast strain**

Vectors pYTH16 (Fuller *et al.*, 1998), pACT2 (Clontech) and the *Saccharomyces cerevisiae* yeast strain Y190 were gifts from Dr Julia White (Glaxo Wellcome Medicines Research Centre, Herts, UK; Table 2.7).

#### 2.3.2. Maintenance of yeast

Solutions required: *YPDA medium*: 2% (w/v) peptone, 1% (w/v) yeast extracts, 2% (w/v) glucose, adenine (60 mg/l); *YPDA Agar*: 2% (w/v) peptone, 1% (w/v) yeast extracts, 2% (w/v) glucose, adenine (60 mg/l), 2% (w/v) agar; *Minimal SD base*: 0.67% (w/v) yeast nitrogen base without amino acids, 2% (w/v) glucose, adenine (60
mg/l); *Minimal SD agar base*: 0.67% (w/v) yeast nitrogen base without amino acids, 2% (w/v) glucose, adenine (60 mg/l), 2% (w/v) agar.

**Yeast Y190 strain**

The *Saccharomyces cerevisiae* Y190 yeast strain used in the yeast-two hybrid experiments has the following genotype: MATα, *ura3-52, his3-200, trp1-901, leu2-3,-112, gal4Δ, gal80Δ, LYS2::GAL1UAS GAL1TATA HIS3, URA3::GAL1UAS GAL1TATA lacZ.

<table>
<thead>
<tr>
<th>Vector</th>
<th>Promoter and fusion</th>
<th>Yeast selection</th>
<th>Bacteria selection</th>
</tr>
</thead>
<tbody>
<tr>
<td>pYTH16</td>
<td>ADH1, GAL4 DNABD</td>
<td>TRP1</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>pACT2</td>
<td>ADH1, GAL4 AD</td>
<td>LEU2</td>
<td>Ampicillin</td>
</tr>
</tbody>
</table>

**Table 2.7. YTH bait and prey vectors.** The encoded fusion, the promoter controlling fusion protein expression and markers for bacterial and yeast selection are shown. GAL4 DNABD: GAL4 (1-147) DNA binding domain. GAL4 AD: GAL4 (768-881) activation domain.

### 2.3.3. Yeast recovery and growth

A sterile spreading loop was used to spread the Y190 glycerol stock (stored at -80°C) on YPDA agar plates. The plates were incubated for 2-3 days at 30°C until colonies had a diameter of 2-3 mm. The plates were sealed with parafilm, stored in the fridge for 2-3 months and used as working stock. A colony was picked every 2-3 months to be streaked onto freshly prepared plates.

### 2.3.4. Transformation of competent yeast cells

Yeast transformations were performed to introduce plasmid DNA into yeast cells. They were carried out using the lithium acetate/single stranded DNA/ polyethylene glycol (LiAc/SS-DNA/PEG) method (Gietz *et al.*, 1995). Solutions required: 1 × TE/LiAc: 10 mM Tris-HCl, 1 mM EDTA, 100 mM LiAc; PEG solution: autoclaved 50% PEG 6000 was added to 1 × TE/LiAc solution to a final concentration of 40% (w/v). From a freshly streaked stock plate, a single large yeast colony was inoculated into 100 ml YPDA medium and incubated overnight at 30°C with vigorous shaking. The following day, a dilution of the overnight culture was made in order to measure the optical density at 600 nm. Using this measurement, an appropriate volume of the overnight culture was inoculated into fresh pre-warmed YPDA medium to obtain an OD$_{600}$ of 0.2. The volume of this overday culture was proportional to the number of
transformation being performed (100 ml per 10 transformations). The yeast culture was grown with aeration at 30°C for 2-3 hours until the OD₆₀₀ value was 0.5-0.6 (1 x 10⁷ cells/ml). Yeast cells were aseptically transferred to sterile 50 ml falcon tubes and pelleted at 2,000 g for 3 minutes. The pellets were then washed in 25 ml sterile dH₂O and subjected to centrifugation at 2,000 g for 3 minutes. The cells were resuspended in 1 x TE/LiAc and centrifuged at 2,000 g for 3 minutes. The 1 x TE/LiAc wash was repeated. The pellet was resuspended in a volume of 1 x TE/LiAc dependent on the number of transformations (1 ml/10 transformations) to give a cell density of 1 x 10⁹ cells. A volume of 100 µl yeast cell suspension was pipetted into microcentrifuge tubes containing the bait plasmid (1 µg), the prey plasmid (1 µg) and 5 µl Herring sperm DNA (10 mg/ml) previously boiled for 5 minutes. Finally, 600 µl PEG solution was added and the components were carefully mixed and incubated at 30°C for 30 minutes with gentle shaking. After a heat-shock at 42°C for 20 minutes, the cells were centrifuged at maximum speed for 30 seconds, the PEG supernatant was removed and pellets were resuspended in 100 µl sterile water. In order to select for HIS3+ transformants, a volume of 60 µl of each transformation was spread onto minimal agar plate lacking leucine, tryptophan and histidine (SD/-Leu/-Trp/-His) supplemented with an appropriate concentration on 3-AT to inhibit the basal expression of the HIS3 gene product. In addition, 40 µl of each transformation were spread onto minimal agar plates lacking leucine and tryptophan in order to test the β-galactosidase activity with the LacZ freeze fracture assay. The plates were incubated at 30°C until colonies appeared (3-4 days).

2.3.5. The LacZ freeze-fracture assay

LacZ freeze-fracture assays were performed to detect β-galactosidase activity in yeast cells. β-galactosidase (encoded by LacZ reporter gene) is an enzyme that hydrolyses the chromogenic substrate X-Gal into galactose and indolyl-blue, forming an intense blue water-insoluble precipitate. Yeast colonies turn blue when the LacZ gene is induced and X-gal hydrolysed (Figure 2.3). Solutions required: X-gal/Z buffer mix: X-gal (BDH) was dissolved in DMF to a final concentration of 100 mg/ml, then an appropriate volume of Z buffer (60 mM Na₂HPO₄.2H₂O, 40 mM NaH₂PO₄.2H₂O, 10 mM KCl, 1 mM MgSO₄.7H₂O) was added resulting in a final concentration of 1 mg X-gal/ml solution. A volume of 2.7 ml β-mercaptoethanol (β-Me) was added per litre of Z buffer used (38.6 mM final concentration). The X-gal/Z buffer/ β-Me mix was
prepared and kept covered from light with aluminium foil in a fume hood. Yeast colonies of approximately 2 mm in diameter were transferred onto Whatman No. 54 paper using a replica plating disc. Filters were then immersed in liquid nitrogen for 10 seconds and laid on the bench (colonies facing upwards). Once thawed, the process was repeated for a total of two freeze-fractures. In the fume hood, filters were placed in Petri dishes lids containing 2 ml of the X-gal/Z buffer/β-Me mix, taking care to exclude air bubbles from beneath the filters. The base of Petri dishes was used to cover the filters which were incubated at 37°C for up to three hours. To terminate the reaction, filters were removed from the plates and dried overnight in the fume hood on a layer of absorbent paper.

![Figure 2.3. Schematic representation of the LacZ assay. X-gal is hydrolysed into galactose and indolyl-blue by the enzyme β-galactosidase, forming a blue precipitate.](image)

### 2.3.6. YTH library screening

A human foetal brain cDNA library (Clontech) in pACT2 was provided as a frozen *E. coli* glycerol stock. Plasmid DNA was prepared from this stock by spreading $1 \times 10^{10}$ cfu onto fresh 132 mm diameter LB agar plates supplemented with ampicillin. Inverted plates were incubated at 37°C for 18-20 hours after which bacteria were scraped off the plates, washed twice in LB medium and used to prepare plasmid DNA using the protocol described in Section 2.1.6.

**Co-transformation of Y190 with pYTH16-GlyT2C and pACT2 cDNA library**

For co-transformation, 50 μg of the pACT2 foetal brain cDNA library, 20 μl denatured herring sperm DNA (200 μg) and 10 μl pYTH16-GlyT2C bait (5 μg) were mixed and transferred into a 50 ml falcon tube containing 2000 μl of competent Y190 prepared as described in Section 2.3.4. Next, a volume of 8 ml of PEG6000 solution (40% (w/v) PEG6000/1 x LiAc/1 x TE) was added to the cells/DNA, mixed thoroughly and incubated at 30°C for 45 minutes with gentle shaking. To increase transformation efficiencies, 500 μl DMSO were added prior to a heat-shock at 42°C for 20 minutes, with mixing every 10 minutes. The cells were then centrifuged at
3,000 g for 1 minute, resuspended in 10 ml of YPD Plus medium (Clontech) and incubated at 30°C for 90 minutes with gentle shaking. The cells were pelleted by centrifugation at 3,000 g for 1 minute and resuspended in 6 ml of 0.9% (w/v) NaCl solution. Aliquots of the cell suspension (350 µl) were plated onto fifteen 132 mm diameter SD/-Leu/-Trp/-His plus 15 mM 3-AT agar plates. The plates were incubated at 30°C until colonies appeared (1 week-10 days). To calculate the transformation efficiency of the library screen, a 30 µl aliquot from the 6 ml library transformation was diluted in 720 µl of 0.9% (w/v) NaCl solution. Then 150 µl of this suspension was plated in duplicate onto SD/-Leu/-Trp agar plates to assay the efficiency of bait/library plasmid co-transformation. These plates were incubated at 30°C for 5-6 days or until colonies appeared.

**Co-transformation of Y190 with control plasmids**

For a positive control transformation, 50 µl cells were pipetted into a 1.5 ml microcentrifuge tube containing 1 µl pACT2-syntenin-1 prey (0.5 µg), 1 µl pYTH16-GlyT2C bait (0.5 µg) and 5 µl denatured herring sperm DNA (50 µg). For a negative control transformation, 50 µl cells were pipetted into a 1.5 ml microcentrifuge tube containing 1 µl pACT2 empty prey vector (0.5 µg), 1 µl pYTH16-GlyT2C bait (0.5 µg) and 5 µl denatured herring sperm DNA (50 µg). A volume of 500 µl PEG solution (40% (w/v) PEG6000/1 × LiAc/1 × TE) was added and the tube content was mixed thoroughly. The tube was incubated at 30°C for 45 minutes with gently shaking, mixing every 15 minutes. 20 µl DMSO were added prior to a heat-shock at 42°C for 20 minutes, with mixing every 10 minutes. The cells were then centrifuged at 3,000 g for 15 seconds, resuspended in 500 µl YPD Plus medium and incubated at 30°C for 90 minutes with gentle shaking. The cells were then pelleted by centrifugation at 3,000 g for 15 seconds and resuspended in 100 µl of 0.9% (w/v) NaCl solution. Aliquots (50 µl) of the positive and negative controls were spread onto SD/-Leu/-Trp and SD/-Leu/-Trp/-His plus 15 mM 3-AT plates and incubated at 30°C for 5-6 days or until colonies appeared.
2.3.7. Analysis of putative positive clones from the library screen

After 5-6 days colonies of 1-4 mm in size (i.e. larger than the overall background), representing putative positive clones were picked and restreaked onto SD/-Leu/-Trp/-His + 15 mM 3-AT. After two days the colonies were replica plated as follows: Whatman No.54 paper circles were gently laid onto the plates using a replica block then ripped off the plates to remove the yeast. The filter was then placed yeast side downwards onto a fresh SD/-Leu/-Trp/-His + 15 mM 3-AT replica plate and then removed, leaving small 'imprints' of the yeast colonies. These replica plates were incubated for 2-3 days at 30°C, after which the yeast colonies regrow. Meanwhile, the Whatman No.54 filters were subjected to \textit{LacZ} freeze-fracture assay. Yeast colonies that grew on the selection plates and turned blue in the \textit{LacZ} assay were selected for rescue of plasmid DNA.

Rescue of plasmid DNA from yeast cells

Solutions required: \textit{Buffer P1}: 10 mM EDTA, 100 μg/ml RNase A, 50 mM Tris-HCl (pH 8.0); \textit{Buffer P2}: 200 mM NaOH 1% (w/v) SDS; \textit{Buffer N3}: 3 M potassium acetate (pH 5.5); \textit{Buffer PE}: 10 mM Tris-HCl (pH 8.5). Low copy number plasmid DNA was isolated from yeast cells using a novel protocol based on the QIAprep Miniprep kit (Qiagen). However, unlike \textit{E. coli}, yeast has tough outer cell walls that need to be initially broken down using enzymatic (lyticase) and mechanical (glass beads) treatments. 50 μl of 425-600 μm glass beads (Sigma) were placed into a 1.5 ml microcentrifuge tube using a 1 ml syringe and 250 μl chilled resuspension buffer P1 and 10 μl lyticase (5U/μl) were added to the beads. A sweep of several \textit{LacZ} positive yeast colonies was taken from the replica plate using a sterile loop and the yeast cells were completely resuspended in the buffer. This mixture was incubated at 37°C for 10 minutes and then vortexed for 20 minutes at 2,500 g on an IKA Vibrax rotating shaker. The preparation was then left to stand for 5 minutes to allow the beads to settle and the supernatant was removed into a fresh 1.5 ml tube containing 250 μl lysis buffer P2, mixed gently by inverting and incubated for 5 minutes at room temperature. Finally, a volume of 350 μl buffer N3 was added to the lysate and the tube was inverted 4-6 times. The sample was centrifuged at 13,000 g for 10 minutes. The supernatant was then applied to a QIAprep spin column by pipetting and centrifuged at 16,000 g for 1 minute. After discarding the flow-through, the column was washed.
with 750 μl buffer PE and the sample centrifuged at 16,000 g for 1 minute. After
discarding the flow-through the sample was subjected to one more minute of
centrifugation at 16,000 g to remove residual wash buffer. The QIAprep column was
placed into a clean 1.5 ml microcentrifuge tube and the DNA was eluted by adding 20
μl Elution Buffer to the column and centrifugation at 16,000 g for 1 minute. Yields of
plasmid DNA isolated during this protocol ranged from 1-10 ng/μl.

Recovery and analysis of pACT2 prey plasmids
Since the bait vector pYTH16 is also an episomal plasmid, in order to select for the
pACT2 prey plasmids, the raw yeast minipreps were digested with SacII restriction
enzyme which linearises pYTH16 but not pACT2. The assumption was made that the
SacII site (CCGCGG) is rare and was unlikely to be present in any of the cDNA
inserts. A volume of 8 μl of purified yeast miniprep DNA was mixed with 0.5 μl SacII
(20 U/μl), 0.5 μl H2O and 1 μl 10 × NEB Buffer 4 and incubated at 37°C for 1 hour.
This digested reaction was transformed into E. coli competent cells following the
protocol described in Section 2.1.4 and plated onto LB agar plates containing
ampicillin. Plasmid DNA was isolated from bacteria using the QuickLyse Miniprep
Kit (Qiagen) as described in Section 2.1.5. These minipreps were then digested with
BglII in order to liberate the cDNA insert from the library vector pACT2. A volume of
8 μl miniprep was mixed with 0.5 μl BglII, 0.5 μl H2O and 1 μl 10 × NEB Buffer 3
and the reaction was incubated at 37°C for 1 hour. The digestion was loaded onto a
1% (w/v) agarose gel in TAE buffer and run for 40 minutes at 90 V using a 1Kb DNA
ladder (Invitrogen) as a marker. Minipreps that appeared to contain an insert were
sequenced using pACT2 forward and reverse primers (Table 2.2) to check the identity
of the insert and to confirm that the open reading frames of the identified interactors
were in-frame with GALAD. DNA sequences were compared to the human genome
using the UCSC browser (http://genome.ucsc.edu/).

2.4. Cell culture methodology
2.4.1. Materials
Bradford reagent was purchased from BioRad Laboratories UK Ltd (Herts, UK).
Cryogenic vials were purchased from Corning (Leicestershire, UK). Acetic acid,
coverslips and methanol were purchased from BDH Laboratory Supplies (Poole, UK).
Dulbecco's Modified Eagle Medium (DMEM), Minimum Essential Medium (MEM) and Opti-MEM were purchased from Gibco (Invitrogen). Hank's Buffered Salt Solution (HBSS), goat anti-rabbit Alexa Fluor 488 antibody, goat anti-mouse Alexa Fluor 546 antibody and TO-PRO-3 were purchased from Invitrogen Ltd (Paisley, UK). Foetal bovine serum (FBS) was purchased from PAA Laboratories Ltd (Somerset, UK). Lipofectamine LTX transfection reagent kit was purchased from Invitrogen Ltd (Paisley, UK). Ammonium chloride, ammonium persulphate, bovine serum albumin (BSA), calcium chloride, dimethyl sulphoxide (DMSO), magnesium acetate, β-mercaptoethanol, octylphenyl-polyethylene glycol (Nonidet P-40), paraformaldehyde, penicillin-streptomycin solution, phosphate buffered saline (PBS) tablets, polyethylene glycol sorbitan monolaurate (Tween-20), poly-D-lysine, sodium chloride, sodium dodecyl sulphate (SDS), tris(hydroxymethyl)methylamine (Tris-base), 4-(1,1,3,3-tetramethylbutyl)phenyl-polyethylene glycol (Triton X-100) were purchased from the Sigma-Aldrich Company Ltd (Dorset, UK). Gel-Saver II tips were purchased from Starlab UK (Bucks, UK). Glycine was purchased from NEN (Boston, US). Tissue culture flasks were purchased from TPP Techno Plastic Products (Trasadingen, Switzerland). Multidishes (6 and 24 wells) were purchased from Nunc (Langenselbold, Germany). Mounting slides were purchased from VWR International Ltd (Poole, U.K.). Vectashield mounting medium was purchased from Vectorlabs (Peterborough, UK).

**Antibodies**

Anti-c-myc (M2) polyclonal antibody and anti-FLAG monoclonal antibody were purchased from Sigma-Aldrich Company Ltd (Dorset, UK).

**Cell lines**

Human Embryonic Kidney (HEK) 293 cells were obtained from the European Collection of Cell Cultures (ECACC), Dorset, UK (http://www.ecacc.org.uk/).

### 2.4.2. The cell culture laboratory and equipment

All cell handling was performed in a class II laminar flow hood. Sterile techniques were adopted to prevent contaminating microorganisms entering the cultures. The cell culture incubator was maintained at 37°C and supplied with 5% (v/v) CO₂ at all times. An inverted microscope (Zeiss, Oberkochen, Germany) was used to monitor cells in
culture. A Denley BS400 centrifuge was used to pellet cells and a water bath (Grant Instruments, Shepreth, UK) was used to warm media to 37°C prior to use. Tissue culture (TC) flasks with a 75 cm² base (T-75 flasks) were used for routine cell culture of adherent cells (TPP). 6 wells multidishes (Nunc) were used for routine transfections.

2.4.3. Preparation of cell culture medium

HEK293 cells (ECACC: 85120602) were cultured in MEM supplemented with 10% (v/v) FBS, 1% (v/v) penicillin/streptomycin (pen/strep; 10,000 U/ml penicillin and 10 mg/ml streptomycin) and 2 mM L-glutamine. Once prepared, the medium was stored at 4°C and pre-warmed to 37°C before use. In the subsequent text serum- and antibiotic-supplemented MEM is referred to as MEM.

2.4.4. Thawing of cryopreserved cells

A vial of frozen cells was removed from -150°C freezer storage and placed in a 37°C water bath until thawed. The cells were resuspended in 1 ml MEM (Gibco) then transferred into a tissue culture flask containing 20 ml MEM and incubated at 37°C overnight.

2.4.5. Maintaining of HEK293 cells in culture

HEK293 cells were grown in flask as a monolayer. Cell confluence on the flask base was estimated by eye using an inverted optical microscope. Cells were routinely passaged at ~70-80% confluence. MEM from a flask containing 70-80% confluent cells was removed with a sterile pipette. After washing in 10 ml pre-warmed HBSS, the cells were detached from the flask using a scraper in 10 ml HBSS. The suspension was removed, transferred to a sterile 50 ml Falcon tube and the cells were pelleted by centrifugation at 355 g for 3 minutes. The supernatant was discarded and a volume of 10 ml MEM was used to resuspend the cell pellet. An appropriate volume of the cell suspension was used to inoculate a sterile flask containing MEM to obtain the desired cell density. The resulting volume of the inoculated MEM was 20 ml. For HEK293 cells a split ratio of 1:5 was performed every 48-60 hours.
2.4.6. Cryopreservation of cells

Cells for cryopreservation were harvested when 70-80% confluent. After removing the culture medium, the cell monolayer was washed with 10 ml PBS. The cells were detached from the flask with a scraper and collected by centrifugation at 355 g for 3 minutes. The pellet was resuspended in 1 ml 10% (v/v) dimethyl sulphoxide (DMSO) in FBS and transferred into a 2 ml cryogenic polypropylene vial. The cryoprotective properties of DMSO protect the cells from disruption during the freeze/thaw process. The vial was placed in a quick-freeze container and frozen at -80°C overnight. Following freezing, the vial was transferred to a -150°C freezer for long-term storage.

2.4.7. Transfection of cells with plasmid DNA

Before plating the cells, coverslips placed in six well multidishes were coated with poly-D-lysine. The objective of this treatment is to enhance cell attachment and growth to glass surface. Both cells and tissue culture glass present negative surface charge. Poly-D-Lysine treatment improves adhesive properties by altering the charge on the glass from negative to positive. Briefly, 500 μl of poly-D-lysine (0.1 mg/ml) was pipetted onto each coverslip and left for one hour at room temperature. The poly-D lysine was aspirated and 1 ml of sterile H₂O was used to rinse the coverslip then aspirated. This process was repeated twice for a total of 3 washes. After the last aspiration, coverslips were left to dry for 3 hours. HEK 293 cells were plated onto Poly-D- coated coverslips. After 12 hours (when 40-50% confluent) the cells were transfected using Lipofectamine LTX reagent (Invitrogen). 1 μg of DNA was pipetted into a sterile .5 ml microcentrifuge tube and. 200 μl of Opti-MEM medium was added to the tube and mixed gently. 2.5 μl Lipofectamine LTX reagent was added to the diluted DNA and the mix was incubated at room temperature for 30 minutes. The culture medium was then removed from the well and replaced with 2 ml Opti-MEM. The DNA-Lipofectamine complex was added drop-wise onto the well and the cells were then incubated at 37°C for 24 hours.

2.4.8. Immunocytochemistry

Solutions required: 0.1% (v/v) Triton X-100 buffer: 10% (v/v) FBS, 0.5% (w/v) BSA in PBS; Blocking solution: 10% (v/v) FBS and 0.5% (w/v) BSA in PBS. 1.5 × 10⁶ cells per coverslip were seeded in six well plates containing poly-D-lysine coated coverslips and incubated at 37°C. When 30% confluent, the cells were transfected
(Section 2.4.7) then incubated at 37°C for 24 hours. Coverslips were then rinsed twice with PBS, and then fixed with ice-cold 4% (w/v) paraformaldehyde in PBS in a fume hood for 2 minutes. After washing twice with PBS, the cells were incubated in 50 mM NH₄Cl for 10 minutes, in order to quench free aldehyde groups. The cells were then washed twice with PBS and permeabilised with 0.1% (v/v) Triton X-100 buffer for 12 minutes. After two washes in PBS the cells were incubated with a blocking solution for 10 minutes. After the blocking incubation the cells were incubated with a primary antibody diluted in blocking solution (1:200) for 1 hour. After three washes in PBS the cells were incubated with a secondary antibody diluted in blocking solution (1:200) for 20 minutes. The coverslips were rinsed three times with PBS and then incubated for 10 minutes with the nucleic acid stain TO-PRO-3 (1:10,000) for 10 minutes in the dark. After rinsing twice with PBS the coverslips were mounted onto a microscope slide using 5 μl of Vectashield (Vectorlabs).

2.4.9. Confocal microscopy

All confocal images were taken using a Zeiss Axioskop LSM 510 Microscope and the planapochromat 63 x 1.4 objective lens (with oil). Images were acquired at 1,024 x 1,024 pixel resolution. An argon laser which emits light at a wavelength of 488 was used to detect Alexa 488 secondary antibody. The HeNe laser which emits light at a wavelength of 543 was used to detect Alexa 546 secondary antibody. The HeNe laser which emits light at a wavelength of 633 was used to detect TO-PRO-3.

2.4.10. [³H]-glycine uptake assays

Solutions required: Krebs buffer: 118 mM NaCl, 1 mM NH₂PO₄, 26 mM NaHCO₃, 1.5 mM MgSO₄ mM, 5 mM KCl, 1.3 mM CaCl₂, 20 mM glucose. HEK293 cells were plated on poly-D-lysine coated 24 well plates (Nunc). When 40% confluent, cells were transfected with the appropriate plasmids at 1 μg/μl per well (single transfections) or 0.5 μg/μl (double transfections) with Lipofectamine LTX reagent (Section 2.4.7) and incubated at 37°C for 24 hours. After transfection, cells were washed twice with 1 ml/well warm Krebs buffer pre-equilibrated with 5% CO₂- 95% air. After 2 minutes, cells were incubated with 1 ml/well [³H]-glycine (NEN) at a final concentration of 300 μM (0.1 μCi/ml) for 5 min. Cells were rinsed twice with ice-cold Krebs buffer pre-equilibrated with 5% CO₂-95% air, then digested with 1 ml/well 0.1 M NaOH for 2 hours. An aliquot (750 μl) of each sample added to 3.5 ml scintillation
fluid, and the radioactivity incorporated was measured by scintillation counting. An additional 50 μl of each sample was used for determining the protein concentration using the Bradford reagent (BioRad). [³H]-glycine uptake was calculated as nmole/min/mg protein and expressed as percentage of that in control cells transfected with the corresponding empty expression vector. All statistical comparisons used an unpaired Student’s t-test.
3. NEW MUTATIONS IN THE GLYT2 GENE (SLC6A5) IN INDIVIDUALS WITH HYPEREKPLEXIA
3.1. Identification of new hyperekplexia mutations in the human GlyT2 gene (SLC6A5)

The finding that hyperekplexia can be caused by mutations in the human GlyT2 gene (Rees et al., 2006; Eulenburg et al., 2006) represented the first example of a disease caused by mutations in a Na\(^+\)/Cl\(^-\) dependent transporter for a classical fast neurotransmitter. However, one immediate question arises: how common are GLRA1 versus SLC6A5 mutations in hyperekplexia?

Since GlyT2 mutations are relatively newly discovered, we contacted other researchers working in this field for additional 'unresolved' hyperekplexia cases, which resulted in the additional recruitment of DNA samples for genetic analysis. All individuals included in this second study conformed to standard diagnostic criteria: a history of neonatal hypertonia, a nose tap response and an exaggerated startle reflex with preservation of consciousness (Andrew and Owen, 1997; Koning-Tijssen and Brouwer, 2000). A second round of SLC6A5 genetic screening, conducted by Prof. Mark Rees (University of Swansea) and Dr. Jean-François Vanbellinghen (University of Liège, Belgium) was carried out on additional DNA samples from the United Kingdom, Netherlands, Spain, Italy, France and Middle East. Due to the high SNP content of certain SLC6A5 exons found in initial genetic studies of SLC6A5 (Rees et al., 2006; Eulenburg et al., 2006) all coding exons were analysed by direct DNA sequencing of PCR amplicons.

This new round of genetic screening identified seven new individuals with missense, nonsense and frameshift mutations in SLC6A5 (Table 3.1; Figure 3.1). In most cases, mutations were inherited as compound heterozygous or homozygous states, confirming that SLC6A5 is associated with recessive forms of hyperekplexia. However, three patients (individuals 1, 4 and 6) harboured only one mutation in SLC6A5, suggesting that either i) these mutations produce GlyT2 proteins that exert dominant-negative effects on wild-type GlyT2 or ii) that additional genetic analysis is required to understand the exact mechanisms of disease in these individuals.
<table>
<thead>
<tr>
<th>Individual</th>
<th>Global origin</th>
<th>Mutation</th>
<th>Expected Subcellular location</th>
<th>Possible Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Netherlands</td>
<td>A275T</td>
<td>Cell surface</td>
<td>Missense mutation</td>
</tr>
<tr>
<td>2</td>
<td>UK</td>
<td>R439X</td>
<td>Cytoplasmic</td>
<td>Protein truncation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R439X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Spain</td>
<td>P108L+fs25</td>
<td>Cytoplasmic</td>
<td>Protein truncation</td>
</tr>
<tr>
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<td></td>
<td>I665K+fs1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Spain</td>
<td>W151X</td>
<td>Cytoplasmic</td>
<td>Protein truncation</td>
</tr>
<tr>
<td>5</td>
<td>France</td>
<td>L198R+fs123</td>
<td>Cytoplasmic</td>
<td>Protein truncation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L237P</td>
<td>Cell surface</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Italy</td>
<td>R439X</td>
<td>Cytoplasmic</td>
<td>Protein truncation</td>
</tr>
<tr>
<td>7</td>
<td>Middle East</td>
<td>P243T</td>
<td>Cell surface</td>
<td>Missense mutation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P243T</td>
<td></td>
<td>Missense mutation</td>
</tr>
</tbody>
</table>

**Table 3.1. New hyperekplexia mutations in SLC6A5.** X stands for stop codon.

<table>
<thead>
<tr>
<th>Amino Acid Sequence of Human GlyT2 indicating the Relative Positions of New SLC6A5 Hyperekplexia Mutations</th>
</tr>
</thead>
</table>

| MDCSAPKEMNNKLPNAPSEAAAQQHGDPCAPRTSPEQELPAAAAPPPR 50 | VPRASSTGAQTQADARACEARPGVGSKKLRSPQAASALRDLREA 100 |
| W151X | VNMSQSTVVGTDGITSVLPSVATVATQDEEQDENDKNAGYSS 200 |
| IIIMVGVGVLNWRFPYLFNGCGAFLIPLYMIGLALGIFPPLEV 250 |
| LGFCILAQGCCGAMLILISVIAVNYVCYTLFYPA 300 |
| SFVSVLPAGSCNNPNTPEKDKTLLSDLSCVIDHPKIQKINSTFCMTA 350 |
| YPNVTMVNTSQANKTFVSGSEEFYFVFKKISAGIEYPEGIRWPALACL 400 |
| FLAWVITYSALLKGTSGKVVTTTVPYVILFVTLPGAGACI 450 |
| WYFITPKEKLDTVWMKAQASSATGGLNIXWKFHKNHNC 500 |
| Q509S S510R TM7 R439X | G491C | R432M TM5 V432Q+fs97 550 |
| PEALLRPI SPEWAIIFPMLLLGLDTMHATITETVSIDEFPKYLTY 600 |
| HKPVIFLCICIFIMFIFPMFTQQGITYWTLDYASYIAYLIAF 650 |
| GLSVYGLRQFCEHLMNMIGFQPNIWKVCAVFPTTLTILFLCFSPYQ 700 |
| JEGMRKQKAFILVREIKERKNIENVVADOGPIAGFYV 750 |
| VCPSPIDPGFLAQHERGYNMIDPLGTSSLGLKLPVKEQDELTQC 797 |

**Figure 3.1.** Amino acid sequence of human GlyT2 indicating the relative positions of new SLC6A5 hyperekplexia mutations. Mutations described by Rees et al., (2006) are shown by black highlighting, whilst newly discovered mutations are indicated by blue highlighting. X denotes a stop codon. Coloured boxes represent the 12 putative transmembrane domains (TM1-TM12). Open and filled blue triangles indicate residues likely to coordinate sodium ions Na1 and Na2 respectively. Black circles indicate amino acid predicted to be involved in glycine binding.
3.2. Study aims

The aim of this study was to understand the mode of inheritance and functional consequences of these new GlyT2 mutations for transporter subcellular distribution and uptake activity. In particular I intended to:

- Assess possible explanations for the occurrence of individuals with only one defective GlyT2 allele, focusing on alternative splicing of SLC6A5 mRNAs
- Examine the subcellular localisation of recombinant epitope-tagged GlyT2 mutants in mammalian expression systems using confocal microscopy
- Study the effect of new GlyT2 mutations on [3H]-glycine uptake in mammalian expression systems, including potential dominant negative effects of substitutions W151X, A275T and R439X.

3.3. Identification of novel N- and C-terminal exons in the GlyT2 gene using 5' and 3' rapid amplification of cDNA ends (RACE)

3.3.1. Alternative splicing of human GlyT2 transcripts

As highlighted above, in three of the additional hyperekplexia cases, only one coding mutation in SLC6A5 was identified. Since most mutations in the GlyT2 gene show recessive inheritance, often in the compound heterozygous state (different mutations on each allele), it is possible that: i) the single ‘orphan’ mutation exerts a dominant-negative effect on the other unaffected GlyT2 allele or ii) that uncharacterised alternative splicing of SLC6A5 transcripts has excluded some exons from mutation screening. In order to examine the latter possibility, the existence of alternative splicing in the human GlyT2 mRNA was investigated. Rapid amplification of cDNA ends (RACE) was used to amplify and clone GlyT2 cDNA sequences using known exons as starting points. In this way, cDNAs representing unusual transcripts such as those created by alternative splicing can be selected for cloning (Frohman et al., 1988).

3.3.2. 5' RACE reveals two novel GlyT2 N-termini

5' RACE was performed using the GeneRacer kit (Invitrogen) and was used to amplify 5' ends of human GlyT2 gene as detailed in Section 2.2.12. Briefly, human Poly(A)+ RNA (Clontech) from different brain regions (adult spinal cord, embryonic
Spinal cord, pons, medulla oblongata, cerebellum and hippocampus) was treated with CIP to remove 5' phosphates, then with TAP to remove the 5' cap structure. The GeneRacer RNA Oligo was ligated to the 5' end of the decapped mRNA in order to provide a known priming site for GeneRacer PCR primers. This 5' end ligated mRNA was reverse transcribed using Superscript III RT and the GeneRacer Oligo dT Primer.

The resulting first-strand cDNA was subjected to a first round of PCR amplification using the GeneRacer 5' primer and the reverse gene specific primer hGlyT2 5' RACE1. One-fiftieth dilution of the product of the first round of PCR was used as a template for a second PCR amplification using GeneRacer 5' Nested Primer and the gene-specific primer hGlyT2 5' RACE2. Amplification conditions are detailed in Section 2.2.12. When separated on agarose gel, the PCR products from all brain regions gave rise to multiple bands, indicating the presence of cDNA of various sizes (Figure 3.2).

![Figure 3.2. Agarose gel showing 5' RACE PCR products (adult spinal cord).](image)

Multiple bands indicate the presence of PCR products of various sizes. M: 1 Kb DNA ladder (Invitrogen). 1: product of first round PCR amplification. 2: product of second round PCR amplification.

Slices of agarose containing the PCR products were cut from the gel and gel purified using the Sephaglas BandPrep kit (Amersham Biosciences), as described in Section 2.2.6. The resultant elution products containing the purified DNA were cloned into the pCR-Blunt II-TOPO vector (Figure 3.3) using the TOPO cloning kit (Invitrogen). TOPO-cloning was chosen as a simple and efficient strategy for cloning PCR products for sequence analysis and is described in detail in Section 2.2.13. The complete
reaction volume was used to transform \textit{E. coli} competent cells and a number of minipreps were prepared.

\begin{figure}
\centering
\includegraphics[width=0.8\textwidth]{plasmid_map}
\caption{Plasmid map of pCR-Blunt II-TOPO. pUc ori: pUc origin of replication; \textit{P}_{\text{lac}}: lac promoter; \textit{lacZ}a ccdB: ccdB gene fused to the C-terminus of \textit{lacZ} \textit{a}; Kanamycin: kanamycin resistance gene; Zeocin: zeocin resistance gene.}
\end{figure}

Minipreps were subjected to restriction enzyme analysis with i) \textit{EcoRI}, which cleaves at both sides of the cloned insert in the TOPO vector in order to verify the presence of an insert; ii) \textit{SacII}, which cuts in exon 2 of the \textit{SLC6A5} gene, to select for GlyT2 cDNA clones. Only minipreps harbouring a cDNA insert that were also linearised by \textit{SacII} were analysed by DNA sequencing. Sequence analysis revealed the existence of two novel GlyT2 mRNAs, both containing an in-frame initiation codon which would produce two proteins of 5 and 10 amino acids longer than the previously characterised isoform, encoded by exon 1a (Figure 3.4). Comparison of the genomic DNA sequence with the 5\textsuperscript{\prime} RACE clones revealed that the two new isoforms arose from alternative splicing of 5\textsuperscript{\prime} exons 1b and 1c to exon 2 (Figure 3.4).

\begin{verbatim}
acgcacaacagcagccccccccccctccggctggagtgacaactggccagcatactcttagctgtgccttttataactggtaatctcaagggggtattatcataaacgaag

\textcolor{red}{M} tccaccagtccagtctgtgcctgtgctcagactgggctgtgcgttgttgcttctttgcttttttttctttaaaggggcttcggagttgctctgggtcc

\textcolor{red}{M L} ccagatgccggtcacagagcggaggtttgtgctctgtctgctggctacagacac gagctactgagacacaattctctgcgctgttga

\textcolor{red}{A E A E} gctgaagccagagtacccagattttgtgcgaatattctcggtctgtttgtga
\end{verbatim}
3.3.3. 3' RACE reveals an additional C-terminal GlyT2 isoform

3' RACE was performed using the GeneRacer kit (Invitrogen) and was used to amplify human GlyT2 cDNAs as detailed in Section 2.2.12. Briefly, human Poly(A)+ RNA (Clontech) from different brain areas (adult spinal cord, embryonic spinal cord, pons, medulla oblongata, cerebellum and hippocampus) was reverse transcribed using Superscript III RT and the GeneRacer Oligo dT primer. The resulting first-strand cDNA was amplified by PCR using the GeneRacer 3' primer and the gene-specific primer hGlyT2 3' RACE1. One-fiftieth dilution of first amplification reaction was subjected to nested PCR using GeneRacer 3' Nested Primer and the gene specific primer hGlyT2 3' RACE2. Amplification conditions are detailed in Section 2.2.12. PCR products from all brain regions, when separated on agarose gel, gave rise to multiple bands revealing the presence of transcripts of various sizes (Figure 3.5).
products were gel purified (Section 2.2.6) and subcloned into pCR-Blunt II-TOPO vector (Section 2.2.13). Minipreps were prepared and subjected to restriction enzyme analysis with i) EcoRI, in order to verify the presence of an insert and ii) NsiI, which present a restriction site in the exon 15 of SLC6A5, to select GlyT2 cDNAs for DNA sequencing. Sequence analysis revealed the existence of a new GlyT2 C-terminus, 25 amino acids shorter than the previously known C-terminus. The novel isoform was named hGlyT2-NFS after the last three amino acids. The two C-termini were generated by the alternate use of exons 15 and 18 (known hGlyT2-TQC isoform) or exons 15, 16 and 17 (new isoform hGlyT2-NFS), as shown in Figure 3.6.

Figure 3.5. Agarose gel showing 3’ RACE PCR products (embryonic spinal cord). Multiple bands indicate the presence of PCR products of various sizes. M: 1 Kb DNA ladder (Invitrogen). 1: product of first round PCR amplification. 2: product of second round PCR amplification.

Known isoform: hGlyT2-TQC: Exon 15 + Exon 18

| W | S | M | V | L | G | W | L | M | L | A | C | S | V | I | W | I | P | I | M |
| TGGTCCATGGTGCTCGGATGGCTAATGCTCGCCTGTTCCGTCATCTGGATCCCAATTATG | 60 |
| FV | IK | MH | L A | P G | R F | IE | RL | KL | LV C |
| TTTGTGATAAAAATGCTCTGGCCCCCCTGAAGATTATTGTAGGGCTGAAATTGCTG |
| NsiI |
| SP | QP | PD | WG | PF | LA | QH | RG | ERY | KN |
| TC GCCA AGC GGA CGT GGGGCCCATTCTTAGCTACACACCCGCGGAGCGCTGAAATTGCTG |
| 120 |
| MID | P | LG | TSS | LG | KL | LP | VK | KDL | L |
| AGATCGACCTCCTGGAGACCTCTGCTGAGAGCACGTGAAGATTATTGCT |
| 180 |
| LG | T | QC | * |
| CGGGCACTCAGTGCAGTTGCTGAGAGCACGTGATAGCATCTGCTG |
| 240 |
| CTTCGCTCCTCCTAGTAGGATGTGATAGGCTGCAAGAGCTGATAGCATCTGCTG |
| 300 |
| ACATTCGCTCCTCCTAGTAGGATGTGATAGGCTGCAAGAGCTGATAGCATCTGCTG |
| 360 |
| TGCTCAGCAGAAGACACGCACACAAGCAGGACAGGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA |
3.4. Further characterisation of the novel isoform GlyT2-NFS

Three different N-terminal variants (specified by exons la, lb and lc) and two different C-terminal variants were identified by 5' and 3' RACE revealing several new exons for genetic analysis. However, while the N-terminal variations are minor, and have no obvious functional consequence, the new C-terminal variant (designated hGlyT2-NFS) lacks the C-terminal class III PDZ-domain binding motif (TQC) found in hGlyT2, which is known to be crucial for correct synaptic localisation of GlyT2 (Armsen et al., 2007). For this reason, hGlyT2-NFS was selected for further functional analysis.

3.4.1. Cloning of pRc/CMV-hGlyT2-NFS

A partial cDNA encoding the C-terminus of hGlyT2-NFS was amplified from human adult spinal cord cDNA using a two-stage PCR amplification. The first round of PCR was carried out using the primers hGlyT2Cnael3 and hGlyT2Cxbal3. The product of the first amplification was used as a template for the nested PCR, performed using primers hGlyT2Cecoi and hGlyT2Cxbal1. Amplification conditions are detailed in Section 2.2.3. PCR products were cloned into pCR-Blunt II-TOPO vector, sequenced and subsequently subcloned into the Ecobl and Xbal sites of the pRc/CMV-hGlyT2 expression vector (kindly provided by Dr Stéphane Supplisson, Ecole Normale
Supérieure, Paris, France), in order to replace the C-terminus of GlyT2-TQC with the corresponding sequence in GlyT2-NFS.

3.4.2. Functional activity of the hGlyT2 variants

The ability of novel isoform hGlyT2-NFS to transport glycine was investigated by transfecting HEK293 cells with pRc/CMV-hGlyT2-NFS construct and measuring [³H]-glycine-mediated uptake. Briefly, cells were transfected with four independent pRc/CMV-hGlyT2-NFS constructs using Lipofectamine LTX reagent as described in Section 2.4.7. Transfections with empty vector pRc/CMV and with pRc/CMV-hGlyT2-TQC construct were carried out as negative and positive controls respectively. 24 hours after transfection, cells were washed with Krebs buffer pre-gassed with 5% (v/v) CO₂/air, and then incubated for 5 minutes in 0.1 μCi/ml [³H]-glycine (60 Ci/mmol, NEN) at a final concentration of 300 μM, as described in Section 2.4.10. Uptake was terminated by the addition of ice-cold buffer followed by aspiration. Cells were digested and used for scintillation counting and for determination of protein concentration using Bradford reagent. [³H]-glycine uptake was expressed as percentage of that in control cells transfected with the empty vector pRc/CMV. It should be noted that low level of endogenous glycine uptake was detected in HEK293 cells (Tunnicliff, 2003; Rees et al., 2006). As shown in Figure 3.7, HEK293 cells transfected with hGlyT2-TQC showed an approximately six-fold increase in [³H]-glycine uptake relatively to cells transfected with the empty vector. However, hGlyT2-NFS mediated uptake was not significantly different from the empty vector control, suggesting that either this new variant does not target to the cell membrane, or that it is non-functional.
Figure 3.7. Transport activity of hGlyT2-TQC and hGlyT2-NFS isoforms. Glycine uptake was measured in HEK293 cells transiently expressing pRc/CMV, pRc/CMV-hGlyT2-TQC and four independent pRc/CMV-hGlyT2-NFS constructs after incubation with [³H]-glycine. [³H]-glycine uptake was expressed as percentage of that in control cells transfected with the empty pRc/CMV vector. Data are means ± s.e.m. (n = 4). * indicates significantly different from control, P<0.01.

3.5. Functional characterisation of new mutations in SLC6A5

The effects of new SLC6A5 hyperekplexia mutations were determined by examining the subcellular localisation and functionality of recombinant epitope-tagged GlyT2 proteins expressed in mammalian cells. All mutations were introduced into the known hGlyT2-TQC isoform, which is now referred to as simply hGlyT2.

3.5.1. Generation of pRc/CMV-hGlyT2 and pRK5myc-hGlyT2 mutants

New SLC6A5 mutations were introduced into the mammalian expression constructs pRK5myc-hGlyT2 and pRc/CMV-hGlyT2 (Figure 3.8) by site-directed mutagenesis (Section 2.2.14). Pairs of complementary mutagenesis oligonucleotide primers (Section 2.2.14) were designed to (i) contain the desired mutation flanked by 10-12 bases of unmodified nucleotide sequence and (ii) anneal to the same sequence on opposite strands of the template DNA. PCR products resulting from mutagenesis
reactions were transformed into *E. coli* (Section 2.1.4) and a number of minipreps for each mutant were prepared (Section 2.1.5).

**Figure 3.8. Plasmid maps of pRc/CMV-hGlyT2 and pRK5myc-hGlyT2.** CMV_p: cytomegalovirus promoter; BGH pA: bovine growth hormone polyadenylation signal; F1 ori: F1 origin; SV40_p: SV40 promoter; SV40 poly: SV40 polyadenylation signal; Neomycin: neomycin resistance gene; ColEl: ColEl origin of replication; Amp: ampicillin resistance gene; SP6: SP6 promoter; myc: myc tag.

DNA sequence analysis of the entire hGlyT2 coding region confirmed the incorporation of the mutations and ensured that no other unwanted changes introduced during PCR were present. Maxipreps of hGlyT2 mutant constructs were prepared, resulting in sufficient amounts of plasmid DNA for subsequent transfection experiments (Section 2.1.6). The maxipreps were re-sequenced to confirm the presence of the desired mutations and the identity of the constructs.

### 3.5.2. Subcellular localisation of new hGlyT2 hyperekplexia mutants

Immunocytochemical experiments were carried out on transfected HEK293 cells in order to determine the subcellular localisation of hGlyT2 and mutants (Section 2.4.8). Briefly, HEK293 cells seeded on poly-D-lysine coated coverslips were transfected with 1 µg total pRK5myc (vector control), pRK5myc-hGlyT2 or pRK5myc-hGlyT2 mutants DNA using Lipofectamine LTX. After an incubation of 24 hours, transfected cells were washed twice in PBS, fixed for 2 minutes in 4% (w/v) PFA in PBS and quenched in 50 mM NH₄Cl for 10 minutes before permeabilisation in 0.01% (w/v) Triton X-100 for 12 minutes, further washing in PBS and staining with an polyclonal
anti-myc primary antibody, Alexa-Fluor 488 goat anti-rabbit secondary antibody and nucleic acid marker TO-PRO-3. Coverslip-mounted microscope slides were examined using a Zeiss LSM 510 Meta Confocal microscope. An argon laser which emits light at a wavelength of 488 was used to detect the Alexa-Fluor 488 antibody. The HeNe laser which emits light at a wavelength of 594 was used to detect TO-PRO-3 (Section 2.4.8). Confocal microscopy showed that myc-tagged hGlyT2 (Figure 3.9, panel A), as well as the L237P and A275T mutants (Figure 3.9, panels D and F), were readily expressed at the cell surface. By contrast, W151X, L198R+fs123, P243T, R439X and I665K+fs1 were cytoplasmic (Figure 3.9, panels B, C, E, G and H).

Figure 3.9. Subcellular localisation of myc-hGlyT2 and hyperekplexia mutants. Confocal microscopy of HEK293 cells transfected with myc-tagged constructs (panel A: GlyT2 wild-type; panels B-H: GlyT2 mutants). Green labelling corresponds to myc-GlyT2, whilst red labelling is for the nuclear marker TO-PRO-3 (TP3). Note that for myc-hGlyT2 and mutants L237P and A275T small GlyT2 clusters can be observed on the cell surface, whereas P243T, L198R+fs123, I665K+fs1, W151X and R439X show cytoplasmic localisation. Ideally, cell membrane expression would have been verified with an antibody raised against an extracellular epitope, but no such antibodies currently exist for GlyT2. Scale bar: 10 μm.

3.5.3. Functional characterisation of hGlyT2 mutants

Functional consequences of new GlyT2 mutations were investigated performing $[^3]$H]-glycine uptake experiments on HEK293 cells transiently expressing pRe/CMV (negative control), pRe/CMV-hGlyT2 (positive control) and hyperekplexia mutants, as described in Section 2.4.10. As shown in Figure 3.10 A, substitutions W151X, L198R+fs123, L237, P243T, R439X and I665K+fs1 abolished $[^3]$H]-glycine uptake.
Mutation A275T was partially functional, but did exhibit a significantly decreased uptake compared to hGlyT2. In order to understand the mechanisms underlying the clinical phenotype in individuals 1, 4 and 6, the presence of a dominant-negative effect was tested by co-transfection, reproducing the heterozygous state. Mixing W151X, A275T and R439X with hGlyT2 in a 1:1 ratio did not significantly alter glycine uptake, as seen in Figure 3.10 B.

This result allowed us to exclude a dominant-negative effect for those three mutations, suggesting the presence of deletions or promoter mutations in the second SLC6A5 allele as possible cause of the disease phenotype in patients 1, 4 and 6. As mutant A275T appeared to be functional, further studies were carried out to determine the detailed kinetic properties of this mutant. pRc/CMV-hGlyT2 and the pRc/CMV-hGlyT2 A275T plasmids were transiently expressed in HEK293 cells and incubations were carried out using a varying concentration of unlabelled glycine (3, 10, 30, 100, 300, 1000 µM). The dose-response curve shows that both hGlyT2 and A275T-mediated uptake was saturable (Figure 3.11 A). An Eadie-Hofstee plot (a graphical representation of kinetics in which reaction velocity V is plotted as a function of the velocity vs. substrate concentration ratio S) of these data was used to determine the Michaelis constant (K_m) and maximum velocity (V_max), as shown in Figure 3.11 B. The Eadie-Hofstee plot of the data revealed that K_m of A275T-mediated transport was significantly increased compared to hGlyT2, indicating a reduced affinity of A275T for glycine (63 µM for cells expressing the mutant versus 35 µM for cells expressing the wild-type). The total transporter activity observed for both the mutant and the wild-type was the same (1.4 nmol/min/mg of protein).
Figure 3.10. Transport activity of hGlyT2 and hyperekplexia mutants. A: [³H]-glycine uptake in HEK293 cells transfected with pRC/CMV, pRC/CMV-hGlyT2 and hyperekplexia mutants after incubation with [³H]-glycine. B: transport activity of selected combinations of hGlyT2 and hyperekplexia mutants, mimicking the heterozygous state. [³H]-glycine uptake was measured in HEK293 cells co-transfected with pRC/CMV-hGlyT2 combined with W151X, A275T and R439X mutants in a 1:1 ratio. X denotes a stop codon. Cells transfected with empty vector pRC/CMV were used as control. Glycine uptake was expressed as percentage of that in control cells transfected with the empty pRC/CMV vector. Data are means ± s.e.m. (n = 8). * indicates significantly different from control, † indicates significantly different from GlyT2, P<0.01.
Figure 3.11. Kinetic properties of hGlyT2 and A275T mutant. [$^3$H]-glycine uptake was measured in HEK293 cells transfected with pRc/CMV, pRc/CMV-hGlyT2 and hGlyT2-A275T after incubation with a range of substrate concentrations. A: Saturation curves for hGlyT2 and A275T. B: Eadie-Hofstee transformation of the data represented in A. The velocity of the reaction V is plotted on the vertical axis as a function of the velocity vs substrate concentration ratio [S] on the horizontal axis.
3.6. Discussion

3.6.1. Identification of new hyperekplexia mutations and splicing patterns in the human GlyT2 gene

Novel mutations in the human GlyT2 gene SLC6A5 were identified in seven hyperekplexia patients from the Netherlands, the United Kingdom, Spain, Italy, France and the Middle East. A recessive mode of inheritance was observed for patients 2, 3, 5 and 7 (compound heterozygous or homozygous inheritance of a single allele), confirming the observation that SLC6A5 mutations are commonly associated with recessive hyperekplexia (Rees et al., 2006). However, in three cases only one coding mutation was detected, so the mechanisms underlying the clinical phenotype in individuals 1, 4 and 6 were ambiguous. I considered that either the mutations W151X, A275T and R439X exert a dominant-negative effect on the other unaffected GlyT2 allele or that uncharacterised alternative splicing of SLC6A5 transcripts might have excluded some exons from mutation screening. I considered that alternative splicing of SLC6A5 at the N- and C-termini was most likely, since a previous study reported the identification of three GlyT2 isoforms, named mGlyT2a, mGlyT2b and mGlyT2c, in the mouse brain (Ebihara et al., 2004). 5' RACE allowed the identification of a total of three hGlyT2 N-termini, generated from alternative splicing of exons 1a, 1b and 1c to an acceptor site flanking exon 2. The three N-termini may result from a single GlyT2 promoter, or alternative developmentally or tissue specific promoters (Section 3.3.2; Figure 3.4). By contrast, 3' RACE revealed the existence of an additional C-terminal variant of GlyT2, which I termed GlyT2-NFS. Genomic sequence analysis suggested that GlyT2-NFS also results from alternative splicing or exon 15 to new exons 16 and 17. By contrast, the previously characterised hGlyT2 variant is made by splicing exons 15 and 18 (former exon 16, Section 3.3.3; Figure 3.6). However, no further mutations were detected in these novel exons in individuals 1, 4 and 6, suggesting possible dominant-negative effects of mutations W151X, A275T and R439X.

3.6.2. GlyT2-NFS: a new C-terminal GlyT2 variant

The novel C-terminal hGlyT2 isoform hGlyT2-NFS encodes a shorter protein that carries a sequence that diverges from the known hGlyT2 isoform in the last 27 amino acids. The ability of the new isoform to transport glycine was investigated by
transfecting HEK293 cells with a vector containing hGlyT2-NFS and examining $[^{3}\text{H}]$-glycine uptake. Surprisingly, functional analysis revealed that the novel isoform was apparently unable to mediate $[^{3}\text{H}]$-glycine uptake at the cell surface. Sequence analysis revealed that the novel isoform lacks the class III PDZ-binding motif TQC which is crucial for the interaction of GlyT2 with the PDZ-containing protein syntenin-1 (Ohno et al., 2004) as well as the other PDZ domain containing proteins identified in Chapter 4. Even though syntenin-1 and the C-terminal TQC motif have been proposed to regulate trafficking and/or presynaptic localisation of GlyT2 in neurones (Ohno et al., 2004; Armesen et al., 2007), artificial mutants of the GlyT2 TQC motif are still found at the cell membrane in HEK293 cells (Armesen et al., 2007). However, it is possible that alternative splicing may directly alter the subcellular location of the GlyT2-NFS variant. It is also noteworthy that most commercial GlyT2 antibodies (e.g. GlyT21-A from Alpha Diagnostics; AB1771 from Chemicon) recognise the 'standard' GlyT2 C-terminus, but not the hGlyT2-NFS isoform. Indeed, the existence of a similar GlyT2-NFS isoform in other species might explain the discrepancies observed in several tissues (e.g. retina) between GlyT2 immunoreactivity and the observed cellular localisation of EGFP under the control of the mouse GlyT2 promoter (Zeilhofer et al., 2005).

3.6.3. New GlyT2 hyperekplexia mutations affect subcellular localisation and/or functionality of the transporter

The functional consequences of the new SLC6A5 hyperekplexia mutations were investigated using immunocytochemistry and $[^{3}\text{H}]$-glycine uptake assays. Human GlyT2 was clearly expressed at the cell membrane, as indicated by immunofluorescence (Figure 3.9) and robust $[^{3}\text{H}]$-glycine uptake (Figure 3.10). Mutants W151X, L198R+fs123, I665K+fs1, and R439X were located in the cytoplasm, as might be expected since they all result in truncation of GlyT2. In addition, mutant P243T also showed a predominantly cytoplasmic location. Consistent with these results, functional studies revealed that all five of these mutants completely abolished $[^{3}\text{H}]$-glycine uptake (Figure 3.11). Although expressed at the cell surface, mutant L237P was not functional in terms of uptake, consistent with the behaviour of other previously characterised missense mutations such as T425M and Y491C (Rees et al., 2006), which presumably disrupt allosteric mechanisms controlling glycine transporter activity. Interestingly, mutant A275T was expressed at
the cell surface and was capable of $[^3]H$-glycine uptake. However, more detailed kinetic studies revealed that A275T had a two-fold lower affinity for glycine than the wild type.

3.6.4. W151X, A275T and R439X do not exert dominant-negative effects

After excluding the presence of undetected mutations in newly identified SLC6A5 exons, further functional investigations were carried out to understand the genetic causes of hyperekplexia in patients 1, 4 and 6. In particular, mutants W151X, A275T and R439X were examined for potential dominant negative effects by co-transfecting these mutants with hGlyT2 in a 1:1 ratio. If the mutant GlyT2 proteins interfere with trafficking of 'normal' hGlyT2, then reduced $[^3]H$-glycine uptake compared to hGlyT2 would be predicted. This effect was previously observed for the dominant GlyT2 mutation S510R (Rees et al., 2006). However, co-transfection of W151X, A275T and R439X with hGlyT2 did not alter $[^3]H$-glycine uptake, suggesting that they are one of a pair of recessive alleles in these individuals. In support of this theory, it is also noteworthy that patient 2 has inherited R439X from both maternal and paternal alleles, which is consistent with a recessive mode of inheritance, while patient 6 apparently has only one copy of R439X. The most likely explanation is that our current detection methods are not able to detect the defect in the second allele in these individuals. For example, the presence of a large deletion in one allele would not readily be detected by PCR screening, due to the presence of the other allele, which could serve as a template for PCR. The presence of gene deletions needs to be assessed using techniques such as Multiplex Ligation-Dependent Probe Amplification (MLPA), which relies on a quantitative multiplex PCR approach to determine the relative copy number of each exon (Figure 3.12) and reveals alterations that might escape detection using conventional diagnostic techniques (Gille et al., 2002).

Alternatively, since the SLC6A5 promoter has not been characterised in any detail, it is possible that mutations could be present in upstream sequences controlling transcription of one allele. Although characterising neuronal promoters is complex, given the large numbers of synonymous and non-synonymous SNPs found in SLC6A5 exons 2, 5, 8 and 15 (Table 3.2) it might be possible to isolate total RNA from white blood cells in blood samples donated for genetic analysis and amplify short GlyT2 cDNAs encompassing these exons. Where SNP heterogeneity is present in the
genomic DNA of a patient but not in cloned cDNAs for exons 2, 5 and 8, a mutation in the promoter of one copy of SLC6A5 might be suspected.

The two parts of each probe are joined by a thermostable ligase

All probe ligation products are amplified using a single primer pair

**Figure 3.12. Schematic representation of MLPA technique.** Each MLPA probe consists of two oligonucleotides, one synthetic (50-60bp) and one M13-derived single-stranded DNA fragment. For each probe, there is a target specific sequence that can be ligated when correctly hybridised to its target. The MLPA probe mix is added to denatured genomic DNA and the two parts of each probe hybridise to adjacent target sequences. Probes are then ligated by a thermostable DNA ligase. A universal primer pair is used to amplify the ligated probes. The amplification product of each probe has a unique length (130-480 bp).

<table>
<thead>
<tr>
<th>Sequence change</th>
<th>Exon number</th>
<th>Predicted consequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>C266A</td>
<td>Exon 2</td>
<td>A89E</td>
</tr>
<tr>
<td>G304A</td>
<td>Exon 2</td>
<td>G102S</td>
</tr>
<tr>
<td>C336T</td>
<td>Exon 2</td>
<td>S112S</td>
</tr>
<tr>
<td>C352T</td>
<td>Exon 2</td>
<td>L118L</td>
</tr>
<tr>
<td>C371T</td>
<td>Exon 2</td>
<td>S124F</td>
</tr>
<tr>
<td>A951G</td>
<td>Exon 5</td>
<td>T317T</td>
</tr>
<tr>
<td>G1371C</td>
<td>Exon 8</td>
<td>K457N</td>
</tr>
<tr>
<td>G1386T</td>
<td>Exon 8</td>
<td>T462T</td>
</tr>
<tr>
<td>G1387A</td>
<td>Exon 8</td>
<td>D463N</td>
</tr>
<tr>
<td>G2103A</td>
<td>Exon 15</td>
<td>E701E</td>
</tr>
</tbody>
</table>

**Table 3.2. SNPs detected in SLC6A5.** SNPs found in exons 2, 8 and 15 are described in Rees *et al.*, (2006)
4. IDENTIFICATION OF GLYT2 INTERACTING PROTEINS WITH THE YTH TECHNIQUE
4.1. GlyT2 interacting proteins are presynaptic candidates for genetic analysis in hyperekplexia

Despite recent advances in the genetics of hyperekplexia, there are still many patients without mutations in \textit{GLRA1}, \textit{GLRB}, \textit{GPHN}, \textit{ARHGEF9} or \textit{SLC6A5} (Rees et al., 2006), suggesting that other disease genes remain undiscovered. However, the discovery that mutations in the GlyT2 gene affect correct functioning and membrane trafficking of the transporter suggests that genes encoding proteins involved in the correct presynaptic localisation of GlyT2 represent plausible candidates for further genetic analysis (Harvey et al., 2008a). Only two GlyT2 accessory proteins have been characterised to date: Unc-33-like protein (ULIP6) and syntenin-1. ULIP6 is encoded by \textit{DPYSL5} on human chromosome 2p23.3 and is a brain-specific phosphoprotein belonging to the Ulip/collapsin response mediator protein family (Horiuchi et al., 2000). This family of proteins is involved in signalling cascades that mediate axonal guidance and outgrowth and also have a role in endocytosis (Quinn et al., 1999). YTH experiments have shown that ULIP6 interacts with the intracellular N-terminal region of GlyT2 (amino acids 135-184) in a phosphorylation-dependent manner. The GlyT2-ULIP6 interaction is also reasonably specific, since GlyT2 does not interact with ULIP1-5 (Horiuchi et al., 2005). ULIP6 may therefore regulate recycling/internalisation of GlyT2, so genetic defects in ULIP6 could cause hyperekplexia by altering the steady-state levels of presynaptic GlyT2. By contrast, syntenin-1 is encoded by \textit{SDCBP} on human chromosome 8q12.1, and was originally identified as a syndecan binding protein (Grootjans et al., 1997). Syntenin-1 binds to the intracellular C-terminus of GlyT2 via two PDZ domains and colocalises with GlyT2 in brainstem sections. This GlyT2-syntenin interaction has been suggested to regulate the trafficking and/or the presynaptic localisation of the transporter (Ohno et al., 2004; Armsen et al., 2007). However, it is important to note that other studies have shown that syntenin-1 binds to the cytoplasmic domains of several membrane proteins such as AMPA, kainate and metabotropic glutamate receptors, syndecans, neurexin, B-ephrins, neurofascin and interleukin-5 receptor α (Hirbec et al., 2002; Hirbec et al., 2005). Therefore mutations in \textit{SDCBP} would be expected to affect diverse receptor systems.
4.2. Characterising new GiyT2 interacting proteins using the YTH system

While the ULIP6 gene (DPYSL5) represents a plausible candidate gene for further genetic screening in hyperekplexia, it is highly likely that further GlyT2 interacting proteins exist. I therefore decided to identify new GlyT2 interactors to ‘feed in’ to the genetic screening process using a well-characterised technique for discovering protein-protein interactions, the YTH system.

4.2.1. The basis of the YTH system

The YTH system is a powerful genetic assay for detecting specific protein-protein interactions in vivo. This system is based on the modular properties of many eukaryotic transcription factors, which are composed of two physically separable and functionally independent domains: a DNA binding domain (DNA-BD) that directs binding to a promoter DNA sequence and an activation domain (AD) that activates transcription. The system exploits the fact that the DNA-BD of a transcriptional factor is incapable of activating transcription of a gene unless physically associated with, but not necessary covalently attached to, an AD (Van Criekinge et al., 1999). The use of this technique was first proposed by Fields and Song (1989) who tested the system to show the interaction between two yeast proteins that were known to interact: SNF1 and SNF4. The success of this experiment suggested that the technique could be used for identifying unknown proteins, encoded by a cDNA library, that interact with a protein of interest (Fields and Song, 1989). In a typical YTH experiment, plasmids encoding two hybrid proteins, one constituted by the GAL4-BD fused to a protein X (bait) and the other represented by the AD fused to a protein Y (prey), are generated and transfected into an appropriate yeast host strain. Only if the two proteins interact an active transcriptional factor is reconstituted. This leads to the transcription of one or more reporter genes that harbour specific binding sites for the DNA-BD, leading to phenotypic alteration of the yeast host cells (Figure 4.1). The two most commonly used YTH systems are the GAL4 system, in which the DNA-BD and AD of the yeast GAL4 protein are used, and the LexA system, where the DNA binding domain of the bacterial repressor protein LexA and the Escherichia coli B42 AD are used (Causier and Davies, 2002).
Co-transform YTH plasmids into appropriate yeast strain

**Figure 4.1. The YTH system.** One bait construct, encoding protein X fused in-frame to the DNA-BD of a transcriptional factor, and a prey construct, encoding protein Y fused in-frame to a transcriptional AD are generated. The plasmids are co-transfected into an appropriate yeast strain where the fusion proteins are expressed. If the proteins X and Y interact, an active transcription factor is reconstituted and binds elements upstream of the reporter gene, activating expression of a phenotypic marker.

### 4.2.2. Yeast reporter strains

Yeast reporter strains contain one or more reporter genes integrated into the chromosomal DNA that harbour specific upstream activating sequences (UAS), regulatory elements recognised by transcription factors. The most common reporter genes are the bacterial *LacZ* gene and the yeast *HIS3* gene, which can be controlled by LexA or GAL4 UAS elements, depending on the system in use. The L40 strain contains two integrated reporters, the yeast *HIS3* gene, under the control of four upstream LexA operators, and the bacterial *LacZ* gene, under the control of eight upstream LexA operators. In the Y190 strain, the *LacZ* and *HIS3* genes are under the control of the GAL4-responsive GAL1 promoter. In the AH109 strain, the three
reporter genes HIS3, LEU2 and LacZ have been placed under the control of the GAL4-responsive promoters GAL1, GAL2 and MEL2 respectively (Figure 4.2).

**Figure 4.2. Genotype of yeast reporter strains L40, Y190 and AH109.** The L40 reporter genes are responsive to LexA-induced transcriptional activation. Y190 and AH109 reporter genes are responsive to GAL4-induced transcriptional activation.

### 4.2.3. The LacZ and HIS3 reporter genes

The bacterial LacZ gene encodes the enzyme β-galactosidase. When LacZ is expressed yeast colonies turn blue in presence of the chromogenic substrate X-gal. β-galactosidase activity is assayed by incubating freeze-fractured yeast colonies with an X-gal containing buffer, resulting in the formation of blue precipitate, dichlorodibromo-indigo (indolyl-blue), when the X-gal substrate is cleaved (Breeden and Nasmyth, 1985). The yeast gene HIS3 encodes imidazoglycerol-phosphate dehydratase, an enzyme involved in the biosynthesis of histidine. Activation of this reporter gene confers histidine prototrophy to yeast strains with a lesion in the native HIS3 gene. This allows the yeast to grow on media lacking histidine, enabling the selection of colonies containing potential interacting proteins. Since HIS3 shows leaky expression in many yeast strains, the basal low-level expression of this gene is inhibited by supplementing the medium with 3-AT, a competitive inhibitor of the HIS3 gene product that suppresses yeast background growth (Van Criekinge *et al.*, 1999).
4.3. Study aims

The aim of this study was to identify novel GlyT2 interacting proteins using the YTH system. This process included the following stages:

- Construct and characterise functional GlyT2 baits by using the GAL4BD fused to the GlyT2 N- or C-terminus. The functionality of the baits was tested by evaluating their ability to bind to prey constructs formed by GAL4AD fused to a known GlyT2 interacting protein, e.g. ULIP6 (N-terminus) or syntenin-1 (C-terminus). This process will also allow optimisation of grown conditions (e.g. level of 3-AT inhibitor).

- Use functional GlyT2 baits to screen a human brain cDNA library in order to find unknown GlyT2 interactors. Transformants were selected for HIS3 reporter gene activity and subsequently tested for LacZ reporter gene activity before yeast plasmid isolation and DNA sequencing.

- Test the specificity of isolated prey constructs for GlyT1, GlyT2 and mutant bait plasmids, e.g. lacking the C-terminal class III PDZ binding motif (TQC).

4.4. Identification of non autoactivating, functional GlyT2 baits

In order to perform a YTH library screen an appropriate vector system, either GAL4- or LexA- based, and a compatible yeast reporter strain were chosen. The bait construct, containing the protein of interest (GlyT2-N or GlyT2-C) fused in-frame to a DNA-BD, was generated by amplifying the corresponding cDNA sequences by PCR and cloning into the GAL4BD vector pYTH16 (Fuller et al., 1998). Known GlyT2 interactors, such as ULIP6 and syntenin-1, were cloned in-frame with an AD into a GAL4AD prey vector (pACT2, Clontech) and sequenced. In a pilot experiment, the chosen yeast strain (Y190) was transformed with the bait and prey vectors and the reporter gene activity were assayed. The detection of an interaction suggests the correct expression, folding and functioning of the bait and prey fusion proteins. Bait and prey vectors were also transformed together with empty prey or bait vectors, respectively, to exclude ‘autoactivation’, a phenomenon where the bait or prey fusion proteins alone initiate the transcription of the reporter gene. The optimal concentration of 3-AT in the medium was also titrated in order to eliminate background growth on -His selection plates due to leaky expression of HIS3 reporter gene. After confirming
that the bait was functional and non autoactivating, and optimising the 3-AT concentration, it was possible to proceed with the library screen.

4.4.1. pYTH16-GlyT2N bait and pACT2-ULIP6 construct testing

A bait construct for the GlyT2 cytosolic N-terminus was generated by cloning the corresponding cDNA sequence into the GAL4BD vector pYTH16. A partial cDNA encoding amino acids 1-200 of GlyT2 was amplified by PCR from human adult hippocampal cDNA using oligonucleotides hGlyT2NSalI and hGlyT2NEcoRI (Section 2.2.4; Table 2.1) and cloned into the SalI and EcoRI sites of pYTH16. The resultant construct was sequenced to confirm in-frame fusion of GAL4BD to the GlyT2 N-terminus. A map of the GlyT2N bait construct is shown in Figure 4.3. In order to test the GAL4BD-GlyT2N bait, a full length cDNA for ULIP6, a protein known to interact with the N-terminus of the transporter, was cloned into the prey vector pACT2. The full-length ULIP6 cDNA was amplified from human adult hippocampal cDNA using the oligonucleotide primers ULIPb-fcoRI and ULIP6-Y/zoI (Section 2.2.4; Table 2.1) and cloned into the EcoRI and XhoI sites of pACT2. Sequence analysis confirmed in-frame fusion of the protein to the GAL4AD sequence. A map of the GlyT2N bait and ULIP6 prey constructs is shown in Figure 4.3.

![Figure 4.3. Map of the bait construct pYTH16-GlyT2N and the prey construct pACT2-ULIP6. ADH₆: constitutive promoter for alcohol dehydrogenase; GAL4BD: GAL4 binding domain; GAL4AD: GAL4 activation domain; HA: haemagglutinin epitope; ColE1 ori: ColE1 origin of replication; pUC ori: pUC origin of replication; ADH₄: alcohol dehydrogenase transcription termination signal; Amp: ampicillin resistance gene; TRP: tryptophan nutritional marker; LEU2: leucine nutritional marker.](image)

Competent Y190 cells were co-transformed with the pYTH16-GlyT2N bait and the pACT2-ULIP6 prey vectors, to test for the known interaction in a small-scale yeast transformation (Section 2.3.4; Table 4.1). pYTH16-GlyT2N plus pACT2 and
pYTH16 plus pACT2-ULIP6 combinations were used to check for bait or prey autoactivation. Transformants were plated on minimal agar lacking leucine and tryptophan (SD/-Leu/-Trp) to enable the selection of co-transformed yeast colonies, and on minimal agar lacking leucine, tryptophan and histidine supplemented with either 15mM or 30mM 3-AT (SD/-Leu/-Trp/-His + 3-AT), to enable the detection of GlyT2-ULIP6 interaction by nutritional selection.

Table 4.1. Pilot transformations of Y190 with pYTH16-GlyT2N and pACT2-ULIP6.

<table>
<thead>
<tr>
<th></th>
<th>pACT2</th>
<th>pACT2-ULIP6</th>
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<tbody>
<tr>
<td>pYTH16</td>
<td>Negative control</td>
<td>Negative control: test prey for autoactivation</td>
</tr>
<tr>
<td>pYTH16-GlyT2N</td>
<td>Negative control: test bait for autoactivation</td>
<td>Positive control: test for bait and prey functionality</td>
</tr>
</tbody>
</table>

After two days (SD/-Leu/-Trp) or four days (SD/-Leu/-Trp/-His + 3-AT) plates were assayed for i) yeast growth and ii) β-galactosidase activity using a freeze-fracture assay for LacZ activity (Section 2.3.5). Unfortunately, I was unable to detect an interaction between the GAL4BD-GlyT2 N-terminus and GAL4AD-ULIP6 (data not shown). Although I excluded problems with both bait and prey constructs by DNA sequencing, it is noteworthy that the original GlyT2-ULIP6 interaction was identified using the LexA YTH system (Horiuchi et al., 2005) and it is likely that either the GAL4BD-GlyT2N bait or the GAL4AD-ULIP6 prey fusion protein did not fold correctly. Since screening a cDNA library with uncharacterised bait is a high risk, the decision was therefore made to focus on the GlyT2 C-terminus.

4.4.2. pYTH16-GlyT2C bait and pACT2-syntenin-1 construct testing

A bait construct for the GlyT2 cytosolic C-terminus was generated by cloning the corresponding cDNA sequence into the GAL4BD vector pYTH16. A partial cDNA encoding amino acids 736-799 of GlyT2 was amplified by PCR from human adult hippocampal cDNA using oligonucleotide primers hGlyT2C'Sall and hGlyT2C'EcoRI (Section 2.2.4; Table 2.1) and cloned into the SalI and EcoRI sites of pYTH16. The resultant construct was sequenced to confirm in-frame fusion of GAL4BD to the GlyT2 C-terminus. A map of the GlyT2C bait construct is shown in Figure 4.4. In order to test the GAL4BD-GlyT2C bait, a full-length cDNA for syntenin-1, a known GlyT2C interactor (Ohno et al., 2004) was cloned into the prey vector pACT2. The
full-length syntenin-1 cDNA was amplified from a cDNA clone obtained from RZPD using the oligonucleotide primers SDB1-\textit{Bam}HI and SDB-\textit{Xho}I (Section 2.2.4; Table 2.1) and cloned into the \textit{Bam}HI and \textit{Xho}I sites of pACT2. Sequence analysis confirmed in-frame fusion of the protein to the GAL4AD sequence. A map of the syntenin-1 prey construct is shown in Figure 4.4.

**Figure 4.4. Maps of the bait construct pYTH16-GlyT2C and the prey construct pACT2-syntenin-1.** ADH$_p$: constitutive promoter for alcohol dehydrogenase; GAL4BD: GAL4 binding domain; GAL4AD: GAL4 activation domain; HA: haemagglutinin epitope; ColEl ori: ColEl origin of replication; pUC ori: pUC origin of replication; ADH$_{\text{trans}}$: alcohol dehydrogenase transcription termination signal; Amp: ampicillin resistance gene; TRP: tryptophan nutritional marker; LEU2: leucine nutritional marker.

Competent Y190 cells were co-transformed with the pYTH16-GlyT2C bait and the pACT2-syntenin-1 prey vectors, to test for the known interaction in a small-scale yeast transformation (Section 2.3.4; Table 4.2). pYTH16-GlyT2C plus pACT2 and pYTH16 plus pACT2-syntenin-1 combinations were used to check for bait or prey autoactivation. Transformants were plated on minimal agar lacking leucine and tryptophan (SD/-Leu/-Trp) to enable the selection of co-transformed yeast colonies, and on minimal agar lacking leucine, tryptophan and histidine supplemented with either 15mM or 30mM 3-AT (SD/-Leu/-Trp/-His + 3-AT), to enable the detection of GlyT2-syntenin-1 interaction by nutritional selection.

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<thead>
<tr>
<th></th>
<th>pACT2</th>
<th>pACT2-syntenin-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>pYTH16</td>
<td>Negative control</td>
<td>Negative control: test prey for autoactivation</td>
</tr>
<tr>
<td>pYTH16-GlyT2C</td>
<td>Negative control: test bait for autoactivation</td>
<td>Positive control: test for bait and prey functionality</td>
</tr>
</tbody>
</table>

**Table 4.2. Pilot transformations of Y190 with pYTH16-GlyT2C and pACT2-syntenin-1.**
After two days (SD/-Leu/-Trp) or four days (SD/-Leu/-Trp/-His + 3-AT) plates were assayed for i) yeast growth and ii) β-galactosidase activity using a freeze-fracture assay for \textit{LacZ} activity (Section 2.3.5; Figure 4.5). The bait construct pYTH16-GlyT2C alone did not activate expression of the reporter genes, as shown by the absence of blue colonies on SD/-Leu/-Trp or growth on SD/-Leu/-Trp/-His + 3-AT (pYTH16-GlyT2C/pACT2 plates; Figure 4.5, filters 3 and 7). Similar results were obtained for GAL4BD plus GAL4AD (pYTH16/pACT2 plates; Figure 4.5, filters 1 and 5) and for GAL4BD plus syntenin-1 prey (pYTH16/pACT2-syntenin-1 plates; Figure 4.5, filters 2 and 6). The specific interaction between the GlyT2C bait and the syntenin-1 prey was confirmed by the presence of blue colonies on SD/-Leu/-Trp and robust yeast growth on SD/-Leu/-Trp/-His + 3-AT (pYTH16-GlyT2C/pACT2-syntenin-1 plates; Figure 4.5, filters 4 and 8). Transformants were plated on SD/-Leu/-Trp/-His medium supplemented with both 15 mM and 30 mM 3-AT. While 15 mM 3-AT suppressed non-specific background while retaining specific interaction (e.g. GlyT2C + syntenin-1), 30 mM 3-AT also suppressed the growth of GlyT2C + syntenin-1 (data not shown). Therefore, 15 mM 3-AT was chosen as the optimal amount for cDNA library screening.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure4.5.png}
\caption{\textit{LacZ} freeze-fracture assay on SD/-Leu/-Trp plates (left panels, filters 1-4) and growth on SD/-Leu/-Trp/-His + 3-AT plates (right panels, filters 5-8). Note that yeast transformed with all vector combinations grow on SD/-Leu/-Trp plates due to the presence of the \textit{TRP} and \textit{LEU} genes on bait and prey vectors respectively. However, blue colouration of yeast colonies is only observed for the GAL4BD-GlyT2C and GAL4AD-syntenin-1, due to a reconstitution of Gal4 activity and activation of the \textit{LacZ} reporter gene. In nutritional selection experiments on SD/-Leu/-Trp/-His + 15mM 3-AT, yeast colonies are only observed for the GAL4BD-GlyT2C and GAL4AD-syntenin-1 combination due to the activity of the \textit{HIS} reporter gene.}
\end{figure}
4.5. cDNA library screening with pYTH16-GlyT2C bait

The steps involved in the preparation for a YTH screen are represented in Figure 4.6.

4.5.1. Choice and amplification of a cDNA library

For effective YTH screening, it is important to choose a cDNA library prepared from a tissue in which the protein of interest is known to be biologically relevant, in order to reduce the number of false positives. In this study, a human foetal brain Matchmaker cDNA library (Clontech) was utilised, where inserts were cloned in-frame with the GALAD in the pACT2 vector (Figure 4.7). The library was amplified in order to obtain enough plasmid for library screening in yeast (Section 2.3.6).

![Figure 4.6. A flowchart depicting the steps involved in a YTH library screen.](image)

Firstly an appropriate vector system, either GAL4- or LexA- based, and a compatible yeast reporter strain are chosen. The bait (GlyT2C) and prey (syntenin-1) constructs are generated using standard PCR and cloning techniques. In a pilot experiment the chosen yeast strain was transformed with bait and prey vectors and the reporter gene activities were assayed. The detection of an interaction indicated the correct expression, folding and functioning of the bait and prey proteins. The bait was independently transformed to exclude the presence of autoactivation (ability to initiate the transcription of the reporter gene in absence of the interacting prey protein). The optimal concentration of 3-AT in the medium was titrated in order to eliminate background growth on -His selection plates due to leaky expression of HIS3 reporter gene. After confirming that the bait was functional and non autoactivating and optimising the 3-AT concentration it was possible to proceed with the library screen.
Figure 4.7. Map of the pACT2-cDNA library plasmid. ADH$_p$: constitutive promoter for alcohol dehydrogenase; GAL4AD: GAL4 activation domain; HA: haemagglutinin epitope; ColE1 ori: ColE1 origin of replication; pUC ori: pUC origin of replication; ADH: alcohol dehydrogenase transcription termination signal; Amp: ampicillin resistance gene; LEU2: leucine nutritional marker. Note that the cDNA inserts (indicated by yellow shading) are of variable size and sequence.

4.5.2. Library transformation

The methodology used for screening a cDNA library is detailed in Section 2.3.6. Competent *S. cerevisiae* Y190 cells were co-transformed with the pYTH16-GlyT2C bait and the pACT2 human foetal brain cDNA library. The transformed cells were spread on 150 mm SD/-Leu/-Trp/-His library plates supplemented with 15 mM 3-AT. For transformation efficiency controls, transformants were spread on two SD/-Leu/-Trp plates to select for cells co-expressing the library and the bait. The transformation efficiency was calculated using the following formula:

\[
\text{# colonies on SD/-Leu/-Trp plate (average) } \times 1000 = \text{# transformants/ 0.5 \mu g pACT2 transformed}
\]

Transformation efficiency for YTH screen was $1.2 \times 10^6$ transformants/ 0.5 \mu g pACT2. As a positive control for the screen, competent Y190 cells were co-transformed with previously tested pYTH16-GlyT2C and pACT2-syntenin-1 combination. Co-transformation of competent Y190 cells with pYTH16-GlyT2C and pACT2 was also performed as negative control. Positive and negative controls were spread on SD/-Leu/-Trp/-His agar plates supplemented with 15 mM 3-AT.
4.5.3. Selection of positive clones and analysis of plasmid DNAs

After 5-6 days, colonies larger than the background became apparent on the library plates. Each HIS+ colony represented a putative positive for protein-protein interaction and was subjected to the following steps:

1. Restreaking on SD/-Leu/-Trp/-His + 15 mM 3-AT plates
2. LacZ freeze fracture assay
3. Isolation of plasmid DNA from yeast
4. Minipreps of plasmid DNA
5. Restriction enzyme analysis of minipreps
6. Sequencing analysis of cDNA clones

The procedure followed to select putative positive clones is detailed in Section 2.3.7. HIS+ colonies were restreaked onto fresh SD/-Leu/-Trp/-His + 15 mM 3-AT plates. Resultant colonies were replica-plated by transferring them from the agar onto Whatman No. 54 paper, in addition to fresh SD/-Leu/-Trp/-His + 15 mM 3-AT plates, which were incubated at 30°C. Filters were subjected to LacZ freeze-fracture assay. When a replica plate was LacZ positive, a sweep of positive colonies were used to isolate plasmid DNA from yeast cells as described in Section 2.3.7. Minipreps of plasmid DNA from yeast were prepared using the Qiaprep Miniprep kit (Qiagen) and subjected to restriction enzyme digestion with SacII to eliminate the episomal pYTH16 plasmid and select for the library plasmid. The digested DNA was transformed into E. coli and several minipreps for each bacterial clone were prepared.

In order to assess the sizes of the cDNA inserts, E. coli plasmid minipreps were subjected to restriction enzyme digestion with BglII. This enzyme cuts either site of the cDNA cloning site in pACT2, liberating the insert, but also has a single site in the bait pYTH16-GlyT2C. Thus following BglII digestion, miniprep DNAs containing the bait vector appeared as a single 7.4 kb band representing the linearised vector on agarose gels. By contrast, miniprep DNAs for pACT2 library clones resulted in two distinct bands, one of 8.1 kb representing the vector and the other for the cDNA insert. Figure 4.8 represents an example of BglII restriction enzyme analysis of miniprep DNA for clones 1.1 and 1.2. pACT2 plasmids were subjected to sequencing to reveal the identity of the cDNA insert and to check that the encoded protein was in-frame with the GAL4AD. All cDNA inserts were sequenced using both forward and reverse pACT2 sequencing primers to determine the exact insert limits (Section 2.2.11; Table
2.2). Following sequence analysis, cDNA inserts were used to search online nucleotide databases such as BLAT (www.genome.ucsc.edu) and BLAST (www.ncbi.nlm.nih.gov) in order to identify the encoded protein.

<table>
<thead>
<tr>
<th>Clone 1.1</th>
<th>Clone 1.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kb</td>
<td>1</td>
</tr>
<tr>
<td>1018</td>
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<tr>
<td>1018</td>
<td></td>
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<tr>
<td>1018</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 4.8. Restriction enzyme analysis of miniprep DNA derived from HIS+ colonies.**

Minipreps 1-8 (clone 1.1) and 1-5 (clone 1.2) were digested with BglII to reveal the size of the cDNA insert within the library plasmid.

**HIS+ colony 1.1**

Restriction enzyme digestion and DNA sequencing revealed a 1.27 kb insert (clone 1.1) encoding syntrophin-α1 (accession number NM_003098). The clone encoded amino acids 94-505 of the full-length protein (Figure 4.9).

**Figure 4.9. Sequence analysis of clone 1.1.** The amino acid sequence of syntrophin-α1 is shown, with the region interacting with GlyT2C highlighted in grey.

**HIS+ colonies 1.2, 5.4, 5.5, 9.4, 9.6, 11.3, 12.1, 12.5**

Restriction enzyme digestion and DNA sequencing revealed inserts of 1.49 kb (clones 5.5, 12.5), 1.12 kb (clones 5.4, 9.4, 9.6, 11.3, and 12.1) and 1.04 kb (clone 1.2) encoding the ligand of numb protein X-1, also called LNX1 (Accession number: NM_032622). The cDNA clones encoded amino acids 135-632 (5.5, 12.5), 258-632 (5.4, 9.4, 9.6, 11.3, 12.1) and 284-632 (1.2) of the full-length protein (Figure 4.10).
Figure 4.10. Sequence analysis of clones 1.2, 5.4, 5.5, 9.4, 9.6, 11.3, 12.1 and 12.5. The amino acid sequence of LNX1 is shown, with the minimal region interacting with GlyT2C highlighted in grey.

**HIS+ colonies 2.1, 14.2**

Restriction enzyme digestion and DNA sequencing revealed a 1.98 kb insert encoding synapse-associated protein 102, also called SAP 102 (Accession number: NM_021120). The two clones encoded amino acids 156-817 of the full-length protein (Figure 4.11).

Figure 4.11. Sequence analysis of clones 2.1 and 14.2. The amino acid sequence of SAP 102 is shown, with the minimal region interacting with GlyT2C highlighted in grey.

**HIS+ colonies 3.2, 3.11, 3.12, 4.3, 4.4**

Restriction enzyme digestion and DNA sequencing revealed a 3.16 kb insert encoding the formin-like 2 protein FMNL2 (Accession number: NM_052905). All clones encoded amino acids 95-1092 of the full-length protein (Figure 4.12).
Figure 4.12. Sequence analysis of clones 3.2, 3.11, 3.12, 4.3 and 4.4. The amino acid sequence of FMNL2 is shown, with the minimal region interacting with GlyT2C highlighted in grey.

**HIS+ colony 5.2**

Restriction enzyme digestion and DNA sequencing revealed a 2.3 kb insert encoding the membrane associated guanylate kinase, WW and PDZ domain containing 1 protein, also called MAGIl (Accession number: NM_015520). The clone encoded amino acids 518-1286 of the full-length protein (Figure 4.13).

Figure 4.13. Sequence analysis of the clone 5.2. The amino acid sequence of MAGIl is shown, with the region interacting with GlyT2C highlighted in grey.
**HIS+ colonies 7.1, 11.1, 12.2**

Restriction enzyme digestion and DNA sequencing revealed a 1.25 kb insert encoding a protein interacting with protein kinase C1 (PICK-1, Accession number: NM_012407). All clones were full-length (Figure 4.14).

**Figure 4.14. Sequence analysis of clones 7.1, 11.2 and 12.2.** The amino acid sequence of the hypothetical protein PICK-1 is shown, with the minimal region interacting with GlyT2C highlighted in grey.

**HIS+ colony 14.3**

Restriction enzyme digestion and DNA sequencing revealed a 1.39 kb insert encoding the *Homo sapiens* synapse-associated protein 97, also called SAP97 (Accession number: U13897). The clone encoded amino acids 441-904 of the full length protein (Figure 4.15).

**Figure 4.15. Sequence analysis of the clone 14.3.** The amino acid sequence of SAP97 is shown, with the region interacting with GlyT2C highlighted in grey.

**HIS+ colonies 1.5, 3.10, 4.2**

Restriction enzyme digestion and DNA sequencing revealed a 0.91 kb insert encoding COP9 constitutive photomorphogenic homolog subunit 5 (COP9S5, accession number NM_006837). Both clones encoded amino acids 31-334 of the full-length protein (Figure 4.16).
Figure 4.16. Sequence analysis of clones 1.5 and 3.10. The amino acid sequence of COP9S5 is shown, with the region interacting with GlyT2C highlighted in grey.

HIS+ colonies 5.3, 6.1, 8.2, 9.2, 10.4, 11.4

Restriction enzyme digestion and DNA sequencing revealed 1.34 kb inserts encoding the eukaryotic translation initiation factor 3, subunit E (EIF3S6, accession number NM_001568). All the clones were full-length (Figure 4.17).

Figure 4.17. Sequence analysis of clones 5.3, 6.1, 8.2, 9.2, 10.4 and 11.4. The amino acid sequence of EIF3S6 is shown, with the minimal region interacting with GlyT2C highlighted in grey.

HIS+ colonies 3.1, 3.3, 3.4, 3.5, 5.1, 7.2, 10.3, 14.1, 15.1

Restriction enzyme digestion and DNA sequencing revealed 1.96 kb inserts encoding the RAN binding protein 9 (RANBP9, accession number NM_005493). All clones encoded amino acids 135-728 of the full-length protein (Figure 4.18).

Figure 4.18. Sequence analysis of clones 3.1, 3.3, 3.4, 3.5, 5.1, 7.2, 10.3, 14.1 and 15.1. The amino acid sequence of RANBP9 is shown, with the minimal region interacting with GlyT2C highlighted in grey.
4.5.4. Eliminating false positives

False positives are GAL4AD fusion proteins that directly bind and activate the \textit{LacZ} and \textit{HIS3} reporter genes whether or not the specific DNABD/target protein is present. In order to eliminate false positives, the candidate AD/library plasmids were retransformed with the empty bait vector pYTH16 or with pYTH16-GlyT2C and tested for reporter gene activation. Truly positive AD/library plasmids would only activate the reporter genes in presence of the GlyT2C bait. Briefly, all putative positive clones listed above were co-transformed in Y190 cells with pYTH16 empty vector or the pYTH16-GlyT2C bait to test the library clones for autoactivation in a small scale yeast transformation (Table 4.1). The transformants were plated on minimal agar lacking leucine and tryptophan (SD/-Leu/-Trp) and on minimal agar lacking leucine, tryptophan and histidine (SD/-Leu/-Trp/-His) supplemented with 15 mM 3-AT and incubated at 30°C for three days.

<table>
<thead>
<tr>
<th>pACT2</th>
<th>pACT2-library clone</th>
</tr>
</thead>
<tbody>
<tr>
<td>pYTH16</td>
<td>Negative control</td>
</tr>
<tr>
<td>pYTH16-GlyT2C</td>
<td>Negative control</td>
</tr>
</tbody>
</table>

\textbf{Table 4.3. Small scale transformation strategy to eliminate false positives.}

\textit{LacZ} freeze fracture assays and growth on nutritional selection plates revealed that clones 3.1, 3.3, 3.4, 3.5, 5.1, 7.2, 10.3, 14.1 and 15.1, encoding RANBP9, activated both \textit{LacZ} and \textit{HIS3} reporter genes when co-transformed with pYTH16. RANBP9 was thus recognised as a false positive and excluded from further experiments. Although COP9S5 and EIF3S6 passed this autoactivation test, they are also commonly found in other YTH screens using unrelated baits and were therefore excluded from further analysis. The remaining seven interactors also passed this autoactivation test (Figure 4.19; Table 4.3) and because of their apparent biological relevance it was decided to investigate their interaction with GlyT2 in further detail.
<table>
<thead>
<tr>
<th>Clone</th>
<th>Accession number</th>
<th>Sequence Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlyT2A 1.1</td>
<td>NM_003098</td>
<td>Syntrophin-α1 (PDZ domain)</td>
</tr>
<tr>
<td>GlyT2A 1.2</td>
<td>NM_032622</td>
<td>LNX1 (PDZ domain)</td>
</tr>
<tr>
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<td>COP955</td>
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<td>NM_021120</td>
<td>SAP102 (PDZ domain)</td>
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<tr>
<td>GlyT2A 14.3</td>
<td>U13897</td>
<td>SAP97 (PDZ domain)</td>
</tr>
</tbody>
</table>

Table 4.4. GlyT2-interacting proteins identified in the GlyT2C YTH screen. For each YTH 'hit' the clone number, accession number and encoded proteins are indicated.
4.6. The PDZ binding motif in GlyT2C is necessary and sufficient for interactions with diverse PDZ domain containing proteins

Analysis of the domain composition of the seven new GlyT2 interactors using PFAM (http://pfam.wustl.edu/) revealed a common motif in six out of seven proteins: the PDZ domain (Figure 4.20). PDZ domains are common protein-protein interaction modules composed of two α-helices and six β-sheets. The name derives from the first three proteins in which those domains were found: PSD-95 (postsynaptic density protein-95), DLG (discs large) and ZO-1 (zonula occludens-1). The primary function of PDZ domains is to recognise specific motifs that occur at the C-terminus of target proteins (Harris and Lim, 2001). PDZ domains have been classified into three classes according to the C-terminal motif they recognise: class I PDZ domains interact with the consensus motif S/T-X-V/I/L, class II recognises the motif Φ-X-Φ and class III
interacts with the motif X-X-C, where Φ represents a bulky hydrophobic amino acid and X represents any amino acid (Harris and Lim, 2001). In different studies, PDZ containing proteins have shown to play crucial roles in the regulating the synaptic distribution and functional properties of ion channels and neurotransmitter receptors (Ohno et al., 2004). Since the cytoplasmic C-terminus of GlyT2 carries a class III PDZ binding motif (TQC), I tested whether this motif was specific and sufficient for the interaction with the PDZ proteins and with the only non-PDZ protein identified, FMNL2. A new bait construct lacking the last three amino acids was generated and tested for interaction with the previously identified GlyT2C interacting proteins in the YTH system.


The new GlyT2C bait lacking the C-terminal three amino acids (TQC) was generated by amplifying the DNA sequence encoding the C-terminal region of GlyT2 by PCR from human adult hippocampal cDNA using oligonucleotide primers hGlyT2C<sup>SalI</sup> and hGlyT2<sup>EcoRI</sup>TQC (Section 2.2.4; Table 2.1) and cloning the resulting PCR product into the <sup>SalI</sup> and <sup>EcoRI</sup> sites of pYTH16. The resultant construct was sequenced to confirm in-frame fusion and deletion of the three C-terminal amino acids. The GAL4BD-GlyT2delTQC bait was tested against the seven GlyT2
interacting proteins in a small-scale yeast experiment. Briefly, pACT2 clones containing cDNAs for GlyT2 interactors were co-transformed with the pYTH16-GlyT2CdelTQC bait vector. Co-transformation of pACT2 clones with pYTH16-GlyT2C bait was also performed as positive control. Transformants were plated on minimal agar lacking leucine and tryptophan (SD/-Leu/-Trp). LacZ freeze fracture assays were used to detect the interactions. As shown in Figure 4.21, deletion of the last three amino acids completely abolished interaction between GlyT2 and all the PDZ proteins. By contrast, the GAL4BD-GlyT2delTQC bait still interacted with the only non-PDZ domain containing protein FMNL2.

In order to examine the selectivity of the interactions between the two known glycine transporters GlyT1 and GlyT2 with the newly identified interactors, a bait construct
encoding the carboxy-terminal region of GlyT1 was utilised. The C-terminus of GlyT1 harbours a type I PDZ-binding motif (SRI; Cubelos et al., 2005c). The pGBK7-GlyT1C bait construct, encoding amino acids 563-638, was kindly provided by Prof. Francisco Zafra (Cubelos et al., 2005c; Figure 4.22). The plasmid was prepared as described in Section 2.1.6 and sequenced to verify that the transporter C-terminus was fused in-frame with the GAL4AD of the bait vector pGBK7 (Clontech).

**Figure 4.22 Plasmid map of pGBK7-GlyT1C.** ADH₃: constitutive promoter for alcohol dehydrogenase; GAL4BD: GAL4 binding domain; HA tag: haemagglutinin epitope; ADH₄: alcohol dehydrogenase transcription termination signal; Kan: kanamycin resistance gene; TRP: tryptophan nutritional marker.

pACT2 clones containing cDNAs for GlyT2 interactors were co-transformed with pGBK7-GlyT1C in Y190 in a small scale yeast experiment. Co-transformation of GlyT2 interactors with pYTH16-GlyT2C bait was used as positive control. Negative controls included co-transformation of GlyT2 interactors with the pGBK7 empty bait vector and of pGBK7-GlyT1C with empty pACT2 prey vector. Transformants were plated following the same conditions as described above and interactions were tested by LacZ freeze fracture assay. As shown in Figure 4.23, only pACT2-syntrophin-α1 interacted with the GlyT1 bait, whereas the other interacting proteins did not result in the activation of the LacZ reporter gene in the presence of the GlyT1 bait.
Figure 4.23. Interaction of syntrophin-a1, but not other GlyT2 interacting proteins, with a GlyT1 bait. Novel GlyT2 interactors were tested for interaction against a GlyT1 C-terminal bait. Co-transformations with the empty bait vector pGBKT7 were performed as negative controls.

4.8. Discussion

Identification of seven potential novel GlyT2 interacting proteins

To identify brain proteins that interact with GlyT2, a YTH screen was performed, using the cytoplasmic C-terminal region of GlyT2 as bait. Screening of a human foetal brain cDNA library led to the isolation of cDNA clones for seven new GlyT2 interacting proteins that had not previously been reported. Bioinformatic analysis of these proteins highlighted the presence of one or more PDZ domains in six out of seven interactors, together with other functional domains potentially involved in protein-protein interactions or activity of the interactors. The following section will briefly describe the general features of the newly identified GlyT2 interacting proteins.
4.8.1. Syntrophin-α1

Syntrophins are a family of modular, signal transduction proteins that present a common domain structure. All five family members, α, β1, β2, γ1 and γ2 contain two PH domains and a PDZ domain (Adams et al., 2000). Through these protein-protein interaction motifs, syntrophins link signalling molecules to the dystrophin-associated protein complex (DAPC) in muscular and neuronal cells (Okumura et al., 2008). Syntrophin-α1 is the predominant isoform in skeletal and cardiac muscle, where it is present on the sarcolemma of all fibers and is involved in the formation and stabilisation of the DAPC. Syntrophin-α1 binds directly to members of the dystrophin protein family (dystrophin, utrophin and dystrobrevin) through the second PH domain, whereas the first PH domain binds phosphatidylinositol lipids. *In vitro* experiments have shown that the PDZ domain of syntrophin-α1 can associate with neuronal nitric oxide synthase (nNOS) and this interaction is necessary for proper localisation of nNOS to the sarcolemma, where it regulates blood flow during muscle activity (Adams et al., 2001). Furthermore, the C-terminus of syntrophin-α1 is involved in binding of the muscle voltage-gated sodium channels SkM1 and SkM2 (Gee et al., 1998). All those interactions suggest a role for syntrophin-α1 in the stabilisation of the sarcolemma. However, syntrophin-α1 also associates with α1D-adrenergic receptors via a PDZ domain-mediated interaction, maintaining its stability at neuromuscular synapse (Chen et al., 2006). In the mammalian central nervous system, DAPC proteins localise to perivascular astrocyte endfeet at the blood-brain barrier, where syntrophin-α1 is required for proper expression and localisation of the water channel aquaporin-4, thus playing a key role in the homeostasis (Bragg et al., 2006).

4.8.2. LNX1

LNX1 was first identified as an interactor of Numb, a protein implicated in the control of cell fate decision during neurogenesis by antagonising the Notch signal pathway. Dho et al., (1998) proved this interaction with a YTH screen, performed in an attempt to elucidate a functional pathway for Numb and revealed that this association was mediated by the phosphotyrosine-binding (PTB) domain of Numb (Dho et al., 1998). In addition to the Numb PTB-domain binding motif, LNX1 contains also four PDZ domains and an N-terminal RING finger domain. This domain confers LNX1 an E3 ubiquitin ligase activity, enabling it to target interacting proteins for degradation
through ubiquitination. \textit{In vitro} and \textit{in vivo} experiments showed that LNX1 targets Numb for proteosomal degradation causing an increase of gene transactivation by the Numb-target, nuclear Notch (Nie \textit{et al}., 2002). LNX1 is also involved in the degradation of Np9, a protein exclusively expressed in tumour tissues and Armbruester \textit{et al}., (2004) suggested an interrelation between Np9 and LNX/Numb/Notch pathway (Armbruester \textit{et al}., 2004). LNX1 interacts with ErB2 receptor, thereby regulating neuregulin-1/Erb signalling critical for maintaining perisynaptic Schwann cells at synapses (Young \textit{et al}., 2005). Recent studies showed that LNX1 is down-regulated in human gliomas, suggesting that this protein might be considered as a diagnostic marker and a potential therapeutic target for those tumours (Chen \textit{et al}., 2005).

4.8.3. MAGI1

MAGI1 belongs to the membrane-associated guanylate kinase (MAGUK) family, a group of proteins characterised by a common modular structure that consist of either one or three PDZ domains, a single SH3 domain and a GuK domain. MAGUK proteins localise to regions of cell-cell contact, such as tight junctions in epithelial cells and synaptic junctions in neurones, where they participate to the assembly of multiprotein complexes via their protein-protein interaction modules. MAGI1 presents three features that distinguish it from all other known members of the family: i) the GuK domain is at the N-terminus rather than at the C-terminus; ii) the SH3 domain is replaced by two WW domains; iii) it contains six PDZ domains instead of the usual one or three (Dobrosotskaya \textit{et al}., 1997). Several studies showed that MAGI1 is localised at tight junctions in polarised epithelial cells, where it functions as a scaffold protein at cell-cell contacts. In kidney glomeruli and in intestinal epithelial cells the complex of MAGI-1 and the integral protein JAM4 is involved in the assembly of components at tight junctions, where MAGI1 is targeted through association with β-catenin (Sakurai \textit{et al}., 2006). Association of MAGI1 with the regulators for small GTP-binding proteins mNET1 and RapGEP might accumulate those molecules to the tight junctions (Hirabayashi \textit{et al}., 2003). Interaction with the two actin-bundling proteins synaptopodin and alpha-actinin-4 suggest that MAGI1 may play a role in actin cytoskeleton dynamics within polarised epithelial cells (Patrie \textit{et al}., 2002). In developing neural tubes, MAGI1 is localised at apical processes extending from both proliferating progenitors and neuronally fated precursors, where it recruits the Notch-
receptor ligand Delta-1. It has been suggested that this interaction stabilises Delta-1 to the surface of adherens junctions, where it activates Notch on neighbouring cells (Mizuhara et al., 2005). Furthermore, studies in zebrafish suggested that the Delta-1-MAGI1 interaction might play a role in the control of neuronal migration (Wright et al., 2004).

4.8.4. SAP97

Synapse-associated proteins (SAPs) have recently emerged as a central player in the molecular organisation of synapses. Because of their domain composition those proteins belong to the MAGUK family described above. SAPs are localised at pre- and postsynaptic terminals of excitatory and inhibitory synapses. In the former case, they are involved in clustering of glutamate receptors (GluRs) and anchoring these clusters in the subsynaptic scaffolds by association with cytoskeleton components. Furthermore those proteins bind and modulate several voltage-dependent K⁺ channels in various organs (Fujita and Kurachi, 2000). SAP97 is a 97kDa protein localised in pre- and postsynaptic terminals of excitatory synapses in the hippocampus and along unmyelinated axons in the cerebellum and spinal cord. Furthermore, SAP97 is also present in epithelial cells, where it localises at the lateral membrane between cells (Müller et al., 1995). Structurally this protein is composed of three N-terminal PDZ domains, followed by a SH3 and a GK-like region (Leonard et al., 1998). Functionally, SAP97 acts as a scaffold protein that assembles multiprotein complexes facilitating signal transduction (Sabio et al., 2005). In epithelial and endothelial cells, SAP97 is required for adherens junction assembly and differentiation by associating with the cytoskeleton components E-cadherin, F-actin and α- and β-catenins (Fujita and Kurachi, 2000). At glutamatergic terminals, SAP97 is involved in localising AMPARs at postsynaptic sites by interacting with GluR1 subunit via the first PDZ domain (Leonard et al., 1998). Gardoni et al., (2003) showed that the SAP97 PDZ1 domain also binds the NR2A subunit of NMDARs. This interaction is regulated by CaMKII-dependent phosphorylation and controls the synaptic targeting of the NMDAR (Gardoni et al., 2003). SAP97 levels are significantly decreased in prefrontal cortex of schizophrenic patients and this reduction is paralleled by a decrease in GluR1 protein levels. It has been suggested that loss of SAP97 contributes to the impaired expression of AMPARs, resulting in dysfunction of glutamatergic neurotransmission typical of pathophysiology of schizophrenia (Toyooka et al., 2002).
4.8.5. SAP102

SAP102 is also member of the SAP family and, along with other neuronal MAGUKs, is composed of three PDZ domains at the N-terminus, a SH3 domain and a C-terminal GK domain. SAP102 is localised in the postsynaptic density (PSD) of excitatory synapses in the hippocampus and is the major MAGUK expressed during early brain development (Tarpey et al., 2004). Immunocytochemical studies also detected SAP102 expression in rat retina and in endocrine and glandular tissues, such as pancreas, thyroid and trachea (Koulen et al., 1998; Kuwahara et al., 1999). Several lines of evidence have suggested a role for SAP102 in the clustering and targeting of NMDARs in excitatory PSDs. Lau et al., (1996) found that SAP102 associates with the NR2 subunit of NMDARs and proved that all three SAP102 PDZ domains bind to the cytoplasmic tail of NR2 (Lau et al., 1996). The first two PDZ domains of SAP102 also bind Sec8, a member of the exocyst complex, and this interaction is probably involved in the trafficking of NMDARs to the plasma membrane (Sans et al., 2003). Furthermore, SAP102 can form a heteromeric complex with Ca\(^{2+}\)/calmodulin and the other MAGUK PSD-95/SAP90 and this complex might lead to clustering of NMDARs during synaptic activity (Masuko et al., 1999). SAP102 expression is required for early expression of NMDARs and the dramatic enrichment of SAP102 at young synapses, when other MAGUK family members are not detected, may indicate a role for this protein in synaptogenesis (Fukaya et al., 1999).

4.8.6. PICK-1

PICK-1 was initially identified using the YTH system as a binding partner of PKC \(\alpha\) (Staudinger et al., 1995) and is mainly expressed in the brain. However, modest levels of protein are also found in testis, heart, lung, liver, spleen, kidney and muscle. In most cells PICK-1 has perinuclear localisation but in neurones the protein localises to synapses. PICK-1 contains an N-terminal PDZ domain and a BAR domain spanning half of the protein. This domain structure is rather unique, since PICK-1 is the only known protein to possess both a PDZ domain and a BAR domain (Xu and Xia, 2006, 2007). In contrast to most PDZ domains, the PICK-1 PDZ domain interacts with both type I and type II PDZ-binding motifs, mediating interaction with a broad range of proteins, including tyrosine kinases, AMPA and kainate receptors and metabotropic GluRs, ion channels, G-protein coupled receptors, aquaporins and ADP-ribosylation
factors. PICK-1 has been suggested to cluster such proteins to the plasma membrane or to target them to the perinuclear region (Madsen et al., 2005). It has been suggested that PICK-1 localises activated PKC to the plasma membrane, bringing it in close proximity with its specific targets (Staudinger et al., 1997). Torres et al., (2001) showed that PICK-1 interacts with the C-terminus of the dopamine transporter DAT and this interaction results in an increase in cell surface expression of DAT and enhanced dopamine uptake via a PKC-mediated mechanism (Torres et al., 2001). Furthermore, it has recently been shown that PICK-1 binds the glutamate transporter GLT1b and that this interaction regulates the modulation of GLT1 function by PKC (Bassan et al., 2008). Polymorphisms of PICK-1 were found to associate with schizophrenia, suggesting a role for this protein in the disorder. As some PICK-1 interacting proteins, such as GluRs, DATs and serine racemase, have been associated with the illness, it is possible that one cause of increased susceptibility for schizophrenia is the altered interaction between PICK-1 and associated synaptic proteins (Dev and Henley, 2006).

4.8.7. FMNL2

FMNL is part of a human gene family comprising the formin-like proteins 1, 2 and 3 (FMNL1, FMNL2 and FMNL3). These proteins are characterised by FH domains (Katoh and Katoh, 2003). Whereas the function of the FH2 domain is unknown, the proline-rich FH1 domain has been shown to directly interact with proteins containing WW-domains, SH3-domains and profilin (Miyagi et al., 2002). Profilin is a small actin monomer binding protein that facilitates actin multimerisation and acts as a key molecule mediating signal transduction and cytoskeletal re-arrangements (Schlüter et al., 1997). Because of these binding properties, FH domain proteins are generally though to play crucial roles in cytoskeletal processes, especially cytokinesis and establishment of cell polarity in yeast and limb formation in vertebrates (Frazier and Field, 1997). Furthermore, FMNL proteins contain an N-terminal GBD that can bind Rho family GTPases promoting the formation of microfilaments and microtubule rearrangements. FMNL1, 2 and 3 mRNAs have been detected in several types of tumour and their expression might be implicated in polarity control or tumour invasion through regulation of the Rho family GTPase signalling pathways (Katoh and Katoh, 2003). A mouse homologue of FMNL2, named man, was found to be highly expressed in spinal cord, kidney and developing limbs (Cui et al., 2005).
However, very little is known about the role of FMNL proteins in brain: no mouse knockouts exist for these corresponding genes and no human diseases have been associated with FMNL1, FMNL2 or FMNL3.

4.9. The specific interaction of GlyT1 and GlyT2 with diverse PDZ domain containing proteins is mediated via C-terminal type I and type III motifs

Since GlyT2 carried a C-terminal type III PDZ binding motif (TQC), it seemed plausible that the interactions between GlyT2 and the six PDZ domain proteins identified by YTH screening relied on this region. Generation of a GlyT2 bait lacking this motif revealed that syntrophin-α1, SAP97, SAP102, LNX1, PICK-1 and MAGI1 all interacted with the carboxy terminus of GlyT2 via this motif. This is consistent with the observation that PDZ-domain containing proteins bind to specific consensus sequences found in the last three or four amino acids of their target proteins (Torres et al., 2001). By contrast, FMNL2, that lacked identifiable PDZ domains, still associated with GlyT2 even in absence of the PDZ binding motif, suggesting that this interaction involves another as yet unidentified site in the GlyT2 C-terminus. To examine the selectivity for GlyT2 of the interactors identified by YTH screening, a bait for GlyT1 was also tested against the seven new GlyT2 interacting proteins. GlyT1 also contains a C-terminal PDZ binding motif (SRI), that has been reported to bind both PSD-95 and Sec3 (Cubelos et al., 2005a; Cubelos et al., 2005c). However, GlyT1 contains a class I PDZ binding motif, and would be predicted to have a different binding specificity to the type III motif present in GlyT2. Consistent with this classification, no interactions were detected between GlyT1 and LNX1, MAGI1, SAP97, SAP102, PICK-1 or FMNL2, suggesting that GlyT2, but not GlyT1, associates with those proteins in a reasonably selective manner. Interestingly, the GlyT1 bait did interact with a prey for syntrophin-α1, suggesting a potential novel role for either GlyT1 or GlyT2 in the perivascular astrocyte protein scaffold at the blood-brain barrier (Bragg et al., 2006). However, having identified seven new GlyT2 interacting proteins, further functional analysis in mammalian systems was required to narrow down suitable candidates for genetic screening in hyperekplexia.
5. PRIORITISING NEW GLYT2 INTERACTING PROTEINS FOR GENETIC ANALYSIS IN HYPEREKPLEXIA
5.1. Biological relevance of the novel GlyT2 interacting proteins

The presynaptic localisation of GlyT2 implies that accessory proteins must be involved in the targeting and anchoring of the transporter to the correct neuronal compartment (Armsen et al., 2007). For this reason, genes encoding GlyT2 interacting proteins are strong candidates for genetic screening in the remaining individuals with hyperekplexia who do not have a mutation in any of the known causative genes (Rees et al., 2006; Harvey et al., 2008a). In the previous chapter, I identified seven new potential GlyT2 binding partners by YTH screening of a foetal brain cDNA library with the GlyT2 C-terminus as bait. Bioinformatic analysis revealed that the majority of newly identified GlyT2 interactors contained at least one PDZ domain. During the last decade, a large number of studies have established the importance of PDZ domain-mediated interactions in the correct localisation and clustering of several classes of ion channels, neurotransmitter receptors and transporters and effector proteins (Torres et al., 2001). However, YTH data alone is not sufficient to support the suggestion that these interactions occur in vivo. For this reason co-expression experiments in mammalian expression systems might shed light on mechanisms involved in the regulation of transporter activity.

5.2. Study aims

The aim of this work was to prioritise candidate genes for genetic analysis in remaining hyperekplexia cases based on the following criteria:

- A bioinformatic assessment using the Mouse Genome Informatics (MGI) website at the Jackson laboratory and Online Mendelian Inheritance in Man (OMIM) database to search for gene locations, existing mouse knockout models or human diseases that could exclude candidate genes from further analysis.

- Investigating the gene structure of FMNL2, cloning a full-length cDNA and assessing possible co-localisation of GlyT2 and FMNL2 in mammalian cells using immunofluorescence and confocal microscopy.

5.3. Bioinformatic study of novel GlyT2 interacting proteins: gene locations, knockout mouse models and associated human diseases

A bioinformatic analysis of the genes for GlyT2 interactors was undertaken in an attempt to narrow down the possible number of candidates for future genetic screening in hyperekplexia. Firstly, the MGI website at the Jackson laboratory (http://www.informatics.jax.org/genes) was used to search for mouse gene symbols, chromosomal locations and associated mouse knockouts (Table 5.1). In particular, I was interested to find out if any knockout models existed with phenotypes similar to GlyT2 knockout mice (Gomeza et al., 2003b) or whether candidate genes could be eliminated on the basis of other overt phenotypes. Syntrophin-α1 knockout mice generated by two different groups (Kameya et al., 1999; Adams et al., 2000) displayed no gross histological changes. However nNOS, a component of the dystrophin protein complex, was absent from the sarcolemma of these mice. Furthermore, the neuromuscular junction of syntrophin-α1 knockout mice presented undetectable levels of postsynaptic utrophin and reduced levels of nicotinic acetylcholine receptor (AChR) and acetylcholinesterase (AChE). For this reason Adams et al., (2000) suggested that mutations in the syntrophin-α1 gene Sntal could be the primary defect in cases of congenital myasthenia gravis where mutations in AChR genes have been excluded. Knockout mice for SAP97 exhibited a severe phenotype characterised by craniofacial defects, abnormalities in reproductive, renal and urinary systems, impaired smooth muscle contractility and decreased body length, and died shortly after birth of respiratory distress. These results provide novel insights into the function of SAP97 and suggest a crucial role for this protein not only in the CNS but also in the development of urogenital organs (Mahoney et al., 2006; Iizuka-Kogo et al., 2007). Knockout mice for SAP102 were viable but exhibited reduced synaptic plasticity and impairments in learning spatial tasks, associated with slightly altered locomotor phenotype. For this reason Cuthbert et al., (2007) suggested that SAP102 is involved in coupling NMDAR with the ERK pathway and hypothesised that NMDA-induced activation of this pathway was suppressed in SAP102 mutants (Cuthbert et al., 2007). PICK-1 knockout mice were also viable and showed no gross developmental defects, but presented a reduced Ca^{2+}-permeable AMPAR plasticity, with a redistribution of GluR2 from cell bodies and dendrites to extrasynaptic regions in spines probably caused by aberrant receptor trafficking (Gardner et al., 2005).
Furthermore cerebellar long-term depression (LTD) was absent in PICK-1-deficient mice (Steinberg et al., 2006). A new knockout model where the mGluR7a-PICK-1 interaction was destroyed was recently characterised; in this study PICK-1 knockout mice and rats presented symptoms of absence epilepsy, a neurological disorder characterised by loss of consciousness and electroencephalogram discharges (Bertaso et al., 2008). To date, no knockout mice have been characterised for LNX1, MAGI1 and FMNL2.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Location</th>
<th>Protein</th>
<th>MGI ID</th>
<th>Associated mouse knockout</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sntal</td>
<td>Chr 2</td>
<td>syntrophin-a1</td>
<td>101772</td>
<td>Structurally aberrant neuromuscular synapses deficient in AChRs, AChE and utrophin. Absence of nNOS1 at the sarcolemma (Kameya et al., 1999; Adams et al., 2000)</td>
</tr>
<tr>
<td>Lnx1</td>
<td>Chr 5</td>
<td>LNX1</td>
<td>1278335</td>
<td>Unknown</td>
</tr>
<tr>
<td>Dlg1</td>
<td>Chr 16</td>
<td>SAP97</td>
<td>107231</td>
<td>Peri/postnatal lethality due to respiratory distress, abnormal development of urogenital organs and reproductive system, decreased body length, cleft palate, impaired smooth muscle contractility (Mahoney et al., 2006; Iizuka-Kogo et al., 2007)</td>
</tr>
<tr>
<td>Dlg3</td>
<td>Chr X</td>
<td>SAP102</td>
<td>1888986</td>
<td>Abnormal spatial learning, cognitive and locomotion deficits (Cuthbert et al., 2007)</td>
</tr>
<tr>
<td>Magi</td>
<td>Chr 6</td>
<td>MAGI1</td>
<td>1203522</td>
<td>Unknown</td>
</tr>
<tr>
<td>Pick1</td>
<td>Chr 15</td>
<td>PICK-1</td>
<td>894645</td>
<td>Disruption of Ca(^{2+})-permeable AMPAR plasticity and cerebellar LTD (Gardner et al., 2005; Steinberg et al., 2006); absence epilepsy characterised by loss of consciousness and seizures (Bertaso et al., 2008)</td>
</tr>
<tr>
<td>Others</td>
<td>Chr 2</td>
<td>FMNL2</td>
<td>1918659</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

Table 5.1. Novel GlyT2 interactors: mouse models of dysfunction. For all GlyT2 interactors, gene symbols, chromosomal locations (http://genome.ucsc.edu/) and identifiers and associated mouse knockouts or mutants (http://www.informatics.jax.org/) are shown.

A similar search was carried out in the OMIM database, in order to identify those GlyT2 interactors already associated with human diseases and to exclude them from further genetic analysis in hyperekplexia (Table 5.2). However, the genes for most interactors do not currently appear to be linked to human disorders. The only two exceptions were Dlg3 and Dlg1, encoding SAP102 and SAP97, respectively. Loss of function mutations in the human Dlg3 gene were found in families with moderate to severe nonsyndromic X-linked mental retardation (Tarpey et al., 2004). The disease
mutations identified in this study all truncated SAP102 within or before the third PDZ domain, probably impairing SAP102 interactions with NMDAR and/or other proteins involved in the NMDAR signalling pathway. Willatt et al., (2005) found that Dlg1 gene is deleted in the 3q29 microdeletion syndrome, a disease characterised by mild to moderate mental retardation associated with slightly dysmorphic facial features. In this study the researchers suggested that the loss of SAP97 might contribute to the disease phenotype since Dlg1 is the autosomal homolog of the X-linked mental retardation gene Dlg3.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Location</th>
<th>Protein</th>
<th>OMIM</th>
<th>Associated human disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDZ domain proteins</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SNTA1</td>
<td>Chr 20</td>
<td>syntrophin-α1</td>
<td>601017</td>
<td>unknown</td>
</tr>
<tr>
<td>LNX1</td>
<td>Chr 4</td>
<td>LNX1</td>
<td>609732</td>
<td>unknown</td>
</tr>
<tr>
<td>DLG1</td>
<td>Chr 3</td>
<td>SAP97</td>
<td>601014</td>
<td>3q29 microdeletion syndrome (Willatt et al., 2005)</td>
</tr>
<tr>
<td>DLG3</td>
<td>Chr X</td>
<td>SAP102</td>
<td>300189</td>
<td>X-linked mental retardation (Tarpey et al., 2004)</td>
</tr>
<tr>
<td>MAGI1</td>
<td>Chr 21</td>
<td>MAGI1</td>
<td>610638</td>
<td>unknown</td>
</tr>
<tr>
<td>PICK1</td>
<td>Chr 22</td>
<td>PICK-1</td>
<td>605926</td>
<td>unknown</td>
</tr>
<tr>
<td>Others</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FMNL2</td>
<td>Chr 2</td>
<td>FMNL2</td>
<td>unknown</td>
<td>unknown</td>
</tr>
</tbody>
</table>

Table 5.2 Novel GlyT2 interactors: human diseases. For all GlyT2 interactors genes symbols, chromosomal location and identifiers and human diseases (http://www.ncbi.nlm.nih.gov/omim) are shown.

In conclusion, knockout mice for GlyT2 interacting proteins syntrophin-α1, SAP97, SAP102 and PICK-1 present distinctive phenotypes, none of which resembles GlyT2 knockout mice. In humans, only mutations in the genes for SAP97 and SAP102 have been previously associated with mental retardation. Thus, my bioinformatics analysis allowed me to exclude these genes from further genetic analysis in hyperekplexia. However, the lack of mouse and/or human genetic studies for LNX1, MAGII and FMNL2 suggests that any of these genes could potentially be involved in hyperekplexia. Due to limitations of time, I decided to focus on FMNL2 because of the high levels of this protein in the spinal cord and because it was the only non-PDZ protein identified in the screening.
5.4. FMNL2 gene structure, alternative splicing and cDNA cloning

Since the cDNA clones obtained for FMNL2 in YTH screening were partial cDNAs, I decided to isolate a full-length cDNA for further functional studies. Searching the University of California Santa Cruz (UCSC) genome browser (http://genome.ucsc.edu/) revealed that the FMNL2 gene is located on the long arm of human chromosome 2 (2q23.3). Further analysis initially suggested the presence of four alternatively spliced isoforms of FMNL2, named A, B, C and D, presenting alternative C-termini (Figure 5.1).

These were listed under NCBI accession numbers: NM_001004421 (FMNL2A), NM_052905 (FMNL2B); NM_001004422 (FMNL2C); NM_001004417 (FMNL2D). However, all accession numbers for variants apart from FMNL2B have recently been withdrawn and FMNL2A, C and D are no longer thought to represent true splice variants. The Expert Protein Analysis System (ExPASy) website (www.expasy.org) was used to confirm potential motif sequences within FMNL2B. This confirmed the presence of three functional domains in FMNL2B, GBD (amino acids 23-276), FH3, and FH2 domains are highlighted in red, green and yellow respectively. The part of FMNL2 encoded by the YTH preys residues is underlined. The four potential C-termini, corresponding to isoforms A, B, C and D, are highlighted in grey.
(amino acids 278-484) and FH2 (amino acids 617-982). The GlyT2 interacting residues corresponded to amino acids 107-516 and spanned part of the GBD domain and the whole FH3 domain (Figure 5.1).

5.4.1. Generation of a FLAG-tagged FMNL2 construct

A full-length FMNL2B cDNA was amplified from the KIAA1902 cDNA clone purchased from Kazusa DNA Research Institute (Japan) using the oligonucleotide primers FMNL2-Xmal and FMNL2-Xbal, as described in Section 2.2.3. The resultant PCR product was cloned into the Xmal and Xbal sites of the mammalian expression vector pRK5FLAG to generate the pRK5FLAG-FMNL2 construct that encodes N-terminally FLAG-tagged FMNL2 (Figure 5.2). Sequence analysis confirmed that the protein was in-frame with the FLAG epitope tag (DYKDDDDK).

![Figure 5.2. Plasmid map of pRK5FLAG-FMNL2. CMVp: cytomegalovirus promoter; SP6: SP6 promoter; SV40p: SV40 promoter; SV40 poly: SV40 polyadenylation signal; Amp: ampicillin resistance gene; FLAG: FLAG tag.](image)

5.5. Investigating possible co-localisation of GlyT2 and FMNL2 in mammalian cells using immunofluorescence and confocal microscopy

To investigate whether GlyT2 and FMNL2 co-localised in a mammalian cell system, immunocytochemistry experiments were carried out using HEK293 cells co-transfected with pRK5myc-GlyT2 and pRK5FLAG-FMNL2, either in combination or alone, as described in Section 2.4.7. Transfections with pRK5myc and pRK5FLAG empty vectors were carried out as controls. After an incubation of 24 hours, transfected cells were washed twice in PBS, fixed for 2 minutes in 4% (w/v) PFA in
PBS and quenched in 50 mM NH$_4$Cl for 10 minutes before permeabilisation in 0.01% (w/v) Triton X-100 for 12 minutes, further washing in PBS and staining with polyclonal anti-myc and monoclonal anti-FLAG primary antibodies, Alexa-Fluor 488 goat anti-rabbit and Alexa-Fluor 546 goat anti-mouse secondary antibodies (Invitrogen) as described in Section 2.4.8. Coverslip-mounted microscope slides were examined using a Zeiss LSM 510 Meta Confocal microscope as described in Section 2.4.9. GlyT2 and FMNL2 presented different distribution patterns when expressed separately in HEK293 cells (Figure 5.3). When expressed alone (with the empty myc vector) FMNL2 was located intracellularly with diffuse staining throughout the cytoplasm (Figure 5.3, panels A-C). GlyT2 alone (co-transfected with the empty FLAG vector) was localised at the cell surface (Figure 5.3, panels D-F) with some intracellular staining as described before (Section 3.6.3).

**Figure 5.3. Subcellular localisation of FMNL2 and GlyT2 expressed separately in HEK293 cells.** A-C: HEK293 cells co-transfected with pRK5FLAG-FMNL2 (red labelling) and pRK5myc control vector (green labelling). FMNL2 is diffusely distributed throughout the cytoplasm (panels B and C). D-F: HEK293 cells co-expressing pRK5myc-GlyT2 (green labelling) and pRK5FLAG control vector (red labelling). GlyT2 is predominantly expressed at the cell surface with some intracellular staining (panels B and C). Scale bar: 10 μm.

When FMNL2 was co-expressed with GlyT2, FMNL2 was recruited to the cell surface where it partially co-localised with the transporter, as shown in Figure 5.4. This subcellular relocation of FMNL2 by GlyT2 is consistent with the interaction observed in the YTH system.
Figure 5.4. Subcellular localisation of FMNL2 and GlyT2 co-expressed in HEK293 cells. When co-expressed with myc-GlyT2 (green labelling), FLAG-FMNL2 (red labelling) re-distributes to the plasma membrane (panels B, C), where it partially co-localises with myc-GlyT2 (panel C). Scale bar: 10 μm.

5.6. Functional consequences of co-expression of GlyT1 or GlyT2 with PICK-1 and FMNL2 using [3H]-glycine uptake assays

Potential effects of co-expression of GlyT2 with PICK-1 and FMNL2 were investigated by performing [3H]-glycine uptake assays on HEK293 cells transfected with pRC/CMV-GlyT2 and appropriate expression constructs for PICK-1 and FMNL2 proteins (Section 2.4.7). I decided to focus on these interactors because: i) PICK-1 is known to interact with other neurotransmitter transporters (e.g. DAT, GLT1b, Torres et al., 2001; Bassan et al., 2008) and therefore might also affect the targeting and/or regulated trafficking of GlyTs (Torres et al., 2001; Bassan et al., 2008); ii) FMNL2 is a large cytoskeletal protein highly expressed in spinal cord and was the only non-PDZ domain containing GlyT2 interactor identified. YTH experiments had suggested that PICK-1 and FMNL2 did not directly interact with GlyT1. However, since PICK-1 and GlyT1 have been linked to schizophrenia (Dev and Henley, 2006), the potential effect of the co-expression of the GlyT1 with PICK-1 and FMNL2 was studied in parallel with GlyT2-uptake experiments.

A mammalian expression construct for PICK-1, pEGFP-PICK-1 (Figure 5.5) and a mutant form in which the lysine 27 residue of the C-terminal PDZ domain is mutated to a glutamate (pEGFP-PICK-1 KE; Dev et al., 2004) were kindly provided by Prof. Francisco Zafra (Universidad Autonoma de Madrid). The K27E mutation abolishes binding of type II PDZ binding motifs to PICK-1 (Dev et al., 2004).
HEK293 cells were co-transfected with pRc/CMV-GlyT1/GlyT2 and pEGFP-PICK-1 (wild-type and KE mutant), in various combinations (Section 2.4.7). Transfections with pRc/CMV-GlyT1/GlyT2 and empty vectors were carried out as controls. Uptake experiments were performed as described in Section 2.4.10. As shown in Figure 5.6, co-expression of GlyT1 and PICK-1 caused an enhancement of glycine uptake in HEK293 cells. Interestingly, this effect was abolished when the KE mutant form, designated as PICK-1(M), was co-expressed with GlyT1, suggesting an involvement of PICK-1 PDZ domain in the interaction with GlyT1. PICK-1 did not appear to affect GlyT2-mediated glycine uptake. Further experiments were carried out to determine kinetic parameters of glycine transport in cells co-expressing PICK-1 and GlyT1. pEGFP-PICK-1, pEGFP-PICK-1(M) and pRc/CMV-GlyT1 were transiently expressed in HEK293 cells and incubations were carried out using varying concentration of unlabelled glycine (3, 10, 30, 100, 300, 1000 μM). Overall, the functional characteristics of GlyT1 and GlyT2 were comparable to those previously described in the literature (e.g. Geerlings et al., 2002). The affinity of GlyT1 for glycine appeared to be slightly lower than for GlyT2 (K_m values of 55 μM and 35 μM for GlyT1 and GlyT2 respectively). A large difference in V_max was measured between the two transporters, consistent with the higher glycine uptake routinely obtained for GlyT1 compared to GlyT2 (V_max values of 8.7 and 1.4 nmol/min/mg protein for GlyT1 and GlyT2 respectively). Examining the effect of PICK-1, the dose-response curve shows an enhancement in the total uptake activity (V_max) in cells co-transfected
with GlyT1 and PICK-1 compared with cells transfected with GlyT1 alone. No significant changes in glycine transport were observed when GlyT1 was co-expressed with PICK-1(M) (Figure 5.7). The Eadie-Hofstee plot of the data revealed that in presence of PICK-1, GlyT1-mediated uptake was enhanced by almost two-fold ($V_{\text{max}}=15.6 \text{ nmol/min/mg protein}$ for cells expressing both proteins versus $V_{\text{max}}=8.7 \text{ nmol/min/mg protein}$ for cell expressing GlyT1 alone). A decrease in the affinity of glycine for GlyT1 was observed in presence of PICK-1 ($K_m= 104 \mu\text{M}$ for cells expressing both proteins versus $K_m= 55 \mu\text{M}$ for cells expressing GlyT1 alone). These results suggest an increase in the number of transporters at the cell membrane, resulting from an enhancement of cell surface expression of GlyT1 in presence of PICK-1 or a change in the turnover rate of the transporter.

![Figure 5.6. Functional consequences of PICK-1 on GlyT1 and GlyT2-mediated transport activity.](image)

Figure 5.6. Functional consequences of PICK-1 on GlyT1 and GlyT2-mediated transport activity. $[^{3}H]$-glycine uptake was measured in HEK293 cells transfected with expression vectors for GlyT1+PICK-1, or GlyT1+PICK-1(M), or GlyT2+PICK-1 or GlyT2-PICK-1(M) or GlyT1, GlyT2, PICK-1, PICK-1(M) alone after incubation with $[^{3}H]$-glycine. Cells transfected with empty vector pRc/CMV were used as control. Glycine uptake was expressed as percentage of that in control cells transfected with the empty pRc/CMV vector. Data are means ± s.e.m. ($n = 8$). * indicates significantly different from control, † indicates significantly different from GlyT1, $P<0.01$. 

...
Figure 5.7. Effect of PICK-1 wild-type and mutant on the kinetics of GlyT1-mediated glycine transport. A: saturation analysis of glycine uptake in HEK293 cells transfected with GlyT1, GlyT1+PICK-1 wild-type or GlyT1+PICK-1(M) after incubation with a range of substrate concentrations. B: Eadie-Hofstee transformation of the data represented in A. The velocity of the reaction $V$ is plotted on the vertical axis as a function of the velocity vs substrate concentration ratio [$S$] on the horizontal axis.
This functional analysis was in clear contrast with my previous YTH studies, where PICK-1 interacted with GlyT2 but no interaction was observed between PICK-1 and GlyT1 (Section 4.7). Post-translational modifications of proteins (phosphorylation, glycosylation, ubiquitination, etc) might occur in yeast affecting association with binding partners. In particular, yeast-specific kinases with no homologues in humans might phosphorylate YTH baits, so impairing potential association with other proteins. I used a yeast-specific phosphorylation site predictor, NetPhosYeast, to assess whether this was the case for the GlyT1 bait. The GlyT1 and GlyT2 C-termini were used to interrogate both the NetPhosYeast database (http://www.cbs.dtu.dk/services/NetPhosYeast/) and the NetPhos database (http://www.cbs.dtu.dk/services/NetPhos/), which predicts phosphorylation sites in mammalian proteins (Figure 5.8; Ingrell et al., 2007; Blom et al., 1999). Interestingly, the GlyT1 bait was predicted to be phosphorylated in yeast. In particular, phosphorylation of serine 631 in the PDZ binding motif (SRI) might impair GlyT1 and PICK-1 interactions in YTH experiments. Notably, S631 is not predicted to be phosphorylated in mammalian cells, suggesting that the GlyT1-PICK-1 association is not affected by phosphorylation in mammalian cells. By contrast, the GlyT2 PDZ-binding motif was not predicted to be phosphorylated in yeast (Figure 5.8), which might explain why this bait interacts with multiple PDZ proteins in yeast.

The effect of co-expressing FMNL2 on GlyT1 and GlyT2 uptake activity was also tested as described above (Section 2.4.10). As shown in Figure 5.9, co-expression of FMNL2 with GlyT1 and GlyT2 in HEK293 cells did not result in a significant change in the transporters mediated uptake. This may reflect that in HEK293 cells, the primary effect of the FMNL2-GlyT2 interaction is a re-localisation of FMNL2, rather than affecting the efficiency of transporter function.
GlyT1

Yeast kinases (NetPhosYeast prediction results)

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Mammalian kinases (NetPhos Phosphorylation prediction results)

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GlyT2

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Mammalian kinases (NetPhos Phosphorylation prediction results)

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IERLKLVCSPQPDPWGPFLAQHRGERYKNMIDPLGTS SLGLKLPVKDLELGTQC

Figure 5.8. Prediction of phosphorylation sites in GlyT1 and GlyT2 baits. Prediction results are provided for each bait in both yeast and mammalian systems. Table columns correspond to sequence name, residue position, phosphorylated residue (S: serine, T: threonine, Y: tyrosine), score (if above 0.5 the residue is predicted to be phosphorylated), kinase (not specified in the current version of the program) and answer or prediction reflecting the likelihood of phosphorylation (Yes, or 'Y'). Potential residues subject to phosphorylation in yeast are highlighted in yellow.
**Figure 5.9.** Functional consequences of FMNL2 on GlyT1 and GlyT2 mediated transport activity. \(^{[3]}\text{H}\)-glycine uptake assays were performed in HEK293 cells transfected with expression vectors for GlyT1+FMNL2, or GlyT2+FMNL2 or GlyT1, GlyT2 and FMNL2 alone after incubation with \(^{[3]}\text{H}\)-glycine. pRc/CMV empty vector represented negative control. Glycine uptake was expressed as percentage of that in control cells transfected with the empty pRc/CMV vector. Data are means ± s.e.m. (n = 8). * indicates significantly different from control, \(P<0.01\).

5.7. Discussion

In this chapter, I performed a bioinformatic analysis of the genes for GlyT2 interacting proteins and further functional tests on selected interactors in an attempt to narrow down the possible number of candidate genes for future genetic screening in hyperekplexia. This analysis revealed that certain PDZ domain-containing proteins are either associated with mouse knockout models that differ drastically in phenotype from the mouse GlyT2 knockout mouse (Gomeza et al., 2003b) or are already associated with other human diseases. Knockout mice for syntrophin-α1, SAP97, SAP102 and PICK-1 presented a phenotype considerably different from hyperekplexia, suggesting that mutations in these genes are unlikely to cause startle disease (Table 5.1). However, no knockout models were found for LNX1, MAGI1 and FMNL2. In humans, mutations in the SAP97 and SAP102 genes were associated with mental retardation syndromes, thus these genes can safely be excluded from further genetic analysis in hyperekplexia patients (Table 5.2).
5.7.1. Functional effect of PICK-1 on GlyT-mediated uptake

Kinetic parameters measured for GlyT1 and GlyT2 mediated glycine transport were consistent with previous studies (e.g. Geerlings et al., 2002); in particular, a considerably higher $V_{\text{max}}$ value was observed for GlyT1, suggesting a higher level of expression at the plasma membrane for GlyT1 than GlyT2. The potential effect of PICK-1 on GlyT1 and GlyT2 activity was evaluated by performing glycine uptake experiments (Section 5.6). Interestingly, co-expression of PICK-1 significantly enhanced GlyT1-mediated uptake; this stimulatory effect could be attributed to an interaction of the transporter with the PDZ domain of PICK-1, since no effect was observed when cells were transfected with mutant form carrying a KE substitution in the PDZ motif. PICK-1 is already known to regulate cell surface expression and trafficking of DAT and GLT1b (Torres et al., 2001; Bassan et al., 2008) and both PICK-1 and GlyT1 are expressed at NMDAR mediated glutamatergic synapses, supporting a role for this interaction in the brain. Recent genetic studies revealed an association between two SNPs in PICK1 and schizophrenia (Dev and Henley, 2006). Interestingly, both the SNPs reported in schizophrenia cases occurred in the PDZ domain of PICK-1, suggesting that altered interactions between PICK-1 and selected binding partners may underlie this psychiatric disorder.

Interestingly, dysfunction of glutamatergic transmission mediated by NMDARs has been implicated in pathophysiology of schizophrenia (Tsai et al., 2004a). In turn, several studies have suggested a crucial role for GlyT1 in the regulation of glycine concentration in the microenvironment of NMDARs (Supplisson and Bergman, 1997; López-Corcua et al., 2001). Assuming that PICK-1 is involved in regulation of GlyT1 function, variations in the PDZ domain of PICK-1 in schizophrenia patients might lead to altered surface expression and/or trafficking of GlyT1 at excitatory synapses, affecting glutamate-mediated neurotransmission. Further studies will be required to investigate whether the regulation of GlyT1 surface expression via PICK-1 takes place through a PKC-dependent mechanism, as seen for other transporters (Torres et al., 2001; Bassan et al., 2008). Although no interaction between PICK-1 and GlyT1 was observed in yeast (Section 4.7), this might be due to cryptic post translational modification of the GlyT1 bait in yeast, which obscures functionally-relevant interactions. Analysis of potential phosphorylation sites revealed that the
GlyT1 bait used in YTH experiments is likely to be phosphorylated by yeast-specific kinases. However, this modification is not predicted to occur in mammalian cells, consistent with the functional effects observed in this system. Unfortunately, the type I PDZ binding motif (SRI) does not lend itself to mutagenesis studies to test this hypothesis, as substituting serine 631 by alanine would remove the phosphorylation site, but would also destroy the PDZ binding motif.

Despite the strong interaction observed in YTH studies, PICK-1 did not show any significant effect on GlyT2-mediated[^H]-glycine uptake. This lack of effect might suggest that the interaction observed in yeast is not functionally relevant in a mammalian expression system. For this reason, immunohistochemical studies on brain stem sections will be carried out in the future to characterise this interaction in more detail. In conclusion, YTH experiments can be considered as the first step in the attempt of identification of proteins regulating transporter function but do not always support functional data obtained in a mammalian cell system.

5.7.2. **FMNL2: a new candidate gene for genetic screening in hyperekplexia?**

To investigate the possible association of GlyT2 with the only non-PDZ domain containing protein, FMNL2, I cloned a full-length cDNA for FMNL2 into the vector pRK5FLAG. This expression construct was used to co-express FLAG-tagged FMNL2 with myc-tagged GlyT2 in mammalian cells. Confocal microscopy revealed that co-expression of the two proteins resulted in re-distribution of FLAG-FMNL2 and partial co-localisation of myc-GlyT2 and FLAG-FMNL2 at the cell membrane, consistent with the interaction detected in the YTH system. However, uptake experiments in HEK293 cells revealed that FMNL2 co-expression with GlyT2 had no obvious effect on GlyT2-mediated[^H]-glycine uptake. Further work is needed to confirm this interaction in more detail. For example, co-immunoprecipitation studies from solubilised spinal cord extracts or synaptic co-localisation in spinal cord sections might confirm this interaction but would rely on the generation of specific antibodies for FMNL2, which are currently a limiting factor. Despite the results in the[^H]-glycine uptake assays, in my view the in vivo spinal cord enrichment and the biological function of FMNL2 are likely to be consistent with a potential role for this protein in hyperekplexia. Schütz et al., (1997) proposed a role for formin-related
proteins in connecting the microfilament system with the GTPase-related signalling cascade. Small GTPases of the Rho family are actively involved in the regulation of microfilament-based processes, and formin-related proteins are likely to be downstream effectors of Rho in this cascade. FMNL2 is known to bind both Rho, via its GBD, and the actin-binding protein profilin, via FH2. Upon binding of Rho, FMNL2 might connect GlyT2 via its FH2 domain both to actin cytoskeleton and to various signalling molecules containing SH3 domains and WW domains. At present, FMNL2 is being screened in individuals with hyperekplexia devoid of mutations in the GLRA1, GLRB and SLC6A5 by collaborators in Swansea in order to reach a definitive conclusion on this matter. These may justify further functional studies on the FMNL2-GlyT2 interaction.
6. GLYT2 DYSFUNCTION IN CONGENITAL MUSCULAR DYSTONIA TYPE 2
6.1. Congenital Muscular Dystonia type 2 (CMD2)

Livestock productivity has seen a considerable improvement in the last 50 years, due to reproductive methods rooted in quantitative genetics, such as artificial insemination (AI) and multiple ovulation and embryo transfer (MOET). However, the widespread utilisation of elite sires with desirable traits has lead to the emergence of recessive defects in livestock, causing drastic reductions in the population size and raising significant economic and animal welfare issues (Charlier et al., 2008). For instance, in the 1990s about 14% of Holstein-Friesian bulls were shown to carry a missense mutation in ITGB2 (leukocyte β2 integrin subunit CD18) causing bovine leukocyte adhesion deficiency (BLAD), a lethal immunodeficiency disorder affecting 0.2% of newborn calves with an estimated annual cost of $5 million in the USA (Shuster et al., 1992). Furthermore, in recent years about 25% of Holstein-Friesian bulls proved to be carriers of a missense mutation in the Golgi UDP-N-acetylglucosamine transporter SLC35A3, causing complex vertebral malformation (CVM), a disease characterised by misshaped vertebrae and scoliosis (Thomsen et al., 2006). A common ancestral sire, widely used for AI, was identified as responsible for the occurrence of both genetic defects in the population, underlying the importance of genotyping animals for disease genes in order to 'avoid risk' matings.

Congenital muscular dystonia (CMD) is a putative recessive inherited disorder with high prevalence in Belgian blue cattle (0.1-0.2%). All animals with CMD have episodes of generalised muscle contractures, but clinical investigations suggested two different forms, CMD1 and CMD2, based on phenotypic differences (Charlier et al., 2008). Calves affected by CMD1 show impaired swallowing, fatigue upon stimulation or exercise and muscle myotonia resulting in an inability to flex limbs and injurious falling. CMD1 calves usually die within a few weeks after birth as a result of respiratory complications. By contrast, calves with CMD2 experience episodes of extreme muscle stiffness and tremor upon acoustic or tactile stimulation and die within a few hours of birth from respiratory difficulties (Figure 6.1; Supplementary videos 1 and 2). CMD2 is reminiscent of inherited congenital myoclonus (ICM) in Poll Hereford cattle (Gundlach et al., 1988; Pierce et al., 2001) which is caused by a nonsense mutation in the bovine GlyR α1 subunit gene (resulting in a Y24X substitution). In Poll Hereford cattle, ICM is also characterised by spontaneous and
startle-evoked muscle stiffness, but calves can survive for at least 10 days after birth and death results from an inability to stand and feed. In order to identify the causative gene for CMD2 and other emerging defects in cattle breeds, whole-genome screening techniques were used on DNA samples from affected animals and controls using either the 23K Affymetrix SNP panel or a custom made 58K Illumina panel (Charlier et al., 2008). In this way, a candidate chromosomal region for each genetic defect was identified. The CMD2 locus was located to a 3.61 Mb segment on bovine chromosome 29, which encompassed 13 genes including one strong candidate: SLC6A5, encoding the glycine transporter GlyT2.

Figure 6.1. CMD2 in Belgian blue cattle. A sequence of images showing a newborn Belgian blue calf affected by CMD2. Limb stiffness and muscle rigidity affect the ability of the animal to stand and feed. Images kindly provided by Prof. Frédéric Rollin, Clinical Science Department, Faculty of Veterinary Medicine, University of Liège, Belgium.
6.2. Study aims

My contribution to this collaborative project, performed in collaboration with Carole Charlier, Frédéric Rollin and Michel Georges (University of Liège, Belgium) was to aid the identification and characterisation of any mutation in *SLC6A5* that could be potentially responsible for CMD2. In particular my aims were to:

- Predict the structure of the bovine GlyT2 gene (*SLC6A5*) and the corresponding cDNA and protein sequences, to facilitate the identification of any potential pathogenic mutation in this candidate gene.
- Confirm the presence of any pathogenic mutation in *SLC6A5* in genomic DNA samples from homozygous CMD2 and heterozygous carrier animals.
- Introduce the CMD2 mutation into a GlyT2 expression construct and investigate the effects on subcellular localisation and \(^3\)H-glycine uptake.

6.3. Prediction of the bovine GlyT2 gene, cDNA and protein sequences and identification of a *SLC6A5* mutation in CMD2

In October 2007, a draft assembly of the bovine genome (Btau_4.0, 7-fold coverage) was released by the Baylor College of Medicine Human Genome Sequencing Center (http://www.hgsc.bcm.tmc.edu/projects/bovine/). This sequence is accessible via the UCSC genome browser website (http://genome.ucsc.edu/). Searching the bovine genome with BLAT using either the human GlyT2 cDNA or protein sequence revealed the structure of the bovine GlyT2 gene (Appendix 1), which consisted of 16 exons on chromosome 29. This allowed prediction of the bovine GlyT2 cDNA and protein sequences (Appendix 2; Figure 6.2). Surprisingly, the bovine and human GlyT2 protein sequences are very highly conserved (95.6% identity) and most of the observed amino acid differences (31 out of 35) are found in the long intracellular N-terminus of GlyT2. Using this predicted gene structure, *SLC6A5* was fully sequenced in CMD2 cases and controls by Carole Charlier at the University of Liège, Belgium. This analysis revealed a missense mutation (T809C) in exon 4 of *SLC6A5*, leading to a L270P substitution in the third membrane-spanning domain of the bovine GlyT2 protein. Further genotyping revealed that 2.3% of Belgian blue cattle and 4.6% of AI sires were carriers of the defective C809 allele, although always in the heterozygous
state. However, all confirmed CMD2 cases were homozygous for this mutation, confirming the recessive nature of this disorder.

Figure 6.2. Alignment of the human and bovine GlyT2 proteins. Human and bovine GlyT2 proteins were aligned using ClustalW (http://www.ebi.ac.uk/). Differences between the two proteins are indicated by grey highlighting, and putative transmembrane domains (TM1-TM12) are highlighted using coloured boxes. The position of the bovine L270P substitution in TM3 (equivalent substitution in human GlyT2 is L269P) is highlighted by white text on a black background (Charlier et al., 2008).
6.4. Amplification and TOPO cloning of SLC6A5 exons 4 and 5 from bovine genomic DNA

To independently confirm the presence of the T809C mutation, genomic DNA from a CMD2 case (sample 1477) and a heterozygous carrier (sample 1482) was used to amplify exons 4 and 5 of bovine SLC6A5 using Pfx DNA polymerase (see Section 2.2.3 for PCR conditions) using specific forward and reverse PCR primers (bGlyT2Ex4+5A and bGlyT2Ex4+5B, see Section 2.2.4). Resulting PCR products were TOPO cloned and fully sequenced. Briefly, slices of agarose containing the two 0.9 kb PCR products corresponding to Exons 4 and 5 from samples 1477 and 1482 were cut from the agarose gel and purified using the Sephaglas BandPrep kit as described in Section 2.2.6. The resultant elution products containing the purified DNA were added to the TOPO vector pCR-Blunt II-TOPO (Figure 3.3) and a TOPO cloning reaction was performed. The complete reaction volume was used to transform E. coli, and cells were plated on LB agar plates supplemented with kanamycin to select for transformants. A number of miniprep DNAs for both the homozygous and the heterozygous state were prepared and sequenced as described in Section 2.2.11. DNA sequencing revealed that as expected, four independent clones from sample 1477 (CMD2 calf, homozygous) all contained the mutant C809 allele. By contrast, only two out of four clones in sample 1482 (CMD2 carrier, heterozygous) corresponded to C809, while the other two clones had the normal T809 allele (Figures 6.3 and 6.4).

Sample 1482: Heterozygous state (CMD2 carrier)
- bGlyT2 1482 1F GTCTGTGTGGAAGGCCATCCCAGCCGCAAGGTGAGTGTTCCTGCTTCCCA
- bGlyT2 1482 2F GTCTGTGTGGAAGGCCATCCCAGCCGCAAGGTGAGTGTTCCTGCTTCCCA
- bGlyT2 1482 3F GTCTGTGTGGAAGGCCATCCCAGCCGCAAGGTGAGTGTTCCTGCTTCCCA
- bGlyT2 1482 4F GTCTGTGTGGAAGGCCATCCCAGCCGCAAGGTGAGTGTTCCTGCTTCCCA

Sample 1477: Homozygous state (CMD2 affected)
- bGlyT2 1477 1F GTCTGTGTGGAAGGCCATCCCAGCCGCAAGGTGAGTGTTCCTGCTTCCCA
- bGlyT2 1477 2F GTCTGTGTGGAAGGCCATCCCAGCCGCAAGGTGAGTGTTCCTGCTTCCCA
- bGlyT2 1477 3F GTCTGTGTGGAAGGCCATCCCAGCCGCAAGGTGAGTGTTCCTGCTTCCCA
- bGlyT2 1477 5F GTCTGTGTGGAAGGCCATCCCAGCCGCAAGGTGAGTGTTCCTGCTTCCCA

Figure 6.3. Confirmation of the T809C mutation in CMD2 and carrier animals. Part of the DNA sequence contig from SLC6A5 exons 4 and 5 showing the mutated codon highlighted in green (normal T809 allele encoding L270) or red (mutant C809 allele encoding P270).
Figure 6.4. DNA sequencing chromatograms showing the CMD2 mutation. Upper row: chromatogram sample 1482 miniprep 2 showing the normal T809 allele. Lower row: chromatogram sample 1477 miniprep 5 showing the mutated C809 allele. The nucleotide affected by the T809C change is indicated by a black arrow.

6.5. Characterisation of the functional consequences of the bovine CMD2 mutation

Bioinformatic analysis revealed that L270 is not predicted to be involved in glycine or Na\(^+\) binding in the transporter (Rees et al., 2006). However, comparison with other vertebrate GlyT2 sequences revealed that leucine is highly conserved at this position, suggesting the structural and functional importance of this residue (Figure 6.5).

![Sequence alignment of the GlyT2 TM3 domain in vertebrate species](http://www.ebi.ac.uk/).

Unlike other amino acids, proline confers local structural rigidity to the three-dimensional structure of proteins. In some locations, the introduction of a proline residue can be tolerated with little or no detrimental effect. However, at others it is
destabilising and can result in accelerated degradation or aggregation of the mutant protein (Bajaj et al., 2007). In order to assess the functional consequences of the L270P mutation, the corresponding substitution (L269P) was made in the mammalian expression constructs pRK5myc-hGlyT2 and pRc/CMV-hGlyT2 by site-directed mutagenesis. PCR (Section 2.2.14) was performed using primers hL269P1 and hL269P2, Table 2.5). After transforming PCR products into *E. coli* competent cells, minipreps and maxipreps of the mutant were prepared. Sequence analysis confirmed that only the desired mutation had been introduced.

**6.5.1. Subcellular localisation of the hGlyT2 L269P mutant**

To investigate the subcellular localisation of the hGlyT2 L269P mutant, HEK293 cells seeded on poly-D-lysine were transfected with 1 µg total pRK5myc (vector control), pRK5myc-hGlyT2 or pRK5myc-hGlyT2 L269P using Lipofectamine LTX, as described in Section 2.4.7. After an incubation of 24 hours, transfected cells were washed twice in PBS, fixed for 2 minutes in 4% (w/v) PFA in PBS and quenched in 50 mM NH₄Cl for 10 minutes before permeabilisation in 0.01% (w/v) Triton X-100 for 12 minutes. After washing in PBS, immunostaining was performed using an anti-myc primary antibody, Alexa-Fluor 488 secondary antibody and nucleic acid marker TO-PRO-3 as described in Section 2.4.8. Coverslip-mounted microscope slides were examined using a Zeiss LSM 510 Meta Confocal microscope. An argon laser which emits light at a wavelength of 488 was used to detect Alexa 488 antibody. The HeNe laser which emits light at a wavelength of 594 was used to detect TO-PRO-3 (Section 2.4.9). This analysis revealed that myc-hGlyT2 and myc-hGlyT2 L269P were both capable of localising to the cell surface, suggesting that the L269P mutation does not affect the membrane trafficking of the transporter (Figure 6.6).

![Image](https://example.com/image.png)

**Figure 6.6. Subcellular localisation of hGlyT2 and L269P mutant.** Confocal images of transfected HEK293 cells showing localisation (green labelling) of myc-hGlyT2 (A, B) and the L269P mutant (C, D) counterstained with the nuclear marker TO-PRO-3 (red labelling). Note that both proteins show membrane localisation (Charlier et al., 2008). Scale bar, 10 µm.
6.5.2. [3H]-glycine uptake is impaired in the L269P mutant

In order to assess whether the L269P mutation affected glycine transporter activity, [3H]-glycine uptake assays on HEK293 cells transfected with pRc/CMV (vector control), pRc/CMV-GlyT2 or two independent pRc/CMV-GlyT2 L269P constructs were performed (Section 2.4.10). Briefly, cells were transfected using Lipofectamine LTX reagent, washed with Krebs buffer pre-gassed with 5%CO2/air, then incubated for 5 minutes in 0.1 μCi/ml [3H]-glycine (60Ci/mmol, NEN) at a final concentration of 300 μM. Uptake was terminated by the addition of ice-cold buffer followed by aspiration. Cells were digested and used for scintillation counting and for determination of protein concentration using Bradford reagent. Glycine uptake was calculated as nmol/min/mg protein and expressed as a percentage of the pRc/CMV-transfected control. This analysis revealed that the L269P mutation completely disrupts glycine uptake (Figure 6.7).

Figure 6.7. Lack of [3H]-glycine uptake in hGlyT2 mutants L269P(1) and L269P(2). pRC/CMV-hGlyT2 and pRC/CMV-hGlyT2L269P constructs 1 and 2 were transiently expressed in HEK293 cells. [3H]-glycine uptake was expressed as a percentage of that in control cells transfected with pRc/CMV (empty expression vector). Note that L269P(1) and L269P(2) do not show any significant [3H]-glycine uptake compared to controls (Charlier et al., 2008). Data are means ± s.e.m. (n = 8). * indicates significantly different from control, P<0.01.
6.6. Discussion

6.6.1. A missense mutation in SLC6A5 causes CMD2

The prediction of the bovine GlyT2 gene, cDNA and protein sequence, facilitated the identification of a missense mutation (T809C) in SLC6A5, resulting in a L269P substitution in TM3 of bovine GlyT2. The functional consequences of this mutation were assessed by introducing the corresponding amino acid change (L269P) into human GlyT2 expression constructs and performing cell-surface localisation and [³H]-glycine uptake assays. Although hGlyT2L269P was present at the cell surface, the L269P substitution abolished [³H]-glycine uptake mediated by recombinant hGlyT2 expressed in HEK293 cells. These results are consistent with the effect of most missense mutations identified in the human GlyT2 gene (Rees et al., 2006; Chapter 3) and strongly suggest that the L270P missense mutation is causative for CMD2, disrupting presynaptic uptake of glycine by GlyT2.
7. GENERAL DISCUSSION
This chapter provides an overview of the results contained in this thesis, and offers a perspective on possible future research directions where appropriate.

7.1. GlyR versus GlyT2 mutations in human hyperekplexia

Hyperekplexia is a rare neurological disorder, but is now gaining increased recognition in clinical fora, in part due to studies identifying new causative genes (Rees et al., 2006). This has resulted in an increase in the number of referrals for genetic analysis in Swansea and the growing recognition that hyperekplexia affects diverse nationalities and ethnic groups. Hyperekplexia has considerable genetic heterogeneity for a rare disease, with two major causative genes (GLRA1 and SLC6A5), three minor genes (GLRB, GPHN and ARHGEF9) and potentially more loci still remain to be discovered (Rees et al., 2006; Harvey et al., 2008a). Although mutations in the GlyR α1 subunit gene (GLRA1) still represent the most common known cause for hyperekplexia, it should be noted that mutations in the GlyT2 gene (SLC6A5) have only been recently described (Rees et al., 2006; Eulenburg et al., 2006). The fact that SLC6A5 mutations are commonly inherited in a recessive manner may explain some 'idiopathic' cases of hyperekplexia, where parents are apparently unaffected, since they only carry one defective allele. In addition to the eleven published mutations (Rees et al., 2006; Eulenburg et al., 2006), it is likely that additional SLC6A5 variants will be uncovered through re-examination of hyperekplexia cases world-wide that are negative for GLRA1 defects.

7.2. Identification of new hyperekplexia mutations and exons in the human GlyT2 gene

In support of this view, in collaboration with Prof. Mark Rees at the University of Swansea and Dr. Jean-François Vanbellinghen at the University of Liège, Belgium, I have characterised seven new missense, nonsense and frameshift mutations in SLC6A5 in individuals with hyperekplexia. The identification of further mutations in the human GlyT2 gene reinforces the idea that SLC6A5 is a second major gene for hyperekplexia, and has provided a rich resource for future structural, biochemical and electrophysiological analyses of neurotransmitter transporter function. In addition, many families affected by hyperekplexia have now been provided with an unequivocal explanation for their condition and can receive genetic counselling.
Immunocytochemical and functional studies were carried out in order to characterise these alterations and understand how they affect GlyT2 activity. This analysis revealed that new GlyT2 mutations resulted in defective subcellular localisation and/or glycine uptake, consistent with the reported effects of previously identified GlyT2 mutations (Rees et al., 2006; Eulenburg et al., 2006). Interestingly, kinetic studies showed that mutation A275T is partially functional, and exhibits a reduced affinity for substrate. Two-electrode voltage-clamp analysis of A275T in *Xenopus laevis* oocytes is currently been carried out by Prof. Stéphane Supplisson (Laboratoire de Neurobiologie, CNRS, Ecole Normale Supérieure, Paris, France) to further characterise this mutant. Although most newly identified mutations appeared to be recessive in nature, in three cases the genetic mechanisms underlying the pathological phenotype are still unclear. A lack of a parental phenotype and \(^{3}H\)-glycine uptake experiments excluded a dominant-negative effect for W151X, A275T and R439X. Thus, mutations in uncharacterised *SLC6A5* exons, large deletions or promoter mutations in the second allele were the most likely explanation in these cases. Although 5' and 3' RACE experiments allowed the identification of novel N- and C-termini exons, utilised during alternative splicing of GlyT2 transcripts, no further mutations were found in these exons. Future genetic research should focus on detecting deletions in human *SLC6A5* using techniques such as MLPA and characterising the human GlyT2 promoter region in fine detail. In this regard, it is curious that three different N-terminal exons were identified by 5' RACE, suggesting that further splicing takes place upstream to a common exon prior to a single promoter or alternatively that different GlyT2 promoters may exist.

A new GlyT2 C-terminal isoform (hGlyT2-NFS) was discovered lacking the C-terminal class III PDZ-domain binding motif (TQC) found in GlyT2, which is known to be crucial for correct synaptic localisation of GlyT2 (Armsen et al., 2007). However, in functional studies, I was unable to show trafficking of GlyT2-NFS to the cell membrane or measure \(^{3}H\)-glycine uptake for this isoform. Although the reason for this is currently unclear, it is possible that this new isoform of GlyT2 is present on internal membrane compartments (e.g. synaptic vesicles) rather than the external cell membrane. A specific antibody against the GlyT2-NFS C-terminus is currently being generated to address this important question.
7.3. Identification of GlyT2 interactors reveals a new candidate gene for hyperekplexia

The discovery that hyperekplexia can have presynaptic as well as postsynaptic causes of disease suggested that GlyT2 accessory proteins could be further candidates for genetic screening in patients still awaiting a definitive diagnosis. For this reason, YTH screening was employed in order to identify new GlyT2 interacting proteins. Six out of seven newly identified interactors (syntrophin-α1, LNX1, SAP97, SAP102, MAGI1 and PICK-1) contained one or more PDZ domains, and interactions were shown to be dependent on the C-terminal class III PDZ-domain binding motif (TQC) found in GlyT2. However, the corresponding genes are not obvious candidates for genetic screening in hyperekplexia, as most have multiple other interactors (e.g. PICK-1, SAP97 and SAP102 are associated with AMPARs; Leonard et al., 1998; Lau et al., 1996; Madsen et al., 2005). Currently, another interactor, the cytoskeletal protein FMNL2, appears to be the best candidate for screening. GlyT2, but not GlyT1 interacts with FMNL2 in a manner that is not dependent on the C-terminal PDZ binding motif. In addition, the orthologous mouse protein, also called 'man' was detected at high level in brain and spinal cord, suggesting a role for this protein in neuronal function (Cui et al., 2005). It is also interesting to note that there are no existing knockout mice models for FMNL2 and that so far this protein has not been associated with any human disease. GlyT2 has been reported to traffic to/from the cell surface and membrane expression levels are the result of a dynamic equilibrium between GlyT2 membrane delivery and internalisation (Fornés et al., 2004). Recent studies have also suggested that active GlyT2 is located in cholesterol-rich membrane rafts and that the lipid environment is involved in the modulation of the transporter activity (Nuñez et al., 2008). The same group showed that treatment with phorbol 12-myristate 13-acetate (PMA) causes GlyT2 inhibition by displacing it from membrane rafts (Fornés et al., 2008). Fornés et al., (2008) proposed the involvement of the actin cytoskeleton in the mechanisms of PMA action on GlyT2, as already reported for SERT (Sakai et al., 2000). Apparently, the small GTPase Rac1, which is involved in the regulation of the actin cytoskeleton, is responsible for mediating the effect of PMA on GlyT2. However, the proposed mechanism requires the presence of a regulatory protein which acts as downstream effector of Rac1 directly connecting GlyT2 to the GTPase-related signalling cascade. FMNL2 presents a N-terminal GBD, which can interact directly with Rac and/or Rho or any other small GTPases.
promoting microfilament-microtubule alignment and microtubule stabilisation (Schlüter et al., 1997). Through the proline-rich formin homology domain FMNL2 can mobilise actin that has been sequestered by profilin and promote actin polymerisation (Witke, 2004). Due to domain composition, FMNL2 could act as a cytoskeletal GlyT2 adaptor and this interaction might play a crucial role in the localisation of the transporter. For this reason, mutations in FMNL2 on human chromosome 2q22.3 might result in mis-localisation of the transporter, thus impairing efficiency of glycineergic transmission, leading to hyperekplexia.

7.4. Potential role for PICK-1 in GlyT1 function at NMDAR

[^H]-glycine uptake experiments showed that co-expression of PICK-1 and GlyT1 enhanced GlyT1 uptake activity in HEK293 cells. Although further validation is required before one can state with certainty that PICK-1-GlyT1 interaction takes place in vivo, this result is of particular interest considering the involvement of both PICK-1 SNPs and the glutamatergic system dysfunction in schizophrenia (Dev and Henley, 2006). Reported SNPs affecting the PDZ domain of PICK-1 might alter PICK-1 interactions synaptic proteins. Assuming that PICK-1 is the protein involved in the localisation and clustering of GlyT1 at glutamatergic synapses, it is possible that altered PICK-1 might affect surface expression and/or trafficking of GlyT1, so affecting glutamatergic neurotransmission (Dev and Henley, 2006). One important aspect for future studies is the involvement of PKC in PICK-1 interactions with GlyT1. Acute PKC mediated transporter down-regulation is well established for most neuronal SLC6 gene family members, including DAT, SERT and GLYT (Melikian 2004; Fornés et al., 2004) and represents the most studied mechanism of transporter regulation. For example, Sato et al., (1995) showed that activation of PKC system by phorbol esters results in a decrease of GlyT1 mediated uptake. Since PICK-1 is a substrate for PKC phosphorylation, it is possible that PKC exerts some effects on GlyT1 expression by altering the interaction between PICK-1 and the transporter (Deken et al., 2001).
7.5. GlyT2 dysfunction in animals

CMD2 can now be considered as a new example of GlyT2 dysfunction in animals. Calves affected by CMD2 have a behavioural phenotype reminiscent of ICM in Poll Hereford cattle (Gundlach et al., 1988; Pierce et al., 2001; Heal et al., 2002). Hence, the convergence of presynaptic GlyT2 deficits and postsynaptic GlyR disruption towards similar phenotypes has now been reported in mice (Gomez et al., 2003b), humans (Rees et al., 2006) and cattle (Charlier et al., 2008). However, it is noteworthy that GlyT2 dysfunction in mice and cows is lethal, whereas in humans with GlyT2 mutations, symptoms often resolve in the first year of life. The extreme phenotype observed in mice and cows could indicate a lack of a compensatory action by the inhibitory GABAergic system, suggesting that glycinerergic and GABAergic systems might have a more sophisticated level of integration in humans. Genotyping of 2,000 healthy Belgian blue animals revealed that 2.3% of Belgian blue cattle and 4.6% of artificial insemination sires were carriers of the defective L270P allele, whilst all 21 confirmed CMD2 cases were homozygous for L270P, confirming the recessive nature of this disorder. Interestingly, the identification of the causative mutation for CMD2 mutation had important practical consequences. Whilst one might imagine that affected sires are automatically culled, in fact this is not the case. Genotyping information is being used to avoid 'at-risk' matings of carrier animals, since the cattle are valuable, and the traits passed on by a given sire might outweigh the potential penalty of being a CMD2 carrier. This practical application demonstrates the importance of genome-wide studies in domestic animal populations, as in this instance, our study has allowed the rapid control of an emerging recessive genetic defect, so preventing further animal suffering (Charlier et al., 2008).

The identification of genetic defects in CMD2 has also prompted the collection of DNA and RNA samples for other 'unresolved' genetic defects where a defect in glycinerergic transmission has been implicated, including familial reflex myoclonus in labrador retrievers (Fox et al., 1984), inherited myoclonus in Peruvian Paso horses (Gundlach et al., 1993) and congenital myoclonus in Holstein cattle (Schulze et al., 2006). Future genetic and functional studies should reveal whether GlyR or GlyT2 gene defects are responsible for these inherited disorders.
APPENDIX 1

Predicted bovine GlyT2 gene sequence

**Bovine genome chr29:19760533-19814188 (reverse complement)**

gatctcttccctcaagttgtttcatcggggatggtggggagcagggggagtgggggggagggggggtggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggggg
cagagcgcctccacagcctgattttttttcaagtcgaagtatagttgtattacccacatgttaat

Exon 7
Exon 14
tagacttttttttcaacatatcgtccctttaattttacttttattattattacatctctcaacttttttttattgcttttgaggactatcttatcatcctcaagtgtgatgatgacatttttaagatcatctgacattcatcttcttaaagatcagagcccttcctataccagtaaatctgcatagggactcatacatgcagctcctgtcagctgctcctttattgtggcttagcttctggctttgcctccacccactctctaagcacagtaattgacaagagacctgtctttcttcctccctggcttgtggtcaaaggctgcaggaagctggctgaagttcctgctgatcccagaaggtggcactagaaaccagattaagacccttgtttcgctgctccgtaacagcttttttaaagcaacatgacagtaaaacagtaaagaaaacagatattttccttaaaaaaaaaaaaacaaaaaacaaaacaaaaacccccaaaactggcacaaaatgagttttaatcaaaacatctccatttctttcggaaaaagaagtttagacagaaaaaacattcagtatttccctccctcggcattgacaagagacattattttagaagccacatctgtgtgcacatctgtatctgatatcacctatcagaggctgcaatttgtctgagaagtcccaccagaccggggacatgagagttgaaaaggattaagatagatttttcagtttcttgaattcaggacgcgttttagccacctaccgaggaaaacaggagacgcccccgcccccgccccccccccccctctgctgtctgcctctgctgttttccctatctctgctgagcttcgccagatcctcctgttactactacacacgttttattttgggtgaaatcaaagttagaatctgtcccagattctttcgacctttgcttcttgtcttagaacagattaacacaagctttgactctcataatctcttactcaaatctcagctacatcttctgatattttctgatggaacgagttgagaaggtggagctttttttttttttgtgagaagtgcctcaactgggctgtgctttactcagtttccattataaaactctccacagaagcctgtggcaaggccccacaatcagaaaatactttccgccaaatcctccttctgtgtttgggagctagagacaagaagaagtggtcagttccatcattggcatcaccttctgctgccatcagctgaatgtcaggtggacctggctccatggctaaattaadctttctcatggccaggttcatctgagagcagggttttatcaaagctgatttggcagggtgaaatcgatgcagaaacaagtttacacagataaatttgatatgatacccgtgtaacaggaaatggtatcagaggagcaacacataacacttcctctttggtccatttgcccttctgtaacacggttatcttgtaggagactagactgagctgagattatgggtttcaatgacttcaaaacccctgaaataaaattgatcatttttatatgtgccttcatgacataattatatagagctataaatattctgcatgcaattattgtatacataggcagaacatgttactgacttaggcacacataagcagaggtattgctatttatagtaaatgaaatggagcaacttaccataattacattaggccatttccaacagactgttaataaagcttataactctaggggcaaatgttctaaagggggcctagagtagatatatctcttataggagcagtatcttcaaccattgtcttcaggctcttgctgctttgaatccatataattgaattactgttgcattggaataatcacttgccccggcagcccgggctttgcagcaatcataaagatgcttacatttgtataatgcttcccacttttccagaatacttccatgtattatctttcctgacgtaattccc
APPENDIX 2

Predicted bovine GlyT2 cDNA sequence

AACAGCGAGCCCCCTCCGGCTGAGTGACACTGGCCAGTGTACTCTCAGCTGATGTCCC 60
TTTAAAACGACATTACAAAGGCGGTTAAGATTACATTCCTACATTTAGCAAGAAAATGGTC 120
AAACGAGGAGCCCTCTGCTTTGCACTGACGCAAACCCGGCCTTTCCCAAGGAGATATAGA 180
AAGGGCTCTTTGCTGAGCCAAACCCAGTCTTGTCAATAGCTGGTCTCATCAGCTACG 240

M D C S A P K E M N K Q 12
TTGACTCTGTTGCCGTTCAGCATGGATCTGCTCTCCCAAGGAAATGAAATACAAA 300

P A N S L E A A V P S G H D G P C A P 32
CCAGCAACAGCTTGAGAGGGCGGGAGTGCCTCCCTGAGGACACACAGATGGCCCGTCGCCCAACC 360

R T S P E Q E L P A A T A A P P P R V P 52
AGGACGAGCCCGGAGGAGCCAGACCTCGGGCAGGCGAGCGAGTCCGAGGCCACCC 420

R S A S T G A Q T F H A A D A R A C E A 72
AGGTCCGCTTCCACCGGCGCTTCGCTGGAGCCAGGACGAGCTGGTCTCATCAGCTACG 480

E R P G G A G A C K L S L R S P A A S 92
AGGCGGCTCTCGGGGATGGAGGAGGGCGCACGGTGCCGGGAGGCTTCGCGGGCCCGTCG 540

A A L R D W S E A H G A Q T A P P S G G 112
GCAGGTCCGCTGGATGAGCCAGGACGAGGCGGGCTTCGAGGAGGCTTCGCGGGCCCGTCG 600

A G P G N A L P C K I T A L R G L E G D 132
GCCGGCCCAGGAAACCCTGCTCTTCTG7AAGATTACAGCCCTGCAGGGGATTAGGAGGAT 660

A N V S V G K G T L E R N N T P A V G W 152
GGCAATGTGAGTGGAGGAAAGGGACGGCGGCTTCGAGGAGGCTTCGCGGGCCCGTCG 720

V N M S Q S T V V L G T G I T S V L P 172
GTGAAATATGAGCAGACCCAGAGCTGGTCTGCGGACCCAGATGGAGAATCGGTCCGGTCTC 780

G S V A T A A T Q E D E Q G D E N K A R 192
GCCAGCAGGGCCAGCCCGCTCCAGCCAGGGCCAGACCGAGGAGGATGAAATAAGGCGCGA 840

G N W S S K L D F I L S M V G Y A V G L 212
GGGAACGGCTGACGAGACTTGACTCCATCTGCTGCAGTGTTGGGATAGCAGGAGGCGTGT 900

G N V W R F P Y L A F Q N G G G A F L I 232
GGCAATGTGAGTGGAGGAAAGGGACGGCGGCTTCGAGGAGGCTTCGCGGGCCCGTCG 960

P Y L M M L A L A G L P I F F L E V S L 252
CTTCATCTTGAGAGGCGGCTTCGCTGCGAGGAGGCTTCGCGGGCCCGTCGAGGTGCCTG 1020

G Q F A S G Q G P V S V W K A I P A L Q G 272
GGCCAGGGCAGCCAGGGGCGGGCGGGGCTGGTGCTGGTGAGGAGGAGGCTCCAGCCAGCCG 1080

C G I A M L I S V L I A I Y Y N V I I 292
TGTGGCATTGCAATGGCTGATCATCCCTGGGTCATGTTAGGAGGTAGCTATC 1140
Q P N I F W K V C W AF V T P I L T F 692
CAGCCAAACATTTTCTGGAAAGTCCTGCTGGCCCTTTTGACATCCGACCATTAAAACTTTT

I L C F S F Y Q W B P M T G S Y R Y P 712
ATCCTTTGTCTGCTTCATCCAGTGGGAGCCCATGACCCTATGTGCTCTTACCGCTACCT

N W S M V L G W L M L A C S V I W I P I 732
AACTGGTCCATGGGCT CGGATGCGATCGCTAGTTCGCTGGCTCCGCTTGCACTGCTGGGATT

M F V I K M H L A P G R F I E R L K E V 752
ATGTTTGTGATAAAAAATGCACTCGGCTGCTGAGATTTATTGAGAGGCTGAAGTTGGTG

C S P Q P D W G P F L A Q H R G E R Y K 772
TGCTCGCGCGACCCGGATGCGCTCTTTCTTAGCTCAACCCTGGAAGCTCAAG

N M I D P L G T S L S L G K L P V K D L 792
AACATGATCGACCCCTTGGGACCTCTTCGCTGGGACTCAAATCGCATTTGGAAGATCCTG

E L G T Q C * 798
GAACCTAGGCACCCAGTCTAGTCTGATGCGATGGGAGATGGGCCCCAGACTTTGATTCTCTTTCT

CCTCTCTGCC 2710
BIBLIOGRAPHY


PUBLICATIONS

Some of the work described in this thesis is described in the following publications:


POSTERS AND PRESENTATIONS


LIST OF ABBREVIATIONS

3-AT 3-amino-1,2,4-triazole
AChE Acetylcholinesterase
AChR Acetylcholine Receptor
AD Autosomal dominant
ADHp Alcohol dehydrogenase promoter
ADHt Alcohol dehydrogenase transcription termination signal
ADP Adenosine diphosphate
AI Artificial insemination
Amp Ampicillin
AMPA Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR AMPA receptor
AP Anchor primer
AR Autosomal recessive
ARHGEF9 Cdc42 guanine nucleotide exchange factor 9 gene
ATP Adenosine triphosphate
BAR Bin/Amphiphysin/Rvs domain
β-Me β-mercaptoethanol
BLAD Bovine leucocyte adhesion deficiency
BLAT Basic Local Alignment Tool
BSA Bovine serum albumin
CH Compound heterozygosity
CIP Calf Intestinal Phosphatase
CMD Congenital muscular dystonia
CMD1 Congenital muscular dystonia type 1
CMD2 Congenital muscular dystonia type 2
CNS Central nervous system
ColEl ori ColEl origin of replication
COP9S5 COP9 constitutive photomorphogenic homolog subunit 5
CSF Cerebrospinal fluid
DAPC Dystrophin-associated protein complex
DAT Dopamine transporter
dHPLC Denaturing high-performance liquid chromatography
DMEM Dulbecco's Modified Eagle Medium
DMF Dimethylformamide
DMSO Dimethyl sulphoxide
DNA Deoxyribonucleic acid
DO Dropout
DPYSL5 Dihydropyrimidinase-like 5 gene
DTT Dithiothreitol
EB Elution buffer
EDTA Ethylenediaminetetraacetic acid
EGFP Enhanced green fluorescent protein
EIF3S6 Eukaryotic translation initiation factor 3 subunit E
EL Extracellular loop
ENU Ethynitrosourea
ExPASy Expert Protein Analysis System
FBS   Foetal bovine serum
FH2   Formin Homology domain 2
FH3   Formin Homology domain 3
FMNL2 Formin-like 2
FRET  Fluorescence resonance energy transfer
GABA  γ aminobutyric acid
GAL4AD GAL4 activation domain
GAL4BD GAL4 binding domain
GAT   GABA transporter
GBD   GTPase Binding domain
GCS   Glycine cleavage system
GDP   Guanosine diphosphate
GEF   Guanine nucleotide exchange factor
GK    Guanylate Kinase
GLDC  Glycine dehydrogenase decarboxylase
GLRA1 Glycine receptor alpha subunit gene
GLRB  Glycine receptor beta subunit gene
GluR  Glutamate receptor
GlyR  Glycine receptor
GlyT  Glycine transporter
GPHN  Gephyrin gene
GSP   Gene specific primer
GTP   Guanosine triphosphate
HA    Haemagglutinin
HBSS  Hank’s Buffered Salt Solution
HEK293 Human Embryonic Kidney 293
His   Histidine
ICM   Inherited congenital myoclonus
JAM4  Junctional adhesion molecule 4
LB    Luria-Bertani
Leu   Leucine
LeuT  leucine transporter
LiAc/SS Lithium acetate/single stranded
LNX1  Ligand of Numb protein X-1
LTD   Long-term depression
MAGII Membrane associated guanylate kinase WW and PDZ domain containing 1
MAGUK Membrane-associated guanylate kinase
MEM   Minimum Essential Medium
MGI   Mouse Genome Informatics
MLPA  Multiplex Ligation-Dependent Probe Amplification
mNET1 Nucleotide exchange factor
MOCO  Molybdenum cofactor
MOET  Multiple ovulation and embryo transfer
MOPS  3-(N-morpholino)propanesulfonic acid
NET   Noradrenaline transporter
NMDA  N-methyl-D-aspartate
NMDAR NMDA receptor
nNOS  Neuronal nitric oxide synthase
OMIM  Online Mendeliane Inheritance in Man
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PDZ</td>
<td>postsynaptic density protein-95 discs large zonula occludens-1</td>
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<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
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<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
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<td>PH</td>
<td>Pleckstrin Homology</td>
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<td>PICK1</td>
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<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
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<td>PPI</td>
<td>Prepulse inhibition</td>
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<td>PSD</td>
<td>Postsynaptic density</td>
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<td>PTB</td>
<td>Phosphotyrosine-binding</td>
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<tr>
<td>pUC ori</td>
<td>pUC origin of replication</td>
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<tr>
<td>RACE</td>
<td>Rapid amplification of cDNA ends</td>
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<td>RANBP9</td>
<td>RAN binding protein 9</td>
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<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>Sc</td>
<td>Spinal cord</td>
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<td>SAP102</td>
<td>Synapse-associated protein 102</td>
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<td>SAP97</td>
<td>Synapse-associated protein 97</td>
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<tr>
<td>SD</td>
<td>Synthetic dropout</td>
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<tr>
<td>SDCBP</td>
<td>Syndecan binding protein gene</td>
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<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<td>SERT</td>
<td>Serotonin transporter</td>
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<td>SH3</td>
<td>Src Homology</td>
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<td>SHMT</td>
<td>Serine-hydroxymethyltransferase</td>
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<td>SLC6A5</td>
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<td>SNPs</td>
<td>Single nucleotide polymorphisms</td>
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<td>TAE</td>
<td>Tris-acetate EDTA</td>
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<td>TAP</td>
<td>Tobacco Acid Pyrophosphatase</td>
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<td>Tris-EDTA</td>
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<td>Trp</td>
<td>Tryptophan</td>
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<td>UAS</td>
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<td>University of California Santa Cruz</td>
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<tr>
<td>VIAAT</td>
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<td>X-Gal</td>
<td>5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside</td>
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<tr>
<td>YPD</td>
<td>Yeast extract peptone dextrose</td>
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<tr>
<td>YTH</td>
<td>Yeast two-hybrid</td>
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