Molecular and biophysical characterization of the glycinergic inhibitory system.

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Molecular and Biophysical Characterization of the Glycinergic Inhibitory System

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Abstract

Glycinergic neurotransmission is a major inhibitory influence in the CNS and defects are associated with paroxysmal neuromotor disorder, hyperekplexia with mutations in subunits of the inhibitory glycine receptor which facilitates postsynaptic ligand-binding, ion-channels. This study investigates the human glycinergic system by*: 1) Mutation analysis of glycinergic candidate genes in hyperekplexia:* the DNA sequencing of *GLRA1* in 88 hyperekplexia patients revealed 30 sequence variants; 21 were inherited in recessive mode or part of compound heterozygosity, indicating that recessive hyperekplexia is more common than previously expected. Further screening of the glycine transporter-2 gene (*SLC6A5*) as a candidate gene, 12 *SLC6A5* mutations were found in 7 human hyperekplexia cases inherited predominantly by compound heterozygosity. *2) Biophysical analysis and molecular modelling of GLRA1 mutations:* which demonstrated that subcellular localisation defects were the major mechanism underlying recessive mutations. Other mutants typically show alterations in the dose-response curve for glycine suggestive of disrupted signal transduction. This study reports the first hyperekplexia mutation associated with leaky current suggesting tonic channel opening as a new receptor mechanism and fully-supported by molecular modelling. *3) Molecular and immunoreactive analysis of gephyrin heterogeneity in human brain:* gephyrin encodes a multifunctional cytoplasmic protein important for organizing glycine and $GABA_A$ receptors at the postsynaptic membrane. Gephyrin has many different transcript isoforms and the study describes the population / distribution of gephyrin isoforms in neuronal tissues using molecular and immunohistochemical techniques. The heterogeneity of gephyrin cassettes indicates that each cassette is temporally and spatially regulated with unique patterns of glycine receptors co-localisation and we hypothesise that different gephyrin isoforms exhibit differential binding specificity affecting protein-protein interactions. This thesis describes that hyperekplexia is definitively a glycinergic disorder with several mechanism of molecular pathogenicity. Moreover, the underlying complexity of proteins, such as gephyrin, reveals further challenges in interpretating the functional significance of the neuronal heterogeneity.

Declarations

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

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Publications 2005 – 2009

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Abbreviations

ALS	amyotrophic lateral sclerosis
AD	Alzheimer's disease
AON	accessory olivary nuclei
CNS	central nervous system
ChAc	chorea-acanthocytosis
Cl	chloride
DAB	3,3'-diaminobenzidine
DC	detergent compatible
dHPLC	denaturing high-performance liquid chromatography
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DMEM	dulbecco's modified eagle's medium
DTT	dithiothreitol
EC50	half-maximal concentration
ER	endoplasmic reticulum
GABAAR	a-type gamma-aminobutyric acid receptor
GABARAP	GABAR -associated protein
GCS	glycinergic cleavage system
Geph-E	gephyrin e domain
Geph-N	gephyrin n domain
GLRA1	glycine receptor alpha subunit
GLRB	glycine receptor beta subunit
GPHN	gephyrin
GRIP1	glutamate receptor interacting protein 1
HD	huntington's disease
HE	hyperekplexia
HERG	human ether a go go related gene
HN	hypoglossal nucleus
HEK-293	human embryonic kidney cells
HPRT	hypoxanthineoquanin phosphoribosyl transferase
IO	inferior olive
IPTG	isopropyl-β-D-thiogalactopyranoside
LB	Luria-Bertani
LBD	ligand binding domain
LGIC	ligand-gated ion channel
LQTS	long qt syndrome
nH	hill coefficient
M	mole
Mena	mammalian enabled
Mg	magnesium
mg	milligram
µg	microgram

ml	microlitre
Na	sodium
ng	nanogram
NMNX	dorsal motor nucleus of the vagus
PBS	Phosphate Buffered Saline
PCR	polymerase chain reaction
PDB	protein data bank
PFA	paraformaldehyde
PTX	picrotoxin
RAFT1	rapamycin and fkbp12 target 1
RE	restriction enzyme
RFLP	restriction fragment length polymorphism
RT	room temperature
SIDS	sudden infant death syndrome
SLC6A5	glycine transporter-2 gene
SN	solitary nucleus;

Chapter 1 Introduction

Fast synaptic neurotransmission in the central nervous system (CNS) is mediated by members of the ligand-gated ion channel superfamily, which includes the glycine receptor (GlyR), acetylcholine receptor (AchR), gamma-aminobutyric acid receptor (GABAR), and glutamate receptors (Celesia, 2001). These channels share similar structures and conduct neuronal signaling by permitting ionic flux which changes the membrane potential (Breitinger and Becker, 2002). Mechanisms of the excitatory neurotransmission and/or inhibitory neurotransmission have been intensively studied in many neurological diseases such as Alzheimer's disease, Parkinson's disease, hyperekplexia and psychiatric disorders (Cyr et al., 2002; Grossberg, 2002; Moghaddam, 2002; Segovia, 2002; Brotchie, 2003; Spedding et al., 2003) In particular, inhibitory transmission pathways have been pharmacological targets to relieve neurological and psychiatric symptoms such as depression, epileptic seizures, and psychosis (Ketter and Wang, 2003; Lynch, 2009). However, the mechanisms underlying inhibitory neurotransmission are not well understood. This thesis aims to provide better understanding of the glycinergic inhibitory system using the following two approaches: 1) mutation analysis of a neurological disorder, hyperekplexia, which is associated with defects in the inhibitory glycinergic molecules; 2) molecular investigation of a multifunctional scaffolding molecule, gephyrin, which is important for organizing of inhibitory GlyRs and GABA type-A receptor ($GABA_AR$) at the postsynaptic membrane of neurons (Betz, 1998).

1.1 Inhibitory system

Glycine receptor (GlyR)s and GABA type-A receptor ($GABA_AR$)s are the two major inhibitory determinants in the central nervous system (CNS) and regulated expression of these receptors at the postsynaptic cell membrane surface is crucial for the balance between neural excitation and inhibition (Lynch, 2004). Defects in several determinants in the glycinergic system (Shiang et al., 1993; Rees et al., 1994; Rees et al., 2002; Harvey et al., 2008) are associated with startle disorder or hyperekplexia (OMIM:149400) which can provide an excellent opportunity to study the dynamics and mechanisms underlying the inhibitory system. Before we examine the molecular basis of hyperekplexia (HE) later in this chapter (section 1.2), the glycinergic system will be briefly reviewed in this section.

1.1.1 Glycine receptor

Glycinergic neurotransmission mediates postsynaptic inhibition mainly in the spinal cord and the mammalian central nervous system (Figure 1.1) (Betz et al., 1999). Binding of a neurotransmitter (or ligand), such as glycine, to GlyR mediates a conformational change which then leads to the opening of the channel gate followed by influx of Cl^- and hyperpolarisation of the cell (Lynch, 2004). GlyRs are a member of the Cys-loop ligand-gated ion channel (LGIC) superfamily and GlyR consists of five subunits with each of which comprising a large N-terminal extra-cellular domain, followed by four membrane-spanning domains (M1-M4) and a short extracellular C-terminus (Celesia, 2001).

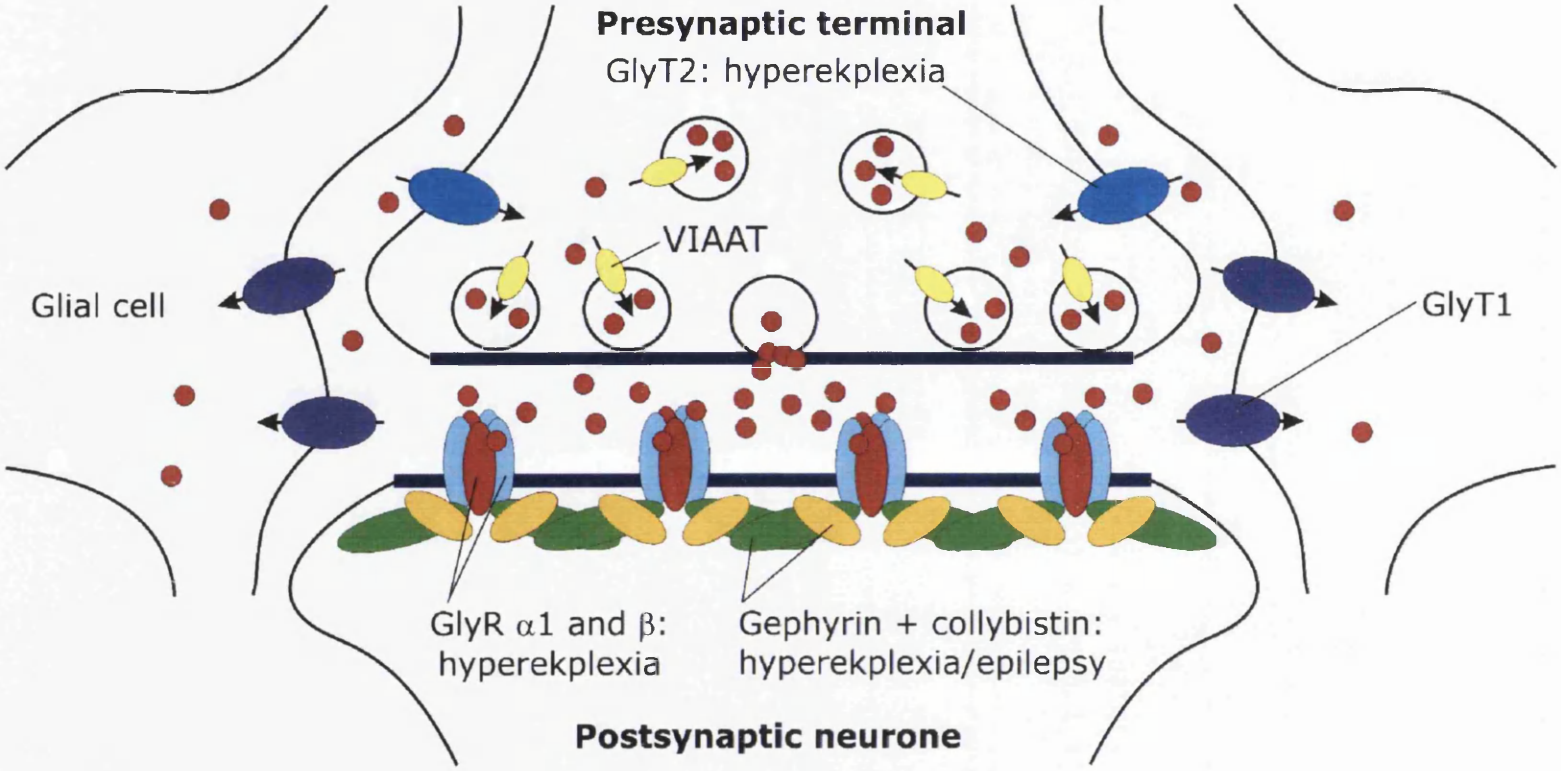


Figure 1.1 Schematic diagram of glycinergic system. *The GlyR's are presented as a pentamer consisting of 2α and 3β. The clustering of GlyR at the postsynaptic cell surface is mediated by the interaction of β subunit with gephyrin. In the presynaptic terminal, the vesicular inhibitory amino-acid transporter (VIAAT) packages glycine (indicated as red dots) into vesicles prior to its release to the synaptic cleft. The binding of glycine activates the GlyRs and results in influx of Cl- and hyperpolaziation of the cell. Once glycine is dissociated from the GlyR, glycine can either be re-transported back into the presynaptic terminal via glycine transpoter2 (GlyT-2) for recycling or it can be removed from the synaptic cleft into glia cells via the glial glycine transporter (GlyT-1) for the termination of the neurotranmission. This figure is a modified version of Figure 1 in Harvey et al 2008 with permission from the author.*

Like other members of the cys-loop LGIC superfamily, GlyR subunit has ligand-binding sites at the interface of two adjacent N-terminal domain, and a channel pore-forming M2 region (Breitinger and Becker, 2002; Lynch, 2004). The adult human glycine receptor has at least four subunits, α1, α2, α3 and β (Betz et al., 1999) and the expression of GlyRs are spatially and developmentally regulated: the neonatal isoform is mainly a homopentamer of α2 subunits encoded by the *GLRA2* gene (Lynch, 2004; Lynch, 2009), whilst the most abundant adult isoform is a hetero-pentamer consisting of ligand-binding α1 and structural β subunits at a 2α1:3β stoichiometry (Grudzinska et al., 2005). The heteropentameric α1β GlyR is the most abundant form of GlyRs in the adult human brain, however the α1 GlyR subunits are able to generate a functional homo-pentameric receptor, whereas the β-subunit requires alpha subunits to form a functional channel (Lynch, 2009).

The formation of GlyR clusters at the postsynaptic sites requires the interaction of the β-subunit with a multifunctional scaffolding protein named gephyrin (Meyer et al., 1995). Gephyrin in turn binds to a variety of structural molecules including microtubules, collybistin and microfilaments to form the subsynaptic cytoplasmic matrix or lattice (reviewed in Fritschy et al., 2008). Mice with complete loss of gephyrin generated by antisense primers or transgenic gene knockout, resulted in the abolished postsynaptic clustering of GlyRs (Kneussel et al., 1999). The importance of gephyrin is further discussed in detail later in this chapter (section 1.3).

1.1.2 Glycinergic system

The glycinergic system is established throughout the brain but does play a subservient role relative to GABAergic neurotransmission. GlyRs are highly-enriched in the medullary brainstem and spinal cord with other regions in the basal ganglia and auditory pathways also heavily innervated with glycinergic inputs (Baer et al., 2003). On the molecular level expression of GlyRs at the postsynaptic cell-surface and controlled release of neurotransmitters such as glycine, are critical aspects of maintaining the normal inhibitory function (Lynch, 2004).

In the synaptic cleft, glycine is removed by two discretely-localized Na+/Cl- dependent glycine transporters, GlyT1 and GlyT2 (Harvey et al., 2008). GlyT1 is mainly expressed in glial cells and terminates neurotransmission by removing excess glycine for enzymatic degradation via glycinergic cleavage system (GCS) (Gomeza et al., 2003). GlyT1 is also involved in controlling glycine concentration at the excitatory *N*-methyl-D-aspartate synapses (Gabernet et al., 2005). Alternatively, GlyT2 transports glycine into the presynaptic terminal for repackaging into synaptic vesicles by the low-affinity vesicular transporter (VGAT/VIAAT) (Supplisson and Roux, 2002; Gomeza et al., 2003) (Figure 1.1). The knockout mouse for GlyT2 has a hyperekplexia-like phenotype whilst the GlyT1 knockout is an embryonic lethal (Gomeza et al., 2003).

GlyR α1 knockout mice also have a lethal outcome, in contrast with human hyperekplexia patients with null mutations in the GlyRα1 subunit where a lethal outcome is avoided through a theoretical compensatory GABAergic mechanism (Kling et al., 1994). This is partially supported by the action of benzodiazepine drugs which enhance the GABAergic inhibition and are the main frontline drug in

alleviation of hyperekplexia symptoms. GlyRs also co-exist with GABARs in the spinal cord neurons and glycine can be co-released with GABA from the axonal presynaptic terminals (Triller et al., 1987; Maxwell et al., 1995). Therefore it is likely that inhibitory influences in the brain and spinal cord are sometimes a mixed influence of GABAergic and glycinergic effects, but the reason for this is unclear. Although it may go some way to explain the relative moderate effects of human knockout phenotypes.

1.2 Hyperekplexia

Hyperekplexia (HE), also known as startle disease, is the first human disease identified as a result of mutations within a neurotransmitter gene in the CNS (Andrew and Owen, 1997). From the findings of this study/ thesis, hyperekplexia is also the first human neurological disorder with mutations in a cognate neurotransporter gene (Rees et al, 2006). The startle response is a normal and basic reaction to sudden unexpected stimulation. The reaction, however, can be present in a pathologically excessive, abnormal form and this clinical entity was initially termed "Hyperexplexia" meaning 'startle' in Greek (Gastaut and Villeneuve, 1967). Hyperexplexia was then replaced by "Hyperekplexia" using the correct Greek word for startle (Gastaut and Villeneuve, 1967). Hyperekplexia is a relatively rare, but potentially fatal, neurological disorder characterized by an abnormal startle reflex and an exaggerated muscle stiffness in response to sudden, unexpected auditory or tactile stimuli (Gastaut and Villeneuve, 1967)

1.2.1 Clinical aspects of hyperekplexia

Symptoms

The disorder is characterised by exaggerated startle and stiffness due to an abnormal increase in muscle tension and the reduced ability of a muscle to stretch (hypertonia) (Gastaut and Villeneuve, 1967). The symptoms are most severe in neonates and infants and the affected newborns suffer from essentially continuous, but variable muscular rigidity and the intense hypertonicity can lead to breath-holding spells and prolonged cyanotic attacks. They are also at risk of sudden death from apnea or aspiration (Nigro and Lim, 1992; Praveen et al., 2001). Several cases of sudden infant death syndrome (SIDS) were reported in association with hyperekplexia (Giacoia and Ryan, 1994). Delay in the acquisition of motor skills is common in infants with hyperekplexia due to hypertonicity. The hypertonia – which is predominantly in the trunk and lower limbs - often diminishes during the first year of life, but the pathological startle response, which can provoke unprotected falls and result in injury, remains throughout development and into adulthood (Nigro and Lim, 1992; Praveen et al., 2001). Patients of all ages experience brief attacks of generalized, intense spasm of skeletal muscles in response to sudden, unexpected noises or tactile stimulation (Zhou et al., 2002). Startle induced spasms often cause unprotected falls without loss of consciousness, resulting in severe injuries.

Classification

Hyperekplexia is a primarily hereditary non-epileptic paroxysmal disorder, transmitting in dominant or recessive manner. Sporadic cases of HE, symptomatic HE, have also been reported, particularly in association with other brain disorders, such as brainstem lesions or frontal lobe dysfunction (Brown et al., 1991; Kellett et al., 1998;

Gambardella et al., 1999; Ruprecht et al., 2002; Gaitatzis et al., 2004). Abnormal startle response can be associated with other neuropsychiatric and neurophysiological disorders such as the congenital stiff-man syndrome, startle epilepsy, myoclonic seizures, cerebral palsy, neuromyotonia, Tourette syndrome, Swartz Jampel syndrome, and Coffin-Lowry syndrome (see Appendix B).

Diagnostic criteria

The diagnosis of hyperekplexia can be established by presence of the following features: 1) neonatal hypertonia; 2) excessive startle reactions followed by general muscle stiffness in response to sudden and unexpected tactile/auditory stimuli; 3) abnormal startle reactions induced by nose tapping; 4) fall-down startle reactions (Matsumoto et al., 1992; Meinck, 2006). A detailed diagnostic inclusion criteria is included in Appendix A. Startle attacks may simulate pseudo-epileptic seizure episodes. Genotype-phenotype correlations have not yet been established in hyperekplexia.

Treatments

Benzodiazepines, which enhance $GABA_AR$ activity, are currently the treatment of choice. Clonazepam often, but not always, has a dramatic and sustained effect on the symptoms of hyperekplexia (Scarcella and Coppola, 1997; Stewart et al., 2002). However, despite a successful treatment of the excessive startle response, further attention is needed to approach the residual problems that may arise from the fear of falling, such as anxiety disorders, depression, and the side-effects clonazepam may cause. Interestingly, it was reported that enhancing GlyR activity has temporarily diminished the hyperekplexia phenotype in mutant mice (Kung et al., 2001). Further investigation into development of drugs that enhance GlyR response may provide potential therapeutic options for hyperekplexia patients.

1.2.2 Pathogenesis of hyperekplexia

Hyperekplexia was first described in 1958 (Kirstein and Silfverskiold, 1958) and the pathogenesis of HE was unclear for many decades. Recent advances in molecular genetics have revealed that HE is associated with mutations in genes involved in the glycinergic inhibitory pathway (Shiang et al., 1993). Hyperekplexia is the first neurological disease identified as a result of mutations within a neurotransmitter gene, GLRA1 and Na+/Cl- dependent neurotransmitter transporter, GlyT2 (Rees et al 2006 and this study). Defects in these genes can result in decreased Cl^- currents and consequently impaired glycinergic inhibitory transmission which may lead to excessive startle reaction and muscle stiffness (Harvey et al., 2008)

To date, five genes are associated with hyperekplexia: *GLRA1, SLC6A5, GLRB,* gephyrin - *GPHN*, and collybistin - *ARHGEF9* have been identified using a positional cloning strategy and an animal model / candidate gene approach. Of these genes 1) *GLRA1* and *GLRB* encode α and β subunits of inhibitory glycine receptor (GlyR), respectively (Shiang et al., 1993; Rees et al., 2002); 2) *SLC6A5* encodes for the glycine transporter subtype-2 (GlyT2) which plays an important role in neurotransmitter reloading of synaptic vesicles at glycinergic synapses (Rees et al., 2006); 3) *GPHN* encodes the peripheral membrane protein, Gephyrin, that is involved in clustering and anchoring of two major inhibitory receptors, GlyR and GABAR (Rees et al., 2003); 4) *ARHGEF9* encodes collybistin which is necessary for clustering of gephyrin on postsynaptic sites. *GLRA1* is regarded as the most prevalent gene associated with hyperekplexia - accounting for 70% of all hyperekplexia mutations identified thus far. Mutations in *GLRB, GPHN*, and *ARHGEF9* are rare causes of HE with only one case per gene reported– albeit with

functional evidence of pathogenicity (Rees et al., 2002; Rees et al., 2003; Harvey et al., 2004) (Table 1.1). *SLC6A5* was identified as the second major gene in hyperekplexia as part of this study and the findings will be discussed in Chapter 3.

Table 1.1 Genes associated with hyperekplexia.

Gene	*Protein*	*Locus*	*Incidence*
GLRA1	GlyR α1 subunit	5q33-q35	> 70 %
SLC6A5	GlyT2	11p15.1	20%
GLRAB	GlyR β-subunit	4q31.3	3%
GPHN	Gephyrin	14q23.3	3%
ARHGEF9	Collybistin	Xq11.2	<2%

1.2.2.1 Hyperekplexia associated genes – *GLRA1*

GLRA1 consists of 9 exons and spans 100 kb of genomic sequence (Shiang et al., 1993) and hyperekplexia is the only phenotype associated with mutations in *GLRA1.* After the identification of the first hyperekplexia mutation, R271Q, in dominant hyperekplexia families in 1993, over 29 distinct *GLRA1* mutations have been identified in 56 index cases from various studies and involving missense, nonsense and frame shift mutations, including 3 cases of compound heterozygotes and 15 presenting as recessive hyperekplexia cases (Table 1.2). Interestingly, an analysis of the transgenic mouse expressing the mutant human *GLRA1* (R271Q) showed that this mutation diminished both GlyR and GABA-A receptor mediated inhibitory transmission (Becker et al., 2002). This indicates that the expression of a mutant gene affects the entire postsynaptic mechanism of inhibition.

Table 1.2 Human hyperekplexia associated mutations

Mutation	Classification of Mutation	Protein Position	Mode of Inheritance	Functional Consequences	Reference
			GLRA1		
del ex1-6	Deletion		R	loss of GlyRa1	Brune et al., 1996 ; Gilbert et al., 2004 ; Becker et al., 2006,
W68C / R316X	Missense / Nonsense	N-terminal / M3-M4	R	NA	Tsai et al., 2004
R72H	Missense	N-terminal	R	NA	Coto et al., 2005
1 bp delC / M147V	Frameshift	M1	R	M147V= no change in agonist responsiveness	Rees et al., 2001
Y202X	Nonsense	M1	R	loss of GlyRa1	Brune et al., 1996; Rees et al., 2001
R218Q	Missense	M1	D*	Agonist sensitivity ↓, membrane expression ↓, Affect Channel gating	Miraglia Del Giudice et al., 2003; Castaldo et al., 2004
Y228C	Missense	M1	R	NA	Forsyth et al., 2007
S231R	Missense	M1-M2	R	Trafficking defects	Humeny et al., 2002
W239C	Missense		D*	NA	Gilbert et al., 2004
I244N	Missense	M1-M2	R	Partially disrupts the agonist transduction mechanism. Channel current ↓ , Channel affinity ↓	Rees et al., 1994; Lynch 1997

P250T	Missense	M1-M2	D	Glycine induced chloride conductance ↓, Reduced ligand affinity↓, Rapid desensitization	Saul et al., 1999; Breitinger et al., 2001
R252H / R392H	Missense	M1-M2 /M3-M4	CH	Loss of channel current	Vergouwe et al., 1999; Rea et al., 2002
V260M	Missense	M2	D	Agonist sensitivity ↓	del Giudice et al., 2001; Castaldo et al., 2004
Q266H	Missense	M2	D	Impaired channel gating - reduced channel open times	Milani et al., 1996; Moorhouse et al., 1999; Castaldo et al., 2004
S267N	Missense	M2	D	Impaired channel gating, alcohol sensitivity ↓	Becker et al., 2008
S270T	Missense	M2	D	NA	Lapunzina et al., 2003
R271Q	Missense	M2-M3	D	Channel conductance ↓, diminished agonist responsiveness	Shiang et al., 1993 ; Langosch et al., 1994; Rees et al., 1994; Elmslie et al., 1996; Kwok et al., 2001; Rajendra et al., 1994; Laube et al., 1995; Kung et al., 2001
R271L	Missense	M2-M3	D/D*	Channel conductance ↓, diminished agonist responsiveness	Shiang et al.,1993 ; Rees et al., 2001; Rajendra et al., 1994; Laube et al., 1995; Maksay et al., 2002
R271P	Missense		D	NA	Gregory et al., 2008
K276E	Missense	M2-M3	D	Reduced channel open times	Elmslie et al., 1996; Seri et al., 1997; Doria Lamba et al., 2007; Kang et al., 2008: Lewis., et al. 1998
K276Q	Missense	M2-M3	D*	NA	Kang et al., 2008

Y279X	nonsense		R	Protein Truncation	Gilbert et al., 2004
Y279C	Missense	M2-M3	D	Signal transduction ↓	Shiang et al., 1995 ; Kwok et al., 2001; Lynch et al., 1997
Y279S	Missense	M2-M3	D	NA	Poon et al., 2005
S296X		M3	D*	NA	Bellini et al., 08
G342S	Missense	M3-M4	D	No deleterious effect on GlyR function. Incomplete penetrance	Rees et al., 2001
			GLRB		
G229D /IVS5+5G-A	Missense/ splice site error	N-terminal	CH	Agonist sensitivity ↓ / Splicing effect	Rees et al., 2002
			GPHN		
N10Y	Missense	N-terminal	D	No affect on GlyR-gephyrin binding	Rees et al., 2003
			CBS		
G55A	Missense	SH3 domain	X-linked Dominant	Trafficking defects	Harvey et al., 2004

*: de novo mutation; D: dominant, R: Recessive, CH: Compound Heterozygous, NA; Not Available

All nonsense and frameshift *GLRA1* mutations identified to date are associated with recessive cases of hyperekplexia with asymptomatic parental carriers, proving that hyperekplexia is not susceptible to haploinsufficiency (Brune et al., 1996; Rees et al., 2001). In contrast to other diseases caused by dysfunction of ion channels (channelopathies) or in murine hyperekplexia models (Buckwalter et al., 1994; Traka et al., 2006), patients with recessive or null mutations in *GLRA1* were not associated with particularly severe cases of hyperekplexia. This indicates that the complete loss of GlyR α1 function in humans may be tolerated due to compensatory mechanisms possibly by other inhibitory influences (Harvey et al., 2008).

1.2.2.2 Hyperekplexia associated genes – *GLRB*

GLRB consists of 9 exons and spans 95 kb of genomic sequence (Milani et al., 1998) and currently hyperekplexia is the only disorder associated with mutations in *GLRB*. *GLRB* in addition to *GLRA3* are candidates genes for autism and idiopathic generalized epilepsy (Sobetzko et al., 2001; Ramanathan et al., 2004). To date only one compound heterozygote has been identified in a transient hyperekplexia case (Table 1.2) (Rees et al., 2002). A missense mutation in this case leads to a GlyR with a reduced agonist sensitivity, whereas a splice site error results in exon skipping. Both mutations did not produce a hyperekplexia phenotype in heterozygous asymptomatic parents.

1.2.2.3 Hyperekplexia associated genes – *GPHN*

GPHN consists of 27 exons and spans 700 kb of genomic sequence. *GPHN* encodes the peripheral membrane protein, gephyrin, which is involved in organizing and clustering of the two major inhibitory receptors, GlyR and $GABA_AR$, to the postsynaptic membrane. Gephyrin mediates GlyR anchoring by binding to the β subunit of

GlyR. One missense mutation was described in a transient HE case (Table 1.2) (Rees et al., 2003). Functional studies of the *GPHN* mutation shows that it did not affect interactions with GlyR, but it remains unknown if the mutation affects interactions with other gephyrin-binding proteins.

1.2.2.4 Hyperekplexia associated genes - *ARHGEF9*

ARHGEF9 encodes collybistin, a RhoGef protein which is required for clustering of gephyrin on postsynaptic sites (Kins et al., 2000). *ARHGEF9* consists of 10 exons and spans 120.18 kb of genomic sequence. One *ARHGEF9* missense mutation, G55A, was identified in a young patient with a severe case of hyperekplexia and epileptic encephalopathy phenotypes who died at the age of four (Harvey 2004). Functional studies of *ARHGEF9* G55A indicated that the mutant collybistin disturbed the localization of gephyrin and GlyR at the postsynaptic sites.

1.2.3 Functional analysis of *GLRA1* mutations

Recessive mutations are scattered throughout the α1 GlyR channel, whereas, dominant mutations are specifically clustered around the M2 domain containing an ion-selectivity filter and flanking regions (Figure 1.2). Previously-identified dominant *GLRA1* mutations have all been missense mutations predominantly located either in the M2 pore-lining domain of the GlyR α1 subunit (V260M and Q266H, S270T), or in the M1-M2 / M2-M3 loops adjacent to the M2 domain (P250I, R271L/Q, K276E, and Y279C/S) (Shiang et al., 1993; Elmslie et al., 1996; Milani et al., 1996; Saul et al., 1999; del Giudice et al., 2001; Lapunzina et al., 2003; Poon et al., 2006; Becker et al., 2008; Gregory et al., 2008). The functional consequences of dominant GLRA1 mutations have been extensively studied and heterologous expression studies demonstrate that

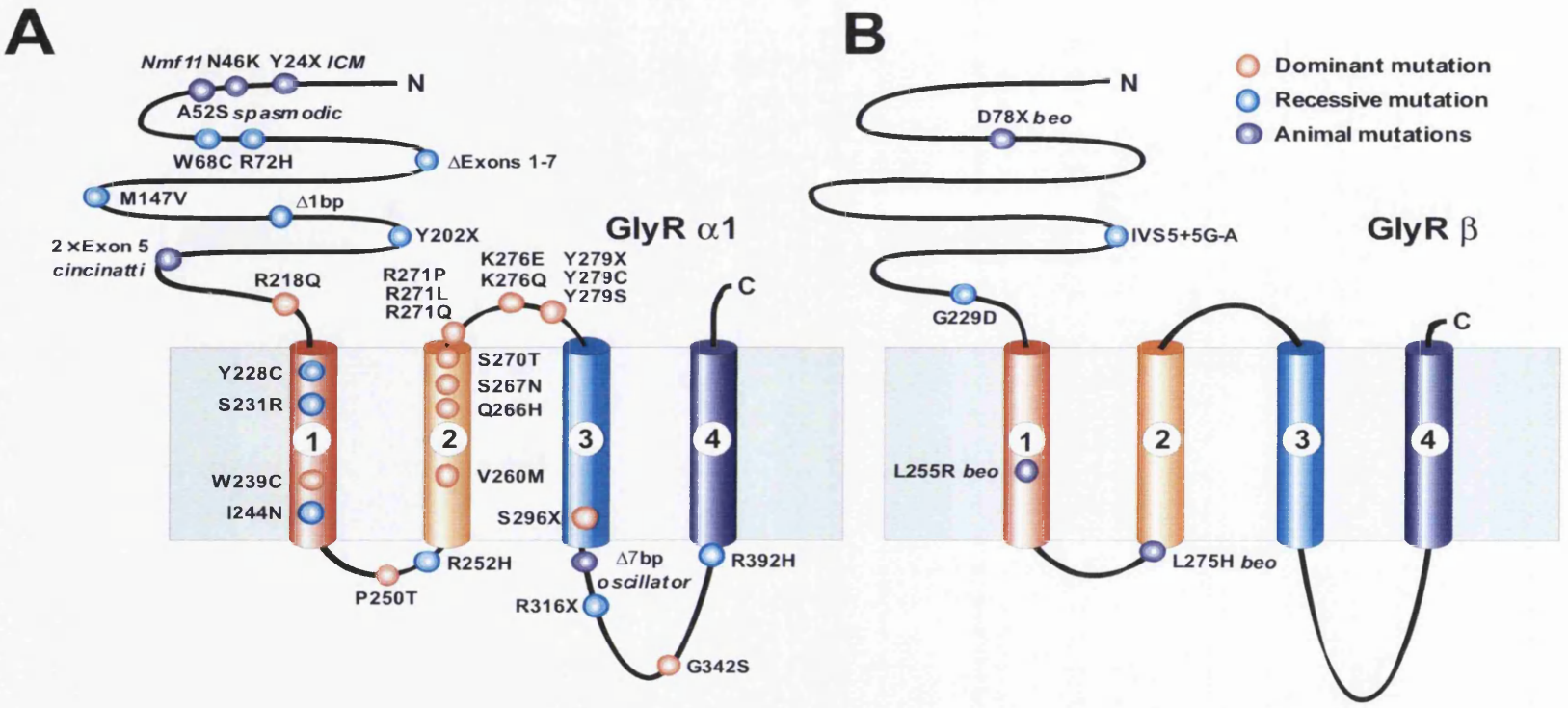


Figure 1.2 *GLRA1* and *GLRB* mutation spectrum for hyperekplexia mapped onto GlyR α1 / β subunit representations. *A) 2-D schematic representation of a single GlyR α1 polypeptide which consists a large N-terminal extra-cellular domain, followed by four membrane spanning domains (M1-M4) and a short extracellular C terminus. Human and animal GLRA1 mutations published to date are indicated. B) 2-D schematic representation of a single GlyR β polypeptide which shares the similar structure with α1 subunit. Human and animal GLRB mutations published to date are indicated. These figures are modified from Figure 1 in Harvey et al 2008 with permission from the author.*

mutations in these regions exert dominant-negative effects by altering the channel gating process, conductance or agonist sensitivity (Rajendra et al., 1994; Laube et al., 1995; Saul et al., 1999; Breitinger et al., 2001; Breitinger et al., 2004).

Ion selectivity of the ligand-gated ion channels is controlled by the charged residues in the pore lining region and the size of pore diameter. The smaller the pore diameter, the higher the permeability of anions (Lynch, 2004). The substitution of proline to threonine at the P250T mutation increases the pore diameter, thereby altering the channel conductance. The high-frequency R271Q mutation (identified in at least 18 independent families with dominant hyperekplexia), which substitutes a positively charged highly hydrophilic arginine to glutamine, can alter the channel conductance by changing polarity (Langosch et al., 1994).

All nonsense and frameshift *GLRA1* mutations identified to date are associated with recessive cases of HE with asymptomatic parental carriers, proving that hyperekplexia is not susceptible to haploinsufficiency (Brune et al., 1996; Rees et al., 2001). Identification and functional studies of two recessive missense mutations, I244N and S231R revealed a reduction in whole cell channel current possibly due to the defects in receptor trafficking or a decrease of membrane expression (Lynch et al., 1997; Rees et al., 2001; Humeny et al., 2002). Thus, it was suggested that the compensation mechanism is induced by a reduction of cell surface GlyR numbers, but not by the alteration in GlyR function (Humeny et al., 2002).

Further functional studies, however, are necessary to investigate whether the traffic defects and reduced number of cell-surface GlyR numbers are the initiating factor for the compensatory mechanism.

Recessive mutations interfere with some aspect of receptor expression, folding, assembly, trafficking, or stability. A $GABA_AR$ gamma-subunit mutation, R43Q, interferes with receptor assembly, trafficking, or surface expression (Kang and Macdonald, 2004)

1.2.4 Hyperekplexia animal models

Defects in glycinergic genes are also associated with hereditary motor disorders with exaggerated startle responses and increased muscle tone in a variety of mammalian species (Table 1.3) (Buckwalter et al., 1994; Kingsmore et al., 1994; Ryan et al., 1994; Kling et al., 1997; Becker et al., 2000; Becker et al., 2002). A LINE-1 transposable element in the *Glrb* results in aberrant splicing of the β subunit transcripts in the *spastic* mouse (Kingsmore et al., 1994). In *spasmodic,* a point mutation causing a A52S missense change in *Glra1* causes ligand sensitivity problems. In homozygous *spastic* and *spasmodic* mice, when the neonatal isoform of GlyRs change over to the adult isoform of the GlyRs (at about 2 weeks of age), a severe neuronal motor phenotype develops.

A microdeletion in the *Glra1* leads to a truncated protein devoid of the M3-M4 loop and M4 of the protein in *oscillator* mouse (Buckwalter et al., 1994). A severe neuromotor phenotype was also observed in homozygous *oscillator* 2 weeks after the birth and they usually die within a week. Mutations in glycinergic genes are also responsible for hyperekplexia in cows, dogs, goats, and various interbred mammals (Harvey et al., 2008). Gephyrin-deficient mice displayed phenotypes similar to human hereditary molybdenum cofactor deficiency and hyperekplexia further suggesting that gephyrin is essential for synaptic clustering of glycine receptors as well as molybdenum enzyme activity (Feng et al., 1998).

Table 1.3 Animal models of hyperekplexia

Gene	Genotype	Mode of inheritance	Functional consequences	Reference
Glra1	***Murine***			
	A52S	Recessive (*Spasmodic, spd*)	Reduced agonist sensitivity	Ryan et al., 1994 ; Saul et al., 1994
	microdeletion	*Recessive (Oscillator)*	loss of M3-M4 loop and M4 domain	Buckwalter et al., 1994; Kling et al., 1997
	S276Q			
	R271Q		Reduced GlyR expression	Becker et al., 2002
	Bovine			
	Tyr 24 X	Recessive		Pierce et al., 2001
Glrb	***Murine***			
	LINE 1insertion	Recessive (*Spastic, spa*)	down-regulation of *Glrb* mRNA	White& Heller, 1982; Kingsmore et al., 1994; Mulhardt et al., 1994; Becker et al., 1995,
	Rat GLRB in spa	*spa/spa* TG456	Low expression of rat *Glrb*	Becker et al., 2000
Gephyrin	***Murine***			
	deletion of exon1& upstream sequences		No gephyrin expression	Feng et al., 1998

1.3 Gephyrin

Gephyrin, meaning 'bridge' in Greek, plays two distinctive and independent roles; 1) clustering inhibitory Glycine and $GABA_A$ receptors at the postsynaptic membrane of neurons; 2) molybdoenzyme activity in non-neuronal tissues and glial cells in the CNS (Feng et al., 1998; Stallmeyer et al., 1999; Smolinsky et al., 2008). Rare defects in gephyrin can result in a neurological startle disorder, hyperekplexia, hereditary molybdenum cofactor deficiency and is disrupted in specific leukaemia translocation breakpoints (Reiss et al., 2001; Rees et al., 2003; Macaya et al., 2005). Gephyrin is encoded by a single-copy gene located on the chromosome 14 (14q23.3) in human and it has at least 29 exons, eleven of which undergo alternative splicing generating multiple isoforms (David-Watine, 2001; Rees et al., 2003).

The diversity of human gephyrin isoforms are mainly generated by alternative splicing of C3/C4cassettes in the central linker region of gephyrin (Rees et al., 2003) whereas in rodents, splicing exons in the N or E terminal regions also account for generating multiple isoforms (Meier et al., 2000; Fritschy et al., 2008) (Figure 1.3). Studies on rodent gephyrin isoforms demonstrated that the presence or absence of central linker region can interfere with the postsynaptic clustering of glycine receptor (Meier et al., 2000; Schrader et al., 2004; Bedet et al., 2006). Mice with complete loss of gephyrin by antisense primers or transgenic gene knockout resulted in severe depletion of GlyRs (Kneussel et al., 1999).

1.3.1 Gephyrin heterogeneity

Alternative splicing is one of the major mechanisms underlying the functional diversity of proteins and at least two thirds of multi-exon

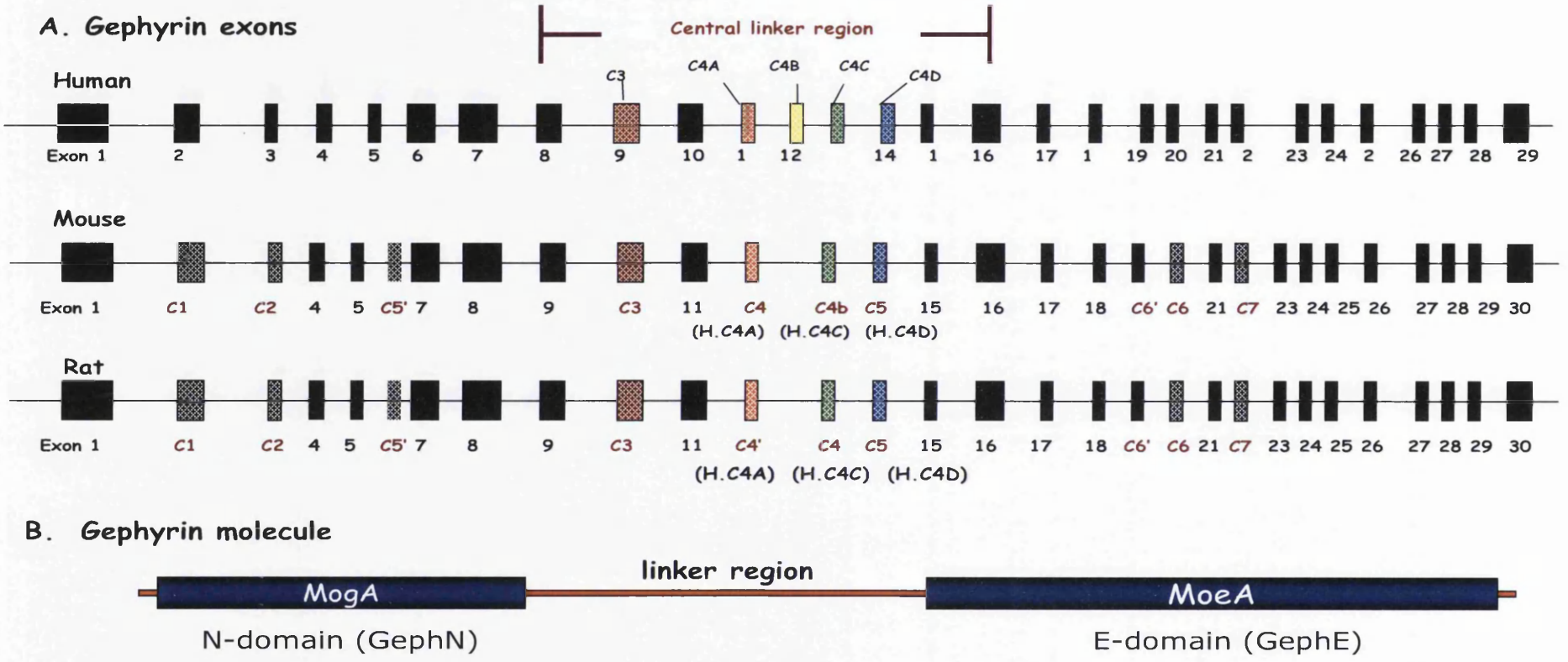


Figure 1.3 Schematic diagram of gephyrin gene-structure. *A) Gephyrin has at least 29 exons and eleven of which are subject to alternative splicing generating multiple isoforms in mouse, rat and human. In human, the diversity of gephyrin isoforms were mainly generated by alternative splicing of C3/C4 regions which constitute the central region of gephyrin. B) The N- and E-domain are homologous to Escherichia coli MogA and MoeA enzymes and are joined by the central linker region. N and E terminal regions are involved in the synthesis of molybdenum cofactor, whereas the linker region contains biding sites for multiple proteins and confers uncharacterised neuronal context to gephyrin transcripts.*

genes are subject to alternative splicing (Lander et al., 2001; Modrek et al., 2001; Johnson et al., 2003; Ben-Dov et al., 2008; Melamud and Moult, 2009). Particularly, the tissue-specific splicing events are most common in the nervous system to accommodate the functional diversity observed in neurological systems (Xu et al., 2002). Gephyrin, a multifunctional cytoplasmic protein, provides an outstanding example of tissue-specific alternative splicing in the neuronal context (reviewed in Fritschy et al., 2008).

Previous studies demonstrated that human neuronal tissues express over a dozen variants of gephyrin isoforms, generated from alternatively spliced exons, whereas non-neuronal tissues express a single gephyrin transcript (David-Watine et al., 2001; Rees et al., 2003). Functional studies on rodent gephyrin isoforms suggested different gephyrin isoforms exhibit different binding specificity and thereby affect protein-protein interactions (Meier et al., 2000; Bedet et al., 2006; Smolinsky et al., 2008). The variety and biological significance of gephyrin heterogeneity in the human brain, however, remains to be characterised and justified. Further analysis of gephyrin transcripts will lead to better understanding of the mechanisms underling the expression of specific splice variants and their functional roles in the dynamics of inhibitory neurotransmission.

1.3.2 Functional effects of gephyrin isoforms

The central region contains several potential protein-interaction domains whereas both N and E terminal regions, homologous to *Escherichia coli* MogA and MoeA enzymes, are involved in the synthesis of molybdenum cofactor (MoCo) (Kneussel and Betz, 2000; Fritschy et al., 2008). Recent findings suggest that gephyrin is involved in the modulation of signaling pathways by interacting

with several neurological partners including Mena/VASP (mammalian enabled/vasodilator stimulated phosphoprotein), dynein light chain, RAFT1 (rapamycin and FKBP12 target 1), collybistin, or the actin-binding protein profilin (Sabatini et al., 1999; Kins et al., 2000; Fuhrmann et al., 2002; Giesemann et al., 2003; Bausen et al., 2006). Thus, the activity of the central linker region is regarded as essential for brain-specific functions and studies in rodents suggest that heterogeneous gephyrin isoforms enable differential binding-specificity and thereby affect protein-protein interactions (Meier et al., 2000; Bedet et al., 2006). Gephyrin interacts with GlyR β subunit through its E-domain but recent studies on rodent gephyrin isoforms demonstrate that the presence or absence of central linker region can interfere with the postsynaptic clustering of GlyRs (Meier et al., 2000; Schrader et al., 2004; Bedet et al., 2006).

Previous studies demonstrated that gephyrin is ubiquitously present in the human brain and it is co-localized with the majority of GlyRs (over 50%) in the human brainstem and spinal cord (Baer et al., 2003; Waldvogel et al., 2003; Waldvogel et al., 2007). Although GlyRs are also identified in the upper brain regions, such as basal ganglia, auditory cortex and amygdala, the greatest concentration of glycine and GlyRs are located in the brainstem, spinal cord and retina (Greferath et al., 1994; Maksay et al., 2002; Waldvogel et al., 2003; Waldvogel et al., 2007).

1.4 Aims and Objectives of Research

1. To assess the *GLRA1* mutation spectrum in a large hyperekplexia cohort.

2. Investigate if a new candidate gene, *SCL6A5,* is responsible for hyperekplexia in *GLRA1*-negative cohorts.

3. Establish the functional basis of *GLRA1* mutation pathogenicity using biophysical assays, cell-surface experiments and molecular modelling.

4. Investigate the extent and baseline of gephyrin isoform heterogeneity in specific areas of the human brain, the spinal cord and retinal tissues.

5. To complement and accompany objective 4, investigate the distribution of gephyrin isoforms in the human brain and spinal cord using new custom-made gephyrin antibodies.

6. Immunofluorescent co-localisation studies with the new gephyrin cassette antibodies with GlyR receptor subunits and constitutional full-length gephyrin in normal human brain samples.

1.5 Justification of Research

Although, the mechanisms of inhibitory neurotransmission have been intensively studied in many neurological diseases such as Alzheimer's disease, Parkinson's disease, hyperekplexia and psychiatric disorders (Cyr et al., 2002; Grossberg, 2002; Moghaddam, 2002; Segovia, 2002; Brotchie, 2003; Spedding et

al., 2003), the mechanisms underlying the inhibitory network are still relatively undefined.

The structural and physiological properties of GlyRs have been extensively investigated using random site-directed mutagenesis approach (Shan et al., 2002; Nevin et al., 2003; Shan et al., 2003; Han et al., 2004; Hawthorne et al., 2006; Press et al., 2007; Yang et al., 2007; Heads et al., 2008; Press et al., 2008; Yang et al., 2008; Chen et al., 2009; Press et al., 2009), however, these studies do not provide us with clinical perspective and the in-vivo biological significance of the receptor activity. Furthermore, the hyperekplexia literature is dominated by small studies or single case-studies where it is difficult to arrange meaningful cohort-based research or establish translational genetics trends. There is also the false perception that hyperekplexia is a dominant disease when slowly, but surely, the evidence-base is indicating that it is a recessive disorder on a world-wide basis.

With 88 patients gathered over 15 years from centers around the world, this study regards our assembled cohort as a compelling resource to bypass the numbers limitation to establish trends. The outcomes of the genetic screening will create 2 research opportunities; the first is the pathophysiological characterization of the gene-positive cases, and the second being further gene-discovery work in those cases who remain gene-negative. This genetic triaging has the advantage of respective patient audit to establish phenotypic trends that will improve future study inclusion criteria.

The discovery of any clinically-relevant mutation in receptor biology triggers an opportunity to define pathophysiological mechanisms and a direct cause of disorder onset. This also can provide insights

in other channelopathy disorders (e.g. epilepsy, arrhythmia, migraine) where similar gene-family structures (e.g. GABA, ACh receptors) can adopt principles from other channelopathy findings. As the research on inhibitory systems becomes more developed then the baseline research can be used in the development of new interventions and also provide insights into drug-resistance and side-effect causes in existing interventions.

The controlled expression and clustering of glycine receptors at the postsynaptic sites are vital for normal inhibitory function. Recent studies demonstrated that the stable clustering of GlyRs at the synaptic sites can be achieved through interacting with gephyrin as well as the controlled expression of gephyrin isoforms (Bedet et al., 2006). The altered level of gephyrin has been implicated in neurological disorders such as Alzheimer's disease (AD), chorea-acanthocytosis (ChAc) and amyotrophic lateral sclerosis (ALS) (Kurano et al., 2006; Lorenzo et al., 2006; Agarwal et al., 2008). In this study, the heterogeneity of gephyrin splicing cassette at the human brain will be examined in both mRNA and protein levels. Understanding the mechanisms of the startle disease and the characterization of the essential scaffolding molecule gephyrin, may ultimately provide important new insights into the pathophysiology and treatment of other neurological disorders that are correlated with inhibitory neurotransmission.

1.6 Experimental Strategy

From the beginning of this thesis work in 2005 there has already been a shift in technology platforms and so this must be considered in context with the following experimental approaches.

1.6.1 Mutation analysis (Chapter 3)

Based on the small cohort sizes and fragmented evidence-base for the genetic contribution of *GLRA1* in hyperekplexia, we have screened the entire coding regions of *GLRA1* gene for our eighty-eight hyperekplexia samples. Although *GLRA1* is currently the major gene for hyperekplexia, more than two-thirds of screened patients are gene-negative. Therefore, using the candidate gene approach, we have selected a second gene to screen based on the hyperekplexia-like phenotype observed in GlyT2 knockout mice (Gomeza et al., 2003). The GlyT2 gene (*SLC6A5)* was analysed in all GLRA1-negative cases using Transgenomics dHPLC technology and direct sequencing due to the expense of mutation screening of both *GLRA1* and *SLC6A5* genes. dHPLC has now been superseded by the Idaho technologies LightScanner platform which is now used in continuing screening efforts.

1.6.2 Functional analysis of GLRA1 mutations (Chapter 4)

As new GLRA1 genotypes were identified the next step was to prepare for the biophysical characterization of the new gene variants. The experiments were conducted as a visiting RA at laboratories in London and Brisbane as part of a multi-centre collaboration. The generation of mutant GLRA1 constructs and immunostaining experiments were hosted by the School of Pharmacy (with Professor Robert Harvey) in London. The electrophysiological studies of mutants were conducted at the University of Queensland during a total of nine months of stay in 2007 and 2008. In addition to the molecular genetics, the cell-surface biotinylation experiments and molecular modeling was completed within ILS, Swansea. The aim here was to provide convincing multi-disciplinary proof for *GLRA1* pathogenicity.

1.6.3 Molecular basis of gephyrin isoforms in human brain (Chapter 5)

To investigate the heterogeneity of gephyrin isoforms and biological basis behind GlyR clustering, cassette-specific primers and antibodies were generated. The expression level of cDNA transcripts were determined using RT-PCR and real-time PCR analysis. Whereas the distribution of the gephyrin proteins containing specific-cassettes was examined using immunohistochemical analysis of human brain sections from medulla oblongata and the cervical spinal cord. Further double-labelling immunocytochemical analysis was adopted to establish co-localisation trends between gephyrin cassettes and GlyRs.

Chapter 2 Materials and Methods

This chapter will describe the methods and technical details of the experiments undertaken to deliver the experimental objectives outlined in Chapters 3-5. Several appendices have been prepared to add some background and indirect context to the work.

2.1 Mutation analysis of hyperekplexia

The molecular genetic analysis of candidate genes uses the techniques outlined in this section and relates to the results described in chapter 3.

2.1.1 Patients

With informed consent procedures in place, patients with suspected hyperekplexia were ascertained by referral from neurologists, paediatricians or clinical geneticists from centres around the world. Eighty-eight anonymized index patients were included in this study following phenotypic evaluation. Clinical inclusion criteria states that phenotypes such as non-habituating startle response (positive nose tap test), history of infantile hypertonicity and an exclusion of mimics such as startle-epilepsy (see Appendix A) are markers for human hyperekplexia. The 88 patients were then tested by PCR amplification methods and genomic sequencing in two hyperekplexia candidate genes.

2.1.2 DNA extraction from blood

DNA was extracted from the patient's blood using standard phenol-chloroform extraction. Approximately 5 -10mls of the patient's blood was placed into a 50ml falcon tube and red cell lysis buffer

was added to constitute a total volume of 50ml. The solution was placed on ice for 15 minutes and then centrifuged for 15 minutes at 3000 rpm. The supernatant was discarded into a dedicated disposal bottle and a further 40ml of red cell lysis buffer was added. The tube was gently vortexed to break up the pellet, and centrifuged for 10minutes at 3000rpm. After the removal of the second supernatant, 4.5ml of proteinase K resuspension, 250µl of 10% SDS and 100µl of proteinase K was added. Following gentle agitation of the digest mix, the solution was incubated in a 37°C water bath overnight. All DNA samples were stored at -20°C.

2.1.3 Polymerase Chain Reaction (PCR)

The genomic sequences of *GLRA1* and *SLC6A5* were determined using BLAST 2.2.14 (NCBI) software. The exonic fragments and exon-intron boundaries of *GLRA1* and *SLC6A5* were amplified using primers designed using the Primer 3.0 program (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). To prevent allelic dropout, all primers were placed in intronic regions that were devoid of known SNPs as revealed by the SNP database at NCBI (Tester et al., 2006) and care was taken to avoid obvious short-repeat and alu-like structures. Primer sequences for both genes are presented in table 2.1.

Optimal PCR protocols were established for *GLRA1* and *SLC6A5* exons using two control DNA samples with Qiagen PCR solution (Table 2.2). Genomic DNA was diluted to 8 ng/µl and PCR was performed in a total volume of 25 µl of a solution containing 80 ng of genomic DNA, 10 pmol of each forward primer and reversed primer, 200 µM deoxynucleotide triphosphates (dNTPs), 1 Unit Taq DNA polymerase (Qiagen) using a PTC-200 thermal cycler (MJ Research Inc). Amplification conditions were an initial denaturation at 94°C for 5 minutes followed by 30 – 38 cycles at 94°C for 30

Table 2.1 PCR primers for GLRA1 and SLC6A5

GLRA1 Primers		
Exon	Forward Primer 5'-3'	Reverse Primer 3'-5'
1	GAAATATACCCACCCCCAAAC	GGTAGCCTCCGTACTCTTTCC
2	TAACCTGGGCCTTACCTCATT	CTGCTTGCTGCTTTAATCTGG
3	CAAGGATCTCCTCCACCAAA	TGGAGACCAATGCAGAGGATA
4	ACCCCCATATAAGATGCAACC	TTTGGCCCCTCTTTTTAGAGTC
5A	CCTATCCTGGGCAACTGATTTT	GCCTATCCCATGGGTAAAAAG
5B	CCTGGTCTCACAAGTTCCATC	AAATGACCTCTGGTCCTGGTT
6	TGTGAGACTGAACCAGGACTCT	TGTTTTAGGCAGAGCAAGGAA
7	ATGTGGGGAATTACCGAAGAG	GAAGGATGGACCATTGAAACA
8	GAAATATACCCACCCCCAAAC	TGCATCACTGCATTTTGCTAT

SLC6A5 Primers		
EXON	Forward Primer 5'-3'	Reverse Primer 3'-5'
1	CTCTACAGCCCGATCCACTCT	AAACGAATCTGCTTTCCCTGT
2	TAAAAGCTGTTGTGACTTTGTTTT	GACACTGTGCGGGCCGTAAT
3	CTTGCTGGGAAGGACCCCTA	TCCCACCCAACCCTCAGGTG
4	CAGGAATGGAGCTAAATTGTCC	GACAGAGTAAGAAAGGGCCTGA
5	TCCATTCTGTACAAGAGAGCCTA	CTCTGTGTTCCCAGAACCTAGT
6	GCAAATGTTTTTGGCATTTGT	CACCTCTGGTCTGCAAATTGA
7	TGTGATTAGGTTCCTGATGGTG	CAGCTCCTGACTCTCTTTCCA
8	CCCTGATGTGCTCTCTGTCAT	GTCTAACCCTTTTTGCCCAAG
9	TTTCCCCTGGAAACATGATA	GTGGCCTCAGGTGTCTGATAA
10	GCACACCTAATGGAAAACTCTG	TCCTCCACCCTCTATCCTGTT
11	ACCAACAGTGGAAGCAGCATA	AGGGGGAGTCTTCAAGAGGAG
12	GCCATCCTAAAAACCAAACCT	CATGGAAATGGTAGTGATTTGC
13	TCCTGTTTGCACCTGACTCTT	CATGAATGCCTTACCGACACT
14	GCTTGAGTGAGGGGCTCTAGT	ACGTATGCAAGGTGCTGTCTG
15	CGAAAGCATCAAACTCATAACG	GGCAACTGTAATCGCTTCATC
16	CAAGGACTCTGGTCAAAGTGG	AAATGGGAGGAGAGCTATGGAA

Table 2.2 Amplification conditions for GLRA1 and SLC6A5

GLRA1			
Exon	Amplimer Size (bp)	PCR Temp (oC)	PCR Cycles
1	453	60*	35
2	347	60	32
3	267	60	32
4	448	60*	32
5A	243	60	32
5B	321	60	32
6	392	60	32
7	347	60	32
8	585	55Q*	35

SLC6A5			
Exon	Amplimer Size (bp)	PCR Temp (oC)	PCR Cycles
1	491	60	32
2	620	55Q	34
3	300	60	32
4	306	60	32
5	400	60	32
6	326	60	32
7	379	60	32
8	325	60	32
9	287	55	32
10	386	60	32
11	294	60	32
12	379	60	32
13	272	60	32
14	239	60	32
15	397	60	32
16	350	60	32

*Q= addition of Q solution (Qiagen) required, *=45 seconds annealing time required. All exons underwent 32 cycles of denaturation at 94 °C s, extension at 55-64 °C s and annealing at 72 °C s unless otherwise stated.*

seconds, 55-64°C annealing temperature for 30 seconds, and 72°C extension temperature for 30-45 seconds.

2.1.4 Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate and isolate DNA fragments. The percentage of agarose in the gel was selected based on the target size of DNA. Agarose gels at 1.5 % were selected for the majority of gels, as this was the optimal concentration for separation of DNA fragments between 200 bp and 500bp in length, a range encompassing the majority of the exons in the hyperekplexia gene assays. For restriction enzyme digests (see section 2.1.5), 2% gels were used to separate smaller fragments produced by cutting of the PCR fragments.

To generate 1.5 % agarose gels, 1.5 g of dried agarose was dissolved in 100 ml of 0.5 X TBE (tris-boric acid-edta) buffer by boiling and after cooling the solution to about 70 °C, the gel was poured into a casting tray containing a well-forming comb. The gel was allowed to solidify at room temperature. Set agarose gels were submerged in 0.5 X TBE buffer (Sigma) in a horizontal electrophoresis apparatus and the DNA samples mixed with 6X gel loading dye (Sigma) were pipetted into the sample wells. Electrophoresis conditions were usually set at 80 –120 V for about 1 h, depending on the size of the gel. DNA fragments were then visualized under UV light by staining gels with SYBR-Green (1:10,000, Invitrogen), a fluorescent dye that intercalates between bases of double stranded nucleic acids.

2.1.5 Denaturing High-Performance Liquid Chromatography (dHPLC)

Transgenomic dHPLC analysis was used to rapidly identify mutations in *SLC6A5* gene, since at the time it was considered the optimal method for mutation detection due to a high degree of automation and high-throughput detection rate (Xiao and Oefner, 2001). dHPLC detects single nucleotide polymorphisms (SNPs) based on the melting temperature differences between hetero- and homo-duplexes of wild - type and mutated DNA and the sensitivity of the analysis is maximized by maintaining the column temperature for successful heteroduplex separation (Figure 2.1).

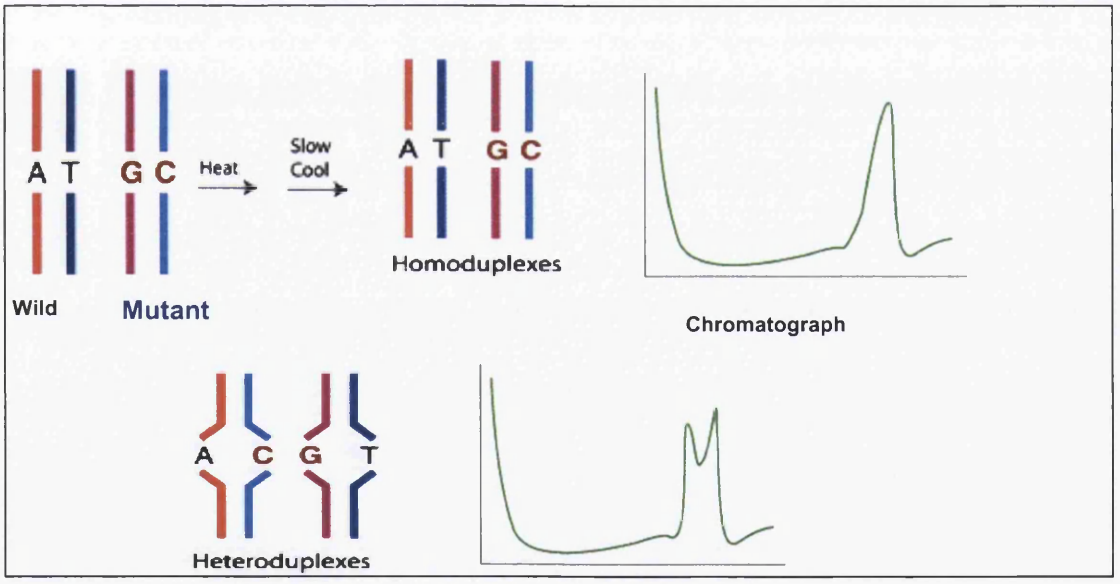
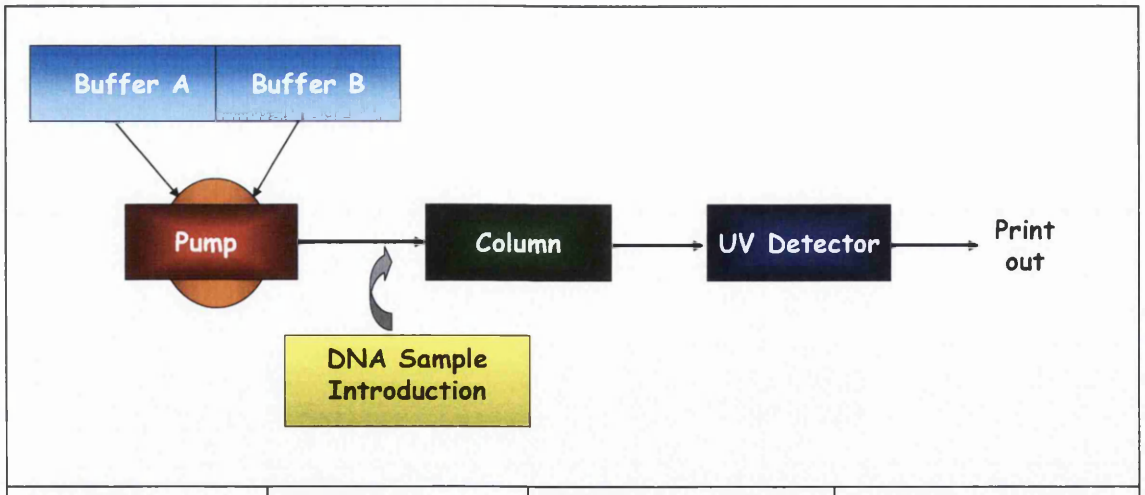


Figure 2.1 Hetero and homoduplexes resolution on the dHPLC. *When an individual is heterozygous for a mutation or polymorphism in a sequence, heating the DNA to 94°C and cooling of the PCR product results in the formation of heteroduplex molecules between the wild-type and mutant sequences. This heterozygosity can be identified by dHPLC when analyzed under partially denaturing temperatures. Mutation detection was based on the visualization of chromatogram shape differences between homozygous and heterozygous samples.*

For each of the exon assays, the optimal partial denaturing temperatures were determined using interpretation of the DNA melting properties by the Navigator version 1.5.1 software.

Prior to dHPLC, 5 µl of PCR product was checked on a 1.5% agarose gel to verify the yield and purity of the amplified DNA fragment. The remaining 20 µl of PCR product was then heteroduplexed in preparation for dHPLC analysis. This was achieved by denaturing at 95°C for 5 minutes and slowly cooling to 4°C (0.1°C/sec) using an automated program on the PTC-200 thermal cycler to generate either homoduplex or heteroduplex molecules if a mismatch of base pairs is present. dHPLC analysis was performed on the Transgenomic 2100 WAVE^{R} DNA fragment analysis system (Transgenomic Inc), using a DNASep HT cartridge and Navigator version 1.5.1 software.

The heteroduplexed sample (5 µl) was automatically injected on a preheated column and eluted on a linear acetonitril gradient with a constant flow-rate of 1.5 ml/min (Figure 2.2). Heteroduplex molecules were separated from homoduplex DNA molecules by ion-pair reverse-phase liquid chromatography in a partial denaturing environment. At the partial denature temperature, the mismatch region of the heteroduplexes begins to melt and the DNA becomes less hydrophobic causing heteroduplexes to elute earlier than homoduplexes. The difference in elution time between hetero- and homo-duplexes, produces distinct patterns of elution profiles with heterozygous samples including double or multiple peaks, peak shift, plateau, or curves. Any sample that displays a variant chromatogram profile was selected for ABI 3100 sequencing.



BUFFER A	BUFFER B	BUFFER C (syringe washing solution)	BUFFER D (column wash solution)
Acetonitrile: 250µl TEAA: 50ml H_2O (dHPLC grade) ~ 950ml	Acetonitrile: 250ml TEAA: 50ml H_2O (dHPLC grade): 700ml	Acetonitrile: 80ml H_2O (dHPLC grade): 920ml	Acetonitrile: 750ml H_2O (dHPLC grade): 250ml

Figure 2.2 Schematic diagram of dHPLC system flowpath. *dHPLC uses a polyacrylamide gel matrix to separate homo- and hetero-duplex species like conventional heteroduplex analysis, but in a denaturing environment, to enhance the sensitivity of detection between the two species. Aliquots of PCR product were automatically loaded on the DNA SepR column packed with polystyrene-divinylbenzene polymers, and stationary phase occurs due to binding of the positively charged TEAA (triethylammonium acetate) ion pairing molecules and partially unwound DNA fragments with the negatively-charged backbone exposed (Xiao and Oefner, 2001). DNA was then eluted from the stationary phase by using a linear acetonitrile gradient formed by mixing buffer A and buffer B. DNA which is progressively eluted from the column is detected using a ultra-violet detector, which allows the DNA fragments to be collected. Recipes for buffer solutions for Transgenomic 2100 Wave DNA fragment analysis dHPLC machine are also shown (Transgenomic Inc.)*

2.1.6 Sequencing analysis.

PCR products of DNA samples were electrophoresed on 2 % agarose gels (Roche Applied Sciences) and then purified with QIAquick Gel Extraction / QIAquick purification kits (QIAGEN Inc). The purified DNA fragments were directly sequenced using Big Dye Terminator and ABI automated sequencer (ABI3100; Applied Biosystems). DNA samples and primers were submitted at 5 ng/µl

and 1.5 pmol/µl respectively. The sequence of nucleotides on the chromatograph was compared to the normal established sequence using a mutation surveyor (vergion.3.2, SoftGenetics, State College, PA) and any changes were investigated for mutational consequence.

2.1.7 Restriction Fragment Length Polymorphism (RFLP) tests

Mutations identified by sequencing analysis were confirmed by a RFLP test. Genetic mutation due to a single or multiple base pair changes could result in the loss or gain of specific restriction enzyme sites. Digestion of the DNA fragment containing the restriction site with an appropriate restriction enzyme could distinguish alleles or variants based on resulting fragment sizes via electrophoresis, and this type of polymorphism was thus referred to as 'restriction fragment length polymorphism'.

A restriction map of the exon was established for both the normal and the mutant sequences using the restrictionmapper software (http://www.restrictionmapper.org/). Comparison between the normal and mutant maps reflects the differential loss and gain of restriction enzyme cut sites. A restriction enzyme was chosen based on availability and price when more than one enzyme was applicable. A total of 25 µl of the PCR products were digested overnight at the required temparature with 1U of enzyme in a total volume of 5µl. DNA fragments were separated on a 2 % agarose gel to assess SNP or mutation frequency in control samples. RFLP mapping can also be applied to the screening of family members of a proband with a known mutation, as a confirmatory assay to accompany the sequencing data.

2.2. Functional analysis of GLRA1 mutations

As a consequence of mutation discovery, the following techniques were adopted and correspond to the research findings in Chapter 4 of this thesis.

2.2.1 Mutagenesis and expression of cDNAs

The full-length human GLRA1 α1 and β-subunits were cloned into the pRK5 vector (provided by Prof Rob Harvey, University of Pharmacy, London) and the pIRES2-EGFP plasmid vector (Clontech, Mountain View, CA, provided by Prof Joe Lynch, QLD Brain Institute, University of QLD, Australia), respectively. Mutant GlyR α1 subunits were constructed using a QuickChange Site-directed mutagenesis system according to the manufacturer's protocol (Stratagene, La Jolla, CA, USA). All cDNA constructs were confirmed by sequencing the entire coding region. Wild-type (WT) or mutant GlyR α1 subunits were transiently expressed in human embryonic kidney cells (HEK-293) using Qiagen's Effectgene transfection reagent or a calcium phosphate technique (Sambrook et al., 1989). HEK 293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, La Jolla, CA) supplemented with 10% of fetal calf serum (Invitrogen) and 5% of a mixture of penicillin and streptomycin (100 U/ml penicillin ; 100 g/ml streptomycin; Invitrogen) at 37°C in 5% CO_2. Homomeric GlyR α1 subunits were co-transfected with empty pEGFP vector (Clontech, Mountain View, CA) as a transfection marker in a 1:1 ratio. For heteromeric GlyRs, α1 and β subunit were transfected at a DNA ratio of 1:10 (Lynch et al 2008). Homomeric alpha1 subunit or heteromeric alpha and beta subunits of GlyRs were distinguished by applying 100μM picrotoxin (PTX), which inhibits homomeric GlyRs but not heteromeric GlyRs (Lynch, 2004). Twenty four hours after transfection, cells were washed in Phosphate Buffered Saline

(PBS) twice, and electrophysiological recordings were made at room temperature within the following 24-48 hours.

2.2.2 Electrophysiology - Patch clamping

Whole-cell patch-clamp recordings were performed on HEK293 cells placed in an external solution consisting of: 140mM NaCl, 5mM KCl, 2mM $CaCl_2$, 1mM $MgCl_2$, 10mM HEPES, 10mM glucose, adjusted to pH 7.4 with NaOH. Electrodes were pulled from borosilicate glass hematocrit tubing (Vitrex, Modulohm, Denmark) with a horizontal puller (P97, Sutter Instruments, Novato, CA, USA) and heat-polished. The electrode has resistances of 1 - 3 MΩ when filled with an internal pipette solution consisting of: CsCl 145mM, $CaCl_2$ 2mM, $MgCl_2$ 2mM, HEPES 10mM, EGTA 10mM, adjusted to pH 7.4 with NaOH. Glycine-gated currents were recorded using the whole-cell patch-clamp configuration at a holding potential of −40 mV using Axon instruments (Union City, CA, USA). Solutions were applied to whole cells via gravity through parallel micro-tubules. The perfusion system was under the control of a manual micromanipulator and solution exchange was routinely complete within 100 ms between adjacent tubes. Currents were digitized at 1 kHz, filtered at 500 Hz and digitally recorded on a computer using an Axopatch 1D amplifier and pClamp9 software suite (Axon Instruments, Union City, CA).

The half-maximal concentration (EC_{50}) and the Hill coefficient (n_H) values for activation were calculated for an individual cell separately using the Hill equation (SigmaPlot 9.0, Systat Software). Currents from individual cells were normalized to the maximum response at saturating glycine concentrations. The averages of the current amplitudes from individual cells were used to construct a dose-response curve by fitting data into the Hill equation through a

nonlinear least squares analysis (SigmaPlot 9.0, Systat Software). For tonic open channel activity, the voltage-clamp protocol used to determine the current-voltage (I-V) curves was voltage-ramps from a holding potential of −80 mV to a target potential of +20 mV over 2-s interval.

2.2.3 High-throughput fluorescent system

Forty eight hours after transfection, HEK293 cells were screened using an automated high-throughput system using the yellow fluorescent protein (YFP) mutant, YFP-I152L as previously described (Kruger et al., 2005; Gilbert et al., 2009). Briefly, HEK293 cells were co-transfected with mutant/WT α1 and YFP-I152L within the pcDNA3.1 vector. Twenty four hours after transfection, cells were plated into a 384 well plate (approximately 2.5 × 103 cells/well). Within the following 24-32 hours, cells were incubated in 25μL standard control solution (NaCl 140mM, KCl 5mM, $CaCl_2$ 2mM, $MgCl_2$ 1mM, HEPES 10mM, and glucose 10Mm; pH 7.4 using NaOH) for one hour. Ten different glycine concentrations (1μM-30mM) were prepared in NaI solution (NaI 140mM, KCl 5mM, $CaCl_2$ 2mM, $MgCl_2$ 1mM, HEPES 10mM, and glucose 10Mm; pH 7.4 using NaOH). Using the automated high-throughput system, fluorescence images of each well were obtained twice before and after the application of NaI solution containing a varying concentration of glycine (Figure 2.3). Individual concentration responses were constructed by pooling results from 2-wells exposed to NaI solution containing the same glycine concentration. The averages of the fluorescence from individual cells were used to construct a dose-response curve by fitting data into the Hill equation (SigmaPlot 9.0, Systat Software). For each mutant, screening was replicated at least 3 times.

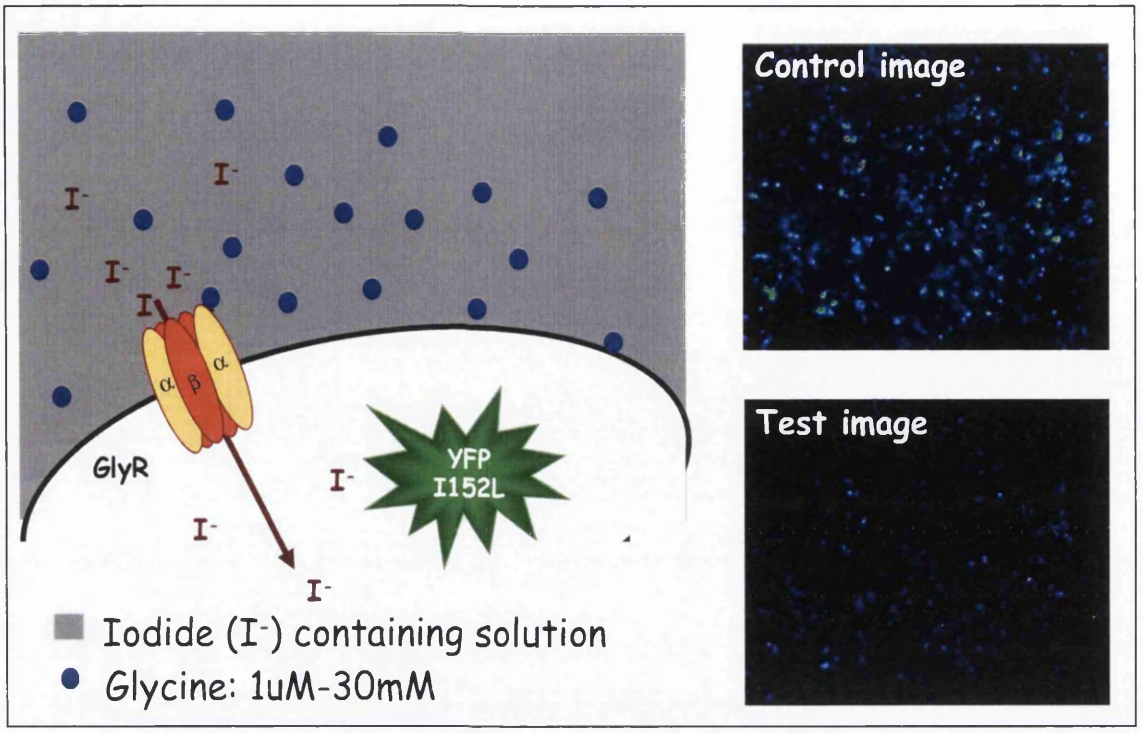


Figure 2.3 Automated high-throughput fluorescent system. *The anion-sensitive yellow fluorescent protein (YFP) mutant, YFP(I152L), highly sensitive to I⁻ and transfection of HEK 293 cells with YFP(I152L) leads to fluorescence cells (Control image). EK 293 cells transfected with YFP(I152L) and GlyRs can be quenched by influx of I⁻ through anion channels such as GlyRs (Test image). A typical image contained average of 500 fluorescence cells that can be used for analysis. Glycine concentration-response relationships can be fitted with the following equation:* $F = F_{init} / (1 + (EC50/[Glycine])nH)$*, where F is the fluorescence level following the application of a particular glycine concentration, [glycine];* F_{init} *is the initial (or control) fluorescence.*

2.2.4 Biotinylation- cell surface expression assay

Two days after transfection, surface expression of GLRA1 subunits in HEK293 cells were investigated using a cell membrane-impermeable reagent Sulfo-NHS-LC-Biotin (Pierce Biotechnology, Rockford, IL, USA) based on the manufacturer's protocol. Briefly, transfected HEK293 cells in a 100ml dish were washed twice with ice cold PBS buffer and incubated in 10ml of PBS containing 0.25mg/ml Sulfo-NHS-SS-biotin for 30 min at 4 °C with gentle

shaking. Excess biotinylating reagent was then removed by adding Quenching solution followed by washing the cells twice with TBS. Cells were lysed using a lysis buffer supplemented with protease inhibitors. To remove non-soluble fraction, the lysate was centrifuged at 14,000 x g for 15 min at 4°C and then the clear supernatant removed for further analysis. The total protein concentration was measured with a DC (detergent compatible) Protein Assay (Bio-Rad, Hercules, CA) and 50µg of total protein was removed for immunoblotting analysis. To separate biotin-labelled surface proteins, 500µg of protein was incubated with 125µl of immobilized biotin-binding protein, NeutrAvidin gels for 1h at room temperature (RT). The biotin protein attached beads were then washed four times with a wash buffer. The surface proteins were isolated from NeutrAvidin linked biotin molecule by incubating with 100µl of a sample buffer containing 50mM of reducing reagent dithiothreitol (DTT) for 1h at RT. The surface proteins were then eluted by centrifugation at 1000 x g for 2 minutes. Total protein lysates were also obtained from cells transfected with mutant or WT GlyR α1 and β subunits by lysing cells as described above, but without the biotinylation labelling. The expression levels of GlyRs were analyzed using anti-human GlyR α1 antibody (1:1500; Millpore, Billerica, MA).

Proteins in the whole cell lysates or cell surface proteins were separated on 4-12% Bis-Tris gels (Biorad) and transferred to nitrocellulose membranes (Invitrogen). The blots were blocked with a blocking buffer (Invitrogen) then incubated with an appropriate antibody at 4°C overnight / 1hour at room temperature. After three washes with Tris-Buffered Saline Tween-20 (TBST), the blots were incubated with secondary anti-mouse or anti-rabbit IgG (GE healthcare, UK) for 1hr at room temperature. Proteins were visualized with a gel imaging system (Biorad). An

anti β-actin antibody (1:7000; ABcam) was used as a control to confirm that the intracellular proteins were not labelled with biotin. The intensity of immunoreactivity signal was quantified with ImageJ software (http://rsb.info.nih.gov/ij/)

2.2.5 Statistical analysis

Data were analysed using Sigma plot (Systat Software, version 9.0; Point Richmond, CA, USA) and GraphPad Prism version 3.02 (GraphPad Software Inc. San Diego. CA) and expressed as mean± SEM. Statistical significance was determined by Student's t-test and considered to be significant at $P<0.01$.

2.2.6 Immunostaining

To examine the subcellular localisation the GLRA1 mutants, HEK293 cells were transfected with the α1 construct using Qiagen's Effectgene transfection reagent. For live-staining, 24 hours after transfection, cells were washed twice with PBS, then incubated in 10% (vol/vol) FCS + 0.5% (wt/vol) BSA (FCS/BSA) in PBS for 15 min at RT. Cells were then incubated with a primary antibody against GlyR α1 subunit (1:400 dilution, Millipore) for 1-hour at RT. After three washes with FCS/BSA in PBS, the cells were incubated with secondary fluorochrome conjugated antibody (1:200, Alexa 488 goat anti-rabbit, Invitrogen) for 30 min at RT (Figure 2.4). Cells then washed three times with FCS/BSA in PBS, followed by two washes with PBS. After fixing cells in 4% paraformaldehyde (PFA) in PBS for 5 min, fixed cells were incubated with the quenching solution containing 50 mM NH_4Cl for 10 min followed by a further wash with PBS. For normal

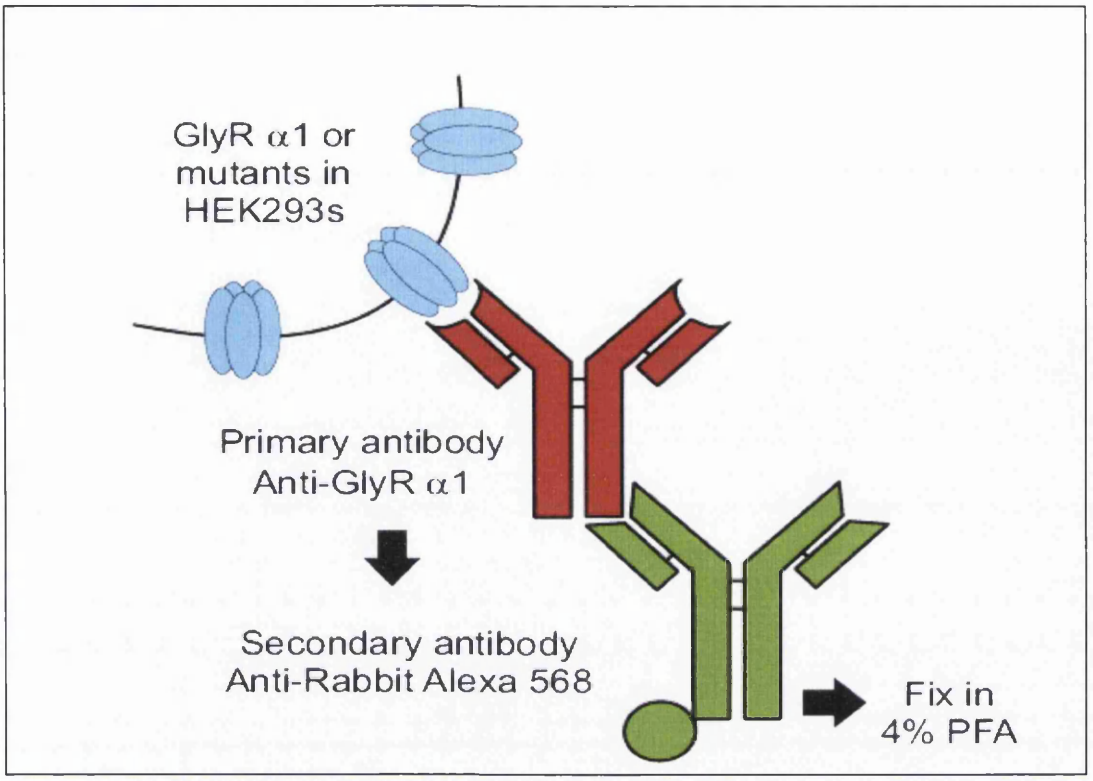


Figure 2.4 Schematic diagram of immunostaining of HEK 293 cells transfected with GlyRs. *GlyR αl subunit expressed on HEK293 cells were detected against the primary antibodies against the N-terminal regions of GlyR αl subunit, and subsequently detected using secondary antibodies. In contrast to normal staining method where the cells were fixed with PFA before the antibody incubation, by fixing cells with PFA after the antibody incubation, only cell surface expressed GlyR αl can be detected.*

staining, cells were fixed with 4% PFA prior to the primary antibody incubation.

2.2.7 Molecular modelling

Structural modelling of wild-type and mutant GlyR α1 was carried out using a homology modelling pipeline built with the Biskit structural bioinformatics platform (Grunberg et al., 2007), which scans the entire protein data bank (PDB) for candidate homologies. The best homology attained for GlyRα1 was based on 26% identity with the crystal structure of the nicotinic acetylcholine receptor

(PDB: 2BG9). Our pipeline workflow incorporates the NCBI tools platform (Wheeler et al., 2007), including the BLAST program (Altschul et al., 1990) for similarity searching of sequence databases. In this case, protein sequences corresponding to the PDB of protein structures were searched for homology with the C-terminus of GlyT1 in order to identify putative structural homologues. A multiple sequence alignment software T-COFFEE (Notredame et al., 2000) was used for alignment of the test sequence with the template. Homology models were generated over 10 iterations of the MODELLER program (Eswar et al., 2003), and the DSSP algorithm (Kabsch and Sander, 1983) applied for secondary structure validation. All models were visualized using the molecular graphics program Chimera (Pettersen et al., 2004).

2.3 Analysis of Gephyrin heterogeneity

This section details the techniques used to deliver the research outputs for Chapter 5 with supporting literature (Rees et al 2003; Waldvogel et al 2006)

2.3.1 RT- PCR analysis of gephyrin isoforms

Primers were designed to target the C3/C4 linker region of Gephyrin, forward, 5'-CAGTGGTGTTGCTTCAACA GA-3'; reverse,5'-TCAGAGGAA AAGAGCATGC-3'. Total RNA from human adult brain, fetal brain, spinal cord and retina (Clontech, Mountain View, CA,) were reverse-transcribed using 1µl of total RNA, 10pmol of oligo (dT) primers and SuperScript III (Invitrogen, La Jolla, CA, USA), and then PCR-amplified. Amplification conditions were an initial denaturation at 94 °C for 5 minutes followed by 30 – 38 cycles at 94 °C for 30 seconds, 55-64 °C annealing temperature for 30 seconds, and 72 °C extension temperature for 30-45 seconds. PCR

products from the DNA samples were electrophoresed on 1.5 % agarose gels and C3/C4 band was extracted from the agarose gel using scalpel blades, then purified with QIAgel agarose gel extraction purification kit (QIAGEN Inc). Purified PCR products of C3/C4 regions were cloned into pGEM-Easy vectors (Promega, USA) by incubating the PCR products with T4 ligase (Promega) and the pGEM-Easy vector for 1 h at RT. 1µl of ligated reactions was then transformed into TOP20 competent cells (Invitrogen) and plated on plates containing ampicillin (1µg/ml), isopropyl-β-D-thiogalactopyranoside (IPTG) (80 µg/ml) and 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-gal) (20 mM) for white and blue colony selection. A total of 1,500 colonies were picked from different neurological sources and transferred into 96 well plates containing 20µl of LB (Luria-Bertani) complemented with ampicillin (1µg/ml). 1 µl of the sub-cloned colony / LB solutions was used to PCR screen for size heterogeneity on a 2% agarose gel fragments and a selection was sequenced using Big-Dye Terminators and an ABI automated sequencer (ABI3100; Applied Biosystems).

2.3.2 Q-PCR analysis

Primers for Q-PCR analysis of gephyrin cassettes were designed with the Primer-3 programme: The following reverse primer was used for amplification of all C3 and C4A-C regions, 5'-TCAGAG GA AAAGGAGACAT GC-3'; Forward primers for each cassette are 1) targeting C3 region, 5'-C AGTCCTGCTGTTGTCATGG-3'; 2) targeting C4A region, 5'-ATTAGACGGCCGGATGAAAG -3'; 3) targeting C4B region, 5'-CTCCATCGAAAGCTGGAGGA -3'; 4) targeting C4C of gephyrin, 5'-TTCCCTCGTGCTCATCTACC-3'; 5) targeting C4D, 5'-G ATTGGAAGGGCTTAAAGATG A-3'; 5) targeting invariant G-domain region of gephyrin (GephG), forward, 5'-CAAGGAAACCCTGATAGA

TTGG-3'; reverse,5'-CTGGTGCTTCCCGTTCTATTA-3'. 6) targeting invariant E-region of gephyrin (GephE), forward, 5'-CAACCATCATC AAAGCAAGGT-3'; reverse, 5'-AGACGGCTGCTCATTTGATTA-3'. Total RNA from human adult brain, fetal brain, spinal cord, retina and heart was reverse transcribed into cDNA as described above and used as template in a Q-PCR experiment. β-actin and hypoxanthineoquanin phosphoribosyl transferase (HPRT) mRNA levels were used as an internal standard. Biorad IQ system was used with SYBRgreen as the reporter dye. Q-PCR was performed in triplicates using the IQSYBR Green supermix (Biorad) on Biorad IQ system (Biorad). A 25μl reaction includes 1μl of cDNA, 10pmol of each forward and reverse primers and 12.5μl of 1x SYBR Green mastermix (Biorad) and PCR conditions are as follows: at 95 °C for 5 min, 40 cycles of denaturation at 95 °C for 10s, anneal at 60°C for 30s, followed by 95 °C for 1 min, 55 °C for 1 min, 81 cycles of 55 °C for 10s (fluorescence acquiring). Primers were optimized to have ~100% efficiency. To determine the fold gene expression level of each cassette, the Ct values of the target gene to the Ct value of the control gene (β-actin) were compared using the following formula: $2^{-(Ct(Target)-Ct(Control))}$.

2.3.3 Immunohistochemical procedures

Adjacent series of from human upper medulla and the cervical level of the spinal cord were processed as a free-floating format in 6-well tissue culture plates and prepared for immunohistochemical procedures using standard protocols outlined below, but also covered in precise detail by Waldvogel et al (Waldvogel et al., 1999; Waldvogel et al., 2004; Waldvogel et al., 2006; Waldvogel et al., 2007). For each primary antibody, dilution factor was optimized using dilution series. Each gephyrin cassette antibody

was then incubated with the antigenic peptide which can block the binding of the primary antibody to test the specificity of the antibody.

2.3.3.1 Brain tissue

The human brain tussue for this study were obtained from the Neurological Foundation of New Zealand Human Brain Bank (Department of Anatomy with Radiology, University of Auckland). with the written informed consent from the families. Ethical approval for this study was obtained from The University of Auckland Human Participants Ethics Committee. Brain tissue was obtained from 5 neurologically normal cases including 1 female and 4 males (Table 2.3), with no history of neurological disease and no evidence of neuropathology. The cases have an average age of 61.2 years (range 35-98 years), and a mean post *post-mortem* interval of 15.2 hours (Table 2.3). Blocks from upper medulla and the cervical level of the spinal cord were frozen with powdered dry-ice and sectioned at a 50-70μm thickness with a microtome. Sections were stored in PBS with 0.1% sodium azide at $4^{o}C$ for the immunohistochemical studies.

Table 2.3 List of human cases used in this study.

Case	Age(yr)	Sex	Postmortem delay (h)	Cause of death
H148	64	M	7	Ischemic heart disease
H149	48	M	21	*CO* poisoning
H187	98	F	15	-
H183	61	M	13	-
H184	35	M	20	-

2.3.3.2 Primary antibodies

1. Custom-made polyclonal antibodies for gephyrin cassettes. Rabbit polyclonal antibody production for cassettes C3, C4A, C4C and C4D was performed by Mimotopes Pty Ltd, Victoria 3168, Australia (www.mimotopes.com). The antigens used correspond to the human gephyrin gene cassettes are as follows: VVMAHGEQPIPGC for C3; QIRRPDESKGVAC for C4A; ARLPSCSST YSV for C4C; G LKDELWRNRGYC for C4D (See Figure 5.3). Two rabbits were injected intradermally with synthetic peptide conjugated to carrier protein followed by a booster injection. The antisera were affinity-purified from a 50/50 mixture of both hyperimmune sera. The antibody titer of the affinity-purified antisera was determined by ELISA assay according to standard manufacturer's procedures (Mimotopes) yielding a titre of 19,413 for C3; 54,281 for C4A; 5,567 for C4C; and 20,766 for C4D; corresponding to a concentration of 104 µg/ml for C3; 319 µg/ml for C4A; 59 µg/ml for C4C; and 119 µg/ml for C4D. Rabbit polyclonal antibody production for cassette C4B was performed by NeoMPS, Inc., San Diego, California (www.neomps.com) using the human C4B peptide sequence LHRKLEELRDHLEGNVKGYC. Three New Zealand white rabbits were injected intradermally with purified peptide conjugated to carrier protein followed by a booster injection. The antisera were affinity-purified, and the antibody titer was determined by ELISA assay according to standard manufacturer's procedures (NeoMPS) yielding a titer of 940,500 and a concentration of 3.42 mg/ml for C4B.

2. GlyRs were detected using a monoclonal antibody Mab4a (Synaptic Systems; Germany) raised against amino acids 96-105 in GlyR α_1 subunit and recognizes both α_1 and β GlyR subunits (Pfeiffer et al., 1984; Perez-Leon et al., 2003). This antibody has

been characterized in immunohistochemical studies on human brain (Waldvogel et al., 2003; Waldvogel et al., 2007).

3. A monoclonal antibody 3B11 (Synaptic Systems, Germany) raised against the first half of the E-domain of the gephyrin protein was used to detect the full-length gephyrin. 3B11 has been recently characterized by immunoreactivity rat spinal cord cells (Smolinsky et al., 2008). At the time of this study there was no viable gephyrin N-domain antibody available.

2.3.3.3 Single immunoperoxidase labelling (DAB staining).

Pretreated sections were incubated with primary antibodies for 48 –72 hrs at 4°C with gentle shaking at the following dilutions: mouse monoclonal antibody mAb4a at 1:2,000; mouse monoclonal antibody 3B11 at 1:1000; C3 at 1:500; C4A at 1:500; C4B at 1:15,000; C4C at 1:200; C4D at 1:500. The sections were washed three times with PBST for 15 min, then incubated with biotinylated secondary antibodies (at 1:400; Veatastain) overnight at 4 ^{o}C. Following three washes with PBS-Triton (PBST), the sections were incubated with Avidin-Biotin Complex kit (at 1:250, ABC kit, Vectastain Elite kits) for 4 h at RT. After three further washes with PBST, the sections were incubated with DAB (3,3'-diaminobenzidine) solution for 15-30 min to generate a brown coloured product.

2.3.3.4 Immunofluorescent double labelling

Sections were incubated in a combination of primary antibodies for 48 -72 hrs at 4°C with gentle shaking; the mAb4a antibody (diluted at 1:2,000) or 3B11 antibody (diluted at 1:1,000) was co-

incubated separately with gephyrin cassette antibodies against C3 (diluted 1:250), C4A (diluted 1:500), C4B (diluted 1:5,000), C4C (diluted 1:50), or C4D (diluted 1:250). The sections were washed three times with PBST for 15 min and then incubated with fluorescent secondary antibodies coupled to Alexa 488 or 568 (1:2500; Invitrogen, La Jolla, CA, USA) for 4 h at RT. The sections were again washed three times with PBST for 15 min, and then mounted on glass slides with Prolong Gold (Invitrogen). Each primary antibody was incubated with both secondary antibodies and no cross-reactivity was observed between the secondary antibodies. In control sections where the primary antibody was omitted, no immunolabelling was obtained.

2.3.3.5 Immunohistochemical analysis

Single immunoperoxidase labeled sections were analysed with light microscopy using a Zeiss *Axioskop* upright microscope (Zeiss, Jena, Germany) equipped for epi-fluorescence and a *Axiocam* digital camera. Double immunofluorescent sections were assessed by laser scanning confocal microscopy (Zeiss LSM 510 Meta, Jena, Germany) by using dual-channel recording of AlexaFluor 568 (red emission) and AlexaFluor 488 (green emission). Digital images were processed and merged for color colocalisation using the Zeiss LSM5 Image Examiner software (Zeiss, Jena, Germany).

To determine the immunoreactivity for the gephyrin cassette, for each cassette, at least three independent experiments were conducted on brainstem and spinal cord sections obtained from 3 different cases (Table 2.3). The level of immunoreactivity was semi-quantitatively analysed by counting the number of puncta from randomly selected cell bodies and dendrites from a total of 402 neurons. Then the level of immunoreactivity of each cassette

expressed as the ratio of that of C3. For analysis of co-localisation, colour images were merged, and the position of each gephyrin cassette was compared with that of the GlyR- or Geph-E clusters. The level of co-localisation was then represented to the level of each cassette analysed.

Chapter 3

Mutation analysis of hyperekplexia

Glycinergic neurotransmission is a major inhibitory system in the central nervous system (CNS) and defects in glycinergic genes are associated with the startle disorder, hyperekplexia. This rare, but potentially fatal, neurological disorder is primarily a hereditary disorder, typically associated with hypertonia, non-epileptic drop-down attacks in response to audible or tactile stimuli and psychogenic consequences. At inception of this thesis and study, dominant mutations in the glycine receptor α1 subunit (*GLRA1*) was the accepted perception from the literature. This study however, describes one of the largest studies in the molecular genetics of hyperekplexia where we not only find an increased incidence of recessive mutations in *GLRA1*, but also describe a new gene in hyperekplexia (at the time) in the form of the glycine transporter GlyT2 (*SLC6A5)* gene. The choice of *SLC6A5* as a candidate gene in hyperekplexia was due to the reporting of a GlyT-2 knockout mouse in 2005 that had all the features of startle disease (Gomeza et al 2005). In this study, we have analysed the entire coding regions of two major glycinergic genes, *GLRA1* and *SLC6A5* through the 88 index-cases.

3.1 Patients

Eighty-eight hyperekplexia index-cases were recruited for hyperekplexia mutation screening having passed the diagnostic inclusion criteria (Appendix A). All samples were accompanied by informed consent from patients, parents, carers or guardians. The samples have originated from healthcare systems from across the

globe and referred from centers/specialties ranging from neonatal paediatrics to adult neurology. Patients were numbered according to the order in which blood samples were received and cohorts were assembled into a manageable size (n<16) for effective mutation analysis. Patient samples were initially screened for *GLRA1* mutations using direct sequencing, with all *GLRA1*-negative patients progressing towards dHPLC screening (and later direct sequencing) of *SLC6A5* (Table 3.2).

3.2 GLRA1 analysis

GLRA1 was a recognized diagnostic gene in hyperekplexia at the start of this study, although the disorder was perceived as a mainly dominant disorder. Eighty-eight index cases of hyperekplexia were screened for genetic variation in the entire coding regions and splice regions of *GLRA1*. Direct sequencing analysis revealed 19 mutations within 30 hyperekplexia index cases, of which 12 mutations were novel and a further 7 mutations have been described in other studies; whilst seven of the novel and recurrent mutations were discovered in more than one index case (Table 3.1, Figure 3.1). This compendium of mutations included 13 missense mutations (9 novel), 3 nonsense mutations (1 novel) and 3 frameshift deletions (2 novel). The majority of mutations (21/30, 70%) were inherited in a recessive mode, including four cases of compound heterozygote inheritance (Table 3.1) thereby significantly increasing the number of pathological recessive hyperekplexia alleles in the literature. Consequently, on a large population, index-case ascertainment basis, hyperekplexia is predominantly recessive, and not dominant as reflected by an ascertainment bias in the literature (Harvey et al., 2008).

Table 3.1 List of patients with GLRA1 variants identified in this study

Case	Sequence	Classification of Mutation	Mutants		Protein Position	Mode of Inheritance	References
1	ΔExons 1-7 (Homo)	deletion	del ex1-7		N/A	R	Brune et al., 1996; Gilbert et al., 2004
2	ΔExons 1-7 (Homo)	deletion	del ex1-7		N/A	R	
3	ΔExons 1-7 (Homo)	deletion	del ex1-7		N/A	R	
4	ΔExons 1-7 (Homo)	deletion	del ex1-7		N/A	R	
5	ΔExons 1-7 (Homo)	deletion	del ex1-7		N/A	R	
6	ΔExons 1-7 (Homo)	deletion	del ex1-7		N/A	R	
7	G574T	missense	R65L	M	N-terminal	CH	Novel
	ΔExons 4-7	deletion	del ex4-7	P	N/A	CH	Novel
8	C573T (Homo)	missense	R65W		N-terminal	R	Novel
9	Δ 931-932CT(Homo)	deletion	L184fs21X		N-terminal	R	Novel
10	Δ 931-932CT	deletion	L184fs21X	P	N-terminal	CH	Novel
	G687A	missense	E103K	M	N-terminal	CH	Novel
11	A764G	missense	Y128C		N-terminal	D	Novel
12	A874G	missense	D165G		N-terminal	R	Novel
13	C971A	nonsense	Y197X	P	N-terminal	CH	Novel
	C986A	nonsense	Y202X	M	N-terminal	CH	Rees et al., 2001
14	C971A (Homo)	nonsense	Y197X		N-terminal	R	Novel

15	C986A (Homo)	nonsense	Y202X		N-terminal	R	
16	C986A (Homo)	nonsense	Y202X		N-terminal	R	Rees et al., 2001
17	C986A (Homo)	nonsense	Y202X		N-terminal	R	
18	G1074A	missense	S231N	}*	M1	CH	Novel
	C1257A	nonsense	S296X		M3	CH	Bellini et al., 08
19	C1128A	missense	P250T		M1-M2	D	Saul et al., 1999
20	C1134T (Homo)	missense	R252C		M1-M2	R	Novel
21	G1141A (Homo)	missense	G254D		M2	R	Novel
22	G1141A (Homo)	missense	G254D		M2	R	
23	C1174T	missense	T265I		M2	D	Novel
24	G1192A	missense	R271Q		M2-M3	D	Shiang et al., 1993; Langosch et al., 1994; Rees et al., 1994; Schorderet et al., 1994; Shiang et al., 1995; Elmslie et al., 1996; Kwok et al., 2001; Gaitatzis et al., 2004;Kimura et al., 2006
25	G1192A	missense	R271Q		M2-M3	D	
26	G1192A	missense	R271Q		M2-M3	D	
27	G1192A	missense	R271Q		M2-M3	D	
28	G1404A	missense	G342S		M3-M4	D	Rees et al., 2001
29	G1404A	missense	G342S		M3-M4	D	
30	G1555A (Homo)	missense	R392H		M3-M4	R	Vergouwe et al., 1999

*N/A; Not Applicable, D: dominant, CH: Compound heterozygous, R: Recessive, P: paternal, M: maternal, *: Parental DNA not available, however, case 18's unaffected male sibling has only one of the heterozygous mutations, namely S296X, indicating by default that S231N and S296X are present on different alleles.*

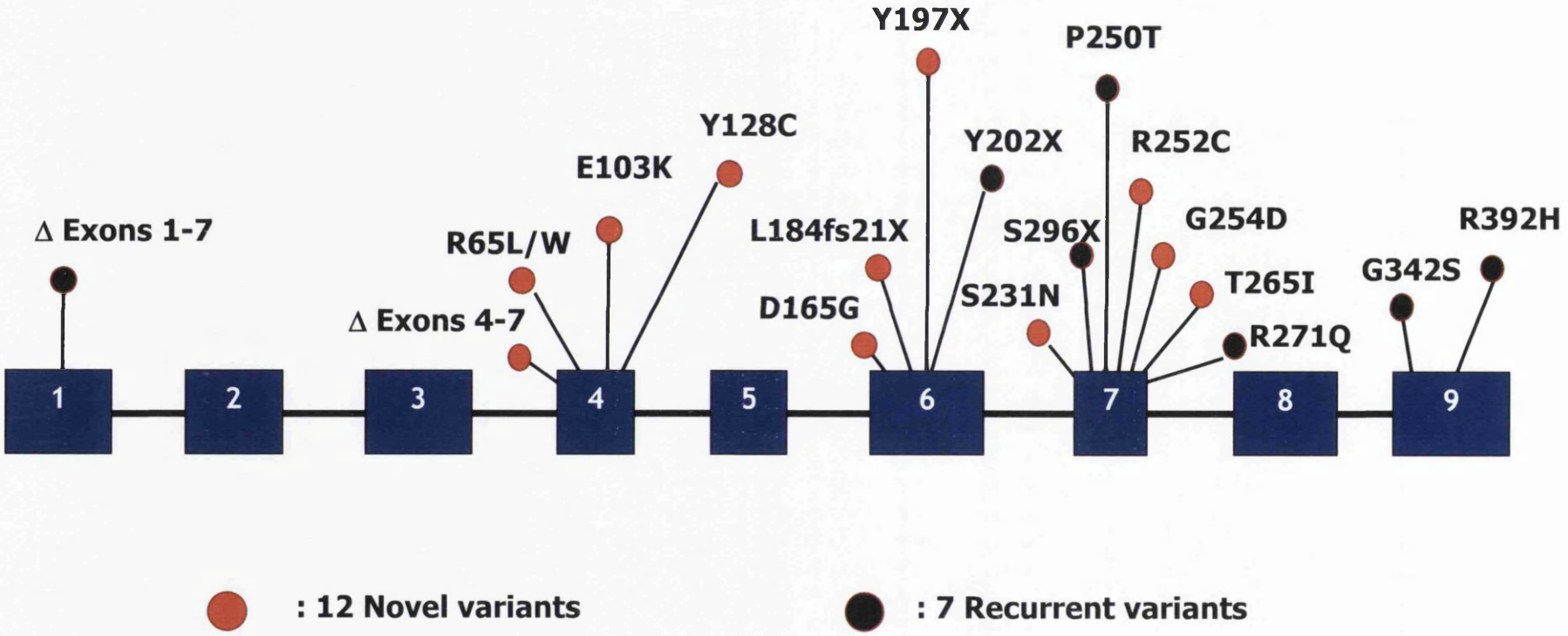


Figure 3.1 GLRA1 variants identified in this study. *Schematic diagram of GLRA1 genomic structure and exonic location of GLRA1 mutations.*

Consistent with previous studies, all nonsense and deletional frameshift mutations were associated with recessive cases of HE, whereas missense mutations transmit either as dominant or recessive traits depending on their relative position in the polypeptide subunit or co-inheritance in compound heterozygotes. Recessive mutations were scattered throughout the GlyR α1 subunit, whereas, dominant mutations are specifically clustered around the M2 domain containing an ion-selectivity filter and flanking regions (Figures 3.2 and 3.3). As revealed from previous studies (Brune et al., 1996; Gilbert et al., 2004; Becker et al., 2006), clinical phenotypes of patients with recessive mutations were not as severe as the phenotype of HE animal models where recessive mutations are associated with more severe outcomes leading to premature death (Buckwalter et al., 1994; Kling et al., 1997; Traka et al., 2006), indicating the existence of compensatory mechanisms. However, they tend to have more complex phenotypes with developmental delay and/or learning difficulties (Appendix C). Parents that were heterozygous carriers of the recessive/compound mutations did not present with clinical symptoms reminiscent of hyperekplexia. Similarly, dominant mutations in index-cases were associated with one affected parent within the family structure.

3.2.1 *GLRA1* recessive variants

Five novel (R65W, D165G, R252C and G254D) and one recurrent (R392H) recessive missense mutations were identified in the N-terminal, intracellular and the transmembrane regions. Of the 30 mutation-positive cases, 13 index-cases were identified with null/non-functional truncated GLRA1 alleles (Table 3.1, Figure 3.4). The established homozygous deletion of exons 1 to 7 (Δexon

1-7) was identified in six patients of Turkish origin with consanguineous parents and is identical to the previously- reported deletion (Becker et al., 2006). The Δexon 1-7 genotype

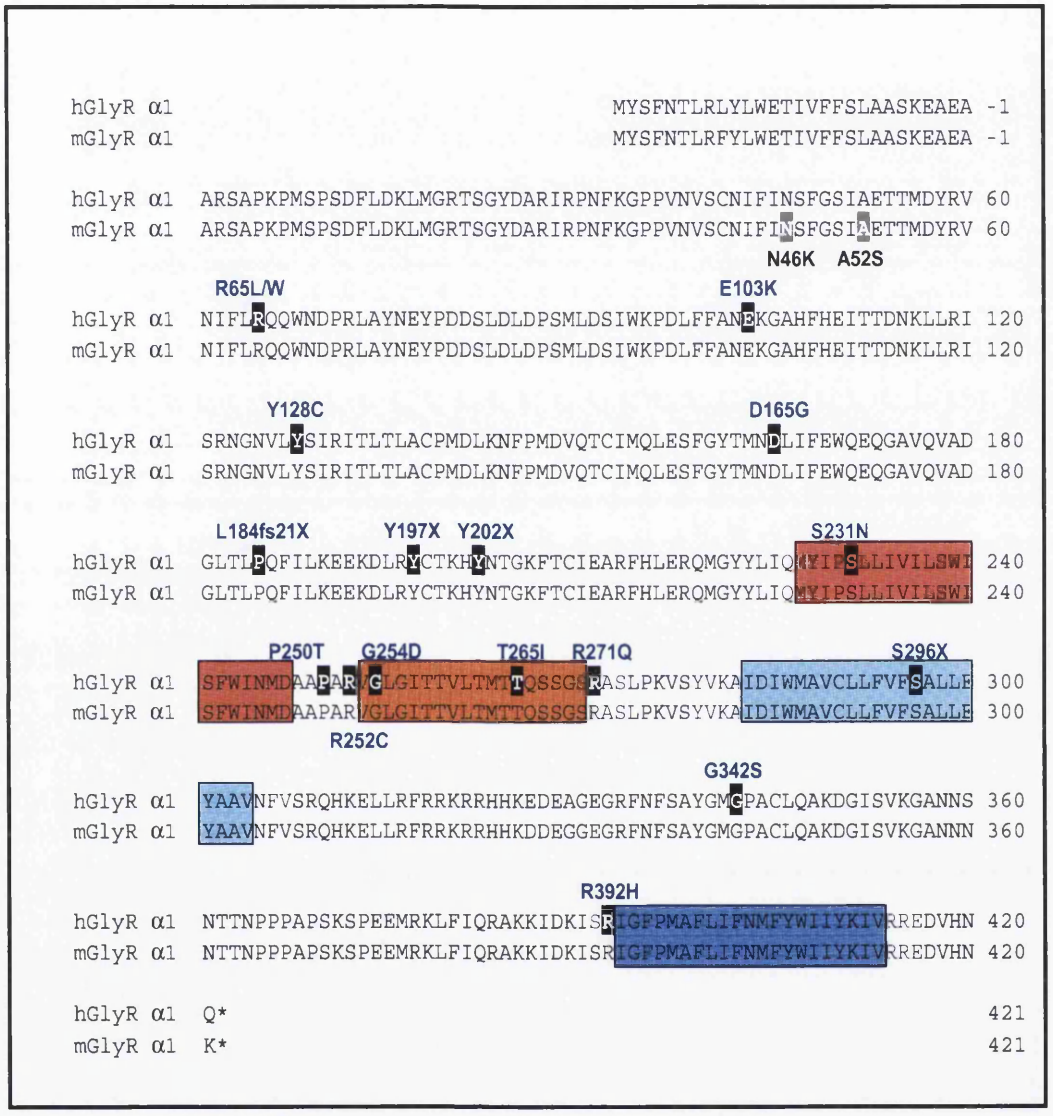


Figure 3.2 Amino acid sequence of GlyR α1 subunit with hyperekplexia variants identified in this study. *Amino acid sequence alignment of human and mouse GlyR α1 with locations of human GLRA1 mutations identified in this study and mouse Glra1 missense mutations published to date (grey shading). Residues on the transmembrane domains (M1-M4) are shaded in colour and correspond to transmembrain (TM) domains in Figure 3.3.*

has been identified in another 13 hyperekplexia patients, all from Turkish decent (Brune et al., 1996; Gilbert et al., 2004). Homozygous premature stop codon alleles, Y197X and Y202X, were also identified in five unrelated Pakistani and Jordanian patients where the homozygous recessive inheritance in 4 patients originated from consanguineous parents.

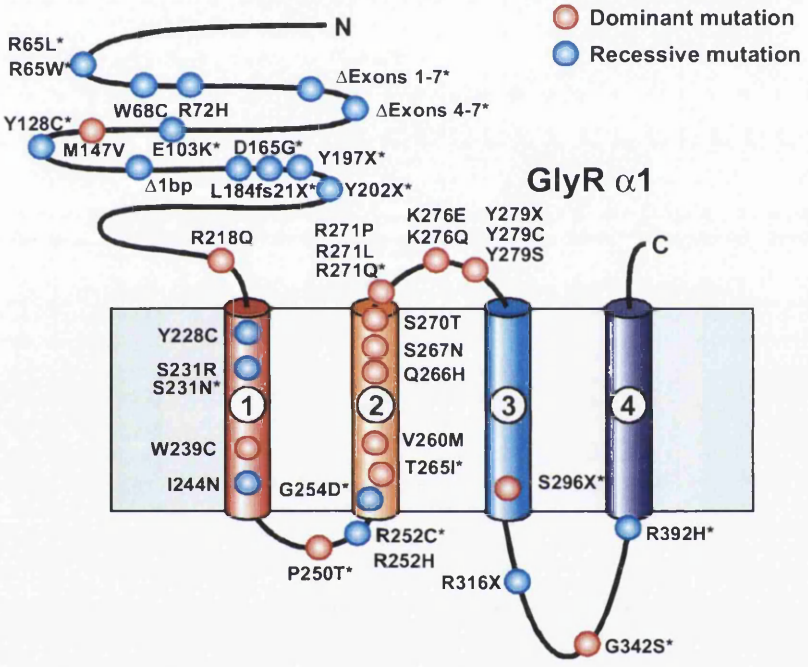


Figure 3.3 Schematic diagram of predicted GlyR αl subunit and relative locations of hyperekplexia mutations. *2-D schematic representation of a single GlyR αl polypeptide which consists of a large N-terminal extra-cellular domain, followed by four membrane spanning domains (M1-M4) and a short extracellular C terminus. Human GLRA1 mutations published to date are indicated. * denotes mutations identified in this study. This figure is a modified version of Figure 1a in Harvey et al 2008 with permission from the author.*

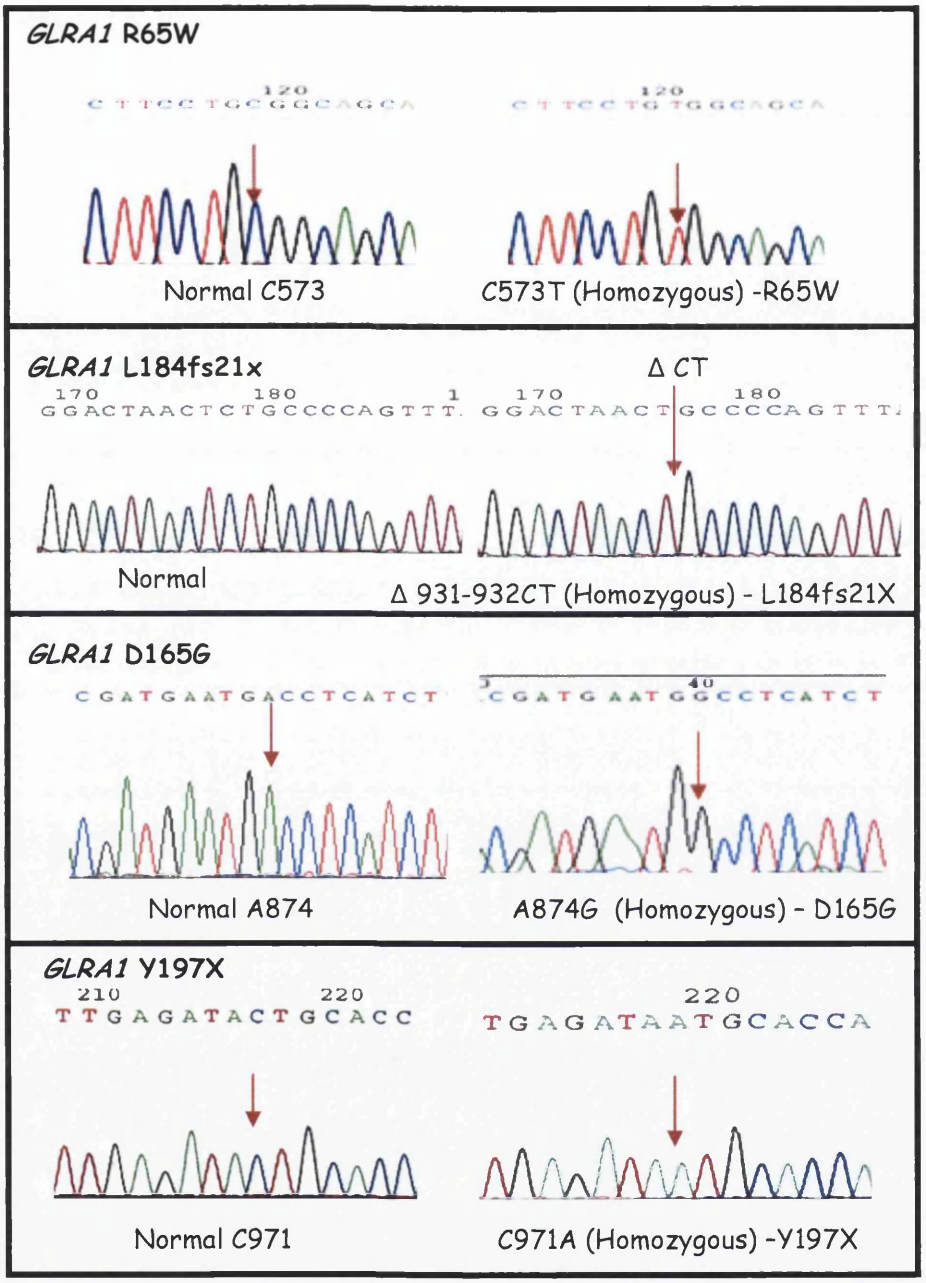


Figure 3.4 Sequence analysis of novel recessive GLRA1 variants identified in this study. *Sequence chromatograms of the normal alleles (left, red arrow) were compared with the homozygous mutations (right, red arrow). The sequence chromatograms are shown in the sense direction. L184fs21X and Y197X were also identified as a part of heterogygous compound mutations, in cases 10 and 13, respectively.*

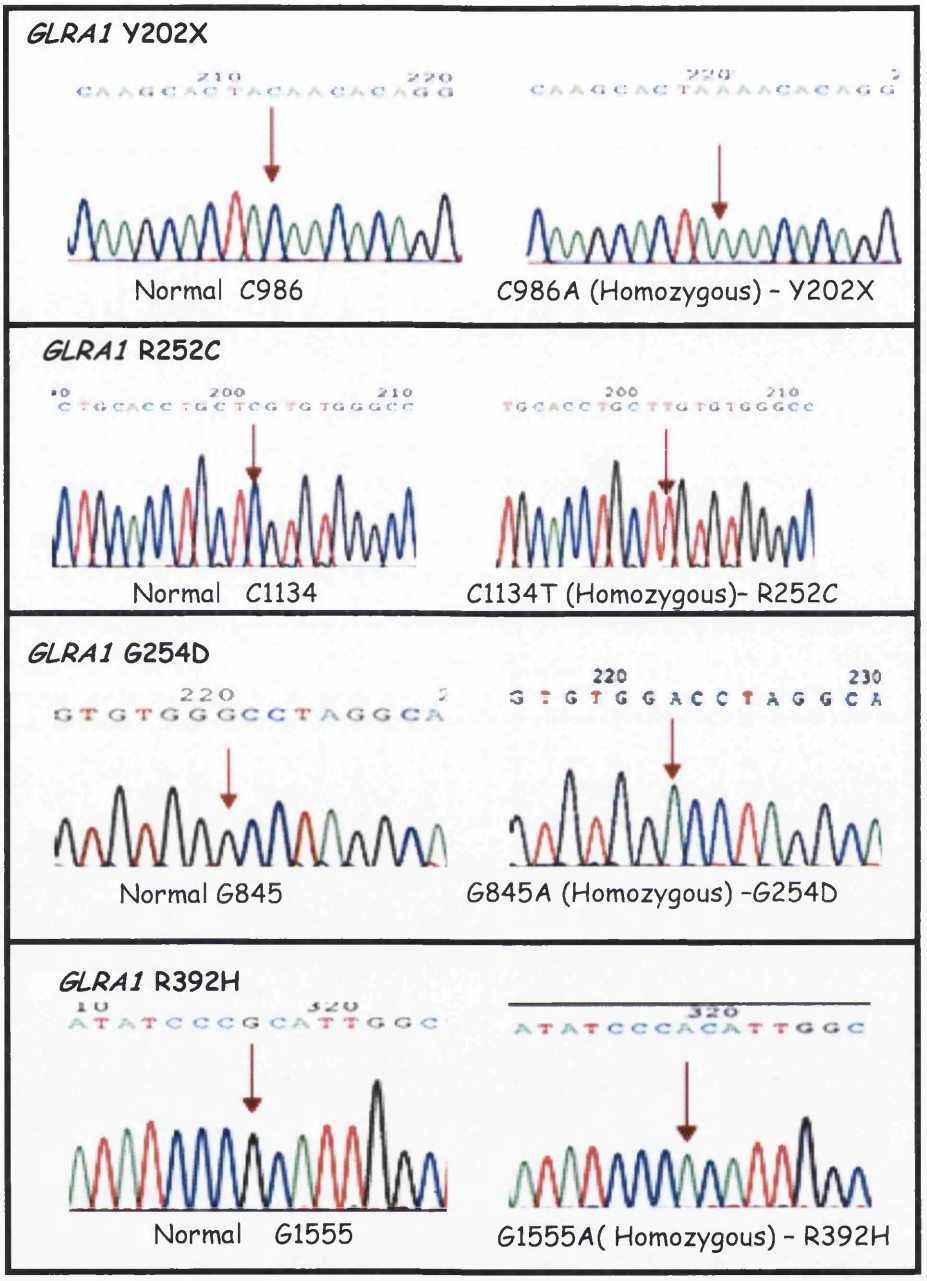


Figure 3.5 Sequence analysis novel and recurrent recessive GLRA1 variants identified in this study. *Sequence chromatograms of the normal alleles (left, red arrow) were compared with the homozygous mutations (right, red arrow). The sequence chromatograms are shown in the sense direction. Y202X was also identified as a part of a heterogygous compound mutation finding (case 13).*

3.2.2 GLRA1 compound heterozygosity variants

Novel compound mutations were identified in four unrelated patients with asymptomatic parental carriers (Table 3.1, Figure 3.6), effectively doubling the reporting of compound heterozygosity in *GLRA1* (Vergouwe et al., 1999; Rees et al., 2001; Tsai et al., 2004); 1) In patient 7, a large deletion (Δexon 4-7) is co-inherited with N-terminal missense mutation R65L. Δexon 4-7, a novel deletion detected by MLPA, is the second largest *GLRA1* deletion identified in a HE case and is expected to produce a non-functional allele. R65L is a novel missense mutation, the effect of which is described in Chapter 4 on functional platforms along with the missense mutations of patients 10 and 18 (E103K and S231N); 2) Novel mutations, L184fs21X and E103K, were identified in patient 10 where the heterozygous frameshift L184fs21X allele generates a premature stop codon at amino acid position 205; 3) Compound mutations, Y197X and Y202X, were detected in patient 13, and both alleles are expected to produce truncated nonsense proteins devoid of transmembrane domains; 4) A novel missense mutation, S231N and recurrent nonsense mutation S296X were identified in patient 18.

Assuming that Δexon 4-7 in patient 7, L184fs21X in Patient 10, and S296X in patient 18 result in non-functional alleles, then corresponding R65L, E103K and S231N respectfully are hemizygous alleles by default and represent the only α1-subunits available for in-vivo α1β GlyR assembly (Figure 3.7). Previously, three compound heterozygotes for *GLRA1* mutations have been reported in the literature: 1) R252H / R392H (Vergouwe et al., 1999); 2) 1bp delC (601-605)/ M147V (Rees, et al., 2001); 3) W96C / R344X (Tsai et al., 2004).

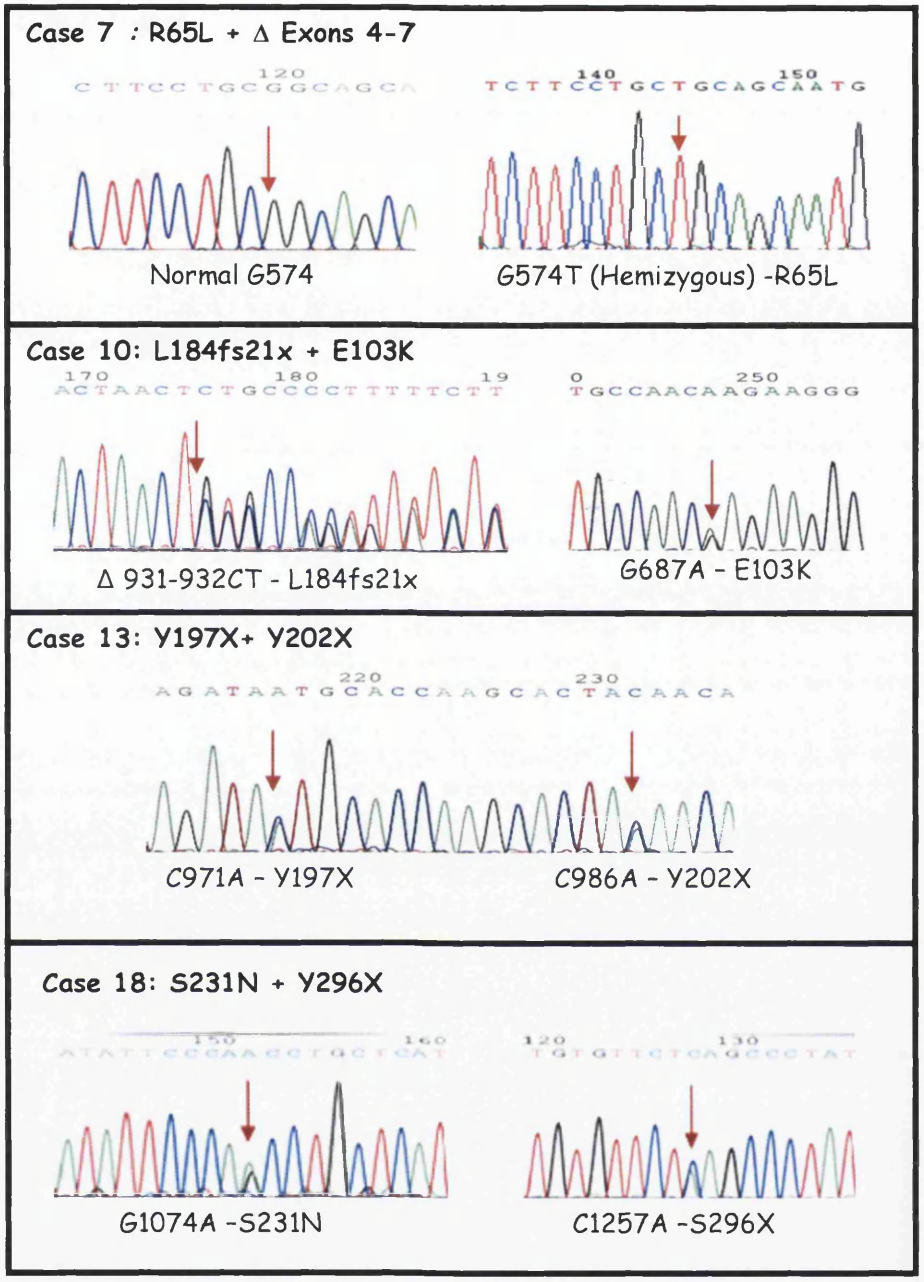


Figure 3.6 Sequence analysis of compound GLRA1 variants identified in this study. *Sequence chromatograms of four cases of compound heterogygous mutations (see red arrows). R65L is the only available allele in Case 7 due to the deletion (Δ Exons 4-7) of the second allele and is compared with wild-type allele. The sequence chromatograms are all shown in the sense direction.*

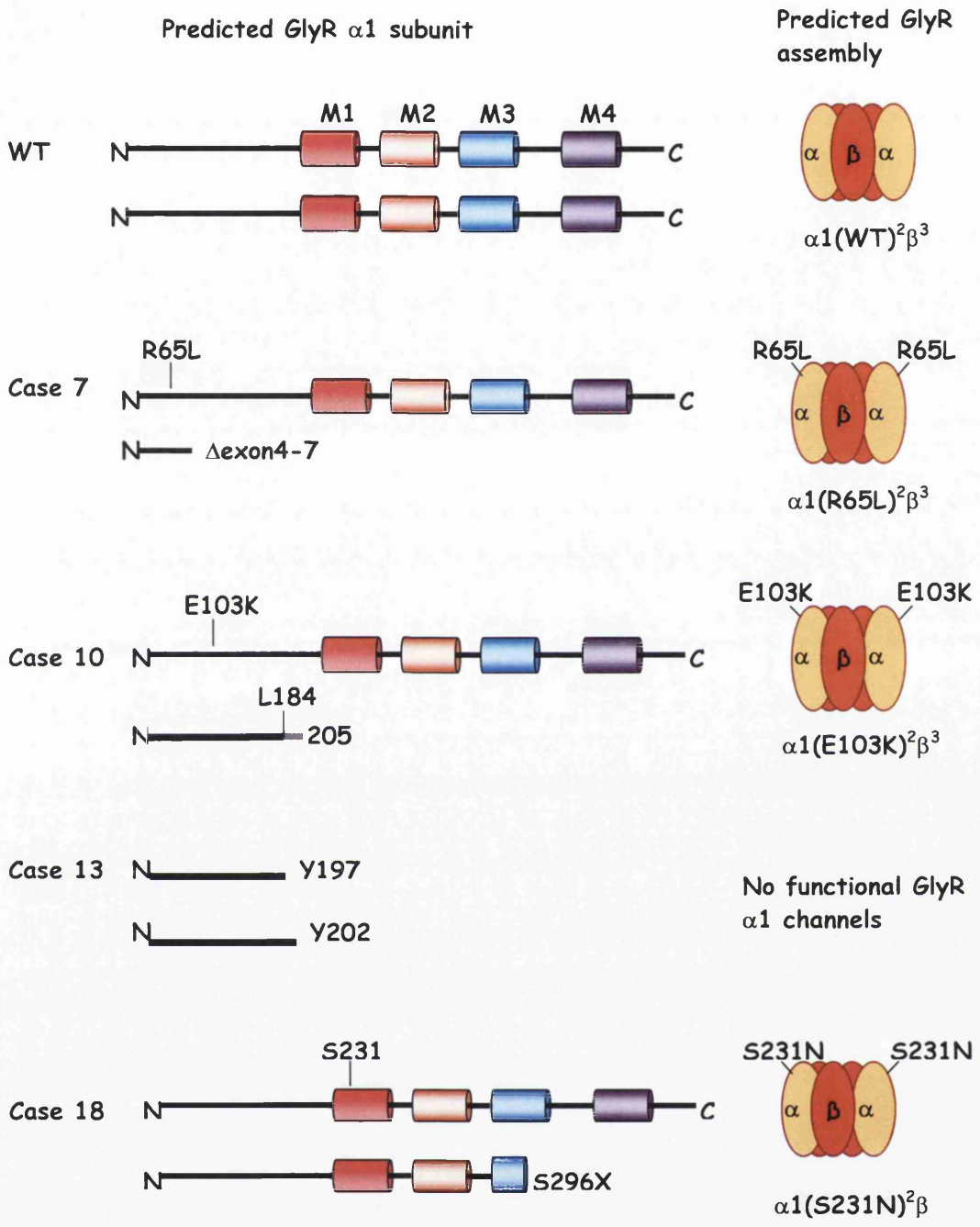


Figure 3.7 Predicted in-vivo outcomes of compound mutations and resulting pentameric assembly components. *Four index-cases were identified with compound mutations in this study. In cases 7, 10 and 18, the missense mutations R65L, E103K and S231N respectively, are expected to represent the only surface-expressed GlyR α1 subunits available for in-vivo assembly. Whereas in case 13, both alleles are expected to produce truncated protein, resulting in no functional GlyR α1 on the cell-surface.*

3.2.3 *GLRA1* dominant variants

Two novel dominant mutations, Y128C and T265I, and two recurrent missense mutations, P250T and R271Q, were identified in 7 hyperekplexia patients (Table 3.1). R271Q was the first *GLRA1* mutation associated with hyperekplexia and has been identified in 18 independent families / index cases with dominant hyperekplexia (Harvey et al., 2008) with a further four independent cases identified in this study. Extensive functional assessment of R271Q and P250T have described their pathophysiological basis (Rajendra et al., 1994; Laube et al., 1995; Lewis et al., 1998; Moorhouse et al., 1999; Saul et al., 1999; Breitinger et al., 2001; Maksay et al., 2002). The previously-identified G342S variant was identified in two patients with an ambiguous functional consequence (Rees et al., 2001) and consequently is being re-examined in this study along with novel alleles Y128C and T265I. However, subsequent to the functional assessment further expanded population studies have revealed that G342S is a rare SNP present in controls (frequency = 0.01). The functional data remains in this study as a virtual negative control for pathogenicity although conceding that G342S may still remain an allelic risk-factor which may exert an effect in tandem with other mutations in GLRA1, as is the case in other disorders (Kubota et al., 2001; Mitra et al., 2003).

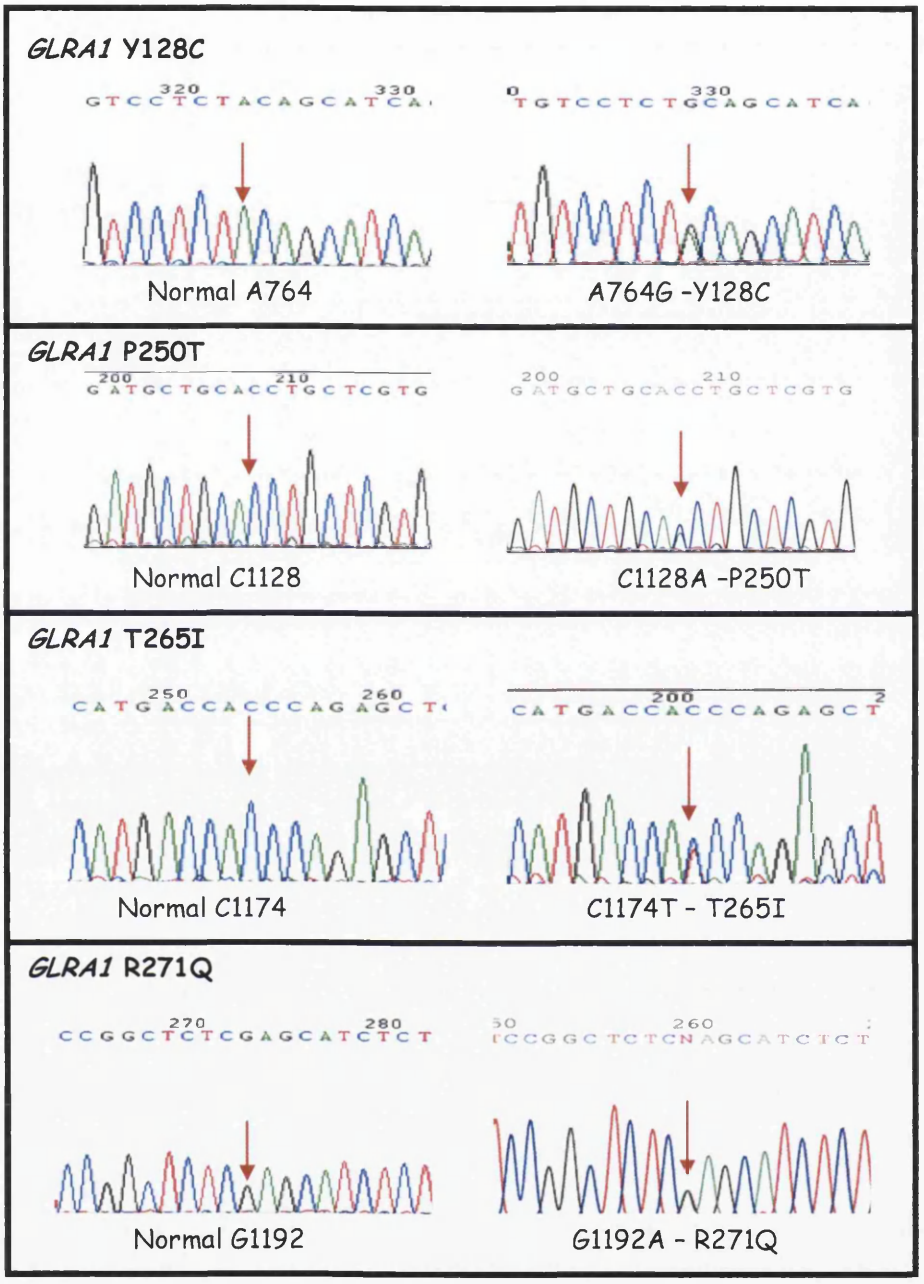


Figure 3.8 Sequence analysis of dominant GLRA1 variants identified in this study. *Sequence chromatograms of the normal alleles (left, red arrow) were compared with the homozygous mutations (right, red arrow). The sequence chromatograms are shown in the sense direction.*

3.3 *SLC6A5* Analysis

Fifty-eight patients, excluded from *GLRA1* mutations, were screened for genetic variation in the coding regions and splice regions of *SLC6A.* This was achieved by using denaturing high-performance liquid chromatography (dHPLC) initially, and repeated by direct sequencing, despite the experimental expense and low ratio of assays to variant discovery. For *SLC6A5* analysis, 16 exons were screened through the 58 patients, and 12 *SLC6A5* mutations were discovered in 7 index cases (Table 3.2). Patients 31 and 32 were identified with recessive mutations, R439X and T425M, respectively (Figure 3.10). R439X is a novel mutation identified after the first report of *SLC6A5* mutations (Rees et al., 2006). The detailed clinical information for patients with *SLC6A5* mutations is included in appendix D (Rees et al., 2006).

The vast majority of *SLC6A5* mutations identified in this study were inherited as compound heterozygotes (Rees et al., 2006); 1) A nonsense mutation Y377X and a missense plus frameshift mutation (V432F+fs97X) were identified in patient 33. Both mutations are expected to produce truncated nonsense proteins. The family members with Y377X were asymptomatic, whereas V432F+fs97X was associated with a partial HE (with nocturnal myoclonus and a nervous disposition); 2) Compound mutations, Y491C and Q630X, were identified in patient 34. Parents who were heterozygous carriers of the compound mutations did not present with HE symptoms; 3) A missense mutation W483R and a missense plus frameshift mutation P108L+fs25X were identified in patient 35 with unaffected parents. 4) Patient 36 was detected with two missense mutations, L306V inherited from the mother and N509S from the paternal side. In this study, only one case of a dominant mutation,

Table 3.2 List of patients with SLC6A5 variants identified in this study

Case	Sequence	Classification of Mutation	Mutants		Protein Position	Mode of Inheritance	References
31	C1315T	Nonsense	R439X		M6-M7	R	Novel
32	C1274T	Missense	T425M		M5	R	Rees et al., 2006
33	C1131A	Missense	Y377X	P	M3-M4	CH	Rees et al., 2006
	G1294T + Ins[T]1295	Missense plus frameshift	V432F+fs97X	M	M5		Rees et al., 2006
34	A1472G	Missense	Y491C		M6-M7	CH	Rees et al., 2006
	C1888T	Missense	Q630X		M10		Rees et al., 2006
35	T1444C	Missense	W482R		M6	CH	Rees et al., 2006
	ΔC[319–324]	deletion	P108L+fs25X		N-terminal		Rees et al., 2006
36	C916G	Missense	L306V	P	M3-M4	CH	Rees et al., 2006
	A1526G	Missense	N509S	M	M7		Rees et al., 2006
37	T1530G	missense	S510R		M7	D	Rees et al., 2006

D: dominant, R: Recessive, CH: Compound heterozygous, P: paternal, M: maternal

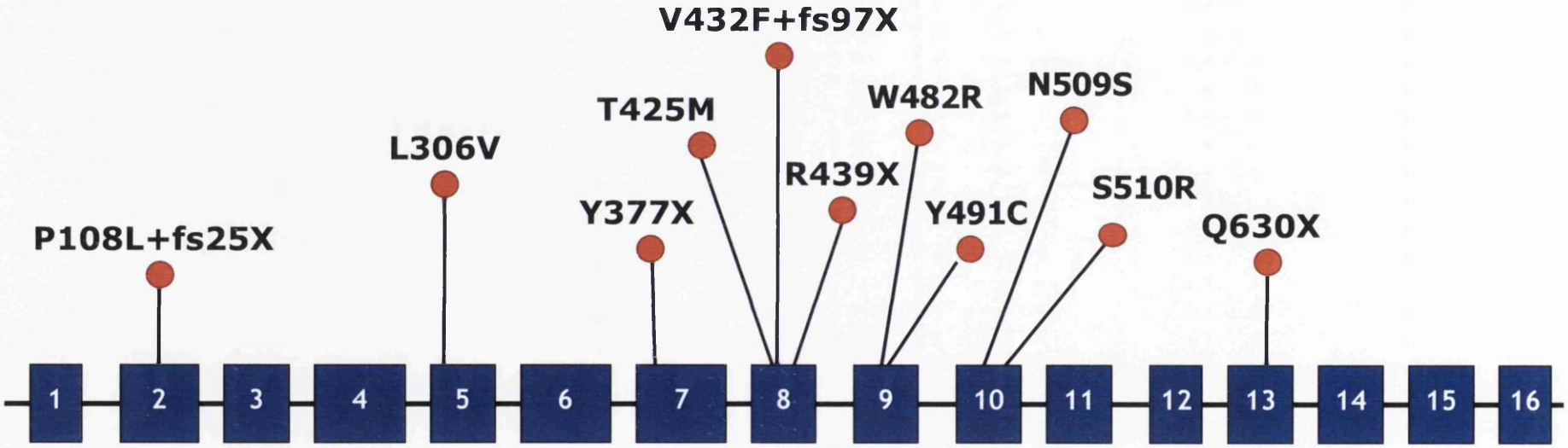


Figure 3.9 SLC6A5 variants identified in this study. *Schematic diagram of SLC6A5 genomic structures and location of SLC6A5 mutation*

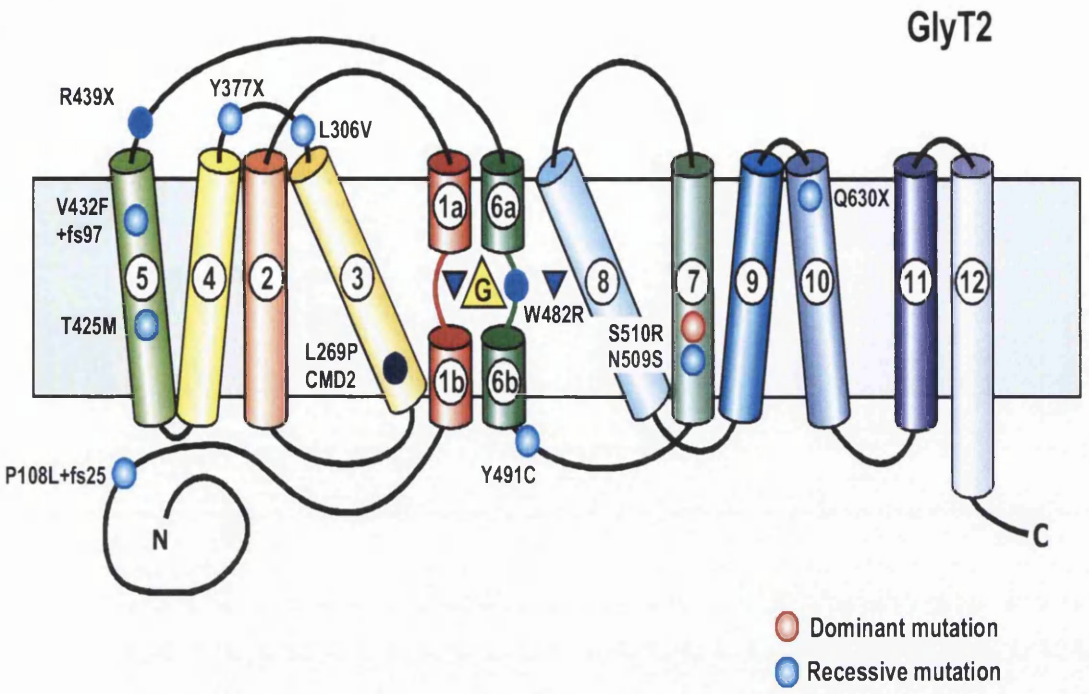


Figure 3.10 Schematic diagram of predicted GlyT2 and relative locations of hyperekplexia mutations. *2-D schematic representation of a single GlyT2 polypeptide which consists a large N-terminal intra-cellular domain, followed by twelve membrane spanning domains (M1-M12) and a short intra-cellular C terminus. SLC6A5 mutations identified in this study are indicated. This figure is a modified version of Figure 1b in Rees et al 2006 with permission from the author.*

was identified (S510R in case 37), further supporting the notion that recessive mutations are the most common cause of hyperekplexia.

All *SLC6A5* mutations, except the novel R439X, were functionally characterized using a glycine-uptake assay, immuno-staining of expression constructs and elecrophysiological validation through our collaboration (Rees et al., 2006). A surface localisation assay showed that all nonsense or frameshift mutations were not able to express on the cell-surface possibly due to traffic defects, whereas the majority of missense mutations, were able to reach the cell-surface (Rees et al., 2006). However, the surface expressed missense mutations displayed a significantly reduced level of

glycine uptake (Figure 3.11). This data was kindly provided with consent from Professor Robert Harvey (School of Pharmacy, London) and Dr Stephan Supplison (INSERM, Paris) within the multicentre collaboration. The data in Fig 3.11 was not generated by the author of this thesis.

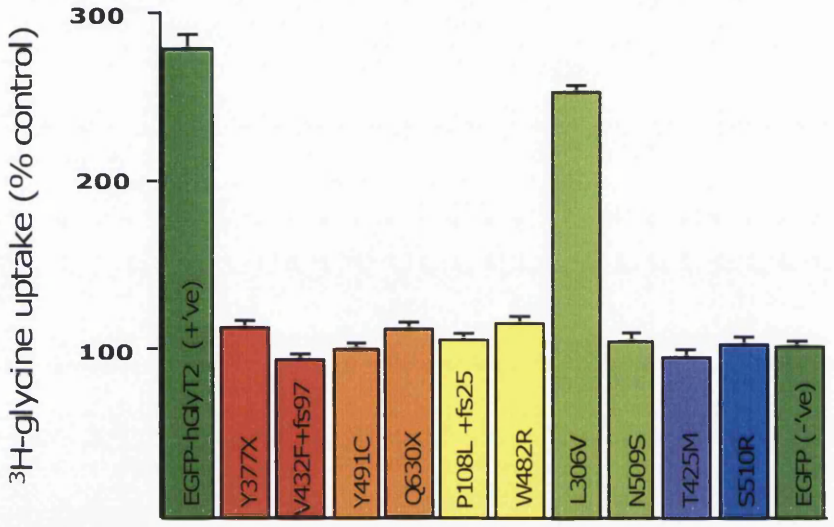


Figure 3.11 Transport activity of WT and mutant GlyT2. *To determine the kinetics of 3H-glycine uptake, cells were transfected with the pEGFP-hGlyT2 construct and incubations were carried out with varying concentrations of unlabelled glycine (1 - 1000 μM). The glycine uptake was expressed as a percentage of that in cells transfected with an empty pEGFP vector. Data are represented as means ± SEM (n = 6-20). This figure is a modified version of Figure 3a in Rees et al 2006 with permission from the author.*

3.4 Summary

Hyperekplexia is a neuromotor disorder characterized by exaggerated startle reflexes and muscle stiffness in response to sudden, unexpected auditory or tactile stimuli. This rare, but potentially fatal disorder is primarily a hereditary disorder and typically caused by mutations in the genes encoding GlyR alpha1 subunit (*GLRA1*) and the glycine transporter GlyT2 (*SLC6A5*).

Systematic DNA sequencing analysis of *GLRA1* in 88 new hyperekplexia patients revealed 19 sequence variants within 30 index cases, of which 21 were inherited in a recessive mode or part of compound heterozygosity. Consistent with previous studies, all deletion and nonsense mutations were associated with recessive onset of phenotype, whereas missense mutations could exert an effect either as dominant or recessive traits depending on the position of the mutation in the GlyR alpha1 subunit. The mutation analysis of the glycine transporter-2 gene (*SLC6A5*) in hyperekplexia also displays predominantly recessive inheritance and compound heterozygosity. *SLC6A5* variants were identified using denaturing high-performance liquid chromatography (dHPLC) and confirmed by sequencing. Screening of 58 GLRA1 negative index cases revealed eleven *SLC6A5* variants within 7 independent families. Collectively, *GLRA1* and *SLC6A5* mutations analysis have a positive outcome in 37 patients in total, reflecting a 42 % of detecting rate, leaving 58 % phenotype positive / genotype negative hyperekplexia without a molecular explanation.

This study indicates, that on a population basis, recessive hyperekplexia is more common than expected and that the previous label and reference towards a 'dominant disorder' was an ascertainment bias on familial presentation and founder linkage analysis cohorts. In contrast to other diseases caused by dysfunction of ion channels or murine hyperekplexia models, patients with recessive mutations/null hyperekplexia mutations in *GLRA1* were not particularly associated with severe cases of hyperekplexia. The explanation for tolerance of null *GLRA1* gene function in humans is likely due to a compensatory mechanism by other neuro-inhibitory mechanisms.

In chapter 4, an account is given on the functional analysis of a proportion of novel *GLRA1* mutations in a recombinant heteromeric GlyR α1β expression system representing the native form of adult GlyRs (Langosch et al., 1988). The pathogenicity of *SLC6A5* mutations were determined through collaboration and only the gene-discovery work was undertaken by this author at Swansea University. Chapter 4, therefore, will be restricted to the analysis of the new *GLRA1* mutations where further progress is made in understanding the pathophysiology of hyperkeplexia.

Chapter 4

Functional analysis of *GLRA1* mutations

Taking forward the genetic findings described in Chapter 3, we prepared expression constructs of 11 *GLRA1* mutants to initiate studies into the functional context of the hyperekplexia mutations. There is no further functional work described for *SLC6A5* since much of this was completed by other investigators as part of a collaboration between Swansea, London and Paris (Rees et al., 2006). Although discussed in Chapter 6, the genetics and functional context of *SLC6A5* (GlyT2) mutations remain in the summarized account given in Chapter 3.

The functional properties of the novel recessive and dominant *GLRA1* mutations were further investigated using in-vitro electrophysiology, fluorescence-based imaging, immunostaining and biotinylation of cell-surface membranes, plus the latest molecular modelling algorithms. This chapter will now describe the outcome of these experiments and demonstrates a multi-faceted explanation for *GLRA1* pathogenicity.

4.1 Preparation of mutagenesis constructs

The human GLRA1 α1 cDNA within the pRK5 vector was kindly provided by Prof. Robert Harvey (The School of Pharmacy, London, UK). Eleven *GLRA1* mutations listed in Table 4.1 were introduced into the WT α1 cDNA plasmid using the Quickchange site-directed mutagenesis kit (Stratagene). The coding region of

α1 subunit was otherwise confirmed intact by sequencing for all α1 constructs used in this study. The pIRES2-EGFP plasmid vector with the full-length human β-subunit cDNA and YFP-I152L within the pcDNA3.1 vector was kindly provided by Prof. Joe Lynch (Queensland Brain Institute, University of Queensland, Australia). Both GlyR-β and YFP-I152L constructs were fully-validated in previous studies (Kruger et al., 2005; Hawthorne et al., 2006; Yang et al., 2007; Yang et al., 2008; Gilbert et al., 2009)

4.2 Electrophysiological analysis of α1 GlyR hyperekplexia mutations

To determine the pathogenic effects of *GLRA1* mutants, WT or mutant human α1 GlyRs were transiently expressed in HEK293 cells either as homomeric α1 GlyRs or as heteromeric α1β GlyRs with WT β subunits (Table 4.1). Transfected HEK293 cells were analysed using whole-cell patching electrophysiology and a high-throughput YFP-based screening assay. Electrophysiology was used to examine the effects of mutations at high-precision in single cells, whereas the fluorescence assay was used to provide an indication of the proportion of cells that expressed mutant GlyRs.

4.2.1 Recessive Variants

When expressed in HEK293 cells, the majority of recessive mutations (R65W, R252C, G254D) failed to generate currents either as homomeric or heteromeric GlyR receptors upon application of upto 30mM of glycine (Figure 4.1). A novel recessive mutation, D165G, induced detectable, but significantly ($p<0.001$) reduced maximum currents compared to WT, regardless of whether it was expressed as a homomer or heteromer (α1(D165G)β:

Table 4.1 Electrophysiology results of novel GLRA1 hyperekplexia mutations expressed in HEK293 cells

	α1	Homomeric α1		Heteromeric α1 β		Functional effects
		Glycine EC50 (mM)	nH	Glycine EC50	nH	
	WT	20.74±7.27 (6)	1.86 ± 0.3	23.24 ± 7.59 (6)	1.17 ± 0.31	
Autosomal	**Y128C**	_	_	_	_	Tonic opening of channel
Dominant	**T265I**	302.37 ± 97.38 *(3)	0.84 ± 0.15	774.83 ± 219.53 *(6)	1.01 ± 0.1	Shift of EC50
	G342S†	62.26±6.0 (6)	2.40±0.18	84.07±11.69 (11)	1.8±0.24	
Recessive	**R65W**	_	_	_	_	Trafficking
	D165G	145.33± 23.20 # (6)	1.49±0.14	212.88±56.83**(4)	1.66±0.4	Trafficking
	R252C	_	_	_	_	Trafficking
	G254D	_	_	_	_	Trafficking
	R392H	_	_	164.79 ± 35.73**	2.07±0.21	Trafficking
Compound	**R65L**	_	_	_	_	No current
	ΔExons 4-7	ND				
Compound	**E103K**	540.7 ± 148.3 * (8)	1.05±0.13	757.52±147.72 *(6)	1.11±0.05	Shift of EC50
	L184fs21X	_	_	_	_	Trafficking
Compound	**S231N**	262.15 ± 31.89 **(5)	1.32±0.16	383.8.1±132.57** (5)	1.75±0.39	Shift of EC50
	S296X	_	_	_	_	Trafficking

Statistically different from Wild type GlyR α1 / GlyR α1 β (p <0.001, *p<0.01, # p<0.5), EC_{50}: half-maximal concentration, nH: the Hill coefficient, ND: Not Determined. † Note G342S, previously reported as a possible dominant mutation is revealed as a SNP in this study.*

Imax=700.90±250.05pA, n=11; vs WT α1β: Imax= 16051.78±868.67pA, n=16). In addition, the glycine EC50 was significantly increased in cells where a recording was possible (Figure 4.2) indicating that glycine sensitivity in these mutants are markedly decreased. The mutation R392H, identified as a homozygous recessive mutation in this study, was previously-identified as a compound heterozygote (R252H and R392H) (Vergouwe et al., 1999). Consistent with the previous study, expression of homomeric R392H mutant in HEK293 cells induced no currents (n=20 for whole cell patching clamp, n>2000 for automated screening). However, expression with GlyR β subunits produced small but detectable currents (Figure 4.1) that exhibited a significant increase in glycine EC50 (Figure 4.2).

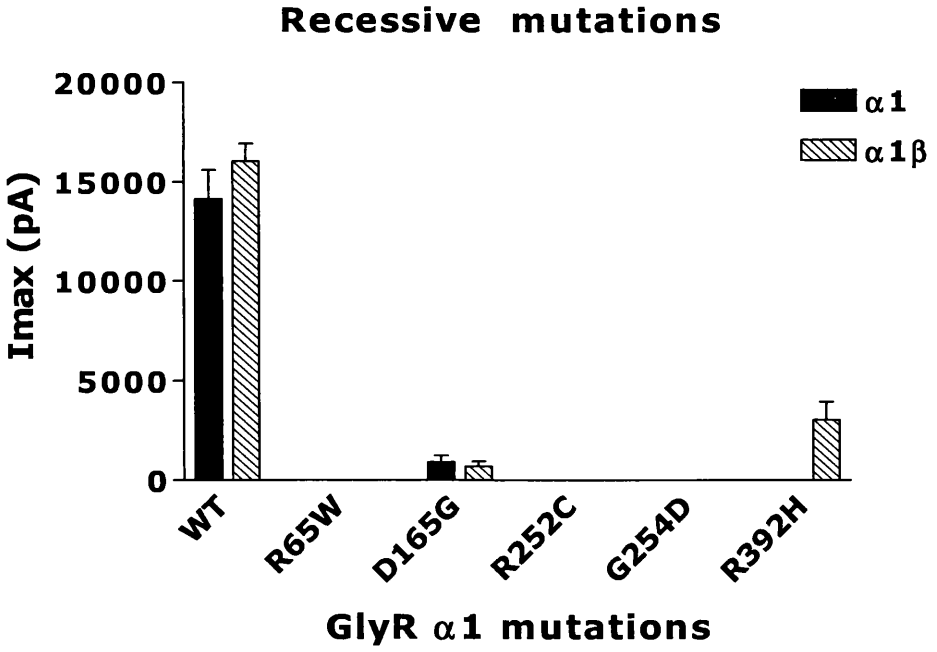


Figure 4.1 Imax values of recessive mutations. *Maximal GlyR currents were obtained from HEK293 cells expressing wild-type (WT) or mutant GlyR α1 individually or with the GlyR-β subunit at saturating glycine concentration (up to 30mM). The majority of recessive mutants generated no/ significantly reduced current ($p<0.001$ vs wild type). Note a significant increase of maximal currents were observed in α1 R392H upon co-expression with β subunit. Error bars represent SEM.*

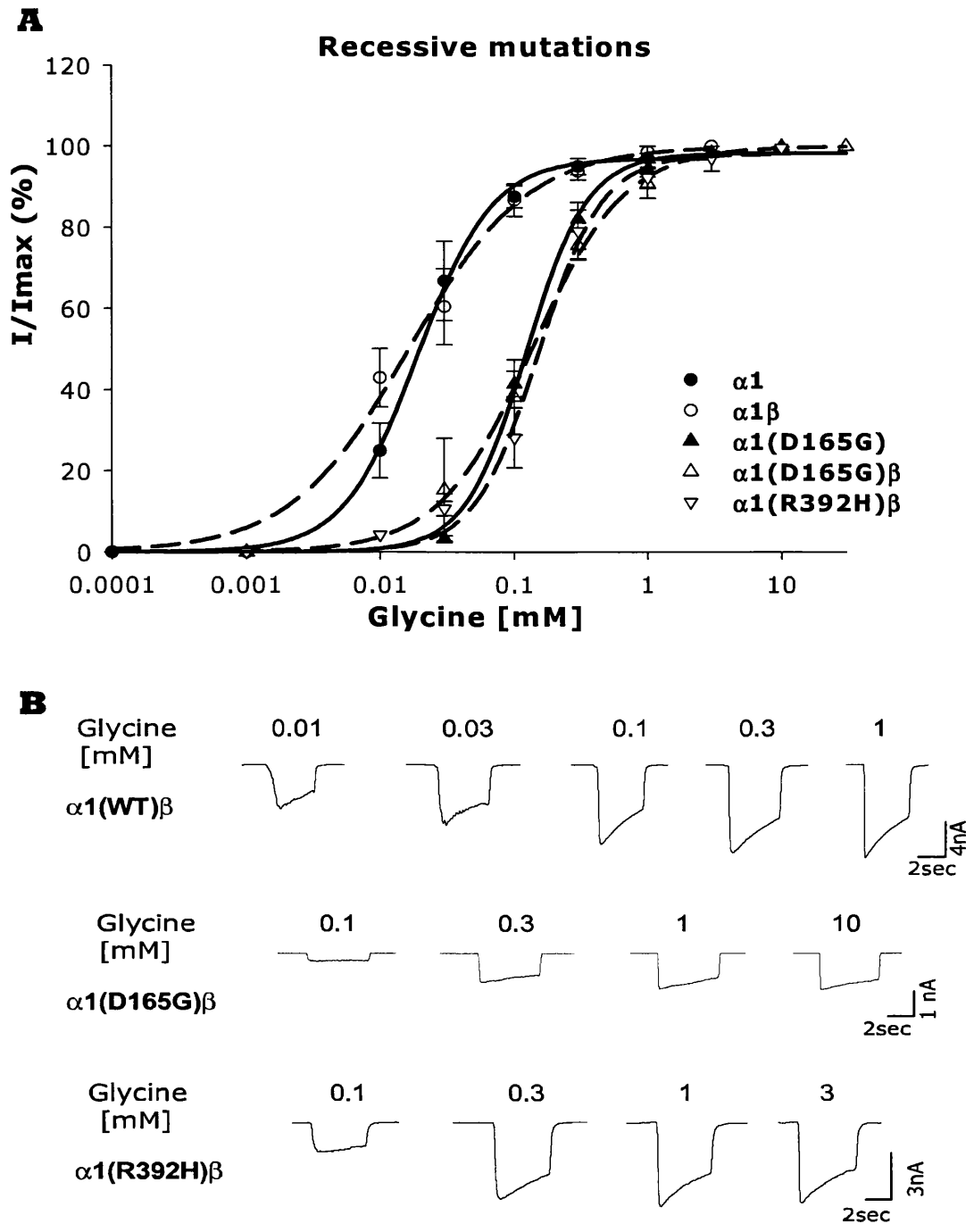


Figure 4.2 Dose-response characteristics of recessive mutations. *Currents were obtained from HEK293 cells expressing WT or mutant GlyR α1 subunit either as homomeric α1 subunits or heteromeric α1β GlyRs at varying concentrations of glycine (0.01–10 mM). The currents are shown as a percentage of the maximal current for each cell.* ***A)*** *While the majority of recessive mutations failed to generate glycine induced currents, two recessive mutations, D165G and R392H, produced small but detectable currents. Both mutants create a large right shift of the EC50 for glycine. R392H, however, was able to generate currents only upon expression with β-subunit.* ***B)*** *Examples of glycine-induced currents produced by WT or mutant GlyR α1 and β subunit.*

4.2.2 Compound Heterozygosity

Hemizygous missense mutations, R65L, E103K and S231N, identified in individuals with co-inherited second-hit nonsense mutations (compound heterozygosity in cases 7, 10 and 18 – table 3.1) were tested. R65L, co-inherited with Δ exon 4-7, generated no current when expressed in HEK293 cells (Figure 4.3). The positively-charged R65 residue is an important determinant for interacting with α-amino groups of glycine (Grudzinska et al., 2005).

Mutations E103K and S231N behaved differently from conventional recessive missense mutants despite mimicking the in-vivo consequences of recessive alleles (Figure 3.7) where α1(E103K) and α1(S231N) are the only functional copies of GlyR subunits at the cell-surface. In contrast to recessive mutants expressing no or small currents, both S231N and E103K, either as homomeric α1 GlyR or heteromeric α1β GlyR, generated fully-functional channels with Imax currents compatible with WT at glycine concentration 30mM (Figure 4.3). However, glycine EC50 was significantly ($p < 0.001$) increased in both mutants (α1(E103K)β = 757.52±147.72, α1(S231N)β=383.8.1±132.57; WT α1β= 23.24 ± 7.59) (Figure 4.4). Unsurprisingly, both S296X and L184fs21X mutants failed to generate any functional channels (Figure 4.3).

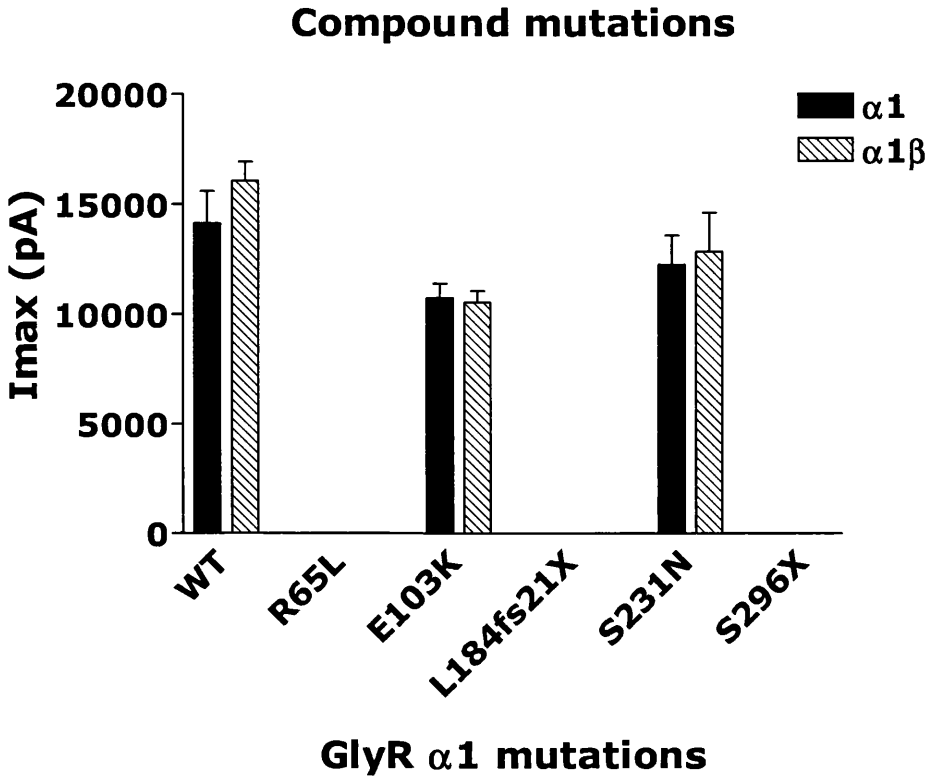


Figure 4.3 Imax values of compound mutations. *Maximal GlyR currents were obtained from HEK293 cells expressing wild-type (WT) or mutant GlyR αl individually or with the GlyR-β subunit at saturating glycine concentration (up to 30mM). HEK293 cells that did not generate any glycine-induced current were excluded from the analysis. Peak amplitudes of E103K, S231N glycine currents were similar to that of WT for either heteromeric or homomeric expression. Error bars represent SEM.*

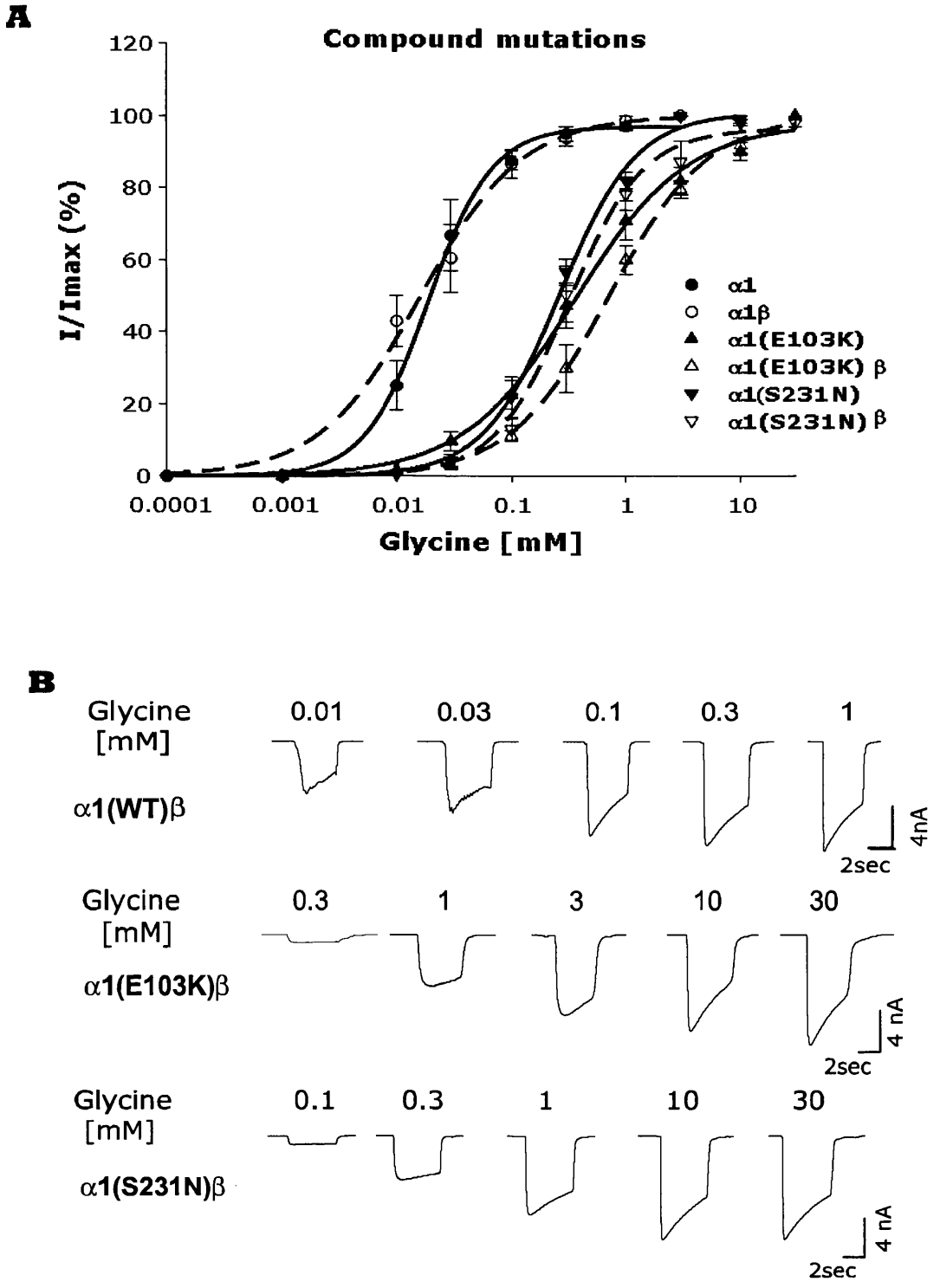


Figure 4.4 Dose-response characteristics of compound mutations. *Currents were obtained from HEK293 cells expressing WT or mutant GlyR α1 subunit either as homomeric α1 subunits or heteromeric α1β GlyRs at varying concentrations of glycine (0.01–10 mM). The currents are shown as a percentage of the maximal current for each cell. A) Compound missense mutations, E103K and S231N, show significant right shift of glycine EC50. B) Examples of glycine-induced currents produced by WT or mutant GlyR α1 and β subunit.*

4.2.3 Dominant Variants

Two novel dominant mutations, Y128C and T265I, showed a significant ($p<0.001$) reduction of Imax compared to WT when expressed as heteromers with the β subunit (α1(Y128C)β = 2276.93±74.5, α1(T265I)β = 3583.38±1218.30; WT α1β = 14578.58±814.20) (Table 4.1, Figure 4.5). When expressed as homomers, T265I generated barely detectable glycine currents, and in those cells where a measurement was possible Imax was 170 ± 58 pA (n= 3, $p<0.0001$ vs WT). The n_H value for α1(T265I) were significantly reduced (Table 2), suggesting a reduction in gating efficacy (Colquhoun, 1998).

However, when α1(T265I) was co-expressed with WT glyR β subunit, the heteromeric glycine-mediated current was easily detectable and a 20-fold increase in Imax (=3583.38±1218.30pA) was observed (which can be used to distinguish α1(T265I) from α1(T265I)β), although it remained significantly less than values observed in WT α1β ($p< 0.001$, Figure 4.5). In both homomeric and heteromeric states, T265I has a significantly increased glycine EC50 ($p<0.001$, Figure 4.6). The recurrent mutation G342S, identified in two dominant hyperekplexia index cases in this study and in previous studies (Jungbluth et al., 2000; Rees et al., 2001), induced Imax currents and glycine EC50 compatible to that of WT, and no evidence of a trafficking defect was observed. Within the limitations of these experiments, we have no evidence to suggest that this *GLRA1* sequence variant is pathogenic.

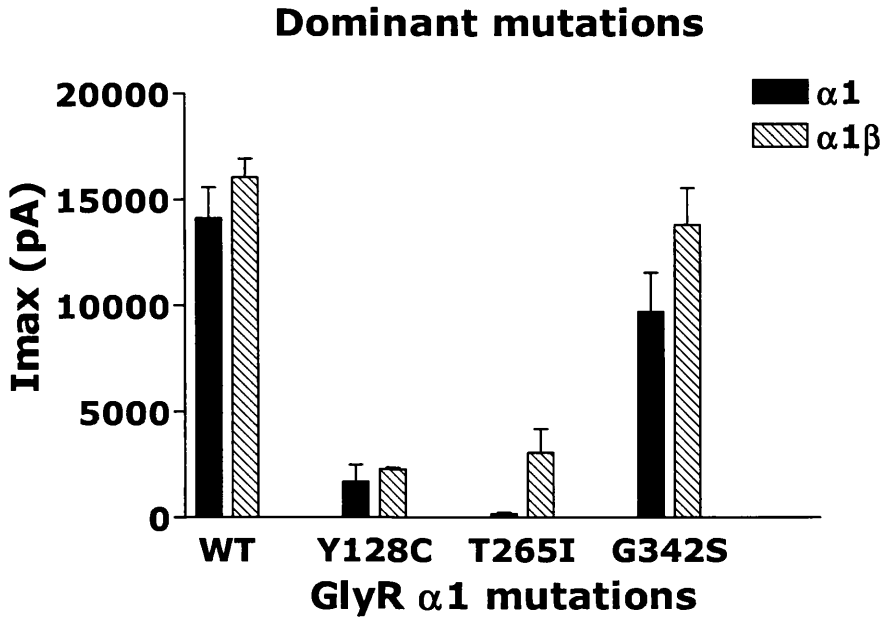


Figure 4.5 Imax values of dominant mutations. *Maximal GlyR currents were obtained from HEK293 cells expressing wild-type (WT) or mutant GlyR α1 individually or with the GlyR β subunit at saturating glycine concentration (upto 30mM). The peak glycine currents were significantly reduced in Y128C and T265I ($p<0.001$ vs wild type). Note a significant increase of maximal currents were observed in α1 T265I upon co-expression with β-subuni ($p<0.01$). Peak amplitudes of G342S currents were similar to that of WT for either heteromeric or homomeric expression. Error bars represent SEM.*

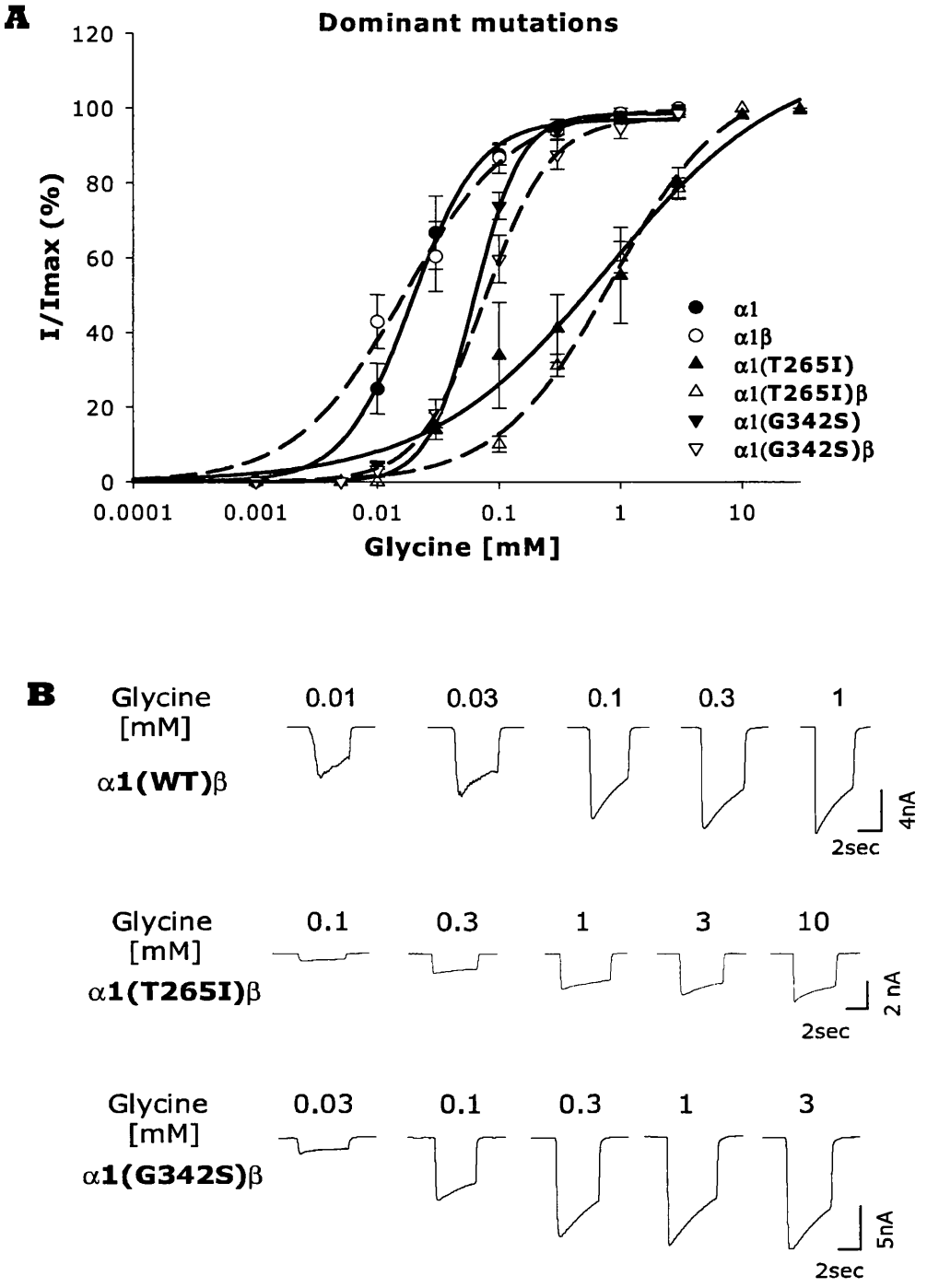


Figure 4.6 Dose-response characteristics of dominant mutations. *A) Currents were obtained from HEK293 cells expressing WT or mutant GlyR α1 subunit either as homomeric α1 subunits or heteromeric α1β GlyRs at varying concentrations of glycine (0.01–10 mM). The currents are shown as a percentage of the maximal current for each cell. A) A dominant mutation T265I shows a significant right shift of glycine EC50 ($p<0.01$) . B) Examples of glycine-induced currents produced by WT or mutant GlyR α1 and β subunit.*

TM2 domain mutation T265I: Interestingly, the glycine-mediated current from HEK293 cells expressing the heteromeric α1(T265I)β GlyR was inhibited by picrotoxin (PTX). PTX, a botanical alkaloid, can inhibit the homomeric α1-3 GlyR subtypes, whereas the heteromeric α1β GlyR subtypes are insensitive to PTX (Pribilla et al., 1992). Thus, PTX is often used in assays to discriminate the homomeric α-GlyR from the heteromeric αβ GlyR.

For WT and other α1 GlyR mutants investigated in this study, PTX (100μM) strongly inhibited currents in cells expressing homomeric α1 GlyRs, but not heteromeric α1β GlyRs. The exception to this was the heteromeric α1(T265I)β GlyR mutant which displayed an anomalously high-sensitivity to PTX. To investigate the gain of PTX sensitivity in α1(T265I)β GlyRs, a range of PTX concentrations (1μM-100μM) were applied in the presence of the glycine EC50 concentration. As shown in Figure 4.7, the glycine-mediated current was reduced by PTX in a concentration-dependent manner. The averaged inhibitory dose-response curve is presented in Figure 4.8. Individual PTX dose-responses in the $\alpha1^{T265I}\beta$ GlyR were fitted with an averaged half-maximal inhibitory concentration (IC_{50}) of 12.7 ± 0.82μM and an n_H of 1.126 ± 0.103. This IC_{50} is significantly lower than the corresponding values of the heteromeric α1 β GlyR, which have previously been determined at 219 ± 28 μM (Hawthorne et al., 2006). Consequently, these results suggest that T265 in the TM2 domain represents an important new determinant of PTX sensitivity.

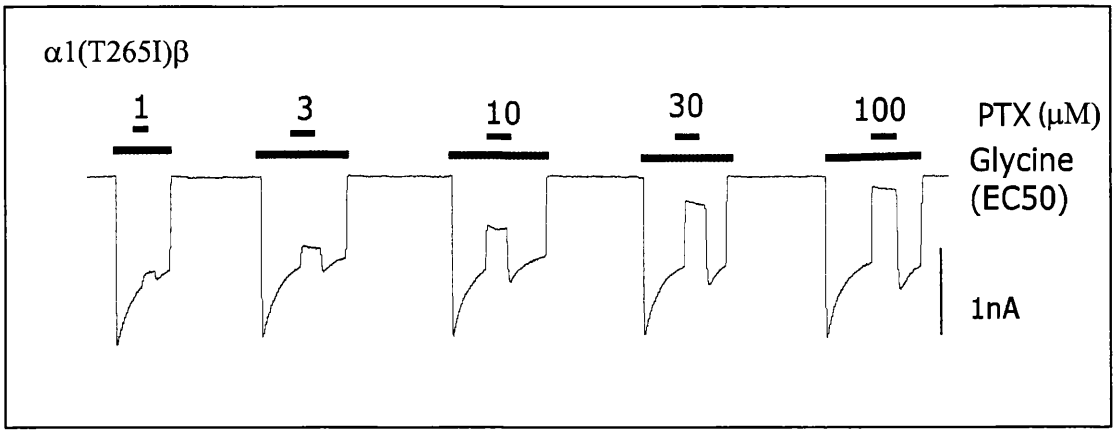


Figure 4.7 αlT265I mutation converted heteromeric αlT265I β GlyRs to PTX sensitive. *Inhibitory dose-response characteristics of PTX-induced currents. The heteromeric αlT265I β was not resistant to PTX and the PTX inhibition shows a dose-dependent manner.The IC50 value of PTX antagonism was 1.49±0.15μM and 6.3 ± 0.7 for α1(T265I)β and α1WT glyRs, respectively. The currents induced by αlT265I was not sufficient enough to generate IC50.*

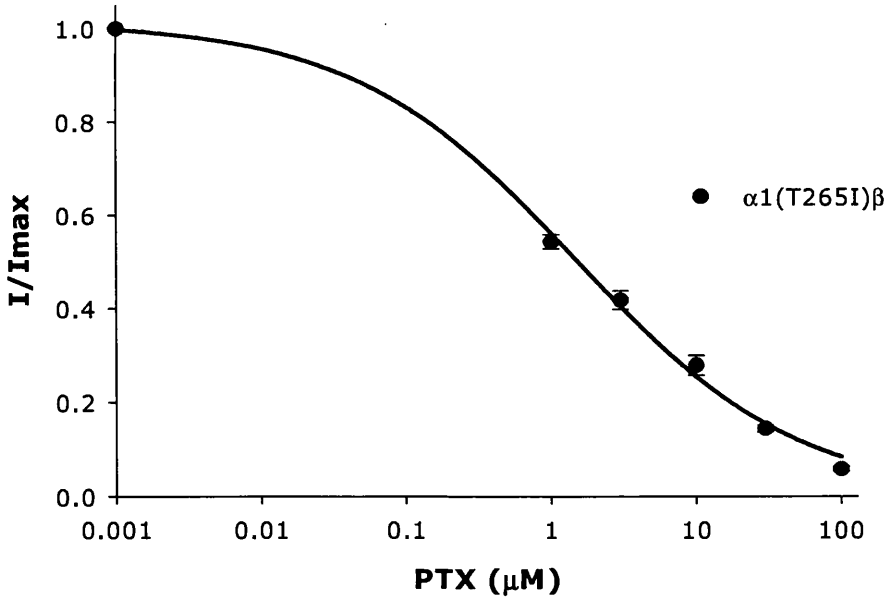


Figure 4.8 TM2-domain dominant mutation T265I. *Examples of PTX current inhibition produced by α1(T265I)β GlyRs when an increasing concentration of PTX was applied in the presence of the EC50 concentration of glycine of the cell. Representative traces of inhibition of PTX on α1WT and α1WTβ in the presence of corresponding glycine EC50 concentration.*

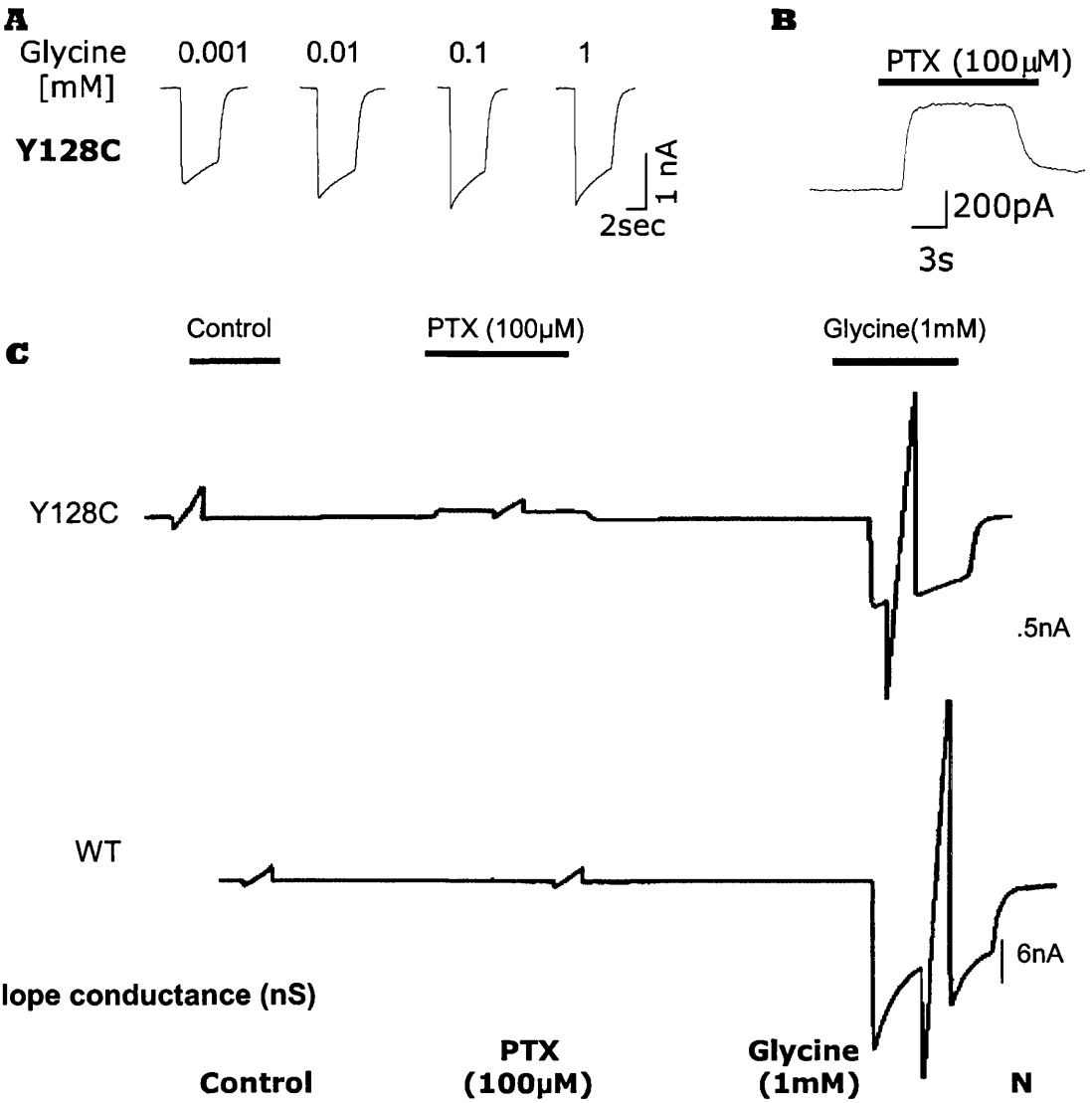
N-terminal mutation GlyR α1 Y128C : The majority of dominant mutations in HE are located in the pore-forming M2 domain or M2 flanking regions of the GlyR α1 subunit. However, the novel mutation Y128C is the second dominant HE mutation located in the N-terminal extracellular domain (Figure 3.3). The only previously-reported N-terminal dominant mutation, R218Q, revealed a decrease in both total and cell-surface expression levels (Miraglia Del Giudice et al., 2003). Consequently, it was intriguing to discover that the Y128C mutant formed spontaneously-opening channels when transiently expressed as homomeric α1 (Y128C) GlyRs or heteromeric α1 (Y128C)β GlyRs (Figure 4.9 A). In the absence of glycine, the competitive antagonist strychnine had no significant effect on leak current in cells expressing α1(Y128C) or α1(Y128C)β GlyRs. In contrast, application of 100μM of PTX, an allosteric inhibitor of GlyRs, significantly reduced inward current (472 ±71 pA, n=25; p<) in the absence of glycine, indicating the closure of spontaneously open channels (Figure 4.9 B).

Both strychnine and PTX did not induce any detectable currents in WT α1 or α1β in the absence of glycine. To further verify the leakage currents of Y128C, voltage ramp protocols as described in the methods section, were performed 1) in the absence of agonist/antagonist (control), 2) in the presence of 100μM of PTX and 3) in the presence of 1mM of glycine (see Figure 4.9 C). In the absence of agonist/antagonist spontaneous opening of α1(Y128C) GlyR was confirmed by unusually larger resting conductances compared to WT; the slope conductance between −80 and +80 mV for Y128C prior to the activation of receptor (resting conductances) was significantly higher compared to WT (Y128C = 2.95±0.48 nS, n=7 ; WT= 0.57±0.20 nS, n=3 ; p<0.001). Application of PTX (100μM) reduced the resting

conductance of Y128C by 35%, whilst resting conductances for WT were similar in the presence or absence of 100μM PTX (Control = 0.57±0.20 pS, PTX= 0.54±0.21). As expected from the smaller glycine current produced by Y128C compared to WT (Figure 4.9), application of 1mM glycine resulted in smaller slope conductance in Y128C than in WT (Figure 4.9 C).

Following the electrophysiological evidence for tonic opening in Y128C, tonic currents induced by cells transfected with α1Y128C were further examined using a high-throughput quantitative fluorescence system (Kruger et al., 2005; Gilbert et al., 2009). To investigate how leak magnitude changed when co-expressed with WT subunits, the α1 GlyR Y128C mutation construct was mixed with different ratios of WT α1 and co-expressed with the I^- sensitive YFP(I152L); (Figure 4.9). HEK 293 cells transfected with YFP(I152L) and GlyRs can be quenched by influx of I^- through anion channels such as GlyRs. Using an automated live-cell imaging system, the glycine dose response characteristics were determined by quantifying the fluorescence change following the application of increasing concentrations of glycine (0.001–10mM).

Following the application of saturating glycine concentration (1mM), over 90% of fluorescent cells transfected with α1 and YFP (I152L) displayed fluorescence quench, indicating over 90% of co-transfection efficiency of α1 and YFP (I152L). The EC50 of WT α1 obtained from cells analysed with the YFP automated system was similar to that of patch-clamped cells (19.3±3.4 for YFP imaging, n>2000; 20.74±7.27 for patching electrophysiology, n= 6). As shown in Figure 4.10, cells transfected with Y128C displayed a maximum fluorescent change in the absence of glycine (n>2000). Cells transfected with an equal amount of Y128C and WT generated



Slope conductance (nS)

	Control	PTX (100μM)	Glycine (1mM)	N
Y128C	2.95±0.48*	1.93±0.31	9.53±1.85	7
WT	0.57±0.20	0.54±0.21	22.87±8.29	3

Figure 4.9 α1Y128C GlyRs generated tonic currents when expressed in HEK 293 cells either as homomeric α1 subunits or heteromeric α1β GlyRs. *A) Examples of glycine-induced currents produced by α1Y128C.* ***B)*** *A representative trace of an upward current induced by PTX (100μM) in the absence of glycine. For WT, no detectable current was induced by PTX or in the absence of agonist (Data not shown).* ***C)*** *Current–voltage curves relationship of WT / Y128C receptors. I-V curves were measured by whole-cell patch clamp recordings using voltage ramp from –80 to +20 mV over 2-s interval. α1Y128C shows unusually large resting conductances in the absence of glycine (Control) (*Statistically different from WT GlyR α1, P<0.001). PTX, GlyR channel blocker, induced outward current and the slope conductance of Y128C was decreased in the presence of 100μM of PTX.*

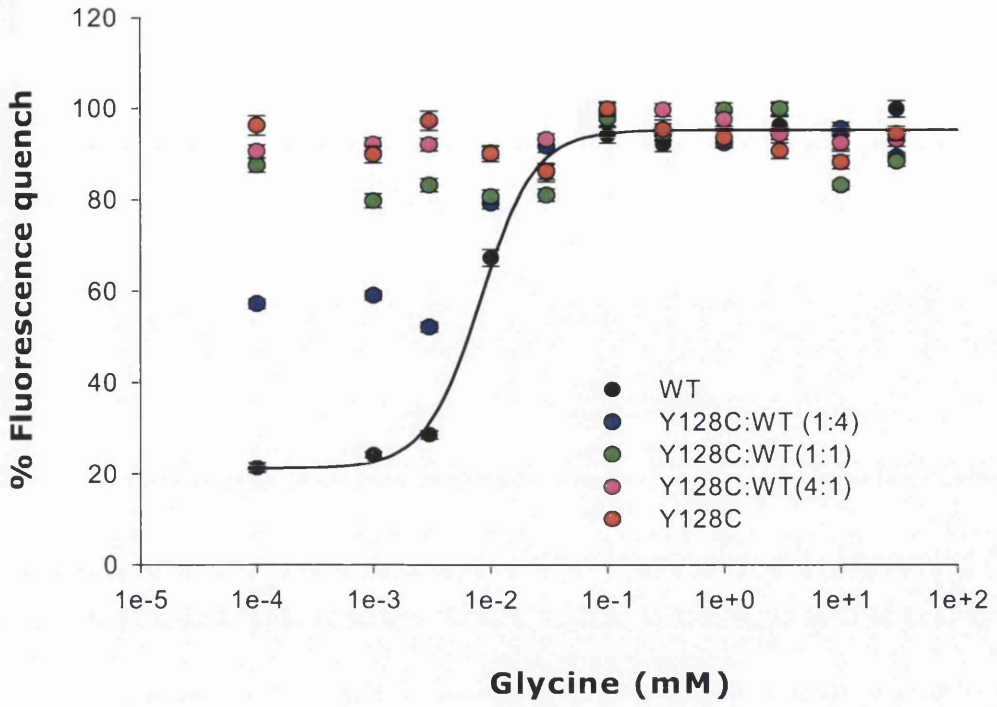


Figure 4.10 Glycine responses of HEK 293 cells transfected with αlY128C cDNA *or with combinations of αlY128C cDNA with αlWT in a different ratio (Y128C:WT = 1:1, 2:1, or 4:1). Over 2,000 cells were analysed using a high-throughput automated system. Y128C:WT at 4:1 and 1:1 resulted in maximum fluorescence quench in the absence of glycine (control) similar to that of Y128C, whilst Y128C:WT at 1:4 generated control fluorescence level in the middle of Y128C and WT.*

a dose response curve similar to that of cells expressing Y128C indicating the dominant-negative effect of Y128C on WT.

4.3 Subcellular localisation with recessive mutations

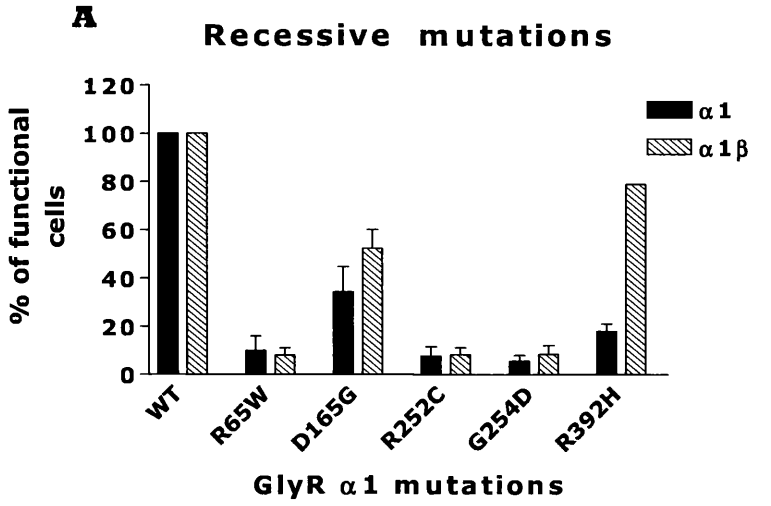
The following experiments were adopted to test whether certain mutations with no evidence of detectable current activity were actually reaching the cell surface or alternatively being retained in the translational pathways of the cellular organelles / or become trafficking mutants.

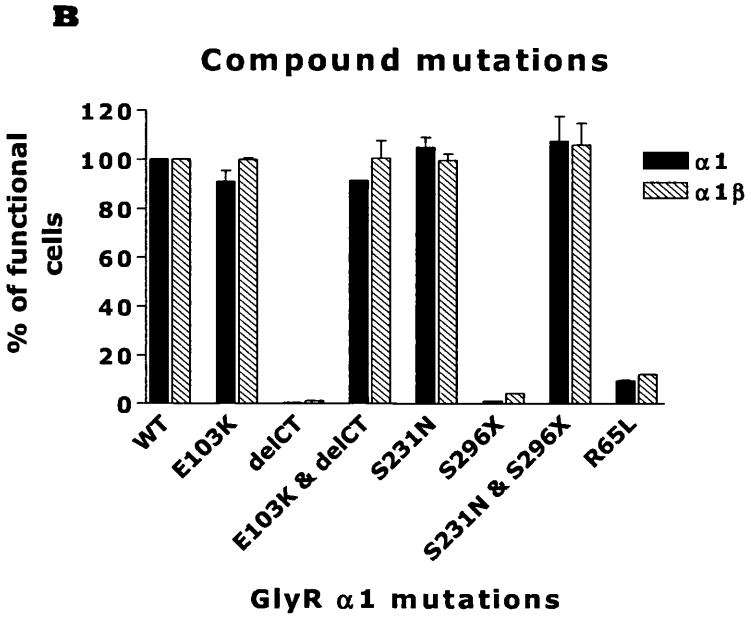
4.3.1 YFP Fluorescence Assay

For each mutant, the number of cells expressing functional GlyRs were determined using the above assay in which HEK293 cells were co-transfected with mutant/WT α1 and YFP. Fluorescent cells that were quenched more than 20% of the initial fluorescence intensity upon the application of a saturating concentration of glycine (maximum of 30mM) were defined as expressing functional GlyRs. Screening of over 1,500 HEK cells transfected with GlyR mutants demonstrated that recessive mutations display a significant reduction in the number of functional channels, further supporting the absence of receptor currents caused by these mutations (Figure 4.11 A).

Dominant mutations, Y128C and T265I and hemizygous missense mutations, E103K and S231N, displayed an expression level compatible to that of the wild-type GlyR channels (Figure 4.11 B, C). To investigate the effects of co-expression of compound mutations, we have screened over 2,000 HEK cells co-transfected with compound mutations (S296X + S231R / E103K + L184fs21X). Both S296X and L184fs21X mutants show loss of function, however, they did not demonstrate any dominant-negative effects on the expression of S231R or E103K respectively (Figure 4.11 B). Previously, S296X was reported to exert dominant-negative effects on the WT GlyR α1 subunit by reducing Cl^- current density from ~170 pA/pF (WT) to ~100 pA/pF (S296X + WT) (Bellini et al., 2007). However, when HEK293 cells were transfected with S296X and WT GlyR α1 and analysed on the YFP automated system (n>2000), no significant change in EC50 or maximum current was observed in comparison to WT.

Figure 4.11 The level of functional GlyRs. *Wild-type human GlyR α1 subunit or hyperekplexia α1 variants were expressed in HEK293 cells, either as homomeric α1 subunit or heteromeric α1β GlyRs, with YFP. The number of cells expressing functional mutant channels were quantified (and compared to that of the wild-type subunit) using a YFP-based high-throughput screening assay. Fluorescent cells that showed >20% of fluorescent change (quench) following the application of a saturating concentration of glycine were considered as expressing functional GlyRs. The % fluorescent change (quench) was calculated using the following equation: (1 – (F_{final}/F_{init}))*100), where F_{init} is the initial (or control) fluorescence value prior to the application of glycine; F_{final} is the fluorescence level after the application of glycine.* ***A)*** *The percentage of fluorescent cells expressing functional Y128C and T265I channels were compatible to that of the wild-type GlyR channels.* ***B)*** *The levels of functional channels for compound missense mutations, E103K and S231N, were similar to that of WT. S296X and L184fs21X do not express functional receptors. When S296X and L184fs21X were co-expressed with E103K and S231N, respectively, the level of functional channel of E103K and S231N were not affected.* ***C)*** *The majority of mutants harbouring recessive mutations (e.g. R65L, R65W, R252C, G254D) have significantly lower levesl of functional GlyRs compared to WT GlyRs.*


A
Recessive mutations
% of functional cells
120
100
80
60
40
20
0
α1
α1β
WT
R65W
D165G
R252C
G254D
R392H
GlyR α1 mutations


B
Compound mutations
% of functional cells
120
100
80
60
40
20
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α1
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delCT
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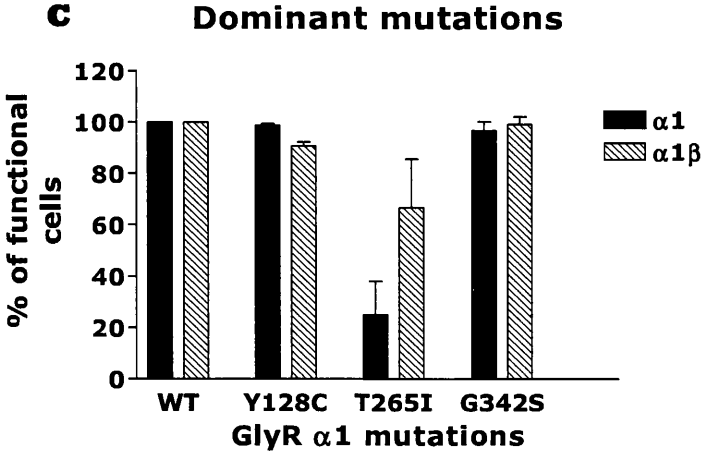
R65L
GlyR α1 mutations


C
Dominant mutations
% of functional cells
120
100
80
60
40
20
0
α1
α1β
WT
Y128C
T265I
G342S
GlyR α1 mutations

4.3.2 Biotinylation labelling assay

Hyperekplexia mutants with no/low level of functional receptors were further investigated for the surface expression of channels. Transfected HEK293 cells were labelled with sulfo-NHS-SS biotin and electrophoresis of cell-surface proteins revealed that the reduction in the number of functional channels observed in recessive mutants is due to the decreased cell-surface expression of channels (Figure 4.12). The whole-cell expression of recessive mutations were compatible to that of WT, however, cell-surface expression levels revealed a significant decrease (Figure 4.12). Positive controls for WT profiles in mutations T265I and R392H displayed the expected WT cell-surface expression (Figure 4.12). The hemizygous mutation, R65L, identified in a compound heterozygous case (Table 3.1, case 7), generated no current but has similar levels of surface protein expression compared with WT.

In contrast, the recessive mutation R65W showed a significant reduction ($p<0.0001$) in the level of surface protein expression but similar level of whole cell expression. The possible explanation for this residue-specific cell-surface difference is described in the molecular modelling section of this study. For the dominant T265I mutation there was no alteration in the cell-surface expression indicating the decreased level of T265I functional channels is due to non-functional surface-targeted channels rather than a reduction in surface-expressed channels.

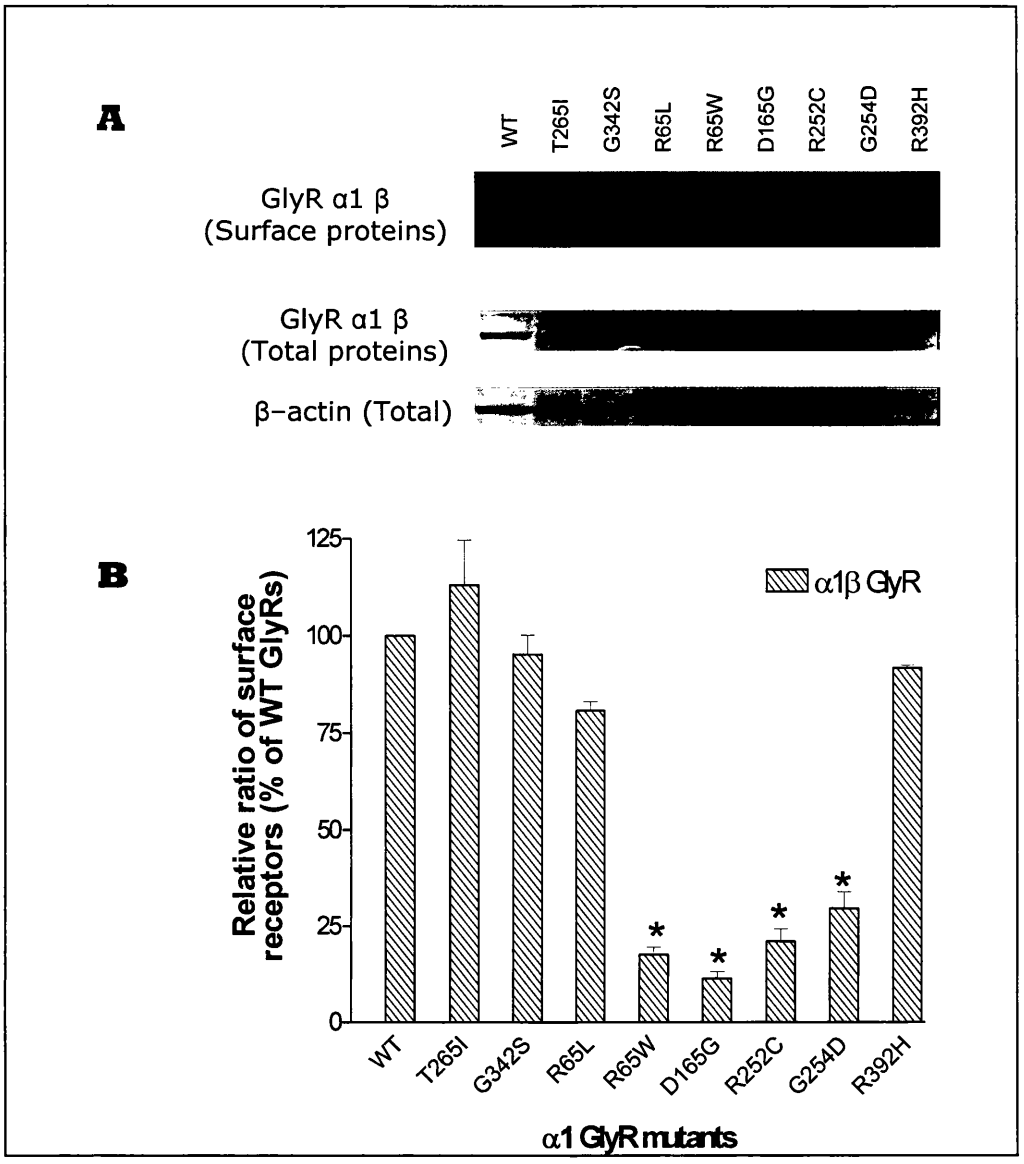


Figure 4.12 Majority of recessive HE mutations resulted in reduced surface expression on HEK293 cells. *HEK293 cells transfected with WT or mutant α1 GlyRs were labelled with sulfo-NHS-SS-biotin, and the cell-surface proteins were analysed by Western blot with an antibody against the GlyR α1 subunit (Millipore).* ***A)*** *Expression of recessive mutations resulted in similar levels of whole cell protein expression (total) but reduced cell-surface protein expression (surface) compared with WT receptors.* ***B)*** *The surface expression of the mutant α1 subunits was quantified using imageJ software and expressed as a percentage of WT α1. The surface level for the recessive mutations, R65W, D165G, R252C, G254D were significantly reduced when compared with WT (*p<0.0001). In contrast, the surface expression of dominant mutation T265I was not reduced compared to WT. 50μg of protein lysates were loaded in the each lane.*

4.4 Immunohistochemistry

The reduction of cell-surface expression of *GLRA1* mutations were also confirmed by surface staining of HEK293 cells with an antibody against the N-terminal of GlyR α1 subunit (Millipore) (Figure 4.13).

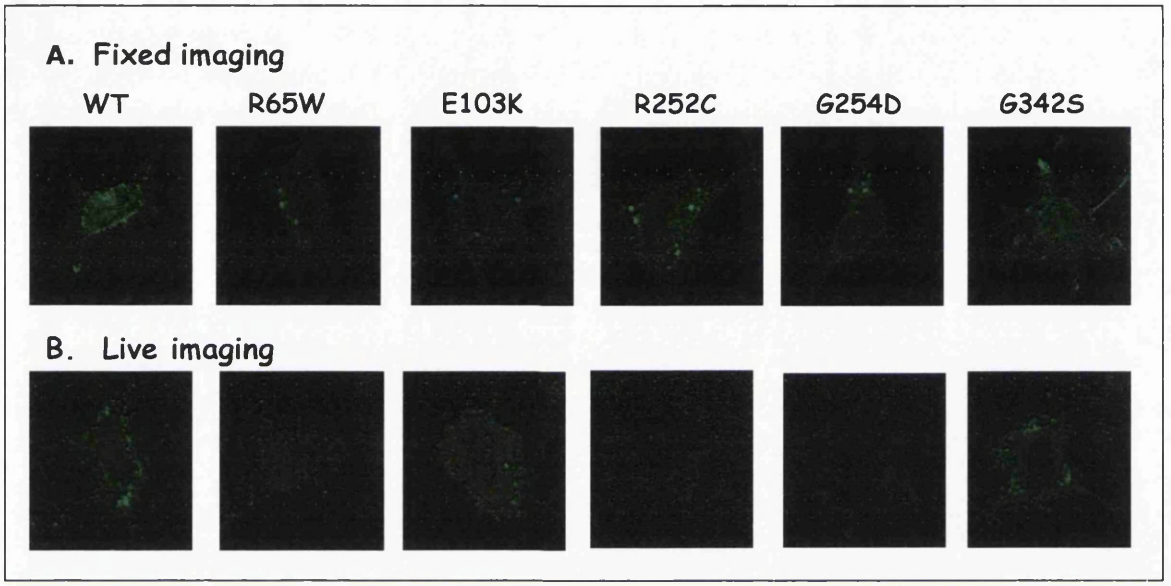


Figure 4.13 Representative confocal fluorescence images of HEK293 cells transfected with wild-type and mutant expression constructs. *The subcellular localisation of the GlyR α1 subunit and hyperekplexia mutants were visualised with anti-GlyR α1 antibody (Millipore). Two different protocols were used to differentiate between cytoplasmic (fixed) and cell-surface GlyRs (live staining). WT GlyR α1 and E103K and G342S GlyR α1 are primarily detected in the cell membrane, while majority of recessive mutations show predominantly cytoplasmic staining with minimal cell-surface localization.*

4.5 Molecular modelling.

Examination of the positions of the new mutations (Figure 4.14A) reveals that they are located in three broad zones – the central region of the extracellular domain, the mid-membrane region and the cytoplasmic loops. The dominant mutations reported involve uncharged residues being replaced by other uncharged residues, while the compound mutations involve residues being replaced by a charged or polar residue, and recessive mutations involve either

the replacement of or replacement with a charged residue (i.e. change of charge). There are no discernable patterns for the location for each type of mutation, rather it is the changes in secondary, tertiary and quaternary structure brought about by individual mutations that determine their functional effects.

The structural modelling of GlyRα1 mutants is summarized in table 4.2 and has proved to be a useful approach in rationalising some of the functional effects of the mutations observed in terms of structural changes brought about by the mutations. Notably, a short alpha helical

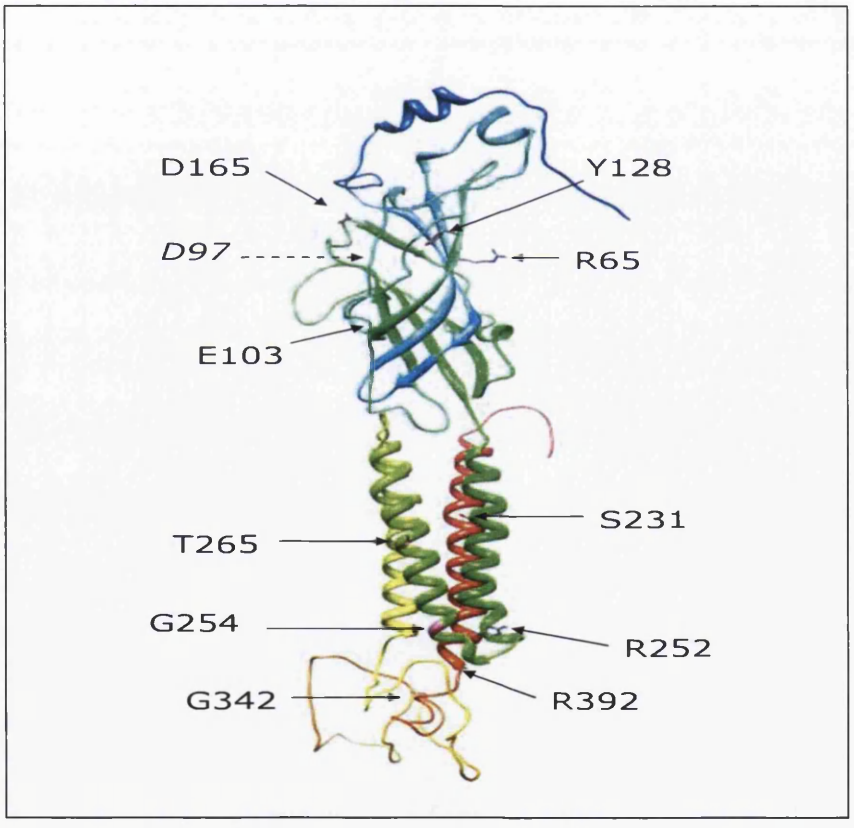


Figure 4.14 Structural modelling of GlyRα1 mutants. *A) The positions of GlyRα1 mutations are superimposed on the modelling structure demonstrating the topology of the variants.*

Table 4.2 Summary of the functional evidence for pathology of novel hyperekplexia mutations

Inheritance	α1 mutant	Electrophysiology -Patch clamping	YFP based High-through analysis	Surface expression	Structural modelling
Dominant	**Y128C**	Tonic opening of channel, reduced current	Number of functional channels are compatible to that of WT	ND*	Formation of short helix overlying membrane domain, relaxed TM region arrangement
	T265I	Shift of EC50, Reduced current	Number of functional channels are compatible to that of WT	OK	Rearrangement of TM regions; relaxation of beta strand structure around glycine binding site
SNP	**G342S**	WT	Number of functional channels are compatible to that of WT	OK	Minor TM region rearrangement
Recessive	**R65W**	No current	Reduced functional channels	Reduced surface receptors	Extension of M2 helical region into the interface with extracellular domain; enhanced beta strand structure in extracellular domain
	D165G	Shift of EC50, Reduced current	Reduced functional channels	Reduced surface receptors	Extension of M2 helical region into the interface with extracellular domain; enhanced beta strand structure in extracellular domain
Recessive	**R252C**	No current	No response	Reduced surface receptors	Extension of M2 helical region into the interface with extracellular domain; enhanced beta strand structure in extracellular domain
	G254D	No current	No response	Reduced surface receptors	Extension of M2 helical region into the interface with extracellular domain

	R392H	Shift of EC50, Reduced current	Number of functional channels are compatible to that of WT	OK	Significant loss of beta strand structure around glycine binding site
Compound	**R65L***	No current	Reduced functional channels	OK	Extension of M2 helical region into the interface with extracellular domain
Compound	**E103K**	Shift of EC50,	Number of functional channels are compatible to that of WT	ND*	Relaxation of beta strand structure around glycine binding site
	L184fs21X	No current	No response	ND*	
Compound	**S231N**	Shift of EC50,	Number of functional channels are compatible to that of WT	ND*	Relaxation of beta strand structure around glycine binding site
	S296X	No current	No response	ND*	

* Hemizygous mutation identified with a Δ Exon 4-7 in case 7; ND: Not determined; OK: surface expression level is compatible to that of WT

structure is predicted to be introduced in the extracellular domain of the Y128C mutant (Figure 4.14B) associated with tonic opening. Several mutant structures displayed marked rearrangement of transmembrane regions, including Y128C, D165G and T265I, which are associate with low current. Conversely, E103K, S231N and G342S mutants show only slight rearrangements of transmembrane regions compared to wild-type, consistent with wild-type current levels. Relaxation of the β-strand structure in the vicinity of the glycine binding site in the extracellular domain was predicted for E103K, S231N, T265I and R392H, and could be aligned with observed increases in EC50.

The R65W, R252C, and G254D mutants displayed extension of either the M2 or M3 helix into the interface with the extracellular domain, and these mutants suffered a lack of expression (Figure 4.14 C). R65L is a second mutation identified on the glycine binding residue R65 and is a part of compound mutations. According to the structural modeling, the main differences between R65L and R65W are in the β strand structure of the extracellular domain, which is enhanced in R65W compared to R65L and wild type i.e. with regards to the β strand structure of the extracellular domain, R65L is much more like wild type, whereas R65W has substantially more strand structure, and therefore with the extended M2 domain helix possesses two significant structural differences compared to wild type. In R65L, the extension of the M2 domain helix is compensated for by the increased flexibility in the extracellular domain, whereas R65W suffers from extension of the M2 helix and less flexibility in the extracellular domain. These structural features occur down the same side of the molecule suggesting that the expression problems encountered by R65W may be caused by an inability to form viable pentameric structures with adjacent subunits, due to this decreased flexibility.

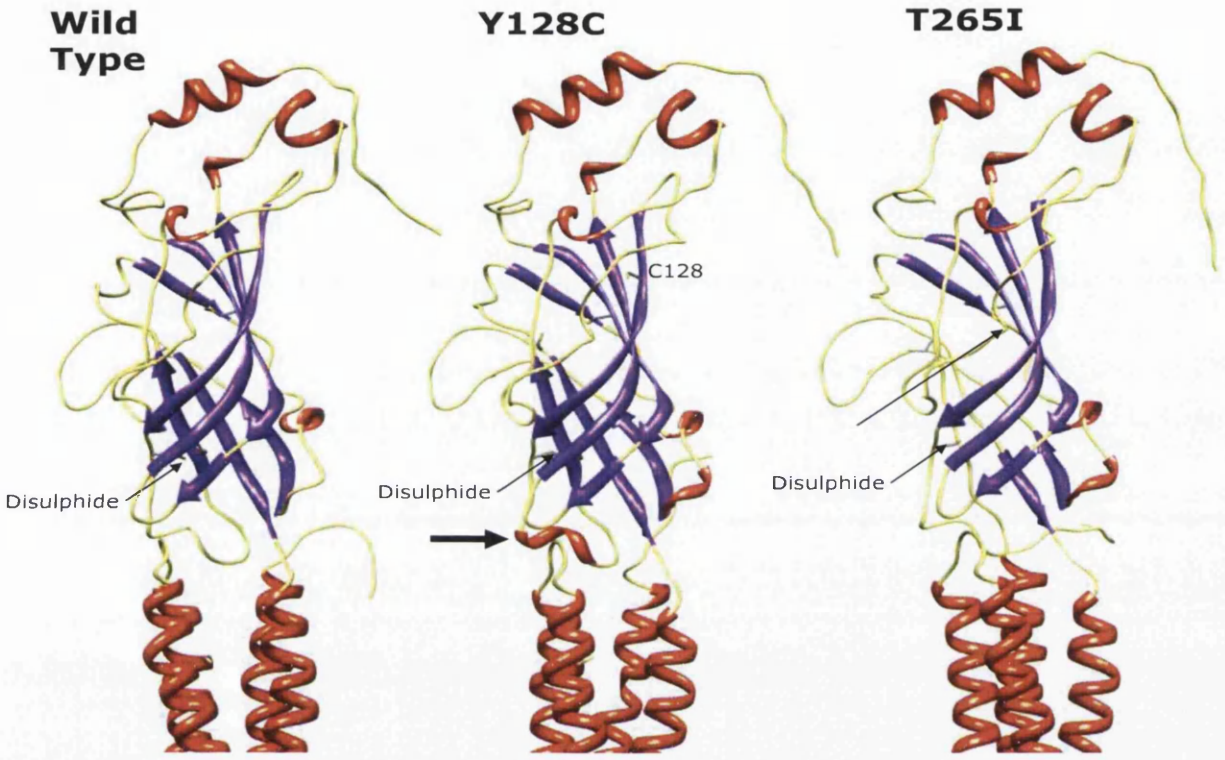


Figure 4.14 B) *Structural modelling of GlyRα1, showing wild type structure, introduction of short alpha helical structure in extracellular domain of Y128C mutant (indicated by thick arrow) associated with tonic opening, marked rearrangement of transmembrane regions in T265I mutant associated with low current; and relaxation of structure in the vicinity of the glycine binding site (regular long arrow), concomitant with observed increases in EC50.*

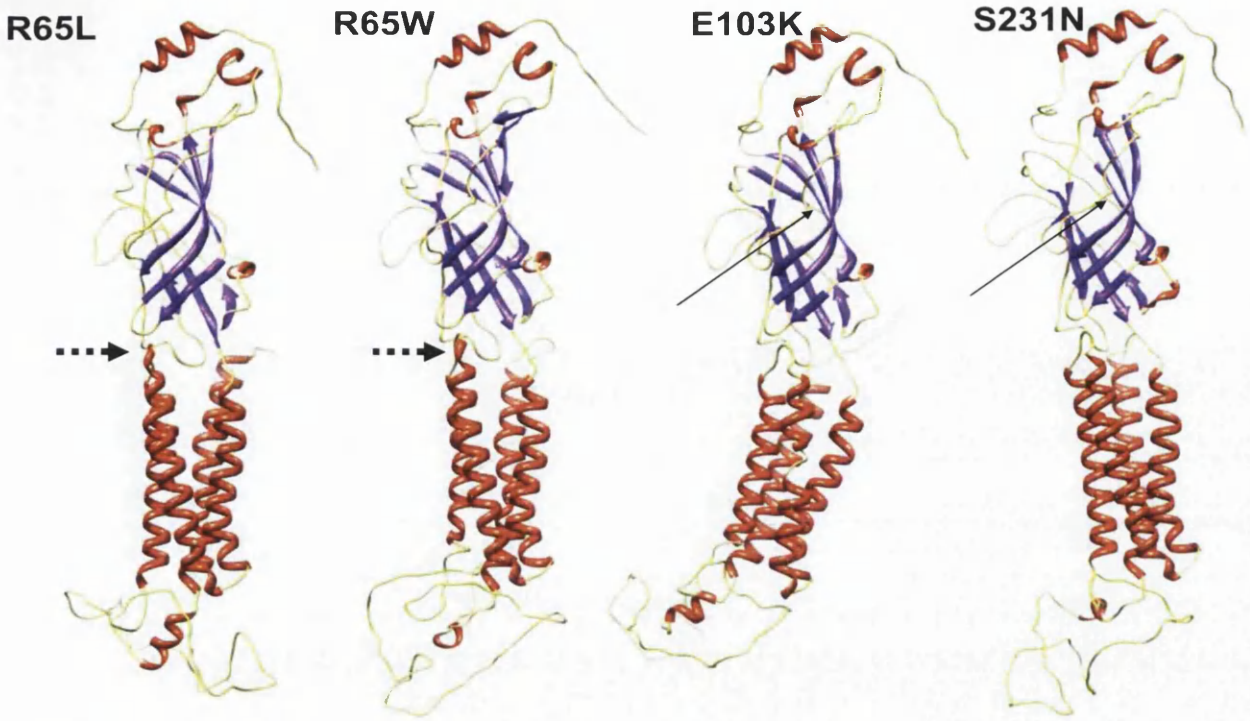


Figure 4.14 C) *R65W mutant displays extension of M2 helical region into the interface with the extracellular domain (dashed thick arrows), associated with lack of expression, whereas R65L mutant displays a less extensive rearrangement of TM helixes. E103K and S231N mutants show only slight rearrangements of transmembrane regions compared to wild-type, and relaxation of structure in the vicinity of the glycine binding site (regular arrows), coincident with observed increases in EC50.*

4.6 Summary

From the 18 novel and recurrent *GLRA1* variants in Chapter 3, we have progressed to investigate the functional effects of eleven novel and two recurrent *GLRA1* mutations. Mutation constructs were prepared using site-directed mutagenesis techniques and the expression level and functional properties of the hyperekplexia mutants were analyzed using patch-clamp techniques and a high-throughput screening system (Table 4.2). The majority of recessive mutants were unable to generate any current due to traffic defects,

where as dominant mutations generated surface expressed but dysfunctional channels.

When expressed in HEK293 cells, either as homomeric α1 subunits or heteromeric α1β subunits of GlyRs, subcellular localisation defects were the major mechanism underlying recessive mutations. Interestingly two mutations identified on the glycine binding residue, R65W and R65L, generated no glycine currents through two different cellular mechanisms; in R65W, identified in a recessive case of HE, the mutant channel was not able to reach the cell surface resulting in no functional channels, whereas R65L, a hemizygous mutation, was expressed on the surface, but the channels were insensitive to glycine activation. GLRA1 mutants without trafficking defects typically show alterations in the dose-response curve for glycine suggestive of disrupted signal transduction.

Whilst the majority of dominant mutations are located in the pore-forming M2 domain or flanking regions of the GlyR α1 subunit, the new dominant mutation Y128C is located in the N-terminal extracellular domain. When transiently-expressed in HEK293 cells, either as homomeric α1 GlyRs or heteromeric α1β GlyRs, the Y128C substitution resulted in spontaneously-opening channels. Although, tonic channel opening has been recognized as one of the mechanisms underlying channelopathies, this is the first report of a hyperekplexia mutation associated with leaky current.

We also demonstrate that T265 on the pore lining region is an important residue on determinant of PTX sensitivity and one amino acid change on the residue was sufficient enough to convert the heteromeric α1β GlyRs to PTX sensitive. PTX is widely-used in recombinant expression studies of cys-loop channels, however, its complex inhibitory mechanism is not yet clear. The analysis of PTX

action on T265I could reveal the new features of pore structural and pharmacological differences between $\alpha 1$ and β subunits.

Chapter 5 Gephyrin heterogeneity in human brain

In this chapter, we transfer to a wider perspective of human hyperekplexia and glycinergic biology by examining the relationship between glycine receptors and gephyrin in the human brain. Gephyrin encodes a multifunctional cytoplasmic protein that is important for both organizing of inhibitory glycine and $GABA_A$ receptors at the postsynaptic membrane of neurons and for molybdoenzyme activity in non-neuronal tissues (Fritschy et al., 2008). Mutations in gephyrin are associated with a neurological startle disorder, hyperekplexia, hereditary molybdenum cofactor deficiency and is disrupted in specific leukaemia translocation breakpoints (Reiss, 2000; Eguchi et al., 2001; Kuwada et al., 2001; Rees et al., 2003).

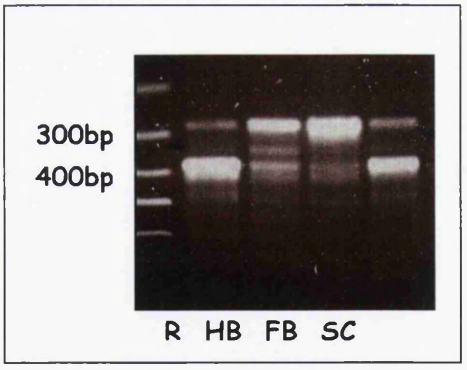
A previous study demonstrated that human neuronal tissues express over a dozen variants of gephyrin isoforms, generated from alternatively spliced exons, whereas non-neuronal tissues express a single gephyrin transcript (Rees et al., 2003). Alternative splicing, which is generally developmentally regulated, is now established as the major mechanism underlying the functional diversity of proteins particularly in the nervous system (Grabowski and Black, 2001), however, the biological significance of the gephyrin heterogeneity in the human brain is not known. Thus, the objective of the experimental approach in this chapter is: 1) to investigate gephyrin transcript heterogeneity among the adult human brain, retina, spinal cord and fetal brain; 2) to examine, using custom-made, cassette-specific gephyrin antibodies, the distribution pattern of each gephyrin cassette in the medullar brainstem region where GlyRs are highly-enriched.

5.1 Molecular analysis of gephyrin transcripts

The genomic structure of gephyrin is detailed on Figure 1.3 and clearly demonstrates the existence of a central linker region and hotspot for differential splicing. Here, the existence of transcript populations in four neurological tissue-sources are assessed and an attempt to quantify their presence in the human brain with Real-time PCR validation.

5.1.1 Transcript analysis of human gephyrin

To detect possible gephyrin splice-variants, C3 and C4 fragments were amplified from human adult brain (HB), fetal brain (FB), retina (R) and spinal cord (SC) RNA (Figure 5.1). This created a ladder effect of several differentially-sized products and an obvious heterogeneity in the patterns observed between tissues. The transcript ladders were excised and sub-cloned into pGem vectors and transformed into ultracompetent cells. Subsequent transcript analysis and sequencing analysis of over 1,500 clones revealed that at least 22 gephyrin C3/C4 transcripts are differentially expressed in the above four areas (Figure 5.2). Ten of 22 splice isoforms were tissue-specific. Consistent with a previous study, in human brain at least 11 distinct neuronal gephyrin isoforms were generated



Fogure 5.1. Heterogeneity of C3-C4 assays in neurological tissues. *C3-C4 regions were amplified from cDNAs of Human adult brain (HB), fetal brain (FB), retina (R) and spinal cord (SC) cDNA..*

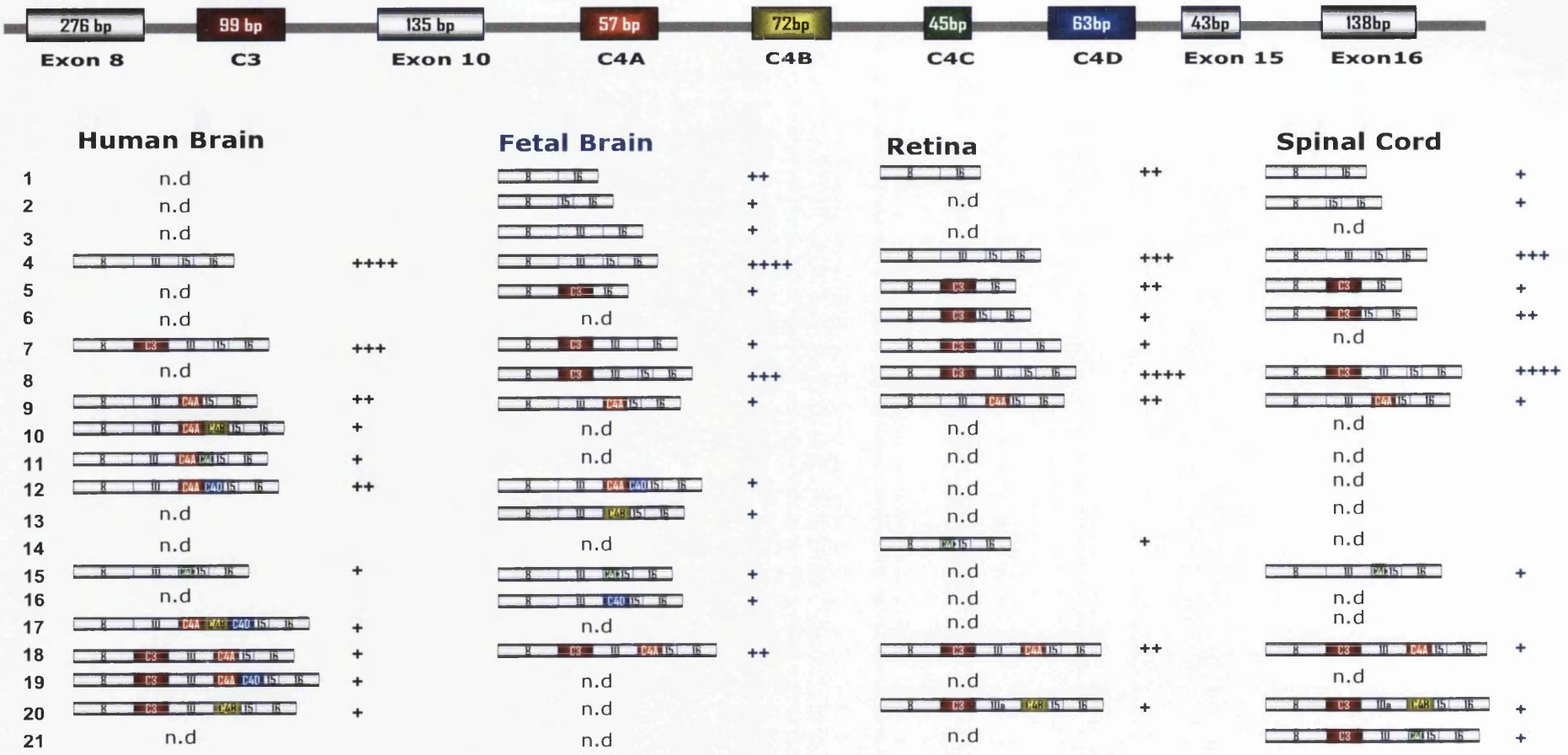


Figure 5.2 Analysis of gephyrin transcripts. *RT-PCR and sequencing analysis of over 1,500 clones revealed distinct tissue specific profiles of gephyrin variants. Of the 22 isoforms identified, some splice isoforms are restricted to adult human brain where a more diverse range of the combinatorial arrangements of C4 exons was observed in comparison to fetal brain, retina and spinal cord. This tissue-specific expression of gephryin variants indicates that the diversity of gephyrin transcripts maybe spatially and temporally regulated.*

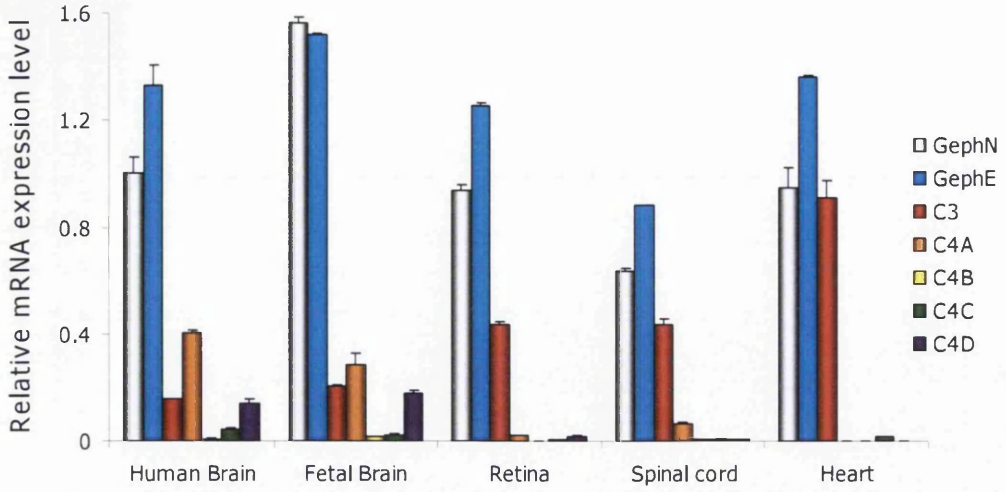
from the alternative splicing of 4 exons (Rees et al 2003), whereas fetal brain has 13 isoforms generated from the alternative splicing of 6 exons. Interestingly, exons 10 and 15 are subject to splicing in the fetal brain and become constitutive in the adult human brain, whereas they remain regulated in the adult spinal cord and retina.

Retina and spinal cord displayed 10 and 11 gephyrin isoforms, respectively and isoforms detected in fetal brain, retina and spinal cord have a single C3 or C4 cassette insertion. Whilst in the adult brain, the majority of C3/C4 isoforms contain multiple C4 cassette insertions, consequently resulting in a more diverse range of the combinatorial arrangements. This experiment underlies the concept of the importance of transcript heterogeneity in the human brain, and the complexity of the interpretation of the biological context in future studies.

5.1.2 Expression of gephyrin splice-variants in mammalian brain tissue

Real-time PCR was conducted to quantify the mRNA levels of the gephyrin cassettes in adult human brain, fetal brain, spinal cord, retina and heart. First, the expression level of each cassette was represented relative to that of full-length gephyrin containing N-domain (GephN) in the adult human brain. As illustrated in Figure 5.3, C4 cassettes were mostly brain-specific, while in the retina and spinal cord, transcripts containing the C3 cassette predominate. Gephyrin molecules containing the E domain (GephE –see Figure 5.3) were more highly expressed than GephN in all tissue-types investigated except fetal brain. This could be explained by the presence of gephyrin molecules containing only E-domain but not N-domain as identified by Y2H experiments (Rees et al., 2003). The occurrence of smaller gephyrin molecules of

A



B

Cassette (%)	C3	C4A	C4B	C4C	C4D
Human Brain	100	264.2	5.2	28.7	92.6
Fetal Brain	100	140.7	7.8	11.5	88.2
Retina	100	5.0	0.2	1.1	4.2
Spinal cord	100	14.9	0.9	1.7	0.6

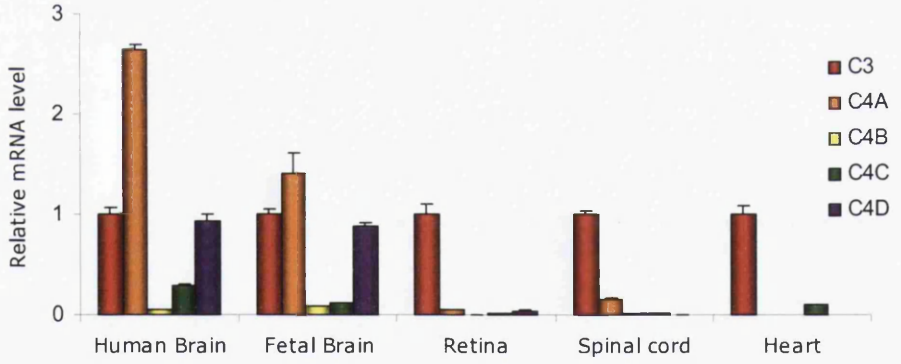


Figure 5.3 Gephyrin splice variant transcript analysis in mammalian brain tissue. *mRNA expression of each gephyrin cassette was determined in human brain, fetal brain, spinal cord, retina and heart using Q-PCR and cassette-specific primers.* ***A)*** *Data are presented as a ratio of expression level of each cassette in different neuronal regions to that of the full length gephryin in the human brain.* ***B)*** *Data are presented as a ratio of expression level of each cassette to that of C3 region in different neuronal regions. In human adult brain, all five splicing exons were identified in a relatively balanced level, while in the retina and spinal cord, C3 was predominantly present.*

various sizes (52- 60kDa) containing E- domain were also identified in rat tissues (Hermann et al., 2001). C4B and C4C containing transcripts were rare in the brain and hardly detectable in retina and spinal cord. When the relative expression level of each cassette was compared to that of the C3 cassette in each tissue, it clearly showed that C4 cassettes were predominantly expressed in the adult and fetal brain, whilst in the retina and spinal cord, C4A, C4B and C4D cassettes had low expression levels but were minimally detectable (Figure 5.3 B). This is consistent with studies on rodent gephyrin isoforms which also reveal variable tissue-specific expression levels of the C4 cassettes (Meier et al., 2000; Smolinsky et al., 2008). Interestingly, C4C cassette was detectable in the heart, albeit to a small extent, but remains the only evidence of C4 cassette involvement outside neurological tissue.

5.2 Immunohistochemical localization of gephyrin isoforms in the human medulla oblongata and spinal cord

The human medulla and spinal cord are highly-enriched with glycine receptors and subject to the glycinergic inhibition of the sensory circuits responsible for the ancient startle response. To begin to understand the neuroanatomy of the human startle response we created cassette-specific, custom-made antibodies targeting the five C3 and C4 gephyrin cassettes (see Figure 5.4). The distribution of gephyrin isoforms containing C3/C4 cassette were investigated in the human brainstem and spinal cord. Previous immunohistochemistry studies have illustrated that

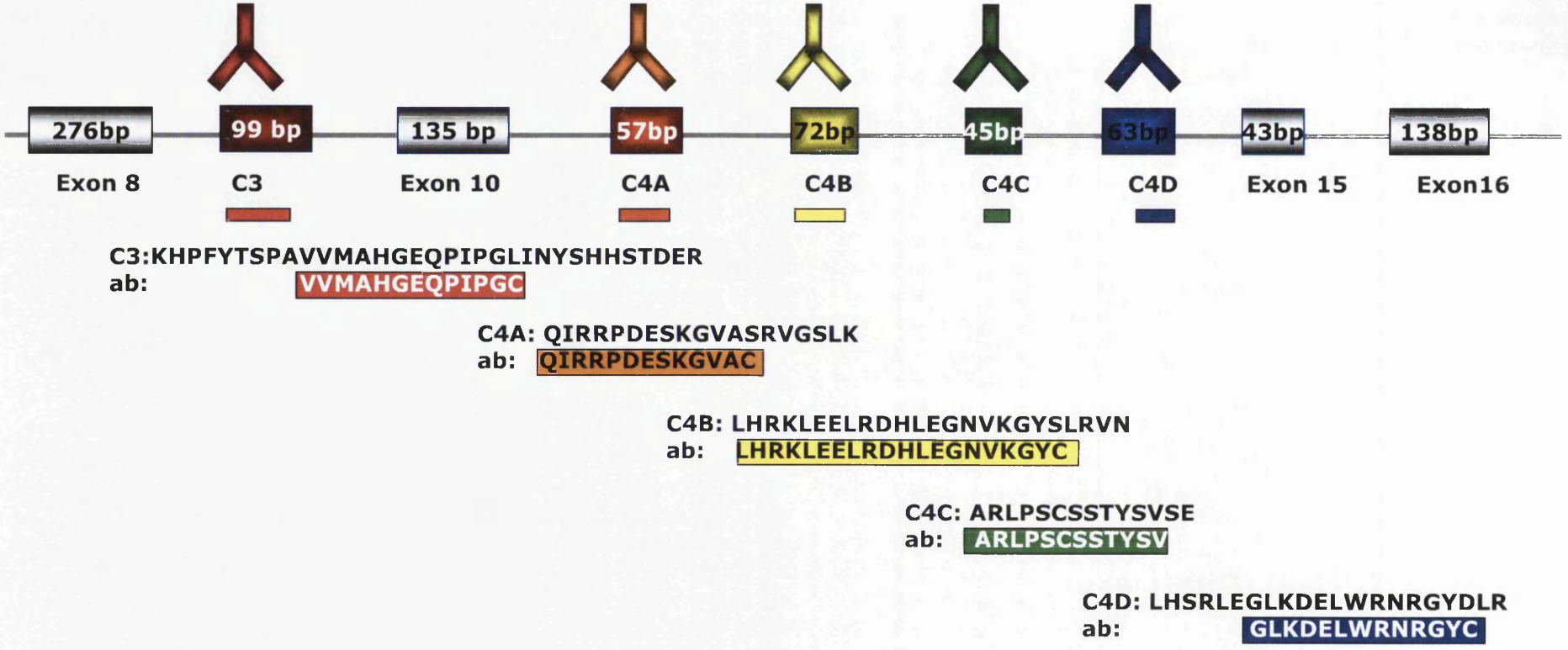


Figure 5.4 Schematic diagram of the human gephyrin splice cassettes and amino acid composition of the C3/C4 exonic fragments. *The epitope boundaries of the C3/C4 gephyrin cassette-specific antibodies are shown below each cassette.*

Table 5.1 Summary of immunoreactivity of GlyR and gephyrin antibodies on human brain medullary and spinal cord sections.

	C3		C4A		C4B		C4C		C4D		GephE		GlyR	
	S	D	S	D	S	D	S	D	S	D	S	D	S	D
Medulla														
DMNX	+	-	+++	+++	+	-	+	-	++	++	+++	+++	++	+++
HN	+	+	++++	++++	+	+	+	-	++	++	+++	+++	++++	++++
SN	+	-	+++	+++	-	-	-	-	+	+	++	++	++	++
STN	+	-	++	++	-	-	-	-	-	-	+++	+++	++++	++++
AON	-	-	++	++	-	-	-	-	+	-	++	++++	++	++++
IO	+	-	++	++	-	-	-	-	+	-	+++	+++	+++	+++
Spinal Cord														
DH	+	+	+++	+++	+	+	+	+	++	++	+++	+++	+++	++++
VH	+	+	+++	+++	-	-	-	-	++	++	++	++	+++	++++

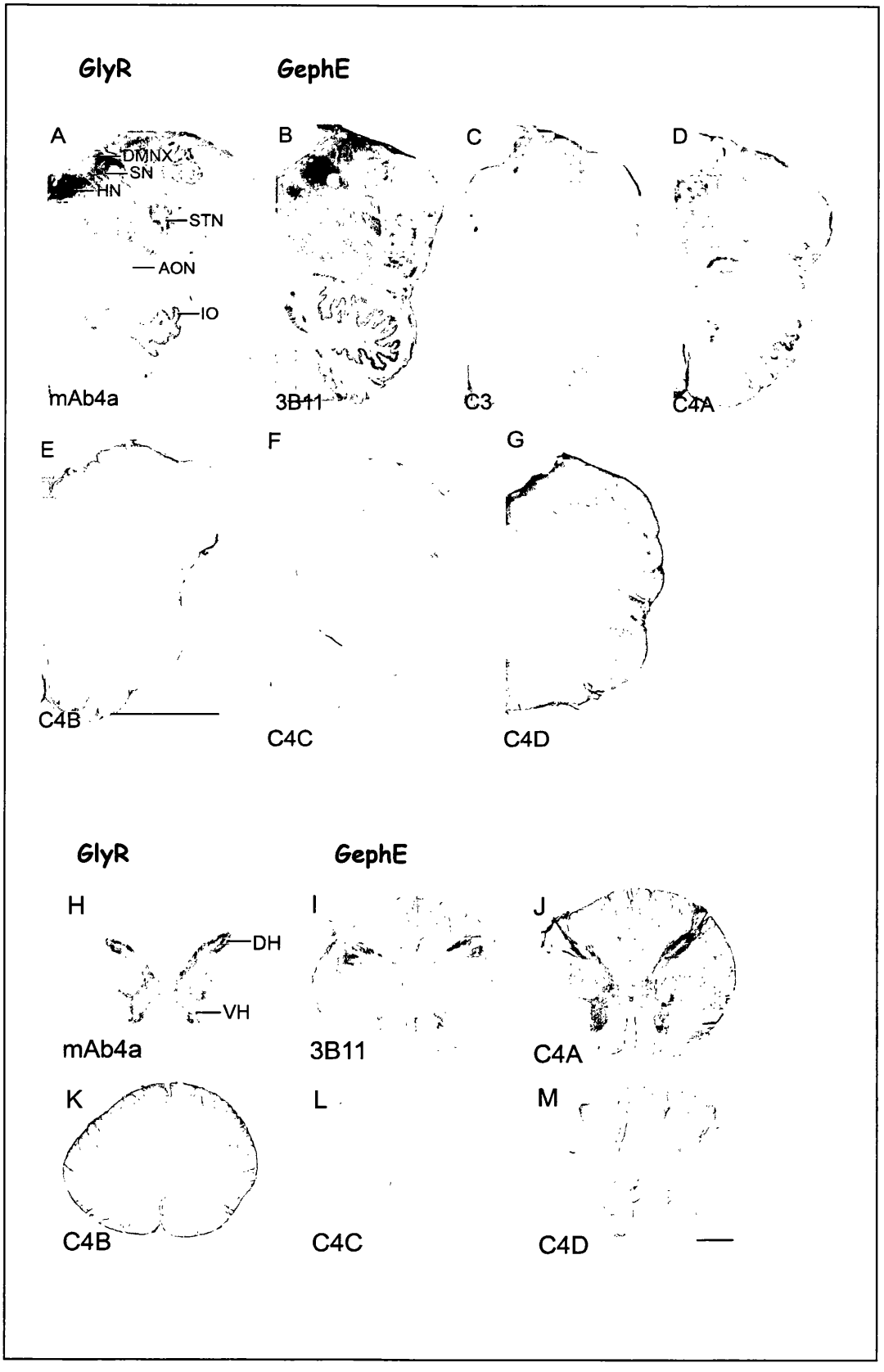
Intensity of the immunoreactivity of gephyrin cassette, Geph, and GlyR in human medulla and spinal cord. Abbreviations: S, soma; D, dendrites; DMNX, dorsal motor nucleus of the vagus; HN, hypoglossal nucleus; SN, solitary nucleus; STN, spinal trigeminal nucleus; AON, accessory olivary nuclei; IO, inferior olive; Density of immunoreactivity: -, not detectable; +, weak; ++, moderate; +++, high; ++++, most intense.

gephyrin has a similar staining pattern compared to GlyRs in the medullar region and spinal cord with a high proportion of gephyrin immunoreactivity co-localizing with glycine immunoreactivity (Baer et al., 2003). Here we have compared the distribution of gephyrin cassettes with that of GlyR or gephyrin E-domain (GephE) in human brain. Monoclonal antibodies, mAb4a (Synaptic Systems; Germany) and 3B11(Synaptic Systems, Germany) were used to detect GlyRs and the invariant GephE respectively. Each gephyrin cassette displays distinct patterns of immunoreactivity and co-localizaton with GephE domain and GlyR in double-labeled experiments (Table 5.1 and Figure 5.5 - 5.9).

5.2.1 Regional localization of gephyrin isoforms

Sections from the upper medulla oblongata and the cervical level of the spinal cord were immunohistochemically labelled for gephyrin cassettes (C3, C4A-C4D), GephE or GlyR using DAB staining (Figure 5.5). Consistent with previous studies (Waldvogel et al., 2003; Baer et al., 2003), GephE and GlyR demonstrate moderate to high levels of immunoreactivity in the hypoglossal nucleus (HN), the dorsal motor nucleus of the vagus (DMNX) and the solitary nucleus (SN) in the upper medulla oblongata (Figure 5.5 A-B). The gephyrin cassettes show weaker immunoreactivity than GephE in the medullar region (Figure 5.5 C-G) and this correlates with lower levels of C3/C4 mRNA compared to total gephyrin mRNA in the spinal cord (Figure 5.3). Each cassette, however, displays distinct staining patterns in the upper medulla oblongata and the cervical spinal cord (Figure 5.5 and Table 5.1).

Figure 5.5 Regional localization of gephyrin isoforms and GlyRs in human medulla and spinal cord. *Macroscopic images of serial coronal sections of human upper medulla (A-G) and spinal cord (H-M) labelled for gephyrin isoforms (C-D; J-M); GlyR (A, H); or gephyrin-E domain (B, I) by DAB staining.* ***A)*** *GlyR and* ***B)*** *gephyrin display intense level of immunoreactivity labelling in the dorsal motor nucleus of the vagus (DMNX), the hypoglossal nucleus (HN), dorsal and medial accessory olivary nuclei (AON), inferior olive (IO) and the spinal trigeminal nucleus (STN).* ***C)*** *C3 shows overall weak labelling with moderate patch staining in the HN and DMNX.* ***D),*** *C4A demonstrates intense levels of immunoreactivity in the HN, SN, AON and moderate levels of labelling in the IO.* ***E)*** *C4B and* ***F)*** *C4C shows relatively weak labelling.* ***G)*** *C4D displays moderate levels of immunoreactivity in the HN, and DMNX.* ***H)*** *In the spinal cord, GlyRs show intense staining in the dorsal (DH) and ventral horns (VH) whereas GephE* ***I)*** *has very intense immunoreactivity in the lamina II of the DH with moderate to high labelling throughout the spinal cord.* ***J)*** *C4A shows intense staining in both DH and VH.* ***K)*** *C4B shows moderate to low-levels of labelling in the DH.* ***L)*** *C4C displays relatively weak labelling in the DH and VH.* ***M)*** *C4D demonstrates weak to moderate levels of labelling in the DH and VH. – (Scale bar) = 0.5 cm in E (applies to A-G); 2 mm in M (applies to H-M).*


GlyR
GephE
A
DMNX
SN
HN
STN
AON
IO
mAb4a
B
3B11
C
C3
D
C4A
E
C4B
F
C4C
G
C4D
GlyR
GephE
H
DH
VH
mAb4a
I
3B11
J
C4A
K
C4B
L
C4C
M
C4D

Of the five gephyrin cassette specific antibodies, C4A showed the highest immunoreactivity in the HN and SN (Figure 5.5A). The staining pattern of C4A was the most similar to that of GlyR and GephE, showing moderate levels of immunoreactivity in the accessory olivary nucleus (AON) and the inferior olive (IO). C3, C4B and C4D displayed overall weak but more diffused immunoreactivity around the HN and DMNX (Figure 5.5 C, E, G).

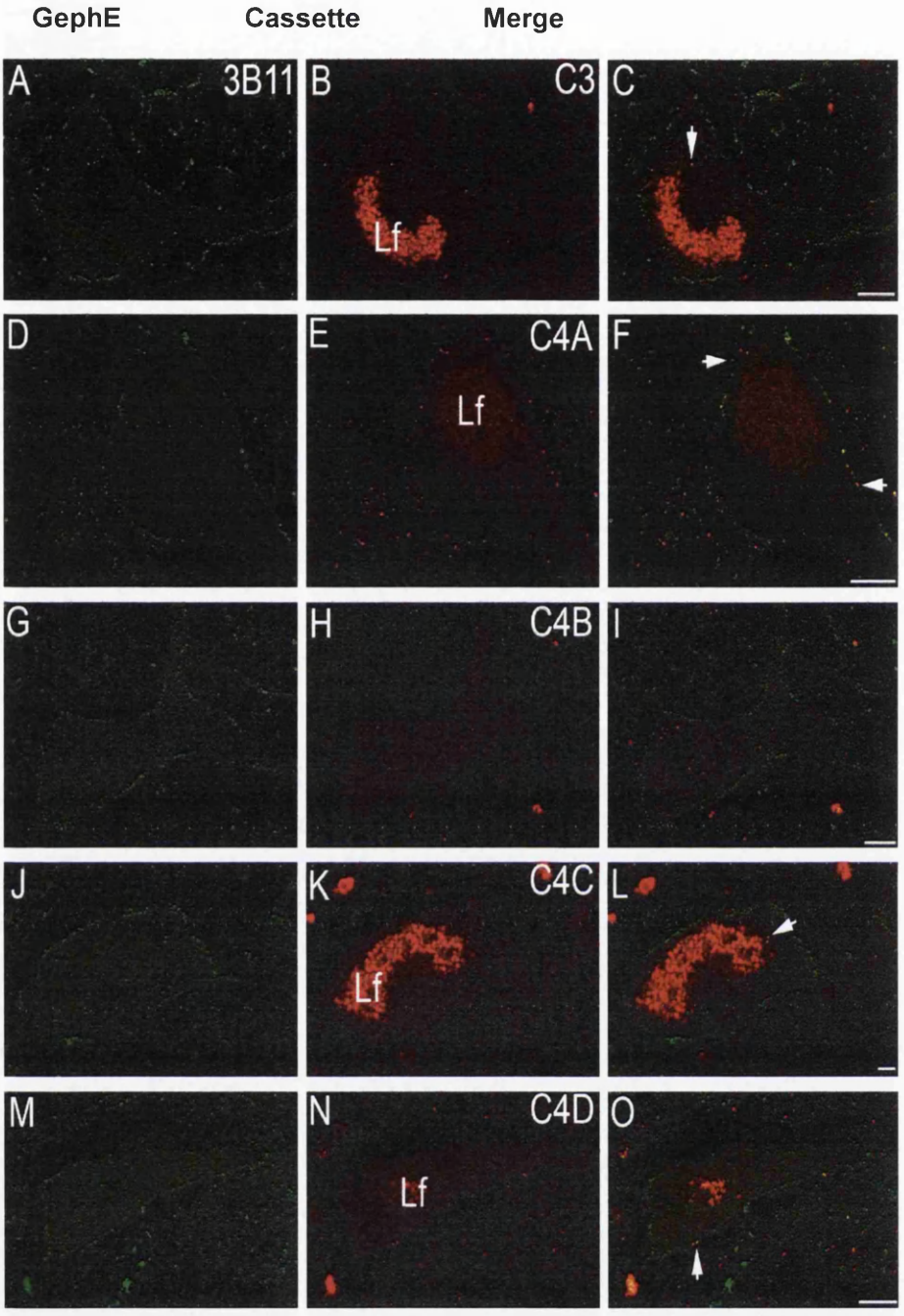
Examples of GlyR and GephE staining in the spinal cord are presented in the Figure 5.5 H-M. The labelling pattern of GlyR and GephE observed here are in accordance with previously described staining immunoreactivity patterns (Baer et al., 2003). Consistent with the staining observed in the medullar area, the immunoreactivity of C4A antibody (Figure 5.5J) demonstrates staining patterns similar to GlyR's with moderate to high-labelling throughout the DH and VH. The C4D antibody (Figure 5.5 M) and C4C antibody (Figure 5.5 L) show weak and moderate labelling throughout the DH and VH respectively. In C4B (Fig. 5.5 K), staining was preferentially localised to the lamina II of the DH.

5.2.2 Cellular localization of gephyrin isoforms

The distribution of gephyrin isoforms containing C3/C4 cassette (C3/C4 variants) was further investigated by confocal immunofluorescence labelling. Although gephyrin binds to GlyR-β subunit through its E-domain (Kneussel et al., 1999; Rees et al., 2003; Schrader et al., 2004), a recent study on rodent gephyrin isoforms indicated that presence or absences of C4 cassette can interfere with the polymerization of GephE and GlyR and gephyrin interaction (Bedet et al., 2006). Here, we have investigated the co-localization patterns of each of the gephyrin cassettes with GlyR or GephE. Figures 5.6 and 5.8 represent the high-resolution

images of medulla oblongata and spinal cord sections double-labelled for each gephyrin-cassette with GephE or GlyR respectively. As observed in previous studies (Baer et al., 2003; Waldvogel et al., 2007), GlyR displayed high-level of punctate immunoreactivity along the membranes of cell bodies and dendritic processes of HN neurons (Figure 5.8 left panel) whereas GephE exhibited punctate staining on the membranes in addition to the cytoplasmic regions (Figure 5.6 left panel). Consistent with Q-PCR data (Figure 5.3), the level of gephyrin cassette staining was less than that of GephE immunoreactivity. All gephyrin cassettes showed puncta immunostaining, the characterization of gephyrin staining (Meier et al., 2000). In contrast to GephE which exhibited immunoreactive punctate both on the neuronal membranes and the cytoplasm (Figure 5.6 left panel), immunoreactivity of the gephyrin cassette appeared to be preferentially localized to either neuronal membranes or cytoplasm (Figure 5.6, middle panel). This preferential localization of gephyrin isoforms was also observed in rat spinal cord neurons expressing gephyrin isoforms containing C4 cassette due to its inability to form polymerisation and localise to the cell membrane (Bedet et al., 2006). Interestingly, a significant proportion of gephyrin variants were not associated or co-localised, with GephE immunoreactivity providing partial indirect evidence that some gephyrin species may be fragmented (Figure 5.6). Further work is needed to prove this suspected outcome and ongoing efforts are imminent. C3 showed predominantly a diffuse staining pattern in the cytoplasmic region of HN neurons (Figure 5.6 B, 5.8 B). Rare C3 clusters observed both intracellularly and on the membrane were scarcely co-localized with GephE immunoreactivity (Figure 5.6 C) or GlyR immunoreactivity (Figure 5.8 C). In agreement with DAB staining, at the cellular level, C4A reveals the most similar staining patterns when compared with GlyR and GephE immunoreactivity.

Figure 5.6 Gephyrin immunoreactivity at the cellular level. *A-O, Confocal laser scanning micrograph single scans from sections of the human hypoglossal nucleus and spinal cord after double-labelling with gephyrin E-domain (GephE, 3B11, left column) and gephyrin isoforms (middle column), with merged images (right column). The labelling of each marker is shown separately and arrows point to colocalizing gephyrin E-domain and gephyrin isoform immunoreactivity in each triplet of images. Moderate to high levels of gephyrin E-domain- immunoreactivity (green, A, D, G, J, M) are present outlining the membranes of cell bodies and neuronal processes. Gephyrin isoform- immunoreactivity (red, B, E, H, K, N) is present in low levels with varying degree of colocalization (yellow in merged images in right column).* ***A-C)*** *C3 displays low levels of IR that is detected mainly in the cell body and often colocalized with 3B11- immunoreactivity.* ***D-F)*** *C4A demonstrates moderate to high levels of immunoreactivity with strong punctate immunoreactivity on the membranes of cell bodies and processes often colocalizing with 3B11- immunoreactivity.* ***G-I)*** *C4B and* ***J-L)*** *C4C show low levels of immunoreactivity that is detected mainly in the cell body.* ***M-O)*** *C4D displays moderate levels of immunoreactivity on dendritic and somatic membranes that often colocalizes with 3B11 immunoreactivity. Lf = lipofucsin auto-fluorescence. Scale bars = 10μm (C,F,I,O); 25μm (L).*


GephE
Cassette
Merge
A
3B11
B
C3
Lf
C
D
E
C4A
Lf
F
G
H
C4B
I
J
K
C4C
Lf
L
M
N
C4D
Lf
O

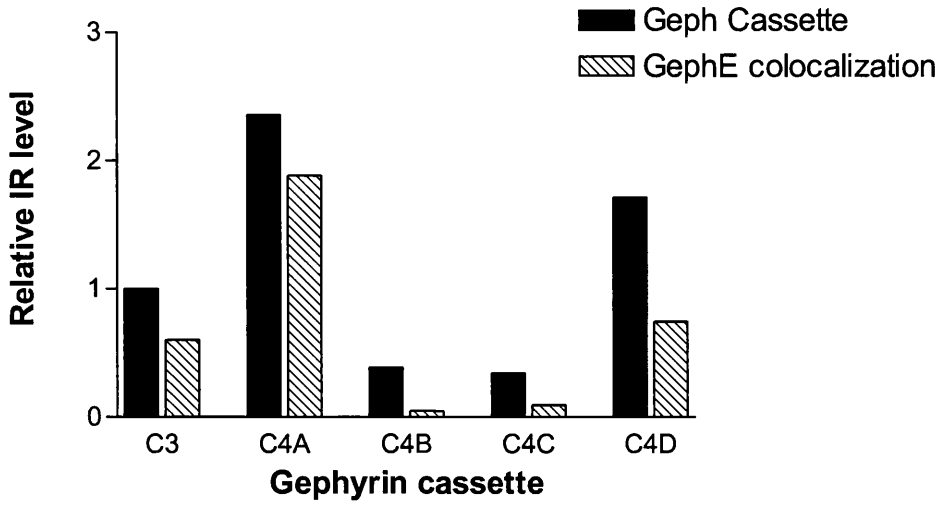
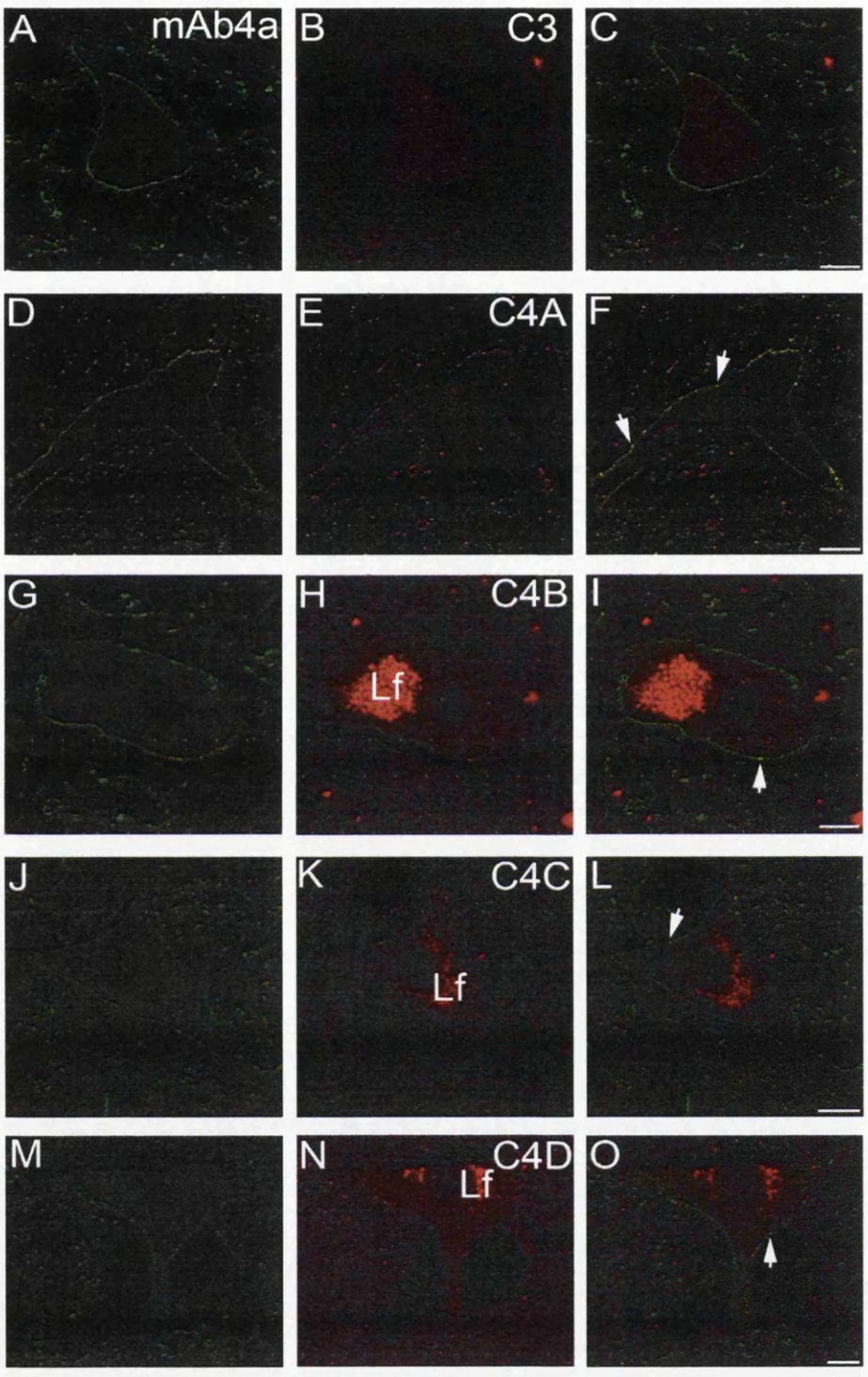


Figure 5.7 Quantitative analysis of gephyrin immunoreactivity at the cellular level. *Semi-quantitative comparison of gephyrin cassette immunoreactivity in HN, Data are presented as a ratio of expression level of each cassette standardised against C3. The ratio of co-localisation with GlyR was represented in a patterned bar.*

The majority of C4A immunoreactive puncta were localized on the membranes of the soma and dendritic processes of neurons in the HN, DH and VH and often co-localized with GephE or GlyR immunoreactivity (Fig 5.6 E-F, 5.8 E-F). C4D has less immunoreactivity than C4A, but presented a similar staining pattern (Fig5.6 & 5.8 N-O). The higher level of immunoreactivity of C4A and C4D compared to that of C3 correlates with the mRNA level detected in the whole human brain. It is interesting to note that the level of C4B and C4C immunoreactivity observed in the neurons expressing GlyR (Figure 5.9) was much higher than the level of mRNA detected in the medulla region (Figure 5.3). This may indicate that the low-levels of C4B/ C4C transcripts in whole brain regions is a function of regulated expression of C4B/C4C gephyrin in discrete populations of neurons in the medullar brainstem region.

Figure 5.8 Gephyrin- and GlyR-immunoreacitivity at the cellular level. *Confocal laser scanning micrograph single scans from sections of the human hypoglossal nucleus and spinal cord after double-labelling with GlyR (mAb4a, left column) and gephyrin isoforms (middle column), with merged images (right column). The labelling of each marker is shown separately and arrows point to colocalizing GlyR- and gephyrin isoform-immunoreactivity in each triplet of images. High levels of GlyR- immunoreactivity (green, A, D, G, J, M) are present outlining the membranes of cell bodies and neuronal processes. Gephyrin isoform- immunoreactivity (red, B, E, H, K, N) is present in low levels with varying degree of colocalization (yellow in merged images in right column).* ***A-C)*** *C3 displays low levels of immunoreactivity that is detected mainly in the cell body and rarely colocalized with GlyR-immunoreactivity.* ***D-F)*** *C4A demonstrates moderate to high levels of IR with strong punctate immunoreactivity on the membranes of cell bodies and processes often colocalizing with GlyR- immunoreactivity.* ***G-I)*** *C4B and* ***J-L)*** *C4C show low levels of immunoreactivity that is detected mainly in the cell body with little immunoreactivity along the neuronal membrane that often colocalizes with GlyR-immunoreactivity.* ***M-O)*** *C4D displays moderate levels of immunoreactivity on dendritic and somatic membranes that often colocalizes with GlyR-immunoreactivity. Lf = lipofucsin autofluorescence. Scale bars = 10 μm (C, F, O); 50 μm (I, L).*


Glycine Receptor
Cassette
Merge
A
mAb4a
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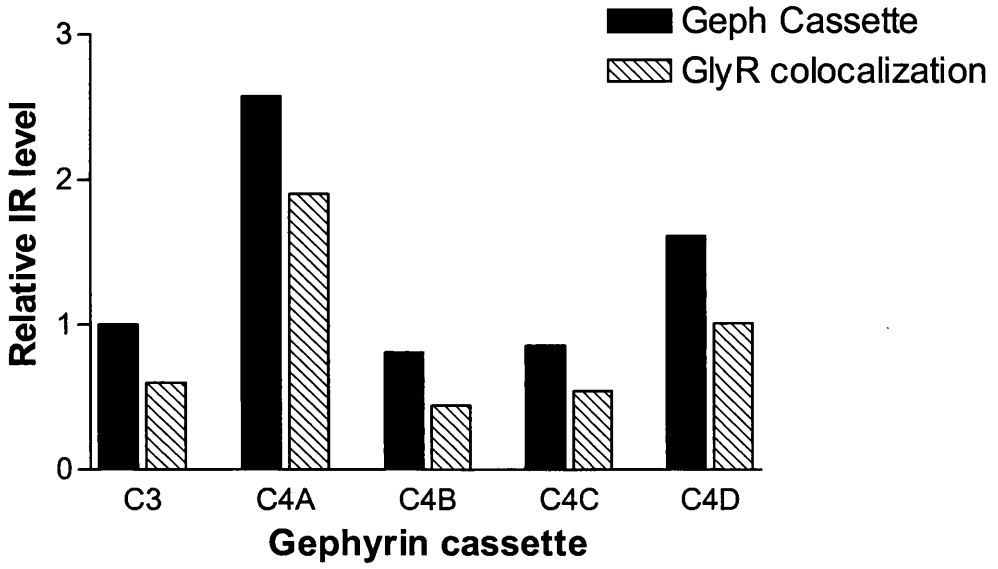

Figure 5.9 Quantitative analysis of gephyrin- and GlyR-immunoreactivity at the cellular level. *Semi-quantitative comparison of gephyrin cassette IR in HN, Data presented as a ratio of expression level of each cassette to that of C3. The ratio of co-localisation with GlyR was represented as a pattered bar.*

5.3 Summary

In this chapter, the distribution of gephyrin isoforms in the human brain and spinal cord was analysed using molecular biological and immunohistochemical techniques. Using RT-PCR and real time analysis, gephyrin transcript heterogeneity was extensively investigated in the adult human brain, retina, spinal cord and fetal brain. RT-PCR transcript analysis and cloning of gephyrin isoforms revealed that some splice isoforms are restricted to adult human brain where a more diverse range of the combinatorial arrangements of exons was observed in comparison to fetal brain, retina and spinal cord. In addition, we have generated novel gephyrin cassette-specific antibodies, that are described for the first time and used to investigate the distribution of gephyrin isoforms in the human brainstem and the cervical spinal cord and their co-localization with GlyR's in these regions.

The heterogeneity of gephyrin cassettes identified in this study indicates that the expression of each cassette is temporally and spatially regulated with unique patterns of co-localization with GlyRs. Functional studies on rodent gephyrin isoforms suggested different gephyrin isoforms exhibit different binding specificity and thereby affect protein-protein interactions. Further analysis of gephyrin transcripts will lead to better understanding of the mechanisms underlying the expression of specific splice variants and their functional roles in the dynamics of inhibitory neurotransmission.

Chapter 6 Discussion

Chapters 3-4 of this thesis have described the genetic basis of human hyperekplexia and we provide further proof of genetic heterogeneity involving the glycinergic inhibitory system. The pathogenicity of the gene mutations in *GLRA1* (this study) and *SLC6A5* (collaboration) have been validated by a range of functional platforms, revealing new mechanisms of receptor / transporter pathology in hyperekplexia. Lastly, in chapter 5, the investigation of gephyrin heterogeneity in the human brain has revealed the plethora of transcript isoforms in the human brain and differential profiles gained from other neurological tissue. The molecular findings correlated well with the neuro-anatomical investigation utilizing new antibodies targeting the exons of the central linker region which are spliced in or out of the gephyrin gene. Collectively, this has advanced our knowledge-base in the themes around human hyperekplexia, inhibitory glycinergic biology and the determinants of GlyR receptor clustering in neural cells.

6.1 Mutations in *GLRA1* and *SCL6A5* cause human hyperekplexia

This study represents the largest genetic screening and molecular characterization of *GLRA1* and *SLC6A5* in hyperekplexia. Our direct sequencing screening program of 88 index-cases, collected over 15 years, identified 19 *GLRA1* mutations within 30 index cases, and a further 12 mutations in *SLC6A5* found in 7 patients negative for *GLRA1* changes. Collectively this represents a 42% detection rate in hyperekplexia leaving a further 58% of index cases without a molecular diagnosis. By defining the genetic hierarchy of this hyperekplexia cohort, we can now triage the cohort into further

gene-discovery experiments for those who are phenotype +'ve / genotype –'ve, as well as validate the pathogenicity of the gene-positive cases.

From the 30 cases with GLRA1 mutations, 18 were inherited in a recessive mode or part of compound heterozygosity (Table 3.1) which is in conflict with the perception that dominant inheritance is the most common form of hyperekplexia. Twelve novel *GLRA1* mutations were identified, all of which were submitted to functional analysis with exception of homozygous stop codon alleles (Y197X) and large deletions (Δexon 4-7) where the outcomes are unambiguous. This study increases the compendium of hyperekplexia associated *GLRA1* mutations by 46% and effectively doubles the number of gene-positive index-cases known in the literature (Harvey et al., 2008).

Consistent with previous studies, all nonsense and intragenic deletion mutations were associated with recessive cases of hyperekplexia, confirming that haploinsuffiency is not a feature in this disorder. Hyperekplexia has traditionally been considered a dominant disorder (Harvey et al., 2008) largely driven by the identification of multiply-affected dominant families with a linked bias of M2 pore domain mutations, and the relative high frequency of R271Q/L alleles in Caucasians. Several studies in recent years, in relative isolation, have been providing an increasing evidence-base for the contribution of recessive alleles, led by the high frequency of Δ exon 1-7 as a founder-effect in the Turkish population (Gilbert et al., 2004; Becker et al., 2006; Siren et al., 2006). This latest study confirms that on an index-case population-basis the recessive inheritance of hyperekplexia is more common than dominant although they are closely-matched in their relative contribution. Combined with the data from *SLC6A5*, the

second major gene of effect in hyperekplexia where compound heterozygosity and homozygous mutant alleles predominate, then hyperekplexia becomes a predominantly recessive disorder.

In addition to the insights of the molecular genetics of hyperekplexia, the functional context of the pathogenic mechanism of *GLRA1* mutations is essential to establish pathophysiological mechanisms underlying the abnormal startle response. The data in this thesis and in the *SLC6A5* collaboration (Rees et al., 2006) greatly enhance the existence of 3 basic glycinergic channelopathy mechanisms.

1. Cell-surface expressed receptors that do not function due to dominant mutations that compromise glycine ligand-binding, alter agonist sensitivity or cause chloride conductance defects.

2. Trafficking mutants where that GlyR pentameric assembly or transition through the transcription/translation processes conspire to cause a deficiency of cell-surface targeting and insertion.

3. Recessive null genotypes, where the creation of functional α1β pentamers is precluded leading to a deficit in glycinergic neurotransmission where compensatory mechanisms likely exist to prevent a lethal clinical outcome.

This study has also identified a new pathogenic mechanism relating to glycine receptors and hyperekplexia. A novel dominant mutation, Y128C, causes spontaneously opening GlyR channels and is discussed below. In addition, we have also identified T265 in the TM2 domain as an important residue for the antagonistic activity of PTX, and 2 substitutions at position 65 of the polypeptide which

have very opposite outcomes in functional analysis and molecular modelling.

6.2 Leaky channels – new mechanism in hyperekplexia

A single dominant missense mutation, Y128C, in the N-terminal of the α1 subunit resulted in tonic channel opening in the absence of glycine agonist. This dominant mutation is the first hyperekplexia mutation identified with spontaneously opening channels or leaky channels as revealed by the biophysical characterisation. This channelopathy mechanism has been recognized as a pathogenic mechanism in other episodic disorders including congenital muscle disease, cardiac arrhythmias and hypokalaemic periodic paralysis (Marks et al., 2002; Paavola et al., 2007; Sokolov et al., 2007; Treves et al., 2008). Constitutive spontaneously opening activity has also been observed in native cys-loop receptors, including $GABA_A$ receptors (Krishek et al., 1996; Birnir et al., 2000; Wagner et al., 2005) and nAChRs (Ferrer-Montiel et al., 1991), in addition to cys-loop receptors with point mutations in regions involved in channel gating such as TM2 and flanking regions (TM2-TM3, TM3) or agonist binding N-domain (Chang and Weiss, 1998; Chang and Weiss, 1999; Corringer et al., 1999; Ueno et al., 1999; Torres and Weiss, 2002; Bhattacharya et al., 2004; Miko et al., 2004; Newell et al., 2004). Site-directed mutagenesis studies of GlyR α1 subunit have identified three residues causing spontaneously opening GlyR α1 channels; N-terminal D97R and A288W in the TM3 domain (Mihic et al., 1997; Beckstead et al., 2002). Structural modelling indicates that Y128 is located in close proximity to D97. Both Y128C and D97R result in similar conformational changes with the formation of the same short alpha helix in the extracellular domain directly overlying the TM regions (Figure 4.14 B) indicating the tonic opening of the channel is mediated by the same mechanism

for both N-terminal mutations. In contrast, A288W, which is located on TM3 domain near the extracellular boundary, appears to achieve tonic opening by an alternative mechanism, i.e. without change in secondary structure. The large tryptophan residue projects outward from the TM3 domain and possibly interferes with the normal packing of the subunits in the pentameric form. It is likely that in this regard the introduction of the short helix in Y128C and D97R has the same effect.

Although the precise mechanisms underlying the channel activation were unclear at present, a recent study revealed that agonist binding on the GlyR α1 initiates rearrangements of the inner β-sheet on the ligand binding domain (LBD) to trigger further movements for removing channel gating (Pless and Lynch, 2009). Y128 is a constituent of the loop E in the inner β-sheet which is subject to conformational change upon ligand-binding and the substitution of tyrosine to cysteine at residue 128 is expected to interrupt the β-sheet structure of the channel transforming (or converting) the channel to favour an open conformational state. This is consistent with the predictions of our modelling studies where the Y128C (and D97R) mutations affect extracellular domain structure immediately adjacent to the membrane domain.

6.3 Dominant M2 mutations impair GlyR channel function

Typical HE dominant mutations are clustered around the pore-forming TM2 and flanking domains with Y128C and R218Q being exceptions from this trend (Miraglia Del Giudice et al., 2003). R271Q and P250T are recurrent mutations in our cohort which have extensive previous characterization in the literature (Langosch et al., 1994; Laube et al., 1995; Shiang et al., 1995; Elmslie et al., 1996; Saul et al., 1999; Breitinger et al., 2001; Kung

et al., 2001; Breitinger and Becker, 2002) and Y128C is already discussed above.

Analysis of a novel dominant mutation, T265I, located in the TM2 domain, revealed a significant decrease of glycine-induced maximal current and increased EC50 mimicking the typical features of dominant *GLRA1* mutations, specifically the reduced glycine sensitivity of membrane expressed receptors (Rajendra et al., 1994; Laube et al., 1995; Saul et al., 1999; Breitinger et al., 2001; Breitinger et al., 2004).

Interestingly, the deleterious effect of the homomeric α1(T265I) subunits were partially rescued by co-transfection of GlyR β-subunits (Figure 4.5). As a α1 homomeric channel, T265I had no detectable current, but upon expression with β-subunit, an 18-fold increase of maximal current was observed. This partial rescue of the heteromeric channel by β-subunit co-transfection was also observed in a recessive mutation R392H in the M3-M4 domain (Figure 4.1). Previously, GlyR channels with defects in glycine binding sites (R65A and E157D) were also rescued by β subunit co-transfection (Grudzinska et al., 2005). Agonist binding and signal transduction occurs by interaction of residues between adjacent domains, thus, it seems the incorporation of WT β-subunits into the mutant α1 GlyRs with defects in agonist binding sites or signal transduction domains have the potential to alter the biophysical properties of the α1β GlyRs. The pharmacological properties of GlyRs can also be altered by the incorporation of β-subunits into α subunits (Supplisson and Chesnoy-Marchais, 2000; Yang et al., 2007; Lynch, 2009).

PTX is widely used to discriminate homomeric α GlyR from heteromeric αβ GlyR, due to its ability to selectively inhibit

homomeric GlyRs (Lynch, 2004). Interestingly, the α1 T265I mutation restored high PTX sensitivity to heteromeric α1(T265I)β GlyR (Figures 4.7 and 4.8), although the mechanism by which this was achieved is not understood at present. Site-directed mutagenesis studies have demonstrated that the structure of the M2 domain is an important constraint for the antagonistic activity of PTX on α1 homomers; mutations on α1 pore lining residues, G254, T258, S267, R271, can reduce the antagonistic effect of PTX (Lynch et al., 1995; Shan et al., 2001; Dibas et al., 2002), particularly, threonine at the residue 258 on α1 subunit is regarded as an essential residue for PTX sensitivity (Shan et al., 2001; Yang et al., 2007). When residue F282 of β subunit polypeptide, which corresponds to T258 on α1 subunit, was substituted to the threonine, the heteromeric α1β(F282T) was converted to picrotoxin-sensitive (Shan et al., 2002).

Structural analysis of cys-loop channels predicted that, T265 forms a part of pore lining residues, along with G254, T258 and S267, and plays an important role for maintaining a minimum pore diameter by forming a hydrophobic bond with Q266 in adjacent TM2 domain (Akabas et al., 1994; Miyazawa et al., 2003) This study demonstrates that T265 is a novel determinant of PTX sensitivity. Analysis of its mechanism of action could reveal new features of pore structural differences and pharmacological differences between α1 and β subunits.

6.4 Recessive mutations cause trafficking defects

Two novel mutations in the pore and the flanking region (R252C in M1-M2 region and G254D in TM2) were associated with recessive cases of hyperekplexia. Carriers of heterogygous R252C or G254D mutations are phenotypically asymptomatic. This indicates some

missense mutations on the pore lining regions may not exert dominant-negative effects and can be tolerated in the heterozygous state. Convergent functional studies of these recessive missense mutations displayed a significant reduction of membrane expression, presumably a defect in channel trafficking, thereby inducing a small or no current by glycine (Table 4.1).

Recessive mutations identified in the N-terminal region, R65W and D165G, also displayed a significant decrease of membrane expression level as displayed in the biotinylation data and the lack of glycine currents.

Hyperekplexia differs from other ion channel disorders in that human patients with recessive/null hyperekplexia mutations do not present with a phenotype as severe as hyperekplexia animal cases or recessive cases in other genetic disorders, indicating some sort of compensatory mechanisms. However, they tend to have a more complicated phenotype including learning difficulties and developmental disorders (Appendix C). Although phenotypic variability is a common feature even among the carriers of a same mutation in complex genetic disorders, generally, recessive or compound mutations in channelopathies are typically associated with more severe phenotype than patients with dominant mutations, as exemplified by mutations in CLC2 or KCNQ1 genes causing myotonia congenita or cardiac arrhythmia respectively (Westenskow et al., 2004; Planells-Cases and Jentsch, 2009).

Defects in the glycinergic system generate HE-like symptoms in a variety of animal models (Buckwalter et al., 1994; Kingsmore et al., 1994; Ryan et al., 1994; Kling et al., 1997; Becker et al., 2000; Becker et al., 2002). Studies indicate that these animal models have impaired glycinergic function linked to hyperekplexia

and experience compensation by enhancement of GLRA subunits or GABAergic neurotransmission (Findlay et al., 2003; Molon et al., 2006). In murine hyperekplexia models, homozygous mutations are generally associated with severe phenotypic outcomes e.g. the murine model of hyperekplexia (*oscillator*, *spd^{ot}*) has homozygous deletion of 7bp which leads to the compete loss of GlyR α1 subunit and fatal phenotypic consequences (Buckwalter et al., 1994). The *spd^{ot}* mice with a heterozygous deletion have a loss of about 50% of α1 subunits, but about 30% reduction in total GlyR levels and are associated with relatively mild phenotype (Kling et al., 1997). This compensatory mechanism was not observed in mutations associated with dominant hyperekplexia animal models (Becker et al., 2002; Findlay et al., 2003). A transgenic mouse expressing the dominant human mutation (R271Q) showed that the mutation diminished both GlyR and GABA-A receptor mediated inhibitory transmission (Becker et al., 2002). This indicates that the expression of a mutant *GLRA1* gene with a dominant mutation may affect the entire postsynaptic inhibitory system.

6.5 Compound missense mutations behave differently

Two hemizygous missense mutations, E103K and S231N, identified as heterozygous compound mutations in this study, do not cause hyperekplexia in the heterozygous status as revealed by asymptomatic parental carriers of the mutations. In contrast to the recessive missense mutations in this study, E103K and S231N expressed fully functional channels with no evidence of trafficking defects. However, both mutations displayed a significant increase of glycine EC50, although they were able to generate maximum currents compatible to WT at saturating glycine concentrations. The pathogenic basis for these mutations are likely to lie in the alteration of glycine binding affinity or gating efficacy (Findlay et

al., 2003), as previously observed with dominant mutations. Hemizygous mutation E103 is located adjacent to the glycine binding residues, A101 and N102 (Lynch, 2004), therefore it is reasonable to speculate that changing a negatively-charged glutamate to a positively-charged lysine in E103K will interfere with the binding of glycine.

In a previously-published recessive hyperekplexia case, a substitution of serine 231 for a charged hydrophilic arginine (S231R) was reported and expression studies of S231R indicated a reduced cell-surface expression level (Humeny et al., 2002). In contrast, S231N seems to alter affinity for glycine binding revealing a substitution-specific effect in GlyR pathology which is also observed with R65L and R65W in this study. The hemizygous R65L mutation does not mimic the EC50 effects of E103K and S231N, and fails to generate currents when expressed in HEK293 cells. However, surface-labelling experiments indicated that the R65L mutants are fully-expressed on the membrane surface, in contrast to R65W which does not reach the cell surface as supported by molecular modelling of the effect on the subunit. Whether or not the R65 mutations have a trafficking outcome, it may all be secondary to the role of residue R65 as a definitive part of glycine binding and the change of polarity in R65L and R65W is expected to alter the stability of glycine binding.

6.6 Gephyrin: a chameleon gene

Gephyrin is a pleiotropic gene which sustains a neuronal clustering function and a non-neuronal metabolic function in the biosynthesis of molybdenum co-factor (Feng et al., 1998; Stallmeyer et al., 1999; Smolinsky et al., 2008). This is reflected in the range of

disorders that gephyrin mutations can cause including hyperekplexia (neuronal context) and molybdenum deficiency syndrome (non neuronal context). There is also an unknown contribution to leukemogenesis through translocations which disrupt the gephyrin gene (Eguchi et al., 2001; Kuwada et al., 2001). Gephyrin is also implicated as a microarray compensator gene observed in Chorea-acanthocytosis (ChAc), a hereditary neurodegenerative disorder caused by loss of function mutations in the chorein gene (*VPS13A*) (Kurano et al., 2006) and in various neurodegenerative conditions (Thompson-Vest et al., 2003; Kurano et al., 2006; Lorenzo et al., 2006; Agarwal et al., 2008; Nithianantharajah et al., 2008; Ryzhikov and Bahr, 2008). Reduced expression of gephyrin was identified in Alzheimer's disease and amyotrophic lateral sclerosis (ALS) (Lorenzo et al., 2006; Agarwal et al., 2008).

To sustain such a varied biological role, whilst encoded by a single highly-conserved gene, the solution lies in the generation of function-specific transcripts and certainly that is the evidence for gephyrin. Gephyrin, typically creates one isoform for the metabolic function in cells, however, generates multiple isoforms in the adult human brain and neurological tissue by alternative splicing of five linker-region cassettes (C3, C4A–D) (Rees et al 2003). This transcript heterogeneity then confers a neuronal context to the function where binding of the E-domain of gephyrin to the β subunit of GlyRs constitutes a major role for gephyrin in glycinergic neurons and some undefined influence in GABAergic neurons (Meyer et al., 1995; Calamai et al., 2009). Tissue-specific gephyrin variants have also been observed in rodents and functional assays indicated that different gephyrin isoforms are involved in a range of different protein-protein interactions (Meier et al., 2000; Bedet et al., 2006; Smolinsky et al., 2008).

To gain insight into the role of human splicing cassette, the expression and distribution patterns of gephyrin variants containing C3/C4 cassettes (C3/C4 variants) were analysed in the human brain regions. Using cassette-specific primers and antibodies, this study has demonstrated that 1) from 1,500 clones, at least 21 gephyrin mRNA transcripts were differentially expressed in the human fetal brain, adult brain, spinal cord and retina (Figure 5.2); 2) gephyrin cassettes were highly brain-specific with C4A and C4D being the most abundantly expressed cassettes and 4B and C4C, though rare, showing highly-localized expression, whilst the C3 variants are predominant in the spinal cord, retina and non-neuronal tissue (Figure 5.3); 3) C3/C4 variants display a distinct distribution pattern in the human brainstem and spinal cord (Figures 5.4 -5.8); 4) C3/C4 aggregates show preferential localization either on the surface membrane or in the cytoplasm with a different level of co-localisation with GephE or GlyR; 5) the diversity of gephyrin transcripts is spatially and temporally regulated and the complexity of gephyrin isoforms is even higher than previously estimated (Rees et al., 2003; Fritschy et al., 2008).

Recent recombinant expression studies of rodent gephyrin indicated that the relative expression of different gephyrin isoforms can regulate the dynamics of GlyRs on the cell membrane (Bedet et al., 2006). Based on the structural analysis of the purified E- and G-domain gephyrin, the full-length gephyrin exists as a more stable trimer status (300kDa) generated by the G-domain aggregation whereas the E-domain alone forms dimers which can interact with GlyRs but are unable to form stable postsynaptic clusters (Sola et al., 2004; Saiyed et al., 2007). Gephyrin variants that failed to form trimerization were not capable of stabilizing GlyR at the cell-surface, thereby disturbing the synaptic stability of receptors (Bedet et al., 2006). The presence/absence of the linker

cassette can interrupt the oligomerization of rodent gephyrin protein and consequently decrease the number of cell-surface GlyRs through the rapid internalization of cell surface receptors (Bedet et al., 2006). Immunohistochemical analysis of human gephryin cassette in this study showed that the variants containing each cassette display distinct immuno-staining pattern with the preferential localization either on the neuronal membranes or in the cytoplasm indicating different C3/C4 variants may exert different oligomerization status and may play distinct roles in the clustering at postsynaptic sites.

Consistent with previous study, the expression level of C3 transcripts displayed a significant difference between neuronal and non-neuronal tissues (Rees et al., 2003). It is present in virtually all gephyrin isoforms identified in heart, whereas its presence is down-regulated in human adult and fetal brain at the mRNA level (Figure 5.3). In neuronal cells, C3 variants were reported to be repressed by nova, a neuronal regulator of pre-mRNA splicing, and the presence of C3 variants in neurones may reflect the requirement for background non-neuronal moco-synthesis, rather than enabling clustering at the postsynaptic sites (Paarmann et al., 2006). As expected, immunoreactive gephyrin clusters containing C3 (C3 variant) are predominantly present intracellularly in the neurons of the HN or DH and virtually no colocalization with GlyR (Figures 5.5 - 5.9). Cytoplasmic clusters of C3 variant were also observed in recombinant gephyrin variant expressed in rat spinal cord neurons (Paarmann et al., 2006). A recent study showed that in the rat brain, cortical neuronal cells display a very low activity of Moco synthesis, whereas glial cells are responsible for the synthesis of Moco in the brain. Consequently, C3 isoforms were most commonly found in rat glial cells and not in the cortical neurons (Smolinsky et al., 2008). This may explain the lower immuno-

labelling of C3 in the medulla oblongata and the cervical spinal cord neurons compared to the high expression of C3 mRNA transcripts in the spinal cord as observed in this study. This is also supported by the expression levels observed in human brain where C3-cassette gephyrin is subservient to the C4 cassette gephyrin which is then reversed in spinal cord and retina (Figure 5.3).

C4A was the most abundant C4 gephyrin cassette both at the mRNA and protein level in the brain. This is consistent with a previous study where C4' (rat gephyrin cassette equivalent to human C4A) was readily detected in the brain tissue but not in the non-neuronal tissues (Meier et al., 2000). C4A variants have a staining pattern compatible to that of GlyR in the medulla oblongata and the cervical spinal cord regions and a significant proportion of C4A is co-localized with GephE and GlyRs on the cell membrane indicating the majority of C4A variants are involved in postsynaptic clustering of GlyRs (Figures 5.5 - 5.9). The interaction of C4A variants with GlyR was also demonstrated in rodent gephyrin isoforms (Meier et al., 2000). In contrast to low expression levels of C4A mRNA transcripts in the spinal cord, immunoflurescence studies showed that C4A is highly-expressed and shows more intense labelling than that of GephE, particularly, in the VH neurons in the spinal cord, indicating the selective localization of C4A variants. C4D is the second most abundant cassette in the brain and is very similar to C4A as evidenced by the convergent data on the mRNA expression pattern, membrane punctate immuno-staining and protein levels. This implicates cassettes C4A and C4D as important developmentally-conserved determinants in the human brain and confers properties onto the gephyrin molecule which triggers / facilitates glycinergic specificity.

C4B is currently a human-specific cassette and was the least abundant cassette at the mRNA level. However, at the protein level, it showed higher level of expression in the glycinergic neurons in the medullar oblongata region. Interestingly, C4B is hardly co-localized with GephE, however the majority of the C4B variants were colocalized with GlyRs on glycinergic neurons. This cell-specific differential expression of gephyrin molecules has been well-documented in rodent tissues (Craig et al., 1996; Simburger et al., 2000). Differential expression of gephyrin in distinct sizes and shapes were even reported in single spinal cord neurons (Gonzalez-Forero et al., 2005).

C4C, is another rare cassette in the human brain, but also showed higher expression level in the discrete populations of glycinergic neurons in the medullar brainstem region. A recent study indicated that the synaptic clustering of GlyR is regulated by gephyrin-gephyrin interaction as well as gephyrin–GlyR interactions (Ehrensperger et al., 2007). Functional analysis of rodent gephyrin isoforms showed that gephyrin variant containing C4 (equivalent of human C4C) can inhibit the polymerization of gephyrin as well as interfere with the glycine and gephyrin interaction by producing a dominant-negative effect (Bedet et al., 2006).

The clear understanding of the functional roles of these cassettes is further complicated by the combinatory transcripts which exist, with multiple cassettes making biological characterization a very difficult task. Based on functional analysis of gephyrin isoforms in rodent (Meier et al., 2000; Smith et al., 2000; Bedet et al., 2006; Paarmann et al., 2006), it is possible that through the regulated expression of different gephyrin C3/ C4 isoforms in different brain regions, gephyrin may interact with distinct proteins and thereby

accommodate the diverse range of functional complications in the human brain.

Gephyrin is involved in clustering of both GABA and glycine receptors (Kirsch and Betz, 1993; Kirsch and Betz, 1995; Craig et al., 1996; Sassoe-Pognetto and Wassle, 1997; Essrich et al., 1998; Giustetto et al., 1998). Both GABARs and GlyRs show subunit specific, distinct regional and a cell-specific expression in the brain with subunit restricted physiological and pharmacological properties (Enz and Bormann, 1995; Sassoe-Pognetto et al., 1995; Lynch and Chen, 2008; Chen et al., 2009). In addition to binding to GABAAR and GlyRs, gephyrin is also interacting with numerous neurological molecules including Mena/VASP, neuroligin, dynein light chain 1/2, RAFT1, collybistin, GABAR associated protein (GABARAP_, Pin1 or the actin-binding protein profilin and tubulin (Sabatini et al., 1999; Kins et al., 2000; Fuhrmann et al., 2002; Giesemann et al., 2003; Bausen et al., 2006), illustrating the diverse role of gephyrin molecules in the CNS.

A recent immunohistochemical study showed that in the human substantia nigra region, some neurons are GABA / glycine specific whereas some have both receptors expressed but without co-locolazing (Waldvogel et al., 2007). Based on the unique distribution pattern demonstrated by different gephyrin cassette, it is tempting to speculate that the differential cellular expression of GABARs and GlyRs observed in these neurons are regulated by the differential expression of gephyrin isoforms. Indeed, a recombinant expression study indicated that the GABAergic postsynaptic sites are not pre-determined for exclusive expression of GABARs, but presence or absence of specific gephyrin cassettes were able to regulate the expression of GlyRs in the GABAergic sites (Meier and Grantyn, 2004). Immunohistochemical analysis

has also revealed that gephyrin is co-localized with the slicing variants of glutamate receptor interacting protein 1 (GRIP1), GRIP1c4–7 or GRIP1a/b, at the vast majority of post-synaptic GABAergic synapses (Yu et al., 2008).

6.7 Study Summary

In this study, we present the largest multi-centre screening programme in hyperekplexia which has revealed novel and recurrent mutations at a rate where functional analysis is beginning to reveal pathophysiological and clinical trends and conclusions. In screening 88 index-cases with unequivocal hyperekplexia, this study confirms that *GLRA1* and *SLC6A5* are genes of major effect in hyperekplexia and have direct clinical translation in 42% of patients. Nevertheless 58% remain gene-negative and more effort is needed to identify the genetic basis of these cases. For the first time, the study confirms that recessive hyperekplexia is more common that dominant hyperekplexia, albeit due to several anthropological and mutation frequency factors.

The functional analysis of the novel *GLRA1* mutations reveals a number of mechanisms in hyperekplexia. This includes compromised ligand-binding, chloride conductance, cell-surface trafficking, agonist sensitivity and functional null scenarios. In addition, new mechanisms of action have been identified, including the first clinical example of tonic opening of GlyR's (Y128C), an important determinant of pictrotoxin binding (T265I), and the first human mutations in the R65 glycine binding site with mutation-specific abilities to reach the cell surface. Several lines of convergent evidence, including biophysical characterisation, cell-surface expression and molecular modelling, all provide a pathophysiological basis in a mutation-specific manner; with the

exception of the G342S variant where no indication of pathology was found, and is now a confirmed population-based non-synonymous SNP variant.

The structural integrity of the GlyR pentamer is one issue in hyperekplexia, but so is the effective targeting and clustering of receptors at the postsynaptic membrane. Gephyrin is an essential determinant in GlyR clustering by providing a sub-cellular scaffolding lattice to support the dynamic process of GlyR cycling and anchoring. This study confirms that gephyrin makes at least 21 transcript editions in human brain, retina and spinal cord and that this is generated by combinatory splicing of the C3/C4 central linker exons. We demonstrate that C4 exons are highly expressed in human adult and fetal brain, especially so for C4A and C4D, and that this trend is repeated by immunoreactivity experiments on postmortem human brain. Immunocytochemical studies also found a high-degree of co-localization between C4A / C4D and GlyRs in the human brainstem region. The next challenge is to unravel the functional context of this heterogeneity and how it affects and specifies glycinergic neurotransmission.

Finally, it must not go unnoticed that this study was only made possible by collaborative, multicentre efforts and the role of consenting patients. Collectively, we are in pursuit of effective diagnostics and drug-control of hyperekplexia – a distressing, life-long condition that can diminish well-being at best and at worst can have fatal consequences; all the result of the dysfunction of an evolutionary-conserved, ancient glycinergic startle response.

Conclusions

- This study describes the ongoing genetic screening program which has identified a new gene in 7 human hyperekplexia cases with 12 pathological mutations in the glycine transporter-2 gene (*SLC6A5*; GlyT2). Functional analysis validated pathogenicity as part of a multicentre collaboration.

- The same study discovered 19 novel & recurrent mutations in *GLRA1* associated with 30 cases of hyperekplexia. Contrary to popular belief, the study indicates that compound heterozygosity and recessive mutations are the most common inheritance pattern in sporadic hyperekpelxia.

- All nonsense and intragenic deletion *GLRA1* mutations were associated with recessive cases of hyperekeplxia, confirming that haploinsuffiency is not a feature in this disorder. In some parts of the world the cultural acceptance of consanguinity is a risk-factor for recessive hyperekplexia.

- Functional analysis of *GLRA1* mutations demonstrates that defects in trafficking appear to be the major mechanism underlying recessive mutations.

- Other hyperekplexia mutants, without trafficking defects, typically show a total absence of channel current or exhibit EC50 alterations in the dose-response curve for glycine, suggestive of disrupted signal transduction.

- Biophysical characterisation and molecular modelling of GlyR-Y129C has confirmed that it is uniquely a leaky channel where the glycine receptor is locked into a position favouring the open channel conformation.

- The T265I mutation confers pictrotoxin sensitivity on α_1(T265I)β heteropentamers which is contrary to the insensitive $\alpha_1\beta$ wild-type counterpart.

- Convergent use of experimental data and molecular modelling reveals the reason behind the cell-surface expression of the glycine binding mutant, R65L; contrary to R65W which displays markedly reduced cell-surface expression.

- The complexity of gephyrin isoforms in humans is even higher than previously estimated; with at least 21 gephyrin mRNA transcripts that are differentially-expressed in the human fetal brain, adult brain, spinal cord and retina.

- The C3 variants are predominant in the spinal cord, retina and non neuronal tissue, whereas the four C4 cassettes were highly brain-specific with C4A and C4D being the most abundantly expressed cassettes.

- By using established antibodies and creating new gephyrin antibodies with epitopes in the C3/C4 region, this study revealed that variant cassettes display a distinct distribution pattern in the human brain stem and spinal cord with different levels of co-localisation with GlyRs.

- The poor representation of cassettes C4B and C4C in generalised real-time PCR, is contradicted by highly-localized immunoreactivity expression of both cassettes; indicating that the diversity of gephyrin transcripts is spatially and temporally regulated in brain cell-types and neural networks.

Limitations of study

1) Due to the time and financial constraints, only *GLRA1* gene -ve samples were screened for *SLC6A5*. However, we cannot exclude the possibility of hyperekplexia cases with digenic mutations in both *GLRA1* and *SLC6A5* genes.

2) Following the ethical guidelines obtained for this study, all patients were anonymised and the detailed phenotypic information of patients were not available for the current study. Further studies are required to establish phenotype-genotype co-relationships in hyperekplexia.

3) The functional impact for the *GLRA1* G342S mutation remains ambiguous; therefore, a causal relationship in patients is not proved conclusively despite being identified in three patients with typical hyperekplexia symptoms.

4) Population studies were not performed for recurrent mutations where credible previous studies have assessed frequencies in normal control populations. In addition, major racial/ethnic groups appear to have different allele frequencies (Botstein and Risch 2003), and our control cohort were typically Caucasian.

5) The pathological outcomes in this study of *GLRA1* and *SLC6A5* mutations were confirmed on a non-neuronal cell line, HEK293. However, other studies have implicated that different experimental outcomes can be observed between HEK293 cells and neuronal cell lines (Kung et al., 2001).

6) Functional analysis of *GLRA1* and *SLC6A5* were performed with constructs based on cDNA of each gene. Therefore, mutations that may cause splicing effects were not considered in the experimental setting and they require new constructs with exonic and the flanking intronic regions containing splicing machinery.

7) Due to the availability of RNA samples, quantitative study of gephyrin isoforms at the mRNA level was performed in the whole brain region whereas the level of gephyrin protein level were investigated in GlyR-rich brain stem regions. To clarify the conflicting results observed in the quantity of gephyrin at the mRNA and protein level in this study, quantitative analysis of gephyrin isoforms on brainstem region RNA is required.

8) The N-terminal specific antibody for gephyrin was not tested in this study. It would be an immediate experiment to test if GephN immunoreactivity shows a pattern relative to the C3/C4 cassettes and GlyRs.

Future Directions

This study has revealed some novel discoveries and attempted to define the causes of hyperekplexia and approach the biological context of receptor dynamics and clustering. However, detailed investigation can lead to more questions than answers and with 58% of the hyperekplexia cases without a molecular explanation, a future challenge is immediately obvious.

The gene-negative samples need further screening in genes that are determinants of the glycinergic proteome. The same level of sequencing detail is required in the genes with rare association with hyperekplexia such as *GLRB, GLRA2, GLRA3* and gephyrin. There is also some justification for screening the GABAergic candidates, particularly since GAD-65 auto-antibodies can cause stiff-person syndrome, although GABAergic mutations are occasionally associated with idiopathic epilepsy rather than paroxysmal startle disorders. Historically, gene discovery from the animal models of hyperekplexia and startle phenotypes have guided human screening efforts. It seems prudent to remain vigilant of new models as they emerge in the literature and react by screening the gene-negative cohorts.

More promise of success will emerge with 3rd generation sequencing and microarray platforms. Molecular genetics is changing from large cohort-based studies to smaller well-defined cohorts matched with the technical feasibility of genome-wide sequencing and transcriptome sequencing. A well-defined hyperekplexia cohort which has all major candidate genes excluded as the pathogenic basis will be well-suited for this high-end technology and this thesis recommends embracing these new

opportunities. Expression microarrays will be more of a challenge since the neuronal context and tissue-type specificity in hyperekplexia means that normal pathology samples such as blood cells or fibroblast lines are unlikely to be informative.

"Let's keep looking where the street lamp standeth" is a paraphrase often used to pay closer attention to what is already known and this applies to *GLRA1* and *SLC6A5*. How? – the effect of intragenic SNP's and changes close to splice-sites that may have a yet undiscovered influence over gene control. The same applies to promoter and enhancer sites for both genes. All these factors need investigating even if it merely contributes to exclusion from the cause of the disorder. Moreover, since auto-antibodies often mimic hyperekplexia symptoms, it is feasible to suggest that glycinergic auto-antibodies may be one of several non-genetic causes of sporadic and late-onset cases.

One of the big challenges to arise from the study is the validation strategy required to assess the functional significance of gephyrin transcript heterogeneity. With 22 transcripts created from multiple combinations of central cluster exons may all have subtle cellular or tissue-type consequences and this is where microarray may be useful as a way forward to analyse effects. Also the preparation of full-length tagged constructs of gephyrin transcripts for transfection experiments in contextual cellular models will be a start to the process of assigning transcripts to specific function.

Although detection sensitivity may be an issue with transcripts, but analysis of the gephyrin C3/C4 cassette antibodies on brain-lysate Western blots is also a future research line to follow.

It is also apparent that other parts of the brain are enriched with glycinergic pathways and the analysis of co-localization patterns would also be a project to fully exploit the unique antibody resource created by this study. Once the constitutional characterisation is complete, there are also other disorders where we could investigate altered patterns of staining and where the disorder is common enough to be represented in brain banks.

Lastly, with a well-defined group of hyperekplexia cases with 2 different genes of effect, then phenotype / genotype correlation studies are warranted. For example, the association of recessive hyperekplexia with learning difficulties is an emerging theme, or the response dynamics to clonazepam treatment in GLRA1 positive versus SLC6A5 positive cases is an intriguing possibility.

Appendix

A. Diagnostics criteria for human hyperekplexia

Required criteria

- ***Startle***: Exaggerated startle reflex to unexpected (particularly auditory) stimuli. The startle response can be prolonged and be present before birth. Consciousness is unaltered during startle episodes. Nose-tap-test is positive (does not habituate).

- ***Stiffness***: Generalized stiffness **immediately after birth,** normalising during the first years of life. The stiffness can be predominantly truncal or lower limb, increases with handling and disappears during sleep. Short period of generalised stiffness following the startle response during which voluntary movements are impossible. This can result in falls in adults.

- ***Exclusion of mimics***: Normal MR imaging, no dysmorphism or congenital deficits noted. Normal EEG during startle episode. Autonomic features of paroxysmal extreme pain disorder absent.

Supportive criteria

- Inguinal, umbilical, or epigastric herniae.
- Congenital dislocation of the hip.
- Hypoxic attacks in infancy.

B. Clinical phenocopies of human hyperekplexia

Phenocopy	Comparisons with human startle
Acquired Hyperekplexia Sub-acute anti-glycine receptor antibody mediated condition that responds to immunosuppresion and plasma exchange (Hutchinson et al., 2008).	***Similarities*** Truncal rigidity, muscle spasms and stimulus induced startle . ***Differences*** Features not present from early life. Immunosuppression clearly efficacious. Apnoea attacks not described.
Paroxysmal extreme pain disorder Autosomal dominant condition recently shown to be a sodium channelopathy involving *SCN9A* (previously known as familial rectal pain syndrome) (Fertleman et al., 2007).	***Similarities*** Onset in neonatal period or infancy, persists throughout life. Dramatic syncopes with bradycardia and sometimes asystole. Tonic attacks are triggered by factors such as defecation, cold wind, eating, and emotion. ***Differences*** Autonomic manifestations predominate initially, with skin flushing in all and harlequin colour change. Later attacks of excruciating deep burning pain often in the rectal, ocular, or jaw.
Jumping Frenchmen of Maine / Latah syndrome Culturally bound neuropsychiatric syndromes thought to be an anxiety / somatisation disorder (Meinck, 2006).	***Similarities*** Excessive response to startle. ***Differences*** Echopraxia (involuntary repetition of another's words or actions) and echolalia (repetitive vocalisations).
Startle Epilepsy Startle epilepsy is a reflex epileptic seizure precipitated by a sudden stimulus; most patients are young and have infantile cerebral hemiplegia (Meinck, 2006).	***Similarities*** Surprising stimuli induce motor reactions – consciousness can be preserved in seizures. ***Differences*** Neuro-imaging will almost certainly be abnormal.
Stiff Person Syndrome Progressive axial stiffness	***Similarities*** Stimulus induced hypertonia, startles and falls.

and intermittent spasms mainly evoked by unexpected stimuli; associated with anti-GAD antibodies in CSF (Meinck, 2006).	Hypertonia can preferentially affect lower-limbs. ***Differences*** Stiffness / hypertonia is much more prolonged than the paroxysmal attacks seen in hyperekplexia.
Tourette's syndrome Motor and vocal tics, associated with an exaggerated startle reflex, behaviour change and stereotypy (Bakker et al., 2006).	***Similarities*** Startle response, symptoms precipitate by stressors. ***Differences*** Vocalisations and obsessive / compulsive behaviours. Motor tics can be complex and appear semi-purposeful.
Crisponi syndrome An autosomal recessive syndrome initially described in 12 different families in southern Sardinia; caused by mutations in the CRLF1 gene (Crisponi, 1996; Crisponi et al., 2007).	***Similarities*** Evident at birth. Marked muscular contraction of the facial muscles in response to tactile stimuli or during crying, contractions slowly disappear as infant calms. Generalised seizures (albeit rare) and mild psychomotor delay in some. Low GABA levels in CSF have been described ***Differences*** Abundant salivation simulating a tetanic spasm. Neck muscle hypertonia. Facial anomalies (large face, chubby cheeks, broad nose with anteverted nostrils and long philtrum). Bilateral camptodactyly. Hyperthermia.
Symptomatic Startle and Myoclonus *Neuropsychiatric* - anxiety states including generalised anxiety disorder, post traumatic stress disorder. *Cerebral* - Children with cerebral palsy, post-traumatic or hypoxic encephalopathy, para neoplastic syndromes. *Brainstem* – particularly pontine pathology eg multiple system atrophy (Bakker et al., 2006).	***Similarities*** Symptoms will be exaggerated by stressors. Stimulus sensitive (e.g. touch) can be seen following hypoxic brain injury. Children with cerebral palsy may have a positive nose-tap test. ***Differences*** Acquired cause often clear, for example late adult onset of multi system atrophy. Hypertonia not a feature of anxiety syndromes.

C. Clinical information for patients with *GLRA1* mutations

Case	Mutation	Sex	AD / AR	Ethnicicity	Family History	Stiff	Startle	Falls	LD or DD	Response to Clonazepam
1	del ex1-ex6	M	R	Turkish	+	+	+	-	-	++
3	del ex1-ex6	M	R	Turkish	+	+	+	-	-	+
8	R65W	M	R	UAE	-	+	+	NA	+	NA
9	L184fs21X	M	R	Turkish	-	+	+	+	+	+
10	E103K & L184fs21X	M	R	Caucasian	-	+	+	NA	+	+
13	Y197X & Y202X	M	R	Jordanian	+	+	+	-	-	+
14	Y197X	F	R	Jordanian	-	+	+	-	-	++
15	Y202X	M	R	Pakistani	-	++	+	-	+	NA
16	Y202X	F	R	Pakistani	+	+	+	+	-	+
17	Y202X	M	R	Jordanian	+	-	+	+	+	x
18	S231N &S296Stop	M	R	White Australian	-	NA	+	NA	++	+
19	P250T	M	D	Irish / Dutch / French	+	+	+	NA	-	NA
20	R252C	M	R	Turkish	+	-	+	-	+	+

21	**G254D**	M	R	Jordanian	+	NA	+	+	-	+
22	**G254D**	M	R	Jordanian	-	+	+	+	-	+
24	**R271Q**	M	D	White Australian	-	+	+	NA	-	+/-
25	**R271Q**	M	D	Caucasian	-	-	+	+	-	+
26	**R271Q**	F	D	White Australian	+	+	+/-	-	-	x
27	**R271Q**	F	D	Caucasian	+	+	+	+	-	+
28	**G342S**	M	D	Caucasian	+	NA	NA	NA	-	NA
29	**G342S**	F	D	NA	+	+	+	NA	-	NA
30	**R392H**	M	R	Italian Canadian	-	+	+	+	+	+

+ = positive, - = not present, +/- = equivocal, ++ = there more than you would expect, more than other cases, x = clonazepam not tried, NA= no information available

D. Clinical information for patients with *SLC6A5* mutations

Case 32 (The Netherlands; homozygote for T425M)

This 9-year-old boy was seen at the out-patient clinic at the age of 2 years. He was the first born of consanguineous parents. The pregnancy was unremarkable. During delivery there was presence of meconium-stained amniotic fluid, but his Apgar scores were 7 and 9 at 1 and 5 minutes. During the first days of life episodes of intermittent generalised stiffness were noted. At the first day of life a generalised tonic clonic seizure was observed lasting 1½ minutes was observed. Treatment with phenobarbital was therefore started. An EEG during a seizure showed bilateral synchronous epileptic activity. During the hospital stay attacks of stiffness with jerks and cyanosis with a duration of 1-3 minutes were observed. Between these attacks myoclonus was noted. Over the years the child suffered from episodes of stiffness and startling. During these episodes, he frequently turned blue and lost consciousness for short periods. These attacks were interpreted as breath-holding spells. On neurological examination this patient showed excessive startle responses with a bilateral pyramidal syndrome. He had persistent delays in cognitive and motor development. While the parents were unaffected, a daughter of a nephew of mother was reported as having startle attacks.

33 (Canada; compound heterozygote for Y377X and V432F+fs97)

The proband was the product of an unremarkable pregnancy to a G3T2L2 woman of Northern European origin and her unrelated Italian/Yugoslavian partner. Foetal movements were described as

unusually jerky throughout the pregnancy. The proband was born at 40 weeks gestation. She had Apgars of 8 and 9 at 1 and 5 minutes. At 90 minutes of age she suffered a generalized tonic-clonic seizure of 50 minutes duration and was successfully treated with phenobarbital. An EEG demonstrated 'non-specific excessive partial sharp waves over the right hemisphere', but no clear evidence of epilepsy. A brain CT scan and MRI were normal. Between seizures 'twitching' was often noted, reflexes were brisk and her startle response was exaggerated. The diagnosis of hyperekplexia was made and she was treated with clonazepam from 10 days of age with marked improvement. During her first year she had episodes of spontaneous stiffening lasting as long as 10 minutes and associated with cyanosis. These could be minimized by picking her up and sometimes aborted by flexing her legs against her body in a specific fashion. The head retraction reflex (HHR) was strongly positive. Over the years the episodes of stiffening diminished. Tripping would precipitate whole body stiffening that prevented her from bracing her fall. At the age of 4 years, she was still requiring 0.08 to 0.09 mg clonazepam administered every 6 hours. Family history revealed that mother, with the V432F+fs97-causing mutation, reported significant sleep myoclonus characterized by stiff jerks of her entire body on a nightly basis, but had no episodes of infantile stiffening or seizures as well as a normal startle response.

34 (USA; compound heterozygote for Y491C and Q630X)

During pregnancy, the mother of the proband was hospitalized on a number of occasions for hyperemesis in the first trimester. At 27 weeks of gestation, she was exposed to pepper spray which caused an allergic reaction and hypoxia. She received both oxygen and steroid therapy which manifested in a case of gestational diabetes. She later developed pre-eclampsia and was put on bed rest. She

was induced at 38 weeks of gestation. The proband, a boy, was delivered with a tight nuchal cord around his neck. He was noted to be experiencing some respiratory grunting but was resolved when treated with a saline bolus. Within 12 hours of birth, the proband was diagnosed as having a massive neonatal stroke which was not confirmed by CT testing. He suffered 47 respiratory arrests in an eight-week period. These episodes were resolved with positive pressure ventilation but caused bradycardia. Treatment with caffeine, phenobarbital and clonazepam did not resolve the apnoea. He also presented with hypertonia and exaggerated startle response to tactile stimuli, resulting in the diagnosis of hyperekplexia. He was tested for the common startle mutations in *GLRA1* but none were found. By nine months of age, the startle response resolved spontaneously and his tone became hypotonic. The proband also was diagnosed with a heart murmur, a large hiatal hernia and GER. By five years of age, his symptoms had resolved. His parents are unaffected.

35 (Australia; compound heterozygote for P108L+fs25 and W482R)

The eldest brother was born after a pregnancy complicated by pre-eclampsia. He had some neonatal episodes consisting of hypertonia and convulsive features considered to be seizures and treated as such. After settling over time the young infant was described as very tense and prone to trembling and episodes of stiffening. In childhood he was prone to episodes of generalised stiffness resulting in fallsthat were provoked by startle. At 5 years of age he was otherwise healthy with retention of the stereotypical nose-tap stereotypes response. The younger brother was also born by Caesarean section after a pregnancy complicated with pre-eclampsia. At 6 hours of life, he began having hypertonic episodes with respiratory obstruction that were treated as seizures. At 13

weeks, he was a very tense, stiff baby who became very rigid when bathed, and some episodes were associated with cyanosis. The generalised hypertonia and prominent nose-tap response were markedly improved with small doses of clonazepam. Treatment was discontinued during infancy without further symptoms.

36 (The Netherlands; compound heterozygote for L306V and N509S)

This 5-year-old girl was born after an uncomplicated pregnancy by vacuum extraction. Despite the presence of meconium-stained amniotic fluid, she had Apgars of 9 and 10 at 1 and 5 minutes. Seven hours post-partum she had her first period of cyanosis. These episodes of cyanosis occurred several times a day during the following days. She was initially treated with luminal lidocaine and clonazepam. During the first days of life periods of intermittent hypertonia and excessive startle responses to unexpected stimuli became evident. After the startle reflexes the stiffness increased and was accompanied by apnoea. Clonazepam was effective for the stiffness and the excessive startle. On neurological examination a head retraction reflex could be elicited. Motor milestones were slightly delayed but caught up. The startle attacks and the stiffness reduced in frequency during the first years of life. The family history for startling and stiffness was negative. MRI and EEG were unremarkable.

37 (UK; heterozygote for S510R)

This first-born male, previously reported as Patient 1 (Stephenson, 1992) developed severe convulsions at age 40 hours, up to six per day, most often precipitated by bathing. After immersion in warm water he would have rapid quivering of his limbs, an interrupted

cry with fast grunting, and then silence with intense stiffening in a semi-flexed posture, leading to deep cyanosis, profound syncope with isoelectric EEG and junctional bradycardia, non-epileptic spasms with forcible urination, and a grey moribund appearance. Surface EMG during episodes showed repetitive giant compound muscle potentials (Pascotto & Coppola 1992) in the "clonic" phase that became more closely spaced in the "tonic" phase. Between these triggered attacks of gross hypertonia, he behaved appropriately, with normal muscle tone. Nose-tapping in the first months of life elicited excessive startle with no habituation. Clonazepam was only given for 48 hours during which time life-threatening syncopes continued: daily baths were then discontinued. After a finding of low CSF GABA (14nmol/l) we had the impression that vigabatrin (0.5g daily from age 7 months) led to marked improvement, insofar as he was then able to have a bath happily for the first time (Stephenson, 1992), but in retrospect this may have been spontaneous improvement, as at age 14 months, when he was on no medication, daily baths did not provoke stiffenings. The abnormal nose-tap response gradually waned and was minimal age 10 years. Aged 13 he no longer has as any excessive startle, and plays prop forward in school rugby. His mother and father have no history of abnormal tone or startles as a baby and do not startle to nose-tap or sudden noise. In summary, this boy had severe life-threatening neonatal hyperekplexia but between attacks was not a stiff baby. There was spontaneous remission in infancy with minimal residual tendency to startle.

E. Ethical approval for Hyperekplexia study

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07 June 2007

Professor Mark. I. Rees
Professor of Molecular Neuroscience
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Dear Professor Rees

Full title of study:	**Genetic basis of neuromotor disorder, hyperekplexia, and associated startle syndromes.**
REC reference number:	**07/WMW02/24**

Thank you for your letter of 18 May 2007, responding to the Committee's request for further information on the above research [and submitting revised documentation].

The further information was considered at the meeting of the Sub-Committee of the REC held on 07 June 2007. A list of the members who were present at the meeting is attached.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation [as revised].

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Application & letter with further information/clarification dated 18 May 2007	1	20 March 2007
Investigator CV	1	02 February 2007
Protocol	1	07 February 2007
Covering Letter	1	19 March 2007
Summary/Synopsis	1	04 February 2007
Compensation Arrangements	Letter of Indemnity - UM Assoc	01 August 2006
Participant Information Sheet	2	17 May 2007
Participant Assent Form: Child	2	17 May 2007
Participant Consent Form: Adult	1	04 February 2007
Response to Request for Further Information		18 May 2007
Letter from MRC to Mr A Patel	1	16 May 2007

R&D approval

All researchers and research collaborators who will be participating in the research at NHS sites should apply for R&D approval from the relevant care organisation, if they have not yet done so. R&D approval is required, whether or not the study is exempt from SSA. You should advise researchers and local collaborators accordingly.

Guidance on applying for R&D approval is available from http://www.rdforum.nhs.uk/rdform.htm.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

Feedback on the application process

Now that you have completed the application process you are invited to give your view of the service you received from the National Research Ethics Service. If you wish to make your views known please use the feedback form available on the NRES website at:

https://www.nresform.org.uk/AppForm/Modules/Feedback/EthicalReview.aspx

We value your views and comments and will use them to inform the operational process and further improve our service.

07/WMW02/24	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely

Roy Evans

Mr Roy L. Evans
Chairman

PP

Please note prior to this ethical approval obtained on 07 June 2007, the extended ethical approval from Auckland council was used for hyperekplexia study.

F. Patient Information Sheet.

You and/or your child have been invited to take part in a research study looking at the:

Molecular Genetics of Startle Syndromes

Before you decide you need to understand why the research is being done and what it would involve. Please take time to read the following information carefully.

Part 1 tells you the purpose of the study and what will happen if you take part. Part 2 gives you more detailed information about the conduct of the study.

Ask if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Part 1

What is the purpose of the study?

The purpose of this study is to identify gene changes that cause startle syndromes.

Why have I been chosen?

You have been invited to take part in this study because your Doctor thinks that you and/or your child may have a startle syndrome. Having a startle syndrome means having an excessive startle. You may startle easily at a sudden sound or unexpected touch or bump and may fall and be injured. If you have any questions about startle syndromes then please ask your doctor for advice.

Do I have to take part?

Whether or not to take part is your decision. In you decide to participate you will be given this information sheet to keep and asked to sign a consent form to show that you have agreed to take part. If you decide to take part you are still free to withdraw at any time without giving a reason. A decision not to take part will not affect the standard of clinical care you or your family receives.

What will happen to me if I decide to take part?

1. If you decide and consent to take part in this research a blood sample will be collected from you and/or your child. About one teaspoonful of blood is the amount that will be collected.

2. The blood sample will be taken for DNA isolation. DNA is the genetic material inside your cells which controls how each cell develops and behaves. A gene is a distinct stretch of that DNA which has a specific job in your body. Sometimes a change or alteration in the DNA of one of those genes can lead to a disease or syndrome like startle syndrome. We will look at your DNA closely to see if we can find a gene change or alteration that causes startle syndrome.

3. If a gene change is found in the sample which is causing the startle syndrome then the research team will write to your doctor with the results.

4. Your Doctor will then contact you to explain what the results are and arrange appropriate support.

What are the possible benefits of taking part?

We hope we will identity the gene change causing the startle syndrome in you and/or your child. This is helpful because it confirms the diagnosis of startle syndrome, and gives your doctor assurance that they are treating your symptoms properly.

Identifying gene changes which cause startle syndromes also helps scientists and doctors to better understand these rare syndromes. This may help people with startle syndrome in the future.

What are the possible disadvantages and risks of taking part?

There are no anticipated risks in taking part.

Will my taking part in this study be kept confidential?

Yes. We will follow ethical and legal practice and all information about you will be handled in confidence. More details are included in Part 2 of this information sheet.

Who has reviewed this study?

This study has been reviewed by the South West Wales Research Ethics Committee.

This completes **Part 1**

If the information in Part 1 has interested you and you are considering participation, please read the additional information in Part 2 before making a decision.

Part 2

Will my taking part in the study be kept confidential?

Yes. When samples are sent to the research team they will be labelled with the name and date of birth of the donor. This is important so that samples do not get mixed up.

Immediately after the sample is received it will be given a unique research reference number. Throughout the research process the samples are identified with the research code only. This means that the sample is anonymous to laboratory staff and in any communications.

When results become available the sample is re-linked to the name, date of birth and referring Doctors name. This is so that your Doctor can be informed of the results. This re-linking of information will be done by an individual within the research team who is aware of their duty of confidentiality.

What will happen to my sample?

The blood sample(s) will be sent to the research team who will look to see if they can find a gene change which is causing the startle syndrome. They will start by looking carefully at the genes where changes have already been found in other people with startle syndromes. This may take up to 6 months to complete.

If the research team discover a gene change in these genes they will write to your Doctor with the results. Your Doctor will then contact you to explain what the results mean for you and arrange appropriate support.

The researchers may not find a gene change causing the startle syndrome in the genes already known to cause startle syndrome. In this case they will continue to investigate the sample in new genes that are not yet linked with startle syndrome. This may take a long time; however, your Doctor will be informed immediately if anything is found.

What will happen if I don't want to carry on with the study?

You are free to withdraw your sample from the study at any time. All you need to do is tell your Doctor that you have changed your mind about the research. If you do decide to withdraw from the study, the samples will be destroyed immediately.

What will happen to the results of the study?

Your doctor will be told if there are any results from the blood sample(s). Your Doctor will then explain these results to you and your family.

Research findings will also be presented for medical journal publication and medical conference presentations. In all cases **identity remains anonymous** and findings cannot be linked back to you or your family.

What do I do now?

Take your time to decide whether or not you would like to take part in the research.

Please feel free to contact us for additional information, or clarification of this information document.

Miss Carrie Hammond, the Research Genetic Counsellor will be happy to hear from you, her contact details are as follows:

Phone Number:	[redacted]
E-mail:	c.l.hammond@swansea.ac.uk
Address:	School of Medicine University of Wales Swansea Singleton Park SA2 8PP

Once you have made a decision then please let your Doctor know.

Thank you for taking the time to read this information

G. ASSENT FORM-Child

Title of Project: Molecular Genetics of Startle Syndrome

Name of Researcher: Professor Mark Rees

Please initial box

1. I confirm that I have read and understand the information sheet for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that participation is voluntary and that we are free to withdraw at any time, without giving any reason, without medical care or legal rights being affected. ☐

3. I agree for my child to give a blood sample for the startle syndrome research. ☐

4. I understand how the sample will be collected and I have had an opportunity to discuss any concerns that I may have about the procedure. ☐

5. I agree for my child to take part in the above study. ☐

_____
Name of Child

_____ _____ _____
Name of Parent/Guardian Date Signature

_____ _____ _____
Name of Doctor taking consent Date Signature

_____ _____ _____
Name of Researcher Date Signature

H. CONSENT FORM -Adult

Title of Project: Molecular Genetics of Startle Syndrome

Name of Researcher: Professor Mark Rees

Please initial box

1. I confirm that I have read and understand the information sheet for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

3. I agree to give a blood sample for the startle syndrome research ☐

4. I understand how the sample will be collected and I have had an opportunity to discuss any concerns that I may have about the procedure. ☐

5. I agree to take part in the above study. ☐

_____	_____	_____	
Name of Participant		Date	Signature
_____	_____	_____	
Name of Doctor taking consent	Date		Signature
_____	_____	_____	
Name of Researcher		Date	Signature

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