The role of gephyrin, collybistin and novel GEFs in the synaptic clustering of inhibitory receptors

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This thesis describes research conducted in the Department of Pharmacology, UCL School of Pharmacy between October 2008 and September 2012 under the supervision of Professor Robert J. Harvey and Dr Kirsten Harvey. I certify that the research described is original and that any parts of the work that have been conducted by collaboration are clearly indicated. I also certify that I have written all the text herein and have clearly indicated by suitable citation any part of this dissertation that has already appeared in publication.

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ABSTRACT

GABA$_A$ and glycine receptors are clustered at inhibitory synapses via interactions with the scaffolding protein gephyrin. In turn, gephyrin is translocated to synapses by collybistin, a guanine nucleotide exchange factor (GEF) for Cdc42. Mutations in the human collybistin gene (ARHGEF9) give rise to a range of symptoms including anxiety, epilepsy and intellectual disability. However collybistin knockout mice revealed a selected loss of clustering of distinct GABA$_A$ receptor subtypes, whilst glycine receptors clustering remained intact. This suggests that other GEFs might also contribute to gephyrin and inhibitory receptor clustering. This thesis describes the identification and characterisation of two novel GEFs that were indentified in a yeast two-hybrid screen using a gephyrin bait: IQSEC2 and IQSEC3. Full-length IQSEC2 did not interact with gephyrin in vitro, and is located at excitatory synapses in vivo, so is unlikely to have a role in GABA$_A$R and GlyR clustering. However, four missense mutations in IQSEC2 were found in families with X-linked intellectual disability (XLID) that impair either calmodulin binding to the IQ-like domain (R359C) or ArfGEF activity (R758Q, Q801P and R863W). By contrast, full-length IQSEC3 did interact with gephyrin in vitro and co-localised with gephyrin and inhibitory receptors in vivo, making this ArfGEF a plausible clustering factor. I also show that a gene fusion affecting IQSEC3 may also result in autosomal intellectual disability associated with behavioural defects. Lastly, I examined the interactions between inhibitory GABA$_A$R and GlyR subunits with gephyrin, mapping binding sites for gephyrin on the GABA$_A$R $\alpha$3 subunit. My analysis revealed that critical determinants of this interaction are located in the motif FNIVGTTPYI in the GABA$_A$R $\alpha$3 M3-M4 domain and the motif SMDKAFITVL at the N-terminus of the gephyrin E domain. Site-directed mutagenesis of the gephyrin E-domain revealed that GABA$_A$R $\alpha$3 and GlyR $\beta$ subunits bind to an overlapping site on the gephyrin E-domain.
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DEDICATION

This thesis is dedicated to the memory of my grandfather, Russell Self. I will always be grateful for his hard work, the dedication his showed his family and the opportunities I had as a result. I hope he would be proud.
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1 INTRODUCTION

1.1 Inhibitory synapses: an overview of structure and function

The junction between two neurons, known as a synapse, is a highly organised structure that is vital for synaptic transmission, a prerequisite for correct brain function and development. Neurotransmitter molecules are released from the presynaptic side of the synaptic junction, the postsynaptic side contains ligand-gated channels or G-protein coupled receptors which respond to these neurotransmitters (Smith and Kittler 2010). Inhibitory synapses fulfil the critical function of controlling brain activity via fast inhibition, by decreasing the chances of an action potential occurring in the postsynaptic cell. The predominant inhibitory neurotransmitter in the brain is \( \gamma \)-aminobutyric acid type A (GABA\(_A\)), although glycine is also prevalent in the brainstem and spinal cord. When these ligands are bound to the corresponding ligand-gated ion channels (i.e. GABA\(_A\)Rs or GlyRs), this triggers the rapid influx of Cl\(^-\) ions into the postsynaptic site via an integral ion channel. For fast inhibition to function correctly, both the presynaptic and postsynaptic elements must be precisely structured and carefully aligned to allow the neurotransmitter to reach the receptor. At the postsynaptic site, this relies on the transport of receptors from their site of synthesis at the endoplasmic reticulum (ER) and assembly in the Golgi network to the cell membrane. Receptors must also be correctly located (e.g. synaptic versus extrasynaptic) and are often held in place by different scaffolding proteins. Unsurprisingly, many different trafficking and scaffolding proteins are involved in these processes, and defects in any one of them could result in improper functioning of the synapse.

Until recently, the ‘membrane activation model’ of clustering at inhibitory synapses (Kneussel and Betz 2000) was the prevailing dogma. This model suggests that GABA\(_A\) and GlyRs are clustered at inhibitory synapses by the scaffolding protein
gephyrin. In turn, the translocation of gephyrin to the membrane is dependent on collybistin, a guanine nucleotide exchange factor, or GEF (Kins et al 2000). The suggested mechanism for the localisation of collybistin to the membrane in this model is via the pleckstrin homology (PH) domain, which binds phosphatidylinositol (3,4,5)-triphosphate (PIP₃) present in the membrane. Collybistin is also proposed to activate Cdc42, a small GTPase, causing the reorganisation of the cytoskeleton beneath the synapse membrane by signalling to various protein kinases and Rac1.

Figure 1.1 The membrane-activation model of inhibitory receptor clustering
Vesicles containing neurotransmitter fuse with the presynaptic membrane allowing release of GABA or glycine into the synapse. On ligand binding, inhibitory receptors allow influx of Cl⁻ ions into the postsynaptic cell. The gephyrin scaffold is shown as a submembrane lattice. Gephyrin is translocated to the membrane by collybistin, which is able to bind to the membrane via the PH domain. Based on the model proposed by Kneussel and Betz (2000).
Whilst there is little doubt that gephyrin and collybistin play important roles in inhibitory receptor clustering, the ‘membrane activation model’ is now known to have several flaws. For example, collybistin binds phosphatidylinositol-3-phosphate (PI$_3$P) (Kalscheuer et al 2009; Reddy-Alla et al 2010), rather than phosphatidylinositol (3,4,5)-triphosphate (PIP$_3$). Other evidence suggests that gephyrin is also able to interact with short motifs present in the M3-M4 intracellular domain of selected GABA$_A$ receptor α subunits (Tretter et al 2008; Kerschner et al 2009; Saiepour et al 2010; Tretter et al 2011) rather than via the GABA$_A$R γ2 subunit or GABARAP as previously proposed by others (O’Sullivan et al 2005). Another unexpected finding was the relatively mild phenotype shown by collybistin knockout mice (Papadopoulos et al 2008), which exhibit increased anxiety and impaired spatial learning due to a selected loss of GABA$_A$R subtypes in the hippocampus and the basolateral amygdala, but unexpectedly do not show a comparable loss of synaptic GlyRs. This strongly implies that other gephyrin clustering factors remain to be identified, and that much remains to be understood about the mechanisms and proteins involved in the formation of a functioning inhibitory synapse.

### 1.2 Critical proteins for the assembly and function of inhibitory synapses

#### 1.2.1 Inhibitory GABA$_A$ receptors

Inhibitory synaptic transmission is vital to control the activity of the nervous system - without it, excitatory transmission would result in cells being stimulated continuously. When GABA binds to GABA$_A$Rs at synapses, this decreases the chance of a new action potential being generated in the postsynaptic neuron by allowing an influx of Cl$^-$ ions, hyperpolarising the cell and preventing impulse conductance. If the reversal potential of the synapse is approximately equal to the resting membrane potential then shunting inhibition occurs, the membrane potential is not altered but the depolarisation caused by excitatory inputs can be reduced. It therefore follows that
diseases affecting GABAergic transmission can have severe effects. Without proper inhibitory synaptic function, the balance between excitation and inhibition - the so-called E/I balance - can be altered, leading to epilepsy, psychological and motor disorders (Martin and Olsen 2000). It is therefore unsurprising that GABA_ARs are major targets for several clinically-important drug classes, including barbiturates, benzodiazepines, neurosteroids, alcohol and volatile anaesthetics, which act as allosteric modulators of GABA_A function. GABA_ARs are diverse in structure and pharmacology, there are 16 different subunits (α1-6, β1-3, γ1-3, δ, ε, π, and θ), as well as different splice variants for some proteins, notably the γ2 subunit, which exists in γ2S and γ2L isoforms. GABA_ARs are part of the cys-loop superfamily, which also includes GlyRs, nicotinic acetylcholine receptors (nAChRs) and serotonin receptors (5HT_3). A typical GABA_A is composed of five subunits - the most commonly found form of receptor contains two α subunits of the same isoform, two β subunits of the same isoform and a single γ2 subunit (2α2βγ). Each receptor subunit contains four membrane-spanning domains (M1-M4) and extracellular N- and C-termini (Barnard et al 1998) as shown in Figure 1.2. The M3-M4 intracellular loop provides the opportunity for GABA_ARs to interact with different synaptic proteins.
Figure 1.2 Representation of the arrangement of a single subunit and a pentameric GABA<sub>A</sub> receptor

(A) A single GABA<sub>A</sub>R subunit composed of four transmembrane domains (M1-M4), with external N- and C-termini and a large intracellular loop between M3 and M4. (B) The arrangement of subunits in a GABA<sub>A</sub>R assembled from five subunits positioned within the membrane, showing that the M2 region lines the integral Cl⁻ channel.

The many different possible subunit compositions of GABA<sub>A</sub>Rs give rise to a large variety of pharmacological and functional attributes (Möhler 2009). GABA<sub>A</sub>Rs containing the α1 subunit are a major subtype and found throughout the CNS. Different mutations in the corresponding gene (GABRA1) are associated with various forms of epilepsy, e.g. juvenile myoclonic epilepsy or absence epilepsy (Cossette et al 2002; Maljevic et al 2006). GABA<sub>A</sub>Rs containing the α2 subunit are highly expressed in the nucleus accumbens (NAcc), frontal cortex, and amygdala, regions intimately involved in motivation and reward. They are likely to mediate the anxiolytic effects of barbiturates and benzodiazepines (Dixon et al 2008; Dixon et al 2010). By contrast, GABA<sub>A</sub> receptors containing the α3 subunit are thought to represent only 10-15% of all GABA<sub>A</sub> receptors. However, they are the major GABA<sub>A</sub>R subtype expressed in brain stem monoaminergic nuclei (Fiorelli et al 2008; Corteen et al 2011). Extrasynaptic receptors, which predominantly contain other α subunit types, including α4, α5 and α6 subunits fulfil the role of tonic inhibition.
(Belelli et al 2009). The synaptic versus extrasynaptic localisation of receptors to
different subcellular sites also appears to be linked to their subunit composition, and
may be due to the highly variable M3-M4 intracellular loops of the GABA_\text{A}R subunits
allowing interactions with different proteins within the cell (Olsen and Sieghart 2008).

1.2.2 Clustering of synaptic GABA_\text{A} receptors by gephyrin

There have been conflicting reports on the role that gephyrin plays in the clustering
of GABA_\text{A}Rs. Essentially, some studies suggest a critical role for gephyrin, whilst
others suggest the opposite is true, i.e. that gephyrin is not required for the synaptic
localisation of the most common isoforms of GABA_\text{A}Rs. In gephyrin knockout mice,
a loss of GABA_\text{A}Rs containing the \(\alpha 2, \alpha 3\) and \(\gamma 2\) subunits was observed in brain
sections and cultured hippocampal neurons (Kneussel et al 1999b). The intracellular
pool of GABA_\text{A}Rs was also increased and both GABAergic and glycinergic currents
were observed to be reduced in knockout animals (Kneussel et al 1999b). However,
certain synaptic GABA_\text{A}R subtypes (e.g. containing \(\alpha 1\) or \(\alpha 5\) subunits) still appear to
cluster in neurons lacking gephyrin (Fischer et al 2000; Kneussel et al 2001; Levi et
al 2004). Hence, although the majority of GlyRs are likely to be clustered by
which GABA_\text{A}R subtypes are subject to gephyrin-dependent clustering has
remained unclear. Curiously, the subcellular localization of gephyrin is also
dependent on certain GABA_\text{A}R subtypes. For example, targeted deletion of the
GABA_\text{A}R \(\alpha 1, \alpha 3,\) and \(\gamma 2\) subunit genes results in a loss of synaptic gephyrin and
GABA_\text{A}R clusters (Schweizer et al 2003; Alldred et al 2005; Li et al 2005; Kralic et al
2006; Studer et al 2006) with cytoplasmic gephyrin aggregates indicating disrupted
synaptic targeting.
Given the numerous individual GABA\textsubscript{A}R subunits that could mediate the interaction, different mechanisms have been proposed to explain the complex interactions of GABA\textsubscript{A}Rs with gephyrin, including alternative splicing, intermediate accessory proteins, post-translational modifications or even binding sites formed at intracellular subunit-subunit interfaces. However, a key breakthrough by Tretter et al (2008) revealed that GABA\textsubscript{A}R \( \alpha \) subunits play a key role in this process. Based on targeting experiments in primary neuronal cell cultures, they revealed that a binding motif (AYAVAVANYA) in the M3-M4 intracellular loop of the GABA\textsubscript{A}R \( \alpha 2 \) subunit could mediate a direct interaction with gephyrin. Further work in this area showed the \( \alpha 3 \) subunit also interacts with gephyrin (Saiepour et al 2010), whilst the \( \alpha 1, \alpha 4, \alpha 5 \) and \( \alpha 6 \) subunits did not (Saiepour et al 2010). Interestingly, the GABA\textsubscript{A}R \( \alpha 2 \) subunit was shown to bind to both gephyrin and collybistin using overlapping sites at the start of the gephyrin E domain (Saiepour et al 2010). Furthermore, the GABA\textsubscript{A}R \( \alpha 2 \) subunit was shown to be capable of ‘activating’ collybistin isoforms harbouring the regulatory SH3 domain, enabling targeting of gephyrin to submembrane aggregates. The synaptic adhesion molecule neuroligin 2 has also been shown to activate collybistin in a similar manner (Poulopoulos et al 2009).

1.2.3 Inhibitory glycine receptors

Glycine receptors (GlyRs) are pentameric ligand-gated chloride channels that mediate inhibitory synaptic transmission in the spinal cord, brainstem, cerebellum and retina (Piechotta et al 2001, Harvey et al 2004). The major adult GlyR isoform, consisting of \( \alpha 1 \) and \( \beta \) subunits, has a key role in the control of spinal motor reflex circuits. Mutations in the genes encoding this GlyR subtype cause excessive startle responses, characterized by noise or touch-induced non-epileptic seizures, excessive muscle stiffness and neonatal apnoea episodes in cattle, mice and humans (Harvey et al 2008). However, the biological roles of other GlyR subtypes,
containing the α2, α3 and α4 subunits, are less clear. The embryonic/neonatal GlyR α2 subtype has previously been linked to roles in synaptogenesis (Kirsch and Betz 1998; Lévi et al 1998), cell fate and paracrine transmitter release in the developing cortex and spinal cord (Flint et al 1998; Mangin et al 2003; Scain et al 2010) as well as retinal photoreceptor development (Young and Cepko 2004). Studies on GlyR α3 subunit knockout mice have revealed subtle defects in central inflammatory pain sensitisation and rhythmic breathing (Harvey et al 2004; Manzke et al 2010). Curiously, the gene encoding GlyR α4 is thought to be a pseudogene in humans due to a stop codon in exon 9, causing a protein truncation between membrane-spanning domains M3 and M4 (Simon et al 2004).

GlyRs are clustered at synapses via a direct interaction between the GlyR β subunit M3-M4 domain and gephyrin (Meyer et al 1995). In fact, gephyrin was initially identified as a 93kDa polypeptide which co-purified from rat spinal cord with the glycine receptor (Pfeiffer et al 1982). The E domain of gephyrin was found to contain the GlyR binding site (Rees et al 2003, Schrader et al 2004) and this interaction has been characterised at the level of molecular structure (Sola et al 2004; Kim et al 2006). Since the gephyrin-GlyR β subunit interaction is necessary for clustering, it is likely that synaptic receptors are heteromers, i.e. α1β, α2β, α3β heteromers.

**1.2.4 The multifunctional protein gephyrin – synaptic clustering and molybdenum cofactor synthesis**

Gephyrin is a 93 kDa multimeric protein with multiple diverse functions in both the central nervous system and in non-neuronal tissues. Gephyrin consists of three domains as shown in Figure 1.3, the G domain (~20 kDa), the C domain (~18 to 21 kDa) and the E domain (~43 kDa). This protein exists in multiple isoforms (P1, C3, C4a-C4d) generated by alternative splicing within the central C domain (Prior et al
The key functions of gephyrin are as the major constituent of the scaffold present at inhibitory synapses, where it is responsible for the clustering of both inhibitory GABA<sub>A</sub> and GlyRs at postsynaptic specialisations. However, another key role is in the biosynthesis of molybdenum cofactor (MoCo) in non-neuronal tissues and glial cells (Schwarz 2005; Smolinsky et al 2008).

**Figure 1.3 Domain structure of gephyrin**

Gephyrin contains an N-terminal G domain and C-terminal E domain, numbering indicates the residue at which a domain starts or ends in the rat protein. These are linked by a central C domain. Alternative splicing results in the inclusion of different ‘cassettes’ (C3, C4a-C4d) in this region, as well as in the G and E domains. Cassette sizes are given in brackets (number of residues) *Cassette only described in rodents to date.

Since all organisms require MoCo for molybdoenzymes such as sulphite oxidase, the proteins involved in the production of MoCo are well conserved (Rajagopalan and Johnson 1992). There is high sequence similarity between the bacterial and plant proteins involved in MoCo biosynthesis and two domains of gephyrin. In fact, sequence similarity of the bacterial proteins MogA and MoeA led to the naming of the G and E domains of gephyrin, as shown in Figure 1.4. The G and E domains present in mammalian gephyrin have corresponding domains in proteins from plants (Cnx1) and bacteria (MogA and MoeA).
However neither plants nor bacteria have a protein similar to the central gephyrin C domain. In mammals, MoCo is synthesised in a pathway involving several enzymes (Schwarz 2005). The key steps are the formation of precursor Z, the formation of molybdopterin and the insertion of molybdenum metal ion into the pterin structure.

The proteins involved in catalysing each step are shown in Figure 1.5. Gephyrin is thought to catalyse the insertion of molybdenum metal ion into pterin, i.e. gephyrin catalyses the final step in the production of MoCo. It is thought that the G and E domains of gephyrin catalyse different steps in the process, based on the mechanistic properties of the Cnx1 protein in plants. The Cnx1 G domain binds molybdopterin and catalyses the transfer of an adenyl group to the phosphate group of molybdopterin (Kuper et al 2004; Llamas et al 2004). The adenylated molybdopterin is then moved to the Cnx E domain, where molybdenum is inserted to make MoCo by zinc assisted hydrolysis (Llamas et al, 2006).
Experiments using gephyrin knockout mice confirmed the key role of gephyrin in molybdenum cofactor biosynthesis. Gephyrin knockout mice died within 24hrs after birth showing a hyperextended posture similar to that seen with strychnine poisoning and in human hyperekplexia (Feng et al 1998). However, the cause of death is more likely to be due to the lack of MoCo and resulting toxic build up of sulphites, which severely damage the brain.

Given that there are two distinct functions of gephyrin, it is tempting to assume that the different isoforms produced by the alternative splicing within the C domain could be responsible for different aspects of gephyrin function. In a recent review, the following table of gephyrin splice cassettes was compiled along with suggestions for a simplified naming system (Fritschy et al 2008).

Figure 1.5 The molybdenum cofactor biosynthesis pathway
<table>
<thead>
<tr>
<th>Old Cassette Name</th>
<th>New Cassette Name</th>
<th>Rationale</th>
<th>Species</th>
<th>Corresponding protein sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>G1</td>
<td>Insertion in G domain</td>
<td>Rat</td>
<td>MSFPLSPAFTLLHILV</td>
</tr>
<tr>
<td>C5/C50</td>
<td>G2</td>
<td>Insertion in G domain</td>
<td>Rat</td>
<td>KFPTFPFCGLQKG</td>
</tr>
<tr>
<td>C2</td>
<td>None</td>
<td>Constitutively spliced exon</td>
<td>All</td>
<td>-</td>
</tr>
<tr>
<td>C3</td>
<td>C3</td>
<td>No change needed</td>
<td>Human</td>
<td>KHPFYTPAVVMAHGEQPI PGLINYSHHSTDER</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ape</td>
<td>KHPFYTPAVVMAHGEQPI PGLINYSHHSTDERSEP</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Rat</td>
<td>NHPFYTPAVFMANHGQPI PGLISYSHHATGSADKR</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mouse</td>
<td>KHPFYTPALFMANHGQPI PGLISYSHHATGSADKR</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Bovine</td>
<td>KHPFYTPAVIMAHEQPI PGLISYSHDATGSAEEP</td>
</tr>
<tr>
<td>C40/C4A</td>
<td>C4a</td>
<td>Inconsistent nomenclature</td>
<td>Human</td>
<td>QIRRDPDESKGVASRVGSLK</td>
</tr>
<tr>
<td>C4B</td>
<td>C4b</td>
<td>No change needed</td>
<td>Human</td>
<td>LHRKLEELRDHLEGNVKGY SLRVN</td>
</tr>
<tr>
<td>C4/C4C</td>
<td>C4c</td>
<td>No change needed</td>
<td>Human</td>
<td>ARLPSCSSTYSVE</td>
</tr>
<tr>
<td>C4D/C5</td>
<td>C4d</td>
<td>Inconsistent nomenclature</td>
<td>Human</td>
<td>LHSRLEGKLDELWRNRG YD LR</td>
</tr>
<tr>
<td>C6/C60</td>
<td>None</td>
<td>Constitutively spliced exon</td>
<td>All</td>
<td>-</td>
</tr>
<tr>
<td>C6</td>
<td>E1</td>
<td>Insertion in E domain</td>
<td>Rat</td>
<td>GRHSRQELKQRPQRNAAF WLLSHGLLSSLLAPHAQGWH HP</td>
</tr>
<tr>
<td>C7</td>
<td>E2</td>
<td>Insertion in E domain</td>
<td>Rat</td>
<td>RFMAAG</td>
</tr>
</tbody>
</table>

**Table 1.1 Constitutively and alternatively spliced exons in gephyrin (Fritschy et al 2008)**

This table shows the revised cassette nomenclature. The location of these cassettes within the gephyrin domains can be seen on Figure 1.3.

The C2 and C6 cassette exons appear to be constitutively expressed and encode parts of the G and E domain, the isoform containing these cassettes (but no ‘C3’ or
‘C4’ cassettes) is commonly named P1. The C3 isoform contains the C2, C6 and C3 cassettes. C4 isoforms contain C2, C6 and any of the C4 cassettes. C3 and C4 cassettes do not seem to be commonly found in the same mRNA, so few isoforms contain both C3 and C4 cassettes. Interestingly, the C3 cassette is constitutively spliced into gephyrin transcripts in peripheral tissues (Rees et al 2003) and glial cells, the site of MoCo synthesis in the brain (Smolinsky et al 2008). For this reason, C3 is thought to be linked to the non-neuronal function of gephyrin in MoCo synthesis. Consistent with this finding, it has also been shown that the C3 cassette is repressed in neurons by Nova proteins (Ule et al 2003). Several studies have attempted to define the biological roles of gephyrin variants. It has been shown that different rat organs and tissues contain a variety of sizes of gephyrin polypeptides (Hermann et al 2001). The ability of seven different gephyrin variants to bind the GlyR β subunit M3-M4 loop was tested and showed that some gephyrin cassettes influence the ability of gephyrin to bind the GlyR β subunit (Meier et al 2000). Through the expression of gephyrin variants in gephyrin deficient L929 cells (mouse fibroblasts) it has been possible to identify which gephyrin variants are able to reconstitute MoCo synthesis. Variants containing either G2 insertions or missing the constitutively spliced exons formerly known as C2 and C6 were inactive in MoCo synthesis, showing that intact G and E domains are required for MoCo synthesis. Variants containing the C3 or C4c cassettes in the C domain were found to be twice as active as the others in terms of MoCo synthesis (Smolinsky et al 2008).

Gephyrin is able to form a synaptic scaffold via multimerisation mediated by the G and E domains. A crystal structure of the complete gephyrin molecule has yet to be achieved, but structures of the individual G and E domains have shed some light on gephyrin oligomerisation. Purification of the full-length P1 isoform of gephyrin gave an approximate 300 kDa complex when eluted from a gel filtration column. The complex was chemically crosslinked and run on SDS-PAGE to give a band at 250
As the P1 monomer is 81.3 kDa, this suggested trimeric structure. Chemical crosslinking of recombinant gephyrin E domains results in a 98 kDa product on SDS-PAGE, suggesting that the E domain dimerises as the single domain is around 48 kDa in size. A partial trypsin digest of the ‘gephyrin-300’ complex provided further evidence of E-domain dimerisation, as three stable fragments were produced, a single N-terminal section, and two nearly identical E domain fragments (Sola et al 2004). The G domain of human gephyrin was recombinantly expressed and purified by size-exclusion chromatography, the molecular weight of the fractions suggested the main conformation was trimeric. This purified fraction was used for crystallography and trimers were confirmed (Schwarz et al 2001). Since the E domain has also been determined to form a dimeric structure, a hexagonal arrangement for gephyrin was suggested based on gephyrin domain self-interactions (Xiang et al 2001) as depicted in Figure 1.6.

![Figure 1.6 Representation of the polymerisation of gephyrin](image)

Gephyrin is able to form dimers through the E domain, and trimers via the G domain, which may allow it to form a two dimensional hexagonal scaffold under the cell membrane.
Disruption of domain oligomerisation results in a diffuse spread of epitope-tagged gephyrin in HEK-293T cells, where as the wild-type gephyrin is able to form aggregates. In neuronal cell cultures wild-type gephyrin forms clusters along the dendrites, co-localised with VIAAT (a marker for inhibitory terminals). For gephyrin mutants where either G or E-domain oligomerisation has been disrupted, gephyrin forms abnormal clusters which were not co-localised with VIAAT (Saiyed et al 2007).

1.2.5 The RhoGEF collybistin – a key player in synaptic gephyrin clustering

Collybistin is a brain specific GEF that was identified as gephyrin interactor through yeast two-hybrid screening of a rat brain cDNA library, and confirmed using co-immunoprecipitation (Kins et al 2000). Importantly, this initial study found collybistin was capable of inducing submembrane gephyrin microaggregates that could recruit GlyRs in recombinant systems. Collybistin catalyses the exchange of GDP for GTP on the small GTPase Cdc42 via the RhoGEF domain (Reid et al 1999). Collybistin also contains an N-terminal SH3 domain (able to mediate binding to proline-rich sequences in interactors) and a C-terminal PH domain (able to bind phosphatidylinositol lipids in cell membranes). In humans ARHGEF9 is located at Xq11.1, the rat gene is also on the X chromosome. Alternative splicing of the rat mRNA gives rise to several isoforms of collybistin (CB), as shown in Figure 1.7.

![Figure 1.7 Representation of the three isoforms of rat collybistin](image)

Three different C termini exist in rat, generated by the insertion of 109bp (CB1) or 62bp (CB2) exons, or neither (CB3). The N-terminus contains an SH3 domain,
alternative splicing results in expression of isoforms with and without the SH3 domain. CB2 and CB3 are both expressed in rat brain, whereas in humans only the homolog of CB3 is detected.

Harvey et al (2004) demonstrated that CB1_{SH3+} and CB2_{SH3+} behaved in the same manner when co-transfected with EGFP gephyrin, and localised with cytoplasmic gephyrin aggregates (Figure 1.8A, B). However when CB2_{SH3-} was transfected into HEK293 cells with EGFP-gephyrin, in \sim 40\% of cells collybistin and gephyrin redistributed to submembrane clusters (Figure 1.8C, D) that were also capable of binding to a DsRed-GlyR β subunit M3-M4 fusion protein (Figure 1.8E-H).

Figure 1.8 Expression of myc-tagged collybistin isoforms with EGFP-gephyrin in HEK293 cells

(A, B) Cotransfection of an EGFP-gephyrin and myc-CB2_{SH3+} results in the formation of cytoplasmic aggregates of gephyrin to which myc-CB2_{SH3+} targets. (C, D) Distribution of EGFP-gephyrin to submembrane microaggregates is observed on co-expression with myc-CB2_{SH3-}. A DsRed-GlyR β subunit M3-M4 fusion protein targets to both intracellular and submembrane microaggregates. Scale bars, 10 \mu m.
Since RT-PCR revealed that SH3-domain containing isoforms are more prevalent in the brain (Harvey et al 2004), it appeared that collybistin requires some form of activation to allow submembrane gephyrin clustering. As mentioned above, neuroligin-2 has been shown to be capable of fulfilling this role, ‘activating’ $\text{CB2}_{\text{SH3}^+}$ and enabling the formation of submembrane microaggregates, driving formation of the inhibitory postsynaptic site (Poulopoulos et al 2009). Similarly binding of the $\text{GABA}_A\text{R} \alpha_2$ subunit also induces the change from cytoplasmic aggregates to membranous microaggregates (Saiepour et al 2010). More recently, $\text{CB2}_{\text{SH3}^-}$ isoforms were shown to promote gephyrin and $\text{GABA}_A\text{R}$ clustering at synapses, whilst $\text{CB2}_{\text{SH3}^+}$ isoforms were suggested to be involved in the formation of superclusters of gephyrin and $\text{GABA}_A\text{Rs}$ (Chiou et al 2011). Deletion of the PH domain does not impair gephyrin-collybistin interactions, but does abrogate the formation of submembrane gephyrin microclusters (Harvey et al 2004). By contrast, deletion of the RhoGEF domain results in a loss of gephyrin and collybistin colocalisation (Harvey et al 2004), suggesting that the RhoGEF domain contains the gephyrin binding site. In support of this suggestion, Cdc42 and gephyrin appear to compete for the same binding site on collybistin (Xiang et al 2006). However, when the RhoGEF domain is present but GEF activity is inactivated by mutagenesis, gephyrin clustering is apparently unaffected (Reddy-Alla et al 2010). However, expression of constitutively-active Cdc42 was recently shown to rescue endogenous gephyrin clustering when CB2 lacking the PH domain was expressed in neurones (Tyagarajan et al 2011a). This suggests that Cdc42 activation is an important part of the gephyrin clustering mechanism.

Expression of overlapping gephyrin fragments and alanine mutagenesis scanning has revealed that a critical determinant of the collybistin binding site on gephyrin is situated at the junction of the C and E domains, a motif that is distinct from the GlyR $\beta$ subunit binding site (Harvey et al 2004). Indeed when gephyrin is pulled down
from cell lysates using an immobilised GlyR β subunit fragment (containing the gephyrin binding site) collybistin can also be detected, suggesting gephyrin can bind collybistin and glycine receptors at the same time (Grosskreutz et al 2001). However, collybistin appears to be dispensable for GlyR clustering in vivo, since motor defects were not observed in collybistin knockout mice, which instead showed increased levels of anxiety and impaired spatial learning due to a selective loss of GABA_A Rs in the hippocampus and the basolateral amygdale (Papadopoulos et al 2007). This finding strongly suggests that additional RhoGEFs involved in inhibitory receptor clustering remain to be identified.

1.3 Genetic defects in gephyrin and collybistin genes – link to neurological disorders

1.3.1 Gephyrin mutations in molybdenum cofactor deficiency

In humans, MoCo deficiency is typically inherited as an autosomal recessive trait, and is categorized into two groups, Group A (mutations in MOCS1) and Group B (mutations in MOCS2 or GPHN) (Reiss and Johnson 2003). This results in lack of function of MoCo containing enzymes, the consequences of this are shown in Table 1.2.

<table>
<thead>
<tr>
<th>MoCo containing enzyme</th>
<th>Function</th>
<th>Impact of lack of functional enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulphite oxidase</td>
<td>Metabolism of sulphur containing compounds, allowing sulphate to be excreted.</td>
<td>Toxic levels of sulphite can build up leading to neurological disorders and intellectual disability, invariably fatal.</td>
</tr>
<tr>
<td>Xanthine dehydrogenase</td>
<td>Purine metabolism, conversion of xanthine to urate, which can be excreted.</td>
<td>Low uric acid levels.</td>
</tr>
<tr>
<td>Aldehyde oxidase</td>
<td>Conversion of aldehydes to carboxylic acids.</td>
<td></td>
</tr>
</tbody>
</table>

Table 1.2 The functions of MoCo containing enzymes
The combined deficiency of these enzymes results in a severe phenotype in affected individuals. The most serious damage is caused by the elevated sulphite levels, which cause permanent neurological damage. Early symptoms include untreated neonatal seizures, opisthotonos (severe hyperextension resulting in arched neck and back) and facial dysmorphism. Later, severe neurological abnormalities, dislocated ocular lenses and intellectual disability occur, and in most cases the disease causes death in early childhood. The majority of cases of MoCo deficiency are caused by mutations in MOCS1 and MOCS2, but more recently mutations in the gephyrin gene (GPHN) have been identified (Reiss et al 2001; Reiss et al 2011). Unsurprisingly, gephyrin knockout mice also show molybdoenzyme defects and a phenotype typical of MoCo deficiency (Feng et al 1998). Understanding how MOCS proteins and gephyrin are involved in the synthesis of MoCo could lead to potential therapies. For example, precursor Z has been synthesized in vitro, and would help individuals with MOCS1 mutations (Schwarz 2005). In the first case published where a GPHN mutation resulted in MoCo deficiency, the cause was a deletion of exons 2 and 3, which caused a frameshift after 21 codons. The patient died at three days of age, showing both hypotonia (decreased muscle tension) and hyperreflexia (over-responsive reflexes). Biochemical assays confirmed MoCo deficiency (Reiss et al 2001). In a more recently published case, the patient is now two years old and suffers from daily epileptic seizures and is fed through gastrostomy (a surgical opening into the stomach). MoCo deficiency was diagnosed after a positive sulphite test. No mutations were found in the MOCS genes, but a homozygous missense mutation was found in the gephyrin E domain, which results in a D580A change. This aspartate residue is completely conserved across species. It is suggested that the G and C domains may be functional, so synaptic clustering is intact which is why the phenotype is less severe than the previously described patient without gephyrin expression (Reiss et al 2011).
1.3.2 Collybistin mutations in X-linked intellectual disability

Intellectual disability, also referred to as mental retardation, is clinically defined as ‘significant limitations both in intellectual functioning and adaptive behaviour as expressed in conceptual, social, and practical skills, which are apparent prior to the age of 18’ (AAIDD 2010). A high proportion of proteins known to be mutated in X-linked intellectual disability (XLID) are part of the postsynaptic proteome – a recent estimate was 28% (Laumonnier et al 2007). Since then, the number of XLID proteins identified has increased, but it is likely that the proportion of postsynaptic proteins remains the same if not being higher. This makes the genes encoding synaptic proteins good candidates for genetic analysis in both X-linked and autosomal recessive intellectual disability (XLID and ARID).

The most common cause of X-linked intellectual disability (XLID) is a mutation in FMR1, specifically a trinucleotide repeat expansion. This particular form of XLID is called fragile-X syndrome. Mutations in over 100 genes have now been identified as causes of XLID (Lubs et al 2012), through segregation studies and DNA sequencing in families with XLID. Due to the nature of XLID, it is usually relatively easy to identify the disability, as a higher prevalence of ID in males in a family will indicate the problem is likely on the X chromosome. In cases of ID where there isn’t any obvious male bias, then the cause may be autosomal which makes identifying the gene responsible much more challenging and requires resources – time, money and labour. Whilst many causes of XLID have been identified, in many cases the particular mutation will only be responsible for XLID in one or a few families, and in most affected families the cause will not have been identified. With recent advances in next generation sequencing, it is becoming cheaper and quicker to sequence entire chromosomes or whole genomes. Therefore the rate at which XLID genes are being identified has rapidly increased. In the majority of published cases, only limited functional assays are carried out to prove pathogenicity, since this requires
further funding which is not always justified when only one family has been identified as carrying a given mutation.

Several reported cases have linked defects in the human collybistin gene (*ARHGEF9*) with epilepsy and intellectual disability. The first report was by Harvey et al (2004) - whilst screening hyperekplexia patients for mutations in collybistin, a missense mutation (c.G164C) was identified which results in a p.G55A mutation in the N-terminal SH3 domain. The male patient's symptoms included tonic seizures provoked by tactile stimulation that developed in the weeks after birth. By four years of age the patient had severe intellectual disability and suffered daily seizures, EEGs showed these were both hyperekplexic and epileptic seizures. The patient died aged 4 years and 4 months. To assess how this G55A mutation resulted in this phenotype myc-tagged CB3SH3,G55A was transfected with EGFP-gephyrin into HEK293 cells. The resultant submembrane gephyrin microclusters more closely resembled that seen with CB3SH3 than CB3SH3+, suggesting the G55A mutation had altered the functionality of the SH3 domain. In cortical neurons, wild-type CB3SH3+ was diffusely expressed and did not affect endogenous gephyrin clustering, whereas the G55A mutant showed much tighter aggregation with gephyrin. The G55A mutant also disrupted GABA\_A R clustering in cortical neurones in a dominant-negative manner. The likelihood is that the phenotype seen in the patient was a result of a loss of gephyrin, GABA\_A Rs and GlyRs from synapses (Harvey et al 2004)

Since this initial report several further cases have been published which linked *ARHGEF9* disruptions to X-linked intellectual disability. In 2008, a female patient with XLID and hyperarousal to noise was reported (Marco et al 2008). In this case fluorescent in situ hybridisation (FISH) revealed an inversion within *ARHGEF9*. The levels of *ARHGEF9* mRNA transcript were 10-fold less than in controls. Skewed X-inactivation may explain why this female patient had this clinical phenotype rather
than being an asymptomatic carrier. This group also sequenced \textit{ARHGEF9} in 576 males with XLID, but found only non-synonymous mutations which the Polyphen-2 (Polymorphism Phenotyping) program (http://genetics.bwh.harvard.edu/pph/) and SIFT (Sorting Intolerant From Tolerant) program (http://blocks.fhcrc.org/sift/SIFT.html) predicted were benign or tolerated. This \textit{ARHGEF9} disruption seems to give a much less severe phenotype than the G55A mutation, possibly due to the patient having some degree of rescue from her intact X chromosome (Marco et al 2008). In a further study, a balanced chromosomal translocation which disrupted \textit{ARHGEF9} was identified (Kalscheuer et al 2009). The patient in this case suffered from epilepsy, anxiety, aggression and mental retardation. Karyotyping revealed a \textit{de novo} balanced translocation between chromosomes X and 18, with DNA sequencing revealing that the breakpoint was situated between exons 6 and 7 of \textit{ARHGEF9}. As a result, mis-spliced \textit{ARHGEF9} mRNAs were produced in the patient, which made two groups of truncated proteins, both of which were found to be stable and expressed at levels similar to normal collybistin. Both proteins lack the PH domain but instead of forming gephyrin microaggregates, the proteins colocalised with EGFP-gephyrin in large clusters. When expressed in cultured neurons, the truncated proteins were found to have a more severe effect, and reduced both synaptic gephyrin and GABA\textsubscript{A}R clusters, consistent with a loss of synaptic gephyrin and inhibitory receptors.

More recently a microdeletion and loss-of-function mutation in collybistin have also been linked to cases of XLID. The \textit{de novo} microdeletion, of 1.3 Mb at Xq11.11, covers a region containing \textit{ARHGEF9}, one gene of unknown function (\textit{SPIN4}) and three other putative genes. The individual’s symptoms included seizures at age five months, which were treated with phenobarbital, carbameazepine, clobazam and topiramate. EEG was normal at three years, and seizures decreased at four years of age. At six years the patient had severe intellectual disability, focal epilepsy,
hyperactivity with attention deficit and limited social interaction. This patient was the first reported with a complete loss of ARHGEF9 and therefore a complete loss of collybistin (Lesca et al 2011). The case also supports a role for ARHGEF9 in cognitive development. Finally, the latest case to be reported was a loss of function mutation in collybistin in a patient with severe intellectual disability and epilepsy. A nonsense mutation, p.Q2X was found in exon 1a which is only used in transcript variant 2. The patient experienced seizures at 20 months, and showed early developmental delay. At 5 years old, he could not speak and had severe intellectual disability (Shimojima et al 2011). Taken together, these studies show that disruption of synaptic clustering of inhibitory GABA\(_A\)R and GlyRs or peripheral MoCo synthesis due to loss of gephyrin or collybistin can produce devastating neurological disorders. This is a compelling reason for understanding the proteins involved in gephyrin clustering at synapses, and dissecting the mechanisms involved in the clustering of inhibitory receptors by these molecules.

In summary, gephyrin and collybistin knockout mice models and patient cases strongly suggest that other proteins, yet to be identified, must also contribute to inhibitory receptor clustering. Whilst GlyR anchoring is dependent on gephyrin, it appears proteins other than collybistin are capable of translocating gephyrin to the membrane. Selected GABA\(_A\)R subtype clustering is lost when gephyrin is knocked out - but not all, again suggesting that other proteins are involved. The purpose of this project was to search for proteins capable of fulfilling these roles.
1.4 Thesis aims

- To use yeast two-hybrid library screening to search for novel gephyrin interactors, with a specific emphasis on identifying additional GEFs that may be involved in gephyrin clustering at synapses, or proteins that link to the peripheral role of gephyrin in MoCo synthesis.

- To use bioinformatics, the yeast two hybrid system and \textit{in vitro} functional assays in mammalian cells to characterise the nature and functional role of novel gephyrin interactors

- To assess novel gephyrin interacting proteins as candidate genes for hyperekplexia, epilepsy and/or intellectual disability

- To improve understanding of how gephyrin clusters GABA\textsubscript{A}R and GlyRs at synapses, with particular emphasis on characterising the interactions of GABA\textsubscript{A}R \(\alpha\) subunits with gephyrin
2 MATERIALS AND METHODS

2.1 DNA methodology

2.1.1 Amplification of specific cDNA by the Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) was used to amplify required complementary DNAs (cDNAs). Template DNA containing the sequence to be amplified is required. In some cases, existing plasmid constructs could be used as a template. For other amplifications either rat or human first-strand cDNA was used, made from either brain or peripheral tissues. cDNA was synthesised using the SuperScript® III First-Strand Synthesis SuperMix (Invitrogen 18080-400). The reaction was set up in the following way:

<table>
<thead>
<tr>
<th>Stage</th>
<th>Procedure:</th>
</tr>
</thead>
</table>
| 1)    | The following components were combined in a 0.2ml PCR tube:  
      - up to 5μg poly(A)+ RNA  
      - 1μl Annealing buffer  
      - 1μl Primer (50μM oligo(dT) or 2μM gene specific primer or 50ng/μl random hexamers)  
      - RNase/DNase-free water to a total volume of 8μl |
| 2)    | The reaction was incubated at 65°C for 5 minutes, then placed on ice and centrifuged briefly before proceeding to next stage. |
| 3)    | The following was added:  
      - 10μl 2× first-strand reaction mix  
      - 2μl SuperScript III enzyme mix. |
| 4)    | The mixture was vortexed and then centrifuged briefly. Incubation was at 50°C for 50 minutes if using oligo(dT) or gene specific primers. If using random hexamers, this 50°C incubation was preceded with 5-10 minutes at 25°C |
| 5)    | The reaction was terminated at 85°C for 5 minutes and chilled on ice. cDNA was then stored at -20°C until needed. |

Table 2.1 The stages of cDNA synthesis

To amplify a specific region of DNA forward and reverse primers of between 20 and 30 nucleotides were designed to cover the start and end of the region of interest, including appropriate restriction enzyme sites. Primers were ordered from Eurofins MWG Operon (http://www.mwg-biotech.com/). A typical PCR reaction included 1μl of template, either cDNA or a plasmid construct at 50ng/μl, 1μl each of a forward and reverse primer mix at 10μM and 22μl Accuprime Pfx Supermix (Invitrogen
37

12344-040). For amplification of GC-rich regions of DNA, 5μl of GC melt (Clontech 639238) or 5M betaine solution (Sigma B0300-1VL) was added. After a brief spin in an Eppendorf 5414D microcentrifuge, the PCR reactions were placed in a thermocycler (Thermo Electron Px2). PCR utilises three key stages, shown in Table 2.2, that allow DNA to be copied in an exponential manner.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Temperature</th>
<th>Length of Stage</th>
<th>Additional Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>1 minute</td>
<td>High temperature denatures double stranded DNA, this allow primers access to their complementary sequence on the single strands in the next stage</td>
</tr>
<tr>
<td>Annealing</td>
<td>60°C</td>
<td>1 minute</td>
<td>Lower temperature allows primers to bind the single stranded DNA. For problematic reactions the temperature was lowered to 55°C or 50°C, or alternatively raised to 65°C if non-specific PCR products were forming.</td>
</tr>
<tr>
<td>Extension</td>
<td>68°C</td>
<td>1 minute per 0.5kb</td>
<td>The polymerase enzyme adds nucleotides to the primers in this stage; the time required depends on the length of the amplicon.</td>
</tr>
</tbody>
</table>

Table 2.2 The stages of PCR

By cycling between the three temperatures, a region of DNA was copied. New DNA was denatured at the start of the next cycle and used as a template, allowing for exponential copying. Although the reaction can continue until no more dNTPs are available, 30-35 cycles were used for most reactions to ensure optimal yield without running out of dNTPs. The Pfx Supermix contained all the necessary nucleotides, buffer and enzyme.

2.1.2 Site-directed mutagenesis

Site-directed mutagenesis was used in cases where a specific base-pair needed to be changed in order to introduce an artificial mutation that changed the coding
sequence. A plasmid template containing the wild-type cDNA was used, and primers containing the mutated base pair(s) were designed to anneal to both strands of the plasmid. Pfu Ultra (PfuUltra™ Hotstart PCR Master Mix – Agilent Technologies 600630) or Pfu Turbo (PfuTurbo Hotstart PCR Master Mix – Agilent Technologies 600600) were used as they are proofreading polymerases with low error rates, ensuring that no mutations other than the desired change were introduced. Error rates for each of the polymerases used can be seen in Table 2.3, Pfu Ultra being the most accurate.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Error Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>AccuPrime Pfx</td>
<td>$2.9 \times 10^{-6}$</td>
</tr>
<tr>
<td>Pfu Ultra</td>
<td>$4.3 \times 10^{-7}$</td>
</tr>
<tr>
<td>Pfu Turbo</td>
<td>$1.3 \times 10^{-6}$</td>
</tr>
</tbody>
</table>

Table 2.3 Error rates of DNA polymerases

After the PCR was complete, a methylation-sensitive restriction enzyme DpnI digest (New England Biolabs) was used to remove the wild-type template DNA. The newly synthesised copy of the template is not digested by this enzyme. Amplified PCR products were then transformed into competent *Escherichia coli* (*E. coli*) for repair and propagation. Additional mutagenesis reactions were carried out to create chimeras of two cDNAs. In this case, a template of one gene was used, and long primers were designed which contained sequence of another gene for the desired region, to create a chimeric PCR product.

2.1.3 Agarose gel electrophoresis

After the PCR was complete, products were run on agarose gels, prepared by mixing 1.0-1.5g of ultrapure agarose powder (Invitrogen 16500500) with 100ml of 1× Tris-acetate-EDTA buffer (TAE buffer) and microwaving to dissolve, resulting in a 1.0-1.5% (w/v) gel. 50× TAE buffer was made from 242g Trizma Base (Sigma T6791), 57.1ml glacial acetic acid (VWR 10001CU) and 100ml of 0.5M EDTA pH 8 (Sigma E5134), topped up to 1 litre with dH₂O. The dissolved agarose mix was
cooled to 50°C and then 5µl of SYBR Safe™ DNA Gel Stain (Invitrogen S33120) was added to enable visualisation of DNA fragments. A gel comb was placed in the liquid agarose to create wells. After setting for 10 minutes, gels were placed in the electrophoresis chamber (BioRad) and immersed in 1×TAE buffer. 10µl of a 1 kb DNA ladder (Invitrogen 15615024) was used as a molecular weight marker. Samples were mixed with 3µl loading buffer (40% w/v sucrose solution with trace amounts of bromophenol blue) and loaded into the wells. Gel electrophoresis was carried out at 100 V for 30 minutes. The DNA was then visualised using a Syngene Ingenius Bio-imaging system or Safe Imager Transilluminator (Invitrogen, S37102). Bands were cut from the gels for successful amplifications or transformed directly into *E. coli* for mutagenesis reactions.

### 2.1.4 Extraction and purification of DNA from agarose gels

The QiaQuick Gel Extraction kit (Qiagen 28706) was used for extraction of DNA from agarose slices. Briefly gel slices were weighed and three gel volumes of buffer QG (5.5M guanidine thiocyanate, 20mM Tris-HCl pH 6.6) were added. Slices were incubated at 50°C with shaking until all agarose had dissolved. One gel volume of isopropanol was added and mixed. The mixture was transferred to a QiaQuick spin column matrix and centrifuged at 13,000 rpm for 1 minute. The columns were washed with 0.75ml buffer PE (20mM NaCl, 2mM Tris-HCl pH 7.5, 80% ethanol). DNA was finally eluted from the matrix using 20µl of elution buffer (EB), (10mM Tris-HCl pH 8.5). To check the gel extraction, 5µl of extracted DNA was run on a new agarose gel.

### 2.1.5 Restriction enzyme digests

Purified DNA was digested with the appropriate restriction enzymes. In general, 15µl of DNA fragment was cut with 0.25µl of each enzyme (New England Biolabs), with 2µl of appropriate NEB buffer, 2µl of 10× BSA if required (100× BSA provided with enzymes from New England Biolabs) and made up to 20µl with water. Reactions
were incubated at the appropriate temperature, usually 37°C, for 1-2 hours. 1.5μg of the desired vector was also digested, in a total reaction volume of 20μl. Primer sets for PCR amplifications, mutagenesis and vector DNAs used in this project are listed in the appendix. In most cases, the same enzymes could be used to cut the insert and vector, although in some cases different enzymes that produced compatible ends were used instead.

2.1.6 Phenol/Chloroform/Isoamyl extraction

After the restriction digest incubation, an Ultrapure Phenol/Chloroform/Isoamyl alcohol (PCI) mix (Invitrogen 15593031) was used to remove restriction enzymes. Briefly, 80μl of water was added to each restriction digest to bring the total volume to 100μl. In the fume hood, 100μl of PCI mix was added, the samples were mixed and centrifuged at 13,000 rpm in a microcentrifuge for 5 minutes. To precipitate the DNA, 10μl of 3M sodium acetate (pH 5.2) and 250μl of 96% (v/v) ethanol was placed in a fresh microcentrifuge tube (1μl of glycogen –(20μg/ml) was also added to help visualise the DNA at later stages). The aqueous phase from the centrifuged tubes was added to the sodium acetate/ethanol/glycogen and mixed. The tubes were placed on dry ice until frozen solid. The tubes were then spun at 13,000 rpm in a microcentrifuge for 15 minutes. The ethanol was then removed, taking care to avoid disturbing the pelleted DNA. After washing the pellet in 70% (v/v) ethanol, residual ethanol was allowed to evaporate. The DNA was then resuspended in 7μl of buffer EB (10mM Tris-Cl, pH 8.5) for inserts, 50μl of buffer EB for vectors.

2.1.7 Ligation of DNA inserts into digested vector DNAs

For ligations, 1μl of appropriate vector, 1μl ligation buffer and 1μl T4 DNA ligase (Roche 10716359001) was added to 7μl insert, tubes were spun to mix, the ligations were kept at 4°C overnight. Where possible, an enrichment digest was carried out to linearise empty vector to prevent transformation of non-insert containing plasmids.
into the competent *E. coli*. However, this method required a digest site to be present in the empty vector that was not present in the ligated insert and vector.

### 2.1.8 TOPO cloning

TOPO cloning was used when large numbers of exons needed to be cloned and sequenced. This method allows the PCR amplicon to be cloned without a restriction digest, saving time when all that is required is cloned DNA for sequencing. Two types of TOPO cloning kits were used – blunt cloning (Zero Blunt® TOPO® PCR Cloning Kit with One Shot® TOP10 Chemically Competent *E. Coli*, Invitrogen K2800-20) and TA cloning (TOPO® TA Cloning® Kit for Sequencing with One Shot® TOP10 Chemically Competent *E. Coli*, Invitrogen K4575-01). These allow for cloning of either blunt-ended PCR products, as produced when *Pfx* polymerase was used, or products with an adenine to the 3’ end of the PCR product, as produced when Clontech Advantage 2 polymerase was used. For exons that were hard to amplify, both enzymes were tried, hence the need to use both types of kit. After the PCR products were run on a gel, the bands were excised and gel extraction carried out as previously described. 4.5μl of the eluted DNA was mixed with 0.5μl of TOPO vector and 1μl of salt solution (1.2 M NaCl, 0.06 M MgCl$_2$) and left at room temperature for 5 minutes. The mix was then transformed into chemically competent bacteria as described below.

### 2.2 Bacterial Methodology

#### 2.2.1 Preparation of competent *E. coli* cells

Ligations or mutagenesis products were used to transform competent *E. coli* cells. Briefly competent cells were produced by treatment with calcium chloride solution. Commercially available competent TOP10 cells (genotype: F– mcrA Δ(mrr-hscRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1araD139 Δ(ara leu) 7697 galU galK rpsL...
(StrR) endA1 nupG) were purchased (Invitrogen C404003). To produce our own stock of competent cells, these TOP10 cells were streaked onto agar plates without antibiotics. Single colonies were picked for inoculation into 3ml LB (Invitrogen 12780052) broth for overnight growth. The following morning, 3mls of culture were poured into flasks containing 200ml pre-warmed LB and grown until the culture reached an optical density of 0.95, measured using an Eppendorf biophotometer. At this point the culture was transferred to ice-cold 50ml falcon tubes and then centrifuged at 4000rpm at 4°C for 5 minutes. The supernatant was poured off and the pellets kept on ice. 10 ml of 80mM CaCl$_2$/50mM MgCl$_2$ solution was used to resuspend the pellets, which were pooled into one falcon tube. The cells were incubated on ice for 10 minutes before centrifuging again at 3000rpm at 4°C for 3 minutes. This was repeated twice, so that the cells were washed in the 80mM CaCl$_2$/50mM MgCl$_2$ solution a total of three times. After the final wash, the cells were resuspended in 5.5ml ice-cold 0.1M CaCl$_2$ solution. An equal volume of ice-cold 50% glycerol (Sigma G5150) was added. 550μl of the solution was pipetted into sterile Eppendorf tubes and rapidly frozen in liquid nitrogen. The stock of competent cells was stored at -80°C until needed.

2.2.2 Transformation of competent *E. coli* cells

To transform constructs into *E. coli*, 100μl of competent bacteria and 5μl of ligation were added to pre-chilled Eppendorf tubes and incubated on ice for 30 minutes. The bacteria were then heat-shocked at 42°C for 45 seconds, and placed immediately on ice for 2 minutes. 250μl of LB broth (preheated to 37°C) was added to each transformation. The bacteria were incubated at 37°C for 1 hour, with shaking in order for antibiotic resistance genes to be expressed from plasmid DNAs. The bacteria were then spread on LB agar plates containing ampicillin (Fisher BPE1760-5) or kanamycin (Invitrogen 11815024) using sterile technique and incubated at 37°C overnight. Ampicillin plates contained a final concentration of 100μg/ml of
antibiotic, from an ampicillin stock (100mg/ml). Kanamycin plates contained a final concentration of 50μg/ml of antibiotic, from a kanamycin stock (50mg/ml). The plates were checked the next morning for colonies, and stored at 4°C until miniprep DNAs were made. Ampicillin resistance is conferred from the β-lactamase gene which is able to break down the molecular structure of the β-lactam ring in ampicillin, rendering the antibiotic inactive. The neomycin phosphotransferase II gene is responsible for kanamycin resistance; the phosphotransferase inactivates kanamycin by phosphorylating it.

2.2.3 Small-scale plasmid DNA preparation

Colonies were picked for successful transformations and were grown overnight in 3ml LB broth (Invitrogen 12780-052) with ampicillin (100μg/ml). DNA minipreps were made to extract the plasmids using a Spin Miniprep kit (Qiagen 27106). The bacteria were first pelleted by centrifugation for 1 minute in a microcentrifuge at 13,000 rpm. Bacterial pellets were then resuspended in buffer P1 (50 mM Tris-Cl, pH 8.0, 10 mM EDTA, 100 μg/ml RNase A), and then the cells were lysed using buffer P2 (200 mM NaOH, 1% w/v SDS). In this alkaline lysis step, the NaOH and SDS act to denature plasmid and genomic DNA as well as cellular proteins. Buffer N3 (4M guanidine hydrochloride, 0.5M potassium acetate pH 4.2) was then used to neutralise the solution. Since plasmid DNA is small it can easily re-anneal to become double-stranded, the longer strands of genomic DNA are not able to re-anneal in this short time and remain precipitated. The tubes were then centrifuged for 10 minutes at 13,000 rpm. The supernatant containing the soluble plasmid DNA was then applied to a spin column and centrifuged for 1 minute at 13,000 rpm. The pellet containing proteins and the genomic DNA was discarded. The columns were washed with buffer PE and the DNA was then eluted into fresh tubes in 50μl of elution buffer EB. A Nanodrop 1000 spectrophotometer (ThermoScientific) was used to check the concentration of miniprep DNA.
2.2.4 Confirmation of successful cloning

Diagnostic restriction digests were usually used to check that the miniprep DNAs contained an insert. The digested DNA was run on an agarose gel, as described above, to confirm that an insert of the correct size was present. In some cases, colony screening via PCR was used to check for inserts. This method was employed when a particularly small insert was being cloned, especially if a low copy-number plasmid was used, making it difficult to see inserts on a gel. Colonies were picked from plates and dipped into a PCR tube containing a reaction mix of 0.5μl 50× Advantage 2 Polymerase Mix (Clontech 639201), 2.5μl 10× Advantage 2 PCR Buffer (Clontech 639137), 0.5μl 50× dNTP Mix (10 mM each, Clontech 639125), 1μl each of 10μM forward primer and reverse primer and 19.5μl H₂O. Primers used were either designed to bind within the insert, ensuring amplification was only possible if the insert was present, or bound to the plasmid at the start and end of the multiple cloning site. In the latter case, if the insert was present, the size of the expected band would be larger than the empty cloning site.

2.2.5 DNA sequencing

Plasmid minipreps were sent for sequencing by the DNA Sequencing & Services (MRCPPU, College of Life Sciences, University of Dundee, Scotland, www.dnaseq.co.uk) using Applied Biosystems Big-Dye Version 3.1 chemistry on an Applied Biosystems model 3730 automated capillary DNA sequencer. Based on the sequencing results, suitable samples were grown up in 200ml cultures for maxipreps.

2.2.6 Large-scale plasmid DNA preparation

Maxipreps allow for larger volumes of bacteria to be used, and give a higher yield of DNA. After sequencing had verified that a given miniprep contained the correct insert or mutation, 200ml of LB was inoculated with 200μl of the appropriate
antibiotic, and then with 200μl of the original miniprep culture. The flask was incubated overnight at 37°C with shaking at 300rpm. The following morning, DNA was extracted using a HiSpeed Plasmid Maxi Kit (Qiagen 12663). The broth was decanted into Falcon tubes and the cells pelleted by centrifugation at 4000rpm at 4°C for 15 minutes. The pellet was resuspended in 10ml of Buffer P1, and then 10ml of the lysis buffer P2 was added. The mix was inverted 4-6 times and incubated at room temperature for 5 minutes. Following incubation, 10ml of neutralisation buffer P3 (3.0 M potassium acetate, pH 5.5) was added and the tube inverted to mix. The lysate was then poured into a QiaFilter cartridge and incubated for 10 minutes. During the incubation, a HiSpeed MaxiTip was equilibrated with 10ml buffer QBT (750 mM NaCl; 50 mM MOPS, pH 7.0; 15% isopropanol (v/v); 0.15% Triton® X-100 (v/v)). After 10 minutes, a plunger was inserted into the filter cartridge and the lysate containing the plasmid DNA was pushed through into the equilibrated HiSpeed Maxi Tip. The lysate was left to clear and then 60ml of wash buffer QC (1.0 M NaCl, 50 mM MOPS, pH 7.0, 15% isopropanol (v/v)) was added. After washing, the HiSpeed Maxi Tip was placed over a clean Falcon tube and the DNA eluted with 15ml buffer QF (1.25 M NaCl, 50 mM Tris-Cl, pH 8.5, 15% isopropanol (v/v)). Next, 10.5ml of isopropanol was added to precipitate the DNA and this mix was incubated at room temperature for 5 minutes. During this incubation, a QIA precipitator maxi module was attached to a 30ml syringe. The DNA eluate was poured in the syringe and pushed through the QIA precipitator with a plunger. The precipitator module was then detached and the plunger removed. After reattaching the precipitator module, 2ml of 70% ethanol was added and pushed through with the plunger to wash the DNA. Air was then pressed through the module to prevent any ethanol carryover. The precipitator module was then attached to a 5ml syringe and 500μl buffer EB was pushed through the module and the solubilised DNA was collected in a 1.5ml Eppendorf tube. The eluted DNA was pushed through the precipitator module again to ensure all the plasmid DNA was collected.
2.3 Yeast methodology

2.3.1 The GAL4 yeast two-hybrid system

Yeast two-hybrid (YTH) experiments were used to test for interactions between proteins. A full-length or partial cDNA for the protein of interest was inserted into either a bait vector or prey vector. Two bait vectors, pYTH9 and pYTH16 were used in these experiments. Both encode the GAL4 DNA binding domain, but pYTH9 can be integrated into the yeast genome, whilst pYTH16 is an episomal plasmid. The prey vector pACT2 encodes the GAL4 DNA activation domain. When the bait and prey vectors (containing DNA for the proteins of interest) are transformed into yeast, proteins are produced with the GAL4BD (bait) and GAL4AD (prey) fused to the N-terminus. If the proteins interact, the binding and activation domains will be in close proximity and this activates transcription of several reporter genes in the yeast strain Y190 (Clontech, genotype: MATa, ura3–52, his3–200, ade2–101, lys2–801, trp1–901, leu2–3, gal4Δ, gal80Δ, cyh2, LYS2 : : GAL1::HIS3, MEL1,URA3 : : GAL1::HIS3, TATA::lacZ). The reporter genes used in this study were LacZ and histidine. The bait and prey vectors also contain nutritional selection markers, which allow successfully transformed yeast to grow on media lacking a given nutrient.
Figure 2.1 Diagrams of bait and prey vectors used in yeast two hybrid experiments

The size of each vector used is indicated and the genes encoded by the plasmid are shown. **GAL4BD** = Gal4 transcription factor DNA binding domain

**GAL4AD** = Gal4 transcription factor activation domain

MCS = Multiple Cloning Site (polylinker region containing multiple restriction sites)

**pADH** = ADH promoter (ensures high expression of the fusion protein in yeast)

**tADH** = ADH terminator

**TRP1** = catalyses third step in tryptophan biosynthesis

**LEU2** = catalyses third step in Leucine biosynthesis

**AMPR** = Ampicillin resistance gene

pYTH9 and pYTH16 contain the gene **TRP1**, which allows the yeast to grow on media which is lacking the amino acid tryptophan. pACT2 contains the gene **LEU2**, which allows transformed yeast to grow on media lacking the amino acid leucine. As the bait and prey vectors contain different nutritional selection markers, dropout media was used to determine whether both vectors were successfully transformed into the yeast. If both bait and prey were transformed and there was a protein-protein interaction, then the yeast were able to grow on media lacking tryptophan, leucine and histidine. The competitive inhibitor 3-amino-1,2,4-triazole (3-AT) was also added to the media, to block low levels of imidazoleglycerol-phosphate
dehydratase, an enzyme necessary for histidine production. By blocking this enzyme, the levels of non-transformed background yeast were low, since they were not able to survive on media lacking histidine. Another reporter gene, *LacZ*, was used in a colorimetric assay, which confirms the interaction and was also useful for giving an indication of the strength of the interaction.

### 2.3.2 Transformation of yeast

The yeast strain Y190 was restreaked on new plates of YPD agar (Clontech 630410) supplemented with adenine (Adenine hemisulphate salt, Sigma A-9126) a few days before transformation, so that the yeast were as fresh as possible. A single colony was picked and suspended in 100ml of fresh YPD (Clontech 630409) liquid medium supplemented with adenine (referred to as YPD) in a sterilised flask. The flask was covered with foil and incubated at 30°C, with shaking at 300rpm, overnight. Flasks containing 100-200ml of fresh YPD liquid medium were also prepared and incubated at 30°C overnight so they were pre-warmed in the morning, ready for use as over-day cultures. On the day of transformation, the optical density (OD) of the yeast liquid culture was measured at 600nm, using YPD liquid medium as a blank for the spectrophotometer (Eppendorf BioPhotometer). An appropriate volume of the overnight culture was added to the pre-warmed YPD, so that the new optical density was around 0.25. The cultures were incubated for 2-3 hours until the optical density (OD) measured between 0.4 and 0.6. When the OD was satisfactory, the over day cultures were transferred to 50 ml falcon tubes and centrifuged at 3000 rpm for 3 minutes at room temperature. The supernatant was discarded and the pelleted yeast were resuspended in 25ml dH²O and the pellets were pooled together into one falcon tube. The yeast were then centrifuged and washed twice with 1×LiAc/TE solution (1M Lithium Acetate, 1M Tris-EDTA, pH 7.5). After the second wash, the pellets were dissolved in 1ml/100ml culture of 1×LiAc/TE (i.e. for 150 ml of over-day culture, 1.5ml of 1×LiAc/TE was used). Aliquots of 100µl
were added to Eppendorf tubes containing 1µg bait DNA, 1µg prey DNA and 5µl carrier DNA (10mg/ml Herring sperm DNA, SIGMA D-7290). 600µl of 40% PEG 1×LiAc TE (for 40ml: 32ml of 50% w/v PEG, 1×LiAc (4ml of 10×LiAc), 1×TE (400µl of 100×TE) and 3.6 ml H₂O) was added to each tube, and the tubes were inverted to mix. The tubes were incubated at 30°C for 30 minutes, in a rotating incubator. The yeast were then heat-shocked at 42°C for 20 minutes. The tubes were then spun for 1 minute at 13,000rpm in a microcentrifuge and the supernatant was removed. The pellet was resuspended in 100µl dH₂O. 60µl was spread on labelled nutritional selection plates, made from minimal SD agar base (Clontech 630412), with a dropout supplement (Clontech 630419) so that the plates were lacking leucine, tryptophan and histidine (-Leu/-Trp/-His). 1M 3-Amino-1,2,4-triazole (Sigma A8056 solution) was added to give a final concentration of between 10mM and 30mM depending on the experiment. Only yeast transformed with plasmids harbouring interacting proteins should be able to grow on these plates. The remaining 40µl of yeast was spread on -Leu/-Trp transformation control plates (dropout supplement lacking leucine and tryptophan, Clontech 630417 and minimal SD agar base). Only yeast successfully transformed with both bait and prey plasmids should be able to grow on these plates. The plates were incubated at 30°C for 3 to 4 days until yeast colonies were visible. Transformation with full-length gephyrin were sometimes left for 5-6 days as expressing the full-length gephyrin appears to be slightly toxic to the yeast and slows growth. Sterile technique was used throughout to avoid any bacterial or mould contamination. For all YTH screens, both positive and negative controls were also utilised. Negative controls included empty bait and prey vectors - this also helped to confirm whether any possible ‘interactions’ could be the result of bait or prey auto-activating reporter genes.
2.3.3 The \textit{LacZ} freeze fracture assay

\textit{LacZ} freeze-fracture assays were carried out to check for \(\beta\)-galactosidase expression (Gietz et al 1997). If the bait and prey proteins interact the GAL4 transcription factor domains will brought into close proximity and a functioning transcription factor will bring about the transcription of the reporter gene.

![Diagram of the \textit{LacZ} freeze fracture assay](image)

1. Bait and Prey cDNA is cloned into the YTH vectors

2. Fusion proteins are produced with the GAL4 transcription factor domains

3. Interaction between bait and prey results in an active GAL4 transcription factor and transcription of reporter genes

\textbf{Figure 2.2 The activation of reporter genes in the yeast two-hybrid system}

The bait and prey vectors are transformed into yeast, the proteins of interest are produced with either the activation domain or binding domain of the GAL4 transcription factor. Interaction between the proteins brings these domains together and allows transcription of the reporter genes, \textit{His3} and \textit{LacZ}.

A mix of X-gal (5-Bromo-4-chloro-3-indoyl-\(\beta\)-D-galactopyranoside, Fisher BPE1615-1), dimethylformadine (DMF, SIGMA D-4551), Z buffer (60mM Na\(_2\)HPO\(_4\), 40mM NaH\(_2\)PO\(_4\), 10mM KCl and 0.1mM MgSO\(_4\),\(\cdot\)7H\(_2\)O) and \(\beta\)-mercaptoethanol (Sigma, M-6250) was prepared and 2 ml aliquots were placed in the lids of petri dishes in the fume hood. Whatman 54 filter papers were used to lift yeast from the
plates. The filters were dipped twice in liquid nitrogen and then placed into the X-gal mix. The filters were incubated at 30°C for a maximum of 4 hrs. X-gal is a substrate of β-galactosidase, an enzyme encoded by the *LacZ* reporter gene. If the bait and prey proteins interact, β-galactosidase is produced and converts the X-gal into galactose and 5-bromo-4-chloro-3-hydroxyindole, the indole is oxidised and the product gives a blue colour, the reaction is shown in Figure 2.3.

![Chemical reactions](attachment:image.png)

**Figure 2.3 The *LacZ* assay**

The reaction was stopped after an appropriate time point (1-4hrs) by removing the filters from the X-gal mixture and leaving them to air dry.

### 2.3.4 Custom cDNA library synthesis and screening

Two cDNA libraries were made, from rat spinal cord and liver RNA. These libraries were made as per the Clontech Make Your Own “Mate and Plate” Library System. Moloney Murine Leukaemia Virus (MMLV) Reverse Transcriptase was used to convert poly(A)+ RNA to cDNA. Rat spinal cord PolyA+ RNA and rat liver PolyA+ RNA were purchased from Clontech (636225 and 6710-1), the source was pooled.
RNA from spinal cords or liver of male and female Sprague-Dawley rats aged 8-12 weeks. The RNA was primed with both CDS III primer (an oligo dT primer) and CDSIII/6 (a random primer). The oligo dT primer will hybridize to the poly A 3’ end of the RNA, whilst the random primer can hybridize to many different sequences, by using both primers and combining the synthesized cDNA good coverage of the RNA should be achieved. Both primers contain the CDS sequence which is necessary for recombination with the pGADT7-Rec vector used for library screening. The cDNA synthesis is described in Figure 2.4.

**Figure 2.4 First strand cDNA synthesis from polyA+ RNA**

(A) 1μl of either oligo dT primer or random primer was incubated with 1μl poly A+ RNA (1μg) and 2μl water. The mixture was incubated at 72°C for 2 minutes.

(B) The mix was cooled on ice for 2 minutes. A reaction mix containing 2μl 5x first-strand buffer, 1μl DTT (100mM), 1μl dNTP mix (10mM) and 1μl SMART MMLV Reverse transcriptase (MMLV-RT) was added and mixed.

* Extra incubation step of 10 minutes at room temperature, only for mix with random primer.

(C) Mixture was incubated at 42°C for 10 minutes.

(D) 1μl of SMART III-modified oligo was added to the mix. When MMLV RT reaches a 5’ end on a template, inherent terminal transferase activity adds a few extra
deoxycytidine to the cDNA. The SMARTIII oligos contain an oligo(G) sequence that base pairs with the deoxycytidine stretch allowing it to be incorporated.

(E) Incubation at 42°C for 1 hour to allow MMLV RT to continue to the end of the template. The SMARTIII oligo contains a universal priming site which is used in long-distance PCR to amplify the cDNAs.

(F) Incubation at 72°C for 10 minutes to terminate first strand synthesis. Cool to room temperature and add 1μl RNase H. Final incubation at 37°C for 20 minutes to degrade RNA. The first strand cDNA can now be amplified using long-distance PCR.

For long-distance PCR 2μl of first strand cDNA was amplified using Clontech Advantage 2 Polymerase Mix in a reaction containing the following: 70μl H2O, 10μl 10X Advantage 2 PCR buffer, 2μl 50X dNTP Mix, 2μl 5' PCR primer, 2μl 3' PCR primer, 10μl 10X Melting Solution and 2μl 50X Advantage 2 Polymerase Mix. PCR conditions were as follows; the initial step was 30 seconds at 95°C. This was followed by 25 or 30 cycles of 10 seconds for denaturation at 95°C, then annealing and extension for 6 minutes at 68°C. The extension time was increased from 6 minutes by 5 seconds each cycle. The final step was 5 minutes at 68°C. An ethanol precipitation was used to concentrate the cDNA libraries before use in the YTH screen, as described in section 2.1.6.

2.3.5 Yeast two-hybrid library screening

YTH cDNA library screens were carried out using a similar protocol to above. Where possible, a yeast strain already containing the integrated bait - e.g. pYTH9-gephyrin - was used to increase transformation efficiency. As described in the yeast transformations above, a fresh colony of yeast was picked and grown overnight in liquid YPDA. An over-day culture was set up at OD 0.2, and monitored carefully over 2 to 3 hours until the OD reach 0.52 (the optimal OD for library transformations). Yeast was washed once with water and then resuspended in 3ml of 1×LiAc/TE. The yeast were split between two 1.5ml Eppendorf tubes, which were spun at 13,000rpm
in a microcentrifuge for 15 seconds. The pellets were finally resuspended in 600µl of 1×LiAc/TE. In new Eppendorf tubes, the cDNA library (20µl), linearised library vector (6µl of 0.5µg/µl pGADT7-Rec), bait DNA (if not using an integrated yeast strain, 5µg) and carrier DNA (20µl denatured herring testis DNA) were prepared. 600µl of the competent Y190 yeast was added. In a 15ml tube 2.5ml of 40% (w/v) PEG 1×LiAc/TE was prepared and the yeast/DNA mix was added. The tube was inverted to mix and then incubated at 30°C for 45 minutes; the cells were mixed every 15 minutes. 160µl of DMSO (99.5% (GC)) was added, and the tube was then incubated in a water bath at 42°C for 20 minutes. After the incubation, the yeast were pelleted at 3,000 rpm for 1 minute at room temperature. The pellet was resuspended in 3ml of YPDA and left to shake gently at 30°C for 90 minutes. The yeast were then centrifuged again at 3,000 rpm for 1 minute at room temperature and finally resuspended in 6ml of 0.9% (w/v) NaCl solution. 30µl of this solution was added to 720µl of 0.9% (w/v) NaCl solution. 150µl of the dilution was spread on to transformation control plates; two -Leu SD agar plates and two -Leu/-Trp SD agar plates. From the remaining 5.97 ml, 350µl per plate was spread onto 16 large nutritional selection -Leu/-Trp/-His+10mM 3-AT SD Agar plates. All plates were incubated at 30°C. Library sizes were estimated from the -Leu and -Leu/-Trp control plates. After four-five days, any colonies growing on the large library plates were picked and restreaked onto smaller nutritional selection plates. Once the colonies had grown on these plate filters were lifted, used to produce replica plates, and then used in the LacZ assay.

For colonies where positive interactions were confirmed by the LacZ assay, yeast minipreps were performed to extract the plasmid. The protocol for yeast minipreps is similar to that of bacterial minipreps, but involves pre-treatment of the yeast with lyticase (Sigma L2524, 10µl of 5U/µl stock solution made in EB) to help break down the cell walls. The cells are also vortexed with glass beads for 20 minutes at room
temperature, then the lysis buffers are added as in bacterial minipreps. The resultant DNA yield is too low to provide useful DNA sequences, but can be used to transform *E. coli*. The prey plasmids were then extracted from the transformed *E. coli* and the miniprep products sent for sequencing. The library cDNAs were in a pGADT7-Rec vector so a T7 primer was used for sequencing.

### 2.3.6 Quantitative yeast two-hybrid assay

Quantitative yeast two-hybrid assays allow a more precise measurement of the interaction strength using a liquid phase assay. The strength of the *LacZ* assay was measured using a plate reader (Thermoscientific Original Multiskan EX microplate photometer) to give OD readings at 540nm, which will vary depending on the amount of chlorophenol red-β-D-galactopyranoside (CPRG) substrate, which is a yellow colour, converted to chlorophenol red (dark red) and D-galactose. Yeast were transformed as per the standard protocol and allowed to grow for 3-4 days. Colonies were then picked from transformation control plates (-Leu/-Trp) and inoculated into 10ml of minimal SD media, with adenine and -Leu/-Trp dropout supplement. The cultures were incubated overnight at 30°C with shaking. The following day the OD of each sample was measured at 600nm. A standard of 0.035 was required, so the dilution necessary was calculated for each sample. The 10ml cultures were spun at 3,000rpm for 3 minutes to pellet the yeast and the pellet was resuspended in 1ml of buffer. The buffer contained 50ml Z Buffer, 135μl β-mercaptoethanol and 1 tablet of protease inhibitor (Roche 11873580001). The total volume for the assay was 1ml, so the appropriate amount of re-suspended yeast was added to clean Eppendorf tubes, and made up to 1ml with buffer. 12μl of 0.1% (w/v) SDS and 15μl of chloroform was then added to each tube to lyse the cells. The samples were vortexed briefly to mix and then incubated at 30°C, with shaking at 1000rpm for 15 minutes. After this 10μl of CPRG solution (10mg/ml) was added to each sample. The tubes were inverted to mix and 200μl was then pipetted into the well of a 96-
well plate. Each sample was run in triplicate. Once all samples were loaded, the plate was covered in foil and incubated at 30°C. After 10 minutes, the plate was read at both 540nm to measure the colour assay, and at 620nm to measure the density of yeast in each well. The plates were generally read every hour, and then incubated overnight, with further readings taken the next day until there was little change in the colorimetric assay. Data was imported into Microsoft Excel for analysis. The \( \text{OD}_{540}/\text{OD}_{620} \) was calculated for each well, to normalise the data based on the amount on yeast in each well. The average \( \text{OD}_{540}/\text{OD}_{620} \) was then calculated for each set of triplicates. A negative control was included in each experiment, testing the baits against the empty prey to check for any auto-activation. This value was subtracted from the calculated average for each experiment. A paired student’s t-test was then carried out on the data to test for significance.

2.4 Cell culture methodology

2.4.1 Maintenance of mammalian cell lines

Human Embryonic Kidney 293 cells (CRL-1573) were used for immunocytochemistry. Cells were maintained in Dulbecco’s Modified Eagle Medium (Dulbecco’s modified Eagle medium (DMEM) (1× liquid, high glucose with L-glutamine, D-glucose, sodium pyruvate, Invitrogen 41966052) supplemented with 1% (v/v) penicillin-streptomycin (Penicillin-Streptomycin Solution liquid, 5000 units/ml, Invitrogen 15070063) and 10% (v/v) Foetal Bovine Serum (Foetal Bovine Serum “GOLD” EU approved, PAA A15-151). DMEM referred to from this point forward was always supplemented in this way. Cells were grown in T75 culture flasks (Techno Plastic Products 90076) and split every 1-2 days depending on the rate of growth, cells were split at 50% to 80% confluence. A Zeiss inverted microscope (Zeiss 473012-9902) was used to monitor cell growth. Cell splitting was carried out in a class II laminar flow hood (Gelaire Flow Laboratories TC48), using
sterile technique. To split cells, fresh pre-warmed (37°C) medium was first added to a new flask. The media was then removed from the current cells and 10ml Hanks’ Buffered Salt Solution (1×HBSS liquid without calcium and magnesium, Invitrogen 14170138) was added to rinse the cells. The HBSS was removed and 1ml trypsin (Trypsin-EDTA solution 0.25% Invitrogen 25200056) added. Trypsin is required to cleave protein bonds that form between adherent cells and the flask. After 2-3 minutes of digestion, 10ml DMEM was added to the flask which inhibits trypsin. Finally, 1 or 2 ml of cell solution was transferred to the new flask and growth medium, which was then placed in the incubator at 37°C, 5% CO₂.

2.4.2 Cryopreservation of cells

For long-term storage, cells were kept at -150°C in a Sanyo MDF-1156ATN freezer. To create a stock, nearly confluent cells were rinsed and detached from the flask as normal. 9ml of HBSS was added to re-suspend the cells, which were then transferred to a 15ml falcon tube and spun at 1,000rpm for 2.5 minutes. The HBSS was removed and the cells re-suspended in 2ml of a freezing mixture consisting of 10% (v/v) DMSO, 40% (v/v) FBS and 50% (v/v) DMEM. This was then aliquoted into a 2ml cryovial, which was placed in a freezing device and stored at -80°C overnight. The next day the cryovials were transferred to -180°C for long-term storage. To revive frozen cells, 15ml of warm DMEM was added to a T75 flask. A cryovial of cells was taken from -180°C to the fume hood, was thawed gently by warming with hands. Once the vial was thawed, cells were added to the flask and swirled gently to mix. After 4 to 5 hours of incubation at 37°C, the cells were checked under the microscope, depending on the state of the cells the media was changed to remove dead cells and prevent toxins building up or cells were left to incubate overnight.

2.4.3 Cell transfection

Two methods were employed to transfect plasmid constructs into HEK293 cells for immunocytochemistry. Initially, lipofectamine (Invitrogen 15338100) was used. Cells
were seeded onto coverslips the day before transfection. Coverslips were pre-baked in an oven for 1.5-2 hours, placed in 6-well plastic plates (Nunc 140675) and pre-treated with 1× Poly-D-Lysine solution (PDL, Sigma P7886) to enable cells to attach to the coverslips more easily. The PDL was removed and the coverslips washed with water before being left to dry. Cells were washed and treated with trypsin as described above and resuspended in 10ml DMEM. The cells were transferred to a 15ml falcon tube and spun at 1,000rpm for 2.5 minutes. The cell pellet was resuspended in 1ml DMEM. A 1 in 10 dilution of the cell suspension was then prepared and 10μl of this suspension was placed on a haemocytometer. The number of cells present was calculated and used to calculate the appropriate volume of cell suspension needed to provide the 2×10⁵ cells required per transfection. The cell suspension was diluted in DMEM and 2ml was added to each coverslip. The cells were returned to the incubator overnight.

A total of 1μg of DNA was required per transfection. Labelled Eppendorf tubes were prepared and the appropriate volume of DNA was added, 100μl of Optimem medium (Invitrogen 31985047) was added followed by 2.5μl of Lipofectamine LTX (Invitrogen 15338100). The tubes were tapped to mix the contents and were left at room temperature in the fume hood for 30 minutes. DMEM was aspirated from the 6-well plate containing the seeded coverslips and 2ml pre-warmed Optimem was added to each well, followed by the DNA/lipofectamine/optimem mix. The plates were swirled gently to mix the contents and returned to the incubator for 4-6 hours. After this time, the Optimem was removed and replaced with DMEM. Cells were then returned to the incubator for 24-48 hours before fixing and staining.

Later experiments used electroporation as a transfection technique. Although this method results in high initial cell mortality, surviving cells express moderate levels of transfected proteins compared to lipofection techniques – which often result in
considerable overexpression. Therefore electroporation resulted in clearer and more biologically relevant results in co-localisation and clustering assays. Electroporation was carried out using a Bio-rad Gene Pulser II unit (Biorad 165-2106) with the Capacitance Extender PLUS module (Biorad 165-2108) and Pulse Controller PLUS (Biorad 165-2110). This method does not require cells to be seeded onto coverslips, so cells were grown in T75 flasks as described previously. Approximately $2 \times 10^6$ cells were used per transfection - in general one flask of cells was sufficient for 4-6 transfections. Baked coverslips were coated with PDL, washed with water and left to dry as before. Once the slips were dry, 2ml of DMEM was added to each well. Electroporation cuvettes (BioRad 165-2081 Gene Pulser Cuvettes, 0.4 cm gap) were sterilised in ethanol and washed three times with sterile water before use. 10-30μg of DNA was used per transfection. The appropriate volume of each plasmid construct was pipetted into the bottom of the cuvettes. The cells were then prepared by removing the DMEM, rinsing with HBSS and treating with trypsin. Cells were resuspended in DMEM, centrifuged and counted as described above, suspending the cells in Optimem rather than DMEM. Appropriate dilutions were made to allow for $2 \times 10^6$ cells in 500μl Optimem per transfection. 500μl of cells was added to each cuvette and tapped gently to mix with the plasmid DNA.

The Gene Pulser and Capacitance Extender PLUS were set up with the chamber connected to the output jack on the front of the main Gene Pulser II Unit). The Pulse Controller PLUS module was set to the infinity position. The capacitance was set to 0.125μF. The voltage was then set to 0.4kV. The cuvette was placed into the chamber and slid in until the cuvette made firm contact with the chamber electrodes. The pulse buttons were pressed simultaneously to electropulse the cells, and held just until the tone sounded. The time-constant reading was displayed and checked to be in the range of between 3 and 4. The cuvette was returned to the fume hood
and left to stand for 10 minutes. The cells were then added to 3ml DMEM and mixed. 500µl of this mix were then added to each well containing a cover slip. Cells were left 24-48h before fixation and immunostaining.

2.4.4 Immunocytochemistry

Ice-cold methanol was used to fix cells for immunocytochemistry, methanol acts by disrupting hydrophobic interactions and reducing the solubility of protein molecules. Structural preservation with this method was sufficient to examine co-localisation and clustering. To fix the cells, DMEM was aspirated from the wells, and 1ml of 1×PBS was added to rinse the cells (PBS consists of 1.06mM KH$_2$PO$_4$, 155mM NaCl, 2.97mM Na$_2$HPO$_4$, Invitrogen 18912014, 1 tablet dissolved in 500ml H$_2$O). The PBS wash step was repeated before adding 2ml of ice-cold methanol to each well. The plate was then placed at -20°C for 3 minutes. The methanol was then aspirated and the cells washed with 2ml PBS for 5 minutes. The wash-step was repeated three times. After removing the PBS, the cell membranes were permeabilised with 2ml 0.5% (v/v) Triton X-100 for 10 minutes (250µl Triton-X-100 (Sigma T-9284) in 50ml PBS). Two further washes in PBS were carried out before adding a blocking solution of 2ml of 2% (v/v) BSA (1g BSA, PAA K41-001 in 50ml PBS) to prevent non-specific antibody binding. After 30 minutes incubation in 2% (v/v) BSA in PBS the primary antibody was applied. The concentration of antibody varied; commonly an anti-myc antibody raised in mouse (Abcam ab32) and an anti-FLAG antibody raised in rabbit (Sigma F7425) were used, they both required a 1 in 200 dilution. 2% (v/v) BSA in PBS was used to dilute these antibodies. pEGFP constructs were also used. Since cloning of cDNAs into these vectors resulted in an in-frame fusion of EGFP, no antibody staining was required to detect these recombinant fusion proteins. 100µl of diluted antibody per coverslip was aliquoted onto parafilm and the coverslips were placed cell side down into the solution. The
coverslips were protected from light during 2 hour incubation. Following this, the cover slips were returned to the 6 well plate, cell side up. The cover slips were washed in 2% (v/v) BSA for 5 minutes, this was repeated 3 times.

Alexa Fluor secondary antibodies (Invitrogen) were used appropriate to the host for generation of the primary antibody, e.g. an anti-mouse secondary was used for monoclonal antibodies raised in mice. All secondary antibodies were used at a 1 in 600μl dilution, again in 2% (v/v) BSA in PBS. 100μl of this dilution was aliquoted on to parafilm and the cover-slips were placed cell side down into the solution. For the primary incubation, the coverslips were protected from light. After 1 hour, the coverslips were returned to the 6 well plate and washed three times in PBS. To mount the coverslips, 8μl of melted Kaisers glycerol gelatine (Merck 109242) was pipette onto a clean, labelled slide (VWR Adhesion slides 631-0108). The coverslips were dried by lightly resting on tissue and then placed face down into the jelly and left to set overnight at room temperature. Slides were viewed using a Zeiss LSM710 confocal microscope. The fluorophores used in my experiments were EGFP, AlexaFluor 546 and 633. The excitation and emission wavelengths for these fluorophores are listed in Table 2.4 along with the laser used for imaging.

<table>
<thead>
<tr>
<th>Fluorophore</th>
<th>Excitation wavelength (nm)</th>
<th>Emission wavelength (nm)</th>
<th>Laser – wavelength (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFP</td>
<td>488</td>
<td>507</td>
<td>Argon Laser – 488</td>
</tr>
<tr>
<td>Alexa Fluor 546</td>
<td>556</td>
<td>573</td>
<td>DPSS Laser – 561</td>
</tr>
<tr>
<td>Alexa Fluor 633</td>
<td>632</td>
<td>647</td>
<td>HeNe Laser – 633</td>
</tr>
</tbody>
</table>

Table 2.4 Excitation and emission wavelengths of fluorophores used.
2.5 Bioinformatics

2.5.1 Analysis of DNA sequencing data

Sequencing data was downloaded from University of Dundee Sequencing Service website, www.dnaseq.co.uk, and analysed using the programme Sequencher, version 5.0 (www.genecodes.com). Reference sequences were downloaded from NCBI (http://www.ncbi.nlm.nih.gov/) and imported into Sequencher to use as templates for alignment and to check for desired or unwanted mutations. Sequences were also checked to ensure they were in-frame with the vector to ensure correct expression of the protein with any relevant tag or fusion protein.

2.5.2 Analysis of protein sequences

Several websites were used to find information on proteins based on their amino acid sequence. For example, http://pfam.sanger.ac.uk/ was used to search for domains within a protein, and gain further information about the function of each domain, and the protein family. It was also useful to be able to compare proteins across species to check for conservation of amino acids. Clustalw2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) was used for both DNA alignments and protein alignments.

2.5.3 Ensembl genome browser

The Ensembl genome browser (www.ensembl.org) was particularly useful for identifying coding and non-coding transcripts from a given gene. The browser also allows easy identification of orthologues, members of the same protein family.

2.5.4 UCSC genome browser

The UCSC genome browser (http://genome.ucsc.edu/) was also used to analyse hits from yeast-two hybrid library screening. Once the sequence of a potential gephyrin interactor had been analysed in Sequencher, the insert sequence was
used in a DNA BLAT search against the USCS rat genome to identify the encoded
gene/transcript. The UCSC genome browser also provides a useful display of the
location of genes on a chromosome, allowing easy identification of neighbouring
genres, which was useful for deletion mapping.

2.5.5 Mutation analysis

Several programs were used to analyse the impact of mutations in genes of interest.
For non synonymous single nucleotide polymorphisms (SNPs), the mutation results
in an amino acid change. These programs give a prediction of the impact of this
change, and whether it is likely to be pathogenic. The prediction is made by
comparing the characteristics of the wild type amino acid and the amino acid it is
changed as a result of the SNP. A mutation may have little impact if the residues
properties are similar to that of the wild type residue, for example if they are both
basic residues. The programs used were SIFT and Polyphen-2. SIFT analysis
(http://sift.jcvi.org/) gives a score from 0 to 1. The amino acid change is predicted to
be damaging if the score is less than 0.05, and tolerated if the score is greater than
0.05. Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/) classifies mutations into 3
categories; benign, possibly damaging or probably damaging. Mutation Taster
(http://www.mutationtaster.org/index.html) was also used, as this program is able to
predict the impact of insertions and deletions not just SNPs. The program predicts
whether the mutations is just a polymorphism or if it is disease causing.
RESULTS

3 SEARCHING FOR NOVEL GEPHYRIN INTERACTORS USING THE YEAST TWO-HYBRID SYSTEM

3.1 Background

The yeast two-hybrid (YTH) system is a useful tool for examining protein-protein interactions in a simple eukaryotic system. This method was first described over 20 years ago, and allows numerous specific protein-protein interactions to be studied in a single experiment (Fields and Song 1989). As described in the methods section 2.3.1, this system makes use of transcription activators, and relies on the fact that both the DNA binding domain (BD) and the activation domain (AD) of a transcription factor are necessary for RNA synthesis to be initiated. In several transcription factors, these domains are functionally independent and therefore can be encoded by separate plasmids, which when co-transformed into yeast will produce the separate domains. If these domains are brought together, the transcription factor will be active. The yeast two-hybrid system employed in my experiments uses the GAL4 transcription factor, a native yeast transcription factor that is active when yeast are grown on medium containing galactose. By using a yeast strain in which the Gal4 gene is deleted, then only once the strain is transformed with plasmids encoding the GAL4 domains is the transcription factor produced. Reporter genes in the host system can be engineered to be under the control of GAL4-responsive promoters. By fusing the BD and AD domains to proteins of interest, interactions can be detected. If there is a protein interaction, the transcription factor BD and AD domains are brought together and reporter genes are transcribed. This provides a simple method for identifying or testing potential protein-protein interactions. However, whilst the yeast two-hybrid system has many advantages there are also some experimental limitations that should be taken into account (Van Criekinge and Beyaert 1999). For example, it is important to remember that protein-protein
interactions will take place in the yeast nucleus – an unnatural environment for many proteins. Potential advantages and disadvantages of the YTH system are summarised in Table 3.1.

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
<th>How drawbacks were managed in library screening</th>
</tr>
</thead>
<tbody>
<tr>
<td>It is a eukaryotic <em>in vivo</em> system, making it closer to higher eukaryotic cells than bacterial or <em>in vitro</em> techniques</td>
<td>Bait or prey protein may be able to activate transcription - leading to auto-activation of reporter genes</td>
<td>The pYTH9 gephrin P1 and pYTH16 gephrin C3 bait were tested to ensure that neither is able to auto-activate. Empty prey vectors were included in screens as negative controls</td>
</tr>
<tr>
<td>Only cDNA is required for screening, no purified proteins or antibodies are needed</td>
<td>Proteins of interest must be able to fold correctly in yeast - fusion of the GAL4 AD or BD to proteins of interest could affect correct folding or conformation</td>
<td>Use of positive control preys, an interaction between collybistin and GlyR β subunit preys with the GAL4BD-gephyrin bait was confirmed</td>
</tr>
<tr>
<td>Weak interactions can be detected more easily, since the ADH1 promoter ensures high expression of all fusion proteins in yeast. Reporter genes amplify weak interactions allowing detection</td>
<td>Protein of interest may be toxic in yeast</td>
<td>Expression of full-length gephrin does slow down yeast growth, but not significantly enough to preclude screening</td>
</tr>
<tr>
<td>Libraries of cDNA can be tested in one experiment – high throughput, and high output</td>
<td>A third protein could be acting as a bridge between two non-interaction proteins</td>
<td>This is a problem with many biochemical techniques. However, it is unlikely that a specific yeast protein is able to interact with both fusion proteins in the nucleus</td>
</tr>
<tr>
<td>Known interactions can be analysed in detail, e.g. mutagenesis can identify specific residues needed for protein-protein interactions</td>
<td>Post-translational modifications such as glycosylation, phosphorylation and disulphide bridges may be different in yeast</td>
<td>This is also a problem in bacterial systems. As a eukaryotic system yeast should be closer to mammalian cells. Phosphorylation is the most common problem, but potential phosphorylation sites can be mutated to test if this is an issue</td>
</tr>
</tbody>
</table>
YTH screening is an inexpensive and rapid way to search for novel interactors. Proteins that are normally in different cellular compartments may be shown to interact in yeast, but in vivo would never come into contact. Further in vitro and in vivo assays for plausible interactors should reveal whether novel gephyrin interactors are functionally relevant.

Table 3.1. Advantages and drawbacks of the yeast two-hybrid system and how these issues were addressed in this study.

In my library screens, two bait vectors were used - pYTH9 and pYTH16 (Fuller et al 1998). Both encode the GAL4 DNA binding domain, but pYTH9 can be integrated into the yeast genome, whilst pYTH16 is an episomal plasmid. The prey vector pGADT7-Rec, which contains the GAL4 activation domain, was used in cDNA library construction. The key genes encoded by these vectors can be seen in Figure 3.1. The genotype of the host yeast strain used (Y190: MATa, ura3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-3, 112, gal4Δ, gal80Δ, cyh2, LYS2 : : GAL1 UAS - HIS3, TATA - HIS3, MEL1, URA3 : : GAL1 UAS - GAL1 TATA - lacZ) allows the use of auxotrophic markers to check whether the vectors have been successfully transformed into the yeast strain. Y190 is unable to synthesis tryptophan (due to the trp1-901 mutation) or leucine (due to the leu2-3,112 mutation). Therefore yeast is unable to grow unless the media contains these nutritional supplements. As shown in Figure 3.1 the bait and prey vectors I used contain functioning LEU2 and TRP1 genes allowing successfully transformed yeast to grown on media lacking leucine and tryptophan. This demonstrates that the yeast is transformed with both vectors.
Figure 3.1. Diagrams of bait and prey vectors used in yeast two-hybrid library screen experiments.

The size of each vector used is indicated and the genes encoded by the plasmid are shown.

GAL4BD = GAL4 transcription factor DNA binding domain.

GAL4AD= GAL4 transcription factor activation domain.

MCS = Multiple Cloning Site (polylinker region containing multiple restriction enzyme sites).

pADH = ADH promoter (ensures high expression of the fusion protein in yeast).

tADH = ADH terminator.

TRP1 = catalyses third step in tryptophan biosynthesis.

LEU2 = catalyses third step in leucine biosynthesis.

AMP^R = Ampicillin resistance gene.

To ascertain if the proteins being tested interact, a third auxotrophic marker is used - histidine. The histidine gene in Y190 is under the control of a GAL4 responsive promoter, so histidine is only produced when the GAL4 transcription factor is active. This can be tested for by growing the transformed yeast on media that lacks histidine, as well as lacking tryptophan and leucine. The LacZ gene is also under the control of a GAL4 responsive promoter, which allows for a colorimetric assay to confirm protein interaction. This assay is described in detail in the methods section 2.3.3.
As well as testing known interactions, the YTH system also allows identification of novel protein-protein interactions via library screening. In this variation, a library of fusion proteins corresponding to different cDNAs from a tissue of interest is tested against a specific bait. Several gephyrin interactors, including the GEF collybistin, have previously been identified in yeast two-hybrid screens or *in vitro* pull-down assays (Table 3.2). Whilst some of these interactors are specifically located at inhibitory synapses, others have more general roles in trafficking or post-translational modifications, such as ubiquitination or phosphorylation. In some cases, the relevance of the gephyrin interaction is unclear. However, the appearance of any of these previously confirmed interactors in the library screen would suggest that the yeast two-hybrid library screening approach was producing valid results. Table 3.2 summarises the gephyrin interactors known to date.
<table>
<thead>
<tr>
<th>Gephyrin interactor</th>
<th>Known functions of interactor</th>
<th>Site of interaction on gephyrin</th>
<th>Biological relevance of gephyrin interaction</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlyR β subunit</td>
<td>Inhibitory synaptic receptor subunit</td>
<td>E domain</td>
<td>Glycine receptors are anchored at the membrane via the gephyrin GlyR β subunit interaction</td>
<td>(Meyer et al 1995; Kneussel et al 1999a; Kim et al 2006)</td>
</tr>
<tr>
<td>GABA&lt;div align=center&gt;α&lt;/div&gt;&lt;div align=center&gt;1&lt;/div&gt; subunit</td>
<td>Inhibitory synaptic receptor subunit</td>
<td>C/E domain</td>
<td>Clustering of GABA&lt;div align=center&gt;α&lt;/div&gt;Rs at membrane – phosphorylation dependent interaction</td>
<td>(Mukherjee et al 2011)</td>
</tr>
<tr>
<td>GABA&lt;div align=center&gt;α&lt;/div&gt;&lt;div align=center&gt;2&lt;/div&gt; subunit</td>
<td>Inhibitory synaptic receptor subunit</td>
<td>E domain</td>
<td>Clustering of GABA&lt;div align=center&gt;α&lt;/div&gt;Rs at membrane</td>
<td>(Tretter et al 2008; Saiepour et al 2010)</td>
</tr>
<tr>
<td>GABA&lt;div align=center&gt;α&lt;/div&gt;&lt;div align=center&gt;3&lt;/div&gt; subunit</td>
<td>Inhibitory synaptic receptor subunit</td>
<td>E domain</td>
<td>Clustering of GABA&lt;div align=center&gt;α&lt;/div&gt;Rs at membrane</td>
<td>(Kins et al 2000; Grosskreutz et al 2001; Kerschner et al 2009; Saiepour et al 2010; Tretter et al 2011)</td>
</tr>
<tr>
<td>Collybistin/hPEM2</td>
<td>RhoGEF for Cdc42</td>
<td>C/E domain</td>
<td>Collybistin regulates the localisation of submembrane gephyrin clusters</td>
<td>(Kins et al 2000; Grosskreutz et al 2001)</td>
</tr>
<tr>
<td>Neuroligin 2</td>
<td>Formation of inhibitory synapses, ligand of presynaptic neurexins</td>
<td>E domain</td>
<td>Neuroligin 2 acts as an activator for collybistin, driving formation of the postsynaptic scaffold</td>
<td>(Hoon et al 2009; Poulopoulos et al 2009)</td>
</tr>
<tr>
<td>GABARAP</td>
<td>GABA receptor associated protein</td>
<td>C domain</td>
<td>GABARAP interacts with the GABA&lt;div align=center&gt;α&lt;/div&gt; receptor γ subunits and gephyrin but is involved in receptor trafficking and is not essential for GABA&lt;div align=center&gt;α&lt;/div&gt;R clustering</td>
<td>(Kneussel et al 2000)</td>
</tr>
<tr>
<td>Hsc70</td>
<td>ATPase activity</td>
<td>G domain</td>
<td>Regulation of gephyrin clustering</td>
<td>(Machado et al 2011)</td>
</tr>
<tr>
<td>Mena/VASP</td>
<td>Involved in actin filament formation and bundling.</td>
<td>E domain</td>
<td>Mena/VASP (mammalian enabled / vasodilator stimulated phosphoprotein) with gephyrin might mediate or regulate gephyrin-microfilament interactions</td>
<td>(Giesemann et al 2003)</td>
</tr>
</tbody>
</table>
### Table 3.2 Review of known gephyrin interactors and their biological relevance.

The proteins described above are generally accepted as gephyrin interactors. Many of these proteins are involved in the clustering of GABA$_A$Rs or GlyRs at inhibitory synapses, whilst some play more general roles in the transport and modification of gephyrin within the cell. However, none of the proteins identified to date have a clear role in molybdenum cofactor (MoCo) synthesis.

<table>
<thead>
<tr>
<th>Protein (Interactor)</th>
<th>Domain/Description</th>
<th>Biological Relevance</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pin1</td>
<td>Peptidyl-prolyl isomerase NIMA interacting protein 1</td>
<td>The phospho-dependent gephyrin-Pin1 interaction enhances gephyrin-GlyR β subunit binding by inducing conformational changes</td>
<td>(Zita et al 2007)</td>
</tr>
<tr>
<td>Profilin 1 and profilin 2</td>
<td>Profilins regulate actin polymerization in a PIP$_2$-dependent manner</td>
<td>Profilin-gephyrin interactions may regulate gephyrin-microfilament interactions</td>
<td>(Giesemann et al 2003)</td>
</tr>
<tr>
<td>Protein kinase(s)</td>
<td>Phosphorylation of proteins</td>
<td>Phosphorylation of gephyrin</td>
<td>(Specht et al 2011; Tyagarajan et al 2011b)</td>
</tr>
<tr>
<td>RAFT1</td>
<td>Rapamycin and FKBP12 target (FRAP/mTOR). Affects protein translation through interactions with the S6 kinase.</td>
<td>RAFT1 mutants that no longer interact with gephyrin do not signal the molecules for protein translation, suggesting that gephyrin might act as a hub for signal transduction pathways involving translational control at synapses</td>
<td>(Sabatini et al 1999)</td>
</tr>
<tr>
<td>Dynein light chains 1 and 2</td>
<td>Cargo binding motor proteins</td>
<td>DLC1 and 2 are likely to be involved in the retrograde transport of gephyrin</td>
<td>(Fuhrmann et al 2002)</td>
</tr>
<tr>
<td>Tubulin</td>
<td>Structural component of the cytoskeleton</td>
<td>High affinity interaction in vitro – gephyrin anchoring to cytoskeleton</td>
<td>(Kirsch et al 1991)</td>
</tr>
<tr>
<td>KIF5</td>
<td>Kinesin family motor protein 5</td>
<td>Synaptic transmission may regulating microtubule-dependent cargo delivery</td>
<td>(Maas et al 2009)</td>
</tr>
</tbody>
</table>
3.2 **Study Aims**

Yeast two-hybrid library screening was utilised to search for novel gephyrin interactors, with a specific emphasis on identifying additional GEFs that may be involved in gephyrin clustering at synapses, or proteins that link to the peripheral role of gephyrin in MoCo synthesis.

- Construct a cDNA library from rat spinal cord poly(A)+ RNA in pGADT7-Rec and screen this library using a Y190 yeast strain containing an integrated pYTH9 gephyrin P1 bait.

- Construct a cDNA library from rat liver poly(A)+ RNA in pGADT7-Rec and screen this library by co-transformation of Y190 with a pYTH16 gephyrin C3 bait.

- Recover pGADT7-Rec library plasmids from yeast growing on selective media and identify the fusion proteins produced by DNA sequencing.

- Re-testing of potential bait and prey interactions to eliminate artifactual interactors, e.g. transcription factors.

- Conduct bioinformatics analysis of selected interacting proteins of interest and triage interactors to select candidates for further investigation.

3.3 **cDNA library construction for yeast two-hybrid screening**

The methodology for cDNA library construction is given in section 2.3.4 and summarised in Figure 3.2. The pGADT7-Rec vector, containing the SMARTIII and CDSIII sequences is shown in Figure 3.2A. Both random-primed and oligo(dT)-primed cDNA libraries were amplified, purified and 5 µl of the final product was run.
on a 1% (w/v) agarose gel (Figure 3.2B). Since the individual cDNAs vary in size, the amplified library is seen as a smear of products that varies in size from ~250 bp to well above 13 kb. Each individual cDNA will have the SMART III sequence at one end and the CDS III sequence at the other, which allows recombination with the corresponding sites in pGADT7-Rec (Figure 3.2C), when the library and linearised vector are co-transformed into Y190.

**Figure 3.2. Synthesis of cDNA libraries.**
(A) The pGADT7-Rec vector contains SMART III and CDS III sequences, and is linearised by restriction digest with SmaI. (B) Agarose gel showing amplified cDNA libraries. Lanes 1 and 2 show spinal cord libraries amplified with 25 and 30 cycles in LD-PCR respectively. Lanes 3 and 4 show liver library amplified with 25 and 30 cycles in LD-PCR respectively. A smear of various molecular weights is visible in each lane showing that the resulting cDNAs had a large size range. (C) cDNAs contain SMART III and CDS III sequences at each end, since these sequences are found in the oligonucleotides used for library amplification. This allows directional homologous recombination in yeast co-transformed with the cDNA library and linearised pGADT7-Rec vector.
3.4 Known and novel gephyrin interactors revealed by yeast two-hybrid screening of a spinal cord cDNA library

Y190 harbouring an integrated pYTH9 rat gephyrin P1 bait was used to screen a rat spinal cord cDNA library in the vector pGADT7-Rec. The library size was estimated at 1.88×10^6 co-transformed yeast, based on a fraction of the library plated on -leu-trp selective medium. The main library was plated on -leu-trp-his selective medium (132 mm² plates) containing 10 mM 3-AT, since pilot experiments with a gephyrin bait and a GlyR β subunit prey indicated this was a suitable concentration to limit background growth without preventing interactions from being seen. Due to the large number of yeast colonies observed after screening, yeast colonies from four library plates (out of sixteen) were restreaked on fresh 82mm² nutritional selection plates. Once the yeast had regrown, interactions were confirmed using a second independent assay for LacZ activity. In the vast majority of cases, the filters turned blue indicating a potential interaction, so yeast minipreps were made in order to determine the identity of the fusion protein. The plasmid DNA yield from yeast minipreps was generally too low for sequencing (1-5 ng), but was sufficient to transform competent E. coli. After transformation, bacterial cultures were grown and miniprep DNAs were isolated. These DNAs were digested with EcoRI and Xhol, which digest pGADT7-Rec at sites located just upstream and downstream of the SMART III and CDS III primer sites, as shown in Figure 3.3A. The digested minipreps were run on agarose gel and a large variety of insert sizes were observed, as shown in Figure 3.3B.
Figure 3.3. Yeast two-hybrid screening hits after restriction digestion.

(A) The cloning site of pGADT7-Rec. EcoRI and XhoI sites are located either side of the SMART III and CDS III sequences, allowing inserts to be cut out. (B) Sample gel of minipreps digested with EcoRI and XhoI. A variety of sizes can be seen, indicating a variety of cDNAs successfully recombined into pGADT7-Rec.
Minipreps were sequenced using the T7 primer and the raw trace data was used in a BLAT search against the UCSC genome browser (http://genome.ucsc.edu/) using the rat Nov. 2004 (Baylor 3.4/rattus norvegicus 4) assembly. DNA sequencing revealed that most of the yeast colonies were carrying plasmids harbouring cDNAs for proteins that were in-frame and genuine interactors. Multiple clones corresponded to the known gephyrin interactors dynein light chains 1 and 2 (Fuhrmann et al 2002), presumably because the coding region is short (270 bp for DLC1 and DLC2). PCR screening was therefore used to eliminate these dynein ‘hits’ to obviate the need for repetitive sequencing of dynein light chain clones, as shown in Figure 3.4.

Figure 3.4. Flowchart showing the steps involved in PCR screening to eliminate cDNA clones containing dynein light chain 1 and 2 cDNAs.
The Dyn12A and Dyn12B primers were designed to be capable of amplifying cDNAs for both dynein light chains 1 and 2, resulting in a PCR product of 262bp. From the gel image in Figure 3.5B, it can be seen that ~40% of the plasmids contained cDNA for dynein light chain 1 or 2 (DLC1 or DLC2). cDNA identified as a dynein light chains 1 or 2 are indicated with a white circle. These plasmids were not re-transformed into *E. coli* and were not sequenced. However, this screening allowed me to enrich for non-dynein gephyrin interactors.

![Figure 3.5](image)

**Figure 3.5** PCR screening of yeast two-hybrid plasmid DNAs to identify cDNAs for dynein light chain 1 and 2.

(A) Coding sequence of DLC1 and DLC2, showing the site and sequence of the PCR primers. (B) Agarose gel of yeast two-hybrid interactors that underwent PCR screening with the dynein light chain 1/2 primers. White dots indicate the clones identified as DLC1/DLC2, a clear band at 262 bp.

A selection of the proteins identified as gephyrin interactions from the spinal cord library screen is shown in table 3.3. These include the proteins that appeared most frequently as well as those considered to be of significant interest due to their function or the apparent strength of the interaction. After sequencing of 131 interactors, it became apparent that the sequencing was beginning to show...
diminishing returns. At this point, since three novel GEFs had been identified, it was decided that the best use of time would be to further investigate these GEFs. A further 300 colonies were picked and stored at -80°C should sequencing of more interactors be required at a later date.

3.5 Known and novel gephyrin interactors revealed by yeast two-hybrid screening of a liver cDNA library

The liver screen was carried out using a full-length gephyrin C3 isoform cDNA cloned into PYTH16, as bait. Yeast strain Y190 was co-transformed with PYTH16-gephyrin C3 and the pGADT7-Rec liver cDNA library. The library size was estimated at 2.58 × 10^5 co-transformed yeast, based on a fraction of the library plated on -leu-trp plates. This highlights the difference between using an integrated bait strain versus a co-transformation for yeast cDNA library screening, since the transformation efficiency was 7-fold less than that observed in the spinal cord screen. After four days of growth there were approximately 40 colonies on the 16 large library plates. As for the spinal cord screen, the colonies were transferred to new -leu-trp-his+3-AT plates and LacZ assays were carried out. Only 24 colonies gave positive LacZ results and were taken through to sequencing. Several proteins appeared several times in the screen – DLC2 was the most frequent interactor, although curiously DLC1 did not appear in this screen. There were multiple hits for Kininogen1, serine protease inhibitor member 3k and alpha-2u globulin PGCL4. Kininogen 1 (also known as bradykinin) contains cystatin domains, which are involved in the inhibition of cysteine proteinases. Alpha-2u globulin PGCL1 contains lipocalin domains, which bind cytosolic fatty acids. A full list of the interactors is shown in Table 3.4. However, since these proteins have no obvious relevance to the peripheral role of gephyrin in MoCo synthesis, none of these proteins were selected as candidates for further work.
<table>
<thead>
<tr>
<th>Identity of interactor</th>
<th>NCBI Identifier</th>
<th>Clones</th>
<th>Protein information</th>
<th>LacZ result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dynein light chain LC8-type 2 (Dynll2)</td>
<td>NM_080697</td>
<td>22*</td>
<td>Component of the dynein multi-protein complex, functions as cargo adaptor, many known (guanine-nucleotide-exchange-factor activity).</td>
<td>+++</td>
</tr>
<tr>
<td>IQ motif and Sec7 domain 2 (IQSEC2)</td>
<td>XM_002727537</td>
<td>16</td>
<td>IQ = Calmodulin-binding motif, Sec7 domain (guanine-nucleotide-exchange-factor activity).</td>
<td>+++</td>
</tr>
<tr>
<td>Dynein light chain LC8-type 1 (Dynll1)</td>
<td>NM_053319</td>
<td>14*</td>
<td>Component of the dynein multi-protein complex</td>
<td>+++</td>
</tr>
<tr>
<td>Ankyrin 3, epithelial (Ank3)</td>
<td>NM_031805</td>
<td>5</td>
<td>Ankyrin repeat, ZU5 domain, Death domain</td>
<td>+</td>
</tr>
<tr>
<td>Myelin basic protein (Mbp)</td>
<td>NM_001025291</td>
<td>2</td>
<td>Myelin basic protein</td>
<td>+</td>
</tr>
<tr>
<td>IQ motif and Sec7 domain 3 (IQSEC3)</td>
<td>NM_207617</td>
<td>2</td>
<td>IQ = Calmodulin-binding motif, Sec7 domain (guanine-nucleotide-exchange-factor activity).</td>
<td>+++</td>
</tr>
<tr>
<td>RAS guanyl releasing protein 2 (RasGRP2)</td>
<td>NM_001082977</td>
<td>1</td>
<td>Guanine nucleotide exchange factor for Ras-like GTPases; RasGEF domain, EF hand, Phorbolesters/C1domain - (calcium and DAG-regulated)</td>
<td>+</td>
</tr>
<tr>
<td>Cell division cycle 42 (Cdc42)</td>
<td>NM_171994</td>
<td>1</td>
<td>GTP binding protein</td>
<td>+</td>
</tr>
<tr>
<td>Ubiquitin-conjugating enzyme E2I</td>
<td>NM_013050</td>
<td>1</td>
<td>SUMO-conjugating enzyme UBC9</td>
<td>+++</td>
</tr>
<tr>
<td>Coactosin-like 1 (Cotl1)</td>
<td>NM_001108452</td>
<td>1</td>
<td>Cofilin/tropomyosin-type actin-binding protein. Severs actin filaments and binds to actin monomer</td>
<td>+</td>
</tr>
<tr>
<td>Heat shock 70kDa protein 12A (Hspa12a)</td>
<td>NM_001107445</td>
<td>1</td>
<td>Heat shock proteins act as chaperones in the cell to assist with protein folding</td>
<td>+</td>
</tr>
<tr>
<td>Heat shock protein 2 (Hspa2)</td>
<td>NM_021863</td>
<td>1</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Cell cycle associated protein 1 (Caprin1)</td>
<td>NM_001012185</td>
<td>1</td>
<td>Role in cell proliferation</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 3.3 A selection of potential gephyrin interactors identified from YTH library screening of gephyrin P1 isoform against a spinal cord cDNA library.

*A further 21 hits were identified as dynein light chain LC8 type 1 or 2 by PCR screening. An indication of the strength of colour that developed in the LacZ assay is given, whilst not a precise measure this gives an idea of the strength of the interaction. ++++, strong interaction, ++ medium interaction, + weak interaction.*
<table>
<thead>
<tr>
<th>Identity of interactor/NCBI identifier</th>
<th>Clones</th>
<th>Protein information</th>
<th>LacZ result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dynein light chain LC8-type 2</td>
<td>NM_080697</td>
<td>5 Component of the dynein multi-protein complex, functions as cargo adaptor, many known interactors imply additional functions.</td>
<td>++</td>
</tr>
<tr>
<td>Kininogen 1 (Kng1)</td>
<td>NM_012696</td>
<td>3 Kinin precursor</td>
<td>++</td>
</tr>
<tr>
<td>Serine protease inhibitor, clade A, member 3K (Serpina3k)</td>
<td>NM_012657</td>
<td>2 Secreted acidic glycoprotein that may act as a serine protease inhibitor</td>
<td>++</td>
</tr>
<tr>
<td>alpha-2u globulin PGCL4 (Obp3)</td>
<td>XM_003749949</td>
<td>2 Odorant binding protein, contains lipocalin domains</td>
<td>++</td>
</tr>
<tr>
<td>GTP binding protein 5 (Gtpbp5)</td>
<td>NM_001013924</td>
<td>1</td>
<td>++</td>
</tr>
<tr>
<td>Selenoprotein P Precursor 1 (Sepp1)</td>
<td>NM_019192</td>
<td>1 Extracellular antioxidant/selenium transport</td>
<td>+++</td>
</tr>
<tr>
<td>Retinol binding protein 4 (Rbp4)</td>
<td>NM_013162</td>
<td>1 May play a role in vitamin A transport</td>
<td>++</td>
</tr>
<tr>
<td>Alpha-1-microglobulin/bikunin precursor</td>
<td>NM_012901</td>
<td>1 May play a role in regulation of immune response</td>
<td>++</td>
</tr>
<tr>
<td>Ring Finger Protein 213</td>
<td>XM_221191</td>
<td>1              Associated with Moyamoya disease (constriction of certain arteries in the brain)</td>
<td>+++</td>
</tr>
<tr>
<td>Biliiverdin reductase B (flavin reductase (NADPH))</td>
<td>NM_001106236</td>
<td>1 Liver enzyme – converts biliiverdin to bilirubin</td>
<td>+++</td>
</tr>
<tr>
<td>Albumin</td>
<td>NM_134326</td>
<td>1 Possible role in nitric acid signalling</td>
<td>+++</td>
</tr>
<tr>
<td>Harbinger transposase derived 1</td>
<td>NM_001113793</td>
<td>1 Putative nuclease</td>
<td>+</td>
</tr>
<tr>
<td>Valosin-containing protein</td>
<td>NM_053864</td>
<td>1 Also known as Transitional endoplasmic reticulum ATPase, may be involved in vesicle transport and fusion</td>
<td>++</td>
</tr>
</tbody>
</table>

Table 3.4 Potential gephyrin interactors identified from YTH library screening of gephyrin C3 isoform against a liver cDNA library.

An indication of the strength of colour that developed in the LacZ assay is given, whilst not a precise measure this gives an idea of the strength of the interaction. ++++, strong interaction, ++ medium interaction, + weak interaction.
3.6 Classification of novel gephyrin interacting proteins by biological role

The spinal cord screen produced a large number of gephyrin interactors. These were classified by their biological role to investigate the variety of protein types that are able to interact with gephyrin. Figure 3.6 illustrates the biological roles of the gephyrin interactors from the spinal cord screen, whilst the screen was dominated by dynein light chains, there were plenty of other protein types that interact with gephyrin, including three novel GEFs.

![Pie chart illustrating the biological roles of gephyrin interactors identified by function and their frequency of appearance in the screen.]

Dynein light chains were the most commonly-identified gephyrin interactors, although GDP-GTP exchange factors (GEFs) also made up a large portion of the screen. A large variety of protein types appear to interact with gephyrin, possibly due to the different functions of this scaffolding protein in neurones versus glia or different subcellular compartments, e.g. cytoplasm and membrane. GABA$_A$R and GlyR subunits were noticeably absent from the screen, but this may reflect the fact that baits and preys with hydrophobic membrane-spanning domains do not function well in YTH assays.
Dynein light chains 1 and 2
Dynein light chains 1 and 2 were the major gephyrin interactors found in my library screens, accounting for nearly half the interacting proteins. To a certain extent, this served as an important indicator that the experiment had worked well, since the interaction between dynein light chains and gephyrin was identified using this method (Fuhrmann et al 2002). Dyneins are motor proteins, composed of heavy chains, intermediate chains and light chains. Whilst the heavier chains move along the microtubules, the lighter chains bind the cargo proteins allowing them to be transported towards the cells centre. Gephyrin and dynein light chains 1 and 2 colocalise in mammalian cells, and this suggested a potential role for these motor proteins in regulating gephyrin clustering. However gephyrin is still able to cluster at synapses even when the dynein light chain binding region in the gephyrin C domain is deleted (Fuhrmann et al 2002) so whether dynein is actually involved in the retrograde transport of gephyrin is unclear.

Ankyrin 3
Ankyrin 3 is an adaptor protein that links integral membrane proteins to the spectrin-based cytoskeleton. There are several variants of ankyrin, but ankyrin 3 (also known as ankyrin G) is known to be enriched in neurons and epithelial cells. Interestingly, a recent genome-wide screening study supported a role for sequence variations in the Ankyrin 3 gene (ANK3) in bipolar disorder (Ferreira et al 2008). Ankyrin 3 could have a potential role in localising gephyrin to axon initial segments, linking gephyrin with other neuronal proteins involved in clustering, such as neurofascins (Ango et al 2004; Burkarth et al 2007). More recently, an Ankyrin 3 variant considered to be a significant risk factor for bipolar disorder was also found to link with high startle reactivity (Roussos et al 2011). Given that collybistin, gephyrin and glycine receptor and transporter defects are associated with startle disorders, an interaction between gephyrin and ankyrin could be relevant in vivo.
Cdc42
Cdc42 is a member of the Rho family of small GTPases and has many roles within the cell including cytoskeletal organization, cell cycling and cell morphology. The human homologue of collybistin, hPEM-2 has been determined to be a GEF for Cdc42 (Reid et al 1999). Since Cdc42 regulates the reorganization of actin filaments it has been suggested that collybistin may activate Cdc42 to bring about cytoskeletal rearrangements which assist with gephyrin clustering (Xiang et al 2006). It is perhaps unsurprising that gephyrin also interacts with Cdc42, given the close association between gephyrin and collybistin. The gephyrin-Cdc42 interaction has recently been confirmed in mammalian cells. Although the interaction of Cdc42 with collybistin appears to be dependent on the activation state of Cdc42, the same does not appear to be true for gephyrin, which can interact with Cdc42 regardless of the activation state (Tyagarajan et al 2011a). Constitutively active Cdc42 also appears to rescue gephyrin clustering when co-expressed with a collybistin mutant lacking the PH domain, confirming a key role for Cdc42 in gephyrin clustering. Whilst there is little doubt that Cdc42 is involved in gephyrin clustering, this interactor was clearly involved in the established collybistin-gephyrin clustering pathway and was therefore not a priority for further study.

Heat shock proteins
Two members of the heat shock protein family were identified in the YTH screen (Hspa2 and Hspa12a). Given that this family of proteins are chaperones that are involved in the folding and trafficking of other proteins, their interaction with gephyrin might have been a coincidence. However a recent study (Machado et al 2011) linked a different member of the heat shock family to gephyrin clustering, Heat shock cognate protein 70 (Hsc70) was identified as a gephyrin interactor using pull down assays and mass spectroscopy. When Hsc70 and gephyrin were co-expressed in COS-7 cells, Hsc70 was partially recruited to gephyrin cytoplasmic
aggregates, whereas when expressed alone, Hsc70 showed diffuse cytoplasmic distribution. When GlyRs were also co-expressed, colocalisation of all three proteins was seen. Overexpression of Hsc70 also decreased the intensity of gephyrin clusters in cultured neurons. Hsc70 has ATPase activity and the association of Hsc70 with another protein depends on whether ADP or ATP is bound. Hsc70 binding to gephyrin was highest when ADP was present. Inhibition of the ATPase activity of Hsc70 increased the intensity of gephyrin clusters, but the amount of associated GlyRs was not changed (Machado et al 2011). The heat shock proteins found in my screen, Hspa2 and Hsp12a, are members of the same 70 kDa heat shock protein superfamily, so it is possible they may interact with gephyrin in a similar manner. However, these hits were not studied further due to time limitations.

**Myelin basic protein**

Myelin basic protein is a component of the myelin sheath of oligodendrocytes and Schwann cells. Between them these cell types provide myelination for the axons of nerve cells in the CNS. The myelin sheath increases the speed at which impulses can travel down axons. Demyelination is present in many neurodegenerative diseases, the most well known being multiple sclerosis, the loss of myelination reduces the conductance of impulses along neurons (Harauz et al 2009). Given the known role of this important protein, an interaction between gephyrin and myelin basic protein seems unlikely to be relevant *in vivo*.

**Ubiquitin-conjugating enzyme E2I**

Ubiquitination of proteins targets them for degradation, the process involves ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s) and ubiquitin-protein ligases (E3s). Ubiquitin-conjugating enzyme E2I is homologous to E2 enzymes, but conjugates small ubiquitin-like modifier 1 (SUMO-1) rather than ubiquitin. SUMOylation of a protein can alter subcellular localisation or protein-
protein interactions. Many SUMOylated proteins are neuronal, most identified to date are nuclear proteins, or involved in trafficking of cytosolic proteins to the nucleus (Zhang et al 2002). Whether or not gephyrin is a genuine SUMOylation target and the effect this might have on its protein interactions will require further study.

**Coactosin-like 1**

Coactosin-like 1 (Cotl1) is an F-actin binding protein involved in regulating the actin cytoskeleton. In transfected mammalian cells, Cotl1 colocalises with actin stress fibres (Provost et al 2001a). However Cotl1 does not appear to effect polymerisation or depolymerisation of actin polymers. Cotl1 is also able to interact with 5-lipoxygenase, this interaction may modulate the interaction between Cotl1 and actin (Provost et al 2001b). Until more is known about Cotl1, it is unclear whether an interaction with gephyrin is likely to be relevant.

**Cell-cycle associated protein 1**

Cell-cycle associated protein 1 (caprin1) is an RNA binding protein, suppression of expression of this gene results in a reduced cell proliferation rate, as the cell cycle is slowed. Interestingly this protein is thought to have a more specialised role in the brain and has been shown to be involved in local translation regulation in neurons, regulating synaptic plasticity. RNA granules containing caprin-1 and mRNAs for proteins involved in synaptic plasticity have been found in the dendrites of hippocampal neurons. Translation of the RNAs can then be induced by Brain-derived neurotrophic factor (BDNF), whilst bound to caprin-1 translation is inhibited but BDNF induces dissociation of caprin 1 from the granules (Shiina et al 2005). Given that this protein is an RNA binding protein, the importance of any interaction with gephyrin is unclear at present.
GEFs

The main aim of the screen was to identify novel GEFs that could be involved in gephyrin and/or GABA$_A$R and GlyR clustering, and in this respect the experiment was very successful. We identified partial cDNAs encoding fusion proteins for three GEFs: IQSEC2, IQSEC3 and RASGRP2. Further analysis revealed that these new GEFs are unrelated to collybistin in both sequence and domain structure. Collybistin contains a RhoGEF domain in tandem with a pleckstrin homology (PH) domain that bind the phosphoinositide PI3P (Kalscheuer et al 2009; Reddy-Alla et al 2010), and a src-homology 3 (SH3) domain which binds to neuroligins (Poulopoulos et al 2009) and the GABA$_A$R $\alpha$ 2 subunit (Saiepour et al 2010). By contrast, IQSEC2 and IQSEC3 proteins contain ArfGEF (Sec7) and PH domains, but also contain an IQ-like domain, which binds calmodulin in a Ca2+-dependant manner (Munshi et al 1996). RasGRP2 contains a RasGEF domain, a C1 domain which binds the second messenger diacylglycerol (DAG) and EF hands which also bind Ca2+. Validation checks were carried out on all three fusion proteins to confirm that they interact with gephyrin. Both P1 and C3 baits for gephyrin were screened against the IQSEC2, IQSEC3 and RASGRP2 preys from the library screen. Empty pYTH16 was also included to confirm these preys were not simply auto-activating the LacZ and His3 reporter genes. The results obtained confirmed that IQSEC2, IQSEC3 and RasGRP2 are all potential interactors of gephyrin, and do not result in the autoactivation of yeast reporter genes. The filters from these assays, along with the protein structures and sequences of IQSEC2, IQSEC3 and RasGRP2 can be seen in Figure 3.7, Figure 3.8 and Figure 3.9.
Figure 3.7 Analysis of IQSEC2

(A) Domain structure of IQSEC2. (B) Protein sequence of rat IQSEC2 (XP_002727583) - the fragment found in the YTH screen is underlined. (C) LacZ filter assays showing interactions between IQSEC2 and both P1 and C3 isoforms of full-length gephyrin.
Figure 3.8 Analysis of IQSEC3.

(A) Domain structure of IQSEC3. (B) Protein sequence of rat IQSEC3 (NP_997500), the fragment found in the YTH screen is underlined. (C) \textit{LacZ} filter assays showing interactions between IQSEC3 and both P1 and C3 isoforms of full-length gephyrin.
Figure 3.9 Analysis of RasGRP2.

(A) Domain structure of RasGRP2. (B) Protein sequence of rat RasGRP2 (NP_001076446), the fragment found in the YTH screen is underlined. (C) LacZ filter assays showing interactions between RasGRP2 and both P1 and C3 isoforms of full-length gephyrin.

For both the IQSEC2 and IQSEC3 proteins, the N-terminus was the region found to interact with gephyrin, prior to any known domains in the proteins. In contrast the C-terminus of RasGRP2 containing the EF hand and C1 domain mediates the gephyrin interaction. The genes encoding these proteins also have quite distinct chromosomal locations: IQSEC2 is located on human chromosome Xp11.22,
IQSEC3 is located on human chromosome 12p13.33 and RasGRP2 is located on human chromosome 11q13.1.

IQSEC2 and IQSEC3 both contain an ArfGEF (Sec7) domain, whilst RASGRP2 contains a RasGEF domain, indicating the type of GTPase they are able to activate. ADP ribosylation factors (Arfs) are GTPases involved in regulating vesicular transport and membrane trafficking. In mammals Arfs are subdivided into classes; I, II or III based on their sequence. These classes appear to localise in different subcellular compartments, with class I and II found at the golgi and ER, whilst class III is found at the cell membrane. Arfs are unique among small G proteins in that they have an N terminal amphipathic helix that becomes exposed when they are activated, allowing these GTPases to insert into the membrane and remain localised there (Gillingham and Munro 2007). The IQSEC proteins are one of five families of GEFS able to activate Arfs, with at least 15 identifiable ArfGEFs in the human genome. The large number of ArfGEFs means many different upstream signals can lead to Arf activation. The functions of some Arfs are shown in Table 3.5, the most important roles being in regulating vesicular transport, membrane trafficking and actin dynamics.

<table>
<thead>
<tr>
<th>Class of Arf</th>
<th>Known Arfs in this class</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Arf1, Arf2, Arf3</td>
<td>Localised to golgi, involved in recruitment of coat proteins to golgi for vesicle coating</td>
</tr>
<tr>
<td>II</td>
<td>Arf4, Arf 5</td>
<td>Overlapping functions with Class I</td>
</tr>
<tr>
<td>III</td>
<td>Arf6</td>
<td>Localised to plasma membrane, involved in endocytosis, cytokinesis, and the organization of the actin cytoskeleton</td>
</tr>
</tbody>
</table>

Table 3.5 The Arf family of small GTPases.

Known interactors of IQSEC2 include several synaptic proteins such as PSD95 and NMDA receptors, also the insulin receptor tyrosine kinase substrate p53 (IRSp53)
and proteins from the retinal synaptic ribbon (Murphy et al 2006; Sakagami et al 2008; Katsumata et al 2009; Sanda et al 2009). IQSEC3 has also been shown to interact with PSD95, SAP97 and Homer/Vesl1/PSDZip45 (Inaba et al 2004; Hattori et al 2007).

In contrast RasGRP2 activates the Ras family of proteins, which includes Ras, Rap1 and Rap2. Ras proteins are vital for controlling cell proliferation and differentiation, and disruption of the Ras pathways is common in cancers. Like the Arf proteins there are several families of GEFs which are able to activate Ras proteins. Ras proteins can also be localised to the membrane through palmitoylation. RasGRP2 is specifically able to activate only two of the three isoforms of Ras, N-Ras and Ki-Ras, but not Ha-Ras (Clyde-Smith et al 2000). Two of the key downstream signalling cascades from Ras are the Mitogen Activated Protein Kinase (MAPK) cascade and Phosphoinositide 3-kinase (PI3K) cascade. Both these pathways have been linked to synaptic plasticity and memory formation (Ye and Carew 2010).

3.7 Discussion

The cDNA library synthesis was successful, with cDNAs of a range of molecular weights produced from both spinal cord and liver RNAs. The spinal cord library screening was more efficient, since we had already integrated the pYTH9-gephyrin P1 bait vector into the yeast genome. This meant that only the cDNA library needed to be transformed into the yeast. By comparison, the liver screen was less efficient since pYTH16 gephyrin C3 and the library had to be co-transformed. Despite the lower number of colonies in the latter screen, there were still 24 cDNAs that were sequenced. However, none of these proteins identified had a clear link to MoCo synthesis, so proteins from this screen were not chosen for further investigation. By contrast, the spinal cord screen proved highly successful in both the high quantity of
known and novel interactors identified. We also fulfilled the main aim of this screen, which was to identify novel GEFs that interact with gephyrin. IQSEC2, IQSEC3 and RasGRP2 are all novel gephyrin interacting GEFs that activate GTPases other than Cdc42. Interestingly, none of these proteins had any sequence or structural identity with collybistin, (Figure 3.10). This highlights one of the main advantages of the YTH system, since no assumptions were made about what kind of GEF should interact with gephyrin.

![Collybistin](image1)

![IQSEC2](image2)

![IQSEC3](image3)

![RasGRP2](image4)

**Figure 3.10 Schematic representations of ARHGEF9, IQSEC2, IQSEC3 and RasGRP2.**

A literature review suggested that IQSEC2 was indeed a postsynaptic protein, although published findings suggest that it is a component of excitatory rather than inhibitory synapses (Murphy et al 2006; Sakagami et al 2008). Very little information was available on IQSEC3, but as a member of the same family as IQSEC2, this GEF was considered to be of significant interest, since like IQSEC2, it has a C-terminal PDZ-binding motif that may suggest interactions with PDZ-containing proteins which are enriched at both inhibitory and excitatory synapses. By contrast,
published data on RasGRP2 did not suggest a neuronal role, since a knockout mouse for RasGRP2 has a phenotype similar to leukocyte adhesion deficiency syndrome in humans (Kilic and Etzioni 2009). In order to gain a better understanding of the novel GEFs, I used online resources to examine their expression patterns, to determine if they were expressed in brain or other tissues. Expression data was obtained from www.biogps.org and can be seen in Figure 3.11, Figure 3.12 and Figure 3.13. This data is gathered using Affymetrix chips, which use oligonucleotide arrays to detect specific mRNAs in a given sample, the values seen in the figures relate to fluorescence intensity and the relative expression levels for a gene can then be compared across different tissues. This analysis revealed that RasGRP2 is clearly highly expressed in peripheral blood cells, consistent with a role in leukocyte adhesion deficiency syndrome (LAD). The symptoms of this disease include immunodeficiency and common infections, the most common cause is due to defects in a β-integrin subunit. However in the mouse knock-out model of RasGRP2 the phenotype includes leukocyte and platelet defects similar to LAD-III. Integrins are receptors expressed on the cell surface that form links to the surrounding tissue via contacts with the extracellular matrix. In blood cells these interactions are critical for a proper immune response, for example forming blood clots. In the RasGRP2 knockout model the activation of β-integrins in neutrophils was shown to be impaired, RasGRP2 is required for platelets to form clots (Bergmeier et al 2007). Therefore RasGRP2 is a possible cause of LAD-III in humans.

However it is possible that RasGRP2 also plays a role in the brain. It has been shown to be enriched specifically in the striatum (Kawasaki et al 1998) and has been reported to be down regulated in a mouse model of Huntington’s disease (Crittenden et al 2010). Whilst this does indicate that RasGRP2 may also have a neurological role the literature does not suggest a synaptic role. IQSEC2 and
IQSEC3 show higher levels of expression in brain tissue and have been shown to interact with synaptic proteins, and as such are more promising candidates for synaptic GEFs. It therefore was decided the interactions between gephyrin and the IQSEC proteins should be the priority for further study.
Figure 3.11 Expression of IQSEC2 in different tissues and cell lines

Human IQSEC2 is widely expressed, but the highest levels are clearly found in CNS tissues, skeletal muscle and bone marrow. Colour coding indicates tissue types. mRNA level was measured using Affymetrix chips.
Figure 3.12 Expression of *IQSEC3* in different tissues and cell lines.

Overall, human *IQSEC3* shows lower expression levels than *IQSEC2* but the highest levels are found in CNS tissues. Colour coding indicates tissue types. mRNA level was measured using Affymetrix chips.
Human RasGRP2 shows low expression levels in all tissues except blood cells, where the levels are around 5-fold higher than the expression of IQSEC2 and IQSEC3 in brain tissue. Colour coding indicates tissue types. mRNA level was measured using Affymetrix chips.
4 THE ARFGEF IQSEC2 – A COMPONENT OF EXCITATORY SYNAPSES INVOLVED IN X-LINKED INTELLECTUAL DISABILITY (XLID)

4.1 Background

The ArfGEF IQSEC2 was identified as a possible gephyrin interactor in my yeast two-hybrid library screen. This protein is also known as KIAA0522, BRAG1 and IQ-ArfGEF and is enriched at excitatory synapses (Jordan et al 2004; Peng et al 2004; Murphy et al 2006; Sakagami et al 2008) where it associates with PSD95, also known as SAP-90 (synapse-associated protein 90), encoded by the gene DLG4 (disks large homolog 4). PSD-95 is perhaps the best-studied member of the MAGUK-family of PDZ domain-containing proteins and contains basic three PDZ domains, an SH3 domain, and a guanylate kinase-like domain (GK) connected by disordered linker regions. Importantly, PSD95 is almost exclusively located in the postsynaptic density of neurons, where it is involved in the anchoring of synaptic proteins including neuroligins, NMDA receptors, AMPA receptors and K+ channels (Sheng and Sala 2001). Curiously, IQSEC2 transcripts are localised in both hippocampal neuronal cell bodies and dendritic processes (Sakagami et al 2008), suggesting that local translation of IQSEC2 mRNA in an activity-dependent manner could contribute to synaptic plasticity. Known IQSEC2 binding partners include the 53 kDa insulin receptor tyrosine kinase substrate IRSp53 (Sanda et al 2009) and RIBEYE, a unique scaffolding protein found at retinal ribbon synapses (Katsumata et al 2009).

Several isoforms of IQSEC2 are produced by alternative splicing (Shoubridge et al 2010a), most of which contains several key functional domains, including an IQ-like domain, a catalytic Sec7 domain, a PH domain and a C-terminal PDZ domain-binding motif. In other GEFs, the IQ-like domain has been shown (Farnsworth et al 1995) to bind calmodulin and accelerate exchange activity. The Sec7 domain can catalyse the exchange of GDP to GTP on the Arf family GTPases. Initially, IQSEC2
was shown to activate Arf1 (Murphy et al 2006) but actually activates Arf6 to a greater extent (Sakagami et al 2008). Arf6 is known to have several key roles in neuronal development and synaptic plasticity (Figure 4.1).

Small GTPases of the ADP ribosylation factor (Arf) family play a crucial role in the regulation of vesicular transport, organelle structure, lipid modification, membrane trafficking and actin cytoskeleton dynamics (Myers and Casanova 2008). In mammals, there are six ubiquitously expressed Arf genes, divided into three classes based on sequence similarity (Class I: Arf1-3, Class II: Arf4-5, Class III: Arf6). Class I and II Arfs are localised to the Golgi and endosomal compartments. By contrast, Arf6 is localised to the cell periphery in association with the plasma membrane and a subset of endosomes (D'Souza-Schorey and Chavrier 2006). The exchange of GDP for GTP on Arfs is catalysed by guanine nucleotide exchange factors (GEFs). There are at least fifteen ArfGEFs in the human genome (Gillingham and Munro 2007) characterised by a catalytic Sec7 domain with sequence identity to the yeast ArfGEF sec7p. Active Arf6 (Arf6-GTP) activates several downstream effectors such as phospholipase D (PLD), phosphatidylinositol-4-phosphate 5-kinases (PIP5K) and Rac1, leading to changes in membrane trafficking and actin dynamics. PA, phosphatidic acid; PIP2, phosphatidylinositol bisphosphate. Modified from Shoubridge et al 2010b.
Arf GTPases are unique in that GTP binding induces two conformational switches, the first is the usual activation of the protein, but the second switch brings about binding of the GTPase to the membrane, so it is no longer cytoplasmic (Cox et al 2004). Indeed, increased GTP results in increased Arf6 localisation at the membrane, and transfecting cells with constitutively active Arf6 (Q67L mutant) shows more mutant than wild-type protein localised at the membrane. Importantly, increasing GDP does not stabilise Arf6 as the membrane, showing the importance of ArfGEFs for the activation and localisation of Arf GTPases (Gaschet and Hsu 1999). The IQSEC2 PH domain is likely to bind phosphotidylinositol lipids within membranes and may also mediate protein-protein interactions (Lemmon 2007). Lastly, the C-terminal PDZ domain-binding motif mediates interactions with PSD95 (Sakagami et al 2008).

4.2 Study aims

Given that IQSEC2 is localised at excitatory synapses, it is unlikely that the IQSEC2-gephyrin interaction occurs in vivo. Indeed, IQSEC2 was shown to be absent from inhibitory GABAergic synapses (Sakagami et al 2008). However, I considered that it was important to understand alternative splicing of this gene and investigate the potential involvement of IQSEC2 in neurological disease. In particular, I noted that three non-synonymous variants in IQSEC2 were identified by Tarpey et al (2009) in separate, multigenerational families with non-syndromic X-linked intellectual disability (XLID). The aims of my study were therefore:

• To use bioinformatics approaches to understand the functional domains of IQSEC2 and the molecular variants generated by alternative splicing.

• To determine whether IQSEC2 interacts with gephyrin in the yeast two-hybrid system and mammalian cells, using epitope-tagged IQSEC2 and gephyrin proteins.
• To use bioinformatics and functional assays to investigate the consequences of the \textit{IQSEC2} mutations identified in individuals with XLID.

\textbf{Results}

\textbf{4.3 Bioinformatics of IQSEC2}

A search of Ensembl, NCBI and UCSC databases revealed that \textit{IQSEC2} is located on the X chromosome at Xp11.22. It also became rapidly apparent that more isoforms of IQSEC2 are generated by alternative splicing than previously recognised. These are shown in Table 4.1 and Figure 4.3. The longest isoform, IQSEC2-1 (NCBI code: NM_0011111125), is encoded by 15 exons spanning 88 kb giving rise to a 6,004 bp mRNA transcript, a 4,467 bp open reading frame and a predicted 1,488 amino acid protein (NCBI code: NP_001104595). A shorter isoform, IQSEC2-2 (NCBI code: NM_015075) is encoded by 14 exons and spans 48.74 kb of genomic sequence giving rise to a predicted 5,237 nucleotide mRNA transcript and a shorter 949 residue protein (NCBI code: NP_055890). These two variants differ in exon 1, 2 and 14, with the longer form containing an additional exon, exon 15, with exon 3-13 identical to both variants. The resulting proteins have 906 amino acids in common but vary at both the N- and C- termini (Figure 4.4). The functional domains: IQ-like domain, Sec7 domain, PH domain are contained in the conserved region between the two isoforms, but the PDZ binding motif at the C-terminus is only present in IQSEC2-1. In an alternate transcript, IQSEC2-3, the 30 bp exon 2 is spliced out, resulting in a 1,478 amino acid protein. Using a commercially available human tissue RNA panel, Shoubridge and colleagues (2010a) confirmed high levels of expression of both IQSEC2-1 and IQSEC2-2 isoforms in the human brain and lower expression levels across many other tissues throughout the body. A number of other shorter transcripts were found in different databases (Table 4.1), but the prevalence and significance of these minor transcripts is currently unclear. The rat
IQSEC2 transcripts identified in the gephyrin yeast two-hybrid screen most closely resemble shorter N-terminal transcripts of IQSEC2-2 (Figure 4.2)

MEAGSGPPGPSEGSPNRAVEYLLLNNIIESQQQLLETQRRRIIEEELEGQLDQQLTQENRD LREESQHLRQELHRDPGLGARDSPGQRSEQYQNLRTQFHHERLRESQFHAFQRGYPNRD GAYQNREAIYRDKEASYQLQDTTGVTARERDVACLHLQHFPALGREGREAGPAHP GREKEAGYSAAVVGQQRPRERGQLRASRSSFPAGGQHSTSTSPFATLQERKSDG

Figure 4.2 Peptide sequence of IQSEC2 variant found in yeast two-hybrid library screen

This variant is 310 residues, it is identical to IQSEC2-1 for the first 245 residues, but then the sequence differs (underlined) and terminates before the IQ like motif.
<table>
<thead>
<tr>
<th>Transcript ID</th>
<th>Transcript length (bp)</th>
<th>Open Reading Frame (bp)</th>
<th>Protein ID</th>
<th>Protein Length (aa)</th>
<th>Biotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>IQSEC-012</td>
<td>/</td>
<td>6011</td>
<td>4466</td>
<td>ENSP00000379712</td>
<td>1488</td>
</tr>
<tr>
<td>ENST00000396435 NM_0011111125 (IQSEC2-1)</td>
<td></td>
<td>6021</td>
<td></td>
<td>NP_001104595</td>
<td></td>
</tr>
<tr>
<td>IQSEC2-009</td>
<td>/</td>
<td>4004</td>
<td>2849</td>
<td>ENSP00000364514</td>
<td>949</td>
</tr>
<tr>
<td>ENST00000375365 NM_015075.1 (IQSEC2-2)</td>
<td></td>
<td>5248</td>
<td></td>
<td>NP_055890</td>
<td></td>
</tr>
<tr>
<td>NM_001243197</td>
<td>971</td>
<td>221</td>
<td>NP_001230126</td>
<td>73</td>
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<tr>
<td>IQSEC2-201/ENST00000375368 (IQSEC2-3)</td>
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</tbody>
</table>

Table 4.1 IQSEC2 variants generated by alternative splicing

Table 4.1 shows all the isoforms recorded in either the NCBI or Ensembl databases. Whilst the key isoforms IQSEC2-1, IQSEC2-2 are present in multiple databases, some of the shorter reported transcripts are only in one database.
Figure 4.3 Alternative splicing of IQSEC2/BRAG1/IQ-ArfGEF

(A) The four transcripts reported as protein coding are shown, exons are shown as blue blocks, exon size is to scale, but intron length is not. Exons 3-13 are identical in IQSEC2-1, IQSEC2-2 and IQSEC2-3 (highlighted in yellow). (B) The protein structure of IQSEC2-1, IQSEC2-2, IQSEC2-3 and the variant found in the yeast two-hybrid screen are represented; IQSEC2-2 is truncated at both the N and C terminus compared to the other isoforms.
IQSEC2-1: ENSP00000379712
MEAGSGPPGPGGSESPNRAVEYLLENNIESQQQLETQRRIIEELEGQLDQLTQENRD
LREESQHLRHELHRDPGARSPGQRESQYQNLRETQFHHRLESERQFAQARVGYPNE
GAYQNYREAVTRDKERASYPFLQDTTITGATARDVQCHLHNEPCALERUGREGERAGFAHP
GREKEAGYSAAVGVPFRPRQRLSGPGSSFPPGHHGTSTSSSSTASTTQLQRKSDGE
NRSTVSVEGDAPGSDLSTAVDSPGQPPYRLSQLPSSHGPPAGVGLFWARQLQRPA
AVALKRQEIEEIKRGALSDYELSTDQDLKVEMLERKYGGFLSRRQAARTQTAFQ
YRMKNCERLSSLRSSAESRMRRIILSNMRQFSFEFEEKAYEFAKPNAYEFEKGPSLDEGAMAG
ARSHRERLGPLYGSGGGIDGGGSSVTSESNSFDNILEDOLFQKSVLASEIDALEN
CHPSGPMSSEPPGSAQLEKRESKEQEDSDTSSFDLPLYLDDTVQPPQSPERLPSTEPPPQ
GREWFAPAPLPPPPVPPPVPSTGETRSAEEGTTRGPCLEGDLRRAAHLPLTIEEPS
DSSVDSLRDSGAVRQHEQAEDCPSHGTKLHKPGGPRAPPHRYPAGEPAPAPAP
PLPANPSGTPQSGGARRLKECEAESDGGDESLESSNESSNETINCSSSSRSSRDS
LREPAPATGKLCQYQRETRHSWDSFAFNNDVQHRHRYIGLNLNFKEPEGIQYLIERLIERGS
LSTDPQVAPHFILERGLSRMIQEGFLNGRQKFNQDVLDCVDMEFDSSMDLDLALKFR
QSHIRVQGAEKVERLIEASFQRYVCNPALVRQFRNPDTIFILAFAIILLNTDMSPSVEK
AERKMLDDFPLKYNLRGVDNEDIPRDDLVGYIYRQIGRELRNTHDHSVQAVERMIVG
KPVLSPLHRLVCCQLYEVPDENPQRQLGLHRQEVLFDLLVTKIQKKKILVLTV
FRQSFPLVEMHIMNOSYQFGKLISAVPGGERKVIILNFAIPLNQDRLRTSDLRESI
AEVQEMEKYVSELEQOKGMPVRNSQAOGKAGSDNVMRASSLDNEDYAGDGLKRGAL
SSSLRDLSDQKGRGRNVSGLSDLTEIGSVISSPRHRMPPPPPEEYKQRPVSN
SSFLGSLFPSGRGKGPQFQMMPTQGQAASASSSASSTHHHHHHHGHSHGGLVLPDP
QSKQLAHLQYQPQGPFAPAPLPPQPLPPPQLPPPLPPPAPAPPVGHPR
HFHANHPVGQPYTQLGPRAPPRGAGHHQFAPHRHNLQHPTSPFLPSFAPQHPPA
HKQGKFHFSSHHFMPFAAGGPGSRGPPGGSYHHPFQSPIFSSHPIPSHYFPL
PPSSHPTHSPLSPTSPHGLPSGPGPTANPSSPKAPKSRISTVV

IQSEC2-2: ENSP00000364514
MEPPGGRSSRTASHTLHYQCTQQVQLDMSKLTGPSLRAESSVEGDAPGSDLSTAVDSPG
QPPYRLSQLPSSHSMGPPAGVGLFWARQLRPAVSALVQKEEEEIKRGALSDYEL
STDQDLKVEMLERKYGGFLSRRQAARTQTAFQYRMKNCERLSSLRSSAESRMRRIIL
SNMRQFSFEFEEKAYEFAKPNAYEFEKGPSLDEGAMAGARSHRERLGPLYGSGGGIDGGGS
VTSTSGFSDNITELDLEFSQKVSKLASEIDALCHPSGPMEEPSQAQLEKRESKSEQQ
EDSSATSTQAPLPPQPPQPPQPPQPPQPPQPPQPPQPPQPPQPPQPPQPPQPPQPPQPP
QSRTGEDSUERGRPCLOCERDFLRRAAHLPLTIEEPSDDSSSVDSLDSRDSGSRGVHRLQVLYEADG
CSPHTLHGKHPGRAPIPHRPPAPGAPAPAPLPPAPNPSGVPAGGRDGLKCE
AAGENSEDGGNESSATTERINCSSSSSRSLREPAPATGKLCQYTQERHSSWDP
AFNDNVQQRHYRIGLNLNFKEPEGIQYLIERLIERGS
LSTDPQVAPHFILERGLSRMIQEGFLNGRQKFNQDVLDCVDMEFDSSMDLDLALKFR
QSHIRVQGAEKVERLIEASFQRYVCNPALVRQFRNPDTIFILAFAIILLNTDMSPSVEKAERKMLDDFPLKYNLRGVDNEDIPRDDLVGYIYRQIGRELRNTHDHSVQAVERMIVG
KPVLSPLHRLVCCQLYEVPDENPQRQLGLHRQEVLFDLLVTKIQKKKILVLTV
FRQSFPLVEMHIMNOSYQFGKLISAVPGGERKVIILNFAIPLNQDRLRTSDLRESI
AEVQEMEKYVSELEQOKGMPVRNSQAOGKAGSDNVMRASSLDNEDYAGDGLKRGAL
SSSLRDLSDQKGRGRNVSGLSDLTEIGSVISSPRHRMPPPPPEEYKQRPVSN
SSFLGSLFPSGRGKGPQFQMMPTQGQAASASSSASSTHHHHHHHGHSHGGLVLPDP
QSKQLAHLQYQPQGPFAPAPLPPQPLPPPQLPPPLPPPAPAPPVGHPR
HFHANHPVGQPYTQLGPRAPPRGAGHHQFAPHRHNLQHPTSPFLPSFAPQHPPA
HKQGKFHFSSHHFMPFAAGGPGSRGPPGGSYHHPFQSPIFSSHPIPSHYFPL
PPSSHPTHSPLSPTSPHGLPSGPGPTANPSSPKAPKSRISTVV

IQSEC2-3: ENSP00000364517
MEAGSGPPGPGGSESPNRAVEYLLENNIESQQQLETQRRIIEELEGQLDQLTQENRD
LREESQHLRHELHRDPGARSPGQRESQYQNLRETQFHHRLESERQFAQARVGYPNE
GAYQNYREAVTRDKERASYPFLQDTTITGATARDVQCHLHNEPCALERUGREGERAGFAHP
GREKEAGYSAAVGVPFRPRQRLSGPGSSFPPGHHGTSTSSSSTASTTQLQRKSDGE
NRSTVSVEGDAPGSDLSTAVDSPGQPPYRLSQLPSSHGPPAGVGLFWARQLQRPA
AVALKRQEIEEIKRGALSDYELSTDQDLKVEMLERKYGGFLSRRQAARTQTAFQYRMKNCERL
SSLRSSAESRMRRIILSNMRQFSFEFEEKAYEFAKPNAYEFEKGPSLDEGAMAGARSHRERLGPLYGSGGGIDGGGS
VTSTSGFSDNITELDLEFSQKVSKLASEIDALCHPSGPMEEPSQAQLEKRESKSEQQEDSSSSTSDPILYLDVTQQSSPERLPSTEPQPPQREFWAPAP
LPPVPVPVSSTGDREGREEGTRGPCLECRDFLRRAAHLPLTIEEPSDDSSSVDSLDSGVCY

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Figure 4.4 Peptide sequence of the major isoforms of human IQSEC2

The predicted sequences of the major IQSEC2 isoforms; IQSEC2-1, IQSEC2 and IQSEC2-3. Alternating exons are shown in black and blue text, with residues overlapping splice sites depicted in red. The different N- and C- termini of the proteins are underlined, whilst the majority of the sequence (highlighted in grey) is identical in each isoform.

4.4 Further investigation of the interaction between gephyrin and IQSEC2 in yeast and mammalian cells

Bioinformatics analysis revealed that the IQSEC2 variant found in the yeast two-hybrid library screen did not correspond to the most commonly expressed IQSEC2 isoforms. For this reason, I cloned cDNAs corresponding to the N-terminus (amino acids 1-749) of the longest IQSEC2 isoform (IQSEC2-1) and corresponding N-terminal baits in pYTH16 for IQSEC1 (NCBI code: NM_001134382.2) and IQSEC3 (NCBI code: NM_001170738.1). These constructs included the IQ-like motif but not the Sec7 domain. Given that these proteins contain calmodulin-binding IQ-like motifs, calmodulin cloned into pACT2 was used as a positive control prey in these experiments. Co-transformations with empty vectors were performed to test for auto-activation. This experiment revealed that the only N-terminal bait to interact with a gephyrin prey was that of IQSEC3, and even this was a relatively weak interaction (Figure 4.5). Whilst this may be related to the fact that expression of full-length
gephyrin retards the growth of yeast, it is important to note that IQSEC1 and IQSEC2 baits showed a positive interactions with a calmodulin prey. Curiously, the bait for IQSEC3 did not appear to interact with calmodulin, which may be explained by differences in the sequence of the IQ-like motif. This is discussed in detail in chapter 7. Most importantly, it appears that the most commonly expressed IQSEC2 isoform does not interact with full-length gephyrin in YTH assays.

Potential interactions between IQSEC2 and gephyrin were also assessed in transfected HEK293 cells. Initial transfections of pEGFP-gephyrin (full-length P1 isoform) were carried out to confirm the best transfection method. Transfections using lipofectamine were successful but large gephyrin aggregates were observed that could compromise cell viability and clustering assays. By contrast, electroporation resulted in lower expression levels of EGFP-gephyrin (Figure 4.6A), which would be less prone to artefacts, such as trapping of other recombinant proteins. HEK293 cells were therefore electroporated with 10µg DNA, left for 24 hours and then fixed. FLAG-tagged IQSEC2 was expressed from the vector pCAGGS, which has a chicken β-actin/rabbit β-globin hybrid promoter (AG) and the human CMV-IE enhancer. Co-expression of FLAG-tagged IQSEC2-1 with pEGFP-gephyrin P1 revealed the proteins did not co-localise in HEK293 cells (Figure 4.6B-D). FLAG-IQSEC2 localised at the cell membrane whilst EGFP-gephyrin remained in cytoplasmic aggregates. Taken together, the results of yeast two-hybrid and cellular assays confirmed my suspicion that the interaction between gephyrin and the commonly expressed IQSEC2 isoform IQSEC2-1 is unlikely to take place in vivo.
Figure 4.5 *LacZ* assays measuring potential IQSEC1, IQSEC2 and IQSEC3 bait interactions with gephyrin and calmodulin preys

The yeast two-hybrid filters show blue colour indicating *LacZ* activity for both IQSEC1 and IQSEC2 interactions with calmodulin, and a weaker interaction between IQSEC3 and gephyrin. Transformation controls using empty bait and prey vectors show no auto-activation of the *LacZ* reporter gene.
Figure 4.6 Confocal imaging of HEK293 cells over-expressing EGFP-gephyrin and FLAG-IQSEC2

A: When expressed alone, large cytoplasmic EGFP-gephyrin aggregates are observed (Harvey et al 2004). B-D: Co-expression of EGFP-gephyrin with FLAG-IQSEC2-1. FLAG-IQSEC2-1 was visualised using a rabbit anti-FLAG primary antibody (Sigma F7425) diluted 1 in 200 in 2% (w/v) BSA in PBS. The secondary antibody was Alexa Fluor® 546 Goat Anti-Rabbit IgG (Invitrogen A11010), diluted 1 in 600 in 2% (w/v) BSA in PBS. Images were taken using a Zeiss LSM710 using a Plan-Apochromat 63 ×/1.40 Oil DIC M27 objective. Scale bars: 20μm.
4.5 Bioinformatics analysis reveals novel variants in \textit{IQSEC2} in non-syndromic X-linked intellectual disability

Until recently, identification of causative mutations in human disease has been achieved primarily by positional and functional candidate gene approaches and targeted re-sequencing of specific genes. However, the application of the systematic re-sequencing of entire exomes and genomes has removed bias associated with candidate gene selection. One early application of this technology was the resequencing of the entire X-chromosome exome using Sanger DNA sequencing in 208 families with X-linked intellectual disability (Tarpey et al 2009). This study was a major achievement, and revealed several previously unidentified XLID-associated genes including \textit{SYP}, \textit{ZNF711} and \textit{CASK}. Importantly, Tarpey and colleagues published all single nucleotide variants (SNVs) found in their study as supplementary data, which meant that other researchers could mine this data for possible additional XLID genes.

Examining this large data set, I noted that three non-synonymous missense changes, resulting in R758Q, Q801P and R863W substitutions were found in \textit{IQSEC2}. Although these variants were not detected in controls, or other individuals with XLID, \textit{IQSEC2} was not immediately assigned as a new XLID gene. The main reason for this was that these variants were amongst over 550 unique non-recurrent variants identified in 208 XLID families (Tarpey et al 2009). For this reason, tracking of all variants in all relevant families could not be performed in a timely manner. In addition, the \textit{IQSEC2} variants were not ranked highly in an assessment of likely pathogenicity based on conservation score (Tarpey et al 2009). However, when I performed my own analysis with three bioinformatics programmes, Polyphen-2, SIFT and mutation taster (Table 4.2) these changes were predicted to be either damaging or disease-causing. Examination of the location of these missense
changes revealed that all three were found in the catalytic Sec7 domain (Figure 4.7).

None of the residues appeared to coincide with residues predicted to be involved in GTPase binding (Shoubridge et al 2010a), but R758Q, Q801P and R863W were conserved in several vertebrate species, perhaps reflecting an important structural or functional role.

<table>
<thead>
<tr>
<th>Change</th>
<th>Polyphen-2</th>
<th>SIFT</th>
<th>Mutation taster</th>
</tr>
</thead>
<tbody>
<tr>
<td>R758Q</td>
<td>Possibly damaging, score 0.635</td>
<td>Damaging, 0</td>
<td>Disease causing, p: 0.9981</td>
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<td>Q801P</td>
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<td>Disease causing, p: 0.9999</td>
</tr>
<tr>
<td>R863W</td>
<td>Probably damaging, score 0.98</td>
<td>Damaging, 0</td>
<td>Disease causing, p: 0.9999</td>
</tr>
</tbody>
</table>

Table 4.2 Prediction of the potential pathogenicity of IQSEC2 Sec7 domain mutations

Bioinformatics programmes used were Polyphen-2: http://genetics.bwh.harvard.edu/pph2/, SIFT: http://sift.jcvi.org/ and Mutation taster: www.mutationtaster.org/

Figure 4.7 Conservation of the IQSEC2 Sec7 domain in vertebrate species

Alignment of the IQSEC2 Sec7 domains from several key vertebrate species. Numbering is shown for the human IQSEC2 sequence and the position and conservation of residues R758, Q801 and R863 is indicated by blue lettering. Most
changes are present in the zebrafish IQSEC2 sequence and are indicated by bold black lettering. Amino acids in IQSEC2 predicted to be important for interactions with Arf GTPases, based on alignments with the ArfGEF ARNO (Shoubridge et al 2010a) are indicated in red lettering. IQSEC2 missense changes do not coincide with these residues.

Given our interest in IQSEC2, we contacted the authors of Tarpey et al who referred us to Josef Gecz and Cheryl Shoubridge at the Women’s and Children’s Hospital in North Adelaide, Australia. Further analysis revealed that the IQSEC2 missense changes clearly segregated with the ID phenotype in the respective families (Figure 4.8). Notably, one of these families was MRX1, the first described family with non-syndromic XLID, mapped in 1988. An additional IQSEC2 missense mutation, resulting in a R359C change, was identified in an Australian family (AU128). This change was located in another key motif, the IQ-like domain (Figure 4.8) and was predicted to be damaging using Polyphen-2, SIFT and mutation taster (Table 4.3).

Figure 4.8 Schematic of the human IQSEC2 protein showing location of mutations in non-syndromic XLID families AUS128, US166, MRX1 and MRX18

Schematic of the human IQSEC2 protein showing the regulatory IQ-like motif (green), catalytic Sec7 domain (pink), PH domain (purple) and PDZ binding motif (orange). The relative locations of mutations found in each family are shown. In the family MRX1 (OMIM 309530), a c.2587C>T mutation was identified in exon 8 leading to a R863W substitution. In the MRX18 family, a c.2402A>C change in exon 6 was detected, leading to a Q801P substitution. A third, family from the USA (US166) had a c.2273G>A change in exon 5 leading to a R758Q substitution. In a fourth Australian family (AU128), a c.1075C>T change in exon 4 was detected, leading to a R359C substitution.
Table 4.3 Prediction of the potential pathogenicity of IQSEC2 IQ-like domain mutation

<table>
<thead>
<tr>
<th>Change</th>
<th>Polyphen-2</th>
<th>SIFT</th>
<th>Mutation taster</th>
</tr>
</thead>
<tbody>
<tr>
<td>R359C</td>
<td>Probably damaging, score 1.000</td>
<td>Damaging, 0</td>
<td>Disease causing, p: 0.9999</td>
</tr>
</tbody>
</table>

Bioinformatics programmes used were Polyphen-2: http://genetics.bwh.harvard.edu/pph2/, SIFT: http://sift.jcvi.org/ and Mutation taster: www.mutationtaster.org/

4.6 IQSEC2 mutations in XLID compromise ArfGEF activity

To obtain definitive proof of IQSEC2 dysfunction, we assessed the effect of the three different Sec7 domain mutations on the ability of IQSEC2 to catalyse GDP-GTP exchange on Arf6 in vitro in collaboration with Randall Walikonis and Jessica Murphy at the University of Connecticut. Site-directed mutagenesis (QuikChange, Agilent) was used to induce the desired mutations into wild-type IQSEC2 expression constructs and DNA sequencing verified that only the desired changes had been introduced. The constructs were then sent to our collaborators and used in two assays. The first assay carried out was a guanine nucleotide exchange assay. GST-tagged Sec7 domains (wild-type or mutant) and GST-tagged ARF6 were expressed in E. coli and purified on glutathione agarose beads. These proteins were eluted and the assay carried out by incubating ARF6 with $[^{35}\text{S}]\text{GTP}_{\gamma}\text{S}$ and purified Sec7 domains. Arf6-GTP$_{\gamma}\text{S}^{35}$S was isolated on nitrocellulose membrane and the amount of bound radiolabel was quantified in a scintillation counter. We found that GTP binding mediated by the Sec7 domain mutants R758Q, Q801P and R863W was similar to that of a control dominant-negative mutation (E849A), demonstrating significantly diminished GTP binding to Arf6 compared to wild-type IQSEC2 (Figure 4.9). We then tested the GEF activity of full-length wild-type and mutant IQSEC2 in a cellular assay. This pull-down assay utilised the interaction of Arfs with the adaptor protein Golgi-localized, $\gamma$ ear-containing Arf-binding protein 3 (GGA3). GGAs specifically
interact with active, GTP-bound Arf, but not inactive Arfs. HA-tagged Arf6 and FLAG-tagged wild-type or mutant versions of IQSEC2 (or empty vector) were co-expressed in HEK293 cells, and cell lysates were subjected to a pull-down assay with GST-GGA3 immobilized on glutathione-agarose. Arf6-GTP was stripped from the beads and Arf6 was quantified by Western blotting. Co-transfection of Arf6 with wild-type IQSEC2 resulted in an approximately 12-fold increase in GTP-bound Arf6, but only a 6- to 8-fold increase when co-expressed with IQSEC2 harbouring mutations in the IQ or Sec7 domains (Figure 4.10)
Radiometric analysis of the effects of mutations in the Sec7 domain of IQSEC2 on GEF activity. Recombinant wild-type (dark blue) or mutated versions of the Sec7 domain of IQSEC2 were incubated with recombinant Arf6 and GTPγS to catalyse binding of GTPγS to Arf6. Each XLID mutation in the Sec7 domain (light blue) significantly reduced GTP binding to Arf6. An ANOVA test was carried out, and confirmed significant differences between the mutants, $F(4,15) = 7.669$, $p = 0.001$. Post-hoc analysis with Dunnett’s test showed a statistically significant difference (**$p<0.005$) between the Q801P mutant and wild-type, and between the R863W mutant and wild-type. The R758Q mutants was also significantly different from wild-type, but with a higher p value (*$p<0.01$). Note that the artificial dominant-negative mutant E849A that should reduce GEF activity of the Sec7 domain was not significantly different from wild-type. Figure modified from Shoubridge et al 2010a.

Figure 4.9 Guanine nucleotide exchange assay for IQSEC2 Sec7 domain mutants
Figure 4.10 GGA3 pull-down assay for IQSEC2 IQ-like and Sec7 domain mutants

Guanine nucleotide exchange activity in a cellular assay analysed using a GGA3 pull-down assay. Arf6 and either wild-type (dark blue) or mutated (light blue) full-length IQSEC2 were co-expressed in HEK293 cells. The cells were lysed and Arf6-GTP was isolated on beads coated with the adaptor protein Golgi-localized, γ ear-containing Arf binding protein 3 (GGA), which binds to GTP-bound but not GDP-bound Arfs. Arf6-GTP was stripped from the beads and quantified by Western blotting. Each mutation reduced activation of Arf6 in this cellular assay. Data are mean ± s.e.m. from three independent experiments. An ANOVA test was carried out, and confirmed some significant differences between the mutants, $F(5,12) = 6.9 \ p = 0.003$. Post-hoc analysis with Dunnett’s test showed a statistically significant difference (*$p<0.05$) between the Q801P mutant and wild-type, and between the R863W mutant and wild-type. The artificial dominant negative mutant E849K also showed a significant difference from the wild-type (**$p<0.001$). However, the R359C and R758Q mutants were not significantly different from wild-type. Figure modified from Shoubridge et al 2010a.
4.7 Bioinformatic investigation of the IQ-like domain mutation R359C

A bioinformatics investigation of the IQSEC2 IQ-like domain was conducted using PFAM (http://pfam.sanger.ac.uk/). IQ and IQ-like domains bind to calmodulin (CaM), now recognized as a major Ca\(^{2+}\) sensor involved in regulatory events via interactions with many cellular proteins. Two different classes of recognition motifs exist for many of the known CaM binding proteins: 1) The complete IQ motif is a consensus for \(Ca^{2+}\)-independent CaM binding and 2) IQ-like motifs for \(Ca^{2+}\)-dependent CaM binding. Complete IQ motifs (containing the G and a second basic residue) do not require \(Ca^{2+}\) to bind calmodulin, but binding of incomplete IQ motifs (lacking the second basic residue) is \(Ca^{2+}\) dependent (Houdusse and Cohen 1995; Munshi et al 1996). Comparison of the sequences of IQSEC1, IQSEC2 and IQSEC3 with these consensus descriptions revealed that these ArfGEFs have IQ-like domains and are therefore predicted to bind CaM in a \(Ca^{2+}\) dependent manner. In addition, the R359C mutation disrupts a key basic (R) residue in this consensus sequence.

\[
\begin{align*}
\text{IQ} & \quad (\text{FILV})Q\text{xxx}(\text{RK})G\text{xxx}(\text{RK})\text{xx}(\text{FILVWY}) \\
\text{IQ-like} & \quad (\text{FILV})Q\text{xxx}(\text{RK})\text{xxxxxxx} \\
\text{R359C} & \\
\text{IQSEC1} & \quad \text{RHAARTIQTAFRQYQMKNFE} \\
\text{IQSEC2} & \quad \text{RRAARTIQTAFRQYRMKNFE} \\
\text{IQSEC3} & \quad \text{RRAACTIQTAFRQYQLSKNFE}
\end{align*}
\]

**Figure 4.11 IQSEC1, IQSEC2 and IQSEC3 harbour IQ-like motifs**

Upper panel: Consensus motifs for IQ and IQ-like domains are shown. Characters within parentheses can substitute for each other. The first hydrophobic residue (preceding the Q) is predominantly I, V or L. The G after the first basic residue (almost always R) is not as conserved, nor are the hydrophobic residues following the second basic residue. Note that IQSEC1-3 are missing the key second basic residue found in complete IQ motifs and that the R359C mutation disrupts a key basic (R) residue.
4.8 *IQSEC2* mutation R359C disrupts IQSEC2/calmodulin interactions

To establish whether the R359C mutation disrupts IQSEC2-CaM interactions, I used the yeast two-hybrid system to test the interactions between the wild-type or mutant IQ-like motif and calmodulin. Empty bait and prey vectors did not autoactivate reporter genes, but the wild-type IQ domain bait was able to mediate a robust interaction with a calmodulin prey, which was abolished by introduction of the R359C mutation (Figure 4.12). This strongly suggests that the reduction in GEF activity observed from R359C in the GGA assay (Figure 4.10) is indirect and result from a loss of stimulation of IQSEC2 activity by cellular CaM.

![Figure 4.12](image)

**Figure 4.12** LacZ assays showing the wild-type and R359C mutant IQ-motif bait interactions with a calmodulin prey

The yeast two-hybrid filters show blue colour indicating LacZ activity for IQSEC2 interactions with calmodulin and how this interaction is abolished by introduction of the R359C mutation. Transformation controls using empty bait and prey vectors show no auto-activation of the LacZ reporter gene.
4.9 Discussion

In this part of my study, I investigated whether the interaction between gephyrin and the ArfGEF IQSEC2 identified in my yeast two-hybrid library screen was of relevance for inhibitory GABA\textsubscript{\textalpha} and glycine receptor clustering at the postsynaptic density. Bioinformatics analysis revealed that there are a variety of isoforms of IQSEC2, generated by alternative splicing. The IQSEC2 isoform isolated by YTH screening was an N-terminal truncated variant, lacking IQ, Sec7, PH and PDZ binding domains and the functional relevance of this isoform is currently unclear. Further testing of interactions of the most common IQSEC2 isoform IQSEC2-1 in yeast two-hybrid and cellular co-clustering assays did not reveal a specific IQSEC2-gephyrin interaction, which is in good agreement with other studies suggesting that IQSEC2 is enriched at excitatory synapses and absent from inhibitory synapses (Jordan et al 2004; Peng et al 2004; Murphy et al 2006; Shoubridge et al 2010b).

Our interest in IQSEC2 and bioinformatics analysis led to our involvement in a study that firmly established mutations in IQSEC2 as a new cause of non-syndromic X-linked intellectual disability. The fact that IQSEC2 mutations result in a reduction but not a complete loss of function in exchange and pull-down assays for GEF activity might provide a plausible reason why IQSEC2 variants showed only moderate conservation scores in some bioinformatics tests and highlight the importance of functional testing of missense variants in disease. I have also highlighted a key role for calmodulin in the regulation of IQSEC ArfGEFs, since disrupting calmodulin-binding to IQSEC2 appears to result in a lowering of IQSEC2 GEF activity in cellular assays. This result is in good agreement with studies on other GEFs, where CaM binding to the IQ-like domain has been shown (Farnsworth et al 1995) to accelerate exchange factor activity. Taken together, the results presented here strongly suggest that all four missense changes in IQSEC2 are functionally relevant and likely to be the disease-causing mutations in these families.
Having established that mutations in *IQSEC2* are causative for XLID, a major challenge is to discover the changes in neuronal function triggered by defective IQSEC2. These could include changes in cell morphology, neuronal migration or synapse formation. Additional mutations may remain to be identified and *IQSEC2* is now undergoing additional screening for genetic variants in families with XLID that were not part of recent studies (e.g. the EURO MRX Consortium; http://www.euromrx.com/).

Given their similar domain structure, neuronal expression and localisation at synapses, other IQSEC gene family members, *IQSEC1* and *IQSEC3*, should also be considered as candidate genes in studies of autosomal ID. For example, a maternally inherited 1.65 Mb deletion on 12p13.33 encompassing 10 genes (including *IQSEC3*) was reported (Baker et al 2002) to cause moderate ID and behavioural problems in a 15-year-old boy. However, whether dominant *de novo* mutations or autosomal recessive forms of ID (ARID) result from mutations in *IQSEC1* or *IQSEC3* remains to be determined. Certainly, large-scale re-sequencing studies are now impacting on studies of XLID and ARID, providing a huge amount of data for new follow-up studies. For example, (Najmabadi et al 2011) recently reported over 50 new genes involved in autosomal cognitive disorders, implicating proteins involved in diverse processes such as transcription and translation, cell-cycle control, energy metabolism and fatty-acid synthesis. Certainly, the discovery of new disease genes opens promising new avenues for the development of rational treatments, which together with accurate diagnostic tests provide a brighter outlook for affected individuals and their families.
5 THE ARFGEF IQSEC3 - A GEPHYRIN-BINDING COMPONENT OF INHIBITORY SYNAPSES LINKED TO AUTOSOMAL ID AND AGGRESSIVE BEHAVIOUR

5.1 Background

The ArfGEF IQSEC3 was also identified as a potential gephyrin interactor in my yeast two-hybrid screen. IQSEC3 is also known as KIAA1110, BRAG3 and SynArfGEF. This protein was first described by (Inaba et al 2004) as a brain-specific potential synaptic GEF for Arf, it was thus named SynArfGEF (Po). This study demonstrated that the corresponding gene, IQSEC3, was highly expressed in rat brain, particularly in the olfactory bulb and hippocampal pyramidal cell layers. IQSEC3 was also reported to interact with various synaptic proteins including PSD-95, SAP97 and PSD-Zip45 (Inaba et al 2004). SAP97 (Synapse-associated protein 97) is a member of the same family of proteins as PSD-95 (MAGUK) and is involved in trafficking AMPA receptors and NMDA receptors to the synapse (Howard et al 2010). PSD-Zip45 is a scaffolding protein that interacts with AMPA and NMDA receptors (Usui et al 2003). Taken together, these interactions suggested that IQSEC3 was most likely to be present at excitatory synapses, but as we will find out, this is not the case.

IQSEC3 has a modular structure similar to that of IQSEC2, consisting of an IQ-like domain, a catalytic Sec7 domain, a PH domain and a C-terminal PDZ domain-binding motif. The ArfGEF activity of IQSEC3 has also been investigated, although there is some controversy in the literature. Initial reports suggested that human IQSEC3 activates Arf1 and Arf6 (Hattori et al 2007). However, a recent study using rodent IQSEC3 suggests specificity for Arf6 (Fukaya et al 2011). Activation of Arf1 by rodent IQSEC3 could not conclusively be shown in the same assay, sequence differences between the rodent and human IQSEC3 may explain the difference in
specificity. The generation of IQSEC3 antibodies has recently revealed that IQSEC3 localises to the postsynaptic densities of inhibitory synapses (Fukaya et al 2011). Immunohistochemistry in adult mouse brain revealed colocalisation of synArfGEF and gephyrin in the hippocampus, olfactory bulb and cerebral pyramidal neurons. Interestingly, there was no colocalisation of IQSEC3 with PSD-95, and the interaction between IQSEC3 and PSD-95 reported by Inaba et al (2004) could not be reproduced by (Fukaya et al 2011). Thus, the IQSEC3-PSD-95 interaction is unlikely to occur \textit{in vivo}. Since IQSEC2 and IQSEC3 puncta did not co-localise, IQSEC3 is likely to be present at locations distinct from IQSEC2 (Fukaya et al 2011). Support for the presence of IQSEC3 at inhibitory synapses came from experiments that demonstrated colocalisation of IQSEC3 with gephyrin and the GABA\textsubscript{A}R α1 subunit, but not the GluA2 subunit (Fukaya et al 2011).
Figure 5.1 Immunohistochemistry of IQSEC3 in mouse brain and spinal cord (Fukaya et al 2011)

(A-F) Immunofluorescent localization of synArfGEF in the hippocampal CA3 region. Coronal sections were immunostained for IQSEC3 (A, D) and gephyrin (B) or PSD-95 (E). Note the colocalisation of IQSEC3 with gephyrin but not PSD-95. (G-I) Immunohistochemical localisation of IQSEC3 (G) and the GABA₆R α1 (H) subunit in the cerebellar cortex (J-M) Coronal section of spinal cord were immunostained for IQSEC3 (J), gephyrin (K) and GlyR α subunit (L) Scale bars 5μm

Lastly, YTH screening revealed IQSEC3 interactions with the cytoskeletal proteins utrophin/dystrophin and S-SCAM/MAGI-2, which have been shown to localise at inhibitory synapses (Sumita et al 2007). It is noteworthy that whilst these interactions support a role for IQSEC3 at the inhibitory synapses, the non-specific nature of the localisation patterns for utrophin/dystrophin and S-SCAM/MAGI-2 suggest that these IQSEC3 interactors cannot account for the targeting of IQSEC3 to inhibitory...
synapses (Fukaya et al 2011). Since other factors must be involved in this specific targeting, we resolved to define the nature of the IQSEC3-gephyrin interaction.

5.2 Study aims

Given that IQSEC3 is localised at inhibitory synapses, the IQSEC3-gephyrin interaction revealed by my YTH screen has strong plausibility. However, it is important to establish whether IQSEC3 also exists in multiple isoforms, whether IQSEC3 is capable of gephyrin clustering and which domains of gephyrin and IQSEC3 mediate this interaction. With this information, the consequences of disrupting the IQSEC3-gephyrin interaction could be studied. I also sought to establish possible links between IQSEC3 and neurological disease. Given previous links of collybistin and IQSEC2 to intellectual disability, it was of significant interest that a maternally-inherited 1.65 Mb microdeletion on 12p13.33 was reported to cause moderate ID and behavioural problems in a 15-year-old boy (Baker et al 2002). This deletion encompasses 10 genes, including IQSEC3. The aims of my study were therefore:

- To use bioinformatics approaches to understand the functional domains of IQSEC3 and the molecular variants generated by alternative splicing.

- To establish the nature of the IQSEC3 interaction with gephyrin, using the YTH system and mammalian models of gephyrin clustering, using epitope-tagged IQSEC3 and EGFP-gephyrin.

- To determine whether IQSEC3 interacts with inhibitory GABA_A and GlyR subunits in the YTH system
To use array CGH and next-generation sequencing to investigate the 1.65 Mb deletion on 12p13.33 reported in Baker et al (2002) and determine the likely breakpoints of this microdeletion.

5.3 Bioinformatics of IQSEC3 variants

A search of Ensembl, NCBI and UCSC databases revealed that IQSEC3 is located on human chromosome 12p13.33. Isoforms of IQSEC3 generated by alternative splicing are shown in Figure 5.2. IQSEC3-1 (NCBI code: NM_001170738), consists of 14 exons spanning 111.7 kb which produces a 6,976 bp mRNA transcript. The open reading frame is 3,549 bp with a predicted protein of 1182 residues (NCBI code: NP_001164209). A shorter isoform, IQSEC3-2 (NCBI code: NM_015232), has 13 exons spanning 93.95 kb. The mRNA transcript is 2,701 bp with an open reading frame of 2,280 bp. This shorter isoform is 759 residues (NCBI code: NP_056047).

Exons 4-13 of the longer isoform are identical to exons 3-12 in the shorter isoform, as shown in Figure 5.2. A summary of all reported transcripts is given in Table 5.1. Both isoforms contain the IQ-like domain, catalytic Sec7 domain and PH domain, whilst only IQSEC3-1 contains a C terminal PDZ-binding motif. The rat IQSEC3 transcripts identified in the gephyrin yeast two-hybrid screen are a match for the IQSEC3-1 isoform, although they only cover the N-terminus and do not extend further than the IQ-like domain. The peptide sequences of IQSEC3-1 and IQSEC3-2 are shown in Figure 5.3.
Table 5.1 IQSEC3 variants generated by alternative splicing

This table shows all the transcripts recorded in either the NCBI or Ensembl databases. There are two key protein coding isoforms IQSEC3-1 and IQSEC3-2. Several shorter transcripts have also been reported, although it is unlikely that these transcripts are common or encode fully functional proteins.

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<th>Open Reading Frame (bp)</th>
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<td>IQSEC3-002 / ENST00000538872 NM_001170738 (IQSEC3-1)</td>
<td>7094 6976</td>
<td>3549</td>
<td>ENSP00000437554</td>
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</table>
Figure 5.2 Alternative splicing of IQSEC3/BRAG3/KIAA1110.

The three key transcripts reported as protein coding are depicted. Exons are shown as green blocks and are to scale, intron length is not to scale. Exons 3/4-13 (highlighted in yellow) are identical in IQSEC3-1 and IQSEC3-2. (B) The protein structure of IQSEC3-1, IQSEC3-2 and the variant found in the YTH screen are shown. IQSEC3-2 is truncated at both the N- and C-termini compared to IQSEC3-1.
Figure 5.3 Peptide sequences of the major isoforms of human IQSEC3

Alternating exons are shown in black and blue text, with residues overlapping splice sites depicted in red. The different N- and C-termini of the proteins are underlined, whilst the majority of the sequence (highlighted in grey) is identical.

5.4 Mapping of reciprocal binding sites on gephyrin and IQSEC3

Bioinformatics analysis revealed that the IQSEC3 variant found in the library screen corresponded to part of the N-terminus of the IQSEC3-1 isoform. I therefore cloned a cDNA for the entire N-terminus (residues 1-649) of IQSEC3-1 into pYTH16. The gephyrin-IQSEC3 interaction was confirmed to be mediated via the N-terminus of...
IQSEC3, as shown in Figure 4.5, which also revealed that the IQSEC3 IQ-like domain does not appear to interact with calmodulin, unlike IQSEC1 and IQSEC2. However, the gephyrin-IQSEC3 interaction was relatively weak, possibly due to the toxic effects of expressing full-length gephyrin in yeast. To identify the reciprocal binding sites for the gephyrin-IQSEC3 interaction a variety of yeast two-hybrid constructs were used expressing different fragments for each of the proteins. Initially, the pYTH16-IQSEC3 N-terminal bait was tested against a selection of gephyrin preys expressed from partial gephyrin cDNAs cloned into pACT2. These gephyrin constructs were designed to contain the three different domains of gephyrin, G, C and E, as shown in Figure 5.4.

![Figure 5.4 Domain structure of gephyrin.](image)

Best estimates for the start and end of individual domains were made based on crystal structures of the gephyrin G and E domains (Schwarz et al 2001; Kim et al 2006). Two different C-domain constructs were used, containing two commonly inserted alternatively spliced cassettes (C3 and C4a). A summary of the constructs used can be seen in Table 5.2.
<table>
<thead>
<tr>
<th>Construct</th>
<th>Amino Acids</th>
<th>Further Information</th>
</tr>
</thead>
<tbody>
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<td>pACT2-gephyrin G domain</td>
<td>1-173</td>
<td>A construct of amino acids 1-189 was also made, but the shorter 1-173 construct was found to be sufficient for interactions</td>
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<td>+C3</td>
<td></td>
</tr>
<tr>
<td>pACT2-gephyrin C domain</td>
<td>173-323</td>
<td>Contains C4a cassette: QI RR P D E S K G V AS R V G S L K</td>
</tr>
<tr>
<td>(C4a isoform)</td>
<td>+C4a</td>
<td></td>
</tr>
<tr>
<td>pACT2-gephyrin E domain</td>
<td>323-736</td>
<td>A construct containing the end of the C domain and E domain was also tested (306-736), but the shorter construct (323-736) was found to be sufficient for interactions</td>
</tr>
<tr>
<td>pACT2-gephyrin Full-length P1 isoform</td>
<td>1-736</td>
<td>Full-length gephyrin containing no additional cassettes</td>
</tr>
<tr>
<td>pACT2-gephyrin Full-length C3 isoform</td>
<td>1-736</td>
<td>Full-length gephyrin containing the C3 cassette</td>
</tr>
</tbody>
</table>

**Table 5.2 Gephyrin constructs made in the pACT2 vector for testing against IQSEC3.**

Positive controls for each of the gephyrin domains were included, to ensure that the gephyrin preys were expressing correctly. The GlyR β subunit is known to interact with the E domain of gephyrin (Kim et al 2006), and dynein light chains are known to interact with the gephyrin C domain (Fuhrmann et al 2002). Since there were no known interactors of the gephyrin G domain at the time of testing, a pYTH16-gephyrin G domain construct was made, since the G domain is known to trimerise (Sola et al 2001). The results of testing these pACT-gephyrin constructs against pYTH16-IQSEC3 and control baits can be seen in Figure 5.5.
Figure 5.5 LacZ assays reveal that IQSEC3 binds to the gephyrin G domain.

LacZ assay filters show that an N-terminal IQSEC3 bait is able to interact with the gephyrin G domain. G domain-G domain, DLC2-C domain and GlyR β subunit-E domain interactions were observed as predicted, demonstrating that all gephyrin preys were correctly expressed.
The results clearly show that the N-terminus of IQSEC3 interacts with the gephyrin G domain. Since the IQSEC3 IQ-like domain does not appear to interact with calmodulin in the YTH system, we decided to map the gephyrin binding site on IQSEC3. We constructed baits for the N-terminus of IQSEC3, dividing this domain into 50 amino acid overlapping fragments which were tested against the gephyrin G domain prey. The result of this analysis can be seen in Figure 5.6. The gephyrin binding site on IQSEC3 is located between amino acids 160 and 210, whereas the IQ-like domain is situated at 312-323, as shown in Figure 5.7.

5.5 IQSEC3-gephyrin interactions in mammalian cells

To confirm that IQSEC3 and gephyrin interact in mammalian cells, I co-transfected pEGFP-gephyrin with pCAGGS-FLAG-IQSEC3 (kindly provided by Hiroyuki Sakagami) into HEK293 cells using electroporation. FLAG-IQSEC3 and EGFP-gephyrin showed clear colocalisation, albeit in cytoplasmic aggregates (Figure 5.8). These images resemble cells expressing EGFP-gephyrin together with inactive collybistin isoforms containing the SH3 domain (Harvey et al 2004), suggesting that additional proteins may be required for IQSEC3 to mediate submembrane clustering of gephyrin. Since one obvious candidate was the GTPase Arf6, we co-expressed HA-tagged Arf6 together with EGFP-gephyrin and FLAG-IQSEC3, but this did not noticeably alter the subcellular localisation of either gephyrin or IQSEC3.
Figure 5.6 *LacZ* assays define the gephyrin-binding motif on IQSEC3.

*LacZ* assay results show that residues 160-210 of IQSEC3 are responsible for mediating the interaction with the gephyrin G domain and full-length gephyrin.
Figure 5.7 Rat IQSEC3 protein sequence showing the location of the gephyrin binding site relative to other functional domains.

The gephyrin binding site is distinct from any of the known functional domains of IQSEC3 (IQ-like, Sec7, PH and PDZ-binding motif), and is contained within residues 160-210.
Figure 5.8 Confocal imaging of HEK293 cells over-expressing EGFP-gephyrin, FLAG-IQSEC3 and HA-Arf6

Panel A-C: Co-expression of EGFP-gephyrin with FLAG-IQSEC3-1. FLAG-IQSEC3-1 was visualised using a rabbit anti-FLAG primary antibody and a secondary antibody Alexa Fluor 546 Goat Anti-Rabbit IgG. Panel D-G: Co-expression of EGFP-gephyrin with FLAG-IQSEC3-1 and HA-Arf6. Arf6 was visualised using a mouse anti-HA primary antibody (Cambridge Bioscience MMS-101P-200) and secondary antibody Alexa Fluor 633 Goat Anti-Mouse IgG (Invitrogen A21052). Primary antibodies were diluted 1 in 200 in 2% (w/v) BSA in PBS, secondary antibodies were diluted 1 in 600 in 2% (w/v) BSA in PBS. Images were taken using a Zeiss LSM710 using a Plan-Apochromat 63/1.40 Oil DIC M27 objective. Scale bars: 20μm.
5.6 Investigation of the interaction between IQSEC3 and inhibitory receptor subunits

Although IQSEC3-gephyrin interactions can be observed in both YTH and cellular clustering assays, it would appear that an activator other than Arf6 is required for IQSEC3 to mediate submembrane clustering of gephyrin. Calmodulin was initially considered to be a good candidate to fulfil this role, since calmodulin binding appears to regulate IQSEC2 activity. However, our YTH experiments indicate that IQSEC3 does not appear to interact with calmodulin. Since we considered that the calmodulin IQSEC3 might be Ca$^{2+}$-dependent, we added varying concentrations (0 to 100 mM) of CaCl to the yeast media. However, this did not result in any observable interaction.

Since the GABA$_\alpha$R $\alpha$2 subunit is capable of binding to the collybistin SH3 domain and triggering collybistin-mediate gephyrin clustering (Saiepour et al 2009), we also tested interactions with baits and preys for GABA$_\alpha$R ($\alpha$1-6, $\beta$1-3 and $\gamma$1-3) and GlyR subunits ($\alpha$1-3 and $\beta$). However, we were not able to observe an interaction of IQSEC3 with these baits and preys in the yeast two-hybrid system. IQSEC3 was also tested against the neuroligin family, since neuroligin 2 (NL2) has been shown to be involved in inhibitory receptor clustering (Poulopoulos et al 2009). Unfortunately testing the neuroligin interactions with IQSEC3 in yeast proved to be problematic. Due to high levels of auto-activation of neuroligin C-terminal baits, none of the results were considered valid, despite different YTH vectors being tried (pDS, pYTH16) and both rat and human neuroligins being cloned.
5.7 A microdeletion on human chromosome 12p affecting \textit{IQSEC3} causing autosomal ID and aggressive behaviour

Given the clear association of gephyrin and collybistin in yeast and mammalian systems, I also sought to establish possible links between IQSEC3 and neurological disease. Given the previous association of mutations in \textit{ARHGEF9} encoding collybistin to X-linked intellectual disability (Harvey et al 2004; Kalscheuer et al 2009), it was of significant interest that a maternally-inherited 1.65 Mb microdeletion on 12p13.33, which contains IQSEC3, was reported to cause moderate ID and behavioural problems in a 15-year-old boy (Baker et al 2002). The proband was a 15 year old male (in 2002), he was described as having deep-set eyes, prominent ears, mild thoracic kyphoscoliosis (abnormal curvature of the spine) and a short neck, but otherwise had minimally dysmorphic facial features. Intellectual assessment at 14 years determined that he had moderate intellectual disability. He also had significant behavioural problems and had been suspended from school as a result of violent episodes. Fluorescence \textit{in situ} hybridization (FISH) was carried out for the proband, and both parents and other family members. This revealed that the proband and mother had a deletion at 12p13.33, whilst the results were normal for the rest of the family members tested. The presence of this deletion in the mother was especially interesting, since although she had required some special education when younger, she now works and looks after her family without any problems. However, it should be noted that her intelligence was not formally assessed (Baker et al 2002).

Since the deletion appears to have a much milder, if any, phenotype in the mother, my initial suspicion was that the proband might have inherited a mutation in one of the genes in this region from his father, and would therefore effectively be homozygous for loss of function for a given gene. Another possibility was that the deletion resulted in a fusion between two genes, which might create a fusion protein
that has a deleterious effect. A view of the 12p13.33 region is shown in Figure 5.9, using information from the UCSC genome browser, the deletion start and end point as suggested by Baker et al (2002) is indicated. Further information about the genes in this region is given in Table 5.3.

Of the genes located in the deleted region there were several that were likely to have a neurological function, Baker et al suggested SLC6A12 and WNT5B should be considered as candidate genes contributing to the ID phenotype. SLC6A12 and SLC6A13 are GABA transporters, but more recent data would suggest these are unlikely to be the cause of the phenotype. SLC6A12 has been shown to be predominantly expressed in the liver, and at lower levels in the kidney and brain, its main role is thought to be in the liver, acting with other betaine transporter proteins (Zhou et al 2012). SLC6A13 mRNA is found in brain, lung, kidney and testis so like SLC612 may have a peripheral role or could be acting as a GABA transporter in the brain (Christiansen et al 2007). Additionally ERC1 may be involved in regulating neurotransmitter release so is also a possible cause, along with IQSEC3. Recently mutations in KDM5A/JARID1A have been identified as a cause of autosomal recessive intellectual disability (Najmabadi et al 2011), and other mutations in other histone demethylases are also known to cause ID (Abidi et al 2008). Previously KDM5A/JARID1A has been linked to susceptibility to ankylosing spondylitis (Pointon et al 2011), but as a histone demethylase is likely to be involved in regulating the expression of many genes.
Figure 5.9 Genes located at chr 12p13.33 in the vicinity of the 1.65 Mb microdeletion.

The genes shown are based on information from the UCSC genome browser Human Feb. 2009 (GRCh37/hg19) Assembly. There are 13 genes in the region, the approximate gene size and position is indicated. The deletion as mapped by Baker et al (2002) using FISH is shown by a red arrow.
<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Gene Expression</th>
<th>Predicted Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>IQSEC3</td>
<td>Highest in the CNS, low in peripheral tissues</td>
<td>Guanyl-nucleotide exchange factor activity for the Arf family of GTPases, localised to inhibitory synapses</td>
</tr>
<tr>
<td>SLC6A12</td>
<td>Widely expressed</td>
<td>Solute carrier family 6, (neurotransmitter transporter, betaine/GABA), member 12</td>
</tr>
<tr>
<td>SLC6A13</td>
<td>Widely expressed, highest in retina</td>
<td>Solute carrier family 6 (neurotransmitter transporter, GABA), member 13</td>
</tr>
<tr>
<td>KDM5A/JARID1A</td>
<td>Expression limited to blood cells</td>
<td>Lysine (K)-specific demethylase 5A</td>
</tr>
<tr>
<td>CCDC77</td>
<td>Widely expressed</td>
<td>Coiled-coil domain containing 77</td>
</tr>
<tr>
<td>B4GALNT3</td>
<td>Widely expressed</td>
<td>Beta-1,4-N-acetyl-galactosaminyl transferase 3</td>
</tr>
<tr>
<td>NINJ2</td>
<td>Widely expressed</td>
<td>Nerve injury-induced protein 2 - cell surface adhesion protein that is upregulated in Schwann cells surrounding the distal segment of injured nerve, and promotes neurite outgrowth, may have a role in nerve regeneration after nerve injury</td>
</tr>
<tr>
<td>WNK1</td>
<td>Highly expressed in early erythroid cells</td>
<td>WNK lysine deficient protein kinase 1. The encoded protein may be a key regulator of blood pressure by controlling the transport of Na⁺ and Cl⁻ ions</td>
</tr>
<tr>
<td>RAD52</td>
<td>Widely expressed</td>
<td>Similar to <em>Saccharomyces cerevisiae</em> Rad52, a protein important for DNA double-strand break repair and homologous recombination</td>
</tr>
<tr>
<td>ERC1</td>
<td>Widely expressed</td>
<td>ELKS/RAB6-interacting/CAST family member 1 (ERC1). Member of a family of RIM-binding proteins, which regulate neurotransmitter release</td>
</tr>
<tr>
<td>WNT5B</td>
<td>Widely expressed, highest in prostate.</td>
<td>Wingless-type MMTV integration site family, member 5B (WNT5B), involved in development and differentiation</td>
</tr>
<tr>
<td>FBXL14</td>
<td>Highly expressed in colon and blood cells</td>
<td>Ubiquitin-protein ligase activity</td>
</tr>
<tr>
<td>ADIPOR2</td>
<td>Highly expressed in CNS and blood cells</td>
<td>Adiponectin receptor 2</td>
</tr>
</tbody>
</table>

Table 5.3 Genes present in the 12p13.33 microdeletion region.

Information regarding expression of each gene was gathered from www.biogps.org.
Information regarding function was gathered from http://www.ncbi.nlm.nih.gov/.
Unpublished array CGH data generated by Eric Haan at the Women’s and Children’s Hospital in Adelaide, identified a second chromosomal defect in the proband, in the form of a microduplication of 1.59 Mb at Xp22.31, also inherited from his mother. The genes in this region are HDHD1A (haloacid dehalogenase like hydrolase) and STS (steroid sulphatase isozyme S). The pathogenicity of this microduplication is the subject of some debate in the literature (Faletra et al 2012). It is well documented that a deletion at this point causes X-linked ichthyosis (Carrascosa-Romero et al 2012; Nagtzaam et al 2012), as a result of the loss of the STS gene. The current viewpoint is that whilst there is no clear phenotype that can be shown to result specifically from copy number variation at Xp22.13, it may have an additive effect to other mutations, or could predispose individuals to disease. As such duplication at this point could be considered a risk factor or modifier for ID (Li et al 2010; Liu et al 2011). Therefore, I decided to investigate the deletion at 12p13.33 in more detail, as this is likely to be the major cause of the phenotype seen in this case, although the duplication at Xp22.31 may contribute to overall risk.

5.8 Deletion breakpoint mapping reveals a potential IQSEC3-ADIPOR2 gene fusion and potentially deleterious SNVs

Genomic DNA from the proband was sent for next-generation sequencing (UCL Institute of Child Health). As shown in Figure 5.10, the chromosome 12 region was selectively sequenced using NimbleGen Sequence Capture technology (http://www.nimblegen.com/seqcap/). A standard shot-gun sequencing library was made from genomic DNA and hybridised to a SeqCap EZ oligonucleotide pool targeting the 12p13.33 region. Streptavidin beads were used to pull down the complex of capture oligos and genomic DNA fragments, and unbound fragments were removed by washing. The enriched fragment pool was eluted, amplified by PCR and subjected to high-throughput sequencing on an Illumina HiSeq 2000. This resulted in high sequence coverage >100× over the region of interest.
SNVs discovered in the genes within or flanking the minimal region are shown in Table 5.4. Next-generation sequencing data also allowed identification of intronic homozygous and heterozygous SNVs in the proband. DNA captured from within the deleted region should show only homozygous SNVs, since the genetic information is inherited solely from the father in this region. Whilst it is not possible to identify the precise breakpoint from SNP analysis, due to their random spacing, loss of heterozygosity was observed between exons 1b and 2 of IQSEC3 and exons 3 and 4 of ADIPOR2. CACNA2D4 is the next gene downstream from ADIPOR2, the SNVs in this gene are heterozygous confirming that DNA is inherited from both mother and father at this point; showing the end of the deletion is within ADIPOR2. It can be seen in Table 5.4 that there are many synonymous SNVs in three of the genes in the deletion, IQSEC3, SLC6A13 and WNK1. There are also a few non-synonymous SNVs which were of more interest, the SIFT, Polyphen-2 and Mutation Taster programs were used to examine the impact of the amino acid substitutions caused by these SNVs. The non-synonymous SNV in IQSEC3, which results in P283S, was predicted to be tolerated by SIFT and mutation taster. This residue is at the N terminus of the protein, prior to the IQ like motif, and after the gephyrin binding region, it is therefore unlikely to affect the function of IQSEC3. There are also three non-synonymous SNVs in WNK1. The first of these, which causes A141T in isoform 4 of WNK1, is predicted to be tolerated. This protein contains a protein kinase domain and an oxidative-stress-responsive kinase 1 C terminal. The A141T
mutation is prior to both of these domains. The second SNV in \textit{WNK1} is the insertion of a single nucleotide which results in a frameshift from position V724 of isoform 3. This mutation is after both functional domains, however the frameshift causes a stop codon at position 770, so a truncated protein results. Both functional domains are intact so it is unclear how this will impact the protein. The third SNV in \textit{WNK1} causes the change C1259S in isoform 2 of \textit{WNK1}, again this is after the functional domains and is predicted to be tolerated.

Of the other SNVs identified in the regions after the deletion, the majority were predicted to be tolerated. Of note there was a SNV in the start codon of \textit{VDR}, however the presence of another methionine residue at position 4 means that only the first 3 amino acids are lost. The E972Q in \textit{GLI1} was predicted to be damaging by polyphen-2 but not by SIFT or mutation taster analysis. \textit{GLI1} encodes the \textit{GLI} family zinc finger 1 (GLI1) which has been implicated in several cancers, GLI1 is responsible for regulating the Hedgehog pathway which controls differentiation and cell growth (Carpenter and Lo 2012). It is possible that the mutation is reported as damaging as it may contribute to tumourigenesis. The function of this protein does not suggest this mutation would cause intellectual disability or the behavioural phenotype.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Exonic Function</th>
<th>NCBI identifier: nucleotide change: aa change</th>
<th>SIFT prediction</th>
<th>Polyphen-2 prediction</th>
<th>Mutation Taster prediction</th>
</tr>
</thead>
<tbody>
<tr>
<td>IQSEC3</td>
<td>non-synonymous SNV</td>
<td>NM_001170738:c.C847T:p.P283S</td>
<td>0.95 - tolerated</td>
<td>-</td>
<td>Polymorphism</td>
</tr>
<tr>
<td></td>
<td>synonymous SNV</td>
<td>NM_001170738:c.T3408C:p.G1136G</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SLC6A12</td>
<td>synonymous SNV</td>
<td>NM_001206931:c.T447C:p.T149T</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WNK1</td>
<td>synonymous SNV</td>
<td>NM_001184985:c.T258C:p.C86C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>non-synonymous SNV</td>
<td>NM_001184985:c.G421A:p.A141T</td>
<td>0.18 - tolerated</td>
<td>0.067 - benign</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>synonymous SNV</td>
<td>NM_001184985:c.T1479C:p.D493D</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>frameshift insertion</td>
<td>NM_213655:c.2172_2173insC:p.V724fs</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>synonymous SNV</td>
<td>NM_213655:c.T2268C:p.P756P</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>synonymous SNV</td>
<td>NM_014823:c.G2325A:p.Q775Q</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>synonymous SNV</td>
<td>NM_014823:c.C3303T:p.T1101T</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>non-synonymous SNV</td>
<td>NM_014823:c.G3776C:p.C1259S</td>
<td>1.00 - tolerated</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CACNA2D4</td>
<td>synonymous SNV</td>
<td>NM_172364:c.G2517A:p.A839A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>synonymous SNV</td>
<td>NM_172364:c.A1515G:p.T505T</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>non-synonymous SNV</td>
<td>NM_172364:c.A979G:p.I327V</td>
<td>1.00 - tolerated</td>
<td>0 - benign</td>
<td>Polymorphism</td>
</tr>
<tr>
<td>A2ML1</td>
<td>synonymous SNV</td>
<td>NM_144670:c.T3843C:p.V1281V</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRB4</td>
<td>non-synonymous SNV</td>
<td>NM_002723:c.C347A:p.P116H</td>
<td>0.27 - tolerated</td>
<td>-</td>
<td>Polymorphism</td>
</tr>
<tr>
<td>ARHGDIB</td>
<td>synonymous SNV</td>
<td>NM_001175:c.G504C:p.A168A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VDR</td>
<td>non-synonymous SNV</td>
<td>NM_000376:c.T2C:p.M1T</td>
<td>0 - affects protein function (low confidence prediction)</td>
<td>0.289 - benign</td>
<td>Polymorphism – mutation in start codon, first 3 amino acids lost.</td>
</tr>
<tr>
<td>FAM186A</td>
<td>non-synonymous SNV</td>
<td>NM_001145475:c.T4267C:p.F1423L</td>
<td>0.75 – tolerated</td>
<td>0 - benign</td>
<td>Polymorphism</td>
</tr>
<tr>
<td>Gene</td>
<td>Type of Mutation</td>
<td>Exon/Description</td>
<td>SIFT Score</td>
<td>Polyphen Score</td>
<td>Mutation Taster Score</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------</td>
<td>--------------------------------------------------------</td>
<td>------------</td>
<td>----------------</td>
<td>------------------------</td>
</tr>
<tr>
<td>KRT77</td>
<td>non-synonymous SNV</td>
<td>NM_175078:c.A1597G:p.R533G</td>
<td>-</td>
<td>0 - benign</td>
<td>0.004 - benign</td>
</tr>
<tr>
<td>GLI1</td>
<td>non-synonymous SNV</td>
<td>NM_001160045:c.G2914C:p.E972Q</td>
<td>0.38</td>
<td>0.971 - probably damaging</td>
<td>Polymorphism</td>
</tr>
<tr>
<td>UTP20</td>
<td>synonymous SNV</td>
<td>NM_014503:c.C612T:p.N204N</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GCN1L1</td>
<td>non-synonymous SNV</td>
<td>NM_006836:c.T6463G:p.Y2155D</td>
<td>0.29</td>
<td>0 - benign</td>
<td></td>
</tr>
<tr>
<td>GCN1L1</td>
<td>non-synonymous SNV</td>
<td>NM_198240:c.C3102G:p.D1034E</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP17</td>
<td>non-synonymous SNV</td>
<td>NM_016155:c.G836A:p.R279H</td>
<td>0.19</td>
<td>0.004 - benign</td>
<td></td>
</tr>
<tr>
<td>ULK1</td>
<td>non-synonymous SNV</td>
<td>NM_003565:c.C725T:p.T242I</td>
<td>0.10</td>
<td>0.003 - benign</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.4 SNVs discovered in genes in the Chr12p13.3 microdeletion interval.

The likely impact of non-synonymous mutations was determined through the use of SIFT, polyphen-2 and mutation taster programs. In a few cases it was not possible to give a score as sometimes the programs were unable to find the correct transcript variant.
We also re-analysed the 12p13.33 using array CGH (Figure 5.11), using DNA samples from the mother, father and proband. This aspect of the project was performed in collaboration with Dr Reinhard Ullmann (Max-Planck Institute for Molecular Genetics, Berlin) an expert in the array CGH approach, using the 400K Whole Human Genome CGH array (Agilent Technologies). The array contains 411,056 oligonucleotide probes (60 mers) with a median probe spacing of 5.3 kb. This analysis suggested that the deletion mapped to human chr12: between nucleotides 79,019-1,743,750 (Hg18). Again, this new data places the start of the breakpoint in the intron between exons 2 and 3 of IQSEC3. The data from the previous array (Hahn) also places the breakpoint in this intron, but much further downstream. The other endpoint of the deletion is within ADIPOR2, again the data from the two arrays differs slightly but both place the deletion in the intron between exons 2 and 3 of ADIPOR2 (Figure 5.12).

![Array Comparative Genomic Hybridisation](image)

**Figure 5.11 Array Comparative Genomic Hybridisation.**

Genomic DNA from a test sample and a reference sample are labelled using different fluorophores and hybridised to thousands of oligonucleotide probes derived from most of the known genes and non-coding regions of the genome, printed on a glass slide. The ratio of the fluorescence intensity of the test DNA to that of the reference DNA is then calculated, revealing copy number variants (CNVs) in a particular location in the genome.
Figure 5.12 Mapping of the breakpoints for the 12p13.33 deletion.
(A) Overview of the 12p13.33 region, with the deletion as mapped by Baker et al (2002). (B) The start of the deletion (breakpoint in IQSEC3) as mapped by array CGH array from different groups is shown as a red line. (C) The end of the deletion (breakpoint in ADIPOR2) as mapped by Ullmann and Haan array CGH array is shown as a red line. Black arrows indicate loss of heterozygosity from SNP analysis of sequencing data.
5.9 Discussion

In order to determine if the interaction between gephyrin and the ArfGEF IQSEC3 identified in my library screen was of relevance for inhibitory GABA<sub>A</sub> and glycine receptor clustering, I used several methods to validate this interaction. Initial bioinformatics analysis showed that multiple IQSEC3 were created by alternative splicing, with two key protein isoforms containing all four functionally important regions – IQ-like motif, Sec7 domain, PH domain and PDZ-binding motif. The longest isoform, IQSEC3-1, matched the IQSEC3 variants found in my library screen. The IQSEC3 fragments identified in the YTH screen also suggested that the gephyrin-binding site must lie at the N-terminus of IQSEC3, before the IQ-like domain. Further testing of overlapping preys revealed that a linear binding site for gephyrin was located between residues 160-210 of IQSEC3. The reciprocal binding site for IQSEC3 on gephyrin was determined to be in the G domain. To my knowledge, only one other interactor of the G domain has been reported to date: heat shock cognate protein 70 (Hsc70), which binds gephyrin in an ADP-dependent manner (Machado et al 2011). Interestingly, the binding sites for other gephyrin interactors, such as inhibitory receptors and collybistin are all within the C or E domains (Kins et al 2000; Harvey et al 2004; Saiepour et al 2010; Mukherjee et al 2011; Tretter et al 2011) which suggests that binding of IQSEC3 and these proteins can occur simultaneously on gephyrin.

In cellular clustering assays, I observed colocalisation of EGFP-gephyrin and FLAG-IQSEC3 in cytoplasmic aggregates rather than submembrane clusters. This suggests that IQSEC3-mediated clustering may require activation in a manner similar to neuroligin-2 or the GABA<sub>A</sub>R α2 subunit mediated collybistin activation (Poulopoulos et al 2009; Saiepour et al 2010). Calmodulin, neuroligins and inhibitory GABA<sub>A</sub>R and GlyR subunits were all good candidates for IQSEC3 activators, given their known role in the activation of ArfGEFs (Myers et al 2012) and clustering of
inhibitory receptors (Alldred et al 2005; Poulopoulos et al 2009; Saiepour et al 2010). Unfortunately YTH testing did not reveal an interaction between IQSEC3 and any of these proteins. Fukaya et al (2011) showed colocalisation of IQSEC3 and the GABA_A R α1 subunit and the GlyR α subunits, as well as with gephyrin. Combined with the interactions they report between IQSEC3 and utrophin/dystrophin and S-SCAM/MAGI-2, it is reasonable to suggest a role for IQSEC3 in the localisation of gephyrin and GABA_A R/GlyRs at inhibitory synapses. To assess the role of IQSEC3 in a neuronal context, I have recently generated dominant-negative IQSEC3 constructs by site-directed mutagenesis. Firstly, I have made an IQSEC3 mutant (E749K) that will still bind to gephyrin and other interactors, but will be defective in ArfGEF activity. I also made two additional constructs either over expressing the IQSEC3 gephyrin-binding motif (160-210) or deleting this motif from full-length IQSEC3. These constructs are currently being assessed for their effects on gephyrin and receptor clustering in transfected cortical neurones in collaboration with Angel de Blas (University of Connecticut).

The identification of *IQSEC2* as an XLID gene (Shoubridge et al 2010) led me to suspect that genetic defects affecting *IQSEC3* might be a potential cause of autosomal ID. Further examination of the microdeletion originally identified by Baker et al (2002) allowed us to determine that the start point of the deletion is between exons 2 and 3 of *IQSEC3*, and the end of the deletion is between exons 2 and 3 of *ADIPOR2*. The protein encoded by exons 1 and 2 of *IQSEC3* is shown in Figure 5.13. This does not include the IQ like domain but does include the majority of the gephyrin-binding site. Therefore it is possible that a fragment of the IQSEC3 protein is being produced that has no GEF activity, but can bind to gephyrin.
Figure 5.13 Peptide sequence encoded by exons 1 and 2 of \textit{IQSEC3}.

If exons 1 and 2 of \textit{IQSEC3} are joined to exon 3 of \textit{ADIPOR2} the open reading frame is disrupted so that the protein terminates early after a stop codon is reached in exon 4 of \textit{ADIPOR2}.
Figure 5.14 IQSEC3-ADIPOR2 fusion protein

Peptide sequence encoded by exons 1 and 2 of IQSEC3 (shown in black and blue) followed by exons 3 and 4 of ADIPOR2 (red and green). The change in reading frame results in an early stop codon in exon 4 of ADIPOR2.

Due to the complex nature of this re-arrangement and the SNVs identified in IQSEC3 and WNK1, it is not currently possible to definitively state that IQSEC3 is an autosomal ID gene. However, our next-generation sequencing data suggests that SNVs in IQSEC3 and WNK1, and/or a fusion between IQSEC3 and ADIPOR2 could be responsible for the complex phenotype observed in this individual. Future work will be needed to: i) Validate the SNPs observed in IQSEC3 and WNK1 using Sanger sequencing, follow the inheritance patterns of these changes in the family and establish their frequency in control DNAs; ii) Use PCR to clone the microdeletion breakpoint – this should be possible given that we have narrowed down the breakpoint; iii) To use RT-PCR on first-strand cDNA generated from patient induced pluripotent stem (IPS) cell lines to test for an IQSEC3-ADIPOR2 gene fusion. Certainly, since IQSEC3 is in the same family of proteins as the known XLID gene IQSEC2 and appears to have a specialised role at inhibitory synapases, it is reasonable to assume that defects in IQSEC3 will have a neurological phenotype.
6 INTERACTION OF INHIBITORY GABA\(_A\)R AND GLYR SUBUNITS WITH GEPHYRIN

6.1 Background

Gephyrin is clearly an important scaffolding protein at inhibitory synapses, mediating the clustering of inhibitory GlyRs and selected GABA\(_A\)R subtypes (Kneussel et al 1999b; Sassoe-Pognetto et al 2000; Alldred et al 2005; Kim et al 2006; Tretter et al 2008; Saiepour et al 2010; Tretter et al 2011). Perhaps the most well characterised of receptor/gephyrin association is the GlyR β subunit gephyrin E domain interaction (Prior et al 1992, Meyer et al 1995; Rees et al 2001; Harvey et al 2004), which has even been visualised at the level of crystal structure (Kim et al 2006). This pivotal study also examined the effect of nine gephyrin E domain mutations (D327A, F330A, R653A, I656A, Y673F, Q683A, P713E, P713A/P714A and D729A) on the interaction with the GlyR β subunit. These residues were identified as being located in the binding pocket forming interactions with the GlyR β subunit intracellular loop. Pull-down assays showed that the F330A and P713A/P714A significantly reduced GlyR β subunit binding, whilst P713E completely abolished binding. Interactions were not significantly altered by the D327A, R653A, I656A, Q683A and D729A mutations (Kim et al 2006).

By contrast, understanding the basis of GABA\(_A\)R clustering by gephyrin has proved more challenging - only in the last couple of years have direct interactions between gephyrin and selected GABA\(_A\)R subunits been reported (Tretter et al 2008, 2011; Saiepour et al 2010; Mukherjee et al 2011). This is perhaps understandable given the plethora of GABA\(_A\)R subunits available to mediate this interaction (α1-6, β1-3, γ1-3, δ, ε, θ and π). A key breakthrough came from the laboratory of Stephen Moss, who showed that a direct interaction between the GABA\(_A\)R α2 subunit and gephyrin was necessary for interactions with gephyrin and GABA\(_A\)R clustering (Tretter et al
Residues 336-347 of the GABA\(_\alpha\)R \(\alpha2\) subunit large intracellular loop proved critical for the interaction (Tretter et al 2008), although a subsequent study demonstrated that this region also bound to the SH3 domain of collybistin (Saiepour et al 2010). However, at the start of my studies, no other GABA\(_\alpha\)R subunit had been shown to interact directly with gephyrin.

As mentioned previously, there is some disagreement over the requirement for gephyrin in GABA\(_\alpha\)R clustering (Essrich et al 1998; Kneussel et al 1999b; Levi et al 2004). The gephyrin knockout mouse (Feng et al 1998) has been important for assessing the impact of a loss of gephyrin on the synaptic clustering of different GABA\(_\alpha\)R types. Initially, gephyrin knockout mice were reported to show a loss of GABA\(_\alpha\)R \(\alpha2\) and \(\gamma2\) subunit clustering at synapses in spinal cord and primary hippocampal neurons (Kneussel et al 1999). Immunostaining for these subunits revealed a cytoplasmic localisation, suggesting that gephyrin was required for submembrane clustering (Kneussel et al 1999). Staining for the \(\alpha3\) and \(\beta2/\beta3\) subunits in spinal cord also revealed a significant decrease in clustering of these receptors in the gephyrin knockout mouse, implying a role for gephyrin in clustering of these subtypes. However, other reports suggest that GABA\(_\alpha\)R \(\alpha1\) and \(\alpha5\) subunit clustering is unaltered in gephyrin knockout mice (Fischer et al 2000; Kneussel et al 2001; Lévi et al 2004) demonstrating that gephyrin-independent clustering mechanisms also exist in vivo. Certainly, GABA\(_\alpha\)Rs containing the \(\alpha4\), \(\alpha5\) and \(\alpha6\) subunits are often located preferentially at extrasynaptic sites (Belelli et al 2009) and are likely to be localized by other clustering factors, such as the actin-binding protein radixin (Loebrich et al 2006).
6.2 Study Aims:

Further evidence is clearly required to establish exactly which GABA<sub>A</sub>R subunits interact with gephyrin. Another open question is whether the gephyrin E domain residues critical for the GlyR β subunit-gephyrin interaction are the same as those responsible for GABA<sub>A</sub>R-gephyrin interactions, i.e. is concurrent binding of GlyRs and GABA<sub>A</sub>Rs to gephyrin permitted or mutually exclusive? The aims of my study were therefore:

- To investigate the molecular basis of a possible interaction between gephyrin and the GABA<sub>A</sub>R α1 subunit, including mapping of the gephyrin-binding site on the α1 subunit.

- Investigate the molecular basis of a possible interaction between gephyrin and the GABA<sub>A</sub>R α3 subunit, including mapping of the gephyrin binding site on the α3 subunit, and the reciprocal α3 binding site on gephyrin.

- Examination of residues within the gephyrin E domain that may have a critical role in both GABA<sub>A</sub>R and GlyR binding.

6.3 Phospho-dependent interactions of the GABA<sub>A</sub> receptor α1 subunit with gephyrin

GABA<sub>A</sub>R α1, α2 and α3 subunits are all commonly found in synaptic GABA<sub>A</sub>Rs (Olsen and Sieghart 2009) so were obvious candidates for testing against gephyrin in the YTH system. Given that the GlyR β subunit interaction is mediated via the gephyrin E domain (Rees et al 2003; Harvey et al 2004; Schrader et al 2004), a gephyrin prey corresponding to amino acids 305-736 of gephyrin was used, since this has previously been shown to mediate robust interactions with collybistin and
GlyR β subunit baits (Rees et al 2003; Harvey et al 2004). GABA_\alpha R baits were also tested against a prey for the full-length gephyrin P1 isoform. Empty bait and prey vectors did not autoactivate reporter genes. Results of these assays can be seen in Figure 6.1. Consistent with previous studies, the GlyR β-gephyrin interaction is clearly a strong, robust interaction (Rees et al 2003; Harvey et al 2004). GABA_\alpha R α2 and α3 subunit baits also interact with gephyrin (Saiepour et al 2010), albeit more weakly. However, no interaction was observed between the wild-type GABA_\alpha R α1 subunit bait and gephyrin preys. This result was surprising, so we compared the gephyrin-binding site in the GABA_\alpha R α2 subunit M3-M4 intracellular loop (Tretter et al 2008; Saiepour et al 2010) to the corresponding sequences in the α1 and α3 subunits (Figure 6.2). We also examined whether post-translational modifications of the GABA_\alpha R α1 subunit bait could be occurring in yeast using the NetPhosYeast server: http://www.cbs.dtu.dk/services/NetPhosYeast/
Figure 6.1 LacZ assay filters showing the interactions of gephyrin with GABA\(_\alpha\)R subunits

Results from LacZ assays show that baits for the GABA\(_\alpha\)R \(\alpha_2\) and \(\alpha_3\) subunits interact with both full-length gephyrin and the shorter 305-736 preys. However, a bait for the GABA\(_\alpha\)R \(\alpha_1\) subunit did not interact with either gephyrin prey. Note that GABA\(_\alpha\)R \(\alpha_2\) and \(\alpha_3\) subunit interactions are far weaker compared to that mediated by the GlyR \(\beta\) subunit.
Figure 6.2 Comparison of the sequences of the M3-M4 intracellular loops of GABA\(_R\) \(\alpha1\), \(\alpha2\) and \(\alpha3\) subunits

Residues conserved across subunits and species are underlined. The position of the intracellular loop within each subunit in rat is indicated with residues numbered at the suggested start and end of the loop. The ten amino acids identified as critical for gephyrin binding in the GABA\(_R\) \(\alpha2\) subunit are shown in red, corresponding residues in the GABA\(_R\) \(\alpha1\) and \(\alpha3\) subunits are shown in green and blue respectively. Residues S373 and T375 in the GABA\(_R\) \(\alpha1\) subunit were targeted for site-directed mutagenesis, as they are potential phosphorylation sites that are not present in the GABA\(_R\) \(\alpha2\) subunit. Alignments were carried out using ClustalW2: http://www.ebi.ac.uk/Tools/msa/clustalw2/ using GABA\(_R\) intracellular loops identified based on data in UniProt: www.uniprot.org

This analysis revealed that sequence conservation within the M3-M4 intracellular loop is not particularly high across the GABA\(_R\) subunits, which may help explain the differences in interaction strength with gephyrin. In particular, sequence conservation between subunits in the region corresponding to the putative gephyrin-binding site in the GABA\(_R\) \(\alpha2\) subunit (Tretter et al 2008; Saiepour et al 2010) was...
low (3/10 residues comparing α1 and α2; 1/10 residues between α2 and α3). NetPhosYeast also predicted that S373 and T375 in the GABA\(_A\)R α1 subunit were potential sites for phosphorylation by yeast kinases, with probabilities above threshold (0.500) of 0.666 and 0.792, respectively. Analysis with NetPhosK (http://www.cbs.dtu.dk/services/NetPhosK/) revealed that T375 is a part of a consensus sequence for phosphorylation by proline-directed kinases such as mitogen-activated and cyclin-dependent protein kinases (Dhariwala and Rajadhyaksha 2008).

To examine whether phosphorylation by yeast kinases affected GABA\(_A\)R α1 subunit-gephyrin interactions, I created several α1 subunit mutants by site-directed mutagenesis. T375 was mutated to alanine (T375A) which is the amino acid found at this position in the GABA\(_A\)R α2 subunit. This mutation is predicted to prevent phosphorylation by endogenous kinases. T375 was also altered to glutamate (T375E) and aspartate (T375D), negatively-charged residues that are often used to mimic the electrostatic effects of phosphorylation (Dean and Koshland 1990). S373 was also mutated to the amino acids found at the corresponding positions in the α2 and α3 subunits (S373N and S373T). These mutated α1 subunit baits were again tested against preys for both full-length gephyrin and the shorter 305-706 construct (Figure 6.3). These experiments revealed that T375 is critical for mediating the GABA\(_A\)R α1 subunit-gephyrin interaction. When this residue is converted to alanine (T375A) then GABA\(_A\)R α1 subunit-gephyrin interactions can be observed. Notably, the phospho-mimetic mutations T375D and T375E do not allow interactions. Taken together, these results firmly suggested that phosphorylation of T375 could control GABA\(_A\)R α1 subunit-gephyrin interactions in yeast and mammalian cells.

These findings were subsequently confirmed in two additional assays by collaborators in the Moss and Schindelin laboratories (Mukherjee et al 2011). GST-
fusion proteins carrying the T375A, T375D or T375E mutations were tested for their ability to bind to gephyrin in overlay assays. Mutations of T375D and T375E significantly reduced gephyrin-binding to 68.9 ± 1.0 %, p≤0.001 and 62.5 ± 6.1%, p≤0.03, t-test; n=3). By contrast, mutation to alanine did not appear to reduce the interaction with gephyrin (108.7 ± 5% of control; p=0.2, t-test; n=3). Isothermal titration calorimetry (ITC) was also used to compare the relative affinities of the T375A and T375E mutants for gephyrin. ITC can be used to determine the binding constant, stoichiometry and enthalpy of binding, the technique measures the heat released or absorbed when binding partners interact. For gephyrin and the GABA\(_\alpha\)R \(\alpha1\) subunit this revealed that the affinity of gephyrin for T375E was reduced by approximately 10-fold compared to wild-type \(\alpha1\) (\(K_D = 183 \pm 33 \mu M\), n=2 vs. 17 ± 11 \(\mu M\), n=11). By contrast the affinity for the T375A mutant was similar to that for wild-type \(\alpha1\) (\(K_D =17 \pm 11\), n=11 and 36 ± 11 \(\mu M\), n=2).
Figure 6.3 *LacZ* assay filters showing critical residues for the GABA$_A$R $\alpha$1 subunit-gephyrin interaction

The T375A mutation confers gephyrin-binding ability to the $\alpha$1 subunit in yeast, whilst the phospho-mimetic mutations do not allow an interaction. Neither of the mutations of S373 has any effect, suggesting that T375 is critical for mediating the interaction with gephyrin.
6.4 Mapping GABA<sub>A</sub> receptor α3 subunit interactions with gephyrin

Since the GABA<sub>A</sub>R α3 subunit has also been reported to interact with gephyrin in the YTH system (Saiepour et al 2010; Figure 6.1) I also sought to map the gephyrin binding site on this subunit. A number of GABA<sub>A</sub>R α3 subunit baits were made, as shown in Figure 6.4A. The residues shown in blue in the GABA<sub>A</sub>R α3 subunit correspond to the residues in the GABA<sub>A</sub>R α2 subunit that are critical for interactions with gephyrin. Several deletions were made spanning this region, to establish whether the gephyrin-binding site is in the same relative location in the GABA<sub>A</sub>R α3 subunit. As in previous experiments, GABA<sub>A</sub>R α3 subunit baits were tested against preys for full-length gephyrin (P1 isoform) and the 305-736 construct (end of C domain and the full E domain). Our collaborators in the Tretter laboratory used GST-tagged versions of these α3 deletion constructs and incubated these with radio-labelled gephyrin in overlay assays. This allowed a comparison between the amounts of gephyrin bound by the wild-type GST-GABA<sub>A</sub>R α3 and fusion proteins with deletions across the putative gephyrin-binding region.

YTH assays indicate the residues deleted in the ∆4 and ∆5 constructs are critical for the GABA<sub>A</sub>R α3 - gephyrin interaction (Figure 6.4B). This was supported by overlay assays (Figure 6.4C), which showed that deletions ∆1 and ∆4 most severely impacted on the ability of the GABA<sub>A</sub>R α3 subunit to bind gephyrin. The ∆3 deletion also showed a slight reduction compared to wild-type. To confirm that residues deleted in the ∆4 construct are responsible for gephyrin binding, these residues (396-405) were exchanged with the corresponding region in the GABA<sub>A</sub>R α1 subunit, which does not interact with gephyrin unless T375 is mutated to alanine. As well as testing against full-length gephyrin preys, a variety of gephyrin mutants (Harvey et al 2004) were used to map the reciprocal binding-site for the GABA<sub>A</sub>R α3 subunit on gephyrin (Figure 6.5).
Figure 6.4 LacZ filter assays and overlay assays demonstrating interactions between GABA<sub>A</sub>R α3 subunit and gephyrin

(A) GABA<sub>A</sub>R α3 subunit deletion constructs used in YTH and overlay assays. Residues in blue in the GABA<sub>A</sub>R α3 subunit correspond to the gephyrin-binding site on the GABA<sub>A</sub>R α2 subunit. (B) LacZ filter assays show that residues 396-405 are critical for the interaction with gephyrin. (C) Upper panel: Coomassie staining showing equal loading of each GST-α3 fusion protein; Lower panel: overlay assay after incubation with radio-labelled gephyrin. The graph shows quantification of the overlay assay. Data represent mean ± S.E.M. (n = 3). Initial analysis with an unpaired Student’s t test showed significant differences (*** p<0.001) between the Δ1, Δ3, Δ4 constructs from α3FL. An additional ANOVA test was carried out, and confirmed significant differences between the constructs, F (4,10) = 68.6 p = 3.1E-07. Post-hoc analysis with Dunnett’s test showed a statistically significant difference (p<0.001) between Δ1 and α3FL, and between Δ4 and α3FL, whilst Δ2 and Δ3 were not significantly different from α3FL.
Figure 6.5 Sequences of the gephyrin mutants used for mapping the \( \text{GABA}_A \) \( \alpha_3 \) subunit binding site

Blocks of five alanine residues were substituted sequentially between residues 310 and 348. The gephyrin E domain is generally accepted to start at residue 323 - bases highlighted in green are part of the E domain. The binding site for collybistin on gephyrin has been mapped to this region (Harvey et al 2004), spanning residues 320-329. The suggested binding sites for the \( \text{GABA}_A \) \( \alpha_2 \) (Saiepour et al 2010) and \( \alpha_3 \) subunits are also indicated.

Figure 6.6 shows that insertion of the amino acids suspected to be the gephyrin-binding site in the \( \text{GABA}_A \) \( \alpha_3 \) subunit into the \( \alpha_1 \) subunit prey (\( \alpha_3 \) into \( \alpha_1 \)) confers gephyrin-binding ability, confirming the FNIVGTTPYPI motif in the \( \text{GABA}_A \) \( \alpha_3 \) subunit is sufficient to mediate interactions with gephyrin. The gephyrin A2-A9 mutant preys reveal that alanine blocks A5 and A6 clearly disrupt the interaction with both \( \text{GABA}_A \) \( \alpha_3 \) subunit and \( \alpha_3 \) into \( \alpha_1 \) preys. This suggests residues within the motif SMDKAFITVL at the N-terminus of the gephyrin E domain are responsible for the interaction with the \( \text{GABA}_A \) \( \alpha_3 \) subunit. It is also noteworthy that \( \text{GABA}_A \) \( \alpha_3 \) subunit interactions with the A7 mutant (EMTPV to AAAAA) was weak in comparison to interactions with the A2-A4 and A8-A9 mutants, suggesting that robust \( \text{GABA}_A \) \( \alpha_3 \) binding might also require one or more amino acids in this
sequence. By contrast, the gephyrin-GlyR β interaction was not affected by any of these alanine blocks. The core GABA_A α3 subunit binding motif within the N-terminal part of the gephyrin E domain (SMDKAFITVL) shows partial overlap with the previously determined collybistin (PFPLTSMDKA) and GABA_A α2 (SMDKAFITVLEMPTVGTE) binding motifs on gephyrin (Figure 6.5). Interestingly, two residues (D327 and F330) in the minimal GABA_A α2 and α3 binding sites on gephyrin have been previously implicated in GlyR β subunit - E domain interactions (Kim et al 2006). However, mutation of D327 and F330 in mutants A5 and A6 respectively, does not appear to be sufficient to disrupt GlyR β subunit- E domain interactions in the YTH system.

Finally, to ensure that the binding sites identified in these recombinant studies had relevance in vivo, wild type HA-tagged GABA_A α3 subunit expression constructs, or those containing binding site deletions Δ1, Δ3 and Δ4 were transfected into primary hippocampal neurons. The aim of this experiment, conducted by collaborators in the Tretter laboratory, was to examine the effect of these mutations on colocalisation with gephyrin and any impacts on gephyrin clustering. Wild-type HA-α3 formed submembrane clusters on both neuronal processes and the cell body (Figure 6.7A) that co-localized with endogenous gephyrin (Figure 6.7B-D). Consistent with my YTH data, construct HA-α3Δ1 (removing P357-I377) exhibited a diffuse distribution on the plasma membrane and showed little or no co-localization with gephyrin (Figure 6.7E-H). By contrast, HA-α3Δ2 (lacking T361-T367) showed robust co-localization with gephyrin (Figure 6.7I-L), confirming that this motif is dispensable for both gephyrin binding and synaptic targeting. Lastly, HA-α3Δ4 (lacking the minimal gephyrin binding motif F368-I377) showed a diffuse cytoplasmic distribution with some small puncta that did not co-localize with endogenous gephyrin (Figure 6.7M-P).
Figure 6.6 LacZ assay filters measuring potential interactions between GABA$_{\alpha}$R $\alpha_3$, $\alpha_1$, $\alpha_3\alpha_1$ and GlyR baits with gephyrin mutants

(A) Sequences of the GABA$_{\alpha}$R $\alpha_3$ and $\alpha_1$ and the $\alpha_3\alpha_1$ chimera construct. (B) LacZ assays confirm that residues 396-405 of the GABA$_{\alpha}$R $\alpha_3$ subunit are sufficient to confer gephyrin binding, and that alanine blocks A5 and A6 in gephyrin disrupt this interaction.
Figure 6.7 Expression of HA-GABA<sub>A</sub>δ3 subunit constructs in hippocampal neurons

Transfected 18-21 DIV neurons expressing HA-tagged GABA<sub>A</sub>δ3 (A-D), δ1 (E-H), δ3 (I-L), δ4 (M-P) stained with HA antibodies under non-permeabilizing conditions (green) and after permeabilization with 0.05% TX-100 with mAb7a against gephyrin (red). Note that wild-type GABA<sub>A</sub>δ3 and mutant δ3 display good co-localization with gephyrin, whilst for mutants δ1 and δ4 little co-localization is observed. The third panel in each row represents the merged images and enlargements of the respective dendrites (indicated by white boxes) are displayed in the fourth panel. Scale bar: 25 μm.
6.5 Examination of residues in the gephyrin E domain critical for GABA<sub>R</sub> versus GlyR subunit interactions

YTH results revealed that critical determinants of the GABA<sub>R</sub> α3 subunit-gephyrin interaction are located in the motif FNIVGTTYPI in the M3-M4 domain and the motif SMDKAFITVL at the N-terminus of the gephyrin E domain. However, one curious result was that the GlyR β interaction with gephyrin appeared to be unaffected by the alanine-scanning mutations (Figure 6.6) in contradiction to the suggestions of Kim et al 2006. I therefore decided to test additional mutations of the gephyrin E domain to determine whether GABA<sub>R</sub> and GlyR interactions with the E domain differ. Five of the key gephyrin mutations identified in by Kim et al (2006), D327A, F330A, Y673F, P713E and Y719A were introduced into a gephyrin prey (305-736) and then tested against the GlyR β subunit and GABA<sub>R</sub> α1-α3 subunit baits (Figure 6.8).

Curiously, these E domain mutations appear to have different effects on the GABA<sub>R</sub> subunit interactions with gephyrin. Consistent with our previous alanine block mutagenesis results, mutations D330A and F330A abolished interactions with GABA<sub>R</sub> baits, but did not appear to affect GlyR β subunit interactions with gephyrin. The Y673F mutation appeared to disrupt the GABA<sub>R</sub> α3 subunit-gephyrin interaction, but not GABA<sub>R</sub> α1, α2 or GlyR β subunit interactions with gephyrin. Lastly, mutations P713 and Y719 abolished interactions between all the tested baits and the gephyrin prey. Since the LacZ filter assays only give a qualitative indication of interaction strength, quantitative yeast two-hybrid assays were used to gain further insight into the impact of the E domain mutations on the interaction with GlyR β subunit, as shown in Figure 6.9. Again, this data strongly suggested that mutations D327A, F330A and Y673F did not disrupt GlyR β subunit-gephyrin interactions – in fact, interaction strength was increased about 100% for these mutants. In agreement with the LacZ assays, mutations P713E and Y719A
abolished GlyR β subunit-gephyrin interactions. Unfortunately, equivalent assays were not possible with GABA₆R baits, due to the lower interaction strength.

Figure 6.8. LacZ assay filters showing differential interactions of gephyrin E domain mutants with GABA₆R and GlyR baits

The GlyR β subunit-gephyrin interaction is clearly disrupted by the P713E and Y719A mutations, whilst the GABA₆R α1T375A and α2 subunit interactions with gephyrin are affected by all mutations except Y673F. All E domain mutations disrupt the GABA₆R α3 subunit interactions with gephyrin.
Interaction strength is measured relative to the interaction between wild-type gephyrin and GlyR β subunit. In agreement with the LacZ assays, the P713E and Y719A abolish the interaction, whilst the other mutations appear to strengthen the interaction. All protein interactions were assayed in three independent experiments in triplicate. Initial analysis with a paired Student’s t test (two-tailed) showed significant differences (*: P < 0.05, **: P < 0.01). Error bars represent standard deviation from the mean. An additional ANOVA test was carried out, and confirmed significant differences between the constructs, $F(5,12) = 284.15$ $P = 5.1E-12$. Post-hoc analysis with Dunnett’s test showed a statistically significant difference ($p<0.001$) between all mutants and the wild-type E domain.
6.6 Discussion

Initial YTH assays suggested that the GABA$_A$R $\alpha$1 subunit did not interact with gephyrin (Saeipour et al 2010), but further investigation revealed that lack of interaction is likely to be an artefact of the YTH system. Bioinformatic analysis predicted that T375 in the GABA$_A$R $\alpha$1 subunit bait is modified by yeast protein kinases, preventing interactions with gephyrin preys. I tested this prediction by abolishing potential phosphorylation of this site in the T375A mutation, and mimicking phosphorylation with the T375D and T375E mutations. My analysis, and further pull-down and ITC assays, revealed that the GABA$_A$R $\alpha$1 subunit-gephyrin interaction is negatively regulated by phosphorylation of T375. These findings have significance for other researchers using the YTH system to screen for interacting proteins, since post-translational modifications of baits in yeast may mean that important interactors are not identified in library screens. My initial results on GABA$_A$R $\alpha$1 subunit-gephyrin interaction formed part of a larger study, demonstrating that this interaction is responsible for determining the residence time of GABA$_A$Rs containing the $\alpha$1 subunit at inhibitory synapses (Mukherjee et al 2011). Using ITC, the GABA$_A$R $\alpha$1 subunit was estimated to have a binding affinity of $\sim$20 $\mu$M for gephyrin, significantly weaker than that previously reported for the GlyR $\beta$ subunit-gephyrin interaction ($\sim$0.4 $\mu$M Schrader et al 2004). This is consistent with the differences observed in LacZ freeze fracture assays. When the GABA$_A$R $\alpha$1 subunit gephyrin binding site was replaced with the corresponding region from the GABA$_A$R $\alpha$6 subunit, there was significantly less clustering at postsynaptic sites, and the amplitudes of miniature inhibitory postsynaptic currents (mIPSCs) were reduced (Mukherjee et al 2011). Taken together, this data strongly suggests gephyrin is required for synaptic clustering of GABA$_A$Rs containing $\alpha$1 subunits. Site-directed mutagenesis has indicated that phosphorylation of T375 may be vital for regulating this interaction, adding an extra layer of complexity not yet observed for the GABA$_A$R $\alpha$2 or $\alpha$3 subunits.
GABA\textsubscript{A}R \(\alpha 3\) subunit-gephyrin interaction studies show that the FNIVGTTYPI motif in the \(\alpha 3\) subunit M3-M4 domain and the SMDKAFITVL motif at the N-terminus of the gephrin E domain are vital for the interaction between these proteins. The latter finding is consistent with my studies on gephrin E domain mutants, since the D327A and F330A mutants also disrupted GABA\textsubscript{A}R \(\alpha 1\)-\(\alpha 3\) subunit binding. By contrast, the Y673F mutation appeared to disrupt the GABA\textsubscript{A}R \(\alpha 3\) subunit-gephyrin interaction, but did not interfere with GABA\textsubscript{A}R \(\alpha 1\), \(\alpha 2\) or GlyR \(\beta\) subunit interactions with gephyrin. Lastly, I also identified two mutations (P713E and Y719A) that disrupt interactions with both GABA\textsubscript{A}R and GlyRs in the YTH system. Taken together, my results contradict a recent study that concludes that GABA\textsubscript{A}R and GlyR subunits utilise a common binding site (Maric et al 2011). Binding of GABA\textsubscript{A}R and GlyR subunits are clearly influenced by key residues in the gephyrin E domain, but there are clearly subtle differences in GABA\textsubscript{A}R and GlyR subunit binding to the E domain.

One area where my YTH data is consistent with Maric et al (2011) is that the strength of GABA\textsubscript{A}R subunit interactions with gephyrin is significantly weaker than binding of the GlyR \(\beta\) subunit (Maric et al 2011). Comparisons of ITC assays revealed that the GlyR \(\beta\) subunit interaction was by far the strongest, followed by the GABA\textsubscript{A}R \(\alpha 3\) subunit and the GABA\textsubscript{A}R \(\alpha 1\) subunit. Surprisingly the GABA\textsubscript{A}R \(\alpha 2\) subunit interaction was found to be too weak to detect under the same conditions. A summary of affinity for each receptor subunit and selected mutants is shown in Table 6.1.
<table>
<thead>
<tr>
<th>Gephyrin Fragment</th>
<th>Receptor fragment</th>
<th>Affinity</th>
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</thead>
<tbody>
<tr>
<td>Full length</td>
<td>$\text{GABA}_\alpha R \alpha 1$ M3-M4 (307-393)</td>
<td>++</td>
</tr>
<tr>
<td>G domain + Linker (1-303)</td>
<td>$\text{GABA}_\alpha R \alpha 1$ M3-M4 (307-393)</td>
<td>-</td>
</tr>
<tr>
<td>E domain (318-706)</td>
<td>$\text{GABA}_\alpha R \alpha 1$ M3-M4 (307-393)</td>
<td>++</td>
</tr>
<tr>
<td>E domain</td>
<td>$\text{GABA}_\alpha R \alpha 1$ M3-M4 (307-393)</td>
<td>++</td>
</tr>
<tr>
<td>E domain</td>
<td>$\text{GABA}_\alpha R \alpha 1 \Delta 333-348$</td>
<td>-</td>
</tr>
<tr>
<td>E domain</td>
<td>$\text{GABA}_\alpha R \alpha 1$ 334-348</td>
<td>+</td>
</tr>
<tr>
<td>E domain</td>
<td>$\text{GABA}_\alpha R \alpha 2$ M3-M4 (307-391)</td>
<td>+</td>
</tr>
<tr>
<td>E domain</td>
<td>$\text{GABA}_\alpha R \alpha 3$ M3-M4 (332-429)</td>
<td>++</td>
</tr>
<tr>
<td>E domain</td>
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<td>-</td>
</tr>
<tr>
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<td>+</td>
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<tr>
<td>E domain</td>
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<tr>
<td>E domain</td>
<td>GlyR $\beta$ M3-M4 (387-426)</td>
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</table>

Table 6.1 Summary of affinities between $\text{GABA}_\alpha R \alpha 1$, $\alpha 2$, $\alpha 3$ ICLs and GlyR loop with gephyrin determined by ITC, native gel and pull-down assays (Maric et al 2011).

This table summarises the affinity data for the interactions of $\text{GABA}_\alpha R \alpha 1$, $\alpha 2$, $\alpha 3$ and the GlyR loop with gephyrin, as determined by Maric et al (2011) via several different methods. +++ = $K_D$ of 0.1-1μM, ++ = $K_D$ of 1-20μM, + = interaction too weak to determine $K_D$ via ITC but verified via pull down assay or native gel, - = no interaction detectable via any method.

Incubation of gephyrin with a GlyR $\beta$ subunit peptide containing the core motif needed for binding was sufficient to prevent the $\text{GABA}_\alpha R \alpha 1$ interaction with gephyrin. Similarly when gephyrin was pre-incubated with $\text{GABA}_\alpha R \alpha 1$ and GlyR $\beta$ was subsequently added the GlyR $\beta$ interaction with gephyrin was reduced. This suggests GlyR $\beta$ is occupying the same binding site as $\text{GABA}_\alpha R \alpha 1$. Also in this study gephyrin E domain mutagenesis studies were carried out and showed P713E and F330A weaken GlyR $\beta$ binding, the same was found to be true for these mutations for $\text{GABA}_\alpha R \alpha 1$ and $\alpha 3$ interactions with gephyrin. This partially fits with my yeast two hybrid data which showed that all mutants reduce $\alpha 3$ binding, $\alpha 2$ and $\alpha 1$ binding is unaffected by the Y673F mutations, and GlyR $\beta$ binding is affected by P713E and Y719A. Taken with the data from Maric et al it is reasonable to state that the interactions between gephyrin and these various inhibitory receptors subunits are mediated by at least some of the same residues in the gephyrin E domain, supporting the proposition of a common binding site.
7 GENERAL DISCUSSION

7.1 The IQSEC family of ArfGEFs: new roles at excitatory and inhibitory synapses

The main aim of my yeast two-hybrid library screen was to identify novel GEFs interacting with gephyrin, and in this regard it was highly successful. We identified IQSEC2 and IQSEC3 as novel GEFs that were capable of interacting with the gephyrin P1 isoform in yeast. The IQSEC proteins are GEFs for the Arf family of GTPases (Murphy et al 2006; Sakagami et al 2008). These new GEFs are unrelated to collybistin in structure or sequence, showing the value of an unbiased approach to identifying new gephyrin interactors. Whilst IQSEC2 and IQSEC3 have been the focus of my studies, a synaptic role has also been suggested for IQSEC1, since this ArfGEF is also found in the postsynaptic density and has been linked to AMPAR signalling (Scholz et al 2010). Table 7.1 summarises the current state of knowledge regarding the IQSEC family proteins.
<table>
<thead>
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<th>IQSEC3</th>
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**Human gene location:**
- IQSEC1: Chr 3p25.2
- IQSEC2: Chr Xp11.22
- IQSEC3: Chr 12p13.33

**Type of synapse:**
- IQSEC1: Excitatory
- IQSEC2: Excitatory
- IQSEC3: Inhibitory

**GTPase activated**
- IQSEC1: Arf1, Arf5, Arf6 (Someya et al 2001, Scholz et al 2010)
- IQSEC2: Arf1, Arf6 (Murphy et al 2006, Sakagami et al 2008)
- IQSEC3: Arf1, Arf6 (Hattori et al 2007, Fukaya et al 2010)

**Interactors**
- IQSEC1: GluA2 receptor subunit, α-catenin
- IQSEC2: P53, NMDARs and RIBEYE
- IQSEC3: PSD-95, S-SCAM/MAGI-2, utrophin, dystrophin

**Suggested biological function**
- IQSEC1: Internalisation of synaptic AMPARs upon long term depression and non-synaptic roles
- IQSEC2: Responsible for activating Arf GTPases at excitatory synapses
- IQSEC3: Responsible for activating Arf GTPases at inhibitory synapses

**Relevance in disease**
- IQSEC1: Unknown
- IQSEC2: Missense mutations in IQSEC2 cause XLID in four families (Shoubridge et al 2010a)
- IQSEC3: Partially deleted in patient with ID and severe behavioural problems (Baker et al 2002)

Table 7.1. Comparison of the members of the IQSEC family of ArfGEFs

Interestingly, it has been shown that GEF activity of IQSEC1 on the Arf6 GTPase is amplified when IQSEC1 is co-expressed with the AMPA receptor subunit GluA2 (Scholz et al 2010). Whilst my search for a similar activator of IQSEC3 has been unsuccessful to date, this study supports the theory that there is a similar step required for IQSEC3 activation, which could in turn trigger submembrane gephyrin clustering. Other functions of the IQSEC1/Arf6 pathway have been shown in several non-neuronal roles including angiogenesis, phagocytosis, tumour metastasis and endocytosis of integrins (Dunphy et al 2006; Morishige et al 2008; Someya et al 2010; Hashimoto et al 2011). This indicates that some Arf6 functions are mediated by IQSEC1 activation in non-neuronal tissues. In contrast, IQSEC2 and IQSEC3 activation of Arf6 appears to be limited to synapses. Interestingly each protein appears to interact or colocalise with different subsets of receptors at excitatory or inhibitory synapses, IQSEC1 with AMPARs, IQSEC2 with NMDARs and IQSEC3...
with GlyRs and GABA_\text{A}\text{Rs}. In summary, the IQSEC ArfGEFs appear to be an exciting new family of proteins that play important roles at both inhibitory and excitatory synapses, forming complexes (either directly or indirectly) with ligand-gated ion channels. Since IQSEC2 and IQSEC3 have been implicated in X-linked and autosomal intellectual disability (XLID and ARID), IQSEC1 should also be considered as a candidate gene for ARID.

### 7.2 IQSEC2: a novel component of excitatory synapses involved in X-linked intellectual disability

Although my yeast two-hybrid screen initially suggested the possibility that IQSEC2 interacted with gephyrin, this interaction is unlikely to be relevant \textit{in vivo} for several reasons. Firstly, the IQSEC2 isoform found in my screen is not a common variant, and lacks most key functional domains (IQ, GEF, PH and PDZ-binding motifs). Secondly, when I co-transfected full-length FLAG-tagged IQSEC2 into HEK293 cells with EGFP-gephyrin, the two proteins failed to co-localise. Given that other published studies have shown that IQSEC2 is enriched at excitatory postsynaptic densities and is able to form a complex with PSD-95 and NMDA receptors (Murphy et al. 2006; Sakagami et al. 2008), I conclude that IQSEC2 and gephyrin are not likely to interact \textit{in vivo}.

Although this aspect of the project did not look promising initially, I noted that several \textit{IQSEC2} missense variants were identified in an X chromosome sequencing study of individuals with XLID (Tarpey et al., 2009). In collaboration with Josef Gecz, Cheryl Shoubridge and Randall Walikonis, I investigated these \textit{IQSEC2} variants using a combination of bioinformatics and functional studies. The missense changes identified impaired IQSEC2 GEF activity, either by impairing the function of the Sec7 domain (R758Q, Q801P and R863W), or the IQ-like motif (R359C). Interestingly,
mutations in both domains inhibited the capacity of IQSEC2 to catalyse GDP-GTP exchange on Arf6, suggesting that the IQ-like motif modulates Sec7 activity. Using the yeast-two hybrid system, I was able to show that the R359C mutation impairs the binding of calmodulin to the IQSEC2 IQ-like motif, suggesting that calmodulin might positively modulate the activity of IQSEC2. In support of this theory, Myers et al (2012) recently demonstrated that calmodulin binding brings about a reversible conformation change in IQSEC2. Application of Ca^{2+} to HeLa cells containing mCherry-IQSEC2 caused the formation of IQSEC2 puncta throughout the cell, whilst removal of Ca^{2+} with a chelating agent resulted in the dissolution of the puncta. Using hippocampal neurons, the authors demonstrated that activation of NMDARs resulted in sufficient Ca^{2+} influx to cause IQSEC2 puncta to form on the spines and dendrites, as well as at synapses. Thus, Ca^{2+} influx may not only regulate ArfGEF activity, but also determine the subcellular localisation of IQSEC2. Figure 7.1 depicts a model of this activation scheme.
Figure 7.1 Model of NMDAR activation leading to IQSEC2 activation by Ca\(^{2+}\)

When the NMDARs are activated the resulting influx of Ca\(^{2+}\) induces conformation changes in IQSEC2 that may bring about its relocation to distinct puncta at the synapse and along dendrites. The Ca\(^{2+}\) activates the GEF activity of IQSEC2, resulting in the exchange of GDP for GTP on Arf6.

The authors also suggested that NMDAR-mediated IQSEC2 activation caused depression of AMPAR-mediated transmission via JNK-mediated synaptic removal of GluA1-containing AMPARs. More controversially, the authors suggested that the R359C mutation did not impair calmodulin binding to the IQ-like motif – in direct contradiction to my own findings. A more severe mutation (IQTAFRQYRM to AATAFAQYRM), denoted BRAG1-IQ, did disrupt calmodulin binding in an *in vitro* pulldown assay, but curiously had no effect on ArfGEF activity *in vitro*. It was therefore surprising that this mutant was reported to result in a hyperactivation of Arf6 signalling *in vivo*, causing constitutive depression of AMPA transmission (Myers et al 2012). Although these studies confirm that IQSEC2 plays an important role at
the excitatory PSD and has improved our understanding of how mutations in GEFs can cause XLID, there are still clearly some areas of disagreement that will require further study.

7.3 IQSEC3: a novel component of inhibitory synapses involved in autosomal intellectual disability and aggressive behaviour

IQSEC3 was also identified as a potential gephyrin interactor though yeast two-hybrid library screening. IQSEC3 is located at inhibitory postsynaptic densities and co-localises with gephyrin, GABA\textsubscript{A}Rs and GlyRs in spinal cord and retina (Fukaya et al 2011), making an interaction between gephyrin and IQSEC3 in vivo quite plausible. In support of this theory, I was able to show that IQSEC3 and gephyrin co-localise when co-transfected into HEK293 cells. I also demonstrated that interaction between IQSEC3 and gephyrin is mediated by the N-terminus of IQSEC3 and the gephyrin G domain. However, to date, I have been unable to find an activator to bring about submembrane clustering of IQSEC3 and gephyrin, despite co-transfecting the Arf6 GTPase or examining potential interactions of inhibitory GABA\textsubscript{A}R and GlyR subunits with IQSEC3. Whether IQSEC3 is regulated by calmodulin remains unclear, since the IQSEC3 domain did not interact with calmodulin in my yeast two-hybrid assays. The likely reason for this discrepancy is shown in Figure 7.2, the sequences of the IQ-like motif show slight differences between IQSEC1, IQSEC2 and IQSEC3, and across species.
The IQ-like motif is highly conserved across species for IQSEC1, IQSEC2 and IQSEC3, with the only differences being seen in zebrafish (in red). Slight differences are seen between proteins, but do not correspond to any of the residues known to be important for calmodulin binding (indicated with *). The residues shown in blue in IQSEC3 differ from those found at equivalent positions in IQSEC1 and IQSEC2. R359 in IQSEC2 is underlined. The complete conservation of this residue indicates structural or functional importance, explaining the deleterious effect of the R359C mutation.

The presence of the residues ‘LS’ in IQSEC3 (shown in blue) instead of ‘MN’ may be responsible for IQSEC3 being unable to bind calmodulin. These residues are the only distinct difference between the IQ-like motifs, but properties of these amino acids are not considerably different. Leucine and methionine both have hydrophobic
side chains, although methionine is slightly larger. Serine and asparagine both contain polar uncharged side chains. However, given my experience with phosphorylation of GABA$_A$R subunit baits, one possibility is that S332 in IQSEC3 is phosphorylated in yeast, preventing calmodulin binding to the IQ-like motif. Indeed, analysis using NetPhosYeast ([http://www.cbs.dtu.dk/services/NetPhosYeast/](http://www.cbs.dtu.dk/services/NetPhosYeast/)) generated a score of 0.5, suggesting that S332 might be modified in yeast. Alternatively, since Cl$^-$ flux - rather than Ca$^{2+}$ flux - is the predominant signal expected at inhibitory synapses, it is quite possible that a different regulatory mechanism applies to IQSEC3.

**Figure 7.3 Model of GABA$_A$R and GlyR activation leading to IQSEC3 activation by Cl$^-$**

When the inhibitory receptors are activated the resulting influx of Cl$^-$ may activate IQSEC3 bringing about its relocation from cytoplasmic clusters with gephyrin to submembrane clusters. The activated IQSEC3 is able to exchange GDP for GTP on Arf6.
Investigation of a 12p13.33 microdeletion found in a male proband with intellectual disability and behavioural problems (Baker et al 2002) revealed that the start of the deletion breakpoint is within the IQSEC3 gene. Several non-synonymous mutations were also identified in genes in the deleted region which may also contribute to the phenotype although my preliminary analysis did not suggest any of the changes were likely to be harmful. One possibility is that a gene fusion event takes place, involving IQSEC3 and ADIPOR2, where the deletion ends. Any resulting fusion protein could harbour a gephyrin-binding motif and would be generated under the control of the neuronal IQSEC3 promoter. It is therefore plausible that this protein could interfere with normal IQSEC3-gephyrin interactions at inhibitory synapses in a dominant-negative manner.

7.4 Interactions between GABAᵦR α1 and α3 subunits and gephyrin

I also characterised interactions between the GABAᵦR α1 and α3 subunits with gephyrin. In collaboration with the Moss and Schindelin laboratories, my initial finding that the GABAᵦR α1 subunit - gephyrin interaction was negatively regulated by phosphorylation of T375 in yeast was also extended to mammalian cells (Mukherjee et al 2011). Mutation of T375 to a phosphomimetic, negatively-charged amino acid decreased both the affinity of the GABAᵦR α1 subunit for gephyrin, disrupted receptor accumulation at synapses and reduced the amplitude of mIPSCs. Single-particle tracking also revealed that gephyrin reduced the diffusion of α1-subunit-containing GABAᵦRs at inhibitory synapses, increasing their residence time at these structures. Taken together, the study confirmed that the GABAᵦR α1 subunit shows phosphorylation-dependent interactions with gephyrin that modulate the strength of synaptic inhibition. By contrast, gephyrin-GABAᵦR α3 subunit interactions were not phosphorylation dependent. Using deletion analysis and mutagenesis, I was able to reveal specific motifs responsible for this interaction -
FNIVGTTYPI in the α3 subunit M3-M4 domain and the motif SMDKAFITVL at the N-terminus of the gephyrin E domain. Interestingly, the GABA_A R α3 subunit binding site on gephyrin overlaps with the binding sites for the GABA_A R α2 subunit and collybistin, suggesting that binding of these proteins might be co-operative or mutually exclusive. Lastly, contrary to a recent study suggesting that GABA_A R α subunits and the GlyR β subunit bind to a common binding site on the gephyrin E domain (Maric et al 2011), my own assays revealed subtle differences in the interactions of the GABA_A R α1, α2 and α3 subunits with gephyrin. The GABA_A R α3 interaction with gephyrin appeared to be most sensitive to disruption and was abolished by all of the gephyrin E domain mutations that were tested. However, the gephyrin Y673F mutation did not disrupt the GABA_A R α1, α2 or GlyR β subunit interactions with gephyrin. P713E and Y719A disrupted both GlyR β subunit and GABA_A R interactions with gephyrin.

### 7.5 Current state of knowledge

Figure 7.4 provides a more up-to date model of the inhibitory postsynaptic density. This model is still broadly based on the membrane-activation model put forward by Kneussel and Betz (2000), but several novel proteins can now be added to the model. For example, neuroligin 2 has been shown to interact with gephyrin and activate collybistin (Poulopoulous et al 2009) and the GABA_A R α2 subunit has also been shown to be capable of activating collybistin (Saeipour et al 2010). Neuroligins are able to provide a link to the presynaptic site via their interaction with neurexins, this interaction is enough to trigger the formation of a synapse (Dean et al 2006). IQSEC3 is also included as an interactor of gephyrin and as a GEF for Arf6.
The membrane-activation model can now be updated to take into account developments in the research over the last decade. Neuroligins and neurexins can now be added to the model of an inhibitory synapse, providing a link from the pre-synaptic site to the post synaptic density. In addition to this, the GABA\(_\alpha\)R \(\alpha\)2 subunit has been shown to be capable of directly activating collybistin. Finally IQSEC3 is shown, since this GEF has been shown to be localised to inhibitory postsynaptic densities and interacts with gephyrin.

7.6 Future Prospects

In my view, IQSEC3 is perhaps the most promising protein for further study. As mentioned in chapter 5, I have generated dominant-negative IQSEC3 constructs by site-directed mutagenesis that will either i) disrupt IQSEC3 binding to gephyrin or ii) retain gephyrin binding, but disrupt IQSEC3 ArfGEF activity. These constructs are currently being assessed for their effects in transfected cortical neurons in collaboration with Angel de Blas (University of Connecticut, USA). The results of

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**Figure 7.4 Updated model of an inhibitory postsynaptic density**

The membrane-activation model can now be updated to take into account developments in the research over the last decade. Neuroligins and neurexins can now be added to the model of an inhibitory synapse, providing a link from the pre-synaptic site to the post synaptic density. In addition to this, the GABA\(_\alpha\)R \(\alpha\)2 subunit has been shown to be capable of directly activating collybistin. Finally IQSEC3 is shown, since this GEF has been shown to be localised to inhibitory postsynaptic densities and interacts with gephyrin.
these assays should provide further information on the role of IQSEC3 at inhibitory synapses and whether loss of IQSEC3 gephyrin binding capability or ArfGEF activity results in a change of neuronal phenotype (e.g. gephyrin puncta size, loss of GABA\textsubscript{A}R clustering). This information will be extremely helpful in elucidating the biological role of IQSEC3.

To investigate the potential role of calmodulin binding to IQSEC3, it would be useful to mutate S332 present in the IQ-like motif of IQSEC3 to determine whether phosphorylation of this residue in yeast prevents calmodulin binding. Mutation of S332 to methionine (S332M), the residue present in IQSEC1 and IQSEC2, might be sufficient to restore calmodulin interactions with an IQSEC3 domain bait in the yeast two-hybrid system. It would also be interesting to mutate the serine to a phospho-mimetic residue (S332D or S332E) to see whether phosphorylation of the serine residue explains the inability of IQSEC3 to bind calmodulin. The possibility also remains that a different protein might bind to the IQSEC3 IQ-like motif, regulating ArfGEF activity. This possibility could be investigated by carrying out a yeast two-hybrid screen using the IQSEC3 N-terminus as bait. I would expect gephyrin to be identified as an interactor in this screen, which would serve as a useful control. Alternatively, IQSEC3 interactors could be identified using immunoprecipitation from brain lysates using specific antibodies (Sakagami et al 2008; Fukaya et al 2011; Sakagami et al 2012), in combination with mass spectrometry. To determine whether Cl\textsuperscript{-} flux influences IQSEC3 subcellular distribution or gephyrin clustering activity, it would also be interesting to co-express GABA\textsubscript{A}Rs or GlyRs with IQSEC3 and gephyrin in HEK293 cells. Stimulation of these receptors with glycine or GABA might provide the trigger needed for IQSEC3 activation.

In the case of the 12p33 deletion involving IQSEC3, future work should aim to: i) confirm the precise breakpoint; ii) complete SNP analysis for genes in the deletion
interval and iii) attempt to amplify transcripts for the IQSEC3-ADIPOR2 fusion protein from cell-line mRNA. For the first aim, multiple PCR primers in IQSEC3 and ADIPOR2 near the predicted breakpoints should be designed based on the next-generation sequencing and array CGH data. These can be used in standard PCR reactions to amplify a deleted genomic DNA fragment that will be unique for the maternal deletion. Sanger DNA sequencing should also be used to verify non-synonymous SNVs in genes in the deleted intervals that may contribute to the ID phenotype. Any potentially damaging SNVs should also be assessed by SIFT or PolyPhen-2 before being prioritised for further functional studies. Given the involvement of collybistin and IQSEC2 in X-linked intellectual disability (Harvey et al 2004; Marco et al 2008; Shoubridge et al 2010b; Shimojima et al 2011), IQSEC3 and IQSEC1 should be considered as strong candidate genes for ARID. As the cost of next-generation sequencing continues to fall, the identification of novel genes involved in ID has risen, with a recent study identifying 50 genes involved in cognitive disorders (Najmabadi et al 2011). These studies offer important hope to those families affected by these devastating neurological disorders.
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### List of Abbreviations

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<td>3-AT</td>
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<td>Molybdenum cofactor</td>
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<td>Neuligin</td>
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<td>NMDA receptor</td>
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<td><strong>PCI</strong></td>
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<td>Transmembrane</td>
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<td>5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside</td>
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<td>Abbreviation</td>
<td>Description</td>
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<td>XLID</td>
<td>X-linked intellectual disability</td>
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<td>Yeast extract peptone dextrose</td>
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<td>Yeast two-hybrid</td>
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<tr>
<td>β-Me</td>
<td>β-mercaptoethanol</td>
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Publications

Some of the work described in this thesis has been published in the following peer-reviewed articles/reviews.


Prizes and Awards

*Society for Neuroscience Graduate Student Travel Award* to attend the Neuroscience 2010 meeting in San Diego, USA. These awards are supported by Eli Lilly & Co and are awarded to 'outstanding female and male graduate researchers'.
Posters and presentations


Ramsden SL, Harvey K, Harvey RJ. The role of GEFs and gephyrin in inhibitory GABA<sub>A</sub> and glycine receptor clustering. 15th International Workshop on Fragile X and other early-onset cognitive disorders, Berlin, Germany. 4th-7th Sept 2011. *Poster presentation.*


### Primer Sets and Sequences

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<tr>
<th>Amplification target</th>
<th>Primer Names</th>
<th>Primer sequence (5’-3’)</th>
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<td>TGTCTGTTGCTATGGTGACCCGACTCCCG</td>
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<tr>
<th>IQSEC3</th>
<th>pK5-HA-rIQSEC3</th>
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<tr>
<td>Alpha1 T375D2</td>
<td>CCTGGCTAAGTTAGGGTCATAGCTGGTGCTGTAGGACG</td>
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<td>CAGACAGCCTTCTGCCAGTACCCGATG</td>
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<tr>
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Geph binding site-1: AAGGATTTCAGGCTATGTCCATTGACGCTGTGTCCAGAGATTATGCGTCCCTGACGAGCATCCCGCC

Geph binding site-2: GGCGTCGACTCAGGAGCCATGCATTTGGGA

GBS del1: GCTGCCCGCGGAGCCCTGTGCCAGGCCGGGT

GBS del 2: ACCGGGCTGGGCGACAGGGCTCAGCGCGGACG
<table>
<thead>
<tr>
<th>Chimera Primers</th>
<th>Amplification Target</th>
<th>Template DNA</th>
<th>Primers Names</th>
<th>Primer Sequences</th>
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<tr>
<td><strong>GABA&lt;sub&gt;A&lt;/sub&gt;R α1 subunit with α3 insertion</strong></td>
<td>pYTH16 GABA&lt;sub&gt;A&lt;/sub&gt;R α1 subunit</td>
<td>LongEx-alpha3into1A</td>
<td>CCAAGAAAGTGAAGGATCCTACCAAGAAACAAGCACCACCTCAACATAGTGGA ACCACCTATCTATCAGGAGGACTACCC</td>
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<td>Ex-alpha3into1A</td>
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<td>pYTH16 GABA&lt;sub&gt;A&lt;/sub&gt;R α3 subunit</td>
<td>N-del alpha3A</td>
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