

# Characteristics of the effects of genetic variation in rodent models of risk for schizophrenia and neurodevelopment disorder

A thesis submitted for the degree of

Doctor of Philosophy (PhD)

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2019

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# Acknowledgements

Firstly I would like to thank my wonderful supervisory team: Kerrie Thomas, Jeremy Hall and Lawrence Wilkinson for all their support and guidance throughout this project. I was very lucky to work in such a wonderful research group with such a great atmosphere instilled from the top down. I have learned a great deal from you all and will be forever grateful. I would especially like to thank Kerrie for being such an inspirational woman in STEM to me, and patiently listening to me and encouraging me when I've felt inadequate or incapable.

I also want to extend my gratitude to Adrian Harwood, my previous supervisor in the NMHRI. He gave me a chance when it felt like no one else was willing to, and over the short time I worked for him I gained such much experience and confidence that I felt drastically more able to complete a PhD.

I would like to thank all members, past and present, of the Thomas/Hall lab throughout the years for all their support. In particular, Simon Trent for assistance with various behavioural and molecular techniques, and Anna Moon and Niels Haan for all the friendship and fun, assistance with various things and support when it was tough going. Thankyou to Jo for many excellent baking treats and fluffy kitties! I would also like to thank all the fab people in the NMHRI technical team, especially Craig and Emma, and Trevor Humby for all his guidance with much of the behavioural work in this thesis.

Manni, I know writing anything nice is going to make you super uncomfortable because –emotions- but just know I'm eternally grateful that instead of getting your PhD I got you! To Kira, you wonderful person. I have never enjoyed living with someone so much, thank you for everything. I miss watching Lucifer with you. (#suspiciouslyclose). To Rachael, Jon, Oly, Lee, Ellen and Aurelian. As I've said to you so many times before you guys are the best group of friends I have ever had, I love you all to pieces, thank you for all our adventures! A particular thank you to Jon, Oly and Ellen for introducing me to Frey, Seshra and Azula. There is no going back now!

To my long suffering karate club who have put up with me whinging about being exhausted for this whole process thank you! Hopefully this thesis will be almost not bad! To Matt, my wonderful PT and friend. I honestly dread to think the state my mental health would be in without lifting, and it is largely down to you. I massively

appreciate your ability to push me to do more than I think I can, but if for some reason it just isn't happening change it up so I don't just feel like I failed. Also thank you and Verity for bringing the adorable Holly into my life. Every gym should have a dog.

I also want to extend thanks to two wonderful youtube channels who have come to mean an awful lot to me. To the whole Oxboxtra team (not forgetting James and John!) your videos have always cheered me up, and given me much needed outlets to just forget about everything that is stressing me out. At this point I have been watching for about 3 years and it feels like catching up with old friends. I was very lucky to meet you all in person, everyone was so lovely!

To everyone at Critical role thank you so much for creating this beautiful world in which I have become immersed and invested in, and for being beautiful people in the real world too! The joy and hope your storytelling fills me with feels like sheltering from a storm.

Finally, the biggest thank you, and the one which words will never truly convey goes to my family: my brother, my Nan, Gran and Grandad, but especially Mum and Dad. You guys are my solid ground. There is no way I could have done this without your love and support. It was never a question when I was younger that I would go as far as I could in whatever I wanted because of the belief you instilled in me. And although that has wavered over the years because of various setbacks you were always there to help pick me up again and set me back on my path. There is a line in a Rise Against song that has resonated with my during the PhD "tell me there's a reason to take another step, to get up off my knees, and follow this path of most resistance". You help remind me why I want to keep going. You have always believed in me and I'll never stop trying to make you proud. I love you both so much.

# Summary

Various genetic factors increase the risk of developing a psychiatric disease like schizophrenia. This includes large deletions or duplications known as copy number variants (CNVs). Genetic investigations have converged on specific pathways, implicating them in the development of schizophrenia. These key pathways include synaptic functioning, post-synaptic density (PSD), and glutamatergic functioning. The PSD is important for synaptic plasticity, a process thought to represent the molecular basis of learning and memory. As such many of the cognitive impairments observed in schizophrenia are proposed to occur as a result of abnormal synaptic plasticity.

CNVs affecting Discs large homolog 2 (*DLG2*) have been associated with psychiatric disease in human genetic studies. DLG2 is an important part of the PSD involved in the regulation of glutamatergic functioning through interaction with glutamatergic receptors NMDA and AMPA. Previous deficits were observed in a knock out (KO) *Dlg2* mouse model in complex cognitive processes involving flexibility. Similar phenotypes were observed in humans carrying *DLG2* CNVs.

Research into *Dlg2* mutation is still limited, especially its behavioural impact. This thesis aimed to characterise two different rodent models (mouse and rat) of *Dlg2* heterozygosity. In both models basic molecular characterisation was conducted on the impact of the genetic lesion. In the mouse model, *Dlg2*<sup>tm1a(EUCOMM)Wtsi</sup>, a range of basic behavioural tasks were used that had not previously reported in the strain, discovering two specific learning phenotypes: deficient motor learning, and reduced acoustic startle response and habituation. Impaired habituation to a context was also observed during a locomotor activity task. Further investigation was conducted into the motor learning phenotype across multiple days. From this it was determined that the deficit was most apparent during the earliest phase of motor learning. Cellular investigation partially implicated reduced neuronal activity in a brain region important for motor learning, M1, in this phenotype. The impact *Dlg2* mutation on adult neurogenesis was also examined, which has previously been proposed as a synaptic risk convergent phenotype. In contrast to previous studies in other risk models no changes were found.

Finally, anxiety and motor learning were investigated in the rat. Like the mouse no anxiety phenotypes were found, but in contrast no motor learning impairment was found. Associative learning was probed using a contextual fear conditioning paradigm. No differences were found, including extinction learning, in contrast to the previously reported deficit. A big advantage of the rat over the mouse is the reduced expression of *Dlg2* in the hippocampus and PFC, as opposed to just the PFC. This better mimics the dysfunctional network observed in schizophrenia and can be capitalised on to study behaviours reliant on these regions.

# **Common Abbreviations**

AMPAR	$\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid		
	receptor		
AN	Adult neurogenesis		
ANOVA	Analysis of variance		
ASD	Autism spectrum disorder		
BPD	Bipolar disorder		
cDNA	Complementary DNA		
CFC	Contextual fear conditioning		
CNV	Copy number variants		
CR	Conditioned response		
CRISPR	Clustered regularly interspaced short palindromic repeats		
CS	Conditioned stimulus		
DAPI	4',6-diamidino-2-phenylindole		
DCX	Doublecortin		
DG	Dentate gyrus		
Dlg	Discs large homolog		
DNA	Deoxyribonuleic acid		
DSM-IV	Diagnostic and statistical manual of mental disorders 5		
EPM	Elevated plus maze		
GABA	Gamma-Aminobutyric acid		
GK	Guanylate kinase-like domain		
GWAS	Genome wide association study		
HET	Heterozygous		
ICD-11	International classification of diseases 11 <sup>th</sup> revision		
КО	Knockout		
LTD	Long term depression		
LTP	Long term potentiation		
MAGUK	Membrane-associated guanylate kinases		
mRNA	Messenger RNA		
NMDAR	N-Methyl-D-aspartic acid		
PCR	Polymerase chain reaction		
PFC	Pre-frontal cortex		
PPI	Pre-pulse inhibition		
PSD	Post-synaptic density		

qPCR	Quantitative PCR
RNA	Ribonucleic acid
SCZ	Schizophrenia
sgRNA	Single guide RNA
SH3	SRC Homology 3 domain
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SNP	Single nucleotide polymorphism
SNV	Single nucleotide variant
US	Unconditioned stimulus
WT	Wildtype

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## **1 General Introduction**

Psychiatric disorders accounted for 7% of the world's disease burden in 2016 (Rehm and Shield, 2019). With the advances in genetic investigation of these diseases (discussed in more detail in 1.1) uncovering overlap between disorders interest in the shared aetiology has increased, and has led some to consider these disorders to be on a spectrum, as opposed to being completely independent (Lobo and Agius, 2012; Adam, 2013). These studies have also brought into sharp focus the inadequacies of current boundaries between the diseases and the criteria currently used for diagnosis, given the shared genetic risk. The genetic approach to studying these disorders has also highlighted important pathways that are susceptible, more so in some diseases than others. Of most relevance to this thesis is the particular susceptibility of the postsynaptic density (PSD) and synaptic functioning in increasing risk of developing schizophrenia (Network and Pathway Analysis Subgroup of Psychiatric Genomics Consortium, 2015a). There is also evidence implicating the importance of the PSD in bipolar disorder (BPD), although this is currently more limited in comparison to schizophrenia (Akula *et al.*, 2016).

Schizophrenia affects around 1% of the global population, whilst BPD has a lifetime risk ranging from 1-4% (Kessler et al., 2005). Schizophrenia is widely considered to be a disorder of abnormal neurodevelopment (Murray and Lewis, 1987; Owen et al., 2011). This is largely due to the association between complications during pregnancy and risk of developing schizophrenia (Gupta and Kulhara, 2010), difference in brain structure sizes in first episode schizophrenia patients (Shenton et al., 2001), and foetal gene expression. It was found genes associated with schizophrenia and ASD, but not BPD, were preferentially expressed during foetal life (Jaffe et al., 2015), and additionally that many of the schizophrenia GWAS risk loci directly influence the placenta and can predict presence of pregnancy complications (Ursini et al., 2017). Whilst this hypothesis is initially at odds with the fact the general age of onset is during adolescence it has been argued that the brain is still undergoing development at this point. For example the importance at this age of synaptic pruning, and that any predisposition (ie genetics, smaller brain regions) may negatively interact with the environment and manifesting as the disease (Davis et al., 2016). By contrast in the current literature BPD is not generally described as a neurodevelopmental disorder, as those neurodevelopmental mechanisms, implicated in schizophrenia, are thought to only apply to a subset of BPD patients, if at all (Valli, Fabbri and Young, 2019).

Both diseases fall towards the more severe end of the proposed psychiatric disease spectrum (Adam, 2013), with psychotic symptoms prevalent in both. However, BPD is predominately a disorder of mood whereas schizophrenia has symptoms falling to three clusters; positive (psychotic), negative (changes in affect) and cognitive. The cognitive impairments fall under several domains, including attention, working memory, executive function and verbal learning and memory (Bowie and Harvey, 2006). These deficits often occur much earlier than positive symptoms in those who go on to be diagnosed with schizophrenia (Häfner *et al.*, 1992) and can be considered a maker for the disease (Rund, 1998). Given this unmet need and potential earlier identification of the disease there is much interest in investigating plausible factors involved in the cognitive dysfunction, such as the role of synaptic functioning.

Current treatments have very little effect on cognitive or negative symptoms of schizophrenia (Tripathi, Kar and Shukla, 2018). Schizophrenia, like BPD, can be a life-long chronic condition, which often reduces life expectancy and results in serious impairment in many domains of daily life such as employment, social relationships and independence. These symptoms are often still present in the absence or remission of psychosis.

Many factors are thought to contribute to schizophrenia, with its development being a complex interplay of biological, environmental and social factors. Factors such as migration (Cantor-Graae and Selten, 2005), childhood adversity or abuse (Matheson *et al.*, 2013), and maternal infection during the first and second trimester of gestation (Boksa, 2008) all increase risk of developing schizophrenia. The disease is considered highly heritable, with heritability estimates (the portion of the variance explained by genetic factors) of around 80% (Gejman, Sanders and Duan, 2010). A close genetic relationship to someone with schizophrenia increases the risk of developing the disease (Figure 1) (Gottesman, 1991). Epigenetic mechanisms, such as DNA methylation, might explain the 52% discordance in monozygotic twins (Mill *et al.*, 2008), and have been proposed to mediate the impact of environmental factors (Brown and Susser, 2008; Ellman and Susser, 2009).



Figure 1. The closer the genetic similarity of an individual to someone diagnosed with schizophrenia the greater their risk of also developing the disease (Gottesman, 1991).

High heritability is also seen in other psychiatric conditions, including BPD which has heritability estimates between 60-85% (Smoller and Finn, 2003), and autism (ASD) which has a heritability estimate of 90% (Tick et al., 2016). This shared genetic aetiology has been demonstrated between these psychiatric disorders, as well as major depression and attention deficit hyperactivity disorder (ADHD), implying there are common pathophysiologies affected (Cross-Disorder Group of the Psychiatric Genomics Consortium et al., 2013). Diagnosis of these psychiatric diseases is still largely subjective, based on symptomatic criteria in the DSM-V and ICD-11. There can be overlap in symptom presentation, and often co-morbidity of these disorders, which makes distinct diagnosis challenging (Murray et al., 2004). However, there are also differences in phenotypes, such as brain structure, and risk factors between the diseases. For example, enlarged ventricles and reduced brain volume have been consistently found in brain imaging studies of schizophrenia (Nelson et al., 1998; Wright et al., 2000; DeCarolis and Eisch, 2010; Arnold et al., 2015), and more recently common genetic risk variants are associated with reduced hippocampal (Harrisberger et al., 2016) and white matter volume (Oertel-Knöchel et al., 2015). Additionally complications in pregnancy increase the risk of developing schizophrenia (Geddes et al., 1999; Ursini et al., 2018). None of these, however, are the case for BPD (Verdoux and Bourgeois, 1993; Hoge, Friedman and Schulz, 1999; Ranlund et al., 2018). This led some to propose a susceptibility to a spectrum of psychiatric diseases, with

different risk factors potentially pushing disease trajectory down certain pathways (Figure 2) (Adam, 2013).



Figure 2. Susceptibility to developing psychiatric diseases can be considered on a spectrum of increasing severity and age of onset, with some overlapping risk factors and symptomology (Adam, 2013).

### 1.1 Genetic approaches to investigating psychiatric diseases

Various approaches to investigating the genetic influence in risk of developing these disorders have been used. Initially linkage analysis was conducted in samples from affected families and sibling pairs to determine key genomic regions. Linkage analysis involved examining the degree of co-segregation between phenotypic traits and genetic markers, allowing estimates of linkage between the disease and genomic loci to be determined (Lander and Kruglyak, 1995). Although there were positive findings these proved difficult to replicate (Farrell *et al.*, 2015). Focus then shifted to a candidate gene approach. The targets investigated were selected due to position (from linkage analysis) or function, such as association with dopaminergic function in schizophrenia studies. Overall this approach was also underwhelming, with the pathogenic potential of some major schizophrenia candidate genes such as *DISC1* and *COMT* still being debated (Farrell *et al.*, 2015), and a lack of consistency surrounding candidate genes in BPD (Seifuddin *et al.*, 2012)

### 1.1.1 Common variants

One major hindrance to exploring psychiatric diseases, using these approaches is the polygenic nature of the diseases (Gejman, Sanders and Duan, 2010). More recently genome wide association studies (GWAS) have replaced hypothesis-driven candidate gene studies, instead using case-control comparison studies with thousands of samples to examine single nucleotide polymorphisms (SNPs). This approach facilitates empirical investigation of association between common genomic variants and disease (van der Sijde, Ng and Fu, 2014). These variants are, as the name implies, commonly occurring (a minor allele frequency between 1-5% (Neale and Sklar, 2015a) but have fairly low penetrance for developing a disease. This is in

contrast to rare variants which do not occur regularly but have a higher penetrance (see below). The reasoning behind GWAS studies is that more frequent occurrence of specific allele variants in patients compared to controls is indicative of a genetic association (Henriksen, Nordgaard and Jansson, 2017). A major study combining all available schizophrenia GWAS samples found 108 risk loci, of which 83 were novel, and implicated not only the dopaminergic system through dopamine receptor D2, but also the glutamatergic system, synaptic plasticity and the immune system (Ripke *et al.*, 2014). This has since been expanded to 145 loci (Pardiñas *et al.*, 2018). Subsequently similar studies were conducted in BPD uncovered 30 risk loci using a GWAS approach (Stahl *et al.*, 2017). The smaller number found likely reflects the smaller cohort size compared to schizophrenia studies.

Building upon the GWAS approach pathway analysis studies were employed to uncover molecular pathways underlying the genetic risk implicated by these variants. A key study examined five disorders (schizophrenia, BPD, ASD, major depression and ADHD). Three major pathways were implicated: synaptic functioning, histone modification and the immune system (Figure 3). Effected pathways were shared across BPD, schizophrenia and depression, again reinforcing the hypothesis of increased susceptibility to multiple psychiatric diseases through common factors (Adam, 2013; Network and Pathway Analysis Subgroup of Psychiatric Genomics Consortium, 2015b). Interestingly however there was divergence between the diseases in the prominence of the different pathways. Histone methylation was more strongly implicated in BPD than for schizophrenia, where the synapse and postsynaptic functioning was predominate.



Figure 3. Pathway analysis conducted on GWAS data for five psychiatric diseases uncovered three key pathways affected by pathogenic variation (Network and Pathway Analysis Subgroup of Psychiatric Genomics Consortium, 2015b)

Whilst GWAS studies are useful, the penetrance conferred by the mutations in common variation is very low, with odd ratios of <1.2 (Neale and Sklar, 2015a). Common variants have been estimated to account for between a quarter to a half of the genetic liability in schizophrenia, but not all (Cross-Disorder Group of the Psychiatric Genomics Consortium *et al.*, 2013). Additionally, the majority of schizophrenia associated SNPs were found in non-coding regions, making it more challenging to uncover the biological basis of the association (Neale and Sklar, 2015a). Therefore, other approaches were also required to understand the complex genetics of psychiatric disorders.

### 1.1.2 Rare variants

As well as common variants much rarer (< 1% minor allele frequency) but highly penetrant mutations also confer disease risk, including copy number variants (CNVs), rare single nucleotide variants (SNVs) and small insertions and deletions (indels) (Neale and Sklar, 2015b). CNVs are deletions or duplications in the genome that range in size from one kilobase (kb) to several megabases (Mb) and can involve multiple genes, although some only affect one gene such as *NRXN1* coding for neurexin (Rujescu *et al.*, 2009). Multiple CNVs have been identified which confer increased risk for developing schizophrenia and have much greater odds ratios than

common variants (2.7 to >26), and although none are sufficient to cause schizophrenia alone (Mowry and Gratten, 2013) carriers risk of developing schizophrenia is between 5-30% depending on the CNV (Forsingdal *et al.*, 2018). The CNV conveying greatest risk are deletions within 22q11.2, with approximately 25% of carriers developing psychosis (Sebat, 2013). By comparison whilst still contributing to risk the impact of CNVs in BPD is less than for schizophrenia (Craddock and Sklar, 2013). Some CNVs are inherited (Harrison, 2015), whilst others occur *de novo* (Xu *et al.*, 2008; Malhotra *et al.*, 2011; Kirov *et al.*, 2012). The burden of pathogenic CNVs is greater in people with schizophrenia than controls (Stone *et al.*, 2008; Walsh *et al.*, 2008).

As well as CNV analysis exome sequencing facilitates interrogation of DNA variants in the protein coding regions of the genome at a single base resolution (Henriksen, Nordgaard and Jansson, 2017). Whilst 13 genes were found in a study examining BPD the study failed to reach the exome-wide threshold for association, again likely due to smaller sample sizes than in schizophrenia studies (Husson et al., 2018). A polygenic burden of rare (<1 in 1000) SNVs and indels across many genes was found in schizophrenia (Purcell et al., 2014). As found in GWAS studies (Glessner et al., 2010; Ripke et al., 2014), and de novo CNV studies (Kirov et al., 2012; Fromer et al., 2014), there was an enrichment of mutations affecting components of the postsynaptic density (PSD, see section 1.3), with SNVs and indels affecting proteins important in glutamatergic signalling, including Arc (activity-regulated cytoskeletonassociated protein) and *N*-methyl-D-aspartate receptor (NMDAR) protein complexes (Fromer et al., 2014). The recent convergence of genetic findings implicating glutamatergic functioning further supported the glutamatergic hypothesis of schizophrenia, providing an alternative to the dominant dopaminergic hypothesis. This hypothesis is beyond the scope of this introduction so for a detailed review of the dopaminergic hypothesis of schizophrenia see Howes and Kapur, 2009. As the glutamatergic hypothesis is intricately linked with synaptic functioning it is discussed in more detail in the following section. Additional interest in the glutamatergic hypothesis comes from its potential to provide alterative targets for antipsychotic drug development.

### 1.2 Glutamatergic hypothesis of schizophrenia

The dopaminergic hypothesis has been unable to explain many aspects of schizophrenia so investigation was expanded to other affected systems, including the glutamatergic system. The glutamatergic hypothesis postulates that many of the abnormalities observed in schizophrenia stem from dysfunctional glutamatergic functioning (Stone, Morrison and Pilowsky, 2007; Javitt, 2010). It was initially proposed following studies investigating the effect of a non-competitive NMDA antagonist ketamine in healthy people. Ketamine was found to induce psychotomimetic affects in studies during the 1960's (Howes and Kapur, 2009). Since the 1990's more studies have confirmed that the administration of ketamine, and other NMDA antagonists like PCP, increased positive, negative and cognitive symptoms in both healthy people and those with schizophrenia (Javitt, 1987, 2010; Javitt and Zukin, 1991). The exacerbation of symptoms in those already diagnosed with schizophrenia implies these NMDAR antagonists are affecting an already vulnerable system (Javitt, 2010). The transitory reproduction of psychotic symptoms following administration of these compounds lead to the development of an alternative to the dopaminergic hypothesis of schizophrenia, instead focusing on dysfunction and dysregulation of NMDA receptor mediated glutamatergic transmission (Tsai et al., 1998). Interestingly, in healthy volunteers whilst both ketamine and the dopaminergic antagonist amphetamine induced positive symptoms, only ketamine produced the perceptual changes, negative symptoms and disruptions in delayed recall and working memory similar to those observed in schizophrenia (Krystal et al., 2005). Additionally amphetamine administration to patients it did not exacerbate their cognitive impairments but in fact may actually improve them (Barch and Carter, 2005)

There is convergent genetic evidence implicating glutamatergic functioning, including GRIN2A, which codes NMDAR subunit GluN2A in risk for developing schizophrenia (Ripke *et al.*, 2014). Additionally, other studies in rodents and humans support the dysfunction of glutamatergic system in schizophrenia. In rodents NMDAR antagonist-induced neurotoxicity in prefrontal cortical areas resulted in persistent anxiety phenotypes often associated with schizophrenia (Coleman *et al.*, 2009). A single photon emission tomography (SPET) study found a reduction in NMDAR binding in the hippocampus in unmedicated schizophrenia patients compared to controls (Pilowsky *et al.*, 2006).

Dysfunction of the glutamatergic system has also been proposed as an upstream cause of dopaminergic dysfunction characteristic of schizophrenia (Lisman *et al.*, 2008; McGuire *et al.*, 2008). Dopaminergic neurons are sensitive to alterations in glutamatergic signalling, as they are regulated by glutamatergic projections to midbrain dopamine nuclei (Miller and Abercrombie, 1996). One influential model reconciles glutamatergic, GABAergic and dopaminergic dysfunction in the development of schizophrenia (Lisman *et al.*, 2008). Fast-spiking interneurons are a

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component of a homeostatic feedback mechanism that stabilises glutamatergic pyramidal neuronal activity. Lisman *et al* (2008) argue this is mediated through NMDAR on the interneurons sensing glutamatergic activity, and if for any reason there is hypofunction of NMDAR this would be misinterpreted as reduced activity of the pyramidal neurons. This faulty reading would trigger the normal homeostatic response to perceived inactivity to compensate and return levels to normal. This is achieved via reduced synthesis of GAD67, which means that less GABA is produced. This reduction in GABA is maladaptive in this context and it erroneously leads to overactivation of glutamatergic signalling. In turn this leads to disinhibition of the hippocampal region, and hyper-activation of dopaminergic neurons in the ventral tegmental area (VTA), resulting in an overactive mesolimbic dopaminergic pathway and the hyperdopaminergia characteristic of schizophrenia (Lodge and Grace, 2007; Lisman *et al.*, 2008).

The hyperactive glutamatergic system over-activating the GABAergic interneurons in the VTA may underlie the negative and cognitive symptoms, whilst over-inhibition of the mesocortical dopamine pathway reduces dopamine supply to the PFC thus causing hypofrontality (Ellaithy *et al.*, 2015).

A dysfunctional glutamatergic system may be the result of direct mutation of proteins important for its function, such NMDAR or AMPAR, but also indirectly through mutations in interacting proteins contained within PSD.

### 1.3 The post-synaptic density

The excitatory PSD is a protein dense region found at the plasma membrane of excitatory synapses. The PSD of individual synapses has been estimated to contain thousands of different component proteins (Sheng & Hoogenraad, 2007; Selimi et al., 2009), many of which are vital to synaptic plasticity – the process that brings about change in the strength of synaptic transmission between neurons (Zheng *et al.*, 2011). The presence of the PSD facilitates regulation of synaptic plasticity through post synaptic mechanisms in excitatory synapses.

The PSD is highly organised structure defined by the interaction of various proteins and the cytoskeleton, resulting in specific localisation of proteins which determines functional microstructures important for synaptic transmission (Colgan and Yasuda, 2014a).



Figure 4. Organisation of the PSD. The receptors and ion channels reside closest to the post synaptic membrane. They are anchored by various scaffolding proteins which lie perpendicularly to the membrane. These proteins are then connected to Shank and GKAP family proteins, which themselves interact with the actin cytoskeleton. Image from Feng and Zhang, 2009.

The first layer of PSD, closest to the post-synaptic membrane, contains the ion channels, membrane receptors and transmembrane cell adhesion molecules (Figure 4). Also within this layer are two key glutamate receptors, *N*-methyl-D-aspartate (NMDA),  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA). Both AMPAR and NMDAR are key receptors for synaptic plasticity. AMPAR form ion channels which primarily conduct sodium and potassium, but depending on subunit composition can also be permeable to calcium (Gouaux, 2004). NMDAR are ligand-gated and voltage-dependant ion channels permeable to calcium, and are considered coincidence detectors, requiring both binding of glutamate and depolarisation of the membrane to release the magnesium ion (Mg<sup>2+</sup>) blocking the channel pore (Dingledine *et al.*, 1999). AMPAR tend to be more laterally distributed in the PSD whereas NMDAR are more centralised (Kharazia and Weinberg, 1997).

Scaffolding proteins are enriched in the second layer of the PSD (Feng and Zhang, 2009a). The largest group of these are the membrane-associated guanylate kinases (MAGUKs) (discussed later in this section), which includes postsynaptic density protein 95/disc large homolog 4 (PSD-95/DLG4), often cited as the fundamental building block of the PSD (Cho, Hunt and Kennedy, 1992; Chen *et al.*, 2011). These scaffolding proteins lie perpendicularly to the PSD membrane and interact directly

and indirectly with the receptors and ion channels in layer one (Figure 4). There is often shared structure between scaffolding proteins, such as the protein-protein interaction PDZ domains (Kim and Sheng, 2004a; Funke, Dakoji and Bredt, 2005). The name is derived from the common structure found in PSD-95, Discs Large and Zona Occludens 1 proteins.

Below these proteins lies the third layer of the PSD. Here the SH3-GK domains of the scaffolding proteins in layer two bind to SH3 and multiple ankyrin repeat domains protein (Shank) and guanylate kinase-associated protein (GKAP) family proteins, which run parallel to the PSD membrane (Feng and Zhang, 2009a). In turn these proteins are connected to the actin cytoskeleton. Other cytoplasmic proteins and enzymes are then able to bind to the protein framework created by the interacting membrane receptors and scaffolding proteins.

A hierarchal assembly of complexes can form as a result of these protein-protein interactions. Individual proteins are starting components, which aggregate into complexes, which in turn associate together to form supercomplexes (Frank *et al.*, 2016; Frank and Grant, 2017). Significantly these supercomplexes can form between scaffolding proteins and glutamatergic receptor. NMDAR complexes, for example, are comprised of NMDAR subunits which form receptor tetramers; tri-heterotetramers contain GluN2A-GluN2B-GluN1, whereas di-heterotetramers contain GluN2A-GluN2B-GluN1, whereas di-heterotetramers contain GluN2A-GluN1 (Frank *et al.*, 2016; Frank and Grant, 2017). These NMDAR complexes then form supercomplexes with two key scaffolding proteins from the MAGUK family, DLG4 and related protein DLG2 (Frank *et al.*, 2016; Frank and Grant, 2017). DLG4 forms part of many supercomplexes, some with NMDAR and some without. Aggregation of these supercomplexes occurs as a result of high DLG4 concentration, resulting in sub-synaptic structures referred to as nanodomains (MacGillavry *et al.*, 2013; Broadhead *et al.*, 2016).

For the NMDAR supercomplexes to form both DLG4 and DLG2 are required, despite the more prominent role of DLG4, on its own it was not sufficient to induce supercomplex formation, nor was DLG2 alone sufficient (Frank *et al.*, 2016). This prerequisite of the presence of all three components is referred to as the 'tripartite rule' (Frank *et al.*, 2016; Frank and Grant, 2017). By contrast the supercomplex assembled between a key plasticity protein, Activity-regulated cytoskeletonassociated protein (Arc), and the inwardly rectifying potassium channel Kir2.3 requires DLG4 but not DLG2 (Frank *et al.*, 2017). This illustrates the possibility a

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mechanism regulating the specificity in composition and type of supercomplex may be in part genetically mediated, as despite the large functional overlap between DLG2, and DLG4 the aforementioned supercomplex does not involve DLG2 (Frank and Grant, 2017).

The structure of the PSD is dynamic, both during development (Feng and Zhang, 2009a) and in response to neuronal stimuli, with PSD proteins assembly or disassembly being associated with different types of synaptic plasticity (Zeng *et al.*, 2018).

Two major forms of plasticity are Hebbian and homeostatic plasticity. Hebbian plasticity is considered to be the mechanism through which information is encoded and retained in synapses and is fundamental to learning and memory (Colgan and Yasuda, 2014b). Homeostatic plasticity is the ability of neurons to regulate their own firing relative to the network in order to maintain a baseline, acting as a safeguard against unregulated excitation or inhibition (Turrigiano, 2011).

The fluidity of the molecular make-up and organisation of the PSD is best illustrated by the changes which occur following different types of Hebbian plasticity - Long-Term Potentiation (LTP) and Long-Term Depression (LTD) (Huganir and Nicoll, 2013; Bosch et al., 2014; Araki et al., 2015), both of which result in long term changes to synaptic strength. Some estimates have suggested at least 2% of the PSD proteasome changes following induction of LTP, although this is probably an underestimation (Zhang, Neubert and Jordan, 2012). LTP has been demonstrated to induce loss or recruitment of different PSD proteins including DLG4, Shank, RNA binding proteins and CaMKII (Yoshimura et al., 2002). Additional AMPAR are incorporated stably into the post synaptic membrane as a result of the PSD remodelling in response to LTP (Malenka, 2003; Nicoll and Roche, 2013). Calcium influx through NMDAR triggers synaptic excitation which activates CaMKII, which relocates into the spine due to its association with GluN2B, and mediates the remodelling of the PSD through phosphorylation of PSD proteins, including AMPAR, NMDAR, DLG4 and Stargazin (Yoshimura et al., 2002). Phosphorylation facilitates movement and can alter binding possibilities between the proteins. For example DLG4 is phosphorylated at serine-73 by CaMKII, which triggers dissociation of binding with Shank, and results in a significant proportion of DLG4 transiently dissociating from the synapse, indicative of PSD remodelling (Steiner et al., 2008).

DLG4, and DLG2, are important for the regulation of LTP and LTD (discussed in 1.3.1) due to their interaction with vital glutamatergic receptors, AMPAR and NMDAR.

Both receptors play key roles in LTP and LTD. The induction of LTP occurs following sustained high level activation of AMPAR facilitating depolarisation of the postsynaptic membrane, thus removing the Mg2<sup>+</sup> block from NMDAR, allowing influx of calcium (Bi and Poo, 2001). This influx then leads to downstream changes in gene expression, and activity-dependant changes in AMPAR trafficking which increase recruitment of AMPAR to the plasma membrane (Bredt and Nicoll, 2003; Huganir and Nicoll, 2013). LTD occurs when prolonged weaker activation induces signalling cascades which result in removal of AMPAR from the plasma membrane. Initially the idea activation of NMDAR and subsequent calcium influx can lead to two opposing processes (LTP and LTD) was confusing. However, manipulation of the relative timing of pre- and postsynaptic activation emphasised the importance of the temporal pattern of calcium release in determining the direction of synaptic response (Bi and Poo, 2001).

AMPAR are additionally vital for homeostatic plasticity, a negative feedback process which prevents chronic over or under activity. This maintains the excitation/inhibition balance in the brain, which is often dysfunctional in neurological diseases including schizophrenia (Wondolowski and Dickman, 2013). If neurons are induced to fire more than normal within hours firing is returned to baseline, and conversely compensation occurs if activity is reduced for any prolonged period time, eventually restoring baseline firing. There are many mechanisms through which this process is achieved; for example some act at a 'local' scale only affecting small groups or individual synapses (Yu and Goda, 2009), and others 'globally' affecting all the neuron's synapses (Turrigiano, 2008). One vital process is synaptic scaling, the adjustment of postsynaptic strength in order to compensate for activity input (Chowdhury and Hell, 2018). There are multiple mechanisms through with synaptic scaling is achieved including phosphorylation of the GluA1 subunit (Oh et al., 2006; Man, Sekine-Aizawa and Huganir, 2007; Joiner et al., 2010), switching between calcium permeable and impermeable AMPARs (Chowdhury and Hell, 2018), and interactions with PSD proteins like Stargazin and DLG4, GRIP1/PICK1, and immediate early genes Homer1A and Arc. These mechanisms can increase or decrease AMPAR at the membrane, alter the AMPAR subunit composition or both (Chowdhury and Hell, 2018).

Synaptic plasticity is considered the cellular basis of learning and memory (Hebb, 1949; Bliss and Lømo, 1973; Whitlock *et al.*, 2006; Takeuchi, Duszkiewicz and Morris, 2014). As discussed above PSD proteins are important for the expression of LTP and LTD. Impairments in LTP have been proposed to underlie cognitive dysfunction in

schizophrenia (McGlashan, 2005; Peled, 2005; Salavati *et al.*, 2015; Bhandari *et al.*, 2016). Therefore it is plausible that mutations affecting PSD proteins, like the glutamatergic receptors and scaffolding proteins already associated with schizophrenia, may be contributing to cognitive dysfunction through abnormal LTP (Harrison and Weinberger, 2005; Frantseva *et al.*, 2008). The importance of the glutamatergic system to this process may also partially explain the lack of effectiveness of current antipsychotics on the cognitive symptoms, given that all current drugs principally target the dopaminergic system (Snyder *et al.*, 1970; Roth *et al.*, 2004; Stone, 2011; Comai *et al.*, 2012), although some atypical antipsychotics additionally target the 5HT receptor (Seeman, 2002).

As with schizophrenia there is convergence of genetic evidence implicating the PSD and abnormal synaptic plasticity to BPD (Pennington *et al.*, 2008; Föcking *et al.*, 2015; Akula *et al.*, 2016), and ASD (van de Lagemaat and Grant, 2010; Chung, Tao and Tso, 2014; Leblond *et al.*, 2014). This implies abnormal functioning of the PSD is a shared risk factor for multiple psychiatric diseases. For example mutations in *SHANK3*, an important scaffolding protein (Kim and Sheng, 2004b), have been linked to schizophrenia, ASD and BDP (Zhou *et al.*, 2016). *Shank3* mutant mice exhibit impaired LTP but normal LTD indicating altered synaptic transmission (Bozdagi *et al.*, 2010; Yang *et al.*, 2012), in addition to impaired social interactions, delayed motor learning and impaired novel object recognition; phenotypes associated with autism and schizophrenia (Yang *et al.*, 2012). *De novo* mutations in *Shank3* have been found in patients with ASD (Durand *et al.*, 2007; Gauthier *et al.*, 2009), duplications have been found in BDP diagnosed patients (Han *et al.*, 2013), and different *de novo* mutations found in patients with schizophrenia (Gauthier *et al.*, 2010).

### 1.3.1 The Discs large homolog (DLG) protein family

The DLG protein family come under the MAGUK superfamily. MAGUKs are defined by the presence of PDZ, SRC homology 3 (SH3) and catalytically inactive guanylate kinase-like (GUK) domains (Godreau *et al.*, 2004). MAGUKs play vital roles in the PSD, anchoring and stabilising glutamatergic receptors to the membrane and coupling their activity to intracellular signalling cascades, therefore through these interactions MAGUKs can potentially regulate synaptic activity strength (Gardoni, Marcello and Di Luca, 2009). MAGUKs interact with other PSD proteins, such as guanylate kinase associated protein (GKAP), encoded by *DLGAP1* (Kim *et al.*, 1997; Naisbitt *et al.*, 1997). GKAP in turn interacts with other scaffolding proteins including Shank and Homer (Tu *et al.*, 1999), both of which interact with actin associated proteins, thus linking the PSD to the actin system in the cytoplasm of dendritic spines (Sala *et al.*, 2001).

The PSD-95 protein family is a group of MAGUKs consisting of four members; DLG1 (SAP97), DLG2 (PSD-93), DLG3 (SAP102), DLG4 (PSD-95) (Elias *et al* 2006; Kim & Sheng 2004b). In vertebrates these genes are paralogs of the *Drosophila* tumour suppressor gene, *Discs large homolog (Dlg)* (Woods and Bryant, 1991). Invertebrates only possess *Dlg*, which codes for two proteins (Mendoza-Topaz *et al.*, 2008). However vertebrates underwent gene duplication around 581-1141 mya, resulting in four paralogs (*Dlg1-4*), of which *Dlg1* is the homolog of *Dlg*, that have accumulated mutations which diversified their structure and function (Ryan and Grant, 2009). The ancestral importance of *Dlg* is conserved across evolution, as *Dlg1* null animals are embryonically lethal, whereas all *Dlg* paralogs are homozygotically viable (Caruana and Bernstein, 2001). DLG4 is a core component of the PSD (Gao, Tronson and Radulovic, 2013) and the number of DLG4 molecules has been found to determine PSD size (Gray *et al.*, 2006).

DLG2 and DLG4 are highly enriched in the PSD whereas DLG1 and DLG3 are found abundantly both at synapses and in the cytoplasm (El-Husseini *et al.*, 2000a). There are also different temporal expression patterns between the DLGs (Oliva *et al.*, 2012). DLG1 and DLG3 expression is highest embryonically and in early post-natal life before decreasing throughout adulthood (Müller *et al.*, 1996; Cai *et al.*, 2008). In comparison, both DLG2 and DLG4 exhibit low expression during early post-natal stages before increasing into adulthood (Sans *et al.*, 2000). Therefore, it appears DLG1, and possibly DLG3, are important for nervous system development and DLG2 and DLG4 less so. DLG2, DLG3, and DLG4 are predominately expressed post-synaptically whilst DLG1 is expressed pre and post-synaptically (Aoki *et al.*, 2001).

All four DLGs contain a variable N-terminal followed by three PSD-95/discs large/zone occludens-1 (PDZ) domains, a Src-homology (SH3) domain and finally a guanylate kinase (GK) domain (Kuhlendahl *et al.*, 1998). The PDZ domains bind to the C-terminal or internal  $\beta$  finger motifs of interacting partners such as receptors, ion channels or enzymes (Lee and Zheng, 2010). The SH3-GK domain have been proposed to mediate oligomerisation of DLGs (Funke, Dakoji and Bredt, 2005; Zheng *et al.*, 2011; Zhu, Shang and Zhang, 2016). There are multiple isoforms of each DLG resulting from alternative transcription start sites and alternative splicing (Zheng *et al.*, 2011) (Figure 5). The isoforms likely have different functions across development, regulating neuronal trafficking and some altering AMPAR functioning whilst others

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regulate synaptic transmission in an activity-dependant manner (Kruger et al., 2013). For DLG2 and DLG4 the  $\alpha$  isoform, which contains a putative palmitoylation motif of N-terminal cysteines, is the primary isoform (EI-Husseini *et al.*, 2000b). The  $\beta$  isoform, is the primary form of DLG1. This isoform in DLG1, DLG2, and DLG4 contains a putative L27 domain (Feng et al., 2004). DLG2 exhibits the most variable N-terminal, with six variations described so far in humans: two palymitoylated isoforms ( $\alpha$ ), L27 containing domain isoforms ( $\beta$ ) and three others ( $\epsilon$ ,  $\delta$ ,  $\gamma$ ) (Parker *et al.*, 2004; Kruger et al., 2013; Reggiani et al., 2017). Further variation in DLG2 isoforms is achieved by alternative splicing of exons encoding the linker region between the SH3 and GK domains (Zheng et al., 2011; Kruger et al., 2013). Dependant on the splice variant the interaction partners able to bind may vary (Kim et al., 1996; Zheng et al., 2011). Due to the presence of multiple isoforms it is plausible there are important functional differences, which is supported by the finding that some isoforms were associated with neurodevelopmental disorder (Reggiani et al., 2017). The role of the palmitoylated isoforms is better understood for DLG4 than DLG2; the process is important for interaction with AMPAR and receptor internalisation (EI-Husseini et al., 2002). Palmitoylated isoforms of DLG2 in non-neuronal cells appeared to influence ion channel clustering (EI-Husseini et al., 2000b) but were not required for synaptic targeting of either DLG2 isoform in neurons (Firestein, Craven and Bredt, 2000a).



Figure 5. Graphical illustration of the various domains found within key isoforms of the different DLGs.

The binding to interacting proteins varies between the DLGs. DLG3 is able to bind to the NMDA subunit GluN2B via any of its PDZ domains (Müller *et al.*, 1996). By comparison binding to GluN2B is only possible to the first or second PDZ domains of DLG2 and DLG4 (Brenman *et al.*, 1996; Kim *et al.*, 1996). Both GluN2A and GluN2B

contain a conserved C terminal PDZ ligand binding motif, ESDV, which regulates this binding. Deletion of the ESDV site in GluN2B disrupts surface and synaptic expression of NMDAR (Lau and Zukin, 2007). In addition to Glun2B inwardly rectifying K<sup>+</sup> channels can bind to PDZ 1 and 2 of DLG2 (Ali *et al.*, 2018). The synaptic functioning of NMDAR is regulated by neuroligins and the Src tyrosine kinase Fyn which bind to the third PDZ domain of DLG2 (Sato *et al.*, 2008; Won *et al.*, 2017). Binding to AMPA receptors is also variable; DLG1 binds directly to the GluR1 subunit (Leonard *et al.*, 1998), whereas the other DLGs interact indirectly through transmembrane AMPAR regulatory proteins (TARPS) such as stargazin (Chen *et al.*, 2000; Schnell *et al.*, 2002; Dakoji *et al.*, 2003). DLG1 is able to rescue deficits in AMPAR currents following DLG2/DLG4 double knockout, but has no effect on transmission if deleted itself (Howard *et al.*, 2010a). The lack of an effect when DLG1 when is deleted further demonstrates that the DLGs do have different roles but that there is capacity for functional compensation between them.

### 1.4 Functional roles of the DLGs

### 1.4.1 The DLGs and disease

The *DLGs* have been associated with various diseases. Mutations in *DLG1* are associated with Crohn's disease (Xu *et al.*, 2014), it is a candidate gene for 3q29 microdeletion syndrome (Willatt *et al.*, 2005), which is also a risk CNV for schizophrenia (Mulle *et al.*, 2010). DLG3 is implicated in X-linked mental retardation (Tarpey *et al.*, 2004). Mutations in *DLG4* are implicated in Williams-Beuren syndrome (Feyder et al. 2010), Alzheimer's disease (Leuba *et al.*, 2008; Bustos *et al.*, 2017) and mice lacking *Dlg4* exhibit phenotypes relevant to autism (Feyder et al. 2010).

*DLG2* was first associated with increased risk of developing schizophrenia through *de novo* CNV studies (Kirov *et al.*, 2012). This approach compares rare variants in trios of schizophrenia patients and their parents, and the study identified likely pathogenic CNVs in *DLG2*. In comparison to other CNV risk genes for schizophrenia, such as the 15q11.2 deletion, the CNVs identified only affected *DLG2* and did not span multiple genes. Additionally, exome sequencing studies identified *de novo* SNVs which resulted in loss of function mutations in *DLG2* associated with schizophrenia (Fromer, *et al.*, 2014; Purcell *et al.*, 2014). Reduced *Dlg2* expression was also discovered in the hippocampus of a NMDAR hypofunction rat model of psychosis (Ingason *et al.*, 2015). In post mortem brains from schizophrenia patients *DLG2* expression was increased in the anterior cingulate cortex but protein expression was decreased, implying abnormal translation and/or accelerated protein degradation

(Kristiansen *et al.*, 2006). Mutations in *DLG2* have since been implicated in risk of developing BPD (Noor *et al.*, 2014) and ASD (Egger *et al.*, 2014; Xing *et al.*, 2016).

Mutations in the *DLGs* produce different phenotypes. In mice acute knockdown of *Dlg1* in slice cultures impairs LTP (Nakagawa *et al.*, 2004), whilst conditional *Dlg1* knockout mice exhibit normal LTP (Howard *et al.*, 2010b), suggesting *Dlg1* is not essential for LTP but may be involved in its regulation. Knockout of *Dlg3* does not affect basal, nor presynaptic function – as might be expected from its postsynaptic localisation, but does impair spatial learning in mice (Cuthbert *et al.*, 2007). *Dlg2* and *Dlg4* share the most overlap in function and localisation of the *Dlgs*, however knockout models exhibit divergent phenotypes, which will be discussed in following paragraphs.

One proposed interaction through which mutations in DLG2 are increasing risk for psychiatric disorders, particularly schizophrenia, is through its interaction with the glutamatergic signalling and influences on synaptic plasticity. Acute knockdown of Dlg2 or Dlg4, with short-hairpin RNA (shRNA) resulted in a ~50% reduction AMPAR mediated synaptic transmission in neuronal cultures (Elias, et al, 2006). A greater reduction (75%) was found following double knockdown of Dlg2 and Dlg4 (Elias, et al, 2006). However, in hippocampal slices from mice with a genetic knockout of Dlg2 and Dlg4, reduced AMPAR mediated plasticity was only observed in double, not single, knockouts. Additionally, expression of Dlg3 was increased in the double knockouts. Taken together these findings suggest that that DLG2 and DLG4 proteins account for the majority of AMPAR trafficking to the synapse, which is required for synaptic plasticity, and that there is compensatory functional redundancy in mice lacking one or more *Dlgs* from birth (Elias, et al, 2006). Further supporting the proposed functional redundancy between DLG2 and DLG4 was the effect of knockout on synaptic scaling, an important form of homeostatic plasticity (Sun and Turrigiano, 2011). Both DLG2 and DLG4 are important for scaling up of AMPAR at the synaptic membrane in *in vitro* cortical neurons, but scaling down was only impaired in *Dlg4* knockdown neurons (Sun and Turrigiano, 2011). DLG2 was not able to compensate the loss of DLG4 in scaling up in 'older' neurons, which had been cultured for longer. Taken together these results imply that there is functional redundancy between the Dlgs but this does not appear to be consistent across all ages, or for all types of plasticity (i.e. the reliance for downscaling is solely on DLG4).

Given this apparent redundancy, particularly between *Dlg2* and *Dlg4*, it is therefore intriguing that mutations affecting DLG2 seem to influence risk for schizophrenia, and

other diseases. There are, however, other findings demonstrating despite the functional overlap there are divergent roles for DLG2 and DLG4. In hippocampal slices basal AMPAR-mediated synaptic transmission in CA1 was normal in Dlg2 KO mice but impaired in *Dlg4* KO mice (Carlisle *et al.*, 2008). This contrasts with Elias et al. (2006) who found KO of *Dlq4* did not affect AMPAR-mediated synaptic transmission. Protocols probing NMDAR-dependent LTP found differential effects in Dlg2 and Dlg4 KO slices. Dlg2 KO hippocampal CA1 slices exhibited impaired LTP but normal LTD, by comparison in slices from *Dlg4* KO mutants impaired LTD and facilitated LTP was exhibited (Carlisle et al., 2008). This suggests a selective role for Dlg4 in LTD and opposing roles for Dlg2 and Dlg4 in LTP. The divergence in LTP phenotypes between the KO suggests that under normal conditions Dlg2 couples NMDAR to MAGUK associated signalling complexes (MASCs) which facilitate LTP, whilst MASCs coupled to NMDAR by Dlg4 suppress LTP induction (Carlisle et al., 2008). As has been discussed above (1.3), the formation of NMDA receptor supercomplexes, important for postnatal development and synapse maturation, require both DLG2 and DLG4 (Frank et al., 2016). This implies that Dlg2 and Dlg4 have independent functions and are not fully functionally redundant, and they can function interdependently.

DLG2 plays an important role in plasticity and has been investigated *in vitro* using both genetic KO cell culture and transient knockdown models. Reduction in *Dlg2* expression perturbed network activity measured by multi-electrode array in mouse primary hippocampal neurons following transient (1 week) siRNA knockdown (MacLaren *et al.*, 2011). An increase in bursting rate and bursting average was observed, implicating *Dlg2* in regulation of normal neuronal network function. This study illustrates differences in observed phenotypes can depend on methodology; reduction in *Dlg2* through acute *in vitro* knockdown demonstrated deficits whereas chronic genetic manipulations did not. This is likely attributable to the opportunity in genetic KO models for compensatory mechanisms to take over throughout development and buffer the effect of any mutations, which is not possible in the shorter time frame of acute knockdown studies. The extent to which compensation is occurring in heterozygous *Dlg2* rodent models, which more closely mimic humans carrying mutations, is unclear. The different findings of the *in vitro* studies of *DLG2* reduction are summarised in Table 1.

Study	Model	Method	Effect
MacLaren et al	Primary culture	siRNA knockdown	↑ burst rate &
2011			bursting average

Primary culture	shRNA knockdown	~50% ↓ AMPAR
	(DLG2 only)	currents
	Double DLG2 &	~75% ↓ AMPAR
	DLG4 KO	currents
Slices	Genetic KO	= AMPAR currents
	(DLG2 only)	
	Double DLG2 &	↓ AMPAR currents
	DLG4 KO	
Slices	Genetic KO	= AMPAR currents
		↓ LTP / = LTD
Primary culture	Genetic KO	Can mediate
		scaling up but not
		down of AMPAR
Primary culture	Genetic KO	Failure of NMDAR
		supercomplex
		formation
Primary culture	Genetic KO	↓ NMDAR
		mediated
		neurotoxicity
		↓ calcium influx
		through NMDAR
		↓ NR2A/NR2B in
		synaptosomal
		fractions
Cerebellum	Genetic KO	= NMDAR subunit
sections		localisation
	Primary culture Slices Slices Primary culture Primary culture Primary culture Cerebellum sections	Primary cultureshRNA knockdown (DLG2 only) Double DLG2 & DLG4 KOSlicesGenetic KO (DLG2 only) Double DLG2 & DLG4 KOSlicesGenetic KOPrimary cultureGenetic KOPrimary cultureGenetic KOPrimary cultureGenetic KOCerebellum sectionsGenetic KO

Table 1. A summary of key findings from in vitro studies examining the impact of DLG2 reduction, primarily focused on the impact on synaptic plasticity.

The importance of the interaction of DLG2 with NMDAR has been further demonstrated in cortical neuronal cultures where knockout of *Dlg2* attenuated NMDAR-mediated neurotoxicity, and reduced calcium influx through NMDAR (Zhang et al., 2010a). The lack of complete abolishment of neurotoxicity again implies developmental compensatory mechanisms between Dlgs. Dlg2 KO cultures were examined to determine whether NMDAR numbers or localisation was affected by the loss of Dlg2. These KO cultures did not show any reduction in total numbers of NMDAR subunits NR2A or NR2B, but did show reduced numbers in synaptosomal fractions, suggesting altered distribution of NMDAR in the neurons (Zhang et al., 2010a). The altered localisation of NMDAR was also observed in the spinal cord and PFC (Liaw et al., 2008). When numbers of NMDAR and their localisation were investigated in the cerebellum of *Dlg2* KO mice no differences were found, either for total numbers or synaptosomal fractions (McGee et al., 2001). This indicates not only was there no change in NMDAR numbers but that receptor localisation was normal, as opposed to studies in vitro. Dlg2 is the only MAGUK expressed in the cerebellum, thus functional redundancy and compensation between the *Dlgs* is unable to account for unaffected localisation of NMDAR. The importance of the Dlg2 interaction with

NMDAR has also been demonstrated *in vivo* in a chronic pain model. *Dlg2* KO mice exhibited loss of morphine analgesic tolerance and jumping activity, a withdrawal behaviour, both of which are NMDAR-dependent (Liaw *et al.*, 2008).

It should be noted that in addition to interactions with NMDAR and AMPAR, *Dlg2* associates with several other key proteins involved in excitatory signalling, such as the inward rectifying potassium channel Kir2.1 (Leyland and Dart, 2004) and ERK2 (Guo *et al.*, 2012). Other interactors of DLG2 have also been associated with schizophrenia. These include neuronal nitric oxide synthase (nNOS) (Brenman *et al.*, 1996), which is increased is post-mortem patient brains (Karson *et al.*, 1996; Nasyrova *et al.*, 2015), as well as ErbB4 (Garcia, Vasudevan and Buonanno, 2000), another gene for which rare variants have been associated with risk for schizophrenia (Walsh, *et al.*, 2008). *Dlg4* also interacts with ErbB4, with a substantial increase in interaction between the two found in post-mortem schizophrenia patient brains (Hahn *et al.*, 2006). Further investigation is required to examine the neuronal and physiological roles of DLG2 interactors, particularly in relation to these less well studied targets. This expanded knowledge will also increase understanding into the role of DLG2 in normal brains, as well as disease states such as schizophrenia.

### 1.4.1.1 DLG2 and cognition

To date less research has examined effects of *Dlq2* mutation *in vivo*. Abnormalities in structure and function of the cerebellum of *Dlg2* KO mice has been investigated in mouse models (McGee et al., 2001). Investigations focussed on the cerebellum due to the inability of other MAGUKs to compensate the loss of *Dlg2*. In the cerebellum the synaptic architecture (morphology and dendritic arborization of Purkinje neurons, general layer structure) in *Dlg2* KO mice was found to be normal (McGee et al., 2001). The cerebellum is an important structure for motor learning and co-ordination (Costa, Cohen and Nicolelis, 2004), but motor co-ordination was found to be normal in Dlg2 KO mice (McGee et al., 2001). In direct contrast to this initial study a more recent one using the same mutant *Dlg2* strain found a severe motor learning deficit in *Dlg2* KO mice (Winkler et al., 2018). This phenotype was also observed in the heterozygotic mice to a less severe extent. The reason for this discrepancy is unclear, although it may be methodological. The mice tested in McGee et al (2001) underwent three 2 minute training sessions on a set speed rotarod before progressing to an accelerating rotarod. Whether the mice in Winkler et al (2018) also had this training period is unclear, as multiple papers with variations in method, including McGee et al (2001), are referenced for rotarod methodology, making it hard to compare between the two.
Additionally, the impact of this training period on the learning process is unclear. Motoric phenotypes are associated with ASD but impaired motor learning may also be predictive of later development of psychosis (Isohanni *et al.*, 2001), therefore addressing this discrepancy is important to understanding the role in *Dlg2* in this process. Additionally, homozygous, but not heterozygous male *Dlg2* KO mice demonstrated hypersociability compared to WT mice, in contrast the hyposociability typically observed in mouse models carrying synaptic mutations associated with monogenetic autism (Winkler *et al.*, 2018).

Most relevant to schizophrenia was a landmark study which found comparable cognitive impairments in both *Dlg2* KO mice and humans with CNVs in *Dlg2* measured using analogous tasks using touchscreens on the Cambridge Neurophysiological Test Automated Battery (CANTAB) (Nithianantharajah et al., 2013). Three knockout mouse models, Dlg2, Dlg3 and Dlg4, and a heterozygotic Dlg1 mouse were tested on a battery of increasingly complex cognitive tasks. The Dlg1 mouse model was heterozygotic due to the embryonic lethality of a full knockout, nevertheless it was shown that one copy of *Dlg1* was sufficient for normal cognition. On the contrary, simple associative learning tasks were impaired in *Dlg4* KO mice, whereas *Dlg2* and *Dlg3* KOs performed normally. However, on more complicated cognitive tasks such as the object-location paired association task, and those that require cognitive flexibility, reversal learning and extinction, *Dlg2* KO mice exhibited deficits in comparison to WT mice. On these same tasks the Dlg3 KO mice demonstrated improved performance compared to WT mice. The divergent phenotypes between the *Dlg* mutants, and the opposite phenotypes observed in *Dlg*2 and *Dlg3* KO mice, reflect the importance of this evolutionary expansion of this family of MAGUKs to more complex behaviours seen in mammals (Nithianantharajah et al., 2013). DLG4 is associated with more synaptic functions than other members of the family, therefore phenotypes associated with its loss are thus perhaps not surprisingly more extreme. Most interestingly cognitive deficits exhibited by *Dlg2* KO mice were also observed in humans carrying DLG2 mutations. Of the CNVs carried by the four patients three were exon disrupting, intronically located deletions, and one person carried multiple exon disrupting duplications (Nithianantharajah et al., 2013). All but one patient had a diagnosis of schizophrenia. Consistent with the Dlg2 KO mouse model, the humans with DLG2 CNVs made more errors than healthy controls in acquisition of visual discrimination and tasks of cognitive flexibility, visuo-spaital learning and memory, and demonstrated decreased accuracy of sustained attention (Nithianantharajah et al., 2013). It is important to note that the people carrying DLG2

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mutations are heterozygotic, in comparison to the full KO mice, therefore it would be important to examine whether heterozygotic rodent models, more reflective of human disease, also exhibit these phenotypes.

#### 1.5 Disease relevant phenotypes

As discussed above, *in vitro* studies implicate Dlg2 in synaptic functioning, and *in vivo* studies implicate Dlg2 in cognitive function in both rodent models and in humans carrying mutations affecting DLG2. However, research into the impact of Dlg2 mutation is still fairly limited. In particular the effects of Dlg2 heterozygosity in rodents which reflect the haploinsufficiency seen in patients with schizophrenia. Therefore, it is important to utilise heterozygotic rodent models to expand upon the initial findings primarily in homozygotes, to uncover psychiatric disease\_relevant phenotypes.

Both Dlg2 KO mice and humans carrying DLG2 mutations demonstrated impaired extinction and delayed reversal learning, processes that require flexibility of cognition (Nithianantharajah et al., 2013). Extinction is an associative learning process observed following classical or operant conditioning (Pavlov 1927; Eisenberg et al. 2003; Suzuki et al. 2004). Classical conditioning involves the pairing of a neutral stimulus (conditioned stimulus, CS) with a biologically relevant stimulus (unconditioned stimulus, US), either an appetitive (e.g. food) or an aversive stimulus (e.g. mild food shock) such that re presentation of the neutral stimulus, the CS, results in similar response (conditioned response, CR) to US (Skinner, 1938). Operant conditioning involves reward or punishment to modify the strength of a specific behaviour (Skinner, 1938). Extinction, measured as a reduction of the CR, occurs when either the CS is repeatedly presented in the absence of the US or the CS is presented for a prolonged period of time. Dysfunctional associative learning has been considered a hallmark of schizophrenia (Bleuler, 1911; Peralta and Cuesta, 2011) and may underlie the development of psychosis (Kapur, 2003b). Impaired associative learning has also been implicated in other psychiatric diseases such as MDD (Mills et al, 2015; Harel et al., 2016). Recently CNVs in patients with schizophrenia were found to be enriched for genes expressed in the CA1 of the hippocampus during the extinction of contextual fear memory in rats, but not the consolidation or retrieval of fear memory that does not result in extinction (Clifton et al., 2017). Aversive (fear) conditioning is the form of associative conditioning that is consistently impaired in humans with schizophrenia (Ax et al., 1970; Ax, 1990; Jensen et al., 2008b). Rodent models have demonstrated aversive conditioning and extinction is dependent on NMDAR activation in the amygdala and hippocampus (LeDoux, 2003a). These data are consistent with a role for selective role for DLG2 in extinction processes of

associate fear learning and memory, potentially mediated through the interaction between Dlg2 and NMDAR. Whether this phenotype is also present in a heterozygous model, or when employing aversive rather than appetitive protocols needs investigation as heterozygous models better replicate the *DLG2* mutations in schizophrenia than homozygous models.

The delayed reversal learning observed in Dlg2 KO mice and human CNV carriers (Nithianantharajah et al., 2013) indicates cognitive inflexibility; a reduced ability to behaviourally adapt to environmental stimuli (Armbruster et al., 2012). Cognitive flexibility is a core executive function and its dysfunction has been demonstrated in rodent models and patients with schizophrenia (Morice, 1990; Brigman, Graybeal and Holmes, 2010), BDP (Morice, 1990; O'Donnell et al., 2017) and ASD (South et al, 2012). This rigidity in responding to the environment may also manifest in other disease relevant phenotypes which involve adaption to the stimuli, such as habituation or sensorimotor gating. Habituation is a simple form of non-associative learning where an organism's response to a stimulus decreases with prolonged exposure (Groves & Thompson 1970; Harris 1943; Thompson & Spencer 1966). When habituation is impaired the stimulus retains its novelty drawing attention which could lead to the formation of abnormal associations (Kapur, 2003b; Martinelli et al., 2018). Habituation deficits have been observed with schizophrenia (Holt et al., 2005; Williams et al., 2013) and ASD (Vivanti et al., 2018). Sensorimotor gating is a process which filters out irrelevant environmental stimuli, preventing information overload and facilitating focus on salient stimuli (Braff and Geyer, 1990). A deficiency in this filtering ability is found in people with schizophrenia Walters & Owen 2007; Gottesman & Gould 2003), BPD (Perry et al., 2001; Kohl et al., 2013) and ASD (Perry et al., 2007; Kremer et al., 2013; Kohl et al., 2014a). In schizophrenia is has been proposed that impaired habituation and a persistent focus on irrelevant stimuli, due to an inability to filter it out, may lead to the formation of abnormal associations, and eventually the development of psychosis (Kapur, 2003b; Jensen et al., 2008b).

#### 1.6 Experimental Aims

There is convergence of genetic risk for schizophrenia and other psychiatric disorders and this shared aetiology suggests common affected pathways and pathophysiology between disorders (Cross-Disorder Group of the Psychiatric Genomics Consortium *et al.*, 2013). Particularly prominently affected gene sets and pathways are synaptic functioning and the glutamatergic system. *DLG2* is an important scaffolding protein found in the PSD and plays a vital role in synaptic plasticity such as LTP, through mediation of NMDAR and AMPAR functioning (Elias et al., 2006; Carlisle et al., 2008; MacLaren et al., 2011; Sun and Turrigiano, 2011). Abnormal synaptic plasticity is associated with schizophrenia and other related disorders McGlashan, 2005; Peled, 2005; Salavati et al., 2015; Bhandari et al., 2016). Dysfunctional LTP at the molecular level may underpin some cognitive deficits seen in schizophrenia as synaptic plasticity is considered the cellular basis of learning and memory (Hebb, 1949; Bliss and Lømo, 1973; Whitlock et al., 2006; Takeuchi, Duszkiewicz and Morris, 2014). In Dlg2 KO mouse models abnormalities of synaptic plasticity were found in vitro and specific cognitive deficits were observed during behavioural tasks. DLG2 KO mice exhibited deficits in multiple tasks which require cognitive flexibility: reversal learning, extinction (Nithianantharajah et al., 2013), and motor learning (Winkler et al., 2018). Genetic studies in humans found association between CNVs in DLG2 and schizophrenia (Kirov et al., 2012; Fromer, et al., 2014), as well as BPD (Noor et al., 2014) and ASD (Egger et al., 2014; Xing et al., 2016). When tested on translationally relevant tasks humans carrying DLG2 CNVs demonstrated similar cognitive impairments to the mouse model (Nithianantharajah et al., 2013). However, the vast majority of research into *Dlq2* mutation in mice has been conducted in full KOs rather than heterozygotic models which more appropriately reflect the human condition. Studying heterozygotic models will provide more insight into the role of Dlg2 in the cognitive processes which are dysfunctional in schizophrenia. With the advances in genetic engineering, particularly CRISPR, it is now possible to generate CNV rat models. Rats offer multiple advantages over mouse models, such as closer physiology to humans (Ellenbroek and Youn, 2016), the opportunity to study more complex cognitive behaviours and less time required training for these tasks (Colacicco et al., 2002; Jaramillo and Zador, 2014). Additionally, CRISPR allows more control over the mutation generated resulting in a genetic lesion that more closely mimics those observed in humans.

In this thesis I will conduct an initial characterisation of our heterozygous *Dlg2* mouse model, on which no research has yet been published, and our completely novel heterozygous *Dlg2* rat model. I will then investigate the impact of a heterozygotic mutation in *Dlg2* on more complex cognitive tasks that probe processes known to be dysfunctional in schizophrenia. Therefore, my aims were as follows:

 Basic molecular characterisation was conducted in both the novel heterozygous *Dlg2* mouse and rat model. In both models it was predicted that there would be a selective decrease in *Dlg2* mRNA expression, but not in any other *Dlg*, in brain regions important in schizophrenia (the PFC and hippocampus) and a control region (the cerebellum).

- Basic behavioural phenotyping was conducted in the heterozygotic *Dlg2* mouse and rat models. Current behavioural literature for *Dlg2* mutation is limited and confined to one mouse strain. Therefore a battery of tasks, including more basic assessments, such as motoric function and anxiety, as well as tasks more associated with schizophrenia, including those involving simple cognition and sensorimotor gating were employed. It was anticipated that the heterozygotes would not be universally impaired, but rather would display specific deficits, on tasks involving elements of cognition.
- Adult neurogenesis was investigated in the mouse model. Dysfunctional adult neurogenesis is a potentially convergent phenotype in synaptic risk models and no research has to date investigated the impact of mutation in any *Dlg* on the process.
- Fear learning and extinction were investigated in the rat model. Extinction learning requires cognitive flexibility and deficits have previously been found in *Dlg2* KO mice and humans carrying *DLG2* CNVs. Additionally, there is a specific association between schizophrenia risk genes and extinction (Clifton *et al.*, 2017). The heterozygotic *Dlg2* rats were predicted to exhibit deficient extinction of fear memories.

# **2 General Materials and Methods**

# 2.1 Animals

# 2.1.1 Ethics

All procedures were conducted in line with Animals (Scientific Procedures) Act (ASPA) (1986) under UK Home Office project license PPL 30/3135 and PIL 131AE7D42.

# 2.1.2 Housing

WT and  $Dlg2^{+/}$  mice were housed with up to 5 littermates of the same sex in standard cages (48cm (L) x 15cm (W) x 13cm (H)). WT and  $Dlg2^{+/}$  rats were housed in groups of 2-4 dependant on weight in standard cages (38cm (W) x 56cm (L) x 22cm (H). Both species were housed on a 12:12 hour light/dark cycle (light phase 8am – 8pm) with *ad libtum* access to standard food (standard chow: mouse (RM3 E) and rat (RM1), Special Services Diet, Lillico, UK) and water. Cages were lined with wood shavings with cardboard tubes and wooden sticks provided as environmental enrichment. Holding rooms were maintained at 45-60% humidity and 19-22°C. Any alternation from standard conditions is described on an individual experimental basis. At the end of experimentation mice were sacrificed via schedule one (cervical dislocation) or perfusion fixation. Rats were sacrificed by either a rising concentration of CO<sub>2</sub> in a home cage culling chamber (Clinipath Equipment Limited, Hull, UK) or by anaesthetic overdose (Euthatal (200mg/ml), Merial, Harlow, UK).

Only breeding of mice was conducted in house. WT x HET pairs and trios were mated, pairs housed in conventional cages and trios in larger cages (45cm (L) x 25cm (W) x 13cm (L)).

# 2.1.3 Genotyping - DNA extraction

Model design is discussed in Chapter 3 (mouse) and Chapter 6 (rat). For genotyping and for both species ear punches were taken during initial animal identification post weaning. Further tail tip biopsies were taken post-mortem for confirmation. All samples stored at -20°C. The Qiagen DNeasy Blood and Tissue Kit (Qiagen, Manchester, UK) was used as per the standard manufacturer's instructions to extract genomic DNA. Either 1 single ear-punch or approximately 0.6cm of tail tissue was lysed overnight at 56°C in 180 µl ATL buffer and 20 µl proteinase K. 200 µl of AL buffer and 96-100% ethanol were added then all liquid was transferred to a spin column and centrifuged at 8000 rpm for 1 minute. The membrane was washed with AW1 and AW2 buffers and then the column was centrifuged at 14,000 rpm for 3 minutes to dry the membrane. 200 µl AE buffer was added directly to the membrane and incubated at room temperature for 1 minute. A final centrifugation step at 8000 rpm for 1 minute eluted the DNA, which was then stored at -20°C. Polymerase chain reaction (PCR) is described separately for each species in Chapters 3 (mouse) and Chapter 6 (rat).

# 2.1.4 Ketamine solution and injection

In Chapter 5 a ketamine-induced hyperlocomotion challenge study was conducted. Ketamine hydrochloride 100mg/ml solution (Ketavet®, Zoetis, UK) was diluted in saline under a sterile hood to 5mg/ml, 10mg/ml and 20mg/ml and was stored at RT until needed. In line with Cardiff University Drug Policy all ketamine was disposed of as directed 30 days after opening. For injection mice were restrained and ketamine solution administered via intraperitoneal (IP) injection. The mice were then transferred to the locomotor activity box, as described in Chapter 5.

# 2.1.5 Perfusion

Quick and uniform fixation of brain tissue was achieved using transcardial perfusion via the left ventricle. In Chapter 4 and 5 perfusion was used for some experiments in the mouse model.

The perfusion pump was set up and a 15-gauge blunted perfusion needle attached. Mice were anaesthetised with 1ml of Euthatal (200mg/ml, IP) injection (Merial, Harlow, UK). The tip of the tail was taken for post mortem genotyping. Transcardial perfusion was then conducted: first 1X phosphate buffered saline (PBS) was flushed through the system until it was clear of blood (approximately 2 minutes), then the solution was switched to cold 4% paraformaldehyde (PFA in PBS) (Sigma-Aldrich, Dorset, UK) (w/v) and perfusion continued until the animal became rigid (approximately 3-5 minutes). Perfusion was then stopped, the brain was dissected out and transferred to fresh 4% PFA for post fixing (either 4-6 hrs at room temperature (RT) or 24 hrs at 4°C). Brains were then transferred to 30% sucrose solution (diluted in ddH<sub>2</sub>0) for several days, before being embedded in OCT (ThermoFisher Scientific, UK) and stored at -80°C.

# 2.1.6 Dissection

Tissue for RT-qPCR was collected from mice that were sacrificed via cervical dislocation or from rats were sacrificed via rising CO<sub>2</sub> concentration. Brains were

removed and the cerebellum, hippocampus and PFC micro-dissected and flash frozen on dry ice. Dissected tissue was stored at -80°C until required.

# 2.2 Behaviour

With the exception of experiments presented in Chapter 7, all behaviour was conducted during the light phase. The basic setup for each behavioural test is described in this section, but full experimental training protocol, manipulations and analysis are described in the appropriate sections of each results chapter. With the exception of experiments discussed in Chapter 7, animals were moved to behavioural testing rooms in their home cages as soon as possible after testing.

# 2.2.1 Mouse behaviour

# 2.2.1.1 Rotarod

Motor learning and motor function were assessed using multiple rotarod paradigms discussed in Chapter 3 and Chapter 4. The rotarod (47600, Ugo Basile, Italy) consists of five 3cm drums with a grippable rubber surface with six flanges dividing into five 5.7cm lanes facilitating simultaneous running of up to 5 mice. The fall height is 16cm. A tray in the bottom each of lane records when a mouse falls from the rod. The speed of the rod can be set to remain consistent (4-50 rpm) or increase across the trial. The latency (s) to the mice falling into the tray or to clinging onto the rod for one full rotation was recorded manually by the experimenter.

# 2.2.1.2 Locomotive activity boxes

Locomotor activity was analysed in two different experiments described in Chapter 3 and Chapter 4. Twelve clear perspex activity boxes (21cm (L), 36cm (B), 20cm (H)) are stacked in a 3 x 4 layout. Each box was fitted with 2 infrared beams, 1 cm above the floor and 3 cm from either end of the box (CeNeS Cognition, Cambridge, UK). Thus up to twelve mice can be monitored simultaneously. Beam breaks are recorded as an activity measure using a custom BBC BASIC V6 programme with additional ARACHNID interfacing (Campden Instruments, UK). Beam breaks refers to a mouse breaking one beam in the activity box.

# 2.2.1.3 Acoustic startle and pre-pulse inhibition (PPI)

Acoustic startle and PPI response were investigated in mice using a SR-Lab<sup>™</sup> Startle Response System (San Diego Instruments, CA). The 30 minute program is described in Chapter 3 in detail.

An isolation chamber contains a clear plexiglass cylinder (35 mm diameter) which is positioned onto of a piezoelectric pressure-sensitive accelerometer sensor. The cylinder is designed to minimise restraint stress by allowing individually placed animals to turn without altering readings by adjusting the sensor. Movement of the mouse breaks the sensor circuit and is recorded. Animals awaiting testing were held in a separate procedure room and after testing animals were held in a separate cage from their home cage until all animals in that cage had been tested.

# 2.2.1.4 Elevated plus maze (EPM)

Anxiety behaviours were investigated using two methods the EPM and open field tasks. The EPM was constructed from white Perspex in a cross formation arrangement of four arms: the two diametrically opposing 'open' arms with no walls and two 'closed' arms, 175 mm (L) x 78 mm (W), with 50 mm high walls (Figure 9). The maze was elevated 300 mm off the floor and illuminated at 15 lux. A computer running Ethovision Observer XT software (Noldus Information Technologies 3.0.15, Netherlands) was connecting to a camera mounted above the maze to record exploratory behaviour. The experimenter was present in the room during the task and manually scored a number of behaviours detailed in Chapter 3. The Ethovision software tracked the mouse's position in the EPM and calculated analysis of time spent in predefined 'zones' within the maze, set up prior to testing by the experimenter and maintained across all trials.

#### 2.2.1.5 Open field

Full description of the protocol measurements are described in Chapter 3 methods. The open field apparatus consisted of a black Perspex floor (750 mm x 750 mm) with white Perspex walls (800 mm high), which was dimly illuminated (15 lux). A camera above the arena was connected to a computer running Ethovision Observer XT (Noldus Information Technologies 3.0.15, Netherlands) software recorded the animal's position (17 frames/s). The software subdivided the arena into a central zone (400 x 400 mm in arena centre) and an outer zone, within 350 mm of the walls.

#### 2.2.2 Rat behaviour

#### 2.2.2.1 Rotarod

As with the mouse model a rotarod task was employed to measure motor learning and function with the full description of the protocol in Chapter 6. The rotarod (47750, Ugo Basile, Italy) consists of four 6cm drums with a grippable rubber surface with five 49cm flanges dividing into four 8.8cm lanes facilitating simultaneous running of up to 4 rats. The height to fall is 30cm. A tray in the bottom each of lane records when a rat falls from the rod. The speed of the rod can be set to remain consistent or increase across the trial (4-50 rpm). As with the mice, the latency (s) to fall into this tray, or clinging onto the rod for one full rotation, was recorded manually by the experimenter.

#### 2.2.2.2 Open field

The open field task was conducted in a black wooden 1 m<sup>2</sup> arena with 50 cm high walls with dimmed lighting (70 lux). A camera was mounted centrally above the arena, which recorded the movement of each animal and was linked up to a computer running EthoVision XT 2.1 software (Noldus, VA). The arena was divided into 'outer' and 'centre' zones. The centre zone was defined as the central 70 cm<sup>2</sup> of the arena, with the outer zone the perimeter of this zone to the wall of the arena. Full description of measurements analysed are described in Chapter 6.

#### 2.2.2.3 Contextual fear conditioning

Contextual fear conditioning experiments were conducted in two standard rat modular test chambers (Med Associates Inc., Vermont, USA). The interior of the chamber measured 30.5 cm (L) x 24.1 (W) x 21.0 (H), with clear polycarbonate door and rear panel and aluminium side walls (Med Associates Inc., Vermont, USA). A 0.5 mA or 0.7 mA scrambled footshock was delivered via a grid made up of 19 equally spaced 1.6 cm above a removable floor tray. A stand-alone aversive bars stimulator/scrambler (Med Associates Inc., Vermont, USA) controlled the delivery of the footshocks. The boxes were housed inside sound attenuating chambers 55.9 cm (L) x 55.9 cm (W) x 35.6 (H) (Med Associates Inc., Vermont, USA). Session programmes were controlled using Med-PC (Version IV) research control and data acquisition system (Med Associates Inc., Vermont, USA). Infra-red cameras (JSP Electronics Ltd, China) suspended centrally above chambers digitally recorded behaviour and viewed using Numeroscope software (Viewpoint, France) for offline analysis. Recording began prior to placement of rats in the chambers. The chambers were cleaned and dried between animals with 50% ethanol to mask odour cues. The manipulations employed to investigate different aspects of contextual fear conditioning, and are described in full in Chapter 7.

#### 2.3 Laboratory Techniques

#### 2.3.1 Quantitative polymerase chain reaction (qPCR)

RT-qPCR is a technique facilitating quantitative mRNA expression analysis through combining PCR amplification and detection.

#### 2.3.1.1 Primer design and validation

FASTA gene sequences from NCBI (http://ncbi.nlm.nih.gov) for mouse and rat were inputted into NCBI Primerblast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primers were designed to meet the following criteria: be approximately 20 base-pairs (bp) long, resulting product ~200 bp, low self-complementation and no potential hairpin generation. Two primer pairs were selected per gene and then tested for in homology elsewhere the genome via BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Primers were then commericially synthesised by Sigma-Aldrich (Dorset, UK). Primer stocks were stored at -20°C and working solution aliquots (10 µm) were used to minimise freeze-thaw cycles. Primer validation was then conducted to assess amplification efficiency and simple amplicon specificity (detailed 2.3.1.3).

#### 2.3.1.2 RNA isolation, DNase treatment and cDNA synthesis

For both species flash frozen PFC, hippocampus and cerebellum samples from dissected brains were removed from the -80 °C and kept on dry ice. ≤30 mg sample was processed using Qiagen's RNeasy kit. 14.3 M ß-Mercaptoethanol was added to the supplied RLT buffer (1:100) and 550 µl of resulting solution added to the sample in a ribotube (MP Biomedicals, UK). The ribotube containing the sample was placed in a ribolyser (Bio-Rad Laboratories Inc., USA) and tissue homogenised using the fast prep homogeniser for 2 x 5 second blasts until the tissue was completely lysed. Samples were then centrifuged for 3 minutes at 14,000rpm for 3 minutes. The solution was then transferred to a 1.5ml Eppendorf, before then centrifuged again for 3 minutes at 14,000rpm to form the pellet. The resulting supernatant was dissolved in 500µl 70% ethanol and 500µl of the supernatant/ethanol mix and loaded into a RNeasy spin column in a collection tube. This column was then centrifuged for 15 seconds at 10,000rpm. The flow through was discarded and this step repeated with the remaining 500µl supernatant. 250µl of buffer RLT + ß-Mercaptoethanol was then added to the pellet and centrifuged for three minutes at 10,000rpm. The supernatant was once again extracted, mixed with 250µl 70% ethanol and added to the same RNeasy column, and again centrifuged for 15 seconds at 10,000 rpm and the flow through discarded. The column was then incubated at RT for 5 minutes. Following this the column was washed by adding 700µl Buffer RW1 to the column and centrifuging for 15 seconds at 10,000rpm and discarding flow through. 500µl Buffer RPE was then added, centrifuged for 15 seconds at 10,000rpm and flow through discarded. Another 500µl RPE was added to the spin column and the column

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centrifuged for five minutes at 10,000rpm to thoroughly wash the membrane. The column was then placed in a new collection tube and span at 14,000rpm for 1 minute to eliminate any possible carryover of Buffer RPE. The spin column was then placed into a 1.5ml collection tube. 30µl of RNase-free water (Ambion Life Technologies, UK) was added directly to the column membrane and incubated for 10 minutes at RT. To elute the RNA, the column was centrifuged at 10,000rpm for 1 minute. RNA content was then measured on a NanoDrop spectrophotometer (Thermo Fisher Scientific, Delaware, USA) to determine concentrations were a suitable level (between 100-10,000ng/ul). RNA purity was then determined by confirmation that the A260/A280 was below 2. A minimum concentration of 75 ng/µl was required for cDNA synthesis.

To remove any contaminating DNA the RNA samples were DNase treated with an Ambion TURBO DNA-free <sup>™</sup> Kit (ThermoFisher Scientific, Delaware, USA). 5 µl 10x TURBO DNAse buffer and 1 µl TURBO DNAse were added to each sample and mixed. Samples were then incubated at 37°C for 30 minutes. 5.5 µl DNAse TURBO Inactivation Reagent was then added to each sample and mixed. Samples were incubated at RT for 5 minutes then centrifuged at 13, 000 rpm for one minute. The supernatant was then transferred to a new tube.

The eluted RNA was then used to generate cDNA. To achieve a reaction volume of 20 µl the volume of eluted RNA required for 1.5 ng/µl was calculated and added to random primer cDNA synthesis tubes (Takara Clontech, France) with RNAse free water (Ambion Life Technologies, UK) making up the volume. Samples were then placed in a thermal cycler (Bio-Rad Laboratories S100, USA) on an optimised cDNA synthesis program: 42°C for 75 mins, 80°C for 15 minutes and infinite sample holding at 8°C. 20 µl cDNA was added to 480 µl qPCR grade water for a 1:15 dilution. Samples were stored at -20°C.

#### 2.3.1.3 Standard Curves

A 1:5 serial dilution series across 6 points of known concentration template cDNA for each species in each brain region investigated (PFC, hippocampus, cerebellum) was used to generate a standard curve for each primer pair for each gene. Each run included measuring the expression of two validated house-keeping genes (Hypoxanthine-guanine phosphoriobosyltransferase – *Hprt*, Glyceraldehyde 3phosphate dehydrogenase – *Gapdh*, Polyubiquitin-C - *Ubc*) (See Table 2 for sequences). The primer sequences of the genes of interest (*Dlg1 – Dlg4*) are detailed in the appropriate Chapters (mouse – Chapter 3 3, rat –Chapter 6).

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Gene	Species	Forward	Reverse
Gapdh	Rat	тстстдстсстссстдтт	TACGGCCAAATCCGTTCAC
		СТ	А
Hprt	Rat	TCCTCCTCAGACCGCTT	ATCACTAATCACGACGCTG
		TTC	GG
Gapdh	Mouse	GAACATCATCCCTGCAT CCA	CCAGTGAGCTTCCCGTTCA
Ubc	Mouse	CCAGTGTTACCACCAAG AAGGT	CCATCACACCCAAGAACAA GC

Table 2. Primer sequences for validated qPCR probes in both species.

To generate standard curves each well of a 96 well plate contained 15 µl reaction mixture (1.9 µl sterile RNase free water, 0.3 µl 10 µM forward primer, 0.3 µl 10 µM reverse primer, 7.5 µl SensiMix (Bioline) and 5 µl cDNA or water (for no template control). After loading, plates were centrifuged at 3,000 rpm for approximately 10-20 seconds before being transferred to Real-Time PCR instrument (Applied Biosystems) on a standard run: 95°C for 10 minutes, followed by 45 cycles of 95°C (15 seconds) and 60°C (1 minute) to allow for duplex denaturing and annealing and elongation, respectively. Finally a melt curve was obtained. Samples were heated to 55°C for one minute and 95°C for 15 seconds. This final stage allowed assessment of single amplicon specificity - when the resulting dissociation curves were visualised as a single peak the primers are considered specific to the target cDNA. The Ct values measured were plotted against initial input amounts on a semi-log10 plot, fitted to a straight line and a gradient generated. This gradient was then inputted into the Thermoscientific efficiency calculator. (https://www.thermofisher.com/us/en/home/brands/thermo-scientific/molecularbiology/molecular-biology-learning-center/molecular-biology-resourcelibrary/thermo-scientific-web-tools/qpcr-efficiency-calculator.html). Primers with efficiencies between 90-110% were considered valid and any primers with abnormal

efficiencies between 90-110% were considered valid and any primers with abnormal melt curves, low efficiency or vastly differing efficiency across regions were redesigned.

#### 2.3.1.4 RT-qPCR Experiments

Each well of a 96-well plate contained 15 $\mu$ l reaction mixture (1.9  $\mu$ l sterile RNAase free water, 0.3  $\mu$ l 10  $\mu$ M forward primer, 0.3  $\mu$ l 10  $\mu$ M reverse primer, 7.5  $\mu$ l SensiMix (Bioline, London, UK)) and 5  $\mu$ l cDNA or water). Only primer pairs previously validated

were used. The gene of interest values were normalised to the housekeeping gene controls (*Gapdh Ubc*). After loading plates were centrifuged at 3,000 rpm for approximately 10-20 seconds before being transferred to the q-PCR machine.

# 2.3.1.5 RT-qPCR Analysis

The cycle threshold (Ct), the value reflects the number of cycles it took for the detection of cDNA signal above the background fluorescence, is outputted by the qPCR machine. There is a negative correlation between the amount of cDNA in the sample and the Ct values. The threshold levels were set at the beginning of the exponential phase and conserved across plates. Quantification was conducted using the comparative Ct method ( $2^{-\Delta\Delta Ct}$  method) to produce fold changes in control and *Dlg2* heterozygotes (Schmittgen and Livak, 2008). The  $2^{-\Delta\Delta Ct}$  method involves subtracting the average housekeeping gene from the gene of interest ( $\Delta$  Ct = Ct <sub>target</sub> – Ct <sub>reference</sub>). The geometric mean of two housekeeping genes was used throughout this thesis.  $\Delta\Delta$  Ct is then calculated by subtracting the experimental group from the control group ( $\Delta\Delta$  Ct =  $\Delta$ Ct <sub>test</sub> –  $\Delta$ Ct <sub>control</sub>) and incorporating standard deviations into the fold change.

#### 2.3.2 Immunohistochemistry

#### 2.3.2.1 Sectioning

Perfused brains were embedded in OCT and frozen. Prior to sectioning they were removed from the -80°C and placed in the cryostat (Leica Microsystems CM1860UV) for 20-30 minutes to warm to temperature (normally between -20°C to -25°C). All brains were coronally sectioned at 40  $\mu$ m. Co-ordinates for the start and end of the brains regions of interest are detailed in appropriate Chapters (4 and 5). Free floating sections were placed into 1x PBS in 12 well plastic plates and stored at 4°C.

#### 2.3.2.2 Immunohistochemical staining

Sections were blocked in 500  $\mu$ l phosphate buffered saline with 1% Tween 20 (PBST) containing 3% normal donkey serum (S30-100ML, Millipore, Hertfordshire, UK) at RT with agitation for 2 hours. Primary antibodies (described in Chapter 4 and 5 methods) were diluted in 500  $\mu$ l 0.1% PBST with 0.2% normal donkey serum (v/v) and incubated overnight with agitation at 4°C. Sections were washed 3 times for 10 minutes in 1x PBS. Alexa Fluor® secondary antibodies (ThermoFisher Scientific,UK) were diluted (1:1000) in 500  $\mu$ l 0.1% PBST with 0.2% normal donkey serum. Sections were protected from light and incubated at RT with agitation for 2 hours. Sections were incubated with the nucleus DNA stain 4',6-diamidino-2-phenylindole (DAPI) (1:1000,

D9542-10MG, Sigma-Aldrich, Dorset, UK) in 500  $\mu$ l 1x PBS at RT with agitation for 5 minutes, then washed 2 x 10 minute in 1 x PBS. Sections were mounted in a counterbalanced manner with 20  $\mu$ l Mowiol® (4-88, Sigma-Aldrich, Dorset, UK) added per slide and glass cover-slipped and stored at 4°C. Image acquisition, and data sampling and analysis for each experiment is detailed in Chapters 4 and 5.

# 2.4 Statistics

Data was analysed using SPSS 23 (IBM Corporation, New York), with an alpha level of P < 0.05 was regarded as significant throughout. All data was tested for normality using the Shapiro-Wilks test, and distribution assessed via visual inspection of histograms. Instances of non-normality are detailed in individual chapters, as well as any transformations applied or outliers removed where appropriate. Outliers were examined by both visual inspection of boxplots and/or studentized residuals of >±3 (https://statistics.laerd.com/). Unless otherwise stated analysis was conducted on untransformed data. In instances of non-normality where no outliers were found, the data is reported untransformed if transformations were attempted but did not result in transformation to normality.

Data was analysed by unpaired two-tailed *t*-test, one-way-ANOVA or mixed ANOVA. For ANOVA where Mauchly's assumption of sphericity was violated, the Greenhouse-Geisser correction was reported and the degrees of freedom corrected. The assumptions of homogeneity of variances and covariances were assessed using Levene's and Box's M tests, respectively and instances of violation are described where found in the results chapters. For *t*-tests that violate homogeneity of variances, Welch's *t* test is used.

Throughout this thesis Laerd Statistics was used as a reference for statistics (https://statistics.laerd.com).

# 3 Validation of genetic mutation and basic behavioural investigation in the mouse model

#### 3.1 Introduction

To date there is limited exploration of *Dlg2* mutations *in vivo*. The majority of previous findings are from primary cell cultures, and there are currently only three studies examining any behaviour. Reported behavioural data in mice primarily derives from one strain, with most data from homozygote knock outs (KO).

The first mouse model with a *Dlg2* mutation was generated by McGee et al (2001). Given that DLG2 is the only MAGUK in cerebellar Purkinje neurons, and to avoid issues of *Dlg* family compensation, the study examined cerebellar structural architecture and functioning. Heterozygocity in this model resulted in a ~50% reduction in mRNA via Northern blot, and a very weak band seen in homozygous mutants that was attributed to alternative splicing skipping the deleted exon. The levels of DIg1, DIg3, and DIg4 were not altered in the brains of DIg2 KO mice, suggesting a lack of compensation at the protein level. There were no reported molecular abnormalities of synaptic architecture, distribution of interacting proteins or impairments of gait or motor coordination measured on a rotarod. The authors argue that although compensation by other *Dlg* family members cannot occur in the cerebellar Punkinje neurons it may occur via other PDZ domain-containing proteins. Given the lack of phenotypes observed the authors proposed that DLG2 was not involved in baseline synaptic signalling in the cerebellum, but rather it may be specialised to plasticity and learning, or extreme physiological situations, although the reason for this interpretation is not clear

A later study using the same mouse model does demonstrate the loss of *Dlg2* has little impact on basic cognition examined but severely inhibited complex cognition. Nithianantharajah *et al* (2013) probed mice carrying mutations members of the *Dlg* family on cognitive tasks. On simpler cognitive tasks, such as visual discrimination, *Dlg2<sup>-/-</sup>* mice were comparable to WT. As tasks increased in complexity *Dlg2<sup>-/-</sup>* mice began demonstrating abnormalities. For more complex object-location paired association learning, *Dlg2<sup>-/-</sup>* mice performed at chance levels. *Dlg2<sup>-/-</sup>* mice also showed a significant impairment in reversal learning of visual stimuli, with the deficit becoming

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increasingly severe as stimuli complexity increased. On an extinction task,  $Dlg2^{-/-}$  mice displayed normal rates of learning during acquisition but impaired extinction. As with the McGee *et al* (2001) paper, the authors suggested that Dlg2 is fundamental for regulating flexibility of learned behaviour.  $Dlg2^{-/-}$  mice also displayed impaired attentional processing, taking longer to reach performance stability and showing reductions in response accuracy and increases in premature responding with decreased duration of stimulus presentation. This study also examined humans with DLG2 mutations in comparable tests, and found the same pattern of impairments as observed in the mice, suggesting conservation of Dlg2 function across rodents and humans.

Most recently a paper was published that predominately focused on *Dlg4* mutant models, but that also conducted some behavioural testing in the McGee (2001) *Dlg2* mouse model (Winkler *et al.*, 2018). Both male and female *Dlg4* KO and heterozygotes were examined but only male *Dlg2* KO and heterozygotes. Similar to the behaviour observed in the *Dlg4* mutants homozygous *Dlg2* mice exhibited a hyper-social phenotype. As the heterozygous *Dlg2* mice did not exhibit this phenotype the authors suggest that DLG2 plays a similar but less prominent role in social interaction than DLG4 (Winkler *et al.*, 2018). No impairments of learning and memory were observed in *Dlg2* mutants using a hole board paradigm or Morris water maze, or anxiety using a light/dark box were found, but deficient motor learning was observed on a rotarod task, with the impairment being more severe in the full KO. Interestingly increased DLG2 protein, but not mRNA expression, was found in the *Dlg4* heterozygotes, suggesting that DLG2 may be partially compensating the loss of DLG4. The converse analysis in *Dlg2* mutants was not reported.

A homozygous KO mouse provides a useful model for understanding the functional roles of DLG2. However, in human psychiatric populations rare recurrent CNVs spanning the DLG2 gene loci are heterozygotic mutations resulting in altered expression levels as opposed to complete loss. Other *Dlg* family members may be differently dysregulated in a knockdown as opposed to a knock out *Dlg2* model. For example, in a double knockout model of *Dlg2* and *Dlg4*, *Dlg3* expression is upregulated, but not for single knockouts (Elias et al 2006).

All characterisation during this thesis will describe studies using *Dlg2* heterozygotes, providing greater translation to the human condition. The model reported during this thesis is a different strain to that used in the studies discussed above.

The model characterised in this thesis, *Dlg2<sup>tm1a(EUCOMM)Wtsi* originates from, and is maintained on, a C56BL/6N background. The model was generated as part the European Conditional Mouse Mutagenesis Program (EUCOMM), aiming to provide knock out models for all protein coding genes. A critical exon common to all transcripts was identified that resulted in a frame shift mutation upon deletion. The genetic lesion in Dlg2<sup>tm1a(EUCOMM)Wtsi</sup> targets exon 14, which encodes the SH3 domain. No papers have been published using this model to date.</sup>

The mouse generated by McGee *et al* (2001) originated from a 129/Sv background, before being crossed with C56BL/6J, with the replacement of a single exon (6) encoding the second PDZ domain with a neomycin cassette.



Figure 6. Schematic diagram demonstrating the difference in genetic lesion locations between the model described in this thesis (a) and the model generated by McGee et al (2001) (b). The transcript depicted corresponds to NM\_011807.3. Image created using Ensembl Release 92 (Zerbino et al, 2018).

The differential localisation of the mutation may influence any molecular or behavioural phenotypes identified (Figure 6). For example if the mutation is downstream of the PDZ domain, where NMDA receptors bind, then this interaction may not be disrupted and thus NMDAR dependant processes may not be affected. The different background strains also need taking into account when comparing findings. For example, C56BL6 strains are consistently reported to be most active in measures of locomotion and less anxious, whereas 129/Sv are more anxious and much less active (Abramov *et al.*, 2008; Mandillo *et al.*, 2008).

Anxiety, responses to novelty or locomotive activity levels (Sousa, Almeida and Wotjak, 2006) can contribute to or confound behavioural measures of cognitive function. As the *Dlg2*<sup>tm1a(EUCOMM)Wtsi</sup> has not been characterised previously it was important to assess baseline behaviours allowing better interpretation of future more complex experiments of cognitive function. This will include basic exploration of motor performance and co-ordination, locomotor activity, context discrimination, startle responses and pre-pulse inhibition, and anxiety. Equally important is the confirmation of reduced *Dlg2* expression in key brain regions and the potential dysregulation of *Dlg* family member expression.

# 3.1.1 Aims

- Confirm a reduction of *Dlg2* mRNA in brain regions important for behaviours affecting in schizophrenia (PFC and hippocampus) as well as a control region (cerebellum) in *Dlg2<sup>+/-</sup>* mice.
- 2. Investigate any potentially compensatory changes in the mRNA expression of other *Dlg* family members (*Dlg1, Dlg3, Dlg4*) in these brain regions.
- 3. To characterise the mouse model on basic behavioural tasks.

# 3.2 Materials and Methods

# 3.2.1 Transgenic Dlg2 Mouse Model

*Dlg2*<sup>tm1a(EUCOMM)Wtsi</sup> mice were generated (first germline transmission 2014) by the Wellcome Trust Sanger Institute, via insertion of the cassette upstream of the critical exon (14) on chromosome 7 on a C57BL/6N-A<sup>tm1Brd</sup> background (Figure 7). This resulted in a frame shift mutation. Both heterozygous and homozygous animals are viable. All procedures were conducted during the light phase of 12:12 hr light/dark cycle.



Figure 7. A diagram of the vector used to generate the knock out first mouse (Skarnes et al., 2011), Dlg2<sup>tm1a(EUCOMM)Wtsi</sup>. The cassette was inserted at 92285553 on chromosome 7, upstream of exon 14. A conditional ready allele can be generated via flp recombinase expression, with subsequent cre expression resulting in the knockout mouse. This is the model used in this thesis. Cre expression in the absence of flp expression generates a reporter knockout mouse. Vector map taken from http://www.mousephenotype.org/data/genes/MGI:1344351.

Experimental cohorts were bred through WT x HET crosses, with WT littermates used as controls. The background strain CB7BL/6NTac has been shown to carry the Crb1<sup>Rd8</sup> mutation, resulting in a mild form of retinal degeneration affecting vision (Pritchett-Corning, 2012). Phenotypic onset is between 2 and 6 weeks of age (Pritchett-Corning, 2012) as the result of a recessive single base pair mutation in the Crb1 gene (Mehalow *et al*, 2003). All data reported in this chapter are from the pure Dlg2<sup>tm1a(EUCOMM)Wtsi</sup> strain. In subsequent chapters to prevent any potential influence of this vision issue experimental cohorts were crossed with C5BL6/J mice.

# 3.2.2 Housing

Mice were housed with up to 5 litter mates as previously described in 2.1.3, with *ad libtum* access to food and water, and environmental enrichment. For breeding, pairs or trios were housed together in conventional caging, as were WT x HET crosses.

Separate cohorts were used for molecular and behavioural analysis.

# 3.2.3 Genotyping by Polymerase Chain Reaction (PCR)

DNA was extracted post weaning as described in 2.1.3 from either ear or tail. The Wellcome Trust Sanger Institute provided primer sequences (Table 3) and a PCR cycling protocol for genotyping. A shared *Tm* for the WT and mutant PCR primers facilitated both simplex and multiplex reactions. Master mixes totalled 20 µl per reaction using the MyTaq<sup>TM</sup> DNA polymerase kit (Bioline, London, UK). For simplex reactions: 9.2 µl ddH<sub>2</sub>0, 4 µl 5x reaction buffer, 0.8 µl each of forward and reverse primers, 0.2 µl MyTaq<sup>TM</sup> and 5 µl DNA. For multiplex reactions: 7.6 µl ddH<sub>2</sub>0, 4 µl 5x buffer, 0.8 µl reverse primer 1 and 2, 1.6 µl forward primer, 0.2 µl MyTaq<sup>TM</sup> and 5 µl DNA template.

Samples were run on a Biorad Thermal Cycler (T100 BioRad<sup>™</sup>, Herts, UK). Conditions for WT and mutant reactions were 94°C for 5 minutes, followed by 34 cycles of 94°C for 30 seconds, 58°C for 30 seconds and 72°C for 45 seconds, with a final extension for 5 minutes at 72°C and held indefinitely at 4°C.

Primer	Reaction	Sequence $(5' > 3')$	Reaction	Expected
			Temp	band size
				(bp)
Dlg2_42053_F	Wild type	CCAGAATGTACTTCAGCACCA	58	312
Dlg2_42053_R		TGTGTGTATGTGTGGCTGTTT		
Dlg2_42053_F	Mutant	CCAGAATGTACTTCAGCACCA		222
CAS_R1_Term		TCGTGGTATCGTTATGCGCC		

Table 3 PCR primer sequences and reaction temperature for genotyping of Dlg2tm1a(EUCOMM)Wtsi provided by the Wellcome Trust Sanger Institute.

A 2% agarose gel was made with 1% Tris-acetate-EDTA (TAE) buffer (w/v) and SYBR Safe Gel DNA Stain (1:1000, ThermoFisherScientific, UK). Analysis was conducted by gel electrophoresis at 95 V for 45 minutes, with 10  $\mu$ I PCR product loaded per well. Gels were visualised using an Omega Lum<sup>TM</sup> G imaging system (Apglegen, San Francisco, USA). For simplex reactions the presence of separate WT (312 bp) and mutant (222 bp) bands indicated a heterozygous animal (Figure 8). For multiplex reactions, WT animals were identified by one band (312 bp) whilst heterozygotes animals were identified by two bands (312 bp and 222 bp). Homozygous animals were identified by a mutant (222 bp) but not WT (312bp) bands.



Figure 8. Example of genotyping for multiplex (a) and simplex (b) reactions to screen for WT and mutant bands in  $Dlg2^{+/-}$  and WT mice. (a) Lanes 1 and 2 are reaction controls. Lanes 3, 4, 6 and 8 have two bands indicative of a heterozygote. Remaining lanes identify V'/T. (b) All extracted DNA has a WT band around 312bp. All but the last lane, indicated with a \* have a lower molecular weight band around 222bp. The \* demonstrates a WT animal due to the lack of a lower weight band. Lanes 1-5 and 6-10 represent the same animals, therefore 1-4 are HET due to the presence of both bands, and 5 is WT.

#### 3.2.4 Molecular Characterisation

# 3.2.4.1 RT-qPCR Analysis

The expression of mRNA was examined by RT-qPCR was in 8 week tissue in three brain regions: the cerebellum, hippocampus and PFC, extracted from 24 mice (12 WT, 12 HET) performed as previously described in section 2.2.1. Whole region extracts from one hemisphere were used to generate cDNA. Both male and female tissue was processed and analysed. Validated primers for *Dlg1* to *Dlg4* were used to compare *Dlg* paralog expression between WT and *Dlg2*<sup>+/-</sup>mice (Table 4).

Primer	Forward Sequence	Reverse Sequence
Dlg2	TGCCTGGCTGGAGTTTACAG	TTTTACAATGGGGCCTCCGC
Dlg1	CGAAGAACAGTCTGGGCCTT	GGGGATCTGTGTCAGTGTGG
Dlg3	GAGCCAGTGACACGACAAGA	GCGGGAACTCAGAGATGAGG
Dlg4	GGGCCTAAAGGACTTGGCTT	TGACATCCTCTAGCCCCACA

Table 4. Primer pairs designed for each Dlg gene previously validated using cDNA from hippocampus, cerebellum and PFC in naïve mice.

Quantitation using the comparative Ct method ( $2^{-\Delta\Delta Ct}$  method) was used to measure changes in *Dlg1-4* expression WT and *Dlg2<sup>+/-</sup>*mice. Data normality was assessed using Shapiro-Wilks test. Where possible appropriate transformations were attempted to correct normality. On examination of boxplots values considered extreme outliers were removed. If significance is changed by outliers being retained this is reported. Normally distributed data was analysed using *t* tests. Homogeneity of variances was assessed by Levene's test. If this assumption was violated Welch's *t* test was used. Where normality could not be corrected Mann Whitney U was used.

#### 3.2.5 Behavioural Characterisation

#### 3.2.5.1 Animals

A cohort of 46 males (27 WT, 19 HET) underwent the behavioural tasks described below in the order of completion, commencing when mice were ~10 weeks old. Two WT animals were euthanised during the course of experimentation on ill health grounds. Mice were handled daily for a week to habituate them to the experimenter prior to water restriction. Apparatus were cleaned between animals with either 1% acetic acid or ethanol wipes to mask odour cues. Mice were transported to testing rooms in their home cages, and were returned to them on task completion. All tasks were completed prior to daily water access and lights off (19:00).

#### 3.2.5.2 Water Restriction

Water access was restricted to 4 hours daily for 2 days (10:00-14:00). Mice were weighed prior to and following access for several days, before further restriction to 2 hours water access (17:00-19:00). Weighing continued daily for 3 weeks, then every other day. At weekends mice had *ad libtum* access to water unless behavioural tests were being conducted.

#### 3.2.5.3 Rotarod

#### Task

Each mouse received 5 training sessions on the rotarod (47600, Ugo Basile, Italy) which accelerated from 5-50rpm over 5 minutes. These sessions took place over 2 consecutive days. Latency (s) to the first fall (fall from the rod or 1 full rotation) was recorded. Accelerating rotarod tasks are considered to assess motor learning. Following completion of the accelerating task motor function was tested using fixed speed trials. Two trials per speed were conducted (5-50 rpm in 5 rpm increments), with an inter-trial interval of 20-30 minutes 20-30 minutes during which mice were returned to home cages. Latency to first fall (s) was recorded, and no more than two speeds per day.

#### Analysis

The normal distribution of all data was assessed Shapiro-Wilks test. Where appropriate transformations were attempted to correct normality. Extreme outliers (±3 studentized residuals) were removed. If their removal affected the significance when the outlier is retained this is reported. Homogeneity of covariances was assessed by Box's M test and homogeneity of variances was assessed by Levene's test. In

instances where the assumption of sphericity was violated the Greenhouse-Geisser correction was applied.

The latency to fall for both accelerating and fixed rotarod tasks was assessed using mixed ANOVA. For fixed speed trials the mean latency to fall for two trials per speed.

#### 3.2.5.4 Habituation and Context Shift

#### Task

Testing was conducted in the locomotive activity boxes described in 2.2.1.1. Laminated sheets with either black squares or spots covered the boxes walls and doors with the pattern alternating by row. Mice were assigned one of the two contexts in a genotypically balanced manner. Mice were brought to the room for 5 minutes prior to the start of the task each day. For four days animals were placed in the same box and allowed to explore freely for 30 minutes. Time of day for experimentation was consistent across days for each group (10:00 - 14:30, lights on 07:00 and 19:00). On the fifth day mice underwent two 5 minute trials, one in the familiar context and the second in the novel. Initial testing context was counterbalanced for genotype. Beam breaks as an activity measure was measured using a custom BBC BASIC V6 programme with additional ARACHNID interfacing (Campden Instruments, UK).

#### 3.2.5.5 Analysis

#### Habituation

The normal distribution of all data was assessed Shapiro-Wilks test. Where appropriate transformations were attempted to correct normality. Extreme outliers (±3 studentized residuals) were removed. If their removal affected the significance when the outlier is retained this is reported. Homogeneity of covariances was assessed by Box's M test and homogeneity of variances was assessed by Levene's test. In instances where the assumption of sphericity was violated the Greenhouse-Geisser correction was applied.

The total beam breaks per day were analysed by mixed ANOVA to assess intersession habituation. Changes in activity between day 1 and day 4 was analysed by *t* test (Day 4 BB total/(Day 1 BB total + Day 4 BB total). Each daily session was split into five 6 minute bins. Intrasession habituation was examined by analysing the 30 minute daily sessions across these bins by mixed ANOVA with the following factors: genotype (WT and HET), 6 minute time bins or quantiles (1, 2, 3, 4, 5) and day (1, 2, 3 and 4). The change in beam break (BB) activity each day between quantile 1 and 5 (Q1 and Q5) was assessed by t test (Q1 BB total/(Q1 BB total + Q5 BB total).

#### Test Day

For the test day the total bream beaks for the 5 minute session in both the novel and familiar contexts were analysed by mixed ANOVA.

#### 3.2.5.6 Acoustic Startle and Pre-Pulse Inhibition (PPI)

#### Task

Animals underwent a 30 minute program in a SR-Lab<sup>TM</sup> Startle Response System (San Diego Instruments, CA) described in 2.2.1.3. Animals awaiting testing were held in a separate procedure room and after testing animals were held in a separate cage from their home cage until all animals in that cage had been tested.. Each session began with a 5 minute habitation to the apparatus and scrambled white noise at background intensity (70 db). The session consists of three blocks of acoustic stimuli, initially 120dB, 105dB and then an increasing range of 80 to 120dB in 10dB increments. Pulse alone trials consisted of 40ms stimulus, whilst pre-pulse trials consisted of a 20ms pre-pulse at 4, 8 or 16dB above background, followed 70ms later by a 40ms stimulus at 120dB or 105dB. Blocks 1 and 2 consisted of 5 pulse alone trials followed by 5 blocks of 2 pulse alone trials, 1 no stimulus trial and 6 pre-pulse trials. The different intensity stimuli were presented pseudorandomly. Pulse alone stimuli of various intensities (80 – 120dB) were presented in block 3 pseudorandomly three times. Between blocks and post block 3 represented three no stimulus trials.

The inhibition of response to the stimulus following pre-pulse presentation was recorded as the average startle response during a 65ms window from startle pulse onset. The first three pulses at 120 dB and 105 dB were averaged and analysed as an index of emotional reactivity, as it is prior to appreciable habituation (Geyer & Dulawa, 2003). To analyse of the habituation of startle response, responses to the first six pulse alone trials at each dB were measured. PPI does not require normalisation as it is a percentage reduction in startle response.

#### Analysis

Due to the measure of startle relying on deflection of the pressure sensitive accelerometer any weight differences between genotypes could influence results, so all startle data is normalised for body weight using Kleiber's 0.75 mass exponent (Kleiber, 1932). The weight adjusted average response amplitude per trial (V avg) was used in all analysis by mixed ANOVA or *t* test.

Normality was assessed for all data using Shapiro-Wilks test. For data constancy all data were analysed untransformed, and only outliers found in multiple tests at each dB were removed from all tests. Homogeneity of covariances was assessed by Box's M test and homogeneity of variances was assessed by Levene's test. In instances where the assumption of sphericity was violated the Greenhouse-Geisser correction was applied.

The average of the first three pulses at 120 dB and 105 dB were analysed by *t* test as an index of emotional reactivity as this is prior to appreciable habituation (Geyer & Dulawa, 2003). The first six pulse alone trials at 120 dB and 105 dB were analysed by mixed ANOVA to assess startle response habituation.

#### 3.2.5.7 Elevated Plus Maze

#### Task

The first task employed to examine anxiety behaviour was the elevated plus maze (EPM) task. The setup is described in 2.2.1.4. A computer running Ethovision Observer XT software (Noldus Information Technologies 3.0.15, Netherlands) was connected to a camera mounted above the maze tracking movement of the mouse during the trial. Mice were placed in the nearest closed arm to the experimenter and allowed to explore freely for 5 minutes. The following behaviours were manually scored by the experimenter: number of head dips (downward movement of the rodent's head over the edge of an open arm), grooming and number of stretch attend postures (defined as an animal stretching forwards into the open arm whilst keeping its hindquarters in a closed arm) (Figure 9). This task is based on the rodent's the conflict between these preferences and their innate motivation to explore novel surroundings. Time spent in the closed arms is indicative of anxious behaviour.

#### Analysis

The normal distribution of all data was assessed Shapiro-Wilks test. Where appropriate transformations were attempted to correct normality. Extreme outliers (±3 studentized residuals) were removed. If their removal affected the significance when the outlier is retained this is reported. Homogeneity of covariances was assessed by Box's M test and homogeneity of variances was assessed by Levene's test. In instances where the assumption of sphericity was violated the Greenhouse-Geisser correction was applied.

Time spent in each maze zone, distance (m) and velocity (m/s), latency to first entry into an open arm (s), and ethological parameters (grooming, head dips, stretch attend postures) were assessed using *t* tests.



Figure 9. Diagram of the elevated plus maze apparatus with dimensions. Image adapted from Cohen, Matar, & Joseph (2013). Drawing not to scale.

# 3.2.5.8 Open Field

#### Task

The open field arena is described in 2.2.1.5 (Figure 10). Mice were consistently placed into the closest corner of the arena to the experimenter, consistently facing the same wall. Animals could freely explore for the duration of the session (10 minutes). A camera above the arena was connected to a computer running Ethovision Observer XT (Noldus Information Technologies 3.0.15, Netherlands) software recorded the animal's position (17 frames/s). Rodents tend to display thigmotaxis, remaining close to the outer walls of the arena.

#### Analysis

The main measures calculated were the duration of time spent in the central zone (the most exposed and therefore the most aversive part of the apparatus). The

velocity (m/s) and total distance travelled (m) were recorded as an indices of activity. All measures were assessed by t tests.



Figure 10. Diagram of the zones in the open field arena. Not to scale.

# 3.3 Results

#### 3.3.1 RT-qPCR

# 3.3.1.1 Reduced Dlg2+/- mRNA expression in the PFC of 8 week old heterozygotic mice.

Cerebellum data was not normally distributed, but removal of two outliers (both HET) resulted in normality. There was no homogeneity of variances so Welch's *t* test was used. Both hippocampal and PFC data was normally distributed and there was homogeneity of variances.

The expression of *Dlg2* mRNA was assessed via RT-qPCR (Figure 11). No reduction in expression of *Dlg2* was found in *Dlg2*<sup>+/-</sup> mice in the cerebellum (Figure 11a), *t* (9.301) = 0.547, *p* = 0.597) or the hippocampus (Figure 11b), *t* (21) -0.238, *p* = 0.815. *Dlg2*<sup>+/-</sup>mice displayed reduced *Dlg2*<sup>+/-</sup> expression in the PFC (Figure 11c), *t* (15) = -4.163, *p* = 0.001



Figure 11. Dlg2 mRNA expression in 8 week old mouse cerebellum (a) hippocampus (b) and PFC (c). Only the PFC displayed a significant reduction in expression. n = Cerebellum 9 (WT) 8 (HET), hippocampus 12 (WT) 11 (HET) and PFC 8 (WT) 9 (HET). Data represent the mean  $\pm$  SEM error bars. \*\*\* P = < 0.001.

# 3.3.1.2 Dlg2<sup>+/-</sup> mice displayed no altered mRNA expression of Dlg family members at 8 weeks across brain regions

All hippocampal data was normally distributed and met all assumptions, as did *Dlg1* and *Dlg3* cerebellum data. *Dlg4* cerebellum data was not normally distributed so Mann Whitney-U was conducted. No PFC data was normally distributed. Mann Whitney U was conducted in all instances.

There was no difference in the expression of mRNA of any the *Dlg* family members, *Dlg1* (Figure 12), *Dlg3* (Figure 13) or *Dlg4* (Figure 14) in the cerebellum, hippocampus or PFC of 8 week old mice, as assessed via RT-qPCR (Table 5).

Gene/Region	Test
Dlg1 PFC	<i>U</i> = 50.00, <i>p</i> = 0.219
Dlg1 Hippocampus	$t_{(7.851)}$ -1.557, $p = 0.159$ ,
Dlg1 Cerebellum	$t_{(10.266)} 0.642, p = 0.\pm 535$
Dlg3 PFC	<i>U</i> = 45.00, <i>p</i> = 0.128
Dlg3 Hippocampus	<i>t</i> (15) -1.107, <i>p</i> = 0.286
Dlg3 Cerebellum	$t_{(12.645)} 0.936, p = 0.367$
Dlg4 PFC	<i>U</i> = 48.00, <i>p</i> = 0.178
Dlg4 Hippocampus	$t_{(15)}$ -1.755, $p = 0.100$
<i>Dlg4</i> Cerebellum	<i>U</i> = 43.00, <i>p</i> = 0.631

Table 5. There were no differences in expression of other Dlg family members in any brain region between WT and Dlg2<sup>+/-</sup> mice.



Figure 12. The fold change in Dlg1 mRNA expression levels assessed in 8 week old tissue by qPCR in the cerebellum (a) hippocampus (b) and PFC (c). n = Cerebellum 9 (WT) 10 (HET), Hippocampus 8 (WT) 8 (HET) and PFC 12 (WT) 12 (HET). Data represent the mean  $\pm$  SEM error bars.



Figure 13. The fold change in Dlg3 mRNA expression levels assessed in 8 week old tissue by qPCR in the cerebellum (a) hippocampus (b) and PFC (c). n = Cerebellum 9 (WT) 10 (HET), hippocampus 8 (WT) 9 (HET) and PFC 12 (WT) 12 (HET). Data represent the mean  $\pm$  SEM error bars.



Figure 14. The fold change in Dlg4 mRNA expression levels assessed in 8 week old tissue by qPCR in the cerebellum (a) hippocampus (b) and PFC (c). n = cerebellum 10 (WT) 10 (HET), hippocampus 8 (WT) 9 (HET) and PFC 12 (WT) 12 (HET). Data represent the mean  $\pm$  SEM error bars.

#### 3.3.2 Behavioural Results

All animals were weighed prior to each task and no differences were found.

# 3.3.2.1 Dlg2+/- mice exhibit a deficit of motor learning deficit but not of motor function.

All accelerating rotarod data was normally distributed, there was homogeneity of covariances, and homogeneity of variances except for Trial one.

The latency to fall across five consecutive trials on an accelerating rotarod was analysed by mixed ANOVA (Figure 15). The assumption of sphericity was met for the accelerating data,  $X^2$  (9) 10.128, p = 0.341. There was an effect of trial, but not genotype on latency to fall (TRIAL:  $F_{(4, 168)}$  35.318, p = <0.001, GENOTYPE:  $F_{(1, 42)}$  1.952, p = 0.170). There was an interaction between trial and genotype on latency to fall (TRIAL x GENOTYPE:  $F_{(4, 168)}$  3.598, p = 0.008).



Figure 15. Latency to fall on accelerating rotarod tasks. The latency to the first fall, or full rotation of the rod, were recorded. Two trials were performed on day 1 and the remaining 3 (from the arrow onwards) were performed on day 2. Data represent the mean  $\pm$  SEM error bars. n = 25 (WT) 18 (HET). \*\* p = 0.01.

To pull apart this interaction, one way ANOVA was conducted between the genotypes at each trial.  $Dlg2^{+/-}$  mice had a shorter latency to fall at Trial 2 than WT mice, ( $F_{(1, 42)}$  9.102, p = 0.004). There was no difference between WT and  $Dlg2^{+/-}$  mice for any other trial.

Each genotype was also examined separately across the 5 trials using repeated measures ANOVA. The majority of data was normal, and all assumptions were met, including sphericity,  $X^2$  (9) 4.985, p = 0.837 and  $X^2$  (9) 8.075, p = 0.528 respectively. Pairwise comparisons were conducted and Bonferroni corrected. WT mice exhibited rapid improvement in latency to fall, as all trials were different to trial 1 but no other trials differed from each other (Table 6).

Trials	Mean Difference (s) ± SEM	ρ
1 and 2	-114.68 ± 157.77	< 0.001
1 and 3	-110.52 ± 14.01	< 0.001
1 and 4	-118.96 ± 14.48	< 0.001
1 and 5	-123.72 ± 14.77	< 0.001

Table 6. Post-hoc analysis following RM ANOVA determined latency to fall for WT mice was increased for each subsequent trial. Differences were only found between first and subsequent trials, no other trials were different to each other. Values are Bonferroni corrected.

In comparison, *Dlg2*<sup>+/-</sup>mice demonstrated a protracted period with a shorter latency to fall, with trial 1 differing from trial 3, 4 and 5, but not trial 2, and trial 2 differing from 4 and 5. There were no differences between any other trials (Table 7).

Trials	Mean Difference (s) ± SEM	p
1 and 3	-84.63 ± 18.28	0.002
1 and 4	-121.95 ± 15.49	< 0.001
1 and 5	-125.95 ± 14.52	< 0.001
2 and 4	-79.58 ± 17.23	0.002
2 and 5	-83.58 ± 21.30	0.01

Table 7. Post-hoc analysis following RM ANOVA determined latency to fall for Dlg2<sup>+/-</sup>mice was reduced for a protracted period. No other trials were different to each other. Values are Bonferroni corrected.

The fixed speed trial data was not normally distributed. Transformations could not be conducted due to some data being positively skewed and some negatively skewed. One outlier (HET) was removed. There was homogeneity of variance for all trials except 5 and 10rpm but not homogeneity of covariances.

The latency to fall was for fixed speed trials was analysed by mixed ANOVA (Figure 16). The assumption of sphericity was violated, X<sup>2</sup> (35) 103.684, p = < 0.001, and so the Greenhouse-Geisser correction was applied. Both genotypes exhibited a decrease in latency to fall across trials (TRIAL:  $F_{(5.696, 233.53)}$  120.649, p = < 0.001, GENOTYPE:  $F_{(1, 41)}$  0.147, p = 0.704, TRIAL x GENOTYPE:  $F_{(5.696, 233.53)}$  0.631, p = 0.697.



Figure 16. Latency to fall on accelerating on the fixed speed rotarod tasks. The latency to the first fall, or full rotation of the rod, were recorded. The mean latency of two trials was reported for the fixed speed task. Data represent the mean  $\pm$  SEM error bars. n = 25 (WT) 18 (HET).

In summary *Dlg2*<sup>+/-</sup> mice fell significantly earlier than WT and took longer to reach asymptotic performance on an accelerating rod, but performed comparably on fixed speed trials of motor function. For both genotypes latency to fall was reduced as rotation speed increased on fixed trials.

# 3.3.2.2 Dlg2<sup>+/-</sup> mice exhibit within session habituation of locomotor activity to a novel environment and comparable responses to a novel context during a context shift task

For analysis of both habituation and test days all data was normal, with the exception of WT familiar data on the test day. In all instances all assumptions were met, with the exception of sphericity, which is reported separately for each test.

Total beam breaks across the four daily sessions were analysed by mixed ANOVA. The assumption of sphericity was violated,  $X^2$  (5) = 25.028, p = < 0.001, so the Greenhouse-Geisser correction was applied.

Total beam break data was analysed to compare intersession habituation to a novel context across the first four days of training between the genotypes, shown in Figure 17a. Despite  $Dlg2^{+/}$  mice appearing slightly more active no difference was found in locomotor activity between the genotypes, nor was there a difference in locomotion across the days (DAY:  $F_{(2.093, 87.925)}$  0.408, p = 0.676, GENOTYPE:  $F_{(1, 42)}$  0.643, p = 0.427, DAY x GENOTYPE:  $F_{(2.093, 87.925)}$ , 0.590, p = 0.590).

No difference was found between the genotypes when intersession habituation was assessed by *t* test for the change in activity between Day 1 and Day 4 (Figure 17 b),

Day 4 BB total/(Day 1 BB total + Day 4 BB total), WT 0.460  $\pm$  0.076, HET 0.480  $\pm$  0.126, t (42), -.0645, p = 0.523. The activity change ratio for both genotypes is around 0.5, demonstrating a lack of habituation. Habituation is reflected in a ratio closer to 0 whilst a ratio closer to 1 suggests sensitisation (Bolivar 2010).



Figure 17. Both genotypes exhibit comparable levels of intersession habituation across the four daily 30 minute sessions (a). The activity change calculated as Day 4 BB total/(Day 1 BB total + Day 4 BB total is comparable between the genotypes (b). Both genotypes exhibit within session habituation for each day over the 30 minute session. Each quantile is the total beam breaks for 6 minutes. On day 1 (c) and day 4 (d) the genotypes are comparable but have begun to diverge slightly by day 4. Data represent the mean  $\pm$  SEM error bars. n = 25 (WT) 19 (HET).

Intrasession habituation was examined by analysing the 30 minute daily sessions in five 6 minute quantiles by mixed ANOVA with the following factors: genotype (WT and HET), 6 minute time bin (Q1, Q2, Q3, Q4, Q5) and day (1, 2, 3 and 4) (Figure 17c, d). The assumption of sphericity was violated for day X<sup>2</sup> (5) 45.230, p = <0.001, time bin X<sup>2</sup> (9) 40.178, p = <0.001, and day\*time bin X<sup>2</sup> (77) 123.941, p = 0.001, so the Greenhouse-Geisser correction was applied in all instances.

There was no effect of genotype or day, but there was a reduction in locomotive activity across the quantiles (DAY:  $F_{(1.812, 74.299)}$  0.504, p = 0.680, GENOTYPE:  $F_{(1, 41)}$  0.324, p = 0.572, QUANTILE:  $F_{(2.557, 104.838)}$  77.073, p = < 0.01). There was no

interaction between day and genotype, (DAY x GENOTYPE:  $F(_{1.812, 74.299})$  0.474, p = 0.606), nor between quantile and genotype, (QUANTILE x GENOTYPE:  $F(_{7.246, 297.077})$  1.144, p = 0.335), nor for day\*genotype\*quantile, (DAY x GENOTYPE x QUANTILE:  $F(_{7.246, 297.077})$  1.182, p = 0.312).

No difference was found between the genotypes when daily intrasession habituation was assessed by *t* test as the change in activity between Q1 and Q5 on each day (Figure 18) Q5 BB total/(Q1 BB total + Q5 BB total) (Figure 18).



Figure 18. The activity change between Q1 and Q5 on day 1 (a), day 2 (b), day 3 (c) and day 4 (d) is comparable between the genotypes. For each day the activity change ratio for both genotypes approaches 0.5, demonstrating a lack of habituation.

Day	t Test
1	<i>t</i> (42) -0.440, <i>p</i> = 0.662
2	<i>t</i> (42) 0.109, <i>p</i> = 0.914
3	<i>t</i> (42) -0.050, <i>p</i> = 0.623
4	<i>t</i> (42)064, <i>p</i> = 0.949

Table 8: Comparison in mean change in total beam breaks as a measure of locomotive activity between Q1 and Q5 on the four habituation days. There was no difference in the change in activity between the genotypes on any day.
For test day analysis a software malfunction resulted in the loss of some data (8 WT, 3 HET).

There was no difference in locomotor activity between the contexts for either genotype (Figure 19) (CONTEXT: F (1, 30) 0.687, p = 0.414, GENOTYPE: F (1, 30) 0.023, p = 0.881, CONTEXT x GENOTYPE: F (1, 30) 0.183, p = 0.672).



Figure 19. Cumulative beam breaks were measured on context shift test day (day 5). Both genotypes demonstrate no difference in activity between novel and familiar contexts. Data represent the mean  $\pm$  SEM error bars. n = 17 (WT) 15 (HET)

# 3.3.2.3 Dlg2+/- startle significantly less than WT mice at 120dB, but exhibit normal PPI.

For all startle response and PPI data at 120 dB 2 outliers were removed (both HET) and at 105 dB 1 WT was removed.

Most increasing dB data was not normally distributed. There was homogeneity of variances for all data except P70, but there was not homogeneity of covariances. Incremental (10 dB) increases in pulse dB from background (70 dB) to 120 db assessed audio acuity of the mice (Figure 20a). The assumption of sphericity was violated for these incremental increases,  $X^2$  (14) = 255.911, p = < 0.001, so the Greenhouse-Geisser correction was applied. As the stimulus intensity increased so did the startle response of both genotypes, although the *Dlg2*<sup>+/-</sup>mice startled less than the WT (Figure 20b), (PULSE: F (1.903, 76.136) 54.450, p = < 0.001, GENOTYPE: F (1, 40) 5.306, p = 0.027, PULSE x GENOTYPE: F (1.903, 76.136) 2.834, p = 0.068).



Figure 20. (a) At greater intensities the startle response between the genotypes begins to diverge with the HETs startling less (b) Graphical summary depicting all pulse alone trials at both startle amplitudes. (1) Startle pulses averaged as an index of emotional reactivity. (2) The first 6 pules at each amplitude are used to calculate habituation to the stimulus. (3) Following this the stimuli are interspersed with lower dB stimuli to assess PPI at each intensity. Data represent the mean  $\pm$  SEM error bars. n = 25 (WT) 17 (HET). All startle response values are weight adjusted using Kleiber's (1932) 0.75 mass exponent. \* p = 0.05

The acoustic startle response was assessed at two stimulus intensities, 120 dB and 105 dB (Figure 20b). The average of the first three pulses at 120dB and 105 dB were assessed as a marker of emotional reactivity and analysed using *t* tests (Figure 21 a, c). All 120 dB data was normal, but all 105 dB was non-normal. All data had homogeneity of variances. For 120 dB *Dlg2*<sup>+/-</sup>mice startled less than WT mice (*t* (40) 3.303, *p* = 0.004). No difference was found at 105 dB (*t* (<sub>41</sub>) 1.694, *p* = 0.098).

Habituation of startle response was assessed over the first six pulse alone trials at 120 dB and 105 dB using mixed ANOVA (Figure 21b and d). The majority of data for 120 dB, and all 105 dB data, was non-normal. For 120 dB there was homogeneity of variances but not covariances, whilst 105 dB data met both assumptions. The assumption of sphericity was violated for 120 dB, X<sup>2</sup> (14) 26.316, p = 0.024 so the Greenhouse-Geisser correction was applied, but was met for 105 dB, X<sup>2</sup> (14) 15.574, p = 0.341. The reduced startle response in the *Dlg2*<sup>+/-</sup>mice compared to WT mice to 120 dB stimulus persisted across the first six trials (TRIAL: F (3.825, 152,983) 0.803, p = 0.520, GENOTYPE: F (1, 40) 7.441, p = 0.009, GENOTYPE x TRIAL: F (3.825, 152,983) 1.250, p = 0.293). At 105 dB the genotypes were comparable, neither displaying habituation to the stimulus (TRIAL: F (5, 205) 1.975, p = 0.084, GENOTYPE: F (1, 41) 1.471, p = 0.232, GENOTYPE x TRIAL: F (5, 205) 1.485, p = 0.196).



Figure 21. (a) The startle response to the first 3 pulses at 120 dB was averaged as an index of emotional reactivity. HETs startled less than WT mice. This difference in startle response persisted across the first 6 pulses at 120 dB (b). HETs startle response is largely unchanged across the 6 pulses. Data represent the mean  $\pm$  SEM error bars. n = 25 (WT) 17 (HET). (c) The startle response to the first 3 pulses at 105 dB was averaged. There was no difference between the genotypes. (d) Neither genotype displayed a change in startle across the 6 trials to the 105 dB pulses. n = 24 (WT) 19 (HET). All startle response values are weight adjusted. \* p = 0.05, \*\* p = 0.01

The startle response on 'pulse alone' and 'pre-pulse' trials was assessed at 120 dB and 105 dB (Figure 22a, c). For pulse alone and pre-pulse trials at both 120 dB and 105 dB most data was non-normal, and there is homogeneity of variances and co-variances. For both 120 dB and 105 dB the assumption of sphericity was violated,  $X^2$  (5) 56.770, p = <0.001 and X2 (5) 53.660, p = <0.001 respectively, so the Greenhouse-Geisser correction was applied.

On the 'pulse alone' and 'pre-pulse' trials at 120dB there was an effect of both pulse type and genotype, but there was an interaction between the two factors (PULSE: F (1.742, 69.692) 5.791, p = 0.007, GENOTYPE: F (1, 40) 5.916, p = 0.020, PULSE x GENOTYPE: F (1.742, 69.692) 5.791, p = 0.007).

To pull apart the interaction, one way ANOVA was conducted between the genotypes for each pulse type, where PPx denotes the dB above background (none, PP4P120, PP8P120, PP16P120). In the absence of a pre-pulse the startle response of *Dlg2*<sup>+/-</sup>

mice was reduced compared to WT at 120 dB (PULSE:  $F(_{1, 40})$  7.908, p = 0.0089), as well as following a minimal pre-pulse at 4dB above background (PULSE:  $F(_{1, 40})$  4.720, p = 0.036), but was comparable for moderate 8dB intensity pre-pulse (PULSE:  $F(_{1, 40})$  3.970, p = 0.053), or high intensity pre-pulse dB (16 dB above background), (PULSE:  $F(_{1, 40})$  2.229, p = 0.143 (Figure 22a).

For 105 dB the 'pulse alone' and 'pre-pulse' trials both genotypes exhibited a reduced startle response as the stimulus intensity increased (PULSE: F ( $_{1.660, 68.050}$ ) 49.730, p = < 0.001, GENOTYPE: F ( $_{1.41}$ ) 2.143, p = 0.151, GENOTYPE x TRIAL: F ( $_{1.660, 68.050}$ ) 2.219, p = 0.125) Figure 22c).

The percentage inhibition in startle response to 120 dB and 105 dB was calculated (Figure 22b, d). For percentage PPI at 120dB most data was normal, whilst most 105 dB was non-normal. There was homogeneity of variances, but not covariances, in both instances. The assumption of sphericity was violated for both 120 dB,  $X^2$  (2) 6.618, p = 0.037, and 105 dB, X2 (2) 9.793, p = 0.007, so the Greenhouse-Geisser correction was applied.

At 120 dB both genotypes demonstrated comparable increases in percentage inhibition as the pre-pulse stimulus intensity increased (Figure 22b) (GENOTYPE: *F* (1, 40) 1.704, p = 0.199, PULSE: *F* (1.730, 69.199) 123.350, p = <0.01, GENOTYPE x PULSE: *F* (1.730, 69.199) 0.238, p = 0.757). A similar affect was found at 105 dB (Figure 23d) (PULSE: *F* (1.643, 67.370) 55.852, p = < 0.001, GENOTYPE: *F* (1, 41) 0.569, p = 0.455, PULSE x GENOTYPE: *F* (1.643, 67.370) 1.918, p = 0.162).



Figure 22. (a) Both genotypes startle less as the pre-pulse stimulus intensity increases in dB, but HETs startle less than WT mice in the absence of a pre-pulse (P120, pulse alone), or when the pre-pulse stimulus is only 4 dB (PP4P120) above background (70 dB). (b) There is no difference between the genotypes for percentage inhibition to an acoustic stimulus at 120 dB, both exhibit an increase in percentage startle response as the pre-pulse intensity increases. Data represent the mean  $\pm$  SEM error bars. n = 25 (WT) 17 (HET). (c) The startle response decreases for both genotypes to a 105 dB pulse as the pre-pulse intensity increases for both genotypes show increased percentage inhibition of startle response to 105 dB pulses with increasing intensity pre-pulses. n = 24 (WT) 19 (HET). All startle response values are weight adjusted. \* p = 0.05, \*\* p = 0.01.

## 3.3.2.4 Dlg2<sup>+/-</sup> mice do not display any anxiety phenotypes

#### **Elevated Plus Maze**

All zone data, except WT middle zone data, and velocity and distance data were normal. Latency to first, head dips, stretch attends and grooming were non-normal so Mann Whitney U was used in these instances. There was homogeneity of variances for all measures except for distance and velocity, so Welch's *t* test was used in these instances.

Both genotypes were comparable in the time spent in each zone of the maze (Figure 23). There is no difference in time spent in the open,  $t_{(42)} = 0.780$ , p = 0.440, closed,  $t_{(42)} = 0.146$ , p = 0.885, or middle, U = 156.50, p = 0.055 zones. The latency to first

entry into the open arms was not significantly different between the genotypes (24a), U = 191.50, p = 0.276.

Both WT and  $Dlg2^{+/-}$  mice travelled similar distances, at similar velocities during the task (t ( $_{36.4}$ ) = -0.471, p = 0.640 and t ( $_{36.32}$ ) = -0.445, p = 0.659 respectively (25b & c).

There was no difference in the number of stretch attend postures, U = 191.50, p = 0.275, periods of grooming, U = 185.50, p = 0.204 or the number head dips, U = 193.50, p = 0.297 (Figure 24d, e & f).



Figure 23. (a) The % total time spent in each zone of the maze during the 5 minute trial. (b) Merged heat maps for each genotype. Warmer colours indicate greater time spent in the maze zone. Data represent the mean  $\pm$  SEM error bars. n = 25 (WT) 19 (HET). Arrows indicate the closed arms.



Figure 24. There were no differences between the genotypes for any of the other measures recorded during EPM trial. (a) The latency of mice to first enter an open arm of the elevated plus maze. (b) Total distance travelled during the 5 minute trial. (c) Velocity of movement during the 5 minute trial. There was no difference in number of head dips (d), stretch attends (e) or periods of grooming (f) between the genotypes. Data represent the mean  $\pm$  SEM error bars. n = 25 (WT) 19 (HET).

#### Open Field

Only inner zone data was normal. Two outliers (1 WT, 1 HET) were removed, resulting in normality for all data. The assumption of homogeneity of variances was met in all instances.

Compared to WT, the  $Dlg2^{+/-}$  mice spent a similar times in the inner,  $(t_{40})$  -0.388, p = 0.351) and outer zones  $(t_{40})$  0.388, p = 0.351) over the 10 minutes in the open field arena (Figure 25). There was no difference in activity levels as indicated by similar distance travelled,  $(t_{40})$  1.167, p = 0.243) or velocity,  $(t_{40})$  1.167, p = 0.241) between the two genotypes (Figure 25).



Figure 25. There was no difference in any measure during the open field task. Both genotypes exhibited thigmotaxis, spending most time in the outer zone and corners of the open field. (a) The percentage time spent in each zone of the arena during the 10 minute trial. (b) Merged heat maps for each genotype. Warmer colours indicate greater time spent in the maze zone. (c) Total distance travelled during the 10 minute trial. (d) Velocity of movement during the 10 minute trial. Data represent the mean  $\pm$  SEM error bars. n = 25 (WT) 18 (HET).

### 3.4 Discussion

A reduction in *Dlg2* mRNA expression was only found in the PFC but not hippocampus or cerebellum of *Dlg2*<sup>+/-</sup> mice. No differences were found between the genotypes in any brain region for other *Dlg* family members (*Dlg1*, *Dlg3*, *Dlg4*).

 $Dlg2^{+/-}$  mice exhibited specific behavioural changes. Both WT and  $Dlg2^{+/-}$  mice exhibited normal motor function but  $Dlg2^{+/-}$  mice demonstrated impaired motor learning, as assessed by the accelerating rotarod. When context discrimination was probed using a locomotive task both genotypes demonstrated intrasession but not intersession habituation across four 30 minute daily sessions, and did not discriminate between a familiar and novel context. The startle response of  $Dlg2^{+/-}$  mice was reduced in comparison to WT mice in response to a 120 dB, but not 105 dB, acoustic stimulus. However, no differences were found in percentage PPI response to either stimulus amplitude. On all measures of anxiety, across two different tasks, no differences were observed between  $Dlg2^{+/-}$  mice and WT mice. No differences in anxiety related behaviours were observed between WT and  $Dlg2^{+/-}$  mice in two anxiety probing tasks. The regionally specific reduction in mRNA expression is reflected in the subtle differences in certain behaviours, as opposed to global impairments.

# 3.4.1 Heterozygote mice have reduced Dlg2<sup>+/-</sup> expression in the PFC with no evidence of compensation from other Dlg family members.

The mRNA expression of *Dlg2* was compared between genotypes in three brain regions, the PFC and hippocampus, which are relevant to potential schizophrenia phenotypes, and the cerebellum (control region) by RT-qPCR. There was a selective reduction in expression of about 50% found in the PFC of *Dlg2*<sup>+/-</sup> mice. No changes in mRNA expression of any other *Dlg* family member was found in the brain regions tested. The mutant *Dlg2* mouse generated by McGee et al (2001) had reduced mRNA in brain tissue as assessed by Northern blot, although *Dlg2*<sup>-/-</sup> mice expressed a very weak band ~7.5 kb. This did not appear to translate into viable protein as Western blot analysis found reduced *Dlg2* protein in the *Dlg2*<sup>-/-</sup> mice was likely attributable to alternative splicing that skips the deleted exon. The regional specificity of the mRNA reduction cannot be compared between models as the *Dlg2* analysis reported was from whole brain extracts, as opposed to discrete regions. Protein analysis of *Dlg1, 3 and 4* was reported for forebrain and cerebellum extracts.

In order to determine whether there is truncated mRNA or transcripts that are unaffected by the mutation, and thus potentially still able to function normally, targeted primers designed to span the PDZ domains can be tested in homozygote tissue from key brain regions. The first and second PDZ domains are where currently available western blot antibodies bind, and are particularly important for binding between *Dlg2* and interactors such as NMDAR. Presence of a band would indicate the presence of mRNA expression upstream of the genetic lesion, suggesting a transcript potentially unaffected by the mutation, similar to the McGee et al (2001) model. The band could then be sequenced and predictive software used to determine the viability of the protein produced.

The lack of a reduction in *Dlg2* mRNA may correspond to normal protein levels in the hippocampus and cerebellum. This could mean biochemistry and behaviour examined which involve these regions may not be negatively affected in  $Dlg2^{+/-}$  mice. However, evidence from human post mortem tissue previously reported differential expression changes, with increased *DLG2* mRNA but decreased protein in schizophrenia patients (Kristiansen *et al.*, 2006). Therefore it is plausible that whilst no changes are observed at mRNA level there may be alterations at the protein level.

# 3.4.2 Reduced Dlg2 expression has no effect on motor function but results in impaired motor learning in rotarod tasks.

A fixed speed rotarod was used to assess motor performance, whilst the accelerating rotarod probed motor learning. Both genotypes demonstrate a comparably reduced ability to remain on the rotarod as the speed increases in fixed increments (Mann and Chesselet, 2015), suggesting differences in motor function in *Dlg2<sup>+/-</sup>*mice. On the accelerating rod, *Dlg2<sup>+/-</sup>*mice eventually reach the same plateau of performance as WT mice but they show a slower trajectory. The *Dlg2*<sup>+/-</sup>mice do not display the sharp increase in latency to fall between their first and second trials observed in the WT mice, potentially demonstrating a slower acquisition in learning the requirement to alter their behaviour in response to the increasing rod speed. This is in contrast to McGee et al (2001) who found no differences in rotarod performance between WT, Dlg2<sup>+/-</sup> or Dlg2<sup>-/-</sup> mice, but is in line with the deficient motor learning observed by Winkler et al (2018). The variation in protocols and genetic lesions between the models needs to be considered. In comparison to the method reported in this thesis, McGee et al (2001) trained these mice on a fixed speed rod and tested on an accelerating rod for four minutes. The decrease in *Dlg* expression in the PFC and lack of change in *Dlg2* mRNA expression in the cerebellum of the mouse model we used suggests that the impairments of motor learning may stem from other cortical brain regions (motor cortex – M1 and M2, striatum, cingulate cortex) involved in the process (Costa, Cohen and Nicolelis, 2004). Therefore assessment of mRNA and protein expression in other regions important for motor learning is required.

Impaired motor learning has previously been observed in other models carrying genetic mutations associated with synaptic function. Impaired performance on the accelerating rotarod and balance beam tasks were observed in *Dlg4<sup>-/-</sup>* mice (Feyder, et al. 2010a), as well as in mice carrying mutations in another scaffolding protein, SHANK3 (Yang et al, 2012). The severity of the impairment observed on the rotarod was greater in SHANK3 mutant males than females, which the authors suggest reflects the male:female ratio of autism observed in humans (Yang et al, 2012). Additionally, mice lacking a key *Dlq4* and *Dlq2* interactor NMDAR1, in the striatum demonstrated severely impaired performance on the accelerating rotarod. These mice displayed comparable performance to WT mice (Dang et al., 2006). Given the similar phenotypes observed in *Dlg2, Dlg4* and SHANK mutant mice, three proteins that contain PDZ and SH3 domains, there is the possibility that the impaired motor learning observed is the result of inability of interactors to bind normally to these domains. Interestingly, however, a study probing a *Dlg4* knockin model where only the PDZ binding domains were altered to retain their structure but were unable to bind ligands, found no difference between WT and knockin (KI) mice on the accelerating rotarod (Nagura et al., 2012a). This could suggest that whilst NMDAR has been demonstrated to play an important role in motor learning (Dang et al., 2006), it is unlikely that the motor learning phenotypes observed in *Dlg2* or *Dlg4* heterozygotes previously, or in this thesis, result from abnormal interaction between Dlg2 or Dlg4 and NMDAR through the PDZ domains.

# 3.4.3 Both genotypes display intrasession but not intersession habituation and no discrimination between a novel and familiar context.

Horizontal locomotion was examined across four daily sessions to one context, before mice were presented with both the familiar and a novel context on day 5. The repeated exposure to the context on days 1 to 4 should result in reduced locomotive activity as the animals habituate to the familiar environment (Bolivar, 2009). Neither genotype displayed intersession habituation of total beam breaks across the four habituation days. Daily intrasession habituation is demonstrated by both genotypes. On test day neither genotype is more active in the novel context compared to familiar, suggesting a lack of context discrimination (Terry, 1979). Altering an aspect of the previously

habituated context, such as the floor surface, has been show to invoke increased activity in multiple mouse strains, including C57BL/6J (Bolivar *et al.*, 2000). The lack of discrimination observed in our experiment may stem from the contexts not being sufficiently different to induce greater exploration in the novel condition. When placed in a novel environment rodents will explore, acquiring an internal representation of that environment in the hippocampus that becomes increasingly complex with more investigation (O'Keefe & Nadel, 1978). The more similar two overlapping contexts are the less distinct the representations will be, resulting in greater difficultly in discriminating between them, potentially leading to a generalisation of the behavioural response (Rolls, 2016). If there was generalisation of context then there would be no need to explore the 'novel' one, given that an adequate representation of the area has been generated.

Repeated exposure to the same context would be anticipated to result in reduced activity across the days, as the environment becomes less novel (Rankin et al., 2009). The lack of intersession habituation demonstrated by both genotypes may have resulted from inadequate initial exploration, requiring prolonged investigation in order to generate a suitable representation. However, the lack of habituation may also represent a strain affect. Greater variability in intersession habituation compared to intrasession habituation between mouse strains has been demonstrated, and suggested that intersession habituation may be more challenging for mice generally given the increased retention interval (Bolivar, 2009). C57BL/6J and C57BL/6Tac were similarly found to display higher levels of intra and intersession habituation when the environment was not overly complex (Bolivar, 2009). Short term intrasession habituation occurs over a single training period, whereas longer term intersession habituation across multiple sessions (Rankin et al., 2009). At its simplest, intrasession is suggested to measure adaptivity, as the learning element occurs in the absence of a retention interval, whereas intersession also probes memory of previous sessions and the environment (Müller et al., 1994).

Overall *Dlg2*<sup>+/-</sup> mice do not demonstrate any differences in their within session adaption or between session habituation when compared to WT mice.

# 3.4.4 Dlg2<sup>+/-</sup> mice startle less than WT to high intensity acoustic stimuli but exhibit normal PPI.

Both genotypes startle more with increasing acoustic stimulus intensity, but begin to diverge at the higher auditory intensities, with  $Dlg2^{+/}$  mice startling less to the 120 dB pulses. The reason for this deviation is unclear. Although there is no evidence linking

*Dlg2* to hearing, the *Dlg2*<sup>+/-</sup>mice might be exhibiting high frequency hearing loss, which can occur with maturation in the background strain, C5BL/6 mice (Willott *et al*, 1994). At the time of acoustic startle testing the mice were 3.3 months old. Therefore, it is unlikely hearing was influenced by age (Willott et al, 1994) and even so it would have been unlikely to have manifested as a genotype difference. To conclusively discount this an auditory brain stem response test could be conducted, but this would be invasive and time consuming.

The reduced startle response of *Dlg2*<sup>+/-</sup>mice persists across both the initial index of emotional reactivity and in their lack of habituation to the 120 dB pulses. The response of the WT mice gradually declined closer to that of the *Dlg2*<sup>+/-</sup>mice. There are several potential explanations for the lack of habituation observed in the *Dlg2*<sup>+/-</sup>mice.

Many factors can influence startle response, such as attention, stress and anxiety (Ray et al. 2009; De la Casa et al. 2016). Previous work demonstrated attentional impairments in *Dlg2<sup>-/-</sup>* mice (Nithianantharajah *et al*, 2013), which was not addressed in the current model. Comparable results between the genotypes on two tests probing anxiety behaviours, discussed below (3.4.5), make it unlikely the differential startle response is due to anxiety levels.

This differential reactivity may prevent accurate assessment of PPI response especially when a high intensity startle response is used. At lower pre-pulse intensities there was a marked difference using a 120dB startle response, with *Dlg2*<sup>+/-</sup> mice startling less. Higher intensity pre-pulses elicited the same reduction in response in both genotypes. However, if PPI is taken as a percentage of the baseline startle, thus negating the differential baseline response, there was no difference in response between the genotypes. This approach reduces the variation associated with manipulation induced changes in startle response (Geyer and Dulawa, 2003). No differences in PPI response between the WT and *Dlg2*<sup>+/-</sup> mutants were also found for the 105 dB stimulus trials where there is no baseline difference in startle response. Therefore both when the genotypes are performance matched for their acoustic startle response (lower intensity, 105 dB) and when startle responses are expressed as a percentage of the pre pulse response (at both 120dB and low 105dB startle stimulus intensities), there were no changes in PPI in the Dlg2<sup>+/-</sup> mice compared to WT.

A *Dlg4* KI model, with abnormal PDZ 1/2 binding domains, displayed a similar pattern of response in PPI tests to that we observed in the current *Dlg2<sup>+/-</sup>* model (Nagura *et al.*, 2012a). Mutant *Dlg4* mice exhibit reduced acoustic startle response but a PPI

response comparable to WT. A similar process may underlie the phenotype observed in  $Dlg2^{+/-}$ . This suggests that the association of Dlg2 and with its interactors might be a basis for this phenotype.

Similar behavioural phenotypes showing a reduced acoustic startle response with a lack of habituation were also observed in a genetic stress model (Dirks *et al*, 2002), and in a loss of function potassium channel knockout (Typlt *et al.*, 2013). Mice (CRH- $OE_{2122}$ ) overexpressing corticotrophin-releasing hormone (CRH) startled significantly less to higher intensity stimuli (110 dB and 120 dB), despite startling slightly more than WT mice at the lowest intensity (75 dB). As with the  $Dlg2^{+/}$  mice, the CRH- $OE_{2122}$  mice did not demonstrate habituation to the startle stimulus (Dirks et al, 2002). Unlike the  $Dlg2^{+/}$ , however, the CRH- $OE_{2122}$  mice also display reduced percentage PPI. However, there were methodological differences that need to be considered as a source of the difference; Dirks *et al* (2002) conducted each component of the startle response as separate experiments, as opposed to one continuous protocol as used in thesis. At the molecular level interaction between MAGUKs, including *Dlg2*, and the CRH receptor, CRHR1, has been demonstrated (Bender *et al*, 2015). This suggests a potentially interesting functional link between *Dlg2* and the CRHR1 receptor.

Voltage-gated and calcium-activated potassium (BK) channel loss of function in mice resulted in a reduced startle response to the highest stimulus, as well as a lack of habituation across trials (Typlt *et al.*, 2013), similar to *Dlg2*<sup>+/-</sup> mice. BK channels help regulate processes such as neuronal excitability (Shao *et al.*, 1999; Brenner *et al.*, 2005), and are proposed to mediate synaptic plasticity that underlies short term habituation of startle (Zaman *et al.*, 2017). *Dlg2* does interact with other potassium channels but currently there is no link with BK channels. Interestingly, however, BK channels which are encoded by the KCNMA1 gene have been implicated in schizophrenia (Zhang *et al.*, 2006; Kendler *et al.*, 2011) and autism (Laumonnier *et al.*, 2006).

Abnormal habituation of startle response is considered a biomarker of schizophrenia, alongside impaired PPI, although habituation deficits might be more intricately linked with acute psychotic episodes than chronic disease (Mena *et al.*, 2016a). However, patients with schizophrenia (Mena *et al.*, 2016b) and autism (Kohl *et al.*, 2014a) have higher acoustic startle responses than control groups as opposed to lower startle responses exhibited by *Dlg2*<sup>+/-</sup> mice. Additionally, abnormal habituation in human cohorts may be influenced by sensitisation, whereas this does not seem to be

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occurring in  $Dlg2^{+/-}$  mice because their startle response was maintained across presentations of the auditory stimulus. Despite the translational differences there does appear to be an altered startle response in  $Dlg2^{+/-}$  mice.

# 3.4.5 Dlg2<sup>+/-</sup> do not display any anxiogenic behaviours in two common paradigms.

For both the EPM and OF measures, there was no indication of a difference in anxiety response between the genotypes. On both tests more time was spent in the 'safer' closed arms of the EPM, or in the corners of the open field, but both mutant and WT mice ventured out into the more risky open arms or arena centre. These findings support the lack of observed anxiety phenotypes in the McGee *et al* (2001) *Dlg2* KO model on open field or light/dark tests (Winkler *et al.*, 2018).

In the EPM there was a trend towards  $Dlg2^{+/-}$ mice spending more time in the middle zone of the maze (p = 0.06). However, there is ambiguity in the meaning and relevance of time spent in this zone as some argue it does not assess anxiety (Rodgers, Dalvi and Anxiety, 1997; Carobrez and Bertoglio, 2005). This could be overcome using an elevated zero maze, which is a continuous circle with open and closed sections, but no central zone (Shepherd et al, 1994). In addition, there was no difference in the so called "risk taking" measures of anxiety, head dips, stretch attends or time spent grooming (Walf and Frye, 2007), nor in terms of distance covered or speed of movement in the  $Dlg2^{+/}$ mice.

Similarly, measuring anxiety in the OF maze, there was no difference in the time spent in either zone of the arena, with both genotypes demonstrating the same levels of thigmotaxis. As with the locomotor activity data obtained during the context shift task, locomotive activity was similar between WT and  $Dlg2^{+/-}$ mice. This indicated that  $Dlg2^{+/-}$  did not exhibit any hyperactivity phenotypes.

Two models of *Dlg4* mutation, the *Dlg4<sup>/-</sup>* (Feyder et al. 2010b) and the *Dlg4* PDZ domain KI mice (Nagura *et al.*, 2012a), found increased levels of anxiety on OF and EPM compared to WT mice. This may reflect a more fundamental role of *Dlg4* in fear and anxiety behaviours compared to *Dlg2*.

## 3.4.6 Strengths and Limitations

For studying hippocampal dependant learning this model may not be ideal given the lack of a reduction in *Dlg2* mRNA in the hippocampus. However, the impact of *Dlg2* mutation on cortical functioning in cognition can be investigated with this model given the mRNA reduction present in the PFC. The PFC itself is important for processes

such as executive function which includes attention, working memory, flexibility and planning. Additionally a reduction in the PFC may influence functioning other cortical areas, for example M1 might be affected given the motor learning phenotype, or downstream regions and systems. For example abnormal innervation of nucleus accumbens from the PFC may play a role in hyperlocomotive phenotypes which are considered as proxy measures of psychosis in rodents. Analysis of mRNA and protein expression in these other regions, such as the motor cortex, would be important to dissociate whether effects are localised, or the result of this potential upstream reduction.

Additionally, as discussed previously if *Dlg2*<sup>-/-</sup> mice still have weak expression of mRNA this suggests the presence of transcripts not affected by the mutation. Alternative transcripts of *Dlg2* are proposed to have different functional importance, therefore only certain processes may be if some transcripts are still functionally expressed, potentially complicating interpretation of phenotypes. Future studies may benefit from functionally inactivating specific transcripts to determine the relative importance of each to different aspects of behaviour.

### 3.4.7 Conclusions

Overall, the mouse  $Dlg2^{+/}$  model investigated in this Chapter was useful for exploring impairment in behavioural domains as a result of Dlg2 mutation. In general  $Dlg2^{+/-}$  mice are comparable to WT mice and are not universally impaired. This is in line with previous research, that found basic learning process, such as acquisition of simple operant conditioning tasks, were intact in mice and humans with Dlg2 mutations (Nithianantharajah *et al.*, 2013). This lack of global impairment in the mice mirrors the exhibition of more specific impairments, rather than a complete inability to function, that is often seen in humans carrying CNV mutations, Nevertheless, selective functional impairments were measured in this  $Dlg2^{tm1a(EUCOMM)Wtsi}$  derived model, in motor learning and in acoustic startle responses. Both behavioural phenotypes observed in the mutant model are similar to abnormalities associated with schizophrenia, although neither are most often associated with the disorder.

# 4 Fast and slow motor learning and psychomotor challenge in the Dlg2+/- mouse

## 4.1 Introduction

### 4.1.1 Motor Learning

In Chapter 3 *Dlg2*<sup>+/-</sup> mice demonstrated specific impairment in motor learning, but not motor function, and reduced acoustic startle response to a 120 dB stimulus. Whilst WT mice demonstrated a rapid increase to asymptote in motor performance, as assessed by the latency to fall from the accelerating rotarod between the first and second trials the *Dlg2*<sup>+/-</sup> mutant mice did not. *Dlg2*<sup>+/-</sup> mice matched the performance of WT mice by Trial 4. This indicates an impairment of motor learning in *Dlg2*<sup>+/-</sup>. This deficit appears independent of motor function, as fixed speed testing on the rotarod did not reveal a performance difference to WT.

A more challenging accelerating rotarod protocol can further probe this learning phenotype, including examining improvement across multiple days, which could also highlight potential deficits in acquisition of motor learning. Another mouse model carrying a mutation in a schizophrenia risk associated synaptic gene, *Cyfip1*, demonstrated impairment of motor learning during an extended motor learning task. Male, but not female, *Cyfip1*<sup>+/-</sup>mice exhibited reduced latency to fall and plateaued much earlier, never matching the performance of the WT mice across seven trials (Bachmann *et al.*, 2019).

Rotarod training provides a simple paradigm in which to assess motor learning. The process of acquiring a new motor skill, "motor learning", occurs in two phases. During the first session there is a rapid increase in ability, the "fast" phase of motor learning, with "slower" improvement across subsequent sessions before reaching a plateau (Karni *et al.*, 1998; Costa, Cohen and Nicolelis, 2004; Luft and Buitrago, 2005). Between-session learning is dependent on protein synthesis selectively in the motor cortex, which if inhibited prevents slow learning (Buitrago et al. 2004; Luft et al. 2004).

The key regions involved in motor learning are the cerebellum, motor cortex (M1 and M2) and dorsal striatum (Costa, Cohen and Nicolelis, 2004). The involvement of the regions changes over the skill acquisition process (Costa, Cohen and Nicolelis, 2004).

In both the motor cortex and the striatum a dramatic increase in the activity of task related neurons was observed during the first trial on an accelerating rotarod. Distinct changes were observed in the motor cortex and striatum as rotarod training progressed. In the motor cortex, more neurons increased firing versus decreased firing, whereas an increase in velocity-correlated neurons was found in the striatum (Costa, Cohen and Nicolelis, 2004). The activity of neuronal ensembles become less pronounced across the training days, which is reflective of the reduction observed in performance improvement in behavioural studies.

After rotarod training an increase in c-Fos positive cells was observed in the dorsal striatum, cerebellum and motor cortex of WT mice (Hirata *et al.*, 2016). The immediate-early gene *cFos* encodes the transcription factor cFos, the expression of which peaks early in learning, and remains elevated during the plateau phase in the motor cortex following motor learning (Kleim *et al.*, 1996). Expression of cFos is used as an indirect measure of neuronal activity, and therefore can be analysed to compare motor learning at the molecular level (Kovács, 2008).

In the motor cortex M1 is primarily referred to in relation to motor learning, and is involved in both fast and slow learning (Costa, Cohen and Nicolelis, 2004). M2 is also vital to the process. Inactivation of M2 in mice did not prevent performance of a rotarod task but did inhibit improvement of stepping pattern (Cao et al. 2015).

Impairments of motor learning have been demonstrated in people with psychiatric disorders, primarily autism and schizophrenia. Differences in blood oxygenation level-dependant (BOLD) signal responses in an important region involved in human motor circuity, the premotor area, were found between schizophrenia patients and controls prior to, and following training of a complex motor task (Kodama *et al.*, 2017). It was proposed that schizophrenia patients exhibited a dysfunction of the neural networks involved during learning and executing complex motor tasks, and that motor learning in the patients was slower, or less efficient (Kodama *et al.*, 2017). Deficits in motor learning and delays in ability improvement may also be predictive of later development of psychosis (Isohanni *et al.*, 2001).

### 4.1.2 Ketamine induced hyperlocomotion

Hyperlocomotion, especially in response to drugs like ketamine or amphetamine, is considered a proxy for studying psychosis. The finding that ketamine, and other NMDAR antagonists like PCP and MK801, transiently induced psychotic symptoms, such as hallucinations, and cognitive deficits in humans formed the basis of the glutamatergic hypothesis of schizophrenia (Lahti *et al.*, 1995; Javitt, 2010). It is

important to note, however, that the hallucinations induced by ketamine tend to be visual in nature as opposed to auditory (Javitt, 2007; Powers *et al.*, 2015).. This pattern is more reminiscent of that seen in acute schizophrenia rather than chronic, established schizophrenia, and may be due to the promiscuity of ketamine (Javitt, 2007). This promiscuity as delineates the glutamatergic hypothesis of schizophrenia (discussed in 1.2) from the ketamine model, despite the glutamatergic hypothesis originally being proposed due the effects of ketamine (Frohlich and Van Horn, 2014). Ketamine has a weak affinity for serotonin 5-HT<sub>2A</sub> receptors, and a proposed equal affinity for dopaminergic  $D_2$  receptors compared to NMDA although this is continuous due to a lack of replication (Kapur and Seeman, 2002).

A single 40 minute infusion with a sub-anaesthetic dose of ketamine (0.5mg/kg) transiently induced psychotic symptoms in healthy people (Krystal, 1994). A single sub-anaesthetic dose of ketamine is sufficient to induce hyperlocomotion in rodents (Hakami *et al.*, 2009), and is associated with increased dopamine release in multiple brain regions in rodents (Kokkinou, Ashok and Howes, 2018). The glutamate hypothesis postulates that the aberrant dopaminergic functioning observed in schizophrenia is downstream of glutamatergic abnormality. NMDA receptor hypofunction is potentially implicated in this process (Roberts *et al.*, 2010).

Ketamine is a non-competitive NMDAR antagonist (Anis *et al.*, 1983) which acts through channel blocking (MacDonald, Miljkovic and Pennefather, 1987). It is less potent the other antagonists like PCP or MK801 due to faster dissociation from the channel (Johnson & Kotermanski 2006). Ketamine is does not act specifically on any particular NMDAR subunits, but there are indications of an increased potency (3 or 4 fold) for NMDARs expressing GluN1/GluN2c, which are preferentially expressed on GABAergic interneurons (Khlestova *et al.*, 2016). This was found in the presence, but not absence, of extracellular Mg<sup>2+</sup>, implying this increase is due to interactions between the channel pore, ketamine and Mg<sup>2+</sup> rather than differential subunit affinity (Johnson & Kotermanksi, 2006). The subunits may also account for different effects of ketamine, for example Glun2D KO mice demonstrated attenuation of ketamine induced hyperlocomotion (Yamamoto *et al.*, 2016).

There are two non-mutually exclusive proposed mechanisms of action through which ketamine is thought to act and increase glutamate levels: the indirect and direct hypothesis. The indirect hypothesis postulates that as ketamine preferentially binds to GluN1/Glun2c containing NMDAR expressed on interneurons it has a disinhibitory effect, resulting in enhanced excitatory pyramidal neuron activity, thus increasing

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glutamate (Miller, Moran and Hall, 2016). This is supported by the finding that NMDAR antagonists decrease GABAergic interneuron function, leading to increased pyramidal cell firing and excitation of dopaminergic neurons, inducing excessive glutamate release (Homayoun, Jackson and Moghaddam, 2005). The direct hypothesis proposes direct inhibition of excitatory pyramidal neuron NMDAR, resulting in protein-synthesis dependant and cell autonomous homeostatic plasticity, although this pathway may be more involved in the antidepressant effect of ketamine (Miller, Moran and Hall, 2016).

The effects of ketamine on the *Dlg2*<sup>+/-</sup> model may be particularly interesting given the interaction between *Dlg2* and the NMDA receptor. Mutation in *Dlg2* may reduce or destabilise NMDAR at the synapse, therefore further stressing a dysregulated pathway with NMDAR antagonism may reveal functional impairments. Previously NMDAR KO mice were found to exhibit a hyperlocomotive phenotype (Mohn *et al.*, 1999; Yasuda *et al.*, 2017).

In primary hippocampal cultures siRNA knockdown of *Dlg2* (~69% reduction) induced hyperactivity in MEA recordings (MacLaren et al. 2011). Hippocampal hyperactivity is proposed to mediate dopaminergic dysfunction (Lodge and Grace, 2007; Wolff *et al.*, 2018). Increased output from the ventral hippocampus can influence dopaminergic activity in the ventral tegmental area (VTA) and increase dopamine release in the nucleus accumbens (NAc) (Lodge and Grace, 2007; Perez and Lodge, 2013). Dopaminergic neurons in the nucleus accumbens have been implicated in mediation of ketamine induced hyperlocomotion in mice (Irifune, Shimizu and Nomoto, 1991), and D2 receptors in the NAc regulate spontaneous locomotion (Hauber and Münkle, 1997). Therefore if *in vitro Dlg2* knockdown is triggering hippocampal hyperactivity these downstream effects may also occur *in vivo*, and may be reflected in a hyperactivity response. Additionally, the *Dlg2*<sup>+-</sup> mice may exhibit a greater hyperlocomotion response to ketamine, or respond at a lower dose than the WT mice.

### 4.1.3 Aims

1. Measure acquisition of fast and slow motor learning across a three day accelerating rotatod protocol.

2. Investigate differences in the activity of M1 between WT and  $Dlg2^{+/-}$  mice during rotarod training by quantifying the expression of the immediate early gene cFos. It is predicted that  $Dlg2^{+/-}$  mice will exhibit reduced expression of cFos after rotarod training compared to WT mice.

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3. Determine whether the observed motor learning deficits are sex specific by conducting the three day rotarod protocol with female WT and  $Dlg2^{+/-}$  mice.

4. Probe altered NMDA receptor function in  $Dlg2^{+/-}$  with a ketamine induced hyperlocomotion challenge.  $Dlg2^{+/-}$  mice are predicted to be hypersensitive to ketamine, and exhibit hyperlocomotion at a lower dose or to a greater extent than WT mice.

## 4.2 Methods

## 4.2.1 Experiment 1 Motor Learning in DIg2<sup>+/-</sup> mice: Animals

A cohort of 35 male mice (18 WT, 17 HET) were aged up to 3 months and housed the same as described previously 2.1.2. Mice were handled daily for a week to habituate to the experimenter prior to testing. All testing was conducted during the light phase (07:00-19:00) between 09:00 and 17:30).

# 4.2.1.1 Experiment 1 Task

Mice were habituated to the testing room for 5 minutes prior to their first trial. All animals completed 7 trials per day on 3 consecutive days. Mice were placed on the rod facing away from the experimenter whilst the rod was rotating at 4 rpm. The rod accelerated incrementally from 4-40 rpm over 5 minutes. During the 5 minute intertrial interval mice were returned to their home cage in the testing room. Latency (s) to first fall was recorded defined as either falling from the rod or one complete rotation of the rod. Animals were returned to their home cages following the last trial. Apparatus was cleaned with ethanol wipes between animals to mask odour cues.

# 4.2.1.2 Experiment 1 Analysis

Normality was assessed for all data using Shapiro-Wilks test. Where appropriate, transformations were attempted to correct to normality. Extreme outliers ( $\pm$ 3 studentized residuals) were removed. If significance is changed by outliers being retained this was reported. For ANOVA homogeneity of covariances was assessed by Box's M test and homogeneity of variances was assessed by Levene's test. In instances where the assumption of sphericity was violated the Greenhouse-Geisser correction was applied. For *t* test Levene's test was used, and if violated then Welch's *t* test is used.

Latency to fall (s) was analysed across the 7 trials on each of the 3 training days between the genotypes using mixed ANOVA with the following factors: Genotype (WT and HET), Day (1, 2, 3) and Trial (1, 2, 3, 4, 5, 6, 7).

Consolidation of motor learning was then analysed by comparing the difference in latency to fall between the genotypes for the last trail on Day 1 to the first trial on Day 2 (D2T1/(D2T1+D1T7) using *t* test.

# 4.2.2 Experiment 2 investigating the cellular basis of impaired motor learning: Animals

A cohort of 24 male mice (13 WT, 11 HET) were aged up to 3 months and housed as described previously 2.1.2. Mice were handled daily for a week to habituate to the experimenter prior to testing. All testing was conducted during the light phase (07:00-19:00) between 09:00 and 17:30).

# 4.2.2.1 Experiment 2 Task

Seven male mice (4 WT, 3 HET) were used as control animals and were single caged in the holding room for 1.5 hrs. Sixteen male mice (8 WT, 8 HET) were single caged prior to testing. All behavioural mice were habituated to the experimental room for 5 minutes prior to their first trial. All mice completed two trials, with the rod accelerating from 4-40 rpm over 5 minutes, and two inter-trial rest periods for 5 minutes. Mice were returned to their holding cage following their first fall, or full rotation of the rod, and for each rest period. Mice were returned to the holding room after completion of the second rest period for 1.5 hrs before sacrifice. The order in which mice underwent rotarod trained was counterbalanced for genotype.

# 4.2.2.2 Experiment 2 Perfusion fixation

Mice were sacrificed through IP administration of 0.1ml Euthatal and transcardially perfused with 1x PBS and 4% PFA (Sigma-Aldrich, Dorset, UK) as previously described 2.1.5. Brains were removed and post fixed for 24 hrs in 4% PFA at 4°C and cryopreserved in 30% (w/v) sucrose solution at 4°C. Brains were embedded in OCT (ThermoFisher Scientific, UK) and stored at -80°C.

# 4.2.2.3 Experiment 2 Immunohistochemistry

Sectioning for immunohistochemistry was conducted as previously described 2.3.2.1. Briefly brains were sectioned coronally in a counterbalanced manner using a cryostat. Sectioning commenced at the emergence of M1 (approx. bregma 2.3 to 2.2). Free floating 40  $\mu$ m sections were taken in 1:10 series, totalling 70 sections per animal, and stored in 500  $\mu$ l 1x PBS at 4°C.

Immunohistochemistry was conducted as previously described 2.3.2. Sections were blocked in 500  $\mu$ I 1% PBST with 3% normal donkey serum (S30-100ML, Millipore, Hertfordshire, UK) at room temperature with agitation for 2 hours. Rabbit anti-cFos (1:5000, Merck Millipore, Hertfordshire, UK) primary antibody was diluted in 500  $\mu$ I 0.1% PBST (v/v) with 0.2% normal donkey serum (v/v) and incubated overnight with

agitation at 4°C. Sections were then washed for 10 minutes 3 times in 1x PBS. Alexa Fluor® (ThermoFisher Scientific, UK) secondary antibodies were diluted (1:1000) in 500  $\mu$ l 0.1% PBST with 0.2% normal donkey serum. Sections were protected from light and incubated at room temperature with agitation for 2 hours. Sections were washed for 10 minutes 3 times, and then incubated with DAPI (1:1000, D9542-10MG, Sigma-Aldrich, Dorset, UK) in 500  $\mu$ l 1x PBS at room temperature with agitation for 5 minutes. Sections were mounted in a counterbalanced manner and 20  $\mu$ l Mowiol® (4-88, Sigma-Aldrich, Dorset, UK) added per slide and sealed with glass coverslips. Sections were stored at 4°C.

# 4.2.2.4 Experiment 2 Imaging and Counting

M1 was imaged in each stained section using a Zeiss confocal microscope running Zeiss Blue software at 20X magnification. Exposure time was constant between sections. A 3 x 3 tile scan of M1 was imaged as a Z stack and analysis was conducted in Fiji (1.52g). Cells were counted as an average intensity projection from the central focal plane and 2 adjacent focal planes in either direction, with a set interval of 1µm between planes. The area of M1 (Franklin and Paxinos, 2012) was measured using the freehand tool and the number of cFos<sup>+</sup> cells were manually counted. The number of cFos<sup>+</sup> cells/mm2 was calculated by dividing the number of counted cells by the area measured.

## 4.2.2.5 Experiment 2 Analysis

Both behavioural and molecular analysis was conducted as described in Experiment 1 (4.2.1.2).

Behaviourally the latency to fall (s) between trial 1 and 2 was compared between the genotypes using mixed ANOVA. Molecularly the number of cFos<sup>+</sup> cells/mm2 was compared between the genotypes using two-way ANOVA.

### 4.2.3 Experiment 3 Motor Learning in Female Dlg2<sup>+/-</sup> mice: Animals

A cohort of 21 female mice (12 WT, 9 HET) were aged up to 3 months and housed as described previously (2.1.2). Mice were handled daily for a week to habituate to the experimenter prior to testing. All testing was conducted during the light phase (07:00-19:00) between 09:00 and 17:30).

## 4.2.3.1 Experiment 3 Task

The experiment was conducted as described in 4.2.1.1 with the addition of daily oestrus swabbing after testing was competed to enable confirmation of oestrus cycle stage.

## 4.2.3.2 Experiment 3 Task Analysis

Normality was assessed for all data using Shapiro-Wilks test. Where appropriate, transformations were attempted to correct to normality. Extreme outliers ( $\pm$ 3 studentized residuals) were removed. If significance is changed by outliers being retained this was reported. For ANOVA homogeneity of covariances was assessed by Box's M test and homogeneity of variances was assessed by Levene's test. In instances where the assumption of sphericity was violated the Greenhouse-Geisser correction was applied. For *t* test Levene's test was used, and if violated then Welch's *t* test is used.

The potential influence of the oestrus cycle on the latency to fall (s) for 7 trials was analysed for each day separately using repeated measures ANOVA.

Latency to fall (s) was analysed across the 7 trials on each of the 3 training days between the genotypes using mixed ANOVA with the following factors: Genotype (WT and HET), Day (1, 2, 3) and Trial (1, 2, 3, 4, 5, 6, 7).

## 4.2.4 Experiment 4 Ketamine induced hyperlocomotion: Animals and Drugs

Following completion of testing on the rotarod the cohort previously tested in Experiment 1 (4.2.1) underwent a ketamine challenge study. Male mice were scruffed daily for 3 days prior to testing to mimic IP injection to habituate them to handling. Ketamine hydrochloride (Ketavet®, Zoetis, UK) was stored, recorded and disposed of in accordance with ASPA 1986. Ketamine solution was made fresh daily prior to testing in a laminar flow hood using filtered, sterile 1x PBS as previously described 2.1.4. All testing was conducted during the light phase (07:00-19:00) between 09:30 and 16:30. Mice were monitored closely following ketamine injection. One WT animal died during the first day of injection. Throughout the course of the study the experimenter took over cage cleaning to limit stress.

### 4.2.4.1 Experiment 4 Task

Mice were habituated to the locomotor boxes in the dark for 2 hrs daily for 5 consecutive days. Prior to testing mice were singly caged and habituated to an adjacent dark behaviour room for 30 minutes. Mice were assigned an activity box

which remained consistent throughout the experiment, as well as session time (09:30-11:30, 12:00-14:00, 14:30-16:30). The groups were counterbalanced for genotype and drug administration. Testing was conducted in clear perspex boxes with beam breaks as the activity measure (described in Chapter 3). Once finished animals were then returned to their home cages and locomotor boxes cleaned with ethanol wipes.

After 5 daily habituation sessions the drug administration schedule began. Mice were singly caged and habituated for 30 minutes in a dark adjacent behaviour room. Mice received 0.1ml/10g of either ketamine hydrochloride (Ketavet®, Zoetis, UK) or saline via IP (eg a 30g mouse received 300µl IP). Ketamine doses used were 5mg/kg, 10mg/kg and 20mg/kg. A cross-over design was implemented such that each mouse received both saline and ketamine at each dose used. This was counterbalanced by genotype for initial injection (SAL or KET), and this order was maintained for subsequent doses. Mice were injected in an adjacent testing room, away from the other animals. Injected mice were transferred to the testing room. When all mice had been injected they were transferred to their designated activity box. Following two hours of testing, mice were returned to their home cages and activity boxes were cleaned with ethanol wipes. Mice were given 1 week wash out between injections.

## 4.2.4.2 Experiment 4 Analysis

Normality of data was assessed as described in Experiment 1 (4.2.1.2).

For the habituation sessions the total daily beam breaks across the 5 daily sessions was analysed by mixed ANOVA. The ratio change in activity between the total beam breaks on day 1 and day 5 (Day 5/(Day 1 + Day 5) was compared between the genotypes using unpaired *t* test.

Daily beam break data was then binned into four 30 minute quantiles, which was then compared across the days between the genotypes using mixed ANOVA with the following factors: GENOTYPE (WT and HET), DAY (1, 2, 3, 4, 5) and QUANTILE (1, 2, 3, 4).

To analyse the effect of ketamine injection on locomotion the total beam breaks across each 2 hr session was compared between the genotypes for each dose (0, 5mg/kg, 10mg/kg, 20mg/kg) using mixed ANOVA. For 0 the mean of all saline trials was used.

Total activity was then binned into four 30 minute quantiles and compared between the genotypes at each dose separately using mixed ANOVA. The change in total activity between during the first quantile of the last habituation day and each dose was then compared (Dose Quantile 1/(Hab Day 5 Quantile 1+Dose Quantile 1) between the genotypes using mixed ANOVA.

### 4.3 Results

### 4.3.1 Experiment 1: Male Dlg2<sup>+/-</sup> exhibit impaired motor learning across 3 days

The latency to fall for 7 trials across 3 days was analysed using mixed ANOVA with the following factors: GENOTYPE (WT and HET), DAY (1, 2, 3) and TRIAL (1, 2, 3, 4, 5, 6, 7). Most data was non-normal but reflect and logarithmic transformation did not correct normality so the data is reported untransformed. Five outliers were removed (4 WT, 1 HET). With the exception of Day 2 Trial 2, 5, 6, 7, and Day 3 Trial 2, 3, 4 and 7 there was homogeneity of variances. The assumption of sphericity was met for TRIAL, but not DAY or DAY\*TRIAL, X<sup>2</sup> (20) 10.701, p = 0.954, X<sup>2</sup> (20) 10.701, p = 0.954, X<sup>2</sup> (20) 10.701, p = 0.954, so the Greenhouse-Geisser correction was applied.

There was an effect of day, trial and genotype on latency to fall (DAY:  $F_{(1.594, 44.630)}$  147.891, p = <0.001, TRIAL:  $F_{(6, 168)}$  16.713, p = <0.001, GENOTYPE:  $F_{1, 28}$  12.059, p = 0.002) (Figure 26). Genotype did not interact with any other factor, and there was a day\*trial interaction but no day\*trial\*genotype interaction (DAY X GENOTYPE:  $F_{1.594, 44.630}$  0.437, p = 0.604, TRIAL X GENOTYPE:  $F_{(6, 168)}$  0.711, p 0.626, DAY X TRIAL:  $F_{6.664, 186.591}$  7.255, p = <0.001, DAY X TRIAL X GENOTYPE:  $F_{6.664, 186.591}$  0.968, p = 0.454).



Figure 26. Both genotypes demonstrate increased latency to fall (s) from the rotarod during 7 trials over 3 days. However a genotypic difference is observed across the days. Dlg2<sup>+/-</sup> mice exhibit impaired motor learning, with a protracted period of a shorter latency to fall (a). This deficit, whilst less pronounced, persists onto day 2 (b) and 3 (c). Data represent the mean  $\pm$  SEM error bars. n = 14 (WT), 16 (HET). \*\* p = 0.01

To understand the DAY\*TRIAL interaction RM ANOVA was conducted for each day separately. As genotype was not involved in any interactions it was not included as a factor. The assumption of sphericity was met for day 1 and 2, but not day 3 so the Greenhouse-Geisser correction was applied,  $X^2$  (20) 18.353, p = 0.567,  $X^2$  (20) 29.507, p = 0.080 and  $X^2$  (20) 40.568, p = 0.004 respectively. Bonferroni corrected pairwise comparisons were conducted where appropriate.

On each day there was an effect of trial (DAY 1:  $F_{(6, 174)}$  15.227, p = <0.001, DAY 2:  $F_{(6, 174)}$  2.785, p = 0.013, DAY 3:  $F_{(4.178, 121.169)}$  2.745, p = 0.030). On day 1 later trials were different to earlier trials, with no difference in trials from trial 3 onwards. On day 2 the only differences were between trial 1 and 5 and 1 and 7. On day 3 the only differences were between trial 1 and 4 and 1 and 6. In summary both genotypes exhibit rapid within session improvement on Day 1, with slower increases observed on Days 2 and 3 before reaching asymptotic performance. Across all days the HET mice demonstrate impairment improvement, which is most pronounced on Day 1.

Consolidation of learning was analysed by t test, comparing the difference in latency between trial 7 on Day 1 and trial 1 on Day 2 between the genotypes (D2 Trial 1/(D1 Trial7+D2 Trial1). All data was normal. For consistency the same outliers were removed as for ANOVA analysis. Inclusion of the outliers did not change significance. There was homogeneity of variances.

Both genotypes demonstrate comparable consolidation of motor learning between the first and second day of the rotarod protocol,  $t_{(28)}$ -1.146, p = 0.261 (Figure 27).



Figure 27. Both genotypes exhibit consolidation of latency to fall from the rotarod between the last trial of day one and first trial of day two, maintaining a similar performance level between the days. Data represent the mean  $\pm$  SEM error bars. n = 14 (WT), 16 (HET).

# 4.3.2 Experiment 2 Investigating the cellular basis of the motor learning deficit in Dlg2<sup>+/-</sup> mice

In Experiment 1 the greatest difference in latency between the genotypes is between the second and third trials on the first day (Figure 26). Therefore, in order to investigate the molecular basis of the deficit observed in  $Dlg2^{+/-}$  mice, rotarod trained mice were sacrificed 90 minutes after completing two trials and inter-trial intervals on an accelerating rotarod. With the exception of T1 WT all data was normal. Removal of 1 outlier (WT) corrected normality. There was homogeneity of covariances, and of variances for T1 but not T2. The latency to fall (s) was greater for trial 2 than trial 1 for both genotypes (TRIAL:  $F_{(1, 13)} 6.602$ , p = 0.023, GENOTYPE:  $F_{(1, 13)} 0.215$ , p = 0.624, TRIAL X GENOTYPE:  $F_{(1, 13)} 1.602$ , p = 0.228) (Figure 28).



Figure 28. The training cohort of mice underwent two trials on an accelerating rotarod prior to sacrifice for cellular investigation. Both genotypes demonstrate an improvement in the latency to fall (s) between the first and second trial of the task. Data represent the mean  $\pm$  SEM error bars. n = 7 (WT) 8 (HET).

The number of cFos<sup>+</sup> cells/mm<sup>2</sup> in M1 was compared between the genotypes by twoway ANOVA. WT control and rotarod tested HET data was non-normal. Squareroot transformation failed to correct normality, and did not alter significance, so data is reported untransformed. There was homogeneity of variances.

There was no effect of condition (behaviour or control) of the number of cFos<sup>+</sup> cells/mm<sup>2</sup>, nor of genotype, and no interaction between these factors (CONDITION:  $F_{(1, 16)} 3.194$ , p = 0.093, GENOTYPE:  $F_{(1, 16)} 3.760$ , p = 0.070, CONDITION X GENOTYPE:  $F_{(1, 16)} 3.541$ , p = 0.078) (Figure 29).

Given the *a priori* hypothesis that  $Dlg2^{+/-}$  mice would have less activation of cFos<sup>+</sup> cells in M1 after rotarod training the number of cFos<sup>+</sup> cells were compared between control and rotarod conditions using *t* tests for each genotype separately. WT rotarod and HET control data were normal, but WT control and HET rotarod were not, therefore a Mann Whitney U was conducted instead. There was an increase in cFos<sup>+</sup> cells in M1 of WT mice following rotarod training compared to controls, U = 12.000, p = 0.038, but no increase was observed in  $Dlg2^{+/-}$  mice, U = 38.00, p = 0.909.

In summary there is a trend indicating reduced neuronal function of M1 in *Dlg2*<sup>+/-</sup> mice during the rapid learning phase of rotarod training.



Figure 29. There is little cFos expression in M1 of control mice of both genotypes (a & b). A trend (p = 0.078) towards reduced cFos<sup>+</sup> expression in M1 of Dlg2<sup>+/-</sup> mice (d) compared to WT mice (c) was found following rotarod training. Data represents mean  $\pm$  SEM. n = 4 (WT CON), 6 (WT ROTA), 3 (HET CON), 7 (HET ROTA). Scale bar measures

200 μm. The nuclei of cells is stained blue (DAPI) and green puncta are cFos<sup>+</sup> cells. White arrows indicate examples of cFos+ cells.

## 4.3.3 Experiment 3 Motor Learning in Female Dlg2<sup>+/-</sup> mice

For the analysis of oestrus cycle stage most data was non-normal but was not corrected by reflect and logarithmic transformation, so the data is reported untransformed. One outlier (WT) was removed.

The latency to fall (s) was then compared for each day between the genotypes using mixed ANOVA. Most data was non-normal but reflect and logarithmic transformation did not correct normality so data is reported untransformed. The same outlier removed in oestrus analysis was removed. With the exception of Day 3 Trial 6 there was no homogeneity of variances.

On day 1 there was no effect of trial or oestrus and no effect between the two (TRIAL:  $F_{(6,96)} 8.819$ , p = <0.001, OESTRUS:  $F_{(1,16)} 0.236$ , p = 0.792, TRIAL X OESTRUS:  $F_{(12,96)} 1.733$ , p = 0.071). For day 2 there is an effect of trial but no effect of oestrus or an interaction between the factors (TRIAL:  $F_{(3.666,54.993)} 2.702$ , p = 0.044, OESTRUS:  $F_{(1,15)} 0.954$ , p = 0.435, TRIAL X OESTRUS:  $F_{(10.999,54.993)} 1.090$ , p = 0.386). For day 3 there was an effect of trial but not oestrus and no interaction between the factors (TRIAL:  $F_{(2.791,41.860)} 0.808$ , p = 0.489, OESTRUS:  $F_{(1,15)} 0.782$ , p = 0.522, TRIAL X OESTRUS:  $F_{(1,15)} 0.782$ , p = 0

As oestrus had no effect on any day it was not included in further analysis. The latency to fall (s) was compared between the genotypes for 7 trials across the 3 days using mixed ANOVA with the following factors: genotype (WT and HET), day (1, 2, 3) and trial (1, 2, 3, 4, 5, 6, 7). The assumption of sphericity was met for day and trial, but violated for day\*trial, X2 (2) 2.513, p = 0.285, X2 (20) 31.238, p = 0.056 and X2 (77) 114.200, p = 0.008 respectively, so the Greenhouse-Geisser correction was applied in this instance.

There was an effect of day and trial, but not of genotype (DAY:  $F_{(2, 36)}$  64.348, p = <0.001, TRIAL:  $F_{6, 108}$  9.027, p = <0.001, GENOTYPE:  $F_{1, 18}$  1.077, p = 0.313) (Figure 30). There were no interactions between any factors (DAY X GENOTYPE:  $F_{(2, 36)}$  0.040, p = 0.960, TRIAL X GENOTYPE:  $F_{(6, 108)}$  0.850, p = 0.534, DAY X TRIAL:  $F_{(5.809, 104.561)}$  1.852, p = 0.098, DAY X TRIAL X GENOTYPE:  $F_{(5.809, 104.561)}$  1.181, p = 0.323). Bonferroni corrected pairwise comparisons determined each day different from the other, and that earlier trials differed from later trials.



Figure 30. The latency to fall (s) for both genotypes increased across the 3 days... Female Dlg2<sup>+/-</sup> mice did not exhibit any impairment across the 3 day accelerating rotarod task. (DAY:  $F_{(2, 36)}$  64.348, p = <0.001, TRIAL:  $F_{6, 108}$  9.027, p =<0.001, GENOTYPE:  $F_{1, 18}$  1.077, p = 0.313). See Appendix 1 for full statistical analysis. Data represent the mean  $\pm$  SEM error bars. n = 11 (WT) 9 (HET).

# 4.3.4 Experiment 4: Dlg2<sup>+/-</sup> mice exhibited abnormal intersession habituation of locomotion across 5 daily sessions

The daily total for 5 habituation sessions were compared between the genotypes using mixed ANOVA. All data except Day 1 HET and Day 5 WT were normal. There was homogeneity of variances and covariances. The assumption of sphericity was met X<sup>2</sup> (9) 16.071, p = 0.066. Both genotypes exhibited reduced locomotion across the days (DAY:  $F_{(4, 132)}$  31.229, p = <0.001, GENOTYPE:  $F_{(1, 33)}$  0.229, p = 636, DAY X GENOTYPE:  $F_{(4, 132)}$  2.264, p = 0.066) (Figure 31 a).

The ratio change in activity was calculated from the daily totals of day 1 and 5 and compared between the genotypes using unpaired *t* test. All data was normal and there was homogeneity of variances.  $Dlg2^{+/-}$  mice demonstrated smaller change in activity between the first and last habituation day compared to WT , *t* (33) -2.750, *p* = 0.010 (Figure 31 b) indicating reduced habituation the environment across multiple days.



Figure 31. The daily total beam breaks reduced across the habituation sessions for both genotypes (a).  $Dlg2^{+/}$  mice exhibited reduced habituation compared to WT mice when the activity ratio for daily beam breaks on day 1 and 5 is examined. Data represent the mean  $\pm$  SEM error bars. n = 18 (WT) 17 (HET). \*\* p = 0.01

The beam break data was binned into quantiles for each day, which was analysed using mixed ANOVA with the following factors: GENOTYPE (WT and HET), DAY (1, 2, 3, 4, 5) and QUANTILE (1, 2, 3, 4) (Figure 32). Most data was normal and no outliers were found. There was homogeneity of variances for all data except Day 5 quantile 2 and 3. The assumption of sphericity was met for day, but violated for bin and day\*bin, X<sup>2</sup> (9) 16.071, p = 0.066, X<sup>2</sup> (5) 43.889, p = <0.001, and X<sup>2</sup> (77) 112.574, p = 0.006 respectively, so in those instances Greenhouse-Geisser correction was applied.

There was a difference of day and bin but not genotype (DAY:  $F_{(4, 132)}$  31.266, p = <0.001, BIN:  $F_{(2.006, 66.194)}$  83.879 p = <0.001, GENOTYPE:  $F_{(1, 33)}$  0.229, p = 0.636). There was an interaction between day and bin, but no interaction between genotype and any other factor or a three way day\*bin\*genotype interaction (DAY X GENOTYPE:  $F_{(4, 132)}$  2.265, p = 0.082, BIN x GENOTYPE:  $F_{(2.006, 2.006)}$  0.766, p = 0.469, DAY X BIN X GENOTYPE:  $F_{(7.480, 246.843)}$  0.999, p = 0.435).





To further interrogate the day\*bin interaction repeated measures ANOVA was conducted separately for each day. As there were no two way or three way interactions involving genotype it was not included as a factor. All assumptions were met at each time bin and Bonferroni pairwise comparisons were conducted were appropriate. The assumption of sphericity was violated for day 1 X<sup>2</sup> (5) 16.469, p = 0.006 and so the Greenhouse-Geisser correction was applied. For days 2 to 4 the assumption was met, X<sup>2</sup> (5) 9.236, p = 0.100, X<sup>2</sup> (5) 4.199, p = 0.521, X<sup>2</sup> (5) 7.511, p = 0.185 and X<sup>2</sup> (5) 7.477, p = 0.188. Bonferroni corrected pairwise comparisons were conducted where appropriate.

There was an effect of bin for each day (Table 9). During earlier initial habituation days locomotor activity was generally different the quantiles. By day 3 onwards generally activity was only different in the first quantile compared to others.

In summary *Dlg2<sup>+/-</sup>* mice exhibited comparable within session habituation, consistent with the finding from a previous Chapter (3) but impaired between session habituation.

Day	F
1	$F_{(2.297, 78.096)}$ 99.747, $p = < 0.001$
2	$F_{(3,102)}$ 44.161, $p = < 0.001$
3	$F_{(3, 102)}$ 41.235, $p = < 0.001$
4	$F_{(3, 102)}$ 16.684, $p = < 0.001$
5	<i>F</i> <sub>(3, 102)</sub> 23.397, <i>p</i> = <0.001

Table 9. RM ANOVA conducted on each day separately determined there was an effect of time bin for each habituation session.

# 4.3.5 Experiment 4: Dlg2<sup>+/-</sup> mice do not display a dose response to increasing doses of ketamine

The effect of increasing doses of ketamine on the total number beam breaks over 2 hrs was examined by mixed ANOVA (Figure 33). Data from one WT rat (5mg/kg group) was lost due to a software issue. Most data was normal. Removal of one outlier (HET) corrected normality for all data except 10mg/kg data. There was homogeneity of variances and covariances. The assumption of sphericity was violated,  $X^2$  (5) 16.977, p = 0.005, so the Greenhouse-Geisser correction was applied. The comparison of most interest is between saline and each dose of ketamine, therefore Bonferroni pairwise comparisons were conducted in order to examine this.

There was an effect of dose, but not genotype nor a dose\*genotype interaction (DOSE:  $F_{(2.185, 65.538)}$  9.635, p = < 0.01, GENOTYPE:  $F_{(1, 30)}$  1.385, p = 0.248, DOSE X GENOTYPE:  $F_{(2.185, 65.538)}$  0.722, p = 0.501). The difference in activity from saline for each dose of ketamine was then examined for each genotype using Bonferroni

corrected pairwise comparisons. For WT mice there was a strong trend towards an increase in activity compared to saline for 20mg/kg (p = 0.053) but not for any other dose. For  $Dlg2^{+/-}$  mice no dose changed activity from saline.



Figure 33. Daily total beam break for increasing doses of ketamine. WT mice exhibited a strong trend towards increased activity (p = 0.053) following administration of 20mg/kg ketamine but no other dose. No dose altered activity compared to saline in the Dlg2<sup>+/-</sup> mice. Saline value is average response to saline across all sessions. Data represent the mean  $\pm$  SEM error bars. n = 16 (WT) 16 (HET).

The beam break activity was then binned into four 30 minute quantiles and the effect of each dose of ketamine separately was then compared between the genotypes. One outlier was found consistently for all analysis and removed (HET).

For saline and 5mg/kg all HET and most WT data was normal. Most 10mg/kg and 20mg/kg data was normal. There was homogeneity of variances and covariances in all instances. The assumption of sphericity was violated for saline, X<sub>2</sub> (5) 24.262, p = <0.001, 5mg/kg X<sub>2</sub> (5) 26.819, p = <0.001, 10mg/kg X<sub>2</sub> (5) 51.190, p = <0.001 and 20mg/kg X<sub>2</sub> (5) 83.467, p = <0.001, so the Greenhouse Geisser correction was applied in all instances.

Activity decreased across the quantiles, and HET mice were more active compared to WT following saline injection, but there was no interaction between these factors (QUANTILE:  $F_{(1.889, 90)}$  25.006, p = 25.006, p = <0.001, GENOTYPE:  $F_{(1, 30)}$  5.669, p = 0.024, QUANTILE X GENOTYPE:  $F_{(1.889, 56.660)}$  0.033, p = 0.962) (Figure 34). This was also the case following 5mg/kg ketamine (QUANTILE:  $F_{(1.995, 59.856)}$  37.999, p = 0.024, p = 0
<0.001, GENOTYPE:  $F_{(1,30)}$ 5.870, p = 0.022, QUANTILE X GENOTYPE:  $F_{(1.995, 59.856)}$ 0.602, p = 0.550) (Figure 34).

However, whilst a similar effect of quantile was observed following 10mg/kg ketamine there was no difference in activity between the genotypes (QUANTILE:  $F_{(1.491, 44.722)}$ 25.639, p = <0.001, GENOTYPE:  $F_{(1, 30)}0.051$ , p = 0.823, QUANTILE X GENOTYPE:  $F_{(1.491, 44.722)}2.151$ , p = 0.140) (Figure 34). There was also an effect of quantile for 20mg/kg ketamine but no effect of genotype (QUANTILE:  $F_{1.235, 37.037}$ ) 24.936, p =<0.001, GENOTYPE:  $F_{(1, 30)}0.208$ , p = 0.652, QUANTILE X GENOTYPE:  $F_{(1.235, 37.037)}$ 0.567, p = 0.491) (Figure 34).



Figure 34. There was a difference in activity between the genotypes across the quantiles for saline and 5mg/kg ketamine, but not for 10mg/kg or 20mg/kg ketamine. Data represent the mean  $\pm$  SEM error bars. n = 16 (WT) 16 (HET). \* p = 0.05.

Given the habituation deficit observed in  $Dlg2^{+./-}$  mice the change in total activity during the first quantile on the last day of habituation and the first quantile at each dose was compared between the genotypes using mixed ANOVA. Most data was normal, there was homogeneity of variances and covariances and the assumption of sphericity was met, X<sub>2</sub> (5) 9.232, *p* = 0.100).

There was an effect of dose on the change in activity, but no effect of genotype or interaction between the two factors (DOSE:  $F_{(3, 90)} 6.881$ , p = <0.001, GENOTYPE:  $F_{(1, 30)} 0.000$ , p = 0.988), DOSE X GENOTYPE:  $F_{(3, 90)} 0.132$ , p = 0.941) (Figure 35). Bonferroni corrected pairwise comparison determined only 20mg/kg increased activity compared to saline.



Figure 35. The change in activity during the first quantile between the last habituation day and each dose of ketamine was comparable between the genotypes. Data represent the mean  $\pm$  SEM error bars. n = 16 (WT) 16 (HET)

#### 4.4 Discussion

Using an extended accelerating rotarod protocol the deficit in motor learning previously observed in  $Dlg2^{+/-}$  mice (Chapter 3) was replicated. The  $Dlg2^{+/-}$  mice again exhibited a delay in the increase of the latency to fall (s) compared to WT mice on day one of rotarod training, maintaining a protracted period of a reduced latency to fall. This protocol also facilitated examination of consolidation of learning across the days. This process appears to be intact in the  $Dlg2^{+/-}$  mice, as demonstrated by retention of latency between the final trail of one day and the first trial of the subsequent day. The cellular basis of this phenotype was investigated by comparing cFos<sup>+</sup> cells in M1 between the genotypes in naïve and rotarod tested animals. There was a strong trend towards decreased cFos staining in M1 of rotarod trained  $Dlg2^{+/-}$  mice compared to WT.

Prior to ketamine challenge, mice underwent five daily habituation sessions to the locomotor activity boxes. Whilst initially comparable in their activity level, towards the end of the habituation period they began to diverge, with  $Dlg2^{+/}$  mice demonstrating reduced between session habituation compared to WT mice. Locomotive responses to increasing doses of ketamine was then compared between the genotypes. When compared to activity following saline injection only 20mg/kg ketamine in WT mice showed a trend towards increased activity. No other dose had an effect in WT mice, and no doses had any effect in  $Dlg2^{+/-}$  mice. When the effect of each dose was analysed separately across four 30 minute quantiles  $Dlg2^{+/-}$  mice were more active in response to saline and 5mg/kg ketamine, but were comparable to WT in response to 10mg/kg or 20mg/kg. Again only 20mg/kg resulted in increased activity when the first quantile of each dose was compared to the first quantile of the last habituation day.

# 4.4.1 Dlg2<sup>+/-</sup> mice exhibited a motor learning deficit on a second accelerating rotarod protocol

As in Chapter 3, male *Dlg2*<sup>+/-</sup> mice from this new cohort exhibited delayed acquisition of improvement of the accelerating rotarod. The increased number of trials per day and extending training across3 days in this experiment facilitates analysis of fast and slow motor learning (Buitrago et al. 2004). This contrasts the protocol in Chapter 3, where less trials were conducted per day, and the number of trials conducted on each day was not consistent. Similar to the results in Chapter 3 the findings presented in this Chapter demonstrate a dramatic increase in ability for WT mice is seen early on, in this instance between Trials 2 and 3 on Day 1. These trials are also the point of

greatest difference between the WT and  $Dlg2^{+/-}$  mice. In comparison to WT, mice the  $Dlg2^{+/-}$  exhibited a shallower and more gradual increase in latency.

This replicated motor learning deficit is in line with Winkler (2018), where *Dlg2* KO mice demonstrated a severe impairment on an accelerating rotarod, with a less slightly less severe deficit found in the heterozygous mice. This pattern of impairment is different to that observed in the *CYFIP1* heterozygous mouse, another important synaptic gene associated with psychiatric disorders. *Cyfip1*<sup>+/-</sup> mice demonstrated minimal improvement and an early plateau in latency (Bachmann *et al.*, 2019). This may reflect a differential role or importance of *CYFIP1* or its interactors in motor learning.

By continuing the training across multiple days it was also possible to interrogate the consolidation of the motor memory in the Dlg2<sup>+/-</sup> mice. The Dlg2<sup>+/-</sup> mice exhibit a similar pattern of improvement on Day 2 to the WT mice, but do not reach a similar latency to fall (s). By Day 3 after the first few trials Dlg2<sup>+/-</sup> mice do eventually reach a similar level of plateau to WT mice. There is no difference between the latency to fall on the first trial on Day 2 and the last trial of Day 1 for either genotype, suggesting that consolidation of the motor memory is intact in *Dlg2<sup>+/-</sup>* mice. Despite the delayed motor learning in the *Dlg2<sup>+/-</sup>* mice, the overall increase in latency observed in both genotypes is consistent with the pattern anticipated with the acquisition of a motor skill (Luft and Buitrago, 2005). The period of rapid learning observed on the first day reflects the "fast" phase of motor skill learning, whilst the reduced degree of improvement across subsequent days before performance plateaus demonstrates the "slow" phase (Karni et al., 1998; Costa, Cohen and Nicolelis, 2004; Luft and Buitrago, 2005). The increased number of trials across multiple days facilitated greater increase in latency, as compared to the protocol employed in Chapter 3 where the latency to fall plateaued around 200 s. To further challenge the consolidated motor memory mice could be retested on the accelerating rotarod after a period of time without training (Buitrago et al., 2004). Once a ceiling effect in performance is observed, in this case running for the full 5 minutes, it would be anticipated that this memory would be retained, so that performance following a break from training would be comparable upon retesting.

In Chapter 3 the importance of NMDAR for motor learning was discussed. A *Dlg4* mouse model was highlighted, which was normal except for the PDZ domain, to which *Dlg4* binds (Nagura *et al.*, 2012a). Unlike full *Dlg4* KO mice (Feyder, et al. 2010) this model did not display impaired motor learning, suggesting the interaction between

*Dlg4* and NMDAR was not driving the phenotype, and that this may be reflected in the *Dlg2* model. In addition to NMDAR AMPAR are important for motor learning. Administration of CNQX, an AMPAR antagonist prior to rotarod training drastically impaired performance, as did injection of CNQX following training suggesting AMPAR are also important for maintaining acquired motor skills (Kida *et al.*, 2016). AMPAR were particularly important during the earlier phase of motor learning (Kida *et al.*, 2016), which is when the deficit is seen in *Dlg2<sup>+/-</sup>* mice. Therefore it is possible that abnormal AMPAR functioning in the motor cortex of *Dlg2<sup>+/-</sup>*mice is involved in the motor learning deficit. Conducting electrophysiology after rotarod training would determine whether in the motor cortex *Dlg2<sup>+/-</sup>* mice AMPAR and NMDAR functioning, or the AMPAR:NMDAR ratio, is abnormal following motor learning (Kida *et al.*, 2016).

# 4.4.2 There is a trend towards reduced cFos<sup>+</sup> cells in M1 of Dlg2<sup>+/-</sup> mice following rotarod training

As detailed in Chapter 3 (3.3.1.1) there is no reduction in *Dlg2* mRNA expression in the cerebellum, implying that the molecular basis of the motor learning deficit in male heterozygotes likely lies in the cortical or striatal regions involved in motor learning (Costa, Cohen and Nicolelis, 2004). Due to the importance of M1 in motor learning, particularly during the earlier "rapid" phase, (Costa, Cohen and Nicolelis, 2004). The neuronal activity in M1 following motor learning was investigated by analysis of cFos, an immediate early gene widely employed as a functional anatomical maker of activated neurons (Bullitt, 1990; Kovács, 2008). Whilst this can be investigated following completion of all training sessions (Hirata *et al.*, 2016) it was decided to examine the point of greatest divergence in latency to fall between the genotypes, as  $Dlg2^{+/-}$  mice demonstrate a more comparable latency to WT mice by the end of the session. This point, as discussed previously (4.3.1), is between the second and third trials.

In terms of the behaviour no genotypic difference was observed in the rotarod trained mice which underwent two trials, although the trajectory of the  $Dlg2^{+/-}$  mice does appear shallower between the two trials. When examining neuronal activity with cFos staining as a proxy marker using ANOVA there was no difference between the conditions, and a trend towards a difference between the genotypes (p = 0.07). The lack of a difference between the conditions is likely masked by the lack of change of cFos expression between  $Dlg2^{+/-}$  control and trained mice. However, the *a priori* hypothesis predicted that there would be an increase in cFos in trained WT but not  $Dlg2^{+/-}$  mice, and so *t* tests when the conducted between the conditions for each

genotype. In this instance there was a significant increase in cFos staining in the WT mice but no change between the conditions for  $Dlg2^{+/-}$  mice. This indicates that during the earliest part of the rapid phase of motor learning the normal increase of neuronal activity in M1 is not occurring the in the  $Dlg2^{+/-}$  mice. As for why this difference was not reflected in the ANOVA it might be attributable to the low *n* in this experiment, with a total of 7 control and 13 rotarod trained mice, or the high variation, particularly in the WT rotarod trained mice. Supporting this conclusion a power calculation using JMP's sample size and power tool (JMP 14.2.0) found that at an alpha level of 0.05 there was a power level of 0.52. In order to have a power of 0.8 at the same alpha level a total sample size of 33 would be required (8/ group). Alternatively this may reflect a need to optimise when the rotarod trained mice are sacrificed. For example completing training up trial 4, the last trial before latency to fall begins to converge between the genotypes previously (Chapter 3), may facilitate greater neuronal activation therefore exacerbating any genotypic deficits.

It is possible that there is a difference in cFos staining across the cortical layers that is being masked by counting the whole section. Motor training has previously been demonstrated to induce LTP in Layer 2/3 neurons in M1 (Rioult-Pedotti, Friedman and Donoghue, 1998; Harms *et al.*, 2008), which in turn provide excitatory input to Layer 5a (Masamizu *et al.*, 2014). Layer 2/3 is proposed to represent co-ordination of signals throughout learning whilst Layer 5a may be involved in evolving the network representing these learnt skills (Masamizu *et al.*, 2014). Therefore co-staining with layer markers, such as Ctip2 (layer 5) (Arlotta *et al.*, 2005) or Cux1 (layer 2/4) (Nieto *et al.*, 2004), may elucidate whether there is a layer specific change in *Dlg2*<sup>+/-</sup> mice.

In addition it would be informative to investigate other important regions, such as M2 and the striatum (Costa, Cohen and Nicolelis, 2004). During rotarod training of Arc-GFP mice more Arc promotor-activated neurons were found in M2 compared to M1, and recruitment of these neurons during training was specific to rotarod training, with different patterns of recruitment found following wheel running (Cao et al. 2015). The striatum could be particularly interesting as a regional difference across the phases of learning has been suggested (Yin *et al.*, 2009). Also it has previously been demonstrated that striatal NMDAR KO mice exhibit impaired motor learning (Dang *et al.*, 2006). Given the interaction between Dlg2 and NMDAR this may underlie the motor learning deficit observed, although the phenotype found in the striatal KO was more severe than the one discussed in this Chapter, as the KO mice plateau very early on and did not show any improvement (Dang *et al.*, 2006). The dorsomedial striatum was preferentially engaged in early phases, and the dorsolateral striatum in

later stages of motor learning (Yin *et al.*, 2009). The motor learning deficit in the *Dlg2*<sup>+/-</sup> mice is most apparent early on. Therefore it could be predicted that when cFos staining analysis is conducted on tissue taken early in the motor learning process, as in the case of the reported experiment, that differences in cFos staining would be observed in the dorsomedial but not dorsolateral striatum.

# 4.4.3 Female *Dlg2+/-* mice do not exhibit a delayed motor learning phenotype

Female rodents are often excluded from behavioural testing due to concerns that the stage of oestrus will impact upon the findings. Although there experiment was conducted with a smaller sized cohort there was no effect of oestrus. This is in line with previous findings that across a behavioural battery there was no effect of oestrus in C57BL/6J mice, although an effect was seen in BALBcByJ females (Meziane *et al.*, 2007). In contrast to the male  $Dlg2^{+/-}$  mice (4.4.2) there was no effect on genotype in females on latency to fall across the sessions. Both genotypes of female mice demonstrated improvement in the earlier sessions, which plateaus by the third session, as was observed in WT male mice. Thus, the early motor learning deficit discovered in the  $Dlg2^{+/-}$  mice is sex specific. Whilst there is one study which previously found a severe motor learning impairment in  $Dlg2^{+/-}$  and KO mice it was only conducted in males, therefore this represents the first indication the deficit is sex specific. Interestingly this male specificity was apparent in the motor learning phenotype observed in another important synaptic risk gene for schizophrenia, *Cyfip1*, heterozygous mouse model (Bachmann *et al.*, 2019).

# 4.4.4 *Dlg2*<sup>+/-</sup> mice demonstrate reduced intersession habituation across five days in a novel context

Across the daily two hour habituation sessions both genotypes did demonstrate intersession habituation of total beam breaks across the days. However, when habituation was assessed as the change in activity between Day 1 and Day 5 WT mice demonstrated a greater reduction in activity compared to *Dlg2*<sup>+/-</sup> mice. When examining the habitation across the four 30 minute bins of each daily session both genotypes demonstrate intrasession habituation on each day, although by Day 5 there is a slight divergence.

In contrast to the WT mice, whose total beam break activity is reduced between Day 4 and Day 5, *Dlg2*<sup>+/-</sup> activity actually increases. By Day 5, *Dlg2*<sup>+/-</sup> mice might be exhibiting sensitisation, paying more attention to the stimulus following repeated exposure and increasing, rather than decreasing, responding to it (Sanderson *et al.*,

2011; Barkus *et al.*, 2014a). This suggests that the impairment is due to abnormal habituation, and given that both genotypes were comparable in earlier sessions, and show a similar level of activity at the start of each session, it is not simply a generalised hyperactivity phenotype.

The genotypic difference in habituation on this task is in contrast to the previous experiment examining locomotor activity in the same boxes, described earlier in Chapter 3. Across all aspects of the context shift task there were no differences in beam break activity between the genotypes, in contrast to the divergence observed in this Chapter. Additionally, whilst within session habituation was observed for both genotypes on both tasks, between session habituation was only observed across the 5 days of the ketamine challenge sessions. This may be due to the difference in task length (2 hrs daily in the boxes in this task vs 30 minutes in Chapter 3), or caging arrangements (in this Chapter mice were singled housed prior to testing in preparation for later ketamine injection, whereas they were taken straight from their home cages in Chapter 3). Also whilst both tasks were conducting during the mouse's light phase the testing in this Chapter was conducted in the dark, whereas in Chapter 3 due to the visual aids employed it was conducted in the light.

A sensitisation response, increased rather than decreased response to repeated exposure, was seen in mice lacking AMPAR subunit *Gria1*, which exhibited short term habituation impairments (Sanderson *et al.*, 2011; Barkus *et al.*, 2014a). Given the interaction between AMPAR and *DLG2* it is possible this interaction plays a role in the impaired habituation phenotype observed in the *Dlg2*<sup>+/-</sup> mice. In addition a habituation deficit the *Gria1*<sup>-/-</sup> mice demonstrated 'inappropriate learning' under some conditions; exhibiting long term memory not seen in WT controls. The authors proposed that this process is reflective of aberrant salience; the attribution of importance to environmental stimuli that are normally considered irrelevant (Kapur, 2003c; Howes and Kapur, 2009).

Aberrant salience. like hyperlocomotion, has been attributed the to hyperdopaminergic state observed in schizophrenia (Kapur, 2003c; Howes and Kapur, 2009). This hyperdopaminergic state results in persistent and inappropriate assignment of salience to stimuli, which can lead to formation of inappropriate associations, and is suggested to underlie psychosis (Kapur, 2003c). Stimulus novelty is important in this process as novel stimuli have a higher salience and so demand attentional focus and promote exploration, as well evoking striatal dopamine release (Rebec et al., 1997; Robinson and Wightman, 2004; Clark et al., 2010; Flagel

*et al.*, 2011). The impaired habituation observed in the *Dlg2*<sup>+/-</sup> mice suggests that the context retained its novelty. This failure of habitation has been observed in patients with schizophrenia, who failed to exhibit suppression of BOLD activity in the medial temporal lobe and hippocampus in response to repeated presentation of fearful faces, in comparison to healthy volunteers (Holt *et al.*, 2005). In some patients a sensitisation response was observed, with increased responding after repeated presentation of the stimuli.

Previously a reduction in *Dlg2* mRNA expression *in vitro* was observed to increase activity in primary hippocampal cultures (MacLaren *et al.*, 2011). If this is replicated in mutant *Dlg2 in vivo* models it may contribute to hippocampal over activity, which has been postulated to underlie dysregulation in schizophrenia (Lodge and Grace, 2007; MacLaren *et al.*, 2011). Whilst *Dlg2* mRNA expression was not reduced in the hippocampus (Chapter 3), and so may not be contributing to potential dopaminergic dysfunction directly (Lodge and Grace, 2007), it might be being mediated due to the mRNA reduction observed in the PFC (Chapter 3). Alterations in the PFC-hippocampal-nucleus accumbens axis can result in a hyperdopaminergic state (Belujon, Patton and Grace, 2014). If this phenotype is the result of dopaminergic dysfunction then it might be rescued by treatment with a dopaminergic antagonist, such as haloperidol (Wiedholz *et al.*, 2008).

# 4.4.4 Dlg2<sup>+/-</sup> mice do not exhibit a dose dependant increase in activity following administration of ketamine

Only 20mg/kg ketamine increased activity in the first quantile when compared to the final habituation day, and was the only dose to trend towards increasing activity compared to saline (p = 0.053) in WT mice. No dose altered activity compared to saline in  $Dlg2^{+/-}$  mice. When the first quantile of each dose was analysed separately  $Dlg2^{+/-}$  mice were more active compared to WT mice following saline and 5mg/kg ketamine. However this difference disappeared at the higher doses.

Initially this could be viewed as  $Dlg2^{+/-}$  mice responding differently to WT mice in response to ketamine – exhibiting increased activity at lower doses (5mg/kg) that may then reach a ceiling so there is less effect of higher doses. For example the genotypic difference during the first quantile disappears at 10mg/kg. This could be due to the fact the  $Dlg2^{+/-}$  mice do not appear to increase activity much between 5mg/kg and 10mg/kg in contrast to WT mice, potentially reflecting an effect of ketamine at a lower dose in the heterozygous mice or having already approached an activity increase plateau. However, this conclusion does not take into consideration the altered

baseline activity of the *Dlg2*<sup>+/-</sup> mice. As a result of the lack of habituation the response to saline already exceeds that of the WT mice. It therefore becomes challenging to pick apart these later differences, as to whether they are truly a different response to a specific dose of ketamine, or instead the ketamine is not having much of an effect but the increased activity observed is still a reflection of the higher activity baseline.

To discriminate between these explanations the experiment could be repeated but requiring the mice to reach a criterion point of habituation before progressing onto ketamine administration. This would remove the confound of increased activity due to impaired habituation that may be responsible for the increased activity following saline or 5mg/kg injection, and provide a clearer demonstration of whether ketamine is differentially effecting  $Dlg2^{+/-}$  mice.

Additionally even in WT mice even 20mg/kg only resulted in a strong trend towards increased activity. Therefore it is plausible that to be able to see any major differences a higher dose would be required. As the effect of ketamine had not been previously investigated in this model it was opted to start with the lower doses reported in the literature and work up, in addition to the fact that it was postulated that the  $Dlg2^{+/-}$  mice might have responded at a lower dose than WT mice.

Investigation into the interaction of NMDAR and *Dlg2*, and the potential reduction in NMDAR that could be exacerbated by an NMDAR antagonist, at the molecular level may provide some answers as to whether ketamine would be predicted to have a differential behavioural response. It is possible *Dlg2* mutation does not alter the number of NMDAR, but rather their localisation or internalisation (Zhang et al., 2010a). NMDAR subunit expression was not altered in rats following ketamine injection, but a higher level of *Dlq4* expression was observed in multiple brain regions and greater functional coupling between NMDAR and Dlg4 was indicated by an increased co-immunoprecipitation of the two in vivo (Lisek et al., 2017). This was proposed to reflect increased targeting of NMDAR to the plasma membrane to restore reduced NMDAR-signalling. Additionally altered interactions have been found between *Dlg4* and PMCA4, a calcium efflux pump, following ketamine administration and may play a role in ketamine-mediated effects on calcium signalling (Lisek et al., 2017). Interactions between Dlg4 and ErbB4 were also transiently increased in the PFC of rats following acute administration of MK-801, and Dlg4-ErbB4-NMDAR association was enhanced following chronic treatment (Li et al., 2013). ErbB4 has been associated with schizophrenia, and interacts with Neuregulin, with is also associated with schizophrenia (Norton et al., 2006; Silberberg et al., 2006; Law et al.,

2007). *Dlg2* also interacts with ErbB4, although this interaction has not been examined in post-mortem schizophrenia brains or following NMDAR antagonism. Both studies investigated ketamine's effects in WT rodents, and it is unknown what effect, if any, reduction in *Dlg2* or *Dlg4* expression may have on these interactions after acute or chronic administration.

### 4.4.5 Strengths and limitations

The impaired motor learning phenotype first observed in Chapter 3 was recapitulated using the more extensive protocol described above. Therefore a deficit in this learning process has been observed in two separate cohorts of  $Dlg2^{+/-}$  mice using two different protocols. Whilst this is in contrast to rotarod experiments with another Dlg2 mouse model it does appear to be a consistent phenotype in the current strain.

As described above (4.3.2) the low *n* may explain the lack of statistical difference in the ANOVA for numbers of cFos<sup>+</sup> cells in M1, or a potential need to optimise the point during the learning process targeted for analysis. Whilst the training point targeted may not have been optimal the delay between the behaviour and perfusion captured peak cFos protein expression, between 1.5 to 2 hrs (Kovács, 1998). Despite cFos expression analysis being a useful tool for investigating neuronal activity it is considered a proxy measure (Kovács, 2008). Future investigation could employ two photon imagining to track neuronal assemblies across multiple rotarod training sessions (Cao et al. 2015; Yang et al. 2009).

In attempting to reduce the novelty response of the locomotor boxes prior to ketamine injection we uncovered a habituation deficit, which may arguably reflect a sensitisation phenotype, in  $Dlg2^{+/-}$  mice. This is the second behavioural task where  $Dlg2^{+/-}$  mice have demonstrated impairments of habituation to a stimulus. A previous cohort did not demonstrate habituation to a 120dB acoustic stimulus, although as discussed in Chapter 3 there are multiple possible explanations for this, together these findings do indicate a subtle deficit of learning processes in these mice.

Interpreting the effect of ketamine on activity was confounded by the altered baseline of the *Dlg2*<sup>+/-</sup>mice, likely due to the habituation deficit observed. As discussed earlier re-designing the experiment would address this issue. Additionally, given ketamine acts through antagonism of NMDAR it will be important to investigate whether *Dlg2* mutation results in any difference in number of NMDAR at the synapse, or whether it alters receptor localisation. This has currently not been investigated in this model.

Finally, due to technological constraints there was an uneven lag time between injection of ketamine and the start of activity recording between the mice. Given the shorter window of action of ketamine this may have therefore confounded any potential genotypic differences in response to ketamine during the first 15 minutes. It was not possible to fully counterbalance for this lag in addition to counterbalancing for genotype and injection solution (ketamine or saline) for time of day. Future drug challenge experiments should aim to reduce this lag, starting recording individually following injection as opposed to as a whole group.

# 4.4.6 Conclusions

The early motor learning deficit in *Dlg2*<sup>+/-</sup> mice initially uncovered in Chapter 3 was replicated in the experiments conducted in this Chapter. This deficit began to normalise across multiple days of testing and was found to be sex specific, only being present in the male mice. The investigation into the cellular basis of this phenotype indicates a reduction in neuronal activity in M1 of the motor cortex, although this needs confirming with an increased cohort size. Additionally investigation into other key regions will determine whether M1 is solely important, or whether this dampened activity is widespread.

Additionally, a habituation deficit was uncovered in a second paradigm, having previously been observed in the acoustic startle test in Chapter 3. Reduced habituation implies that there is less plasticity in the synapses in response to repeated stimuli, and may be indicative of abnormal dopaminergic function and attribution of salience to the environment (Kapur, 2003c). Due to this deficit, however, it is difficult to determine whether  $Dlg2^{+/-}$  mice responded differently to increases doses of ketamine as a result of NMDAR functioning abnormality or preceding hyperactivity as a result of impaired habituation.

# 5 Adult Neurogenesis in the Mouse Model

# 5.1 Introduction

Adult neurogenesis (AN) is the generation of new neurons, in the dentate gyrus of the hippocampus and the olfactory bulb, during adulthood (Kempermann *et al.*, 2004). AN occurs across species, declining with age (Galvan and Jin, 2007), although there is still controversy over the process in humans (Eriksson *et al.*, 1998; Spalding *et al.*, 2013; Boldrini *et al.*, 2018; Sorrells *et al.*, 2018). Increasingly adult neurogenesis is has been proposed to underlie some of the cognitive dysfunction in psychiatric diseases including schizophrenia (discussed below in 5.1.1). There is also evidence suggesting abnormalities of AN in rodent models of genetic risk for schizophrenia involving in synaptic functioning may represent a commonly affected pathway (Westacott, 2016; Haan *et al.*, 2018; Moon *et al.*, 2018).

Most relevant to psychiatric disease is AN occurring in the dentate gyrus (DG) of the hippocampus. Only one cell type is generated by the process; DG granule cells (Kempermann *et al.*, 2004). These neurons are the primary excitatory neurons in the DG, receiving input from the entorhinal cortex and projecting along the mossy fibre tract to hippocampal sub region CA3 (Jonas and Lisman, 2014). There are four phases of adult neurogenesis, with six distinctive milestones, based on cellular morphology and expression of certain markers (Figure 36).



Figure 36. Development timeline of adult neurogenesis. The expansion phase encompasses the pool of cells which may differentiate into neurons. Many of those cells born are eliminated before they can progress to the survival phase. During the survival phase the neurons establish functional connections, undergo synaptogenesis and axonal and dendritic growth, which is then fine-tuned. This process takes ~ 7 weeks in mice. The blue box highlights those cells captured when staining using DCX, the primary marker of adult neurogenesis used in rodent studies.

The initial pool of cells, "type 1", are radial glial like cells that divide asymmetrically, producing a "type 2" daughter cell. The "type 2" population can be further subdivided into "type 2 a" and "type 2 b", with only "type 2 b" cells expressing doublecortin (DCX), having become neuronally fated. From these arise neuronally committed neuroblasts, "type 3" cells which are minimally proliferative. Apoptosis dramatically reduces the number of new neurons within a few days of the neurons expressing NeuN (Kuhn *et al.*, 2005), so most neurons are eliminated before they form connections and integrate into the circuitry. This point in the process is where most regulation occurs, ensuring only a small population of the surplus of new neurons generated actually survive (Kempermann, Song and Gage, 2015). The limited number of neurons that become integrated demonstrates that the function of adult neurogenesis is not to replace older cells (Crespo et al 1986). After a further 2-3 weeks the expression of calretinin switches to calbindin.

#### 5.1.1 Adult neurogenesis in psychiatric diseases

In multiple psychiatric diseases abnormal AN has been implicated in phenotypes and treatment response. Increased AN is proposed to influence response to antidepressants, as the latency between treatment onset and observable improvement corresponds to the maturation time course of new-born neurons (Malberg *et al.*, 2000). Antipsychotics may improve the cognitive impairment associated with schizophrenia, and differences observed between these drugs may be associated with their impact on AN (Chikama *et al.*, 2017). Atypical antipsychotics, but not haloperidol, increased BrdU positive cells (Chikama *et al.*, 2017), an effect which may stem from agonism of the 5-HT1A receptor (Schreiber and Newman-Tancredi, 2014).

In addition to moderating response to drugs AN may underlie aspects of psychiatric disease pathology. One of the earlier phenotypes identified in schizophrenia was a reduction in hippocampal volume, using both post mortem analysis (Bogerts, Falkai, Greve, Schneider, & Pfeiffer, 1993; Bogerts, Meertz, & Schönfeldt-Bausch, 1985; Jeste & Lohr, 1989; Nelson et al, 1998) and MRI imaging (Altshuler *et al.*, 2000; Sim *et al.*, 2006; Goldman *et al.*, 2008). This may indirectly reflect a reduced level of adult

neurogenesis (Adriano, Caltagirone and Spalletta, 2012; Walter *et al.*, 2016). In post mortem schizophrenia patient brains a reduction of PSA-NCAM cells, which stains immature neurons, was found (Barbeau *et al.*, 1995). Others found reduced Ki67, which is expressed only in cells in the cell cycle, marking them as potentially proliferative (Reif *et al.*, 2007; Allen, Fung and Shannon Weickert, 2016). However, the majority of these studies are confounded by the high probability the patients had a long history of antipsychotic use, which can affect AN (Walton *et al.*, 2012; Chikama *et al.*, 2017).

Risk factors for psychiatric diseases, both environmental and genetic, have also been found to alter AN. Mutation in DISC1 is associated with risk for developing schizophrenia (Millar *et al.*, 2000), and was found to impair AN (Mao et al., 2009). DiGeorge syndrome chromosomal region 8 (*Dgcr8*), a candidate gene for 22q11.2 deletion is associated with schizophrenia (Van, Boot and Bassett, 2017) and autism (Ousley *et al.*, 2017). In heterozygous *Dgcr8* mice, impaired AN and hippocampal dependant learning was found (Ouchi *et al.*, 2013). Childhood trauma is a major risk factor for many psychiatric diseases (Schäfer and Fisher, 2011; Carr *et al.*, 2013). Modelled in rodents using early life stress (ELS) paradigms, behaviours associated with schizophrenia and other psychiatric diseases have been observed, as well as inhibition of AN (Lajud and Torner, 2015). Interestingly in females ELS, which show greater resilience to the negative effects the ELS compared to males (Naninck *et al.*, 2015), the normal increase in AN in response to exercise was not observed (Abbink *et al.*, 2017). This suggests an influence of AN on the stress response (Bannerman *et al.*, 2004) that may affect interactions with other risk factors for psychiatric disease.

Reduction of AN in rodent models also induces schizophrenia related phenotypes. In irradiated adult rats deficits in working memory and PPI were observed (Iwata *et al.*, 2008). Deficits in PPI were also observed in a MAM treated rat model (Maekawa *et al.*, 2009).

# 5.1.2 Aims

1. Experiment 1: Conduct investigate into whether there is a baseline difference in number of newborn neurons between WT and *Dlg2*<sup>+/-</sup>mice, using the widely employed proxy marker for adult neurogenesis, DCX staining (Jin et al. 2002; Nacher et al. 2003), during young adulthood (8 weeks) when neurogenesis peaks (Snyder *et al.*, 2009).

 Experiment 2: The impact of age on the number of DCX<sup>+</sup> cells in older (8 month) mice will be compared between the genotypes. Previously learning tasks have been demonstrated to increase AN (Gould *et al.*, 1999), and may counteract the age related decline in AN, therefore naïve 8 month old mice will be compared to a cohort that underwent a battery of behavioural testing (detailed in Chapter 3).

Under my supervision brain sectioning, staining and analysis for experiment 2 was conducted by an undergraduate project student, Alice Pennington.

# 5.2 Materials and Methods

### 5.2.1 Animals

For experiment 1, a cohort of 20 mice (10 WT, 10 HET, 13 male, 7 female) were housed under standard conditions as previously described in 2.1.2, with *ad libtum* access to food and water. The mice only received basic handling pertaining to identification and husbandry before sacrifice at 8 weeks old.

For experiment 2, a cohort of 45 male mice (25 WT, 20 HET) were housed as previously described in 2.1.2. Of these there were 27 naive mice (16 WT, 11 HET) sacrificed at 8 months old having only receiving basic handling associated with husbandry. The remaining 18 (9 WT, 9 HET) were part of the cohort which underwent behavioural testing in Chapter 3. Following the completion of behavioural testing, around 8 months of age, these mice were sacrificed.

# 5.2.2 Perfusion Fixation

For both experiments mice were sacrificed through IP administration of 0.1 ml euthatal (Merial, Harlow, UK) and transcardially perfused as described in 2.1.5 with 1x PBS and 4% PFA (w/v) (Sigma-Aldrich, Dorset, UK). Brains were removed and post fixed for 4-6 hours in 4% PFA at RT and cryopreserved in 30% (w/v) sucrose solution at 4°C until they sank. Brains were embedded in OCT (ThermoFisher Scientific, UK) and stored at -80°C.

# 5.2.2.1 Tissue Sectioning

Brains were sectioned coronally in a counterbalanced manner using a Lecia CM1900 cyrostat (Milton Keynes, UK). Sectioning commenced at the beginning of the hippocampus (approx. -0.82 mm bregma). Free floating 40 µm sections were taken in 1:10 series, totalling 80 sections per animal, and stored in 500 µl 1x PBS at 4°C.

# 5.2.2.2 Immunohistochemistry

Immunohistochemistry was conducted as described previously (2.3.2). Briefly sections are blocked in 500  $\mu$ I 1% PBST with 3% normal donkey serum (S30-100ML, Millipore, Hertfordshire, UK) for 2 hrs at RT with agitation, then incubated with goat anti-DCX primary antibody (1:50, sc-8066, Santa Cruz, Insight Biotechnology, Middlesex, UK) diluted in 500  $\mu$ I 0.1% PBST (v/v) with 0.2% normal donkey serum (v/v) and incubated overnight with agitation at 4°C. Sections were then incubated with Alexa Fluor® secondary antibodies (ThermoFisher Scientific,UK) diluted (1:1000) in 500  $\mu$ I 0.1% PBST with 0.2% normal donkey serum for two hrs with agitation at RT,

then incubated with DAPI (1:1000, D9542-10MG, Sigma-Aldrich, Dorset, UK) in 500  $\mu$ I 1x PBS at RT with agitation for 5 minutes. Sections were mounted in a counterbalanced manner with 20  $\mu$ I Mowiol® (4-88, Sigma-Aldrich, Dorset, UK) added per slide and glass cover-slipped.

# 5.2.3 Imaging of Sections

The DG from all sections were imaged in a counterbalanced manner at 20x magnification on an upright Leica DM6000B fluorescence microscope using the tile scanning module. Exposure times were kept constant between animals.

# 5.2.4 Cell Counting

The number of DCX<sup>+</sup> cells/mm<sup>2</sup> were manually counted in the dentate gyri for each section for 8 sections per animal for 10 WT (7 male, 3 female) and 9 HET (7 male, 2 female) animals using the ImageJ (1.50i) cell counter plugin. DCX is a cytoplasmic stain, so green (DCX 488) ringed DAPI cells, a nuclear stain, were considered DCX<sup>+</sup>. The distribution of new-born adult neurons along the transverse axis on the DG varies, separated into the suprapyramidal and infrapyramidal blades (Synder et al, 2009). Therefore DCX<sup>+</sup> cell counts for each blade were calculated separately, as were any ectopically located cells, and all values summed to give the total count per section. Cells were considered ectopic when localised outside the SGZ, e.g. the hilus. The area of each blade was measured using ImageJ's freehand selection tool and the DCX<sup>+</sup> cell counting and analysis. Data was missing from 1 WT female due to unsuccessful staining and 1 HET female due to damaged sections.

# 5.2.5 Analysis

Experiment 1: The number of DCX<sup>+</sup> cells/mm<sup>2</sup> was compared between the genotypes for the whole DG, each blade individually and the percentage of ecoptic cells using t tests. Additionally, a two-way ANOVA was used to compare DCX<sup>+</sup> density per mm<sup>2</sup> in the whole DG with the following factors: GENOTYPE (WT and HET), SEX (male and female).

Experiment 2: The number of DCX<sup>+</sup> cells/mm<sup>2</sup> was compared between the genotypes and behaviour vs naive by two way ANOVA.

All data was assessed for normality using Shapiro-Wilks. Transformations were attempted to correct normality where appropriate and extreme outliers (±3 studentized residuals) were removed. If significance is changed by the removal of

outliers this is reported. Homogeneity of variances was assessed using Levene's test, and if violated Welch's *t* test used instead.

# 5.3 Results

# 5.3.1 Experiment 1: DIg2 heterozygous mutation has no impact on DCX<sup>+</sup> cell density in the dentate gyrus of 8 week old mice

All data was normal and passed Levene's test. No effect of sex or genotype was found, nor an interaction between these factors (SEX:  $F_{(1, 14)} 0.357$ , p = 0.559, GENOTYPE:  $F_{(1, 14)} 0.168$ , p = 0.688, SEX X GENOTYPE:  $F_{(1, 14)} 0.330$ , p = 0.575, two way ANOVA). As a result subsequent analysis was conducted on combined male and female data.

Data for whole DG analysis was normal, no outliers were found but the data failed Levene's test. WT data for suprapyramidal and infrapyramidal blades and percentage ectopically located cells were non-normal, which was corrected in all instances with the removal of 1 outlier (WT male). Data for both blades and percentage ectopic passes Levene's test, and the removal of the same outlier in the whole blade analysis means data passes Levene's. Whole DG data analysed by ANOVA passes Levene's test.

No difference was found between  $Dlg2^{+/-}$  and WT mice in the number of DCX<sup>+</sup> cells per mm<sup>2</sup> in the dentate gyrus (Figure 36 a-c),  $t_{(16)}$ -0.261, p = 0.798. When split by blade no differences were observed for the suprapyramidal blade,  $t_{(16)}$ -0.373, p =0.714, nor the infrapyramidal blade,  $t_{(16)}$  0.516, p = 0.613 (Figure 37 e & f). Ectopic cells were analysed as a percentage of overall DCX<sup>+</sup> cell numbers in the whole dentate gyrus by *t* test. No difference was found between WT and  $Dlg2^{+/-}$  mice  $t_{(16)}$  -0.399, p = 0.695 (Figure 37 d).



Whole Dentate Gyrus





Figure 37: There was no difference in the density of DCX<sup>+</sup> cells/mm<sup>2</sup> in the whole dentate gyrus of 8 week old Dlg2<sup>+/-</sup> mice (a-c), or the percentage of total of DCX<sup>+</sup> cells that are ectopically located, defined as localised outside the SGZ (d). There is no difference when the dentate is divided into two blades, the suprapyramidal (upper most blade in each image)(e) or the infrapyramidal blade (lower blade in each image) (f).n = 9 (WT), 9 (HET). Data represents mean  $\pm$ 

SEM. Scale bar measures 200 µm. The nuclei of granule cells is stained blue (DAPI) and in DCX<sup>+</sup> cells the cytoplasm is stained green.

# 5.3.2 Experiment 2: DIg2 heterozygous mutation has no impact on DCX<sup>+</sup> cell density in the DG of 8 month old behaviourally tested or naive mice.

No genotype data was normal, nor was the non-behavioural group. One outlier was removed (naive WT) and all data was square root transformed. This corrected normality for WT data but not HET or naive data. There was no homogeneity of variances.

No difference in the density of DCX<sup>+</sup> cells was found between the genotypes or the behavioural groups, and there was no interaction between these factors (Figure 38) (GENOTYPE:  $F_{(1, 40)}$  1.673, p = 0.203, BEHAVIOUR:  $F_{(1, 40)}$  0.654, p = 0.424, GENOTYPE X BEHAVIOUR:  $F_{(1, 40)}$  0.112, p = 0.740).





Figure 38. There is no difference in the density of DCX<sup>+</sup> cells/mm<sup>2</sup> in the whole DG of 8 month old behaviourally naive (control) WT or Dlg2<sup>+/-</sup> mice (a & b). Neither was there any difference in DCX<sup>+</sup> cells/mm<sup>2</sup> between WT and Dlg2<sup>+/-</sup> mice that underwent behavioural tested (c & d). n = naïve 19 (WT) 11 (HET), behaviourally tested mice 9 (WT) 9 (HET). Error bars are SEM. Scale bar measures 200 µm. The nuclei of granule cells is stained blue (DAPI) and in DCX<sup>+</sup> cells the cytoplasm is stained green).

### 5.4 Discussion

Quantification of new born neurons in the DG using DCX staining is a widely used method for investigating adult neurogenesis in rodent models. No difference was found in baseline DCX<sup>+</sup> cell numbers were compared between the genotypes during the peak of adult neurogenesis, 8 weeks of age (Snyder *et al.*, 2009). Adult neurogenesis declines with age (Galvan and Jin, 2007) although this reduction may be attenuated by intervention such as cognitive testing or exercise. Both genotypes demonstrated vastly fewer DCX<sup>+</sup> cells at baseline at 8 months old, and no protective effect of behavioural testing was found.

# 5.4.1 Baseline DCX numbers in the DG are similar between WT and Dlg2<sup>+/-</sup>8 week old mice

For the whole DG there was no difference in the number of DCX<sup>+</sup> cells between the genotypes. Around 8 weeks, young adulthood in mice, is the peak of adult neurogenesis (Snyder, *et al.*, 2009), and in normally comparably developing animals represents the time a difference in cell number may be most apparent. A functional asymmetry has been suggested between the blades, due to different circuit functioning, and that the infrapyramidal blade may be more involved in activating the hippocampus (Scharfman, et al, 2002) whilst the neurons in the suprapyramidal have been demonstrated to be more functionally active (Chawla *et al.*, 2005; Snyder *et al.*, 2009). However, compared to WT mice, even when split into separate blades, no differences in DCX<sup>+</sup> cell number were observed in *Dlg2<sup>+/-</sup>* mice compared to WT. Ectopically located cells were analysed as these cells may not integrate properly into the circuity, which could negatively impact the functioning of the network (Duan *et al.*, 2007). Increased ectopic localisation of DCX<sup>+</sup> cells has been observed in other synaptic risk models, such as DISC1 (Duan *et al.*, 2007) but no difference was found in *Dlg2<sup>+/-</sup>* mice.

Increased or decreases DCX<sup>+</sup> cell number may not translate into altered numbers surviving and having any functional impact. This can be investigated by injecting mice with BrdU daily for 5 days then sacrificed them after 30 days. NeuN<sup>+</sup> mature neurons co-stained with BrdU will demonstrate how many of the neurons born during injections, having incorporated BrdU during their cell cycle, have survived to integrate into the SGZ. The functional importance of these neurons can be probed behaviourally, using pattern separation tasks which are thought to rely on new adult neurogenesis (Sahay *et al.*, 2011), or through electrophysiological studies (Farmer *et al.*, 2004; Schmidt-Hieber, Jonas and Bischofberger, 2004; Song *et al.*, 2005).

# 5.4.2 There is no difference in DCX<sup>+</sup> cells in the DG of aged WT and Dlg2<sup>+/-</sup> mice, nor between behaviourally tested or naïve mice.

The second experiment compared naïve  $Dlg2^{+/2}$  and WT mice which had been allowed to age up to 8 months old and between 8 month old naïve mice and a cohort that had undergone months of behavioural testing (Chapter 3). As rodents age the proliferative capacity for adult neurogenesis decreases (Kempermann, 2015), which is reflected in the lower number of DCX<sup>+</sup> cells/mm<sup>2</sup> counted in the second experiment (100s rather than 1000s).

Physical activity (van Praag, Kempermann and Gage, 1999a; Kronenberg *et al.*, 2003) and cognitive testing (Gould *et al.*, 1999) are positive regulators of adult neurogenesis and may counteract this age related decline in newborn neuron numbers (Kempermann, 2015). Exercise increases adult neurogenesis in the hippocampus, but not the olfactory bulb (Brown *et al.*, 2003), and expands the pool of cells that can mature and integrate (van Praag, Kempermann and Gage, 1999b). However, no protective effect of enhanced activity (following behavioural testing) was observed for either genotype in 8 month old mice. It is possible that the tasks were not intensive enough to promote substantial increases in adult neurogenesis, or that stress associated with regular testing diluted any changes (Joëls *et al.*, 2004; Levone, Cryan and O'Leary, 2015).

### 5.4.3 Adult neurogenesis in other risk models for psychiatric disease

This is the first study to investigate the impact of mutations in any *Dlg* gene or MAGUK's more generally on adult neurogenesis. In contrast to the absence of any AN phenotypes observed in *Dlg2*<sup>+/-</sup>mice, other genetic models of psychiatric risk have been found to show abnormalities of adult neurogenesis. SNPs in the gene encoding the calcium voltage-gated channel subunit alpha1, *CANCA1C*, has been associated with schizophrenia (Nie *et al.*, 2015). In a cKO mouse model reductions in DCX<sup>+</sup> neuron numbers and decreased BrdU staining were observed (Lee *et al.*, 2016). The BrdU reduction was also found in a heterozygous rat model but not decreased DCX<sup>+</sup> numbers (Moon *et al.*, 2018). In another synaptic risk gene model, *Cyfip1*<sup>+/-</sup> 8 week old mice there is an increase in DCX<sup>+</sup> cells in the SGZ, as well reduced migration from the SGZ (Haan *et al.*, 2018). Subsequent work determined the migration phenotype stemmed from abnormal actin polymerisation, whilst the increased DCX<sup>+</sup> numbers were due to aberrant microglial function and impaired apoptosis. AN was also investigated in a KO mouse model of a component of the complement system, C3 and its receptor C3-a (Westacott, 2016). C3 is downstream of the biggest GWAS hit

for schizophrenia C4 (Ripke *et al.*, 2014; Sekar *et al.*, 2016). Both mutant C3 mice models showed increased numbers of DCX<sup>+</sup> in the whole DG, in both supra- and infrapyramidal blades individually, and abnormal morphology (Westacott, 2016). Whilst the phenotypes observed differ slightly it appears that adult neurogenesis may often be affected by mutations that confer risk for psychiatric diseases.

# 5.4.4 Limitations

As there is not a reduction in *Dlg2* mRNA expression in the hippocampus it is possible that any abnormalities in DCX<sup>+</sup> number as a result of mutation may not be apparent in this model. It is possible, however, that other phenotypes may be present that have not be assessed. Although the numbers of DCX<sup>+</sup> did not vary between genotypes there may be morphological differences in these cells. It is also possible that the cells are not functioning normally, or forming normal synaptic connections. Given the importance of the importance of *Dlg2* at the synapse there may be abnormalities of distal connections, which could be examined using retroviral tracing (Vivar *et al.*, 2012).

Additionally AN could be examined with other markers. DCX expression overlaps with another often used marker, PSA-NCAM. DCX is expressed by multiple cell types during neurogenesis, including type 2b, type 3 and early post-mitotic neurons (Kempermann, Song and Gage, 2015). Even if DCX<sup>+</sup> numbers do not change the proportion of different subtypes which express DCX may shift. As these subtypes, except type 3, also express a discreet marker in addition to DCX, the proportion of DCX<sup>+</sup> cells of each subtype can be determined.

# 5.4.5 Conclusions

Using a widely accepted proxy for studying adult neurogenesis, no difference in the number of DCX<sup>+</sup> cells was found between WT and *Dlg2<sup>+/-</sup>* mice in young and old mice. However, this is only one aspect of adult neurogenesis and there may be abnormalities of more specific aspects of the process, as opposed to a more generalised impairment. The lack of altered DCX<sup>+</sup> cell numbers differentiates *Dlg2* from many other synaptic proteins implicated in the risk for psychiatric disease.

# 6 Characterisation of Rat Model

# 6.1 Introduction

There are limited publications using *Dlg2* rodent models *in vivo*. Mutant *Dlg2*<sup>+/-</sup> mouse models in the literature implicate *Dlg2* in cognitive processing. Generally rats are preferably used to study cognition compared to mice in in neuroscience research. However, it has been very challenging to generate KO rat models until more recently, when advances in genetic engineering have facilitated the development of such models. Therefore the benefits of rat models for studying cognition can be combined with this increased ability to produce genetically manipulated models which reflect genetic risk factors in psychiatric diseases. This Chapter will discuss characterisation of a novel *Dlg2*<sup>+/-</sup> rat model.

The mutation in the *Dlg2<sup>+/-</sup>* rat model is in exon 5, resulting in a premature stop codon in exon 6. This is further upstream than the mutation in the mouse model (exon 14) described in Chapter 3. Mutations may alter observable phenotypes through loss of function of the full length protein or the expression of mRNA prior to the mutation site, resulting in truncated protein that acts as a dominant negative, as is observed with the truncated version of the Tropomyosin receptor kinase B (TrkB) receptor (Fenner, 2012). The *Dlg2<sup>tm1a(EUCOMM)WIsi* mouse model was designed to interrupt a critical exon which spans as many transcripts as possible, with some transcriptional start sites being further downstream. The rat model was generated using CRISPR which should allow greater control over mutation creation than traditional methods, given the specificity of binding between target DNA and the engineered complimentary single guide RNA (sgRNA) (Jinek *et al.*, 2012).This facilitates targeting of multiple sites to generate deletions or inversions (Canver *et al.*, 2014), the insertion of specific sequences to generate knock-in models (Wang *et al.*, 2015) or correct disease mutations (Yin *et al.*, 2014).</sup>

Whilst there are similarities between the rodent species, rats should not be considered 'big mice'. There are many aspects in which the species differ which determine the choice of species specific experimental investigations. Rats physiology is considered closer to that of humans, the larger size increases tissue samples potentially reducing the number of animals required, and size also makes surgery easier (Ellenbroek and Youn, 2016). Studies involving optogenetics on the other hand are easier in mice due to the smaller brain allowing easier passage of light into deeper brain regions (Ellenbroek and Youn, 2016). Rats are generally preferred for studying

cognition because they tend to require less habituation and training sessions to perform tasks (Colacicco *et al.*, 2002; Jaramillo and Zador, 2014), and experience less of the associated stress and anxiety than mice. Performance over time is also less stable in mice than rats (Colacicco *et al.*, 2002). Fundamental differences between the species may explain some divergence in ability of the two species. For example mice exhibit greater difficulty during Morris Water Maze tasks, which might be attributable to a difference in the species natural response to water (Whishaw and Tomie, 1996). Wild rats actively spend time in and around water, whereas wild mice actively avoid water. This idea is supported by the lack of difference between the species tested in a 'dry maze' (Whishaw and Tomie, 1996).

As with the mouse model the background strain of the model must be considered when interpreting findings. Long Evans (LE) originate from an outbred rat strain first developed in 1915 by crossing Wistar females with a wild Norway male (Pass & Freeth 1993). LE are often utilised for cognitive testing, as they often require less training to learn self-initiate trials and acquire reversal of contingents than other commonly used strains such as Sprague Dawley (SD) rats (Turner and Burne, 2014). LE have been shown to be more active, explore more, demonstrate reduced anxiety, PPI and improved cognition compared to SD, which are commonly used in psychiatric research (Turner and Burne, 2014). Additionally LE have been found to freeze less than SD during initial and repeated context and tone tests (Graham *et al.*, 2009). Therefore protocols previously developed using other strains may need modification to pull out any genotypic effects.

As this rat model is novel it is important to characterise the basic molecular and behavioural phenotype to determine the similarly of the haploinsufficiency rat and mouse models. In the  $Dlg2^{+/}$  mouse model, characterised in Chapter 3 (3.3.1.1), a reduction of Dlg2 mRNA was only found in the PFC, without any change in expression of Dlg2 and other Dlg family members in any other brain region examined (PFC, hippocampus and cerebellum). Behaviourally male  $Dlg2^{+?}$ -mice exhibited specific impairments in motor learning, acoustic startle response and between session habituation across 5 days. No deficits of motor function, context discrimination, anxiety response or ketamine-induced hyperlocomotion were demonstrated. A full knockout Dlg2 model exhibited impairments of object location paired association, reversal learning, extinction and attention, as did humans with Dlg2 mutations (Nithianantharajah *et al.*, 2013) suggesting conservation of Dlg2 function. Therefore exploring whether phenotypes observed both mouse models are recapitulated in the rat will illuminate the conservation of impairments up the evolutionary tree. This will

have important consequences for the use of this novel *Dlg2* haploinsufficiency rat as a viable model of human brain function.

# 6.2 Aims

- 1. Measure *Dlg2* mRNA in three key brain regions of *Dlg2* heterozygous rats: hippocampus, cerebellum and PFC.
- 2. Determine whether there any compensatory changes in mRNA expression for *Dlg* family members *Dlg1*, *Dlg3* and *Dlg4* in the hippocampus, cerebellum and PFC *Dlg2* heterozygous rats.
- Examine basic motor function and anxiety behaviours in the Dlg2<sup>+/-</sup> compared to wild type (WT) rats

# 6.3 Materials and Methods

### 6.3.1 Generation of Founders

*Dlg2* heterozygous rats were generated on a LE Hooded background by Horizon Discovery (Pennsylvania, USA) using CRISPR

Clustered, regularly interspaced, short palindromic repeat (CRISPR)/Cas9 gene editing is rapidly becoming the most popular method of gene editing due to its ease of use in comparison to methods such as zinc finger nuclease technology, the specificity achievable when generating mutations, and the ability to generate mutations in species that ES manipulation is generally unsuccessful in, including rats (Smalley, 2016).

The technology is adapted from the RNA-mediated CRISPR-Cas9 adaptive immune system of bacteria, such as *Streptococcus pyogenes*, that protect hosts from invasion by foreign DNA (Jinek *et al.*, 2012). The system acts as a genetic memory facilitating the detection and destruction of invaders, as genetic code remnants from past invaders, "spacer" sequences, interrupt the repeating sequences of genetic code. These sequences are transcribed in short RNA sequences that guide the system to matching DNA sequences in subsequent infections and cleaves the DNA causing a double stranded break (DSB). However, unless a protospacer adjacent motif (PAM) is present then the Cas9 will not bind and cleave the DNA. This PAM, a 2-6 base pair sequence immediately after the targeted sequence, prevents cleavage of host DNA as it is only found in the viral DNA. In *S.pyogenes* the canonical sequence is NGG ('5-'3). These PAM sites are found throughout the genome. Recognition of the PAM is thought to destabilise the adjacent sequence, allowing sgRNA interrogation and DNA-RNA binding where the sequence is present (Mekler, Minakhin and Severinov, 2017).

When this process is adapted for gene editing a short guide RNA (gRNA) is designed to match DNA sequences of interest, which guides the machinery to the desired genetic loci and causes a DSB. The DSB triggers one of two repair pathways: nonhomologous ending joining (NHEJ) or homology derived repair (HDR). The NHEJ pathway is more error prone, introducing random insertions/deletions that can alter the reading frame of the coding sequence. This is the pathway utilised in the creation of the *Dlg2*<sup>+/-</sup> rat. The HDR pathway relies in on the inclusion of a donor template allowing the introduction of specific mutations (Figure 39). A concern with the method is the potential for off-target cleavage at unintended sites (Markossian and Flamant, 2016), which must be tested for during model generation.



Figure 39: Image from Ran et al., 2013. Cas9 triggers DSBs which can be repaired via two pathways. The NHEJ pathway is error-prone and can result in indel mutations at the junction site when repaired by endogenous DNA repair mechanisms. The HDR pathway can be triggered with the introduction of a plasmid or single stranded oligonucleotide to act as a repair template. This results in a more precise mutation than the random indels generated via NHEJ.

The model was designed by the NMHRI as part of the DEFINE project. It was then produced and validated by Horizon Discover, as described in this paragraph. A sqRNA (ccagggtcatctccaatgtqAGG) was targeted to exon 5 of Dlg2 using a proprietary bioinformatics software (Horizon Discovery, plc UK). The resulting successful founders had a 7bp deletion (782933-782939 in the genomic sequence) in exon 5, which caused a frame shift and generation of an early stop codon in exon 6 (Figure 40). Confirmation of successful non-homologus end joining (NHEJ) activity was assessed via PCR and sequenced by Horizon Discovery plc, UK. The PCR reaction was as follows; 1µl of DNA in solution (extracted using Epicenter®QuickExtract Solution<sup>™</sup>), 1µm forward (tctgaccttgctgtgtctgc) and 1µm reverse (gcgtcactacagaaagccttg) primers, 12.5µl 1x Sigma Jumpstart TaqReadyMix<sup>™</sup> (Sigma-Aldrich, St Louis, USA) and 6.5µl ddH<sub>2</sub>0. The PCR reaction was run at 95°C for 5 mins, followed by 35 cycles of 95°C for 30s, 60°C for 30s and 68°C for 1 min. The final extension was 68°C for 5 min. PCR products were resolved on 2% agarose gels.

-3	د) (۱۹۹۵) اور	Pre-
782801	21. ARCATURATA CACAGEREGE CAGEGORICTA OFFITTAGAT GACAGEREAT CATITAATAT CORCITATING TITUTACAGE GARATTERES TITUE TIGTACTATE OFGICIENCE OFFICECCEGAT CEARARTER ENGIGENERA GIARATTATA OFFICEARAGATOTEC CITTAAGTEC AGACE	TAAG
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783001	TCACOTANAA AICAAAMGCA TIAAAMIGAC TOGAGCASII GAICCACIGO AICAGGIAGI CAINIITGIA COTIIGGAGA AIGAINAIII IAIAA NOTOCATTII INGITIICGI NATTIINCIG NOCICOICAN CINGGIGACG INGICCATCA GIAINAACAI GGAMACCICI INCININAA MINII	ACTT TGAA
-1	1 he'Vel Ann The Ang The Lew Ang The Be Price Ty Vel Anne Oly The Ole Ide Ole Ty. Ole Price Ole Ole Ide The Lew Ole Ang Oly Anne Ser I	ing Low
801	IGTCAACACA GACACTITES ACACTATICS ITAIGTCAAI GEAACAGAAA ITGAATAIGA AITIGAAGAA AITACAITEG AGAGG <mark>GGAAA ITCAG</mark> ACAGITETET CIGTGAAACC IGTGAIAACE AAAAAAAAA AAAAAAAAAAAAAAAAAAAAAAA	TCIG
+1	1. Giệ Phả Sar Br. Alà Giệ, Giệ, Thr App Aph Pho Hệ Một Thr Leu Alà. Tự Leu Leu Alp Alp Leu Phá Gin Giu Yai Leu Gin Gin Ap Met Al	Aup Se
601	GGATTCASTA TIGCTGGAGS GACAGATAAT COLCALATGA COLCACATA TITATTACGA AGATTATICC AGGAGGTGCT GCAGCAGAGS ATGGC CCTAAGICAT AACGACCICC CIGICIATTA DIACIDIACI GOOACGITAT AAATAATGCI ICIAATAAGS ICCICCACGA CGICGICICC TACCG	GACT
	1. See Dig See. The Ter Val. See. Ops. Dig 🧮 Met Tigs Leve. Met Cips Ang. Phe Pio The Val. Lips. Din. Tip. Ang. Pio: See. Ang. Lips. Ohn. Ann. Leve	Les Phe
701	CAGOSTCAAC GAITCIATCI ISCOSTGAA IGAAGIIGAI GISTCOGAGG IITCOCACAG IAAAGCAGIG GAGGCCCTCA AGGAAGCAGG CICIA GICCCAGIIG CIAACAIAGA ACGCCCACII ACTICAACIA CACAGCCICC AAAGGGIGIC ATTICGICAC CICCGGGAGI ICCIICGICC GAGAI	TGTT

Figure 40: Top: The genomic sequence of the WT rat. The section highlighted in blue is the sequence to be deleted. Bottom: The translated mRNA following the 7bp deletion. Image provided by Horizon Discovery plc UK.

Both genotyping and sequencing were used to confirm the deletion by Horizon Discovery, plc UK in the founders (Figure 41).



Figure 41: The deletion site is marked by a red line. Prior to this line only single peaks occur and the quality (grey bars) is high. After the 7bp deletion the sequence displays a mixed read out from both the WT and deletion alleles, demonstrated by presence of two peaks. Data provided by Horizon Discovery and analysed using Snapgene Viewer 3.3.2.

An online CRISPR guide generation tool from MIT (<u>http://crispr.mit.edu/</u>) ranks all potential guides in relation to their on-target activity as well as providing the top ten off target sites for each sgRNA (Figure 42). Horizon discovery, plc UK used this information to assay for potential off-target effects present in the founders using PCR and SURVEYOR Cel-1 mutation Detection Assay (IDT). None were found.

	Off Target Sequence	genomic coordinates	comments	F primer	<u>R primer</u>	results
Injected gRNA	CCAGGGTCATCTCCAATGTGAGG					
OT Site 1	CCAGGGTCATCTCCAATGTTAAG	chr19: 34563666-34563688	screened by SAGE	aggttagtccgtgcatggtg	gcttgcctacagccgtattt	negative
OT Site 2	CTTGAGTCATCTCCAATGTGTGG	chr11: 75326973-75326995	screened by SAGE	tgtcagtgctgccttttgtc	tcctttgtgtggtgtgggttc	negative
OT Site 3	CCAGGGTGATCTCCAATCTGAGG	chr14: 84150501-84150523	screened by SAGE	ggtaactggcctttgggttt	tctgatttggggcttaggtg	negative
OT Site 4	TTATTGTCATCTCCAATGTGTAG	chr16: 22863405-22863427	screened by SAGE	tacccacttttcacccaagc	ttgcccctttcagagaagac	negative
OT Site 5	CTATGGTCATCTCCAATGGGTAG	chr20: 16439248-16439270	screened by SAGE	aaaccggttatgtcctgtgc	ggaggaagatggaggaaac	negative
OT Site 6	ACAAGCTTATCTCCAATGTGTAG	chr6: 46008366-46008388	screened by SAGE	tgcctaggaaactggcaact	tgtgtcacttggatggatgtc	negative
OT Site 7	CCAGGATAATATCCAATGTGTAG	chr16: 34645325-34645347	screened by SAGE	tgctcactgctgataggtctg	ggatatcattggaccccaca	negative
OT Site 8	TTAGGGTAATATCCAATGTGAGG	chr3: 89806272-89806294	screened by SAGE	tatctcggcccaagaagaag	caaagaccaggatcccaatg	negative
OT Site 9	CAATAATCATCTCCAATGTGCAG	chr4: 164282994-164283016	screened by SAGE	agcaggtcttcagcttggtt	ccagaggccctcaaattaca	negative
OT Site10	CGAAGGTCCACTCCAATGTGCAG	chr17: 19811676-19811698	screened by SAGE	tcgtgggaaggaaagacttg	ggcagtccatgcctgtttat	negative

Figure 42: Off-target table for the top 10 predicted off-targets from the MIT online tool ((http://crispr.mit.edu/) for the sgRNA used to generate the 7bp deletion. Table provided by Horizon Discovery, plc UK.

 $Dlg2^{-/-}$  rats are viable but initially all pairings were WT x HET, resulting in a Mendelian distribution of WT and HET pups, with an average litter size of nine pups. Selected heterozygous founders were sent to Charles River (Margate, UK) and bred to produce experimental colonies.

# 6.3.2 Ethics and Housing

All procedures on the rats were conducted in line with Animals (Scientific Procedures) Act (ASPA) (1986); under UK Home Office project license PPL 30/3135 and PIL 131AE7D42.

Rats were housed as previously described 2.1.2 in groups of 1-4, dependant on weight, with *ad libtum* access to standard food and water on a 12:12 hr light:dark cycle (lights on 07:00). All testing in this chapter was conducted during the light phase. Nesting material, wooden sticks and cardboard tubes provided environmental enrichment.

# 6.3.3 Genotyping by PCR

Ear punches from all weaned animals sent were from Charles River for genotyping prior to experimentation. Post-mortem tail and brain samples were taken for genotype confirmation. DNA extraction follows the protocol described in 2.1.3.

Horizon Discovery plc, UK designed primers (forward – tctgaccttgctgtgtctgc, reverse – gcgtcactacagaaagccttg) and PCR cycling protocols were used. In accordance with manufactures instructions GoTaq<sup>®</sup> Green Master Mix (Promega, Southampton, UK) reaction mix was used for PCR. Briefly, 5µl sample was added to 12.6µl GoTaq<sup>®</sup>, 2µl forward and reverse primers, and 3.5µl ddH<sub>2</sub>0. Samples were run on a BioRad Thermal Cycler (T100 BioRad<sup>™</sup>, Herts, UK) under the following conditions: 95°C for 5 minutes then 35 cycles of 95 °C for 30 seconds, 60 °C for 30 seconds and 68 °C for

40 seconds and a final extension at 68 °C for 5 minutes. Samples were held indefinitely at 4 °C.

A 4% gel was made with 1% TAE buffer (w/v) and SYBR Safe Gel DNA Stain (1:1000, ThermoFisherScientific, UK). PCR products were analysed by gel electrophoresis for 2 hrs at 150v, with 10  $\mu$ l of sample loaded per well. Gels were visualised using iBright CL1000 (Invitrogen, ThermoFisher Scientific, UK). Two bands were indicative of a heterozygous animal, with a mutant band at ~446 bp and a WT band ~421 bp, and single bands ~421 bp were indicative of a WT (Figure 43).



Figure 43: Sample gel for genotyping. HET Animal 2666 and WT 1-3 after DNA extraction from brain tissue sent from Horizon are used as controls. Samples 7-13 are extracted from ear samples sent by Charles River of live animals. The presence of two bands in samples 7, 9, 11 and 13 indicate successful generation of heterozygous rats. The remaining samples are WT.

# 6.4 Molecular Characterisation

# 6.4.1 RT-qPCR Analysis

A cohort of twenty-seven male and female LE rats were used for gene expression analysis (16 HET, 11 WT). The expression of *Dlg1, Dlg1, Dlg2, Dlg3, and Dlg4* mRNA in the cerebellum, hippocampus and PFC was analysed by RT-qPCR using validated primers (Table 10). The tissue was extracted from whole brain hemispheres from 8-week-old rats and ~30g tissue from each region of interest was converted to cDNA as previously described in 2.3.1.2.

Quantitation was conducted using the comparative Ct method ( $2^{-\Delta\Delta Ct}$  method) to measure fold change in expression between WT and  $Dlg2^{+/-}$  rats. Data normality was assessed using Shapiro-Wilks test. Where possible appropriate transformations were attempted to correct normality. On examination of boxplots values considered extreme outliers were removed. If significance is changed by outliers being retained this is reported. Normally distributed data was analysed using *t* tests. Where normality could not be corrected Mann Whitney U was used. Homogeneity of variances was assessed by Levene's test. If this assumption was violated Welch's *t* test was used.

Primer	Forward Sequence	Reverse Sequence		
Dlg2	GGACATCCCCGGATTAGGT G	TGTAGTTTATTTCCTGCCTCGT GA		
Dlg1	CCCAGATGGTGAGAGTGAC G	AGTTACGTGCTTCAGGCCTTT		
Dlg3	GTCTAATCGGGACTTCCCTG G	TGGAACTGCTTTCGCTGTCA		
Dlg4	ACAACCAAGAAATACCGCTA CCA	CCCCTCTGTTCCATTCACCTG		

Table 10. Primer pairs designed for each Dlg gene validated using control rat cDNA from hippocampus, cerebellum and PFC.

### 6.5 Behavioural Characterisation

#### 6.5.1 Animals

A cohort of thirty-eight male Long Evans rats (19 WT, 19 HET) underwent two behavioural tasks, rotarod motor performance and open field anxiety measures. When testing began rats were 3.9 months old. Rats were allowed to acclimatise to the animal unit for 1 week following transport from Charles River (Margate UK), and were then were handled daily for 3 days to habituate the rats to handling prior to testing. Rats were moved and rehoused to an adjoining animal unit between the tasks and were given a week to acclimatise to the new unit. After rotarod testing, 3 cages of 4 rats were separated into 6 cages of 2 to account for an increase in age related weight. Rats were transported to behavioural rooms in their home cages, and returned to them following task completion. All apparatus was cleaned between animals with ethanol wipes or 70% ethanol to mask odour cues. WT littermates were used as controls.

#### 6.5.2 Rotarod

#### Task

Rats were tested on the rotarod as described 2.2.1.1. Rats were habituated to the testing room for 10 minutes prior to testing. Each rat underwent three daily trials for three consecutive days on an accelerating rotarod (47760, Ugo Basile, Italy) which accelerated from 5rpm to 40rpm over 5 minutes. Latency (s) to fall from the rod was taken as a measure of motor performance. Rats were returned to their home cages and moved back to the holding room between trials with an inter-trial interval of 1 hr minimum.

# Analysis

Normality was assessed for all data using Shapiro-Wilks test. Where appropriate transformations were attempted to correct normality. Extreme outliers (±3 studentized residuals) were removed. If significance is changed by outliers being retained this is reported. Homogeneity of covariances was assessed by Box's M test and homogeneity of variances was assessed by Levene's test. In instances where the assumption of sphericity was violated the Greenhouse-Geisser correction was applied.

The latency (s) to fall from the rotarod was analysed by mixed ANOVA with the following factors: Genotype (WT and HET), Trial (1-3) and day (1-3).

### 6.5.3 Open Field

#### Task

The open field task was conducted in the 1 m<sup>2</sup> arena described in 2.2.2.2. Rats were transported in home cages to an adjacent holding room for 20 minutes prior to testing. When taken into the testing room rats were placed in the centre of the arena. Recording was started immediately and the single session lasted for 10 minutes. Rats were returned to their home cages following testing. The percentage time spent in the centre and outer zones was measured as indices of anxiety. Total distance (cm) travelled and maximum velocity (cm/s) were measured as indices of activity.

#### Analysis

Normality was assessed for all data using Shapiro-Wilks test. Where appropriate transformations were attempted to correct normality. On examination of boxplots values considered extreme outliers were removed. If significance is changed by outliers being retained this is reported. Normally distributed data was analysed using t tests. Where normality could not be corrected Mann Whitney U was used. Homogeneity of variances was assessed by Levene's test. If this assumption was violated Welch's t test was used.
### 6.6 Results

#### 6.6.1 RT-qPCR

# 6.6.1.1 Reduced Dlg2 mRNA expression in the hippocampus and PFC but not cerebellum in Dlg2+/- rats

The expression of *Dlg2* mRNA was assessed via RT-qPCR, comparing the genotypes with *t* tests or Mann Whitney U. All cerebellum and hippocampal data was normal and had homogeneity of variances. All WT PFC data was non-normal. One outlier was removed (WT) but this, nor transformation (square-root), corrected normality so Mann Whitney U was used.

 $Dlg2^{+/2}$  rats displayed no change in Dlg2 expression in the cerebellum, t (12) 0.492, p = 0.674 (Figure 44a). There was an approximately 50% reduction in Dlg2 expression in the hippocampus (Figure 44b), t (14) -4.259, p = 0.001, and the PFC (Figure 44c) U = 0.00, p = 0.001.



Figure 44. The relative change in Dlg2 mRNA expression levels assessed in 8 week old rats by RTqPCR in the cerebellum (a) hippocampus (b) and PFC (c). n = 6 (WT) 8 (HET) (cerebellum), 8 (WT) 8 (HET) (hippocampus) and 6 (WT) 8 (HET) (PFC). Data represent the mean  $\pm$  SEM error bars. \*\* p = 0.01.

# 6.6.1.2 There was no change of Dlg1, Dlg3 and Dlg4 mRNA expression in Dlg2+/- rats

The expression of mRNA for *Dlg1*, *Dlg3* and *Dlg4 was* assessed via RT-qPCR, comparing the genotypes with *t* tests or Mann Whitney U. All cerebellum data was normal. There was homogeneity of variances for *Dlg1* and *Dlg3* but not *Dlg4*, so Welch's *t* test was used. Hippocampal data for *Dlg3* was normal and had homogeneity of variances, but all *Dlg1* data was non-normal. Transformation (Log10) failed to correct normality, so Mann Whitney U was used. All hippocampal HET data for *Dlg4* was non-normal, one outlier was found (HET) and removal corrected normality. There was homogeneity of variances. All PFC data for *Dlg1* was normal and had homogeneity of variances. WT PFC data for *Dlg3* and *Dlg4* were non-normal, but

removal of one outlier (both WT) in each instance corrected normality. In both instances there was homogeneity of variances.

There was no difference in the expression mRNA of any the *Dlg* family members, *Dlg1* (Figure 45), *Dlg3* (Figure 46) or *Dlg4* (Figure 47) in the PFC, hippocampus or cerebellum of 8 week old rats, as assessed via RT-qPCR (Table 11).



Figure 45. The relative change in Dlg1 mRNA expression levels assessed in 8 week old rats by RTqPCR in the cerebellum (a) hippocampus (b) and PFC (c). n = 6 (WT) 8 (HET) (cerebellum), 8 (WT) 8 (HET) (hippocampus) and 6 (WT) 8 (HET) (PFC). Data represent the mean  $\pm$  SEM error bars.



Figure 46. The relative change in Dlg3 mRNA expression levels assessed in 8 week old rats by RTqPCR in the cerebellum (a) hippocampus (b) and PFC (c). n = 6 (WT) 8 (HET) (cerebellum), 8 (WT) 8 (HET) (hippocampus) and 6 (WT) 8 (HET) (PFC). Data represent the mean  $\pm$  SEM error bars.



Figure 47. The fold change in Dlg4 mRNA expression levels assessed in 8 week old tissue by qPCR in the cerebellum (a) hippocampus (b) and PFC (c). n = 6 (WT) 8 (HET) (cerebellum), 8 (WT) 7 (HET) (hippocampus) and 6 (WT) 7 (HET) (PFC). Data represent the mean  $\pm$  SEM error bars.

Gene/Region	TEST
Dlg1 PFC	$t_{(10)}$ 0.219, $p = 0.831$
Dlg1 Hippocampus	<i>U</i> = 23.00, <i>p</i> = 0.563
Dlg1 Cerebellum	$t_{(12)} 0.492, p = 0.632$
Dlg3 PFC	<i>t</i> <sub>(12)</sub> -2.218, <i>p</i> = 0.06
Dlg3 Hippocampus	<i>t</i> (13) -1.589, <i>p</i> = 0.136
Dlg3 Cerebellum	<i>t</i> <sub>(12)</sub> -0.297, <i>p</i> = 0.771
Dlg4 PFC	<i>t</i> <sub>(11)</sub> - 0.106, <i>p</i> = 0.918
Dlg4 Hippocampus	$t_{(8.042)}$ 0.548, $p = 0.599$
<i>Dlg4</i> Cerebellum	$t_{(6.873)}$ 0.045, $p = 0.966$

Table 11. The expression of Dlg1, Dlg3 and Dlg4 mRNA was compared between the genotypes from extractions of the PFC, hippocampus and cerebellum of 8 week old WT and  $Dlg2^{+/-}$  rats. No differences were found in the expression of these Dlg isoforms in any region investigated.

### 6.6.2 Behavioural Results

# 6.6.2.1 Dlg2<sup>+/-</sup> rats showed comparable motor performance to WT across a three day accelerating rotarod task

Latency to fall was assessed by mixed ANOVA with the following factors: Genotype (WT and HET), Trial (1-3) and Day (1-3) (Figure 47). All data was non-normal. There was no homogeneity of covariances, but there was homogeneity of variances in most instances. A Log10 transformation corrected normality for all data, as well as homogeneity of variances and covariances. However, Log10 cannot transform zero values, so 3 WT and 2 HET rats with latency (s) of zero were not transformed. The assumption of sphericity was met for trial, day and trial\*day, X<sup>2</sup> (2) 2.917, *p* = 0.233, day X<sup>2</sup> (2) 2.318, *p* = 0.314, and trial\*day X<sup>2</sup> (9) 8.762, *p* = 0.460 respectively.

There was an effect of day and trial, but not genotype (DAY:  $F(_{2, 62})$  11.447, p < 0.001, TRIAL:  $F(_{2, 62})$  18.765, p = < 0.001, GENOTYPE:  $F(_{1, 31})$  1.633, p = 0.21) (Figure 48). Genotype did not interact with either factor but there was a day\*trial interaction (DAY\*GENOTYPE:  $F(_{2, 62})$  0.064, p = 0.938, TRIAL\*GENOTYPE:  $F(_{2, 62})$  2.349, p =0.104, DAY\*TRIAL:  $F_{(4, 124)}$  7.921, p = <0.001). There was no day\*trial\*genotype interaction (TRIAL\*DAY\*GENOTYPE:  $F(_{4, 124})$  0.573, p = 0.662).



Figure 48. Both genotypes exhibit increased latency (s) to fall on Day 1, and a plateau of performance by Day 2. Data represent the mean  $\pm$  SEM error bars. n = 16 (WT) 17 (HET).

As there was no two way or three interaction involving genotype this factor was not included in subsequent analysis. Due to the trial\*day interaction one way ANOVAs were conducted for each day separately. For all one way ANOVAs all assumptions were met and where appropriate followed up by Bonferroni corrected pairwise comparisons. The assumption of sphericity was met on Day 1, 2 and 3, X<sup>2</sup> (2) 0.913, p = 0.634, X<sup>2</sup> (2) 5.738, p = 0.057 and X<sup>2</sup> (2) 0.891, p = 0.641 respectively.

Only on Day 1 was there an effect of trial (TRIAL:  $F(_{2,64})$  41.332, p = <0.001). Latency (s) to fall on Trial 1 was shorter than Trial 2 (-0.403 ± 0.064, p = <0.001) and Trial 3 (-0.509 ± 0.059, p = <0.001), plateauing between Trial 2 and 3 (0.106 ± 0.055, p = 0.187). Both genotypes increase latency (s) to fall on Day 1 across the three trials, reaching asymptotic performance by Day 2.

To examine the consolidation of learning across the three days, the performance on the first trial on each day was compared. This approach also removes the potential confound of fatigue across each session. Trial 1 on each day was compared between the genotypes by mixed ANOVA. There was homogeneity of variances and covariances, and the assumption of sphericity was met X2 (2) 2.113, p = 0.348.

There was an effect of day but not genotype, nor an interaction (DAY:  $F_{(2, 62)}$  19.217, p = <0.001, GENOTYPE:  $F_{(1, 31)}$  3.465, p = 0.072, DAY X GENOTYPE:  $F_{(2, 62)}$  0.254, p = 0.776) (Figure 49). Bonferroni pairwise comparison determined only Day 1 was different from any other day.



Figure 49. Both genotypes demonstrate increased latency (s) to fall on the first Trial on Day 2 compared to Day 1. There is a trend towards an impairment on Trial 1 of Day 3 in Dlg2<sup>+/-</sup> rats (p = 0.072). Data represent the mean  $\pm$  SEM error bars. n = 16 (WT) 17 (HET).

To confirm whether weight had any effect of the rats latency fall a mixed ANOVA was conducted. As there was no difference in weight between WT and HET rats genotype was excluded as a factor. Rat weights were binned into six 50g categories (350-400, 401-450, 451-500, 501-550, 551-600, 601-650). Mixed ANOVA was conducted with the following factors: weight bin (as previous), day (1-3) and trial (1-3).

Data normality was asses by Shapiro-Wilks. Most data was non-normal. Square-root transformation corrected all data except bin 2 D1T1, bin 2 D2T3, and bin 1 and 2 D2T2. Five outliers were found. Removal resulted in normality for all except bin 2 D3T2, and resulted in bin 3 D1T1 becoming non-normal. Box's M test was violated for homogeneity of covariances, but Levene's test for homogeneity of variances was met. The assumption of sphericity was met for trial ( $X_{2=}(2)$  3.817, p = 0.148), day ( $X_{2}(2)$  4.226, p = 0.121, and trial\*day ( $X_{2}(9)$  15.221, p = 0.086).

As in the main data set there was an effect of trial and day (TRIAL:  $F_{(2, 56)}$  8.163, p = .001, DAY  $F_{(2, 56)}$  10.689, p = <0.001). There was no interaction between weight and trial or day (TRIAL X WEIGHT:  $F_{(10, 56)}$  1.093, p = 0.384, DAY X WEIGHT:  $F_{2, 56}$  1.932, p = 0.060. There was a trend towards an effect of weight on latency to fall (WEIGHT:  $F_{(5, 28)}$  2.367, p = 0.065).

# 6.6.2.2 Dlg2+/- rats show are comparable on all measures of anxiety in the open field task to WT rats

A software malfunction resulted in some data being lost (7 WT, 7 HET). All measures were compared between the genotypes by t tests. All data was normal and homogeneity of variances.

The time spent in arena zones was compared between genotypes using *t* tests. The time spent in the centre,  $t_{(23)}$  1.400, p = 0.175 and perimeter,  $t_{(23)}$  -1.400, p = 0.175 of the arena was comparable between  $Dlg2^{+/-}$  and WT over the 10 minutes (Figure 49a). Both genotypes covered a similar total distance (cm)  $t_{(23)}$  -0.995, p = 0.330, at a similar mean velocity (cm/s),  $t_{(23)}$  -1.145, p = 0.264 (Figure 50b, c). Both genotypes spent a similar percentage of time in the centre and perimeter zones of the maze and exhibit comparable levels of locomotion during the 10 minute task.



Figure 50.  $Dlg2^{+/-}$  and WT rats spent a comparable amount of the 10 minute trial in the centre and the perimeter (a).  $Dlg2^{+/-}$  rats cover a similar distance (cm) (b) at a similar velocity to WT rats (c). n = 12 (WT) 13 (HET). Data represent the mean  $\pm$  SEM error bars.

#### 6.7 Discussion

# 6.7.1 Dlg2<sup>+/-</sup> have reduced Dlg2 mRNA expression but comparable Dlg family member expression in two brain regions

A ~50% reduction in  $Dlg2^{+/-}$  expression was found in the both the hippocampus and PFC. This is in contrast to the mouse, where a reduction was only found in the PFC. Neither model demonstrated a reduction in the cerebellum. This suggests a mosaicism of mutation expression between brain regions, which has now been observed in two separate species in models generated using different techniques. The ~50% decrease in *Dlg2* mRNA observed is in line with the anticipated reduction in expression in a heterozygotic model, as the mutation only affects one copy.

As with the mouse model, no differences were found in the rat for mRNA expression of any other *Dlg* family members. Thus, at the mRNA level no compensatory expression mechanism is apparent in the three regions examined in the rat model. Similar to the mouse model the mutation in *Dlg2* in the rat leads to a selective reduction in the expression of the targeted *Dlg* family member. As a reduction in *Dlg2* mRNA expression was found in the hippocampus the rat model provides a better opportunity to examine the effect of *Dlg2* mutation on hippocampal dependant cognition.

# 6.7.2 Dlg2<sup>+/-</sup> rats do not demonstrate an explicit impairment in motor performance during an accelerating rotarod task

Across the three day protocol there was no difference in the latency (s) to fall between *Dlg2*<sup>+/-</sup> and WT rats. Both genotypes exhibit improvement in performance (increased latency to fall) on Day 1 over the three trials, plateauing by Day 2. This pattern is consistent with the acquisition of a motor learning skill, where greatest improvement is seen on the first day before beginning to plateau over subsequent sessions (Luft and Buitrago, 2005). Improvement in motor ability in this manner reflects the role of learning in this process, as opposed to being an exercise phenomena where the animals keep improving as fitness increases (Buitrago et al. 2004). If the improvement would be anticipated daily. Instead, as observed in this model, the greatest improvement is demonstrated on the first day, with some improvement on subsequent days before plateauing, indicative of a learning process (Buitrago et al. 2004).

The consolidation of learning between the days was examined by comparing the first trial on each day, removing the potential confound fatigue in later trials. If

consolidation has occurred the latency for the first trial should be increased on subsequent days before plateauing (Luft and Buitrago, 2005). The latency to fall on the first trial improves across the three days (p = <0.001) and there is a trend towards a difference in performance between the genotypes (p = 0.07), with the Dlg2<sup>+/-</sup> rats diverging slightly from WT rats by Day 3. This might be indicative of a potential deficit in memory formation that could be drawn out using a more sensitive task. The first phase of consolidation occurs between 10 minutes to 6 hrs post training (Walker et al., 2003). Further consolidation is thought to occur during sleep, improving performance, hence the improvement between days (Luft and Buitrago, 2005). The importance of sleep in the consolidation of motor skill learning has been demonstrated in humans (Pereira et al. 2015), although it is less clear in rodents. Following accelerating rotarod training, sleep was found to promote the formation of spines in a subset of branches in layer V motor cortex of mice, and that neurons activated during task acquisition where re-activated during non-rapid eye movement sleep (Yang et al., 2014). This suggests a fundamental role of sleep in the formation of learning dependant synapse formation, which can be prevented if this re-activation is inhibited (Yang et al., 2014). However, sleep following rotarod training was not found to improve learning of the task, but did improve learning of complex wheel running (Nagai et al., 2017).

The lack of an observable phenotype on the accelerating rotarod is divergent from the mouse model, where  $Dlg2^{+/-}$  mice exhibit a persistently impaired learning in two different protocols, a simple and a more extended training paradigm (Chapter 3 and 4). This may reflect a species difference, both in terms of the mutation itself or task suitability for the animal. As discussed above (6.1) there are caveats comparing the species in general, but there are also methodological differences.

The latency (s) to fall for both genotypes never exceeded 90 s on average, suggesting that the rats may exhibit lower performance on the rotarod task than the mice. By Day 3 the majority of the mice were consistently staying on the rod for the entire 5 minute trial, whereas only 1 rat achieved this once across the three days. It is harder to determine whether this is a species issue or a protocol issue. In the literature there is more variability in accelerating rotarod protocols for rats, with the number of daily trials conducted being less consistent across publications, as well as length of intertrial intervals (ITI), compared to the largely consistent literature for mice (normally between 7 to 10 trials a day with 5 minute ITI). The difference in the number of trials between the species means the trajectory of the learning curve for the rats is less observable, making it more challenging to compare between the species, as the

increased number of trials for mice provides greater opportunity for a deficit to be seen particularly in the earlier stages.

There could be a number of reasons for this difficulty of the task for rats. The size of the rats may be inhibiting the ability of larger rats to stay on the rotarod. The largest rats were ~630g, and although no differences were found between the genotypes for weight there is a trend towards an effect on weight on latency to fall (s) on the rotarod (p = 0.065) (see 6.6.2.1). Therefore there may be a minimal contribution of weight to performance on the rotarod. The rats may exhibit less motivation to stay on the rod (Hånell and Marklund, 2014). The innate fear of falling from the rod serves as the motivational drive to keep moving (Dunham & Miya, 1957), but fear may not be sufficient to maintain behaviour if diminished following falls during previous trials attenuated this fear (Brooks and Dunnett, 2009). Modifications to the task to increase motivation can be implemented, such as placing cold water beneath the rod (Difeo, Curlik and Shors, 2015). The rats may also show gait deficits that would affect maintaining balance on the rotarod. Rats alter their gait whilst running on the rod as training progresses (Buitrago et al. 2004). It is possible that the rats struggled to make this shift in gait pattern. Automated gait analysis whilst rats were walking or running (Mendes et al., 2015), or gait analysis during rotarod training (Buitrago et al. 2004), would be able to determine whether this is a factor for impaired motor performance on the rotarod compared to mice.

An alternative approach to measuring motor performance and learning, may be to use a paw reaching or staircase task (Montoya *et al.*, 1991). In addition to motor coordination and learning, also assessed by the rotarod, this task also probes the innate manipulation skill of each paw (Baird, Meldrum and Dunnett, 2001), requiring rodents to reach and grasp pellets in a narrow space from a central platform and has been used for studies in both mice (Chen, Gilmore, & Zuo, 2014) and rats (Pagnussat *et al.*, 2009a). The staircase task removes the potential confound of fatigue and general fitness of the rat, as they are only required to reach down and retrieve a pellet rather than keep running for a prolonged period of time. Additionally the sugar pellet reward may ensure sustained attention and motivation to complete the task, as opposed to fear of falling from a rod. Previously it has been reported rats reach asymptotic performance and retrieve all the available pellets within 10-15 minutes during a test, following 2 weeks of training (Montoya and Astell, 1990). An impairment in motor learning in this task may be reflected by a reduced number of pellets being retrieved compared to WT rats across the training sessions. There may also be a delay in

reaching criteria for the task (number of trials to retrieve 12, 15 and 18 pellets) (Pagnussat *et al.*, 2009a).

# 6.7.3 *Dlg2*<sup>+/-</sup> rats do not exhibit any anxiety phenotypes during the open field test

Both genotypes spend the majority of the trial in the perimeter of the arena, but do venture into the centre and spend around a third of the trial there. Therefore according to the open field task the *Dlg2<sup>+/-</sup>* rats are comparable to WT rats in presentation of anxiety behaviours. This is comparable to the lack of anxiety phenotypes observed in the mouse model characterised in Chapter 3. Nevertheless, the rat model should also be tested using other anxiety probing paradigms, such as the elevated plus maze or light dark boxes, as the tasks may represent different dimensions of anxiety (Ramos et al., 2008). Given that neither species has exhibited any anxiety phenotypes, but only the rats exhibit reduced expression of *Dlg2* mRNA in the hippocampus this may indicate that the function of *Dlg2* in the hippocampus may not be not important for anxiety behaviours. The locomotor activity of  $Dlq2^{+/2}$  rats in the open field was comparable to WT rats, as was also found in the mouse model However, in both models only examining one 10 minute open field task may only assess the model's immediate response to the novelty of the arena (Spruijt et al., 2014). A habituation deficit was uncovered in the  $Dlg2^{+/}$  mice following repeated exposed to a context for five consecutive days (Chapter 4). Investigation is required into whether this impairment of between session habituation is also seen in the rat model.

### 6.7.4 Strengths and Limitations

The expression of *Dlg2* mRNA is reduced in both the hippocampus and PFC of the rat model in comparison to the more selective reduction observed in the PFC of the mouse model. The rat model may thus be more useful than the mouse model for investigating impairments of higher order cognition as a result of *Dlg2* heterozygosity, as not only are the hippocampus and the PFC independently crucial for cognitive processing, but dysfunctional interaction between the two regions may provide a common element involved in their pathophysiology (Godsil *et al.*, 2013).

Additionally LE rats do not appear to have impaired visual acuity (Prusky *et al.*, 2002), unlike the recessive mutation found in some C56BL6/J mice (Pritchett-Corning *et al.*, 2012), removing a potential confound in data interpretation or using tasks dependent on visual perception. Other behaviours that we observed that were impaired in the mice, acoustic startle and PPI response, could also be examined in the rat model to

examine whether there is conservation of that phenotype, and whether loss of *Dlg2* in multiple brain regions influences responding. Additionally western blot analysis could be conducted to examine whether the reduction in mRNA expression translates to the protein level.

### 6.7.5 Conclusions

There is a reduction in *Dlg2* mRNA in the PFC and hippocampus of the rat model, without any change in expression of other *Dlg family* members, in comparison to the specific reduction in the PFC observed in the mice. On two basic behavioural tests the  $Dlg2^{+/}$  rats do not exhibit any impairments, demonstrating comparable ability to WT rats on the accelerating rotarod, as well as locomotion and anxiety response during an open field test. This indicates a potentially smaller effect of mutation in the rat model compared to the mouse model, where deficits of motor learning were observed. However, in both instances further probing with more sensitive testing would be beneficial, to determine whether any deficits were masked during these tasks. Additionally examination of the rat's acoustic startle and PPI response would also be illuminating, due to the relevance of the task to psychiatric diseases, the findings in the mouse model, and potential effect of deceased *Dlg2* expression in two brain regions as opposed to just the PFC.

# 7 Contextual Fear Conditioning in the Rat Model

### 7.1 Introduction

A major advantage of the rat compared to the mouse model is the ability to probe more complex cognitive processes (Colacicco *et al.*, 2002; Jaramillo and Zador, 2014). Associative learning is dependent on the glutamatergic system, and as *Dlg2* interacts with key receptors in glutamatergic function, NMDA and AMPA, deficits may become apparent when employing tasks which behaviourally probe this system.

Since 1911 abnormal associative learning has been considered a feature of schizophrenia (Bleuler, 1911; Peralta and Cuesta, 2011). More recently has been proposed to play a role in psychosis. Abnormal glutamatergic function is proposed to be upstream of the hyperdopaminergic state thought to underlie psychosis in schizophrenia (Howes & Kapur, 2009; Kapur, 2003c). Psychosis is considered to be the result of aberrant salience, the aberrant assignment of importance to external objects and internal representations, resulting in the formation of abnormal associations. Once learnt these inappropriate associations can be reinforced and become fixed, and delusions may be the result of patients attempting to make sense of the aberrant associations (Kapur, 2003a). NMDAR mediated synaptic plasticity is important for associative learning, including aversive learning (Kosmidis, Breier and Fantie, 1999; Hofer et al., 2001; Jensen et al., 2008a). Mice pre-treated with PCP, a NMDAR antagonist, exhibited a lasting impairment of associative learning using a fear conditioning paradigm (Enomoto et al., 2005). Genetically modified mice lacking Grin1, which codes for the essential NMDAR subunit GluN1, demonstrated deficits in learning during Skinner-box tasks (Hasan et al., 2013).

One established way often used to study associative learning processes is through fear conditioning. This involves the formation of an association between an initially neutral stimulus (conditioned stimulus, CS) and an either rewarded or aversive stimulus (unconditioned stimulus, US) such that presentation of the CS alone will elicit a change in behaviour often equivalent to that exhibited when exposed to the US (conditioned response, CR). This process can be investigated through aversive learning paradigms, such as contextual fear conditioning (CFC) where a novel context or training environment acts as the CS. Fear memory can last a lifetime even when only experienced once (Maren, 2005). In single trial tests, a context (CS) is presented

with an aversive stimulus (US), normally a foot-shock. This form of associative learning is rapid, long lasting and produces a measurable, stereotypical behavioural outcome such as freezing – defined as complete immobility except for respiration in response to re exposure to the CS. Fear conditioning is an evolutionary conserved form of associative learning (Milad and Quirk, 2012; Pattwell *et al.*, 2012), Aversive fear learning is mediated through NMDA receptors in the amygdala and hippocampus (LeDoux, 2003).

Re-exposure to the CS in the absence of US tests the recall and expression of the associations formed during conditioning, with exhibition of the fear response without the US occurring indicates successful formation of association. Consolidation of learning for long term memory is a protein synthesis dependent molecular process that occurs over several time scales (Squire *et al.*, 2015), starting with earlier IEG expression peaking and returning to baseline within 1.5-2 hrs (Ressler *et al.*, 2002), and later synaptic structural changes taking 24-48 hrs (Yang, Pan and Gan, 2009a; Clopath, 2012).

Extinction involves prolonged or repeated exposure to the CS after initial acquisition in the absence of the US, and results in the loss of the CR (Pavlov, 1927; Eisenberg *et al.*, 2003; Suzuki *et al.*, 2004). Extinction is considered a new form of learning, competing with the CR during re-exposure to the CS and reduces responding, as opposed to degrading the original association (Bouton, 2004). Due to the competition between the memories it is possible to retrieve the conditioned memory through experimental manipulations, such as a reminder stimulus, after extinction (Bouton, 2004). As a result of the competition between the memories the CS-US memory can be recovered after extinction learning using a reminder stimulus, demonstrating that the initial conditioned memory was not attenuated or overwritten by the extinction memory (Bouton, 2004; Trent *et al.*, 2015a). As with acquisition of fear memory, extinction is NMDAR dependant (Falls, Miserendino and Davis, 1992), with dose dependant impairment of within and between session extinction observed following administration of NMDAR antagonists (Baker and Azorlosa, 1996; Santini, Muller and Quirk, 2001; Sotres-Bayon *et al.*, 2009).

A number of brain regions are involved in both fear conditioning and extinction. The PFC is important for associative fear learning when it involves a temporal or contextual component (Gilmartin, Balderston and Helmstetter, 2014), although its role in contextual fear appears to depend on the predictive value of the context. When the PFC is lesioned and the sole predictor of the shock the fear memory is largely intact

(Corcoran and Quirk, 2007; Gilmartin and Helmstetter, 2010; Zelinski et al., 2010). However, if there are other reliable shock predictors in addition to context, such as light, then inactivation of the PFC impairs the fear memory (Zhao et al., 2005; Gilmartin and Helmstetter, 2010). The hippocampus is important for contextual learning as it binds together various elements to form a contextual representation (Phillips and LeDoux, 1992; Fanselow, 2000). Without this representation learning cannot occur (Maren, Phan and Liberzon, 2013a). Animals immediately shocked when placed in a chamber do not have to time to form this representation and do not exhibit contextual conditioning (Fanselow, 1990; Wiltgen et al., 2001; Frankland et al., 2004). Additionally if the hippocampus is lesioned post-training recently acquired fear memories are impaired, indicating the hippocampal dependant encoding is the default mechanism for contextual learning (Biedenkapp and Rudy, 2009). Remote fear memories are not affected reflecting the selective memory impairments seen in humans with hippocampal damage (Fanselow, 2000; Rudy, Barrientos and O'Reilly, 2002). The amygdala is important in contextual fear conditioning for co-ordination of the fear response (Fanselow and LeDoux, 1999; Lee et al., 2001; Davis, 2006; Kochli et al., 2015). Sensory inputs converge in the basolateral amygdala where protein synthesis dependant formations of association occurs (Maren et al., 2003; Kwapis et al., 2011) between the CS (context) and US (shock) in the basolateral amygdala (Barot et al., 2009). All three structures are also involved in extinction (reviewed in Maren et al. 2013 and Maren 2011).

Aversive associative learning impairments have been observed in people with schizophrenia (Kosmidis, Breier and Fantie, 1999; Hofer *et al.*, 2001; Jensen *et al.*, 2008a; Hall *et al.*, 2009). As well as a deficient response to the CS patients with schizophrenia also exhibit an inappropriate increase in response to control or unconditioned stimuli (Jensen *et al.*, 2008a). Fear memory extinction deficits have also been found in patients with schizophrenia (Holt *et al.*, 2009). The *Dlg2*<sup>-/-</sup> mouse model demonstrated impaired extinction using an appetitive touch screen task (Nithianantharajah *et al.*, 2013). However, reward based associative learning engages different neural circuits and mechanisms to fear (Schultz, Dayan and Montague, 1997).

Of the other *Dlg* family members only the role of *Dlg4 in* fear conditioning has been examined. The expression of *Dlg4* is increased in the amygdala and other brain regions that contribute to the network supporting fear learning (Mao *et al.*, 2013). Formation of extinction memories rapidly reverses this increase (Mao *et al.*, 2008; Mao *et al.*, 2013). Deletion of insulin substrate-2 increases expression of *Dlg4* and

increased fear memory for both context and cues (Irvine *et al.*, 2011). In the mouse *Dlg4* knock in (KI) model exhibited deficient contextual discrimination. Overall *Dlg4* appears to be important for the stability of acquired fear memory, extinction and contextual discrimination in associative learning. It is currently unclear whether *Dlg2* may be comparably important in these processes.

The different importance of each *Dlg* in cognitive function have been demonstrated in full KO mice (Nithianantharajah *et al.*, 2013). In particular the divergence of roles between the paralogs in associative learning have been observed. *Dlg2* and *Dlg3* KO demonstrated opposing extinction phenotypes; *Dlg3* KO mice demonstrated faster extinction compared to WT in opposition to the slower extinction observed in the *Dlg2* KO mice. As discussed above studies into *Dlg2* in fear conditioning are lacking and are therefore beneficial to undertake.

### 7.1.1 Aims

- 1. Determine whether the mutation in *Dlg2* impacts upon acquisition of fear memory during a single shock trial CFC paradigm.
- Assess whether *Dlg2<sup>+/-</sup>* rats exhibit comparable recall of the fear memory to WT 48 hrs after conditioning.
- Examine whether there is any effect of *Dlg2* mutation on extinction of the CR, or recall of the extinction memory.
- Investigate whether the initial conditioned memory can be recovered following a reminder stimulus after multiple extinction sessions, facilitating discrimination between the strength of the CS-US memory and extinction.

### 7.2 Methods

### 7.2.1 Animals

For a pilot study to optimise the CFC paradigm 15 male Long Evans rats (10 WT, 5 HET) were used and were housed as previously described (2.1.2) with the exception of the being held in the reverse dark light room. For the second experiment a cohort of thirty eight male Long Evans rats (19 WT, 19 HET) characterised in Chapter 6 were rehoused in the reverse dark light room (lights off 10:00 – 20:00) after open field testing. Rats were allowed to acclimatise for 1 week before further testing. At start of testing rats were 5.1 months of age. Rats were transported to the behavioural room and conditioning chambers in large transport cages, which was kept consistent between trials, and returned to home cages immediately following testing. All rats transported in box A were tested in chamber A, and all in box B in chamber B. All trials were completed during the dark phase, at least 30 mins after lights off. During each trial the chamber light was on, turning off when the trial finished. The rats were housed in a reverse dark light room but tested in chambers with the light on so that the holding room context was as distinct as possible from the testing boxes.

# 7.2.2 Contextual Fear Conditioning

### Task

All sessions were conducted in two standard rat modular test chambers (Med Associates Inc., Vermont, USA) previously described in 2.2.2.3. All rats underwent each session in the same chamber. During testing sessions the light turned on in the chamber, staying on for the duration and turning off after the program had finished. This contrasted with the holding room, which was in the dark phase during testing, helping to separate the contexts of the holding room and the testing room. To maintain this difference rats were transported from home cages to the testing room in a light proof box and the box used was constant across all manipulations.

# 7.2.2.1 Experiment 1: Pilot

Previous work from our lab using a *Cyfip1*<sup>+/-</sup> rat model on the Long Evans background found that the WT rats did not condition using a 0.5 mA foot-shock (Simon Trent, personal communication, data not shown). Given the shared background strain, a pilot of 15 rats (10 WT, 5 HET) was conducted with a small cohort of WT and *Dlg2*<sup>+/-</sup> rats. Rats were placed in the chambers for 2 mins to acquire a contextual representation (CS) before receiving a 0.5 mA scrambled shock (US) for 2 secs. Rats

remained in the chamber for 1 min post shock before being returned to home cages. All rats were conditioned and tested between 10:30- 12:30.

Two recall sessions were conducted 24 hr and 1 week after conditioning. Rats were returned to the conditioned context for 2 minutes in the absence of the US.

### 7.2.2.2 Experiment 2: Conditioning

For the main experiment the conditioning protocol was similar to the protocol described above but using a 0.7 mA shock. The cohort was divided across two days (n = 20 on day 1, n = 18 on day 2, counterbalanced for genotype) and all training and test sessions were conducted between 10:30-13:00. This timing was maintained for all subsequent trials.

### 7.2.2.3 Experiment 2: Recall and Extinction

Forty-eight hours post conditioning the rats were exposed to the conditioned context (CS) for 10 mins to elicit extinction of the conditioned memory in the absence of the US (Barnes and Thomas, 2008; Barnes, Kirtley and Thomas, 2012; Trent *et al.*, 2015a). Forty-eight hours post extinction, and 28 days post conditioning, the rats were exposed to the conditioned context for 2 minutes in the absence of the US (long term extinction recall test).

### 7.2.2.4 Experiment 2: Reminder session

Eight days after the long term extinction recall rats were exposed to the conditioned context for 2 minutes. This was co-terminated with a 2 sec 0.25 mA scrambled foot-shock as a reminder stimulus (Trent *et al.*, 2015b). Forty hours later the rats were exposed to the conditioned context for 2 minutes.

### 7.2.2.5 Analysis

Offline analysis for freezing behaviour was scored every 10 seconds blind to condition (Barnes & Thomas, 2008; Trent et al. 2015). Freezing is defined as complete immobility, except for respiration, for 1 second. The number of instances of freezing was divided by the total bin number for each session (i.e. for 2 min recall the number was divided by 12) to generate a percentage freezing for each animal. Two experimenters blind to genotype independently scored each video to ensure reliability.

Normality was assessed for all data using Shapiro-Wilks test. Where appropriate transformations were attempted to correct normality. Extreme outliers (±3 studentized

residuals) were removed. If significance is changed by outliers being retained this is reported. Homogeneity of covariances was assessed by Box's M test and homogeneity of variances was assessed by Levene's test. In instances where the assumption of sphericity was violated the Greenhouse-Geisser correction was applied.

For the pilot percentage freezing across sessions conditioning (pre-US and post-US) and recall (24 hr and 7 days) was compared between genotypes by mixed ANOVA.

For the main experiment the percentage freezing was compared between genotypes by mixed ANOVA for each session type: conditioning (pre-US and post-US), extinction (first and last two minute bins), extinction recall (48 hrs and 28 days), reminder sessions (reminder and recall). Additionally, percentage freezing was compared between genotypes across the 10 minute extinction session in 2 minute bins by mixed ANOVA.

### 7.3 Results

# 7.3.1 Experiment 1: A 0.5 mA shock is does not induce sufficient CFM in either genotype

A small pilot was conducted to determine whether a 0.5 mA shock was sufficient to induce a sufficient conditioned fear memory. Pilot data was normal except 7 day WT, 24 hr HET and all pre-US data. There was homogeneity of variances and covariances for conditioning. One outlier was found for recall sessions (HET) and upon removal there was homogeneity of variances and covariances, and corrected normality for 24 hr HET data. The outlier was removed from conditioning analysis without impact.

The percentage freezing pre-US and post-US was compared using mixed ANOVA (Figure 51). Both genotypes exhibited increased freezing post-US (SESSION:  $F_{(1, 12)}$  21.511, p = 0.001, GENOTYPE:  $F_{(1, 12)}$  26.144, p = 0.991, SESSION X GENOTYPE:  $F_{(1, 12)}$  0.036, p = 0.852).

Fear memory recall was compared between the genotypes 24 hrs and 7 days post conditioning using mixed ANOVA (Figure 51). Both genotypes demonstrated a weak CR and similar percentage freezing in the recall sessions (SESSIONS:  $F_{(1, 12)}$ 3.051, p = 0.106, GENOTYPE:  $F_{(1, 12)}$ 0.003, p = 0.950, SESSION X GENOTYPE:  $F_{(1, 12)}$ 3.051, p = 0.106).



Figure 51. Both genotypes exhibited increased freezing following a 0.5 mA shock, but no difference in percentage freezing across 2 recall sessions (24 hr and 7 days). n = 10 (WT), n = 4 (HET). Data represents mean  $\pm$  SEM.

# 7.3.2 Experiment 2: Dlg2+/- rats exhibit normal acquisition, extinction and recall following a single a 0.7 mA foot-shock

Two rats were excluded from analysis due to repeated escaping from the chamber. One further WT was found to be an outlier in all tests and removed. Several videos during the reminder recall session were corrupted (4 HET). Most data was normal. Extinction first and last 2 minutes, and both extinction recall sessions had homogeneity of variances and covariances. Pre-US and post-US had homogeneity of covariances but not variances and reminder and recall session only had homogeneity of variances, not covariances. Following on from the pilot study in order to increase CFM in both genotypes the shock intensity was increased to 0.7 mA.

 $Dlg2^{+/-}$  rats were able to acquire fear memory similarly to the WT rats. The percentage freezing exhibited between  $Dlg2^{+/-}$  and WT rats pre-US and post-US was analysed by mixed ANOVA (Figure 52 a). Both genotypes exhibited increased percentage freezing in the minute post-US compared to pre-US (SESSION:  $F_{(1, 33)}$  108.623, p =

<0.001, GENOTYPE:  $F_{(1, 33)}$  0.048, p = 0.828, SESSION X GENOTYPE:  $F_{(1, 33)}$  0.002 p = 0.962).

Both genotypes demonstrated robust CFM in the first two minutes of the extinction session, but *Dlg2* mutation did not impair extinction or extinction recall. The percentage freezing 48 hrs post conditioning was examined for the first 2 and last 2 minutes of a 10 minute extinction session using mixed ANOVA (Figure 52a). Both genotypes exhibited a reduction in percentage freezing between the first and last 2 minutes (TIME BIN:  $F_{(1, 33)}$  14.298, p = 0.001, GENOTYPE:  $F_{(1, 33)}$  0.031, p = 0.861, TIME BIN X GENOTYPE:  $F_{(1, 33)}$  0.000, p = 0.986). Extinction across the 10 minutes was then examined in 2 minute bins by mixed ANOVA (Figure 52b). The assumption of sphericity was met, X<sub>2</sub> (9) 7.942, p = 0.541. Over the 10 minutes the percentage freezing was reduced for both genotypes comparably (TIME BIN:  $F_{(4, 132)}$  7.471, p = <0.001, GENOTYPE:  $F_{(1, 33)}$  0.008, p = 0.929, TIME BIN X GENOTYPE:  $F_{(1, 132)}$  0.143, p = 0.966).

Extinction recall was then tested 48 hours and 28 days post extinction and analysed using mixed ANOVA (Figure 52a). The CR for both genotypes was lower for both 48 hr and 28 day, demonstrating between session extinction. However, there was no difference in percentage freezing between the two recall sessions nor between genotypes (SESSION:  $F_{(1, 33)} 0.074$ , p = 0.788, GENOTYPE:  $F_{(1, 33)} 0.018$ , p = 0.894, SESSION X GENOTYPE:  $F_{(1, 33)} 0.778$ , p = 0.384).

Finally, neither genotype responded to the 0.25 mA reminder stimulus. The percentage freezing was analysed during the reminder stimulus session and a subsequent 48 hr recall session using mixed ANOVA (Figure 52a). Both genotypes exhibited weak CR in the final recall session after the reminder session, indicating no US mediated recovery of the CR as would be expected (Bouton, 2004; Trent *et al.*, 2015a) (SESSION:  $F_{1, 33}$  1.167, p = 0.288, GENOTYPE:  $F_{(1, 33)}$  1.090, p = 0.304, SESSION X GENOTYPE:  $F_{(1, 33)}$  0.110, p = 0.743).

In summary, *Dlg2* mutation did not affect acquisition of CFM following a 0.7 mA shock, there was no impact on within session extinction and both genotypes showed long term extinction with no spontaneous recovery of CFM 28 days after extinction training. A reminder stimulus was not effective for either genotype.



Figure 52. Dlg2 mutation did not affect percentage freezing in response to a single 0.7 mA shock during CFC, extinction or recall sessions compared to WT (a). Weak intrasession extinction was exhibited by both genotypes during the 10 minute extinction trial 48 hrs after conditioning (b). n = 17 (WT) 18 (HET). Data represents mean  $\pm$  SEM.

### 7.4 Discussion

# 7.4.1 A 0.5 mA shock was not sufficient to produce adequate freezing for manipulation in either genotype

The pilot study demonstrated that both genotypes were able to acquire an association between the CS and the US at 0.5 mA. There was a substantial reduction in freezing between post-US and 48 hr recall which may suggest an impairment in consolidation of fear memory in both WT and HET rats. This low level of conditioned freezing precludes further manipulation to study long term memory or extinction. A similar effect was observed in a Cyfip  $1^{+/-}$  rat model, also from a Long Evans background, with both genotypes exhibiting little conditioning to a 0.5 mA shock (Simon Trent personal communication, data not shown). These experiments demonstrate that Long Evans (LE) rats require a higher intensity US for this protocol than Lister Hooded (LH) rats, the strain with which this protocol was generated with (Simon Trent, personal communication). Strain differences in CFC between Sprague Dawley (SD) and LE rats have been observed, with SD exhibiting more freezing and ultrasonic vocalisations than LE rats (Graham et al., 2009). It was also noted in both the Cyfip<sup>+/-</sup> and WT LE rats that greater freezing post-US and during recall was observed following a 0.8 mA shock, but this shock intensity prevented extinction of the fear memory (Simon Trent personal communication, data not shown). Previously weaker extinction was observed following more intense shocks (Annau and Kamin, 1961). In order to induce a greater freezing response, without inhibiting extinction, a 0.7 mA shock was used with LE rats for subsequent studies.

# 7.4.2 Dlg2 mutation did not affect acquisition, extinction or recall of fear memory following a 0.7 mA shock

Both genotypes acquired CFM following a single 0.7 mA shock. Across the 10 minute extinction session both genotypes demonstrated extinction when analysed in 2 minute bins, and when compared between the first and last 2 min bins.

Both genotypes demonstrate a similarly reduced CR 48 hrs post extinction, which is maintained when tested again 28 days post conditioning. This suggests neither genotype exhibited spontaneous recovery. This refers to the resurgence of the conditioned freezing memory following extinction with the passage of time (Milad and Quirk, 2012). Spontaneous recovery is thought to reflect a failure to retrieve the extinction memory as opposed to its loss (Bouton, 1993).

In addition, neither genotype demonstrated a resurgence of freezing behaviour in the recall session following a reminder stimulus session which co-terminates with a 0.25 mA shock. Normally the CR reappears after a reminder session (Bouton, 2004; Trent *et al.*, 2015a). The reminder stimulus used may have been too low in this instance; the stimulus needs to be sufficient to retrieve the conditioned memory without resulting in new learning (Cai *et al.*, 2006; Myers and Davis, 2007; Blundell, Kouser and Powell, 2008). Given the need to avoid facilitating new learning a stimulus strength previously successful in provoking freezing behaviour was selected (Trent *et al.*, 2015). However, given the previously demonstrated weaker response of LE rats to a 0.5 mA shock a greater intensity reminder may have been required. Alternatively, there may have been no memory to retrieve, given the weaker CR following conditioning.

The ability to learn the association between CS and the US, as well as later extinction of CR, were particularly interesting to examine given the previously observed deficit in extinction in  $Dlg2^{-/-}$  mice (Nithianantharajah *et al.*, 2013). To investigate the mouse's ability to acquire associations the mice underwent operant conditioning, with the selection of the correct image in the correct location triggering a food reward. Extinction was explored by studying how long until response reduced in the absence of reinforcement. The  $Dlg2^{-/-}$  mice learnt the association but demonstrated slower extinction than WT mice (Nithianantharajah *et al.*, 2013). By comparison the  $Dlg2^{+/-}$ rats in our study demonstrated normal acquisition of the CS-US association during CFC and both genotypes exhibited extinction of the fear memory.

Comparing the KO mouse and our rat model there are factors that may influence the different outcomes. Gene dosage may be important, as our rat model is still expressing one copy of *Dlg2* as opposed to the mouse model which previously demonstrated an extinction deficit being a full KO. The presence of one copy of *Dlg2* may be sufficient to prevent impairment in NMDAR dependant processes, such as fear memory acquisition and extinction. Indeed the presence of a single copy of *Dlg1* enabled mice to learn normally, whereas the full deletion is embryonically lethal (Nithianantharajah *et al.*, 2013). Another factor could influence our findings is the weaker CFM induced in both genotypes, which may indicate a less persistent CFM. We observed that LE rats require a high intensity shock to produce a robust CFM, with both genotypes demonstrating around 50-60% freezing post-US following a 0.7 mA shock. Additionally the reminder stimulus failed to recover the CR. Whilst this may be due to a stronger extinction memory that could not be out competed this seems less likely, particularly given the increased difficulty of extinguishing CFM resulting

from higher intensity shocks (Annau & Kamin, 1961). Instead it is possible that the acquired CFM was weaker to start with. The reminder stimulus acts a cue for retrieval of the CS/US memory, but if this memory has been forgotten then there is no memory to retrieve. Given these methodological restrictions it may not have been possible to uncover any genotypic differences.

Furthermore, the lack of an extinction deficit in our rat in comparison to the KO mouse model may be due to differences in appetitive vs aversive learning. Appetitive learning capitalises on the rodent's response to a rewarding stimulus (Itzhak, Perez-Lanza and Liddie, 2014). Positive reinforcement of the response, given in temporal relation to the stimulus, increases the response probability by placing the emphasis on the relationship between the stimulus and response, as opposed to the association between two stimuli (Skinner, 1938; Martin-Soelch et al. 2007). The time taken for the association to form between the positively reinforced stimulus and the reward is much longer, requiring more training, than the fundamental fear response invoked during aversive conditioning. Given the prolonged training period required it is arguable that appetitive learning is more complex than one trial aversive learning paradigms, and as a result provides more opportunity for deficits to become apparent and influence behaviour.

### 7.4.3 Strengths, limitations and future directions

As observed in Chapter 6 the *Dlg2*<sup>+/-</sup> rats are not anxious or hyperactive compared to WT rats (6.6), phenotypes which could have confounded the interpretation of CFC data. For example, immobility (freezing) and hyperactivity are mutually exclusive, whilst anxious rats could exhibit greater innate freezing regardless of learning a CS-US association. The lack of such phenotypes suggests there was no influence of these behaviours on the CFC experiments in this Chapter.

However, strain differences are likely to have impacted upon the findings in this Chapter. Previously it has been shown that LE exhibit less freezing and vocalisation than SD rats during CFC studies (Graham *et al.*, 2009). The authors suggested this may be the result of increased fear expression in SD than LE, or faster and/or stronger fear conditioning in the SD (Graham *et al.*, 2009). The protocol described during this Chapter was initially developed using a third strain, LH rats (Simon Trent, personal communication). As discussed 6.6.2.1 there were issues determining a sufficient shock level to induce robust a CFM in both mutant and WT LE rats, across two different models. Therefore it is quite possible that the paradigm itself needs modification to be able to draw out any genotypic differences in models with a LE

background. This could include increasing the number of shock/context pairing sessions or altering the extinction protocol (multiple sessions as opposed to one long session).

A potential difference in pain sensitivity may exist between genotypes. Antisense knockdown of *Dlg2* was found to attenuate paw withdrawal response to acute thermal and mechanical stimulation during a neuropathic pain test, a response dependant on NMDAR activation in the spinal cord (Zhang *et al.*, 2003). Given the genotypes responded comparably post-US a differential pain response to the shock is unlikely, but could be tested by conducting a tail flick or hot plate test (Bannon and Malmberg, 2007).

The simplicity of the single trial CFC task employed provides a useful starting point to investigating associative learning. Given the association of *Dlg2* and NMDA receptors, and the importance of NMDAR in fear memory and extinction (Falls, Miserendino and Davis, 1992; Baker and Azorlosa, 1996; Santini, Muller and Quirk, 2001; Sotres-Bayon et al., 2009), combining ketamine injection and CFC may reveal differential effects on extinction (Clifton, Thomas and Hall, 2018). As discussed in Chapter 4 this additional manipulation, on a system that may be weakened by Dlg2 haploinsufficiency, may provide more challenge than can be accommodated. Ketamine has already been shown to impairs extinction in WT rats (Clifton, Thomas and Hall, 2018). Another approach would be to modify the protocol. The shock during conditioning could be increased in mA, and to counter the possibly strengthened extinction conduct multiple days of extinction testing. As well as probing recall in the conditioned context examining behavioural responses it in a second context may reveal whether the  $Dlg2^{+/2}$  rats exhibit a generalisation of the fear memory and failure to discriminate between contexts, as has been observed in multiple Dlg4 mutant models (Nagura et al., 2012a; Fitzgerald et al., 2015). Alternatively a discrete CS, such as a tone or light could be used instead of a context, so called cued conditioning (Curzon, Rustay and Browman, 2009). Cued conditioning, like contextual conditioning, involves the amygdala (Phillips and LeDoux, 1992). However, hippocampal lesions do not impair CR acquisition in cued conditioning paradigms, only contextual ones, implying that the hippocampus is more important for the formation of a contextual CS-US association (Otto and Poon, 2006). Another paradigm that could be employed to further explore the role *Dlg2* haploinsufficiency in associative learning is latent inhibition (LI). LI involves pre-exposure to the CS prior to co-occurrence with the US, reducing its predictive value and subsequently the likelihood of association formation (Lubow, 1973). LI is impaired in schizophrenia

patients, thought to result from the inability to filter out irrelevant stimuli (Lubow, 1973; Swerdlow *et al.*, 1996).

## 7.4.4 Conclusions

When tested with a single shock trial CFC mutation in *Dlg2* did not affect acquisition of CFM, nor the extinction of this memory. Any genotypic effects may have been masked by the necessity of increasing the shock to 0.7 mA in order to achieve an anticipated level of freezing. Going forward more complex protocols may be better able to uncover any differences.

# 8 General Discussion

# 8.1 Introduction

Abnormal synaptic functioning has been repeatedly implicated through multiple approaches in the risk of developing psychiatric diseases. *Dlg2* is an important PSD protein involved in the functioning of glutamatergic receptors, which forms part of the molecular basis for learning and memory. Disruption of glutamatergic signalling may contribute to the cognitive deficits observed in schizophrenia. Mutations in *Dlg2* have been associated with schizophrenia, ASD and BPD. Previous research has implicated mutations in *Dlg2* with impaired cognitive flexibility, as KO mice exhibited deficient extinction, reversal learning (Nithianantharajah *et al.*, 2013) and motor learning (Winkler *et al.*, 2018). However, behavioural characterisation of *Dlg2* mouse models is still limited and the vast majority of current literature was obtained using full KO models. All data presented in this thesis is from more clinically relevant heterozygous *Dlg2* rodents, which are more translatable to humans heterozygous for *DLG2* CNVs.

This Chapter will present the key findings from each chapter of this thesis and discuss the potential implications, before going on to outline the limitations of the work and how to take it further with potential future investigations.

# 8.2 Summary of Findings

The characterisation of two heterozygous rodent models was detailed in this thesis. The mouse model employed is a different strain to that of all currently published research on *Dlg2* mutation and the rat model is completely novel. It was therefore important to examine the impact of the mutation in each model. The expression of *Dlg2* mRNA in important brain regions for processes affected in psychiatric diseases (PFC, hippocampus) as well as control regions (cerebellum) was examined. In the mouse model (Chapter 3) a significant reduction in *Dlg2* mRNA expression was found in the PFC, but not the hippocampus or cerebellum. In contrast in the rat model (Chapter 6) a significant reduction was found in both the PFC and hippocampus, but not in the cerebellum. The selectivity of the reduced regional expression in of the mouse, compared to rat model, may have influenced the behavioural phenotypes observed.

Within the *Dlg* family of proteins there is possibility for a functional redundancy between the different proteins (discussed in 1.4.1) therefore it was important to

investigate if there were any changes in expression of *Dlg1*, *Dlg3* or *Dlg4* in the rodent models. No evidence was found of changes in mRNA of the other *Dlg* family members in any brain region of either the mouse (Chapter 3) or rat (Chapter 4) models.

Basic behavioural characterisation was also conducted in each model. No evidence was found in either the mouse (Chapter 3) or the rat (Chapter 4) for altered locomotor activity levels or anxiety phenotypes. Acoustic startle and PPI response, locomotive response to a novel context, and motor function/learning were investigated in the mouse model. Differences compared to WT mice were observed in motor learning on the accelerating rotarod task, acoustic startle responses and habituation to an acoustic stimulus. On an accelerating rotarod task male but not female *Dlg2*<sup>+/-</sup> mice demonstrated impaired motor learning, maintaining a shorter latency to fall for longer before eventually matching performance of the WT mice with further training. In the rat model (Chapter 6) only the accelerating rotarod task was conducted, and in contrast to the mouse no differences were found. *Dlg2*<sup>+/-</sup> mice exhibited a reduced startle response to a 120-dB acoustic stimulus and did not demonstrate habituation to this stimulus.

As part of the ketamine-induced locomotion challenge (Chapter 4), interrogating the interaction between *Dlg2* and NMDAR, mice were given five daily habituation sessions to reduce novelty induced locomotion in response to the context prior to ketamine injection. It was found that  $Dlg2^{+/-}$  mice exhibited reduced habituation across these sessions compared to WT mice. However subsequently, no differences in activity in response to ketamine were found between the genotypes. Whilst the deficient habituation provides further evidence of deficient habituation in  $Dlg2^{+/-}$  mice in a second task it is in contrast to the context shift experiment in Chapter 3 using the same locomotor boxes. In this test neither genotype demonstrated between session habituation, although both demonstrated within session habituation, across four sessions prior to the final testing day.

There are several factors that may have contributed to these conflicting results. The context shift habituation sessions were much shorter than for the ketamine study, 30 minutes versus 2 hrs. It may be that this is not sufficient time daily to result in significant habituation across multiple days, and that more consecutive days would be needed to achieve this. It is also possible that the lighting may have influenced the outcome. Whilst both studies were conducted in the light phase of the day the context shift experiment required the light to be on during the task, so that the mice could observe the patterns on the walls, whereas the ketamine study was conducted in the

dark. Finally, as highlighted in Chapter 3 there is a potential visual issue in the background strain of the mice. The cohort tested in Chapter 3 had not been crossed to eliminate the possibility of this occurring, and so it could be that the mice did not behave as anticipated due to impaired vision and may not have been able to easily see the visual clues. By contrast the cohort in Chapter 4 had been crossed to prevent this potential issue, and were less reliant of visual cues being tested in the dark in clear boxes.

The delayed motor learning phenotype was further investigated in Chapter 4. A separate cohort of mice underwent an extended training version of the accelerating rotarod task which increased the number of daily trails. Additionally, the mice were tested on three consecutive days to examine consolidation of learning. This modified protocol enabled examination of whether the motor learning deficit was specific to fast or slow motor learning. The early motor learning deficit observed in Chapter 3 was replicated. In the extended task, male *Dlg2<sup>+/-</sup>* mice exhibited deficient motor learning during the earlier phase of learning, and motor functioning was not impaired, as with increased training the *Dlg2*<sup>+/-</sup>mice eventually reached a similar performance level as WT mice. Both genotypes exhibited skill learning consolidation across the three days. No deficit was found using this protocol with female mice, implying that the impairment was sex specific. The cellular basis of this phenotype was then investigated (Chapter 4). When comparing neuronal activity in M1 of the motor cortex following rotarod training, *Dlg2<sup>+/-</sup>* mice in the trained group did not express the increase in cFos staining observed in the WT mice, indicating less neuronal activity during early phase motor learning.

The deficits observed in the mouse model were found in fairly simple learning tasks that involved non-associative learning reliant on synaptic plasticity (Chapter 3 and 4). Associative learning and cognitive flexibility, such as extinction, was investigated in the novel rat model using a single shock contextual fear conditioning paradigm (Chapter 7). Neither genotype exhibited sufficient conditioning to the initially tested 0.5 mA shock, so the intensity was increased to 0.7 mA. Following the 0.7 mA shock contextual fear conditioning was found to be intact in  $Dlg2^{+/-}$  rats. Across a 10 minute trial both genotypes exhibited comparable extinction of the fear memory, and no differences were found for recall of extinction in subsequent sessions.

The final investigations in the mouse model examined the potential impact of *Dlg2* mutation on adult neurogenesis in the DG of the hippocampus (Chapter 5), a process that may reflect a convergent phenotype across synaptic risk gene models

(Westacott, 2016; Haan *et al.*, 2018). When comparing expression of the new-born neuron marker DCX, no differences in the basal rate of neurogenesis between the genotypes were found in 8-week-old mice or older 8-month-old mice. Rates of neurogenesis did not differ for either genotype between behaviourally naive mice and those which had undergone behavioural testing.

#### 8.2.1 Does heterozygosity of Dlg2 impact upon brain functioning?

Previous behavioural examination of *Dlg2* mutant models was limited both in abundance and scope. The majority of the literature available focused on KO mice, as opposed to the more disease relevant heterozygotes, probed a limited range of behavioural tasks and was all conducted in one *Dlg2* mutant strain. This thesis is also the first time *Dlg2* mutation has been investigated in a rat model. The behavioural experiments conducted with the *Dlg2*<sup>tm1a(EUCOMM)Wts</sup> strain demonstrated that the male  $Dlg2^{+/-}$  mice are not generally impaired. There was no evidence of phenotypes in general locomotion, motor function, PPI response or anxiety in *Dlg2*<sup>+/-</sup> mice.

Thus, there is no evidence of abnormalities in the mice, such as generalised hypo- or hyperactivity, nor anxiety phenotypes, which could have confounded other tests. This supports a recent study, where neither homozygous or heterozygous male *Dlg2*<sup>-/-</sup> mice exhibited altered anxiety measured during the open field or light/dark tests (Winkler *et al.*, 2018).

However,  $Dlg2^{+/2}$  mice did exhibit behavioural specific deficits; habituation to an acoustic stimulus and deficient motor learning. Acoustic startle and PPI response had not been investigated in Dlg2 mutant mice, therefore this represents a novel observation.

The first paper investigating a *Dlg2* mouse model found no differences between WT and *Dlg2* mutant mice on rotarod tasks (McGee *et al.*, 2001), whereas Winkler *et al* (2018) supports the impaired motor learning phenotype observed in male *Dlg2*<sup>+/-</sup> mice, and found an even more severe phenotype in *Dlg2* KO. Given that this deficit has been seen in two different strains, and across experiments using different motor learning paradigms in this thesis (Chapter 3 and Chapter 4), it is likely that impairment in motor learning is a genuine phenotype of *Dlg2* heterozygosity. Furthermore, the observation of reduced neuronal activation in M1, as measured by cFos expression in *Dlg2*<sup>+/-</sup> mice during motor learning (Chapter 4) points towards a biological basis for the deficit.

In the novel rat model motor learning, locomotor activity and anxiety were investigated (Chapter 6). As with the mouse model there was no evidence of altered locomotor activity or anxiety. Although conducting further anxiety tests, such as light/dark box or EPM, would be beneficial to support this conclusion as multiple tasks may assess a wider spectrum of the emotional profile of the rats (Ramos et al., 2008). An accelerating rotarod task was employed to assess motor learning, although the corresponding motor function task, involving the set speed rotating rod was not conducted due to time constraints, so conclusions cannot be drawn regarding motoric functioning in the rats. In contrast to the mouse model no differences were found in motor performance, and similar to the mice there were no differences in consolidation between the genotypes. There are a number of possible explanations for this discrepancy between the mouse and rat models. In general the rats performance was worse than on the rotarod task than the mice, with the average latency to fall never exceeded 90 seconds for the rat, even after multiple sessions. By comparison the majority of the mice were remaining on the rod for the 5-minute task duration despite undergoing more trials than the rats. The difference in protocols may also have influenced the ability to detect any differences in the rat model. The task for rats had a longer inter-trial interval and less trials per session, therefore the slope of the learning curve is reduced when compared to the 7 trials per session in the mice, making it more difficult to uncover subtle deficits. As suggested in Chapter 6 an alternative task for assessing motor learning, such as the staircase task (Montoya et al., 1991; Pagnussat et al., 2009b), may be more practical for the rats as it removes fatigue as a confound.

Contextual fear conditioning was used to investigate associative learning processes in the rat model in Chapter 7. No differences in acquisition of a fear memory, extinction learning or recall of the extinguished memory were found between the genotypes following a single shock trial. The protocol employed was quite simple, and as such may not have been sensitive enough to draw out deficits in any of the learning processes, which may only become apparent with increasingly complex manipulations. This could be addressed by increasing the complexity of the protocol through introduction of multiple contexts, using different extinction protocols, or employing a different approach, such as latent inhibition (LI). LI relies on the PFC and hippocampus and involves active inhibition of the CS-US association. This inhibition occurs as a CS no-US association is formed during the pre-exposure to the context, which then interferes with the formation of the CS-US association during conditioning

(Escobar et al 2002). This approach may therefore represent an alternative means for assessing cognitive flexibility in the rat model. The lack of any impairment in extinction is in contrast to the delayed extinction seen previously in a *Dlg2* KO mouse (Nithianantharajah *et al.*, 2013). This could be attributed to a species difference, a gene dosage effect (one functional copy in the rat vs a full KO mouse) or task type (aversive learning in our rat vs appetitive learning in the mouse).

Only a few basic behavioural tasks have been conducted so far in the rat model, which demonstrated no current indication of abnormalities in anxiety or locomotor activity. However, as the rat model is completely novel further characterisation should be conducted to explore the impact of the mutation, for example probing acoustic startle and PPI response, habituation to a novel context or different cognitive processes like working memory or spatial memory.

Of the tasks assessed in both species so far in this thesis there has not been convergence of phenotypes. It is possible that species differences may mean some phenotypes will not translate into the rat model, as may be the case with the lack of an extinction phenotype in Chapter 7 that had previously been seen in another Dlq2 KO mouse strain (Nithianantharajah et al., 2013). However, it is also important to recognise that examining the same processes may require optimisation of experimental conditions and training protocols for each species. For example, as discussed above, rotarod tasks may not be the most effective means to investigate motor learning in the rat model. Whilst at present there is no convergence of phenotypes the rat is still a useful model from which important insights could be gained. One major advantage the rat model has over the mouse model described in this thesis is the reduction *Dlg2* mRNA in the hippocampus, which was not found in the mouse. The rat model, therefore, is better suited than the mouse to studying the impact of *Dlg2* mutation on the hippocampus, and hippocampal dependant tasks. For example, it is possible no difference was found in DCX expression in the DG of the hippocampus in the heterozygous *Dlg2* mice because there was no apparent difference in *Dlg2* expression in this region.

The deficits observed in the  $Dlg2^{+/2}$  mice in this thesis involve cognitive processes that are arguably less complex than previously reported phenotypes, delayed reversal learning, delayed extinction, and attentional deficits, in Dlg2 KO mice (Nithianantharajah *et al.*, 2013). This could stem from a gene dosage effect, an

interpretation supported by the increased severity of the motor learning phenotype between *Dlg2*<sup>+/-</sup> mice and KO mice (Winkler *et al.*, 2018).

Habituation is an universal behaviour often described as the simplest form of learning (Harris, 1943; Thompson and Spencer, 1966; Groves and Thompson, 1970). Motor learning is specifically associated with gaining a new motoric skill, and that once learnt it can be automatically executed, as opposed to being cognitively demanding (Dayan and Cohen, 2011; Kal et al., 2018). Arguably this is at odds with the suggestion previously put forward by (Nithianantharajah et al., 2013) that DLG2 is only important for more complex cognitive tasks. Perhaps, it is better to frame the behavioural deficits found the Dlg2 knockdown and knockout models as being reflective of abnormalities of flexibility and adaptivity. For both phenotypes reported in this thesis, impaired habituation and deficient motor learning, responding to the environment is important. The ability to stay on a rotarod at a constant speed was normal in *Dlg2*<sup>+/-</sup> mice. Thus, motor performance was normal when conditions were stable. Only when the speed of the rod increased during a trial and there was a requirement to adapt to this change did a deficit become apparent. Additionally, this deficit begins to normalise as training progresses because the need for a behavioural adjustment in motor coordination begins to diminish with the reduced cognitive demand of the task. Impairments in habituation were demonstrated both in response to an acoustic stimulus (Chapter 3) and an environment (Chapter 4). Habituation to an environment is initially comparable to WT mice but gradually diverges, potentially reflecting a sensitisation phenotype on the last day of habituation that may have become more pronounced with further sessions. Habituation plays a role in filtering input and ensuring only the most relevant and important draws attention, such as to threats or potential rewards (Turatto, Bonetti and Pascucci, 2018). There would be little benefit to expend energy exploring an environment that is already known to be lacking in food, for example. In the Dlg2<sup>+/-</sup> mice the context appears to retain its novelty for a prolonged period of time, meaning some aspect of the habituation process is functioning abnormally.

# 8.3 Relevance of phenotypes observed in Dlg2<sup>+/-</sup> mice to psychiatric diseases

### 8.3.1 Impaired motor learning

Mutations in *Dlg2* have been associated with schizophrenia (Kirov *et al.*, 2012; Fromer, *et al.*, 2014), ASD (Egger *et al.*, 2014; Xing *et al.*, 2016) and BPD (Noor *et* 

*al.*, 2014). Motor learning deficits have been observed in schizophrenia (Isohanni *et al.*, 2001; Kodama *et al.*, 2017), ASD (de Moraes *et al.*, 2017), and recently BPD (Chrobak *et al.*, 2015, 2017).

Motor learning has been found to be slower, or less efficient in schizophrenia patients (Kodama *et al.*, 2017) and may even predict the later development of psychosis (Isohanni *et al.*, 2001). The earlier onset of these motor phenotypes during childhood, such as delays to ability to walk, compared to more traditionally considered symptoms, like psychosis that emerge in adolescence, may therefore provide one aspect with which to stratify children at risk of going on to develop schizophrenia.

Motor deficits and delays in development are common co-morbidities of ASD (Lai, Lombardo and Baron-Cohen, 2014), but are not diagnostic criteria for the disorder (Marko *et al.*, 2015). Children with ASD have demonstrated impaired motor learning on a task which relies on visual error learning, previously found to rely on lobule VI and some of lobule VIII of the anterior cerebellum, which was smaller compared to healthy controls (Marko *et al.*, 2015). A systematic review found there appears to be a specific pattern of deficits in motor learning in ASD, which was likely attributed to slower rates of learning (de Moraes *et al.*, 2017). It is also interesting to note that the most common neurobiological finding in post mortem ASD brains is a reduction in Purkinje cell numbers; *Dlg2* localises to the PSD and dendritic microtubules of Purkinje neurons in the cerebellum (Brenman *et al.*, 1998).

One critical component for motor behaviour, as well as social and communication, is the generation of internal models of action (Shadmehr and Krakauer, 2008; Mostofsky and Ewen, 2011). Following a movement the brain forms an association between the motor command and the sensory feedback so that it can predict the sensory consequences of the self-generated action (Shadmehr and Krakauer, 2008). The developmental nature of ASD suggests that the core deficits observed, including motoric skill, may result from abnormalities in the process of generating these internal models (Provost et al. 2007). Indeed, when the processes involved in this internal model generation were examined in children with ASD it was different to that relied upon by normally developing children (Haswell *et al.*, 2009). Whereas the healthy controls primarily relied upon visual feedback whilst acquiring a novel action pattern those with ASD excessively relied on proprioceptive feedback, discounting the visual (Haswell *et al.*, 2009). Interestingly this impaired motor learning in ASD may be reminiscent of prediction errors in schizophrenia.

Dysfunctional predictive coding in schizophrenia is proposed as an explanation of hallucinations (Sterzer et al., 2018). According to this account, bottom up perceptual signals interact with higher order cognitive processes in order to generate expectations about the environment (Fletcher and Frith, 2009; Nazimek, Hunter and Woodruff, 2012). Received input can then been compared to these expectations. If the stimuli match the expectations then they are suppressed, whereas when discrepancies between the stimuli and prior expectations occur they trigger a mismatch signal, or a "prediction error" (McCleery et al., 2018). These errors demonstrate the predictive model was incorrect and needs to be updated to accommodate the triggering stimuli, to prevent further errors. Hallucinations are argued to result from erroneous environmental expectations and result in dampening of predicative error signalling (Northoff and Qin, 2011; Nazimek, Hunter and Woodruff, 2012; Horga et al., 2014). Incoming sensory input, attributed to aberrant sensory cortex activation as opposed to external stimulation, is consistent with prior expectations and so does not trigger a prediction error. This false perception results from the sensory cortex erroneously anticipating a perceptual event (McCleery et al., 2018). Biologically it has been proposed that these prediction errors are encoded by phasic dopamine signals (Nasser et al., 2017).

In BPD, investigation of motoric phenotypes is much more limited but there appear to be deficits in implicit motor learning tasks using the serial reaction time task (SRTT) (Chrobak et al., 2015, 2017). The SRTT task requires participants to learn a sequential pattern of finger presses, which occurs without conscious recognition this sequence. The sequence is mixed in with random order stimuli, allowing comparison of the participant's reaction time to the random order and repetitive sequence blocks (Tzvi, Münte and Krämer, 2014). This task engages key regions of the brain involved in implicit motor learning; primary motor cortex, PFC, striatum, posteriori parietal cortex and cerebellum (Dayan and Cohen, 2011; Tzvi, Münte and Krämer, 2014). Some studies have found deficits in this task in schizophrenia patients whilst a smaller number found no impairments (Remillard 2014, for review). In right handed participants, the deficits in a BDP cohort were different to those observed in schizophrenia cohort (Chrobak et al., 2017). BDP patients exhibited a deficit performing the task with either hand, whilst schizophrenia patients were able to demonstrate some motor learning with the right hand, but not the left (Chrobak et al., 2017).
#### 8.3.2 Reduced acoustic startle amplitude and habituation

In Chapter 3 *Dlg2*<sup>+/-</sup> mice exhibited a decreased response to the highest intensity stimulus, 120 dB, and lack of habituation to it.

Decreased habituation in response to an acoustic stimulus has been found in some studies of patients with schizophrenia (Braff, Grillon and Geyer, 1992; Parwani et al., 2000; Meincke et al., 2004), although these are contradicted by other studies in which habituation was intact (Kumari, Soni and Sharma, 2002; Aggernaes et al., 2010). Although the flattened habituation response seen in the  $Dlg2^{+/2}$  mice is reflective of the decreased habituation seen in schizophrenia patients, often patients exhibited higher initial responding to the auditory signal, unlike the observation in  $Dlg2^{+/-}$  mice (Parwani et al., 2000; Meincke et al., 2004). There are several mouse models that also exhibit reduced startle response and a lack of habituation, including a genetic stress model (Dirks et al., 2002) and a LoF potassium channel KO (Typlt et al., 2013) (Chapter 3, for more detailed discussion). In patients with schizophrenia, impaired PPI response to an acoustic stimulus is considered a potential biomarker for the disease (Gottesman and Gould, 2003; Walters and Owen, 2007). By contrast to PPI impaired habituation of the acoustic startle response appears to be more intricately linked to the acute phase of schizophrenia. Thus whilst both habituation and PPI were disrupted 5 days after hospitalisation, when tested again 3 months after discharge only PPI response remained abnormal (Mena et al., 2016a).

Reports of altered habituation to acoustic startle in ASD patients are quite variable, and this heterogeneity likely stems from the subgroup of ASD, their sex and age of the patient (Kohl *et al.*, 2014b). In a mixed sex high functioning autism (HFA) adult cohort, no differences were found in habituation or PPI response, despite an initial higher startle amplitude in the HFA group (Kohl *et al.*, 2014b). However, in a male only study a reduction in habituation was observed (Perry *et al.*, 2007), as well as in studies of children with ASD (Kemner *et al.*, 2002).

Startle response data is largely inconsistent in BPD possibly due to variables such as sex or current phase of the disease (mania, depressed, euthymic). In addition, less is known overall about sensorimotor gating in BPD (Kohl *et al.*, 2013). Very few studies have examined the process in BDP patients in the mania phase, with an initial study reporting reduced habituation and PPI in mania patients (Perry *et al.*, 2001), but this was not replicated in a subsequent study (Carroll *et al.*, 2007). In a study addressing phase (depressed and euthymic, but not mania) and sex, females with BDP did not differ to controls for PPI response regardless of state, whereas males

with depression but not euthymic exhibited lower PPI than controls (Matsuo *et al.*, 2018). No evidence of differential habituation was found.

# 8.3.3 Abnormal habituation to a context

In human studies exploring habituation in psychiatric disease they predominately investigate the response to repeated stimuli, for example acoustic or visual, as opposed to in relation to a context. As discussed in 8.3.2 impaired habituation to an acoustic stimulus has been found in schizophrenia patients (Braff, Grillon, & Geyer, 1992; Meincke, et al 2004; Parwani et al., 2000), although there are discrepancies between studies. Impaired habituation to visual stimuli have also been found in a few fMRI studies. For example, one study found reduced habituation in the right anterior hippocampus in response to fearful faces following repeated exposure (Holt et al., 2005), whilst another found deficient habituation in response to neutral faces or objects in the hippocampus and visual cortex (Williams et al., 2013). In ASD there is no real consensus on acoustic startle stimulation, as discussed in the previous section. Impaired habitation to visual stimuli has been observed in ASD (James and Barry, 1980; Vivanti et al., 2018). Patients with ASD required more trials to habituate to a familiar visual stimulus, which consisted of various shapers in different colours, but this may have been confounded by the reduced attention paid to both novel and familiar stimuli during the task (Vivanti et al., 2018). There appears to be a lack of literature explicitly looking at visual habituation in patients with BPD and the findings relating to acoustic stimulus habituation in BPD are contradictory.

For ASD and BPD rodent model's literature examining habituation to a novel context is limited therefore making it challenging to compare to phenotypes observed in schizophrenia. One study employing a prenatal exposure to valproic acid ASD model found decreased exploration and accelerated habituation to an open field in pubertal and adult rats (Olexová *et al.*, 2013). It is possible that fear mediated the reduced exploration of the environment, which in turn may influence the habituation process. In a model reflecting behavioural relevance to BPD, GSK-3 $\beta$ -OX mice, hyperactivity and impaired habituation to a novel context was found (Prickaerts *et al.*, 2006).

Habituation is influenced by many factors, such as attention, initial exploration, general activity level, and novelty. For example the marked hyperactivity of a DAT KO mouse likely interfered with habituation to a novel context, as there was also a lack of habituation in a familiar context and the novelty-induced hyperlocomotion was reversed by antipsychotics (Spielewoy *et al.*, 2000). The delayed habituation observed in the *Dlg2*<sup>+/-</sup> mice (Chapter 4) is unlikely to be simply general hyperactivity,

as the locomotion of the genotypes is comparable during the initial sessions before diverging in later sessions and no evidence of hyperactivity was found in the open field task (Chapter 3).

An alternative basis for the habituation deficit may be an abnormal response to novelty (Barkus et al., 2014). Ordinarily a novel context will induce hyperlocomotion initially before gradually activity decreases as a result of habituation. However, if for some reason the context retains its novelty for a protracted period of time the habituation might be deficient or not occur at all. There is limited research specifically testing whether the balance between the value of novelty and of familiarity is skewed in favour of novelty in people with schizophrenia, and whether this does indeed interfere with adaptive behaviours, such as habituation (Martinelli *et al.*, 2018). However, one study reported that schizophrenia patients preferred newly introduced images (regardless of whether they were truly novel or actually familiar) compared to healthy controls, and this was found to correlate with hallucinatory severity (Martinelli *et al.*, 2018).

Habituation deficits have been observed in models carrying mutations in the subunits which form the interacting partner of Dlg2, AMPAR. Enhanced novelty-induced hyperlocomotion and delayed habituation to a novel context was found in GluA1 KO mice (Procaccini *et al.*, 2011, 2013), as were short term habituation deficits on the Y maze in a GluA1 KO mouse (Barkus *et al.*, 2014a). Additionally short term deficits were also seen in a Cyclin-D2 (CD2) KO hyperactive ventral hippocampus model (Grimm *et al.*, 2018). It has previously been proposed that *Dlg2* reduction could result in hippocampal overactivity (MacLaren *et al.*, 2011). However, in all instances the deficits observed were over the short term, predominately across a single session. By contrast the phenotype observed in the *Dlg2*<sup>+/-</sup> mice is gradual, becoming apparent across the habituation sessions, whereas within session habituation was comparable to the WT mice. Therefore, whilst an abnormal response to novelty may be involved it is not likely the full explanation for the phenotype.

As the activity level of the *Dlg2*<sup>+/-</sup> mice gradually increases following repeated exposure to the context it could be argued that this reflecting more of a sensitisation phenotype or may have progressed into one with subsequent sessions. Sensitisation is a non-associative learning process where repeated exposure to a stimulus induces a progressively amplified behavioural and neurochemical response (Groves and Thompson, 1970).

In order to address whether this is the case extending the number of sessions could illuminate whether the divergent trajectory between the genotypes continued. Subsequent treatment with traditional antipsychotics, which target dopaminergic receptors, could demonstrate whether this phenotype is partly dependant on dopaminergic functioning by essentially rescuing the phenotype. In the GluA1 KO mouse (Procaccini *et al.*, 2011, 2013) and CD2 mouse (Grimm *et al.*, 2018) treatment with antipsychotics normalised the hyperlocomotion observed. It would also be interesting to investigate in the  $Dlg2^{+/-}$  mice whether the abnormal habituation phenotype extends to a task such as novel object recognition and whether there is perseverance of focus on 'familiar' objects that was observed in GluA1 KO mice (Sanderson and Bannerman, 20*12; Barkus et al*, 2014b), which again may indicate dysfunctional dopaminergic functioning .

# 8.4 Limitations

# 8.4.1 Models

Genetically the use of a single gene knockout or knockdown rodent, as with our heterozygous *Dlg2* models, does not address the polygenic nature of schizophrenia. With all models it is important to remember that none of them are able to completely model any psychiatric disease as they result from a complex interaction of factors; genetic, environmental, and social. Additionally, there are aspects of the disease, such as hallucinations and delusions which are not translatable in rodents. It should be therefore be clear that these models are not intended to be considered models of psychiatric disease. Instead single gene knockout models can increase our understanding of the functional consequences of specific mutations, as well as the wider effects on disease associated pathways, such as glutamatergic transmission. This in turn can help uncover the contribution of specific genes to observed phenotypes.

In the  $Dlg2^{+/-}$  mouse model used, the mutation was designed to target all transcripts, which resulted in a further downstream mutation around exon 14. By comparison the Dlg2 mouse model in the literature, where the mutation is in exon 6 (Chapter 3). This may explain why there was no clear reduction in mRNA expression in the hippocampus or cerebellum of our  $Dlg2^{+/-}$  mice. The downstream mutation result in residual expression of some transcript's mRNA, through alternative splicing or lack of nonsense mediated decay of the upstream transcript. Even in homozygous mice developed by McGee *et al* (2001), which has a much further upstream mutation compared to our model, Northern blot analysis found a weak band for *Dlg2*, attributed

to alternative splicing. As the rat model is completely novel it is possible that there are transcripts not affected by the mutation, and thus may be affecting observable phenotypes. For example, recently in mice a novel splice variant of *Dlg2* was found to be highly expressed in immune system plasmacytoid dendritic cells (Ali et al., 2018). In KO mice, generated through deletion of exon 9, expression of this novel isoform was found in the brain and bone of homozygotes. It is unknown whether this newly identified isoform is able to fulfil any of the normal functioning of Dlg2 (Ali et al., 2018). Different transcripts of *Dlg2* are known to have differing roles. For example PSD93α is required for postsynaptic membrane targeting (Firestein, Craven and Bredt, 2000b), and the overexpression of PSD93 $\delta$  or  $\epsilon$ , but not PSD93 $\alpha$ , increases potency of AMPAR and the number at the post synaptic membrane (Kruger et al., 2013). It is therefore possible that phenotypes can be influenced by which transcripts are affected by the specific mutation, and which are likely to be masked in a genetically global knockout model. Generating transcript specific mutants was beyond the scope of this project but representing an interesting opportunity for future investigations.

Western blot analysis of Dlg2 in both the mouse and rat models used is necessary to determine whether there were any changes in protein expression. This is particularly important because in post mortem schizophrenia brains, increased mRNA but decreased protein has been found (Kristiansen *et al.*, 2006). This indicates abnormal expression of DLG2 in schizophrenia brains, and that there is either abnormal translation and/or accelerated degradation of DLG2 occurring (Kristiansen and Meador-Woodruff, 2005). Western blots were conducted for both the mouse and rat models to examine *Dlg2* expression in multiple brain regions. Unfortunately despite trialling a panel of antibodies from various suppliers, as well as two generated previously by a colleague examining *Dlg2 in vitro*, only one antibody produced any bands, which were very weak and inconsistent across experiments. Despite extensive troubleshooting with this antibody, including different preparation methods (RIPA and Synper) and modifications to various aspects of western blot protocol no viable blots were generated.

In addition to determining the expression of *Dlg2* mRNA in key brain regions the expression of the other *Dlg* family members was also examined, investigating whether there were any compensatory changes in heterozygous rodents. No evidence of expression changes for any other *Dlg* family members in *Dlg2*<sup>+/-</sup> rodents was found. This is similar to other published literature. Nevertheless, *Dlg4* expression has been found to functionally compensate for *Dlg2* in *in vivo* models (Elias et al. 2006).

However, in vitro studies have repeatedly found that acute knockdown of Dlg2 has a significant impact on NMDAR and AMPAR functioning (Elias et al. 2006; Sun & Turrigiano 2011; Carlisle et al. 2008; Frank et al. 2016). This suggests that reduction of *Dlg2* has important consequences for synaptic functioning, but that compensation from Dlq4 can ameliorate the effects. The discrepancy between the in vivo and in vitro models is likely an issue of the differing complexity of the systems. Given animals grow up with a chronic reduction in *Dlg2* resulting from genetic manipulation there is more opportunity, and potentially more need, for compensatory processes to develop, which may not occur in the shorter time span of *in vitro* experiments. Employing inducible *Dlg2* KO mice, for example using a tetracycline-controlled transcription method, would be one approach to investigate the impact of acute knockdown in vivo on processes reliant on synaptic plasticity, such as LTP during a learning paradigm. This could determine whether phenotypes would be more marked and/or selective when the opportunity for compensatory processes during development are reduced. This approach could be used to examine the effect of acute *Dlg2* KO during learning, as could intrahippocampal RNAi, exploring if *Dlg2* is more important for some aspects of learning than others, such as extinction.

This project employed heterozygous, as opposed to homozygous, *Dlg2* models which better reflect the occurrence of mutation in humans (Kirov et al. 2012; Purcell et al. 2014). Something that has not been so far investigated, however, is the impact of duplication of the *Dlg2 gene* as opposed to deletion. In the study comparing humans with DLG2 mutations with KO mice three of the four carriers were diagnosed with schizophrenia two of the subjects had deletions within DLG2, but one had two duplications instead (Nithianantharajah et al., 2013). Whether this has any substantial effect on phenotypes compared to deletions is unknown for DLG2. However, so called mirror phenotypes have been observed in humans carrying 16p11.2 mutations (Qureshi et al., 2014; LeBlanc and Nelson, 2016) and rodent models (Arbogast et al., 2016), where deletions and duplications have opposite effects on aspects of cognition, brain structure and metabolism. In the mice, for example, the deletion model exhibited reduced weight, impaired adipogenesis, deficient recognition memory, and hyperactivity, with the opposite found in the duplication model (Arbogast et al., 2016). In humans deletion carriers exhibited increased size on multiple brain measures whereas reduced size was found in those with duplications (Qureshi et al., 2014). Therefore, this may be an avenue of investigation worth pursing in future.

Fundamentally this thesis to characterise and compare an established *Dlg2*<sup>+/-</sup> mouse and a novel*Dlg2*<sup>+/-</sup> rat model in basic behavioural tasks and some that are relevant

for psychiatric disorders. Therefore, there were other factors that could interact with a genetic mutation and potentially modify its impact but it was beyond the scope of the current project to investigate them. For example within cage dominance structures may have influenced behaviour of either of the genotypes. In all the experiments with both rodent species all cohorts were in mixed genotype housing. It has been demonstrated previously that in mice lacking Neuroligin3, a gene associated with ASD, altered the social behaviour of co-housed WT mice (Kalbassi *et al.*, 2017). Given that recently a hypersocial phenotype was observed in homozygous *Dlg2* mice (Winkler *et al.*, 2018), this potential variable may be worth exploring in future experimentation. It is unknown whether a similar phenotype was present during our studies, and whether this impacted co-housed WT mice, as social behaviours were not investigated.

The potential impact of an altered pre or postnatal environment, ie poor maternal care, in breeding females with *Dlg2* mutations was not directly addressed by the experiments. Where possible the *Dlg2* mutation was transmitted through the paternal line for the experimental cohorts to remove this variable. However, it may be worthwhile investigating this experimentally given the link between early life stress and disease risk. For example, the glucocorticoid receptor has been implicated in postpartum depression, and heterozygous deletion in C57BL/6 dams reduced their licking/grooming behaviours (Chourbaji et al., 2011). Pups that received low levels of these behaviours later exhibit elevated stress and anxiety phenotypes as adults (Caldji et al., 1998). There are hundreds of genes which alter maternal care, for example influencing bonding and sociability, and within the genes enrichment was found for genes associated with schizophrenia, BPD and ASD (Gammie et al., 2016). Future examination of disease relevant environmental factors, such as maternal infection, maternal or juvenile stress, in combination with *Dlg2* mutation could uncover whether this interaction would result in more severe phenotypes. This could be especially interesting as whilst the impact of common variants on risk for developing schizophrenia has been proposed to be mediated by presence or absence of pregnancy complications (Ursini et al., 2018), this is unknown for de novo CNVs.

# 8.4.2 Sex

Another important limitation of the findings presented in this thesis is that almost all experiments were conducted in male rodents. Concern surrounding the influence of the oestrus cycle on behaviour often means female mice are excluded from behavioural studies (Meziane *et al.*, 2007; Beery, 2018). All rat experimentation was

conducted in males only in the first instance with the intention of repeating the studies in females in the future. However, as discussed in Chapter 4 a small pilot was conducted to investigate motor learning in female  $Dlg2^{+/-}$  mice. The female mice underwent the same three day rotarod protocol detailed in the first experiment of Chapter 4. In contrast to the male  $Dlg2^{+/-}$  mice the females did not display any deficits. Thus, the early motor learning deficit appears to be sex specific in  $Dlg2^{+/-}$  mice.

Whilst this is only one experiment, it reinforces the importance of studying both sexes. In humans there are sex differences in many psychiatric conditions. In schizophrenia, the prevalence, symptomology and response to treatment is different between the sexes (Kokras and Dalla, 2014). It has also been proposed that oestrogen may exert a protective effect in schizophrenia (Kulkarni et al., 2013). The male-to-female ratio in autism is 4.5:1 (Christen sen et al, 2016) and 1.4:1 for schizophrenia (McGrath et al., 2008). The ratio for BPD by contrast is roughly equal but there are sex differences in disease course, presentation and co-morbidity (Koo and Duman, 2009). Despite the higher occurrence in males there is an increased rare CNV burden females for schizophrenia (Han et al., 2016), and when sex is corrected for the association between these CNVs and schizophrenia is unaffected. Similarly in ASD females possess a high CNV burden (Jacquemont et al., 2014), including an excess of genes disrupted by de novo CNVs (Gilman et al., 2011; Levy et al., 2011; Sanders et al., 2015). The discrepancy between greater mutational burden and lower prevalence of both ASD and schizophrenia in females is consistent with the hypothesis that females require an increased risk factor load in order to manifest a neurodevelopmental disorder (Robinson et al., 2013; Jacquemont et al., 2014). There is also a nonmutually exclusive hypothesis that males may be more susceptible than females to developing ASD, from genetic risk studies (Robinson et al., 2013) and investigation of epigenetic changes (Kim et al., 2016).

Mutations in risk genes related to *Dlg2* may result in divergent phenotypes between the sexes. In *Dlg1* cKO mice, a motor learning phenotype was observed in female, but not male, mice (Gupta *et al.*, 2018). This is the reverse to the motor learning phenotype observed in the *Dlg2*<sup>+/-</sup> male mice, highlighting the difference between phenotypic manifestation between the sexes, and the function of different members of the same protein family.

Given there is evidence indicating sex differences, both in a related gene (*Dlg1*) and from our own data with  $Dlg2^{+/-}$  mice it would thus be important to study female  $Dlg2^{+/-}$  mice in the same behavioural battery as males. Also, as discussed above, there are

often sex differences in disease risk for psychiatric disorders that may well be reflected in rodent models. There is more drive to include both sexes wherever possible in basic research in order to explore where there are divergences.

Of particular interest for the *Dlg2* models would be investigating ketamine induced hyperlocomotion, as sex differences in response to acute administration of NMDAR antagonists has been observed. Following administration of PCP (Vezzani *et al.*, 1989) and MK-801 (Nabeshima *et al.*, 1984a), females exhibited greater behavioural sensitivity, including hyperlocomotion, stereotyped behaviours and ataxia. The differential response to NMDAR antagonists is due to pharmacokinetic differences between the sexes, with lower metabolism of NMDAR antagonists in females resulting in higher levels in the brain and plasma (Nabeshima *et al.*, 1984b; Vezzani *et al.*, 1989; Shelnutt, Gunnell and Owens, 1999). Therefore, whether *Dlg2*<sup>+/-</sup> females are more sensitive to acute ketamine injection than the males and exhibit a different dose response pattern remains an important question. The experiment would permit the assessment of normal locomotion and habituation to a novel environment in the females at the same time.

# 8.4.3 Molecular and Behavioural Studies

As discussed in Chapter 4 the impaired motor learning deficit appears to be partially unpinned by a reduction in neuronal activity in M1. Due to time constraints confirmation of a reduction in *Dlg2* expression in the motor cortex was not measured. This would be important to determine, especially given the lack of a reduction of *Dlg2* mRNA in the cerebellum, another key structure in motor learning (Chapter 3). Due to the difficulty in reliably dissecting out M1 this analysis could be conducted using *in situ* hybridisation of sectioned brains. This method was also provide better spatial resolution of expression changes, allowing examination of individual cortical layers, for example.

As discussed at multiple points in this thesis the interaction between NMDAR and *Dlg2* may be important for cognitive impairments observed in *Dlg2* KO mice. Expression of the NMDAR subunits in the heterozygotes models discussed in this thesis was not conducted. NMDAR expression has only previously been investigated in primary cortical *Dlg2* KO cultures (Zhang *et al.*, 2010b), and not *in vivo*. Winkler *et al* (2018) examined protein expression in hippocampal synaptic fractions from *Dlg4*<sup>+/-</sup> mice and found no change in any NMDAR or GluR1 AMPA receptor subunit expression. They did not conduct this analysis in *Dlg2*<sup>+/-</sup> mice. It would be important to examine NMDAR expression in our *Dlg2* models as receptor density or distribution

may influence observed phenotypes, as discussed in relation to the ketamine challenge study in Chapter 4 for example.

Finally, as discussed previously during this thesis *Dlg2* is most heavily implicated with synaptic plasticity which underpins learning and memory. However, investigation was only conducted on a small number of associative learning tasks, motor learning and CFC. In the mouse model there were additional intended experiments focusing on cognition that were not conducted. For example, no change was found in DCX expression in  $Dlg2^{+/}$  mice, and so a follow behavioural task, pattern separation, was not conducted as it is thought to rely on new-born neurons. This task probes the rodent's ability to discriminate between two squares (in a touchscreen task) or two objects (in a physical task) at variable distances apart. For the rat model again, there was the need to conduct basic characterisation, even more important as the model is completely novel. As discussed in Chapter 7 increasing the complexity of the CFC task, for example employing multiple contexts, in the rat model would potentially uncover aspects of cognition impacted by the *Dlg2* mutation. Even in the literature the majority of the cognitive testing of *Dlg2* models is limited. Future experiments could investigate different cognitive processes that are impaired in patients with schizophrenia, such as working memory, using Y or T mazes or recognition memory using novel object recognition or novel object location, or associative recognition memory with object-in-place tasks. The cognitive deficits associated with schizophrenia can be considered both clinical and prognostic predictors and therapeutic targets, therefore it is vital to investigate them in rodents modelling different risk factors of the disease.

# 8.5 Future directions

# 8.5.1 Further investigation of the early motor learning deficit

The experiments presented in Chapters 3 and 4 demonstrate a deficit in early phase motor learning in male  $Dlg2^{+/-}$  mice, which at the network level may be underpinned by reduced neuronal activity in motor cortex M1. In human imaging studies the anterior cerebellum has been proposed to underlie motor learning deficits in ASD patients (Marko *et al.*, 2015). The presence of a motor learning deficit in the male mice, despite the lack of reduced *Dlg2* mRNA in the cerebellum, implies that it is less likely the cerebellum is involved in the motor learning deficit.. Comparison between males and females would also be important, given the sex specific nature of the phenotype.  $Dlg2^{+/-}$  females would be predicted to exhibit a similar increase in cFos staining during rotarod training to the WT female mice. Additionally, investigating

neuronal activity in both sexes using cFos following a later stage of motor learning would be interesting. The male *Dlg2*<sup>+/-</sup> mice do gradually improve across the motor learning sessions, although never quite reaching the same plateau as WT mice. Therefore, it would be predicted that neuronal activation in regions like M1 would become more comparable to that of WT mice at later stages of the motor learning process, i.e. towards the end of the second day or start of the third day.

Electrophysiological approaches to investigating the motor learning phenotype would also increase understanding of the impact of Dlg2 mutation on synaptic plasticity directly. Rats which had undergone one day of accelerating rotarod training exhibited an increased AMPAR:NMDAR ratio, and increased amplitude, but not frequency of mEPSCs, suggesting involvement of increased postsynaptic AMPAR during early stage motor learning in layer 2/3 of M1 (Kida et al., 2016). Under normal conditions no change in the AMPAR:NMDAR ratio was found in *Dlg2* or *Dlg4* KO slices, although knockdown in cultures using shRNA did result in decreased AMPAR EPSCs (Elias, et al 2006). Preliminary studies from collaborators working on our *Dlg2<sup>+/-</sup>* rat model found reduction in the AMPAR:NMDAR ratio (Simonas Griesius, personal а communication). Given a motor learning phenotype has already been demonstrated in the  $Dlg2^{+/-}$  mice on a rotarod task, which evidence suggests alters the AMPAR:NMDAR ratio during the period of training where we observed the behavioural deficit (Kida et al., 2016), it would be interesting to assess whether the AMPAR:NMDAR ratio is abnormal in the mice. One approach to behaviourally investigating whether abnormal AMPAR functioning is mediating the early motor learning phenotype would be comparing  $Dlg2^{+/-}$  mice to WT mice given an AMPAR antagonist, such as CNQX, prior to training on the accelerating rotarod. If AMPAR functioning is indeed involved in this phenotype the mice treated with CNQX may exhibit a similar deficit to the  $Dlg2^{+/-}$  mice.

An alternative approach to investigating plasticity would be to investigate structural plasticity with motor learning by measuring spine formation. Using two photon imaging of dendritic spines in layer 5, two days of accelerating rotarod training was found to induce regionally specific spine formation in the forelimb area of the motor cortex in mice, whereas a motor task without the learning element did not (Yang, Pan and Gan, 2009b). It has been previously demonstrated that MAGUKs, in particular the PSD-95 family, are involved in the stabilisation of nascent spines (Wang *et al.*, 2014; Lambert *et al.*, 2017). Extrapolating this methodology to this model would allow examination of spine formation during the earlier stage of motor learning, as well tracking the stability of the spines over time, comparing them between WT and *Dlg2*<sup>+/-</sup> mice.

Differences in spine formation or stability may play a role in the motor learning deficit observed in the *Dlg2*<sup>+/-</sup> mice, demonstrating how *Dlg2* mutation may mediate dysfunctional synaptic plasticity. As has been discussed previously in this thesis abnormal synaptic plasticity is believed to be important to the cognitive impairment observed in schizophrenia.

# 8.6 Concluding Remarks

Heterozygosity of *Dlg2* in two different rodent models was not found to result in universal impairment, but instead specific deficits in processes involved in learning. The experiments discussed in this thesis demonstrate a delayed early motor learning phenotype in male, but not female, *Dlg2*<sup>+/-</sup> mice, that may have a basis in reduced neuronal activation in M1. In the male *Dlg2*<sup>+/-</sup> mice across two paradigms (locomotion response to a context and acoustic startle) the simple non-associative learning behaviour of habituation was found to be impaired. As demonstrated by these findings altered *Dlg2* function may therefore be linked to common phenotypes that are shared across some psychiatric disorders. For example motor learning deficits are observed in SZ and ASD, and may also be present in BPD.

This work expands upon the currently limited pool of research into *in vivo* effects of *Dlg2* mutation. The rotarod experiments conducted during this thesis lend support to the previous finding of deficient motor learning in *Dlg2* mutants (Winkler *et al.*, 2018), furthers the understanding of this phenotype by demonstrating that it is sex specific, and uncovers a potential molecular basis. The findings do suggest that whilst some basic learning processes are intact in *Dlg2* mutants (Nithianantharajah *et al.*, 2013), abnormalities may manifest in other types of simple learning such as habituation. Although simpler than the cognitive tasks deficits have previously been found in for *Dlg2* KO mice an element of cognitive flexibility is required for these behaviours. Importantly the research detailed in this thesis was conducted in heterozygotes, arguably more applicable to the human carriers, and demonstrates that deficits still manifest despite the presence of one copy of *Dlg2*.

The novel rat model provides another avenue through which to investigate the impact of *Dlg2* mutation. When compared to the mouse model discussed in this thesis it has a major advantage in that the reduced expression of *Dlg2* in the hippocampus and PFC better mimics the dysfunctional network observed in schizophrenia. This fact can be capitalised on in future studies to investigate behaviours reliant on these regions and exploring more complex aspects of cognition may be simpler or less time consuming than in the mouse model.

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