Understanding psychiatric risk from *DLG2* haploinsufficiency CNVs through the phenotyping of a *Dlg2*$^{+/-}$ rat model

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Acknowledgements

Scientific Contributions from this PhD

Journal articles

· Behavioral training rescues motor deficits in *Cyfip1* haploinsufficiency mouse model of autism spectrum disorders. *Translational Psychiatry* (2019)9:29 https://doi.org/10.1038/s41398-018-0338-9

· Reduced expression of the psychiatric risk gene *DLG2* (PSD93) impairs hippocampal synaptic integration and plasticity. Revise and resubmit, *eLife*. 2021. BioRxiv https://doi.org/10.1101/2021.08.02.454736

· Behavioral and molecular characterization of the *Dlg2* haploinsufficiency rat model of genetic risk for psychiatric disorder. Revise and resubmit, *Genes, Brain and Behaviour*. 2021. BioRxiv https://doi.org/10.1101/2021.10.11.463943

Conference presentations

· 'Measuring hedonic and motivational components of anhedonia in the *DLG2* heterozygous knockout rat model of psychiatric disorder.' Poster at Sociedad Española de Psicología Comparada (SEPC) 2019.

· 'Measuring 'hallucination-like' behavior in the *DLG2* heterozygous knockout rat model for psychiatric disorder.' Talk at Cardiff Psychology Postgraduate Conference 2019.

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· 'Assessment of hippocampal-dependent behaviors in the *Dlg2$^{+/-}$* rat model.' Poster at Federation of European Neuroscience (FENS) 2020.

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Summary

Copy number variants at the 11q14.1 locus are associated with multiple psychiatric conditions (e.g. Kirov et al., 2012; Gao et al., 2018). The candidate gene within this CNV, which is either completely deleted or duplicated, is *DLG2* (coding PSD-93). PSD-93, a synaptic scaffold protein, stabilises effectors in the postsynaptic density of excitatory synapses. If PSD-93 content is altered key synaptic processes may be disrupted.

Phenotyping a rat model heterozygous for *Dlg2* (+/-), which models the deletion CNV in patients, can help isolate endophenotypes with cross-disorder relevance. At the protein level *Dlg2$^{+/-}$* rats show a decrease in PSD-93 without changes to PSD-95 or NR1 expression. Ex-vivo structural MRI scans were analysed for white matter abnormalities and differences in grey matter volume; however no genotype effects were seen.

Behavioural phenotyping was conducted using assays relevant to symptom domains seen in 11q14.1 deletion syndromes including anxiety, social behaviour, PCP-hyperlocomotion, and sensorimotor gating. Memory and learning in a battery of object recognition tasks and water-maze reference memory were assessed as an index of cognitive ability. Hedonic responses to stimuli, appetitive conditioning, and motivation to work for reward were assessed to capture reward processing. Finally, a novel paradigm to assess the propensity towards hallucinations and delusions in rodents was conducted.

Dlg2$^{+/-}$ rats performed as wild-types on almost all measured domains and tasks. In many cases this presents a departure from findings with *Dlg2* homozygous (-/-) knockout mice who demonstrate increased anxiety and deficient social behaviour. With the caveats of drawing conclusions across species and experiments, this highlights the importance of using the most clinically valid (i.e. heterozygous) models when characterising the effects of CNVs. *Dlg2$^{+/-}$* rats showed a potentiated and sustained locomotor response to the psychostimulant PCP, showing that although its effects are subtle, deleting one copy of *Dlg2* in a rat model does result in a compromised system with a possible psychosis susceptibility.

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Abbreviations

ADHD – Attention-Deficit/ Hyperactivity Disorder

AMPA – A-Amino-3-Hydrocy-5-Methyl-4-Isoxazolepropionic Acid

AMPAR - A-Amino-3-Hydrocy-5-Methyl-4-Isoxazolepropionic Acid Receptor

ANOVA – Analysis of Variance

ASD – Autism Spectrum Disorder

CO_2 – Carbon Dioxide

CA1 – Cornu Ammonis 1

CaMKII – Calcium/Calmodulin-Dependent Protein Kinase II

CNV – Copy Number Variation

CR – Conditioned Response

CRISPR – Clustered Regularly Interspaced Short Palindromic Repeats

CS – Conditioned Stimulus

DLG1 - Disks Large Homolog 1

DLG2 – Disks Large Homolog 2

DLG3 - Disks Large Homolog 3

DLG4 - Disks Large Homolog 4

DNA – Deoxyribonucleic Acid

DSM-IV – Diagnostic and Statistical Manual of Mental Disorders 5

EPM – Elevated Plus Maze

GWAS – Genome-wide Association Study

HET – Heterozygous

IP – Intraperitoneal

LTD – Long Term Depression

LTP – Long Term Potentiation

Maguks – Membrane-Associated Guanylate Kinase

mRNA – Messenger Ribonucleic Acid

NMDA – N-Methyl D-Aspartate

NMDAR - N-Methyl D-Aspartate Receptor

PBS – Phosphate Buffered Saline

PCR – Polymerase Chain Reaction

PFA – Paraformaldehyde

PFC – Prefrontal Cortex

PPI – Pre-pulse Inhibition

PSD – Postsynaptic Density

PSD-93 – Postsynaptic Density 93

PSD-95 - Postsynaptic Density 95

qPCR – Quantitative Polymerase Chain Reaction

RNA – Ribonucleic Acid

SEM – Standard Error of the Mean

SH3 – SRC Homology 3 Domain

shRNA – Short Hairpin RNA

shRNA – Short Hairpin RNA

siRNA – Small Interfering RNA

SNP – Single Nucleotide Polymorphism

SNP – Single Nucleotide Polymorphism

SNP – Single Nucleotide Polymorphism

STEP – Striatal-enriched Protein Tyrosine Phosphatase

TBS – Tris Buffered Saline

US – Unconditioned Stimulus

WT – Wildtype

µl – Microlitres

µm - Micrometres

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Chapter 1

General Introduction

1.1.   Investigating genetic risk for psychiatric disorder to specify the determinants of biological and psychological aspects of disease

1.1.1.   Genetic risk and psychiatric disorder

From its earliest description psychiatric illness has been known to run in families. The contribution of one's genetic makeup to psychiatric disorder was confirmed with twin, adoption, and family studies (e.g. Folstein, 1996; Lichtenstein et al., 2009; Shih et al., 2004; Sullivan et al., 2003). Twin studies compare the co-occurrence of disorder in identical twins which share all their genetic variants, and fraternal twins which share only 50% of them, to parse the contribution of genetic and environmental factors to phenotypes. This allows twin studies to specify how much the variation in a given trait can be attributed to genetic variation through heritability estimates. The heritability estimate for disorder in schizophrenia is roughly 80% (e.g. Cardno & Gottesman, 2000), and for autism spectrum disorders (ASD) 90% (e.g. Tick, Bolton, Happé, Rutter, & Rijsdijk, 2016).

This level of analysis only confirms genetic aetiology without the ability to isolate exactly which genes are associated with a disorder on a reliable basis. This is especially true of polygenic conditions such as schizophrenia and ASD. The high heritability of many psychiatric conditions triggered a wave of research to specify the exact genetic contribution genetic variation has to disorders. This critically depends on technological advances in the feasibility of sequencing entire human genomes. The Human Genome Project (2001) to sequence the first human genome took 12 years and cost nearly $3 billion (Abdellah et al., 2004; Lander et al., 2001; National Human Genome Research Institute, 2010), while next-generation sequencing now enables the sequencing of an entire human genome in a single day for around $1000 (Metzker., 2010;Desai et al., 2021).

The clinical utility of next generation sequencing derives from its ability to identify differences between a patient's genome (for example an individual diagnosed with schizophrenia) and a reference genome of a mentally healthy control. Genomic differences known as variants allow researchers to go beyond classifying a condition as hereditary (and potentially quantifying the degree of heritability) and give the possibility of isolating which genes contribute to certain conditions. These findings can be further advanced to look at how different risk genes interact to contribute to risk for disorder (pleiotropy, Chiu et al., 2017) and how variant-determined protein changes interact at pathway and system levels (Nguyen et al., 2019). Thus, genetics has become a new ignition for research into the biological mechanisms of psychiatric disorders, just as the discovery of antidepressants (indicating a possible role of serotonin in depression) or antipsychotics (bringing dopamine into the schizophrenia discussion) once did (Hiroi & Yamauchi, 2019).

1.1.2. Common and rare genetic variation

Early surveys of genetic variation found that two human chromosomes in the population differ at a rate of 0.1% on average (IHC, 2005). These differences (genetic variants) fall into two classes. The first is changes to the DNA sequence of one or a few base pairs (single-nucleotide polymorphisms, 'SNPs', and short insertions or deletions of bases) and the second sub microscopic structural variations affecting more than 1000 DNA base pairs (copy number variants, 'CNVs') (Lee & Scherer, 2010). SNPs outnumber CNVs in the genome by around three orders of magnitude (Malhotra & Sebat, 2012). For example genome wide association studies (GWAS) have identified over 8000 SNPs that are associated with schizophrenia at 145 distinct genetic loci (Pardiñas et al., 2018; Ripke et al., 2013), while only 11 CNVs have been consistently associated with schizophrenia (Rees et al., 2016, 2014).

SNPs entail common variation that individually and subtly raises the risk for psychiatric disorder, with the combined effects of many of these small genetic changes contributing to overall disease risk (Pardiñas et al., 2018; Ripke et al., 2014; Sullivan, Daly, & O'Donovan, 2012). Together SNPs are estimated to contribute to between 30-50% of the genetic predisposition towards schizophrenia, despite individual SNPs typically having odds ratios of around 1.10 each for schizophrenia risk (Ripke et al 2013). Alternatively, CNVs entail rare

genetic changes which span multiple genes and regions, causing larger changes to the genome have a much more catastrophic effect on phenotype. Unlike SNPs these variants are more likely to determine genetic risk individually due to their greater penetrance (Malhotra & Sebat 2012). The odds ratios for neurodevelopmental disorders such as schizophrenia, autism and intellectual disability following certain CNVs are in the range of 10-70 (Cooper et al., 2011; Girirajan et al., 2011; Malhotra & Sebat, 2012; Marshall et al., 2017).

On average there are >1000 CNVs in the genome, accounting for around 4 million base pairs of genomic difference (Conrad et al., 2010; Mills et al., 2011). The structural variation comprising a CNV is heterogenous. These can include inversions or translocations, respectively changing the orientation or location of a DNA segment. There are also CNVs consisting of deletion or duplication of whole sections of DNA, reducing or increasing the usual number of genes in these loci (Weischenfeldt et al., 2013).

CNVs and other rare variants have a higher penetrance than the combined effect of common variants. Yet they only contribute to a minority of cases of schizophrenia and common neurodevelopmental disorders, under 10% (Purcell et al 2014, Rees et al 2014). This raises the question as to whether disorders caused by CNVs are specific syndromes, or cause the same phenotypes as when a disorder is manifested by common variants (Owen, Craddock, & O'Donovan, 2010). Where schizophrenia is concerned there is some evidence towards these different mechanisms causing the same disorder, as schizophrenia symptoms displayed by CNV carriers closely resemble symptoms in patients with no pathogenic CNVs (Bassett, Scherer, & Brzustowicz, 2010). Schizophrenia is also found in CNV carriers in the absence of other disorders, suggesting that in these cases it does not occur as a consequence of another primary disorder (e.g. intellectual disability or epilepsy) (Owen et al 2010). This indicates that investigating CNV-related processes which lead to schizophrenia, and possibly other disorders, could be generalised to forms of these disorders with different aetiological origins.

1.2. Copy number variation and psychiatric disorder risk

1.2.1. What causes copy number variation?

When it proceeds without error, the exchange of genetic material between two strands of DNA containing long stretches of similar base sequences in homologous recombination is essential for maintaining genetic variation. This occurs in meiosis by shuffling genetic material during chromosomal crossover, and in mitosis when repairing double-stranded breaks in DNA. The most common cause of CNVs is through errors in this process, known as non-allelic homologous recombination or unequal crossing over (Kirov, Rees, & Walters, 2015; Malhotra & Sebat, 2012). This is where two highly homologous, repetitive sequences of DNA are misaligned in DNA replication or repair, leading to duplication or deletion products where there are different numbers of copies of the genes contained between repeats.

The size and content of the duplications and deletions will depend on the distance between the long sequence repeats whose misalignment produces them. Due to this recurrent CNVs tend to repeatedly occur in the same genomic locations where highly homologous DNA sequences manifest. These are typically sections of low copy repeats, stretches of duplicated DNA over 1 kb in size sharing a sequence similarity of over 90% (Bailey et al., 2002; Bailey, Yavor, Massa, Trask, & Eichler, 2001). When non-allelic homologous recombination occurs in meiosis a de novo mutation is created (Turner et al., 2008), and when in mitosis a somatic CNV results, present only in a proportion of cells post-conception (Simmons et al., 2012).

There are also non-recurrent CNVs which are randomly distributed throughout the genome. These are thought to be the result of erroneous DNA double-strand break repair known as non-homologous end joining (Malhotra & Sebat, 2012). The breakpoints of CNVs formed by non-homologous end joining are frequently observed within repetitive elements suggesting that this process may be triggered by certain genomic architectures, although extensive sequence homology is not required (Toffolatti et al., 2002). Non-recurrent CNVs can also arise through fork stalling and template switching (FoSTeS), a replication-based genomic

rearrangement mechanism that is induced by errors (i.e. single stand breaks) during the DNA replication process (Hastings, Lupski, Rosenberg, & Ira, 2009; J. A. Lee, Carvalho, & Lupski, 2007).

Finally, CNVs can occur somatically or in the germline via retrotranspositions. This involves retrotransposons, which can 'copy and paste' themselves into different genomic locations (transposons) by converting RNA back into DNA through reverse transcription. CNVs can be generated when the relocation of retrotransposons interferes with existing genes. Nearly 20% of the human genome comprises long interspersed nuclear elements (L1), the only active class of retrotransposons in the human genome, which can cause CNVs by this mechanism (Goodier & Kazazian, 2008). Overall it has been estimated that 70.8% of CNV deletions are attributable to either non-allelic homologous recombination, non-homologous end joining or FoSTeS, while 89.6% of small insertions were attributable to retrotransposition activity (Mills et al., 2011).

With such large odds ratios for psychiatric conditions which deliver impairments in forming and maintaining relationships (e.g. social withdrawal in schizophrenia), it would be expected that CNVs are selected against in the population, as carriers are less likely to produce offspring. This been confirmed (Stefansson et al., 2014). Calculating selection coefficients, a measure of the relative reduction in the contribution that a particular genotype makes to the gametes compared with other population genotypes, Rees, Moskvina, Owen, O'Donovan, & Kirov, (2011) found that each new mutation is eliminated from the population in a very small number of generations: 1-5 on average. Yet CNVs remain present in the general population due to their tendency to occur de novo in low copy repeat loci through non-allelic homologous recombination (Gu et al., 2008). For example, 5% of people with schizophrenia have a de novo CNV (Kirov et al., 2012; Malhotra & Sebat, 2012), 5.8% of people with autism spectrum disorder (Sanders et al., 2011) and an even higher percentage of those with intellectual disability (Rosenfeld et al., 2013).

1.2.2. Mechanisms by which copy number variation contributes to psychiatric disorder

Individual CNVs do not match well with discrete psychiatric disorder diagnoses. A single CNV, for example that at 11q14.1, can result in increased risk for schizophrenia, bipolar, autism, epilepsy and intellectual disability (Egger et al., 2014; Gao et al., 2018; Kirov et al., 2012; Noor et al., 2014). To understand how a particular CNV contributes to disorders it is best to isolate how a particular structural variant might determine biochemical, cognitive, and neurophysiological endophenotypes. This fits well with the current climate of moving away from categorical disorder classification systems in psychiatry and towards network-based views of symptom clusters (Research Domain Criteria: RDoC, Morris & Cuthbert, 2012). This is because breaking down disorder into constellations of co-occurring symptoms allows for the specific relation of genetic, biological, or psychological processes/ abnormalities to individual symptom domains. Where genetic change is the aetiology disruptions in domains are specified as phenotypes. Symptom domains will occur with different frequencies in different psychiatric disorders, allowing the genetic component to insinuate a biological process that is commonly involved across disorders and possibly influential in comorbidity.

Theoretically there are multiple ways a single CNV can influence disease outcome. It could be that many or even all genes in a CNV contribute in some way to a psychiatric disorder, which is consistent with the fact that the severity of the phenotype increases with the size of the CNV (Girirajan et al., 2011; Sanders et al., 2015). Alternatively, there is the possibility that deletion or duplication of a single gene could be what is driving the phenotypic effect of the entire CNV, as is indicated by work discovering that in individuals with autism small de novo CNVs typically contain a single autism-associated risk gene (Sanders et al., 2015). These two possibilities comprise the endpoints of a sliding scale for any given CNV, where theoretically its effect could be due to disruption of a single gene, multiple complete genes, or all genes mutated. The protein products of disrupted genes could interact directly to impair a biological process or determine effects through mere summation of deficits. In every case the final clinical outcome will be determined by an individual's unique mosaic of genetic, biochemical, and environmental risk factors.

Information concerning psychiatric disorder mechanisms can be gained by studying processes which multiple disease-associated CNVs converge on. In schizophrenia, CNVs from patients are enriched for genes involved in neurodevelopmental and synaptic signalling processes (Walsh et al., 2008). Where de novo CNVs are concerned there is enrichment particularly for genes involved in NMDA receptor (NMDAR) postsynaptic signalling complexes (Fromer et al., 2014). Enrichment was also found for genes expressed during fear extinction in the hippocampus, but not genes expressed following fear memory consolidation or retrieval (Clifton et al., 2017). This specifies deficits in extinction learning as pathogenic, potentially contributing to the schizophrenic tendency to retain delusional beliefs even in the face of conflicting evidence (Corlett et al., 2009). There is a further coherence in these schizophrenia gene enrichment studies from the fact that extinction learning is mediated by NMDARs in the amygdala and prefrontal cortex (Davis, 2011), allowing specific hypotheses to be generated about which processes in which brain regions can link genetic risk to psychiatric outcome.

Despite this success, a detailed mapping between CNVs (i.e. genes and combinations of genes) and relevant symptom domains, psychological processes or biological abnormalities has not been outlined. Assessing this by observing which processes are spared and impaired in samples of human CNV carriers is valuable (e.g. linking CNVs with structural MRI findings, Sønderby et al., 2021). However, to go beyond a correlative level of analysis, animal and cellular models are needed to causally demonstrate that a genetic change leads to a particular phenotype. This provides a way to strip apart theoretical mechanisms by which a CNV could generate disorder and investigate exact behavioural or biological processes using single and multiple gene deletion or duplication mutants, tested alongside their naturally occurring wild-type controls.

1.2.3. Copy number variation involving the *DLG2* gene

Using a large international cohort Kirov et al (2012) identified 34 de novo CNVs associated with schizophrenia. One of these was a deletion in the 11q14.1 region, occurring in two patients. This CNV entails complete deletion of the *DLG2* gene, a member of the discs large family of membrane-associated guanylate kinases (MAGUKs) of which there are four

paralogs: *DLG1*, *DLG2*, *DLG3* and *DLG4*. These genes code for proteins which anchor receptors and other effectors in the postsynaptic density, a huge protein complex associated with the postsynaptic membranes of excitatory synapses responsible for the transduction of synaptic signals (see Section 1.3.1 for details). As the only coding region deleted at 11q14.1 relates to *DLG2* it likely that any phenotype results from heterozygous deletion of this gene. While it is likely that not all de novo CNVs isolated by Kirov et al's (2012) large study are pathogenic, there is evidence that a CNV that disrupts *DLG2* may be.

Firstly, genetic disruptions involving MAGUK family members have been associated with a variety of neurodevelopmental disorders. *DLG1* and *DLG2* CNVs have been reported before in schizophrenia (Levinson et al., 2011; Mulle et al., 2010; Walsh et al., 2008), and those encompassing *DLG3* have been associated with mental retardation (Tarpey et al., 2004) and autism (Guilmatre et al., 2009; Pinto et al., 2010).

Secondly, the finding of *DLG2* CNVs in schizophrenia has replicated with Nithianantharajah (2018) finding two *DLG2* deletions and one *DLG2* duplication in a 1,115 participant schizophrenia cohort. Michaelovsky et al (2019) studied family members carrying another schizophrenia associated CNV (22q11.2), demonstrating that the one member of the family with a diagnosis of schizophrenia had both 22q11.2 and 11q14.1 deletions. Further, since its initial isolation this 11q14.1 CNV it has also been isolated in patients with bipolar disorder (Noor et al., 2014), ASD (Ruzzo et al., 2019) and two patients with intellectual disability (Reggiani et al., 2017). A duplication CNV at this locus has also been associated with ASD, with a predicted resultant *DLG2* transcript that would encode an expanded amino acid residue of 999 instead of the typical 749-residue PSD-93 protein *DLG2* codes for (Egger et al., 2014). This link between disrupted gene and coded protein in disorder provides evidence that the mechanism of protein alteration via genetic disruption could be pathogenic. This is often assumed with psychiatric-relevant genetic mutations without direct evidence of protein abnormalities.

Genetic disruption to *DLG2* outside of CNVs is also associated with a broad spectrum of psychiatric disorders. Alemany et al., (2015) isolated a SNP in *DLG2* associated with ADHD in an adult population. This SNP was specifically related to the standard error of hit-reaction

time, a test of response speed consistency relating to impulsivity in patients. A significant downregulation of *DLG2* mRNA has also been found in the dentate gyrus and CA1 hippocampal regions of post-mortem brain tissue from those with major depressive disorder relative to comparison subjects matched for sex, race and age (Duric et al., 2013).

DLG2 may be so frequently implicated in genetic studies as there is evidence that the *DLG2* gene is in a hotspot for L1-associated variants, regions of repetitive DNA sequences that are able to mutate via retrotranspositon. This encourages mosaicism at this location which has the potential to be pathogenic (Erwin et al., 2016). Taken together these findings show that multiple mechanisms of disruption to *DLG2* and related *DLG* genes confer psychiatric risk, implicating the *DLG2* coded protein PSD-93 (and where the other *DLG* paralogs are concerned, its relatives) as having an involvement in mechanisms which precipitate disorder.

Finally, for the case of schizophrenia, disruption to *DLG2* aligns with biochemical hypotheses of the cause of disorder. The CNVs outlined by Kirov et al (2012) are enriched for genes encoding proteins associated with synaptic function. MAGUK proteins, PSD-93 included, associate with the NMDA (Chen et al., 2012; Irie et al., 1997; Niethammer, Kim, & Sheng, 1996) and AMPA receptors (Dakoji et al., 2003). Antagonism of NMDARs is known to induce schizophrenia-like symptoms in humans (Javitt & Zukin, 1991; Snyder, 1980; Tamminga, 1998) and exacerbate positive and negative symptoms in schizophrenic patients (Itil, Keskiner, Kiremitci, & Holden, 1967; Lahti, Koffel, Laporte, & Tamminga, 1995; Malhotra et al., 1997). Building from this, the glutamate hypothesis of schizophrenia postulates that the negative, positive and cognitive symptoms of the disorder stem from aberrant glutamate signalling (Moghaddam & Javitt, 2012). The primary glutamatergic dysfunction is proposed to cause a downstream effect on dopamine, triggering a secondary impairment in the system (Moghaddam & Javitt, 2012). Ingason et al., (2015) report a clear link between glutamate dysfunction and *Dlg2*, with the finding that *Dlg2* expression is significantly reduced in a chronic NMDAR antagonism (MK-801 for 20 days) rodent model of NMDAR hypofunction. Where *DLG2* is the primary component disrupted through genetic aberrations, it follows that a dysfunctional glutamate system may be the result of and

impairment in the proteins responsible for its functioning, such as the NMDA and AMPA receptors, due to alteration to the postsynaptic scaffolds (e.g. PSD-93) that anchor them.

For these reasons *DLG2* is an attractive candidate gene for modelling disease relevant processes such as alterations to synaptic transmission or glutamate signalling. Determining the role of *DLG2* in generating psychiatric endophenotypes can be used to further isolate related proteins and pathways implicated in *DLG2*-associated psychiatric disorders, such as schizophrenia and ASD.

1.3. The postsynaptic density, *DLG2* and disease

1.3.1. The importance of *DLGs* in the postsynaptic density

When viewed through an electron microscope, excitatory synapses can be distinguished by an electron dense tip at their spine heads (Palay, 1956). This is the postsynaptic density (PSD), a layered cluster of proteins responsible for transducing signals relayed across the synaptic cleft. In this 30 to 40 nm macromolecular signalling complex proteins can be roughly divided into classes of cell adhesion proteins, cytoskeletal proteins, scaffolding and adaptor proteins (e.g. MAGUKs), membrane-bound receptors and channels (e.g. NMDAR and AMPAR), G-proteins and modulators and signalling molecules (e.g. kinases/phosphatases) (Husi, Ward, Choudhary, Blackstock, & Grant, 2000; Kennedy, 2000; Klauck & Scott, 1995; Sheng, 2001; Ziff, 1997).

As the large majority of excitatory synapses in the central nervous system are glutamatergic, ionotropic glutamate receptors concentrate at the PSD, where specialized molecules such as PSD-93 anchor them and regulate their trafficking. The dynamic reorganisation of the PSD components and structure is thought to be the structural basis for aspects of the synaptic regulation and plasticity which are crucial for memory formation (e.g. Carlisle & Kennedy, 2005; Hering & Sheng, 2001; Yuste & Bonhoeffer, 2001). Synaptic scaffold proteins such as the DLG paralogs of the MAGUK family are crucial for allowing the postsynaptic neuron to respond to its inputs by altering the structure and constituents of the PSD as they stabilise and relocate other proteins through interaction with the cytoskeleton (Choquet & Triller,

2013). All *DLG* proteins contain three discs large (also known as PSD-95/zona occludens-1 PDZ) domains, a Src-homology-3 domain and a catalytically inactive guanylate kinase domain, just like other MAGUKs. They diverge in the structure of the N-termini of their PDZ domains (Won, Levy, Nicoll, & Roche, 2017) which determines their different binding partners.

Genes for DLG proteins are ancient in origin, appearing in protosynaptic eukaryotes such as choanoflagellates around 550 million years ago (Nithianantharajah et al., 2013). Two rounds of whole-genome duplication and vertebrate-specific duplication of *DLG* genes has resulted in the four *DLG* paralogs (*DLG1*, *DLG2*, *DLG3* and *DLG4*). This expansion provides an example of the diversification of postsynaptic genes thought to be responsible for the broadening of synaptic capabilities and concomitantly cognitive function amongst vertebrates (Nithianantharajah et al., 2013). The *NR2* genes of the NMDA receptor provide another example of this, with gene duplication resulting in four distinct paralogs (*NR2A*, *NR2B*, *NR2C*, *NR2D*) which have also diverged at the protein sequence level (Ryan et al., 2008). Nithianantharajah et al (2013) propose that this duplication may give an expanded and flexible set of cognitive functions but these advances in behavioural repertoire come at the price of increased susceptibility to mental illness from disease-causing mutations in any of the increased number of related genes.

Interestingly no amino acid motif is conserved amongst the *NR2* paralogs except the PDZ domain responsible for interacting with MAGUKs (Ryan & Grant, 2009). Thus, the relationship between NMDAR and MAGUKs is clearly an important one. This is interesting given the known link of NMDAR dysfunction to schizophrenia (e.g. Moghaddam & Javitt, 2012), as the genetic disruption causing alteration to PSD-93 may have its influence through secondary effects on NMDARs.

As mentioned above, the definitional roles of the *DLG* proteins are the stabilisation, recruitment and trafficking of NMDA and AMPA receptors to the postsynaptic membrane (Chen et al., 2000; Kornau, Schenker, Kennedy, & Seeburg, 1995). However, the adaptive function of gene duplication and diversification is in creating proteins with different roles, which there is evidence of between the *DLG*s. There are different regional and temporal

expression patterns of the paralogs. *DLG2* and *DLG4* are enriched in the PSD only, while *DLG1* and *DLG3* are found both at synapses and in the cytoplasm (El-Husseini et al., 2000). Furthermore, *DLG1* is the only paralog expressed presynaptically and postsynaptically, the others only being expressed postsynaptically (Aoki et al., 2001). During development *DLG1* and *DLG3* are highly expressed embryonically and postnatally before decreasing in expression throughout adulthood, while *DLG2* and *DLG4* show the opposite expression pattern (Cai et al., 2006; Müller et al., 1996). Thus, it seems that the *DLG1* and *DLG3* play more of a developmental role than their two relatives.

Other unique functions for specific *DLG* paralogs have been discovered. Unlike other *DLG*s, *DLG3* (protein SAP102) plays a role in clearing NMDA receptors from synaptic sites (Chen et al., 2012). This is likely due to its lack of PDZ-domain palmitolaytion allowing it to move in and out of spines, shuttling NMDARs in and out of the synapse (Zheng et al., 2010). Only *DLG4* (protein PSD-95) has been found to trigger the degradation of STEP (striatal-enriched protein tyrosine phosphatase) in the PSD (Won, Incontro, Nicoll, & Roche, 2016). STEP is a protein known to increase internalisation of NMDARs (Goebel-Goody et al., 2012), thus degradation of STEP by PSD-95 indirectly prevents removal of NMDAR from the postsynaptic membrane.

There is also variation in the manner with which different *DLG*s perform their primary function of glutamate receptor stabilisation. *DLG1* is the only *DLG* to bind AMPA directly via the GluR1 subunit (Leonard et al., 1998) while *DLG2*, *DLG3* and *DLG4* interact with AMPA indirectly through transmembrane AMPAR regulatory proteins (Chen et al., 2000; Dakoji, Tomita, Karimzadegan, Nicoll, & Bredt, 2003; Schnell et al., 2002). Where the NMDAR is concerned *DLG3* binds subunit GluN2B through any of its PDZ domains, while *DLG2* and *DLG4* can only bind GluN2B through their first or second PDZ domains (Brenman et al., 1996; Kim et al., 1996; Müller et al., 1996). This implies that diversification of the *DLG* gene also opened the possibility for functional compensation between the paralogs, i.e. if *DLG1* cannot directly regulate AMPA, the other paralogs can perform this function successfully via a different route.

1.3.2. Isn't it unfair when your sibling gets all the attention: the roles of *DLG2* (protein PSD-93) and *DLG4* (protein PSD-95) at the synapse

The most extensively researched paralog is *DLG4* (PSD-95), shown in the synapse with its key interactors in Figure 1. This is the most abundant in the PSD (Cheng et al., 2006; Cho et al., 1992), and has been linked to similar psychiatric conditions to PSD-93 such as schizophrenia and ASD (Coley & Gao, 2018). Like PSD-93, PSD-95 is predominantly found in the PSD in adult brains rather than the cytoplasm (Cai, Li, Rivera, & Keinänen, 2006). It has been proposed to be a 'slot' protein which determines synaptic AMPA receptor (AMPAR) content (Elias et al., 2006; Keith & El-Husseini, 2008). This is important as AMPA receptors are responsible for the bulk of fast excitatory transmission in the central nervous system, and the main way the postsynaptic response to a stimulus is increased is through the insertion of AMPA receptors into the postsynaptic membrane. This is achieved through both increasing the number of AMPARs at the membrane, and inserting modified Ca^{2+} permeable AMPARs which have a high conductance to the postsynaptic surface (Plant et al., 2006; Terashima et al., 2004). Modifying the connectivity between two neurons via the synapse is a process key to Hebbian plasticity, to allow dynamic connections between neurons relative to experience. It is also essential for homeostatic plasticity which tunes neural networks to balance excitatory and inhibitory network dynamics.

Beyond AMPA stabilisation and trafficking, PSD-95 has been shown to be important in the synaptic localization of many key PSD effectors, as outlined below. While the role of PSD-95 is well elaborated, the synaptic role of PSD-93 remains controversial. PSD-95 and PSD-93 share a 71% sequence homology and the same domain structure (Fiorentini et al., 2009). However they have differing PDZ1 domains that could interact preferentially with different binding partners (Fiorentini et al., 2009), rendering them suitable both for functional redundancy and distinctive biochemical processes.
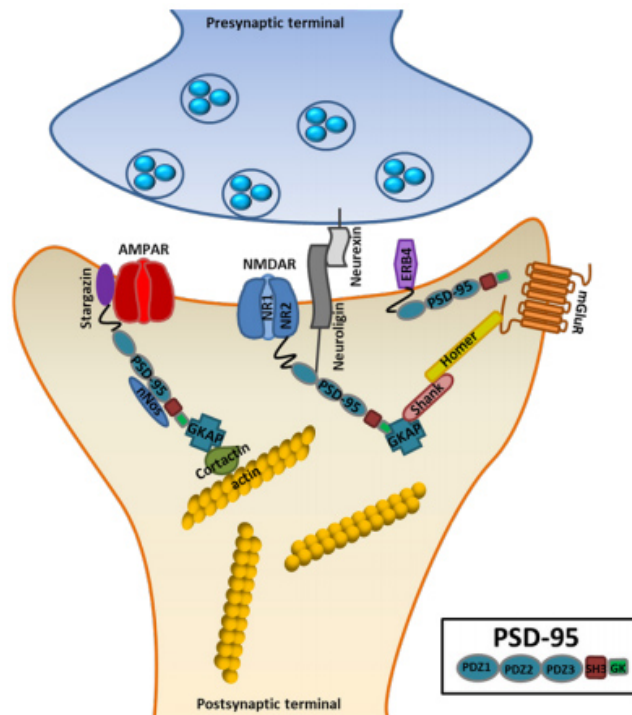
Figure 1: Localisation and key binding partners of PSD-95, the most structurally homologous and functionally redundant PSD-93 related protein, at the synapse. The structure of PSD-93 is schematically the same as PSD-95 with three post-synaptic density domains (PDZ1-3) shown in blue, a SH3 domain and a catalytically inactive guanylate kinase (GK) domain. PSD-93 interacts with NMDARs and Neuroligin directly much as depicted here for PSD-95. PSD-93 interacts with AMPARs indirectly through transmembrane regulatory proteins (e.g. stargazin shown here) much as PSD-95 does. Differences in function between PSD-93 and PSD-95 are outlined in the following sections. Other biding partners of PSD-93 are detailed in section 1.3.5. Image adapted from Coley & Gao, (2018).

1.3.3. AMPAR related processes

An AMPAR-mediated process that involves both PSD-95 and PSD-93 is synaptic scaling. A determinant of homeostatic plasticity, synaptic scaling refers to the mechanism by which neurons regulate their own excitability in response to chronic changes in network activity via bidirectional adjustments in synaptic AMPAR content. Using neuronal cultures transfected with short hairpin RNAs (shRNAs) to knockdown PSD-95, PSD-93 and DLG3, Sun and Turrigiano, (2011) demonstrated that PSD-95 and DLG3 abundance at the synapse were bidirectionally regulated by changes in network activity, while PSD-93 was not. However,

changes in abundance did not appear sufficient to drive homeostatic changes in synaptic strength. Rather PSD-93 and PSD-95 were found to play different roles in synaptic scaling up and down at different neuronal ages. In young neurons PSD-93 and PSD-95 could support scaling up, while PSD-95 alone was required for scaling down. In older neurons both scaling up and down were critically dependent on only PSD-95. It appears that early in development there is a functional redundancy between PSD-93 and PSD-95, with PSD-93 able to compensate for PSD-95 loss during scaling up.

Further evidence that PSD-93 and PSD-95 play different roles in developmental plasticity processes comes from work by Favaro et al (2018). Electrophysiology, western blotting, and immunohistochemistry were performed in visual cortex slices from mice with homozygous (-/-) knockout of either PSD-93, PSD-95 or both proteins. These slices were taken at different developmental timepoints as mice matured, at $P4 \pm 1$, $P11 \pm 1$, $P20 \pm 1$, $P25 \pm 1$ and $P28 \pm 1$. It was found that PSD-93 inhibited maturation of immature AMPAR silent synapses in mouse cortex development, while PSD-95 promoted it. Critical period closure was impaired in PSD-95$^{-/-}$ mice, while it closed precociously in PSD-93$^{-/-}$s. Together it appears that PSD-95 and PSD-93 work together during development as accelerator and brake to determine the pace of maturation, fine tuning networks during critical periods.

Favaro et al., (2018) focused primarily on the visual cortex, yet similar PSD-93 and PSD-95 related maturational deficits to silent synapses were also found in the nucleus accumbens, hippocampus and medial prefrontal cortex. These findings could serve as a template for malfunction in other cortical areas during development that could precipitate neurodevelopmental disorders, particularly as critical periods for language and social behaviour coincide with ASD diagnosis in children (Berger et al., 2012) and critical periods of higher cognitive functions precede clinical diagnosis of schizophrenia in young adults (Selemon & Zecevic, 2015).

At mature excitatory synapses PSD-93 and PSD-95 play a role in maintaining AMPARs. Using methods involving shRNA knockdown and testing homozygous mutants for PSD-93, PSD-95 and both combined in cultured hippocampal pyramidal cells, Elias et al., (2006) demonstrated both functional redundancy and key roles for these related proteins in

synaptic transmission. Mice lacking either PSD-95 or PSD-93 show little deficit in synaptic transmission, reflecting redundancy as double genetic knockouts showed a deficit. Furthermore, it was found that PSD-95 and PSD-93 determine the AMPAR content at separate, largely nonoverlapping subsets of synapses (Elias et al 2006). This has been confirmed using in vivo techniques, which demonstrated that only about 30% of synapses expressed both DLGs (Sans et al., 2000). Functional consequences of this synaptic heterogeneity can be assumed but are difficult to directly assess. These findings imply a mechanism of compensation for loss of either protein from a synapse, where PSD-93 could support transmission in the absence of PSD-95 or vice versa.

The research reviewed here demonstrates a critical role for PSD-93 in development, both in terms of developmental scaling up and down and in activating silent synapses (Favaro et al., 2018). In mature neurons PSD-93 and PSD-95 operate in tandem to facilitate AMPAR related synaptic transmission and may play discrete roles in differing neuronal populations (Elias et al., 2006). These findings concern the role of PSD-93 in basal-state AMPAR processes. In terms of activity-dependent processes it has been demonstrated that none of the NMDAR-associated MAGUKs, PSD-95 and PSD-93 included, are uniquely required for the insertion of AMPARs into the postsynaptic membrane to potentiate neuronal coupling following synaptic strengthening by long-term potentiation (LTP) (Carlisle et al., 2008). The role of PSD-93 and PSD-95 in activity-dependent processes is outlined further in the following section.

1.3.4. NMDAR related processes

PSD-93 and PSD-95 also have unique roles in Hebbian plasticity, the mechanism through which change in synaptic efficacy and structure retains information, a cellular basis for learning and memory (Bliss & Lømo, 1973; Hebb, 1949; Whitlock, Heynen, Shuler, & Bear, 2006). This encompasses LTP, the strengthening of a synaptic connection between neurons, and its counterpart long-term depression (LTD), an activity dependent reduction in synaptic efficacy. Both LTP and LTD are umbrella terms encompassing multiple molecular mechanisms which may be expressed in different neuronal populations, brain regions and at different stages of development. Glutamatergic LTP mostly relies upon AMPAR and NMDAR,

and thus is also related to the proteins that interact with these receptors at the membrane such as PSD-93 and PSD-95.

Changes to synaptic strength can be seen physiologically through reorganisation of the PSD, a process PSD-93 and PSD-95 are crucial to. It has been estimated that at least 2% of the PSD proteasome changes following induction of LTP, although this is probably an underestimation (Zhang, Neubert, & Jordan, 2012). In glutamatergic LTP this rearrangement occurs due to sustained high-level AMPAR activation, facilitating depolarisation of the postsynaptic membrane, removing the $Mg2^+$ block from voltage-gated NMDARs (Bi & Poo, 2001). This allows Ca^{2+} influx through voltage-gated NMDARs, which triggers synaptic excitation thereby activating CaMKII which is associated with the GluN2B NMDAR subunit. CaMKII relocates into the spine and mediates PSD remodelling through phosphorylation of PSD proteins, which facilitates movement or alters protein binding possibilities (Yoshimura et al., 2002). This brings us back to the key PSD remodelling processes, as this LTP cascade results in additional AMPAR being incorporated into the postsynaptic membrane (Malenka, 2003; Nicoll & Roche, 2013).

Glutamatergic LTD occurs predominantly when prolonged weaker neuronal activation induces signalling cascades which result in the removal of AMPAR from the postsynaptic membrane (Lüthi et al., 1999). LTD is also dependent on Ca^{2+} and voltage-gated NMDARs (Bi & Poo 2001). The timing of pre and postsynaptic neural activation determines whether LTP or LTD will occur. For example, according to Hebb's (1949) law strong co-occurring activation would result in synaptic strengthening through LTP, yet activation of only the presynaptic neuron would result in LTD (Bi & Poo 2001).

Both PSD-93 and PSD-95 interact to form NMDAR stabilising supercomplexes (functional groups of bound interacting proteins forming complexes which associate together) (Frank & Grant, 2017; Frank et al., 2016). Neither protein alone is sufficient to induce supercomplex formation (Frank et al., 2016). Other supercomplexes, such as that between Arc and Kir2.3 (an inwardly rectifying potassium ion channel) require PSD-95 but not PSD-93 (Frank & Grant, 2017). This suggests that PSD-93 might have a specific role in NMDAR related processes, which is further evidenced by Elias et al's (2006) finding that single shRNA

knockdown for PSD-95 or PSD-93 selectively reduced AMPAR transmission, whereas simultaneous double shRNA reduced both AMPAR and NMDAR mediated excitatory post synaptic potentials (EPSCs). It has also been shown that PSD-93 deficiency in mouse brain slices reduces the expression of its two direct NMDAR subunit binding partners, NR2A and NR2B (Tao et al., 2003; Zhang et al., 2010). Thus, while either PSD-95 or PSD-93 can stabilise AMPARS, both proteins are key structural elements required for proper expression and stabilisation of NMDARS in the cytoplasmic matrix.

Carslile et al., (2008) set out to determine the role of PSD-95 and PSD-93 in LTP and LTD plasticity processes in the hippocampal CA1 region. They compared different stimulation protocols to induce these forms of plasticity in hippocampal brain slices from PSD-93 and PSD-95 homozygous knockout mice. These entailed LTD induced by low frequency stimulation of 1 Hz over 15 minutes, spike timing-dependent LTP which occurs by back-propagating action potentials that occur within several millisecond after presynaptic action potentials, and TPS-induced LTP generated by theta frequency (5 Hz) stimulation of the presynaptic fibres. LTD was normal in PSD-93 mutants and impaired in PSD-95 mutants, while both TPS-induced and pairing-induced LTP were facilitated in PSD-95$^{-/-}$ slices and impaired in PSD-93$^{-/-}$ slices. This shows partially opposing roles for these paralogs in LTD and LTP forms of Hebbian plasticity.

These researchers found that NMDAR-mediated synaptic transmission, ascertained by assessing the NMDAR generated component of the EPSC under whole-cell voltage-clamp conditions, is normal in PSD-93 and PSD-95 knockouts (Carlisle et al., 2008). This aligns with prior findings that shRNA mediated knockdown of PSD-93 or PSD-95 had no effect on NMDAR-mediated synaptic currents (Elias et al., 2006). These researchers propose that alterations of LTP seen here are not due to changes in the function of NMDARs themselves, but a product of the complexes and biochemical pathways PSD-95 and PSD-93 couple them with. For example, PSD-93 may be critically responsible for coupling NMDARS to signalling molecules that facilitate LTP induction. This could create a network where synapses which mainly express PSD-93 (Elias et al., 2006) could facilitate LTP induction, which would be down-regulated at synapses where PSD-95 is predominant. This is a further example that

diversification of the *DLG* paralogs affords different plasticity processes which could alter the application of a process (i.e. LTP) to provide expansion of functions.

A conflict in findings is apparent with Tao et al (2003) finding NMDAR-mediated excitatory postsynaptic currents to be reduced in PSD-93$^{-/-}$ mouse synapses. This is likely due to the difference in brain regions tested, with Carslile et al. (2008) and Elias et al. (2006) studying NMDAR EPSC in hippocampal slices while Tao et al. (2003) focused on these events in the dorsal horn and forebrain. This further implies that in different brain regions the relationship between NMDAR and PSD-93 may vary, operating directly or through other binding partners and pathways in the differing PSD structures of synapses at varying brain regions. As the majority of information concerning the role of PSD-93 and PSD-95 in LTP and LTD is known through studying hippocampal slice preparations (e.g. Carlisle et al., 2008, Elias et al., 2006) this discrepancy implies that LTP and LTD might have different relationships to these proteins elsewhere in the brain.

Thus far the studies described have focused on characterising plasticity processes in brain slices or cultured cells with homozygous *Dlg2* disruption. This means that there will be no PSD-93 at the synapse, enabling researchers to ascertain synaptic functions which depend on it. However, to understand how PSD-93 may be disrupted, or cause disruption to synaptic processes in disorder heterozygous *Dlg2* mutant models should be studied. This is because heterozygous mutants, which should show a reduction of PSD-93 by ~50% at the synapse, capture the fact that in CNVs associated with psychiatric risk such as that at 11q13.1 only one copy of *DLG2* is deleted.

Investigations into synaptic transmission in the hippocampus of *Dlg2* heterozygous rat brain slices demonstrated an increase in NMDAR-mediated synaptic currents (Griesius et al., 2022). This would be expected to enhance neuronal excitability and synaptic plasticity, but impaired pairing-induced LTP was demonstrated in the model. This LTP impairment was thought to result from an increase in potassium ion channel function, leading to an electrical leak precipitating a decrease in input resistance and reducing associative LTP. Thus, phenotypes seen in the heterozygous model do not recapitulate those seen in homozygous models, as no changes to TPS-induced LTP were shown in *Dlg2*$^{+/-}$ rats unlike the facilitation

of this process, and of pairing-induced LTP, in *Dlg2*^{-/-} mouse slices (Carlisle et al 2008). These differences highlight the importance of studying the most psychiatric-relevant models (i.e. *Dlg2* heterozygotes) if aiming to draw conclusions about processes implicated in disorder.

Griesius et al (2022) also found no changes in AMPAR currents in the heterozygous knockdown model. This is in line with the finding from Elias et al., (2006) that PSD-93 and PSD-95 are both able to support AMPAR related processes even in the absence of the other protein. In heterozygous mutants it is likely that AMPAR processes are supported by either PSD-95 and the remaining PSD-93, possibly together or by either protein in separate groups of synapses.

1.3.5. PSD-93 interactions with other PSD components

PSD-93 association with AMPAR and NMDAR glutamatergic processes is instructive given the key role of this type of neurotransmission in learning processes (Whitlock, Heynen, Shuler, & Bear, 2006) and in schizophrenia (Balu, 2016; Tamminga, 1998), a condition associated with *DLG2* disruption. Yet there are other PSD-93 binding partners which if disrupted could precipitate psychiatric phenotypes, such as proteins involved in potassium channel palmitoylation, cell adhesion, microtubule assembly, and cell signalling: palmitoyltransferase ZDHHC14 (Sanders et al., 2020), Fyn (Nada et al., 2003; Sato et al., 2008), ERK2 (Guo et al., 2012), GKAP (Kim et al., 1997), and MAP1A (Brenman et al., 1996). Uniquely to the MAGUK family, PSD-93 is targeted to the axon initial segment where it regulates neuronal excitability via its interactions with potassium channels (Ogawa et al., 2008; Sanders et al., 2020).

PSD-93 binds neuroligin 1-3 (Irie et al., 1997). Through neuroligins PSD-93 and other DLGs may also indirectly influence presynaptic neuron function and quantal release. Neuroligins branch the synapse and can interact with b-neurexin which is coupled to other effectors which regulate neurotransmitter vesicle release (Dean & Dresbach, 2006). There is evidence of enhanced paired-pulse facilitation in PSD-93 and PSD-95 homozygous knockout mice brains, suggestive of a reduction in neurotransmitter release from the presynaptic terminal Carslile et al., (2008).

Loss-of-function mutations in the gene coding neuroligin-4 and point mutations in its homologue neuroligin-3 are the cause of ASD in some cases of monogenic heritable cases (Jamain et al., 2003; Laumonnier et al., 2004) while mutations in the neuroligin-2 gene have been associated with schizophrenia (Sun et al., 2011). In addition, neuroligin-3 deficit mice display a behavioural phenotype reminiscent of the lead symptoms of autism (Kalbassi, Bachmann, Roberton, & Baudouin, 2017; Radyushkin et al., 2009). This entails, amongst other phenotypes, a reduced preference for interacting with a known conspecific over a novel one and hyperactivity to novel environments (e.g. Radyushkin et al., 2009). On similar tests $Dlg2^{-/-}$ knockout mice show slightly different phenotypes. These include hypoactivity in novel environments (Yoo et al., 2020, Winkler et al., 2018) and reduced preference for socialising with a novel conspecific rather than a novel object (Yoo et al., 2020 but see Winkler et al 2018). The genetic association of these two binding partners, PSD-93 and neuroligins, with disease could demonstrate another general biological process which if disrupted increases the risk of disorder. The phenotypic difference generated by targeted disruption to these related proteins could result from imbalance to this general biological process. However, comparing across different studies with different manipulations to PSD-93 and neurologin-3 does not rule out these seemingly related deficits having different biological origins.

1.3.6. Differing functions of PSD-93 isoforms

Further increasing the multiplicity of the function of the DLGs in the synapse is the fact that they all exist in various isoforms resulting from splice variation. PSD-93 has six N-terminal isoforms, more than any other DLG, including two palmitolayted isoforms (α), a L27 domain containing isoform (β) and three other unique N-terminal isoforms (γ, δ, ϵ) (Parker et al., 2004). Using overexpression of the 6 isoforms in organotypic hippocampal slice cultures, some of which has PSD-95 or PSD-93 knockdown, Krüger et al. (2013) demonstrated that all PSD-93 isoforms enhanced AMPAR function in the hippocampus by different mechanisms. This allows for specific and selective modes to regulate synaptic plasticity. PSD93 α and ϵ enhanced AMPAR function, while β and γ rescued AMPAR function to control cell levels when PSD-95 was knocked down. β enhanced AMPAR function in a way dependent on

excitatory synaptic activity (i.e. environmental input). The authors propose that this role of PSD-93 β may be essential in certain conditions, for example in the developing sensory cortex when immature synapses lack PSD-95 (Yoshii et al., 2003). Variation in isoforms, as with the expansion of DLG paralogs, likely provides a way to broaden synaptic signalling scaffolds to demarcate plasticity, allowing for more complex behaviours but risking damage to specific processes upon disruption (Nithianantharajah et al., 2013).

1.3.7. *DLG2* disruption and neural network properties

The work reviewed thus far focuses on the protein composition and plasticity processes measured in electrophysiological observations from single neurons. Focusing on the network firing properties of multiple neurons may be more indicative of the kind of disruption seen in a neurodevelopmental disorder precipitated by *DLG2* disruption. MacLaren, Charlesworth, Coba, & Grant, (2011) examined neuronal network firing behaviour of primary cultures of mouse hippocampus neurons on multi-electrode arrays, knocking down schizophrenia and bipolar susceptibility genes including *Dlg2* with RNAi. It was found that ~63% *Dlg2* knockdown impairs neuron burst rate, burst duration and a correlation index (the degree of correlation between bursting of pairs of neurons in the array) on days 7 and 8 post plating. These effects recovered by the end of the recording period when the RNAi would have started to wane, and thus this pattern of impairments is more consistent with *Dlg2* having a role in ongoing synaptic function than in development, as knockdown produces recoverable effects. These impairments to network firing resulting from *Dlg2* disruption point to its involvement in another mechanism of cross-disorder aetiology, that of altered neuronal synchrony as observed in schizophrenia (Spellman & Gordon, 2015) and autism (Lajiness-O'Neill et al., 2018).

1.4. *DLG2* and rodent psychiatric disease models

DLG2, through interaction with various synaptic proteins, is implicated in a variety of processes essential for synaptic plasticity and function. Although the molecular and electrophysiological consequences of *DLG2* disruption have been outlined, the effect of this on behaviour, such as learning and cognition, have not been extensively characterised.

Rodent models provide a way to assess the influence of a specific genetic manipulation on psychiatry-relevant behavioural endophenotypes in a controlled *in vivo* environment. This is particularly salient when attempting to understand the pathogenicity of genes deleted in a CNV which is linked to a broad spectrum of psychiatric outcomes (i.e. the same deletion or duplication leading to diagnoses of schizophrenia, autism, bipolar disorder and intellectual disability in different carriers). Rather than modelling a particular condition *Dlg2* deletion in rodents allows parsing of which behavioural and biological phenotypes are spared and impaired by genetic disruption, allowing cross-disorder biochemical pathways and behavioural processes to be isolated.

1.4.1. Behavioural phenotypes in rodent models of *Dlg2* disruption

The first study to assess the behaviour of *Dlg2* homozygous knockout mice did so with the aim of assessing the association of cognitive functions with each *Dlg* paralog, with the hypothesis that the evolutionary expansion of the *Dlg* gene should afford advances in cognition for mammals (Nithiananthrajah et al., 2013). Mutants of all *Dlgs* (*Dlg1*, *Dlg2*, *Dlg3* and *Dlg4*) were compared on a battery of increasingly complex touchscreen tasks adapted from the Cambridge Neurophysiological Test Automated Battery (CANTAB). Although $Dlg1^{-/-}$ was embryonic lethal, *Dlg1* heterozygosity resulted in similar cognitive performance to wild-types, showing that a single copy of *Dlg1* is sufficient for normal cognition. Simple Pavlovian and operant associative learning tasks were impaired in $Dlg4^{-/-}$ mice but were successfully performed by $Dlg2^{-/-}$ and $Dlg3^{-/-}$ knockouts.

When tested on more complex tasks such as extinction, discrimination learning, reversal learning, attention (five choice serial reaction time task) and the ability to learn and remember object-location pairs (visuo-spatial discrimination learning), $Dlg2^{-/-}$ mice exhibited deficits in comparison to wild-types. A dichotomy of function was observed between *Dlg3* and *Dlg2* on attention and the acquisition of reversal and discrimination, where $Dlg3^{-/-}$ amplified learning rate and attention span while $Dlg2^{-/-}$ attenuated learning rate and attention span. The researchers postulate that in a healthy brain *Dlg3* facilitates the acquisition of this kind of learning and attentional allocation while *Dlg2* regulates the flexibility of attention and the use of learned information.

Relying on CANTAB tasks and touchscreens allowed comparison between $Dlg2^{-/-}$ mice and humans with *DLG2* CNVs. Three human carriers exhibited the deletion CNV and one the duplication. All but one had a schizophrenia diagnosis. Just as the mouse model, human *DLG2* CNV carriers made more errors than controls in the acquisition of visual discrimination, cognitive flexibility tasks and visuo-spatial learning and memory and demonstrated decreased accuracy of sustained attention. The translational comparison is striking, but reliance on touchscreen tasks limited learning and cognition to only visual stimuli which may be more challenging to learn about than more ethologically relevant domains to rodents such as scent and sound (Broadbent et al., 2007). Most importantly all humans tested had heterozygous *DLG2* disruption but were compared to homozygous mice. It is essential to know whether the impairments seen in *Dlg2* knockout rodents remain in a more clinically accurate heterozygous *Dlg2* model.

When tested on other disease-relevant domains such as social and motor behaviour, studies of $Dlg2^{-/-}$ mice have produced conflicting results. Winkler et al (2018) found $Dlg2^{-/-}$ mice to be impaired in rotarod and beam balance testing while McGee et al., (2001) report comparable rotarod performance between *Dlg2* homozygotes, *Dlg2* heterozygotes and wild-types. Further, in the social domain, Winkler et al (2018) found $Dlg2^{-/-}$ mice to be hypersocial on tests of dyadic social interaction while Yoo et al (2020) found $Dlg2^{-/-}$s to be hyposocial - investigating the novel conspecific less on dyadic social interaction and the three-chamber social preference and social novelty tasks. Mutants in Winkler et al (2018) and Yoo et al (2020) also demonstrated hypoactivity to a novel environment and increased self-grooming in their home cage and novel environments, the latter of which has been taken as a rodent behaviour analogous to the repetitive behaviours ('stimming') seen in autism (Kapp et al., 2019).

In line with the social behaviour impairments and repetitive behaviour Yoo et al (2020) state that $Dlg2^{-/-}$ mice show key phenotypes associated with autism. Yet with the lack of replication, discrepancies in protocols and generation of mutants could account for these differences. Interestingly when heterozygous *Dlg2* mutants were tested by Winkler et al (2018) their social behaviour was comparable to wild-types, further highlighting the

importance of testing models as close as possible to mutations seen in the human population.

It is clear from this review of previous investigations that heterozygous *Dlg2* models, those which capture the single chromosome deletion of *DLG2* in a CNV (Fromer et al., 2014; Kirov et al., 2012; Purcell et al., 2014; Walsh et al., 2008) or single chromosome loss-of-function mutations (Reggiani et al., 2017; Ruzzo et al., 2019), have been under-studied. Where they have been investigated, they have not shown the same impairments as *Dlg2*$^{-/-}$s, showing normal social preference, locomotor response to a novel environment and grooming (Winkler et al., 2018). While homozygous models elucidate which processes the Dlg2 protein is essential for, heterozygous models provide a more valid window into which phenotypes persist when some PSD-93 protein is present at the synapse, and the mechanisms of compensation (i.e. between PSD-93 and PSD-95) which are clinically relevant. For these reasons the work in this thesis focuses on phenotyping a novel *Dlg2*$^{+/-}$ rat model.

1.4.2. Overview of rodent model and key questions

A major challenge to work using animal models of psychiatric disorder risk factors is that phenotypic outcome will vary with species, background strain of that species, and its rearing and housing environment. Variation of this sort distracts from drawing conclusions about the manipulation of interest (here *Dlg2* haploinsufficiency) on relevant assays and confuses translation of these principles to humans. This work presents the first in-depth characterisation of a novel CRISPR-generated *Dlg2*$^{+/-}$ rat model, having the advantages of using a species and strain amenable to behavioural testing and a genetic lesion that recapitulates those observed in humans.

Rodent models are excellent for investigating psychiatric disease as complex behaviour and cognition can be assessed, unlike models using worms, flies, or fish. Nonhuman primates afford greater translational comparisons but are more expensive to house and breed than rodents. From 1990 to 2015 the proportion of rodent model publications that used mice increased steadily to over 50% (B. Ellenbroek & Youn, 2016). Mice are commonly favoured

due to genetic manipulation tools for mice initially surpassing those for rats, with the first recombinant mouse model described in 1987 (Thomas and Capecchi 1987) and the first recombinant rat model in 2010 (Geurts et al., 2010).

Expansion of the toolbox for gene editing in rats has allowed a renewed interest in rat models, which have several advantages over mice. Rats are easier to handle than mice and are less stressed by the human contact necessary for behavioural experiments, with increased handling in mice leading to stress (Meijer, Sommer, Spruijt, Van Zutphen, & Baumans, 2007) and anhedonia (Clarkson, Dwyer, Flecknell, Leach, & Rowe, 2018). Furthermore mice often need substantially longer habituations and training sessions to perform cognitive tasks than rats do (Colacicco, Welzl, Lipp, & Würbel, 2002; Jaramillo, Zador, & Jaramillo, 2014; Prusky, West, & Douglas, 2000), and in association with this experience more handling-based stress than will influence their task performance. Rats larger brain sizes increase spatial resolution on MRI scans of rat brains (Chapter 2) and facilitate surgical procedures. Rats may also be preferable for investigations of social behaviour (Chapter 3) as they are not limited to strict one-male hierarchies (Van zegeren, 1979), prefer socialising with conspecifics more than mice (Vestal, 1977) and benefit more from communal social structures (Branchi & Alleva, 2006; Branchi et al., 2006; Martinez, Brunelli, & Zimmerberg, 2015).

In addition to these species-based advantages the $Dlg2^{+/-}$ rat model used here has a Long Evans background, unlike Sprague-Dawley usually used in psychiatric research. Long Evans are preferable as they require fewer sessions than Sprague Dawley to learn operant tasks, including signal detection and reversal learning (Turner & Burne, 2014). Choosing the adequate species and strain for psychiatric disorder modelling has been named homological validity, and outlined as a key criteria for establishing validity in animal models (Belzung & Lemoine, 2011).

It is also the case that using clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9 (CRISPR/Cas9) to generate the model in Long Evans embryos avoids variation in phenotype caused by allelic variation in the region of the target gene. In the past this issue has arisen when transgenic models are created by causing

mutations in embryonic stem cells of one rodent strain and crossing these with another high-fecundity rodent strain, creating a genetic background that is biased by the mutated strain in the transgenic compared to wild-types (Hiroi, 2018; Hiroi & Yamauchi, 2020). Quality control checks when generating the $Dlg2^{+/-}$ rat model confirmed that the base sequence surrounding the modified allele allelic sequences in wild-types and heterozygotes were identical, as well as confirming the specificity of the genetic disruption to only the $Dlg2$ gene (see Supplementary Information of Griesius et al 2022).

This work presents the first in-depth characterisation of a novel $Dlg2^{+/-}$ rat model. As the model is not disorder-specific but models genetic risk for multiple psychiatric conditions, behavioural phenotyping was conducted using a variety of assays for psychiatric-relevant domains. This is in line with the Research Domain Criteria (RDoC) view of mental disorders, which departs from the DSM view that disorders comprise discrete nosological entities based on observed clusters of symptoms. RDoC uses triangulation from multiple levels of analysis (from genomics to behaviour and self-report) to describe dimensions of functioning that span the full range of human behaviour. Disorders can be described in terms of varying degrees of function/ dysfunction to these general psychological/biological domains (Anderzhanova, Kirmeier, & Wotjak, 2017; Cuthbert, 2014). Using a rodent model to develop a picture of what is spared and impaired on assays covering multiple key domains of function can illuminate the biological and psychological reasons a risk variant may lead to disorder. The endophenotypes discovered can ultimately serve as disease biomarkers and pave the way to treatment.

The phenotyping carried out in this thesis progresses through many stages of analysis. Protein expression work was intended to answer basic questions concerning the validity of the rodent model in determining whether PSD-93 and related proteins were differentially expressed in grossly dissected prefrontal cortex, hippocampus, cerebellum and posterior cortex (Chapter 2). Genotype based differences in brain structure were explored using MRI (Chapter 2). A gamut of behavioural phenotyping was conducted starting with well-established tasks assessing phenotypes commonly seen in rodent models of psychiatric disorder (Chapters 3 & 4). Symptom domains with cross-disorder relevance to conditions associated with $DLG2$ disruption were assessed including cognitive abnormalities (Chapter

4), reward processing (Chapter 5), and reality testing (Chapter 6). These included the development of new tasks which could allow psychiatric symptom domains previously untestable in rodents to be assessed, such as avolition (Chapter 5) or hallucinations (Chapter 6).

1.5.   Outline of Experiments

In Chapter 2 I begin with an investigation into the biological alterations arising from *Dlg2* heterozygosity in terms of protein expression and brain structural changes to grey and white matter using MRI. Then in Chapter 3 follows a behavioural phenotyping of *Dlg2*$^{+/-}$ rodents starting with a collection of 'standard' assays for phenotypes common to rodent models of schizophrenia and autism including anxiety, sensorimotor gating, habituation, social interaction, and response to the NMDAR-antagonist PCP. These allow for comparison with the aforementioned published literature on the behaviour of *Dlg2* homozygous knockout rodents. Cognitive impairments in *Dlg2*$^{+/-}$ rodents were investigated involving object recognition based on object identity, location, object-location paired associates and temporal order; and reference memory in the water maze. These are outlined in Chapter 4. Reward processing in *Dlg2*$^{+/-}$ rats is described in Chapter 5 including assays of anhedonia and motivation to work for reward. Finally, Chapter 6 describes a novel method of assessing propensity for hallucinations and delusions, reality testing, in rodents and the performance of *Dlg2*$^{+/-}$ rats on such a task.

Chapter 2

Basic biological characterisation of Dlg2$^{+/-}$ **rat model**

2.1. Introduction

2.1.1. Protein expression

In humans DLG2 is mainly expressed in the brain, as shown in Figure 2 for mRNA and Figure 3 for protein. These data were compiled by the Human Protein Expression Atlas, an open-source initiative to map human cellular proteins across tissues using integrated omics technologies, in situ hybridisation and RNA sequencing for mRNA description and immunohistochemistry for protein level and localisation description. Aberrations of these expression levels would be expected if one copy of DLG2 is deleted as in pathogenic CNVs in the 11q14.1 region and would be expected to drive disorder (Kirov et al., 2012; Nithianantharajah et al., 2013; Noor et al., 2014; Reggiani et al., 2017; Ruzzo et al., 2019).
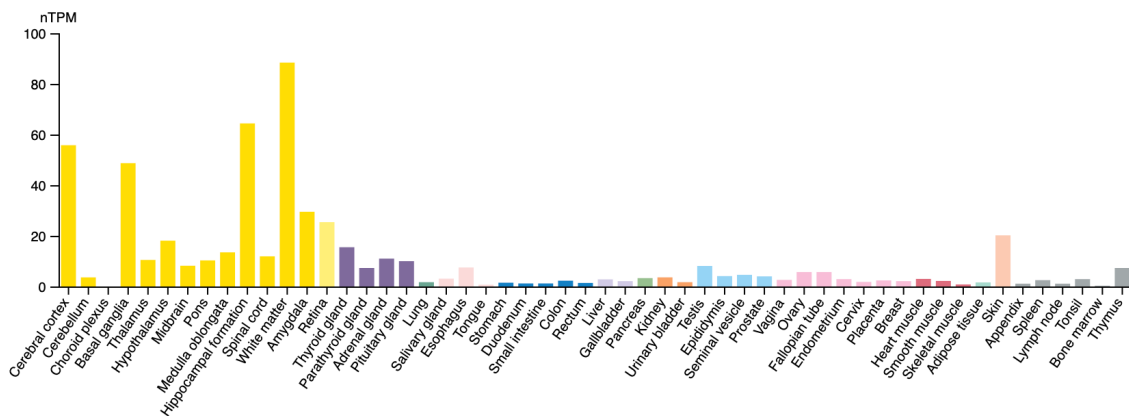
Figure 2: *DLG2* **mRNA expression overview in human tissue compiled by the Human Protein Atlas.** nTPM stands for normalised transcript expression values.
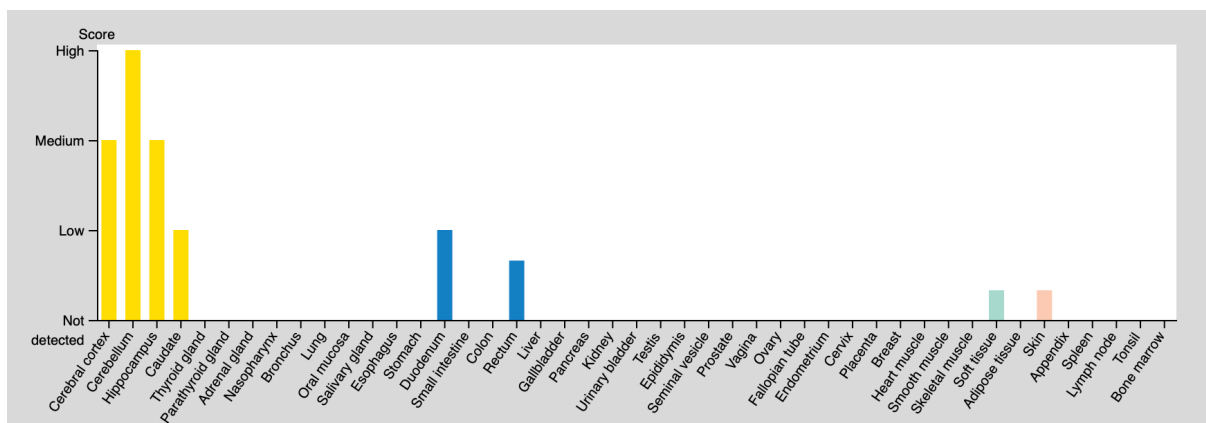
Figure 3: Protein expression overview of PSD-93 in human tissue compiled by the Human Protein Atlas.

Further evidence that a change in PSD-93 protein levels can be pathogenic, there is evidence that even in the absence of direct genetic lesions alteration to *DLG2* transcription and translation is observed in schizophrenia, reinforcing its status as a protein of interest for the disorder. In situ hybridisation and western blotting studies have shown significant differences in *DLG2* expression within the anterior cingulate cortex, but not the dorsolateral prefrontal cortex, of individuals with schizophrenia (Kristiansen, Beneyto, Haroutunian, & Meador-Woodruff, 2006). Specifically, PSD-93 was decreased in the anterior cingulate of schizophrenia patients relative to matched controls, while *DLG2* transcripts were increased, indicating that the loss of protein may trigger a compensatory response at the transcriptional level. It would be interesting to know whether similar expression changes are seen in other disorders related to *Dlg2* such as autism, intellectual disability, bipolar and ADHD. This would set apart DLG2 changes as a cross-disorder biomarker at the protein as well as the genetic level.

In Kristiansen et al (2006) PSD-93 changes were seen to co-occur with differences to expression of NMDA receptors. In the anterior cingulate, but not the dorsolateral prefrontal cortex, there was increased expression of NR1 isoform NR1c2'. Furthermore Zhang et al (2010) observed that PSD-93 deficiency in cultured cortical neurons reduces the expression of NR2A and NR2B NMDAR subunits. This work links abnormal translation and/or accelerated degradation of NMDAR subunits and PSD-93 in schizophrenia. A chain of causality between these is implied by Ingason et al., (2015) who demonstrated decreased expression of rat *Dlg2* in the hippocampus of chronic NMDAR-antagonist (MK-801) treated rats. This indicates that in this case PSD-93 changes may occur downstream of changes to NMDAR function.

The first essential step in characterising a genetic lesion rodent model is to assess whether the expected mRNA and protein levels are decreased (here *Dlg2* and PSD-93) and if there is obvious compensation for this through related proteins. The other *Dlg* paralogs (*Dlg1*, *Dlg3* and *Dlg4*) display some functional redundancy with *Dlg2*, particularly *Dlg4* (PSD-95) (as discussed in 1.3.1 of the General Introduction). Thus, any change in gene or protein expression in the other *Dlg* paralogs could evidence compensation for *Dlg2* decrease by a

related paralog. Previous data has supported a lack of this type of compensation, with *Dlg2* mRNA transcripts decreased in the hippocampus and prefrontal cortex of *Dlg2$^{+/-}$* rats relative to wild types (Chapter 6.4, Pass, 2019) yet no change in *Dlg1*, *Dlg3* or *Dlg4* mRNA expression. Here western blotting for PSD-93 and PSD-95 was carried out to confirm that only PSD-93, and not its most closely related paralog, was reduced at the protein level.

Changes to expression of the NR1 NMDAR subunit were also assessed. The NMDAR is a key binding partner of PSD-93 (e.g. Carlisle, Fink, Grant, O'dell, & O'dell, 2008; Elias et al., 2006; Frank et al., 2016) which is independently involved in psychiatric disorder pathogenesis (e.g. Lakhan, Caro, & Hadzimichalis, 2013; Yamamoto, Hagino, Kasai, & Ikeda, 2015). Looking for NMDAR expression changes in the model can help discern whether *Dlg2* heterozygosity contributes to psychiatric risk through NMDAR dysfunction. It also complements the work discussed above, as here PSD-93 changes would have been primary to any NMDAR changes, and thus interactions between the expression of these proteins can be interrogated in reverse to Ingason et al., (2015).

2.1.2. Structural differences in brain grey and white matter

Schizophrenia and autism spectrum disorder are associated with distinct brain structural changes to grey and white matter which are visible when assessed by MRI (e.g. Glahn et al., 2008; Haukvik, Hartberg, & Agartz, 2013; Pagnozzi, Conti, Calderoni, Fripp, & Rose, 2018; Shepherd, Laurens, Matheson, Carr, & Green, 2012). Focusing on volume changes to grey matter can give an indication of atrophy or abnormalities in specific brain areas. These can be investigated by region-of-interest (ROI) approaches which delineate a structure of interest by masking it on a brain template (usually a representative brain derived from averaging control brains). All brains are then registered to this template to circumvent interindividual differences in brain anatomy, and the volumes of the masked structures are extracted and compared across experimental groups. It is also possible to assess regions of volumetric discrepancy between groups without a priori specification of where these may manifest (i.e. ROIs) using voxel-based-morphometry (VBM) methods. VBM also registers every brain to a template. These brain images are then smoothed so that each voxel represents the average of itself and its surrounding areas, and voxel-wise statistical

comparisons are used to determine regional differences in volume across groups (Ashburner & Friston, 2000; Scarpazza & Simone, 2016).

In schizophrenia several meta-reviews have collated evidence from previous systematic reviews concerning MRI structural brain changes and assessed the quality of this evidence and the systematic review procedure. This allows consistent findings which are well-evidenced to be summarised. In schizophrenia these include ventricular enlargement and grey matter reductions of the anterior cingulate, frontal (particularly medial and inferior) and temporal lobes, hippocampus, amygdala, thalamus, and insula (e.g. Brent, Thermenos, Keshavan, & Seidman, 2013; Haijma et al., 2013; Kuo & Pogue-Geile, 2019; Shepherd et al., 2012). There are many other regional alterations - indeed the volume of more or less all brain structures has in one or more studies been associated with schizophrenia (Haukvik et al., 2013). It has been proposed that many of these may be specific to illness stage or medication status (Haukvik et al., 2013; Shepherd et al., 2012). The fact that these brain changes are present at the onset of schizophrenia provides evidence that this disorder is caused by faulty neurodevelopmental processes rather than being neurodegenerative (Buckley, 2005; Haukvik et al., 2013; Li et al., 2016).

Findings have been heterogenous in autism due to the complications of comorbid conditions and the important modifying factors of the age and IQ of participants (Chen, Jiao, & Herskovits, 2011; Pagnozzi et al., 2018). Meta-analyses taking these factors into account have reported consistencies of findings including increased total brain volume in young children (determined by a ROI approach, Amaral, Schumann, & Nordahl, 2008; Courchesne et al., 2001; Sparks et al., 2002), consistent reductions in cerebrospinal fluid (Pagnozzi et al., 2018) and increased grey matter volume in the frontal and temporal lobes (determined by a VBM approach reviewed by Chen, Jiao, & Herskovits, 2011). Given that ASD is a neurodevelopmental condition longitudinal studies have been conducted to assess abnormalities in the trajectory of brain growth. Scanning infants at multiple timepoints from 1.5 to 5 years old Schumann et al., (2010) found that all regions except occipital grey matter undergo an abnormal growth trajectory. This was also true in a study with a longer scope, scanning to 10 years of age (Hardan, Libove, Keshavan, Melhem, & Minshew, 2009).

In addition, white matter changes have been consistently described in both autism and schizophrenia. These have been assessed by delineating white matter tracts of interest and comparing their volumes in a manner similar to the ROI-based methods outlined for grey matter. A method which does not require manual or semi-manual segmentation of white matter tracts is the analysis of diffusion tensor imaging (DTI) data. The concept behind DTI is that water molecules diffuse differently along brain tissue depending on its type, integrity, architecture, and the presence of barriers. This gives information about its orientation and quantitative anisotropy (directional dependence) (e.g. Beaulieu, 2002; Chenevert, Brunberg, & Pipe, 1990; Douek, Turner, Pekar, Patronas, & Le Bihan, 1991; Moseley et al., 1990). With DTI analysis it is possible to infer for each voxel the molecular diffusion rate (mean diffusivity, MD), the directional preference of diffusion (fractional anisotropy, FA), the diffusion rate along the main axis of diffusion (axial diffusivity, AD) and the diffusion rate in the transverse direction (radial diffusivity, RD). In white matter the diffusion of water molecules is restricted along the axon by myelin, rendering it anisotropic, while grey matter is less directionally dependent and diffusion in cerebrospinal fluid is unrestricted in all directions (isotropic) (e.g. Hagmann et al., 2006; Pierpaoli, Jezzard, Basser, Barnett, & Di Chiro, 1996; Song et al., 2002).

Given that different tissue components contribute to the restriction of water movement in white matter, microstructural changes to these components including cell membranes, macromolecules, myelin sheaths and axon walls can contribute to changes in anisotropy (Beaulieu 2002). Thus DTI parameters allow investigation into the integrity of brain white matter. For example RD increases in response to demyelination (e.g. Song et al., 2005) and dysmyelination (e.g. Nair et al., 2005; Song et al., 2002) while AD is indicative of axonal damage (e.g. Budde, Xie, Cross, & Song, 2009; Song et al., 2003).

White matter microstructure changes in schizophrenia are widespread (e.g. Kubicki & Shenton, 2014; Samartzis, Dima, Fusar-Poli, & Kyriakopoulos, 2014). These include pervasive and significant decreases in FA in patients within almost all major fasciculi (Kelly et al., 2017; Vitolo et al., 2017) with effect sizes larger in peripheral tracts than those in the core white-matter skeleton (Kelly et al, 2017). Meta-analyses have summarised the greatest FA decreases to be in the corona radiata and the body and genu of the corpus collosum

(Gasparotti et al., 2009; Whitford et al., 2010). Significantly higher MD and RD were also observed for schizophrenia patients compared with controls (Kelly et al, 2017).

In ASD DTI studies have consistently reported reduced FA, increased MD and increased RD in the ASD group relative to controls in the corpus collosum (e.g. Alexander et al., 2007; Barnea-Goraly et al., 2004; Barnea-Goraly, Lotspeich, & Reiss, 2010; Brito et al., 2009). These studies and others have also consistently found differences in prefrontal white matter (e.g Barnea-Goraly et al., 2004, 2010; Sundaram et al., 2008), cingulate gyrus (Barnea-Goraly et al., 2004, 2010; Thakkar et al., 2008), and internal capsule (Barnea-Goraly et al., 2004, 2010; Brito et al., 2009; Keller, Kana, & Just, 2007) in ASD participants relative to controls. Barnea-Goraly et al (2004) also reported reduced FA values in brain regions implicated in tasks that involve ascribing mental states to others (theory-of-mind) and social cognition, cognitive domains individuals with ASD typically struggle on. These involved the ventromedial PFC, anterior cingulate, temporoparietal regions, amygdala and superior temporal sulcus. Given the relation of *DLG2* to these conditions volumetric brain changes and alterations to white matter microstructure in the *Dlg2$^{+/-}$* rat may align with these findings.

Specific links between *DLG2* and volumetric brain changes have also been made. Smeland et al., (2018) combined GWAS data on schizophrenia with GWAS data on MRI volumetric measures of brain regions. This allowed the isolation of genes which are implicated in the volumes of specific brain structures and increased risk for schizophrenia. *DLG2* was identified as a loci shared between schizophrenia risk and increased putamen volume, which has been observed in some neuroimaging studies into schizophrenia (Okada et al., 2016). Associations between *DLG2* SNPs and asymmetry of the amygdala, putamen and hippocampus were also observed in Alzheimer's patients (Wachinger et al., 2018). Therefore, a *Dlg2* heterozygous rat may be expected to show volumetric changes to the putamen, amygdala and hippocampus specifically. Similar grey and white matter changes to ASD and schizophrenia are also captured by NMDAR-antagonism rat models. Chronic MK-801 treated rats displaying atrophy of the hippocampus, ventral striatum and cortex alongside microstructural impairments to the corpus callosum (Wu et al., 2016). Similar

structural changes may be expected in the $Dlg2^{+/-}$ rat due to the close interaction between PSD-93 and NMDARs.

Changes in to grey and white matter structure caused by $Dlg2$ heterozygosity were assessed using MRI. Whole-brain comparison of white matter microstructure between $Dlg2^{+/-}$ and wild-type rats was carried out by analysing DTI data using TBSS (Smith et al., 2006). As this analysis is the first to investigate structural changes in brains affected by $Dlg2$ genetic lesions, and because the TBSS protocol lends itself well to this, a hypothesis-free exploratory analysis of white matter differences across genotypes was undertaken. Analysing differences in grey matter could not be carried out in the same exploratory manner due to a lack of brain templates for the background strain of $Dlg2^{+/-}$ and wild-type rats (Long Evans). Therefore, volumetric changes were interrogated by specifying ROIs and comparing the volume of these between genotypes. ROIs included were the hippocampus, perirhinal cortex, ventricles, striatum, and prefrontal cortex.

The hippocampus was chosen as decreased hippocampal volume is frequently observed in schizophrenia patients (Brent et al., 2013; Haijma et al., 2013), and is predicted by hypermetabolism beginning in CA1 and spreading to the subiculum with psychosis onset (Schobel et al., 2013). Acute administration of ketamine, a NMDAR-antagonist, reproduces the primary CA1 finding with chronic exposure shifting the hippocampus to a hypermetabolic basal state with concurrent atrophy leading researchers to conclude that decreases in hippocampal volume caused by hypermetabolism are glutamate-driven (Schobel et al., 2013). Investigating hippocampal volume alteration in $Dlg2^{+/-}$ rats will augment behavioural tasks which rely on processing in this structure (Chapter 4) and work showing alterations to $Dlg2^{+/-}$ rat hippocampal plasticity (Griesius et al., 2022).

Smaller perirhinal cortex volumes are frequently observed in schizophrenia patients (Haijma et al., 2013). In a sub-chronic PCP NMDAR-antagonism rat model of schizophrenia where global brain volume reductions were seen, the volume of the perirhinal cortex was found to positively correlate with rodents ability to recognise objects (Doostdar et al., 2019). $Dlg2^{+/-}$ and wild-type rats were assessed on this task (Chapter 4, novel object recognition) allowing any volume changes to be aligned with behaviour.

The ventricles were chosen as ventricular thinning is seen in humans diagnosed with schizophrenia (e.g. Brent et al., 2013; Cahn et al., 2002; Haijma et al., 2013), and ventricular enlargement in ASD (e.g. Movsas et al., 2013; Vidal et al., 2008). This phenotype also persists in the poly(I:C) maternal immune activation rat model of schizophrenia (Piontkewitz, Arad, & Weiner, 2012), and a genetic mouse model of CNV 22q deletion (Eom et al., 2020).

The striatal components comprising the putamen, ventral striatum (VSTR) and dorsal striatum (DSTR) were manually segmented. This region has multiple links to the *Dlg2* gene. Putamen volume has been linked both to schizophrenia and the *DLG2* gene (Smeland et al., 2018). Electrophysiological studies also determined abnormalities in striatal functioning in *Dlg2*$^{-/-}$ mice (Yoo et al., 2020a). The ventral and dorsal striatum were chosen as the VSTR, but not DSTR, shows grey matter decreases in a NMDAR-antagonism rodent model of schizophrenia (Wu et al., 2016) allowing comparison with the *Dlg2*$^{+/-}$ rat. Finally, the striatum is hypothesised to be involved in generation of psychotic symptoms through aberrant incentive salience (Roiser, Howes, Chaddock, Joyce, & McGuire, 2013), allowing brain-behaviour links to the reality-testing Experiment conducted in Chapter 6.

Finally, the anterior cingulate gyrus and primary motor cortex (M1) were manually defined comprising the prefrontal regions. In the cingulate cortex reductions in volume are typical of schizophrenia and are associated with worse disease outcomes (Mitelman, Shihabuddin, Brickman, Hazlett, & Buchsbaum, 2005). M1 was chosen as previous work has found an impairment in motor learning in *Dlg2*$^{+/-}$ mice with delayed cfos immediate early gene expression after relevant behavioural challenge in this region (Pass, 2019), and any structural changes here would add to this finding.

2.2. Methods

2.2.1. Generation and maintenance of *Dlg2*$^{+/-}$ rat line

Generation and founders

Dlg2 heterozygous rats were generated on a Long Evans Hooded background by Horizon Discovery (Pennsylvania, USA) using CRISPR/Cas9 gene editing technology. Proprietary bioinformatics software (Horizon Discovery, St.Louis, USA) was used to design a short guide RNA (sgRNA – CCAGGGTCATCTCCAATGTGagg) targeting a Protospacer Adjacent Motif (PAM) sequence within exon 5 of the rat *Dlg2* gene on chromosome 1. Successful founders generated by Horizon Discovery has 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 as shown in Figure 4. Confirmation of successful non-homologous end joining activity was assessed by PCR and sequenced by Horizon Discovery, UK. Selected heterozygous founders were send to Charles River (Margate, UK) and bred to produce experimental colonies by breeding male heterozygous rats were bred with female wild-types resulting in a Mendelian distribution of wild-type and heterozygous pups. A full description of the development and quality control of this line can be found in Supplementary Information of Griesius et al., 2022.
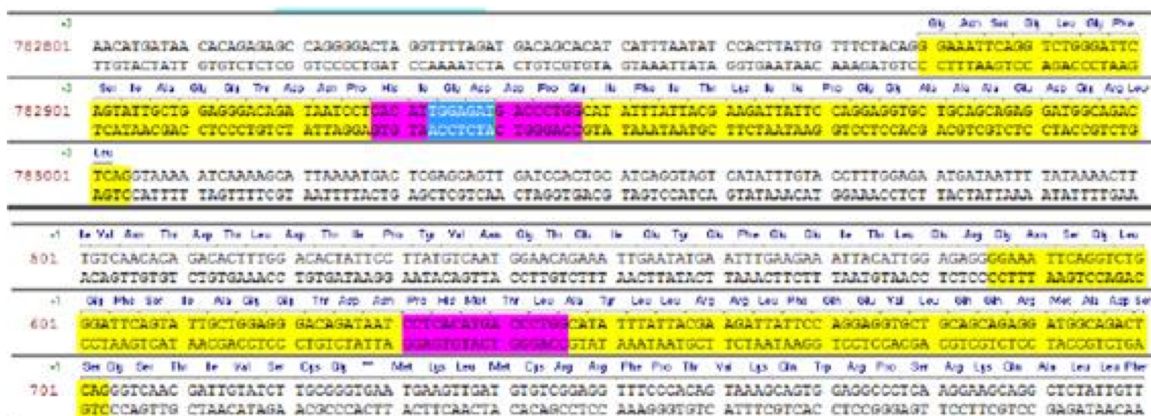
Figure 4: Targeting of exon 5 with deletion shown in blue (top panel) and outcome after non-homologous end joining in purple (lower panel).

Breeding and housing

Selected heterozygous founders were send to Charles River (Margate, UK) and bred to produce experimental colonies. Male heterozygous rats were bred with female wild-types resulting in a Mendalian distribution of wild-type and heterozygous pups, with an average

litter size of nine pups. This breeding protocol should have prevented any influence caused by *Dlg2* haploinsufficiency on maternal behaviour influencing pups.

Once required for experiments *Dlg2+/-* rats and wild-types were shipped to School of Psychology, Cardiff University. Here animals were housed in groups from two to four in standard cages (L × W × H: 50cm × 32cm × 21cm) in rooms with a temperature between 19-23°C maintained on a 12h light-dark cycle. Cages had sawdust and paper nesting and environmental enrichment (wooden chews and cardboard tubes). Research was conducted in accordance with the Home Office regulations under the Animal (Scientific Procedures) Act 1986 under the authority of PPL303243 or PPL303135.

2.2.2. Genotyping by polymerase chain reaction (PCR)

Two methods of DNA extraction were used consistently to attempt genotyping of rats from all cohorts, with infrequent success. For both protocols it was common for no bands to be visible once the gel was imaged even with extensive troubleshooting (detailed below). Due to this Cohorts 2- 4 were genotyped using the following methods and Cohorts 5 and 6 sent to Transnetyx (Corvova, TN, USA) who designed a custom *Dlg2+/-* probe and conducted genotyping remotely from samples provided using real-time PCR. Genotyping was performed both pre and post-mortem, with ear biopsies sent from Charles River for the former and tail tissue for the latter.

DNA extraction with Qiagen DNeasy Kit (Qiagen, Manchester, UK)

Manufacturers standard protocol was used. Approximately 20mg tissue (0.6cm tail tissue or one ear punch) was lysed at 56°C overnight in 180 µl buffer ATL and 20 µl protinase K. 200 µl of buffer AL and 200 µl of 96-100% ethanol was added to the sample before putting through a spin column at 8000rpm for 1 minute. Buffer AW1 was added to the spin column as a first wash and spun through at 8000rpm for 1 minute. Buffer AW2 was then added and spun through at 8000rpm for 1 minute, before a final spin of 14,000 rpm for 3 minutes to dry the membrane. DNA was eluted by adding 200µl of buffer AE to the membrane,

incubating for 1 minute at room temperature and centrifuging at 8000rpm for 1 minute. DNA was stored at -20 °C until required.

DNA extraction with KAPA Mouse Genotyping Kit (KAPA Biosystems, Massachusetts, USA)

Manufacturers standard protocol was used. DNA was extracted from each sample (approximately 20mg of tissue) using a 100 µl master mix containing 88 µl of PCR-grade water, 10 µl 10× KAPA Express Extract Buffer and 2 µl 1 U/ul KAPA Express Extract Enzyme. Lysis was performed by putting each sample on a heat block at 75 °C for 10 minutes followed by 95 °C for 5 minutes. Lysed samples were stored at -20 °C.

PCR

To distinguish wild-type from heterozygote animals a multiplex reaction was set up.

For the Qiagen extracted DNA master mixes were made up to have a total of 24µl reaction mix per sample tested containing: 12.5µl Master Mix (Promega, UK), 1.25µl of forward and reverse primers, and 8 µl distilled MilliQ H_2O and 1 µl crude DNA extract. For the KAPA extracted PCR reactions were set up for each sample using a master mix containing: 12.5 µl 2× KAPA2G Fast Genotyping Mix with dye, 9 µl PCR-grade water, 1.25 µl of each primer and 1 µl of crude DNA extract. Primer sequences can be seen in Table 1.

Samples were run on a BioRad Thermal Cycler (T100TM BioRad, Herts, UK). PCR consists of five key stages, initial denaturation followed by cycles of denaturation, annealing and extension before a final longer extension. These phases had the following conditions for Qiagen extracted samples: 95 °C for 5 minutes, then 35 cycles of 95 °C for 30 seconds, 60 °C for 30 seconds, 68 °C for 40 seconds before 68 °C for 5 minutes and maintaining samples at 4 °C. For KAPA extracted samples PCR was then performed with the following cycling protocol: 95 °C for 3 minutes then 35 cycles of 95 °C for 15 seconds, 60 °C for 15 seconds, 72 °C for 15 seconds before holding samples at 72 °C for 1 minute and maintaining them at 4 °C.

These protocols were frequently altered to troubleshoot the problem of getting no visible gel bands. This was conducted by varying the number of cycles from 40-60, reducing the annealing temperature by 2 – 5 °C, increasing the initial 75 °C lysis time to 15 minutes for the KAPA extracted samples and increasing the extension time in increments from 1 – 5 minutes. Primer and sample concentration and temperature assays were also performed.

Table 1: Primers used in genotyping to differentiate $Dlg2^{+/-}$ and wild-type rats.

Forward primer	5' – TCTGACCTTGCTGTGTCTGC – 3'
Reverse primer	5' – GCGTCACTACAGAAAGCCTTG – 3'

Gel electrophoresis

Samples were run on a 4% agarose gel made from 0.5 x Tris/Borate/EDTA (TBE) buffer (54g of Tris base, 27.5g boric acid and 20ml of 0.5M EDTA, ph 8.3). 5 µl of ethidium bromide was used per 100ml agarose ran. 10 µl of the resulting PCR samples (with 4 µl DNA Gel Loading Dye (6×) (Thermo-Fisher Scientific, Delaware, USA) for the Qiagen extracted samples) was loaded onto the gel and run at 150V for 1.5 hours while the gel was submerged in 0.5 µl TBE. Gels were visualised on a BioRad Gel Doc XR imager (BioRad, Herts, UK). Two bands were indicative of a heterozygous animal, with a mutant band at ~446 bp and a wild-type band at ~421 bp. Single bands at ~421 bp were indicative of wild-types.

2.2.3. Western blotting

Protein extraction

Western blot analysis was conducted on gross-dissected hippocampal, prefrontal cortex, cortex and cerebellar tissue from male wild-type (n = 12) and $Dlg2^{+/-}$ (n = 12) animals from Cohort 2. Extraction was performed on ice to prevent protein degradation. Sample buffer was made up by adding one cOmplete™ Mini Protease Inhibitor Cocktail tablet (Roche, Switzerland) per 10 ml of buffer and Phosphatase Inhibitor Cocktail (Cell Signalling Technology, USA) in a 1:100 dilution to Syn-PER™ Synaptic Protein Extraction Reagent

(ThermoFisher Scientific, UK). Homogenisation of the four grossly dissected brain regions (prefrontal cortex, hippocampus, cortex and cerebellum) was performed using ~ 10 slow strokes in a 2ml Dounce tissue grinder (ThermoFisher Scientific, UK) with the appropriate amount of sample buffer for the weight of tissue, defined as 10 ml of Syn-PER per gram of tissue. The homogenate was first centrifuged at 1200 × g for 10 mins at 4 °C. To obtain synaptosomes and cytosolic fractions for each sample the supernatant was transferred to a new tube and centrifuged at 15,000 × g for 20 mins at 4 °C. Following this the cytosolic fraction (supernatant) was removed and the synaptosome pellet re-suspended in 100 μl sample buffer. Samples were stored at -80 °C.

Bicinchoninic (BCA) protein assay

Protein concentration was quantified for each sample the day before western blotting was conducted. In this assay the Cu2+ reduction to Cu1+ by protein in an alkaline environment which forms a light blue colour is combined with a BCA-Cu1+ reaction to form a dark-purple colour. The dark-purple colour has a strong linear absorbance at 562 nm with increasing protein concentrations.

A range of bovine serum albumin (BSA) standards (Sigma-Aldrich, UK) from 0.125mg/ml – 2mg/ml were prepared with Milli-Q water and 10% sample buffer. 5 μl of synaptosome from samples were diluted in 45 μl Milli-Q water. BCA Reagent A and B from the PierceTM BCA Protein Assay Kit (ThermoFisher, USA) were mixed in a 50:1 ratio to form the BCA working reagent. To all standards and samples 1ml of BCA working reagent were added, mixed by aspiration and incubated at 37 °C for 30 mins. Absorbance readings were then taken on a spectrophotometer at 562nm within 10 mins of incubation which had previously been blanked with Milli-Q water. A standard curve of BSA concentration was plotted by the absorbance vales for the standards, and the resulting equation line of best fit used to determine the sample concentrations.

Western blotting

Samples were defrosted on ice. 2× Bio-Rad Laemmli Sample Buffer (Bio-Rad Laboratories, UK) was added to beta-mercaptoethanol in a 20:1 ratio. Synaptosomes were diluted in sample buffer so that 40ug of protein was loaded per well. Samples were added to Laemmli buffer at a 1:1 ratio and this mixture heated at 96 °C for 5 minutes to denature protein-protein interactions and facilitate antibody bindings. Samples were loaded onto NuPAGE^{TM} 4 to 12% Bis-Tris Midi Protein Gels (ThermoFisher Scientific, USA) along with Precision Plus Protein^{TM} Kaleidoscope Standards protein ladder for calibration. Samples were arranged so that brain regions and genotypes were counterbalanced across gels with a WT standard used on each gel for comparison.

Gel were run at room temperature in NuPAGE^{TM} Running Buffer (Invitrogen, UK) at 85V for 20 mins and then for a further hour at 115V. Protein was then transferred to 0.45 um pore size nitrocellulose membrane (Invitrogen, UK) at 85 V for 2 hours 15 minutes at an ambient temperature of 4 °C in NuPAGE^{TM} Transfer Buffer (Invitrogen, UK) containing 10% 2-propanol (ThermoFisher Scientific, UK). Membranes containing transferred protein were washed in Tris-Buffered Saline (20mM Tris, 150mM NaCl, pH 7.6) with 0.1% Tween 20 (TBST) before blocking in 5% milk for one hour at room temperature with gentle rocking.

Primary antibodies were diluted to appropriate concentrations in 5% milk and incubated with the membrane overnight at 4 °C. These included rabbit anti-PSD93 (1:1000, Cell Signalling Technology, USA), mouse anti-NR1 (1:1000, Merck Millipore, UK), rabbit anti-PSD95 (1:2000, Abcam, UK) and mouse anti-GAPDH (1:5000, Abcam, UK). Membranes were then subject to 3 × 10-minute TBST washes before incubation with the appropriate fluorescent IRDye 680RD secondary antibodies at 1:15,000 dilution in 5% milk at room temperature. After another series of TBST washes membranes were imaged on Odyssey CLx Imaging System (Li-COR, Germany) in the 700nm channel.

Analysis

Densiometric analysis of bands was performed using ImageLab 6.0 (https://imagej.nih.gov/ij/). The densities (with background subtracted) of the protein of

interest were divided by the loading control densities for each sample to provide normalised values. Densities were then averaged by group.

2.2.4 MRI acquisition and analysis

Sample preparation

A summary of all rat Cohorts used throughout the experimental work in this thesis can be found in Appendix 1, including *N*, sex, genotype, and which PPL the work was conducted under. Sixteen heterozygous and seventeen wild-type male rats from Cohort 2 were chosen for MRI. They were all culled at least 2 weeks following participation in behavioural experiments. Rats were culled at around 10 months of age by inhalation of slowly rising CO_2 concentration for 8 minutes (administered by Home Cage Culling Chamber, Clinipath Equipment Ltd, UK) followed by transcardial perfusion with a flow rate of 13 rpm through the left ventricle with ~ 100 ml of phosphate buffered saline (PBS, chemical, pH 7.4). This was followed by an infusion of ~250 ml of 4% paraformaldehyde (PFA chemical) in PBS (pH 7.4). Brains were removed from skulls and post-fixed in 4% PFA in PBS overnight before being transferred to Andrew Stuart for MRI acquisition. For scanning brains were immersed in Galden, a proton-free susceptibility-matching fluid in a 15ml syringe. Use of a syringe allowed any residual air bubbles which may have affected MRI measurements to be pushed out. After scanning brains were returned to PFA solution. Due to signal drop off one rat was perfused and scanned per day until completion to allow equating the time between perfusion and scanning. One heterozygous rat and four wild-types were excluded from the analysis due to missing or incomplete scans resulting in final numbers of 15 *Dlg2*$^{+/-}$ rats and 13 wild-types.

MRI data acquisition

MRI scans were conducted by Andrew Stuart in the Experimental MRI Centre (EMRIC), Cardiff University. MRI acquisition was conducted *ex-vivo* on a 9.4 Tesla (20 cm) horizontal-bore animal system (Bruker Biospin, Germany). This was equipped with BGA12-S (12cm inner bore size, integrated shims) gradients. A transmit 1H 500 Watt echo-planar imaging

(EPI) volume coil was used with a phased array 4-channel surface coil and Paravision software (version 6.1, Bruker Biospin) were used for data acquisition. Anatomical data was collected using a T2 weighted scan and Diffusion Tensor Imaging (DTI) a Diffusion weighted scan. Other measures taken (Quantitative T2 mapping, Quantitative T1/R1 mapping and qMT) are not analysed here. Details of the scan protocols can be found in Table 2.

Table 2: Scan parameters. All sequences were acquired at 9.4 Tesla. For each of the sequences the main acquisition parameters are provided. FOV: field of view; TE: echo time; TR: repetition time.

Structural/ Anatomical Protocol	
Sequence	T2 weighted RARE
Matrix Size	256 × 256 × 90
FOV (mm)	25.6 × 25.6 × 90
Voxel Dimension (mm)	0.1 × 0.1 × 0.35
Rare Factor	6
First Echo (ms)	9
Echo Spacing (ms)	9
Effective TE (ms)	27
Repetition Time (ms)	6500
Averages	9
Diffusion Protocol	
Sequence	Diffusion weighted RARE
Directions	32
B0s	3
Bvals (s/mm^2)	1200
Matrix size	192 × 192 × 75
FOV (mm)	28.8 × 28.8 × 30
Voxel Dimension (mm)	0.15 × 0.15 × 0.4
Rare factor	2
Effective TE (ms)	26
Repetition Time (ms)	9000

Averages	1
DW Gradient Duration (ms)	6.8
DW Gradient Separation (ms)	12.3
Quantitative T2 Mapping	
Sequence	T2 MSME
Matrix Size	160 × 160 × 50
FOV (mm)	21.28 × 21.28 × 35
Voxel Size (mm)	0.133 × 0.133 × 0.7
Number of Echos	10
First Echo (ms)	9
Echo Spacing (ms)	9
Repetition Time (ms)	6000
Averages	4
Quantitative T1/R1 Mapping	
Sequence	3D FLASH
Matrix Size	160 × 160 × 100
FOV (mm)	21.28 × 21.28 × 30
Voxel Size (mm)	0.133 × 0.133 × 0.3
Flip Angles	6, 9, 13, 24, 36, 48
TE (ms)	4
TR (ms)	30
Averages	3
Quantitative Magnetisation Transfer	
Sequence	3D FLASH
Matrix Size	160 × 160 × 100
FOV (mm)	21.28 × 21.28 × 30
Voxel Size (mm)	0.133 × 0.133 × 0.3
Flip Angle	5
TE (ms)	5
TR (ms)	47
Averages	2

Off Resonance Pulses (hz/degree)	0/350, 1000/350, 1500/350, 3000/350, 6000/350, 12000/350, 24000/350, 1000/950, 1500/950, 3000/950, 6000/950, 12000/950, 24000/950

Image processing for volumetric analysis

Advances Normalisation Tools (ANTs Version 2.1.1, http://stnava.github.io/ANTs/) was used to implement registration stages. A template brain was generated by registering separate T2 images of each of the 28 rats to each other to create one average brain (using ANTs buildtemplateparalell). ROIs were manually delineated on coronal slices from rostral to caudal on this template using in-house tools written in MATLAB (Version R2021a) in line with previous literature (see references by structure below) and brain structures highly visible on the image (due to contrast, i.e. ventricles) triangulated by their relation to Bregma in the rat brain atlas (Paxinos & Watson, 2013). Greg Parker was consulted for suggestions and expertise in this analysis.
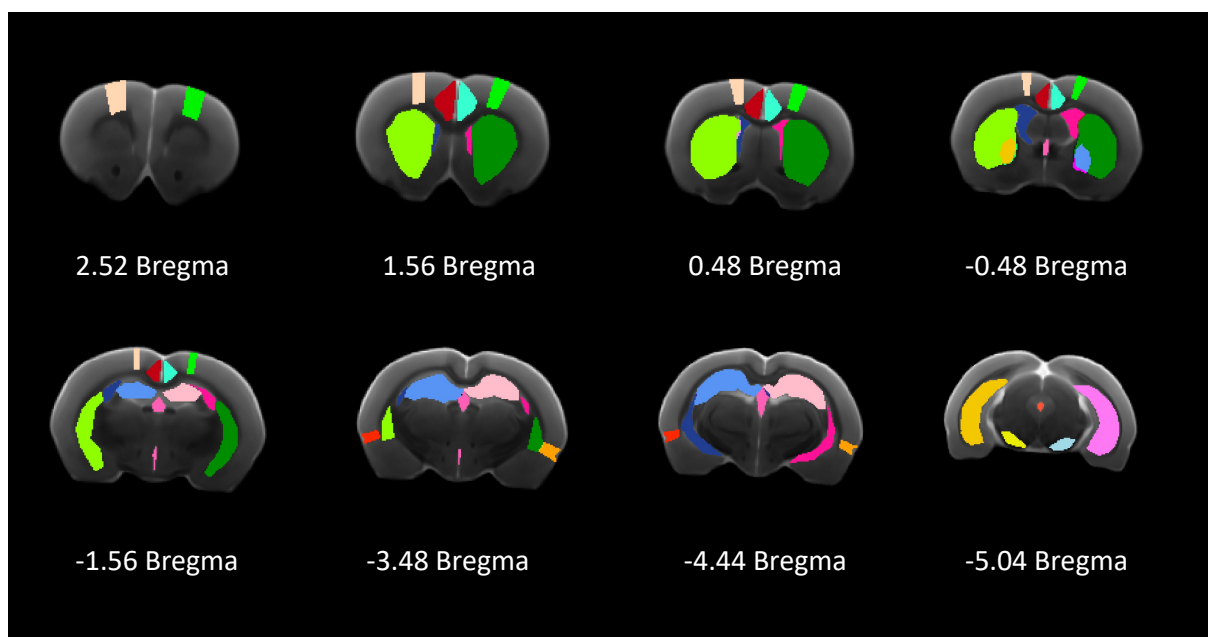
Figure 5: Coronal slices of the template brain at various anatomical locations relative to Bregma. Masked structures are highlighted with colour. Note that the same structure in left and right hemispheres is given a different colour. The template at 2.52 Bregma shows M1

left (pastel orange) and right (green). At 1.56 Bregma M1, caudate putamen left (brightest green), caudate putamen right (darkest green), anterior cingulate left (dark red), anterior cingulate right (turquoise), lateral ventricle left (dark blue), lateral ventricle right (brightest pink). At 0.48 Bregma M1, caudate putamen, anterior cingulate, lateral ventricles. At -0.48 Bregma M1, caudate putamen, anterior cingulate, lateral ventricles, third ventricle (pink), globus pallidus left (pastel orange), globus pallidus right (pastel purple). At -1.56 Bregma M1, caudate putamen, anterior cingulate, lateral ventricles, third ventricle, dorsal hippocampus left (pastel blue), dorsal hippocampus right (pastel pink). At -3.48 Bregma dorsal hippocampus, lateral ventricles, third ventricle, caudate putamen, left perirhinal cortex (red), right perirhinal cortex (orange). At -4.44 Bregma dorsal hippocampus, lateral ventricles, third ventricle, perirhinal cortex. Finally at -5.04 Bregma fourth ventricle (dark orange), ventral hippocampus left (pastel yellow), right (dark pink), substantia nigra and internal capsule left (yellow) and right (light blue).

Wild-type and $Dlg2^{+/-}$ volumes were contrasted for the following structures:

Dorsal and ventral hippocampus

The hippocampus is a landmark brain structure, appearing clearly on T2 weighted images. Manual segmentation of dorsal and ventral subfields was conducted in line with Wolf et al., 2002. The dorsal hippocampus is shown in Figure 5 from -1.56 Bregma as pastel blue (left) and pastel pink (right). The rostral-most slice of the dorsal hippocampus was approximately -2.12 mm from Bregma, consisting of eight 35 mm thick brain slices and ending at approximately -4.20 mm from Bregma. The ventral hippocampus continues from here, being marked by the hippocampus extending in the inferior direction for eight slices until -6.48 mm Bregma with the caudal boundary visible by the absence of the dentate gyrus subfields and the loss of contrast between the external capsule and subiculum. Figure 5 shows the ventral hippocampus at -5.04 Bregma as pastel orange (left) and dark pink (right).

Perirhinal cortex

The perirhinal cortex can be located around the rhinal sulcus in the rat brain atlas (Paxinos & Watson, 2013). The rostral-most slice was defined by the inferior third ventricle becoming visible and the inferior migration of the lateral ventricles at -3.12 mm Bregma. After eight slices the caudal-most slice was defined as where the CA3 subfield of the ventral hippocampus extends in the inferior direction just before the closing of the lateral ventricles, at approximately -4.36 mm Bregma. It can be seen on Figure 5 starting at -3.48 Bregma as bright red (left) and orange (right).

Ventricles

The ventricles are highly visible on T2 weighted images due to the high contrast of water molecules in the image. The lateral ventricle emerges rostrally at 2.04 mm Bregma and ends after 19 slices where the ventral hippocampus takes its space at – 4.36 mm Bregma. Figure 5 shows this from 1.56 Bregma as dark blue (left) and bright pink (right). The third ventricle emerges clearly at -0.48 mm Bregma and ends where the ventral hippocampus extends laterally at -5.16 mm Bregma after 13 slices, and can be seen in Figure 5 at -0.48 Bregma in pink. The aqueduct and fourth ventricle starts rostrally with the end of the hippocampus and complete separation of the two cerebral hemispheres at -6.84 mm Bregma and after 11 slices ends caudally at -8.76 Bregma where the duct separates around the cerebral midline, shown at -5.04 Bregma in Figure 5 as dark orange.

Striatum

Striatal regions were segmented as in Antonsen et al., 2013. The caudate putamen starts rostrally where the corpus callosum visibly joins across hemispheres at 2.28 mm Bregma and after 14 slices ends where the third ventricle opens inferiorly and CA3 becomes apparent at 3.12 mm Bregma. It can be seen in Figure 5 at 1.56 Bregma as brightest green (left) and darkest green (right). The globus pallidus starts where the third ventricle opens at -0.60 mm Bregma and ends at -1.32 mm Bregma after five slices and is shown on Figure 5 at -0.48 Bregma as pastel orange (left) and pastel purple (right). The substantia nigra and internal capsule can be segmented on the eight slices from -4.20 mm to -7.44 mm Bregma

where the ventral hippocampus starts and ends. It can be seen on Figure 5 at -5.04 Bregma as yellow (left) and light blue (right).

Prefrontal cortex

The anterior cingulate was outlined as in Wolf et al., 2002, beginning rostrally at 2.28 mm Bregma where the anterior commissure becomes evident and the corpus collosum joins hemispheres. After 13 slices the causal-most slice of the anterior cingulate gyrus is where the third ventricle becomes circular at the midline (-1.72 mm Bregma). It can be seen on Figure 5 at 1.56 Bregma as dark red (left) and turquoise (right). M1 begins rostrally where the forceps minor becomes discernible around 3.72 mm Bregma, and after 20 slices ends where the third ventricle opens inferiorly at Bregma -3.12 mm. M1 is shown on Figure 5 at 2.52 Bregma as pastel orange (left) and green (right).

Template masks were co-registered to the individual brain scans for each rodent (using ANTs antsRegistrationSyN) and to structure volumes for each rodent were extracted using MATLAB.

Assessment of brain-wise group differences in white-matter microstructure using TBSS

In order to perform a whole-brain analysis to isolate any white-matter microstructure changes in *Dlg2^{+/-}* rats compared to wild-types the TBSS protocol (Smith et al., 2006), modified for use in rodents (Sierra et al., 2011), was used. This analysis was run by C. Casella. All FA maps were submitted to a free-search for a best registration target in order to minimize the image warping required for each brain volume. Specifically, each volume was first registered to every other volume, and the one requiring minimum transformation to be registered to other volumes was selected as the best registration target. This target was used as a template into which the registration was performed. Following registration, a mean FA map was calculated, thinned to represent a mean FA skeleton, and an optimal threshold of 0.2 was applied to the mean FA skeleton to create a binary white-matter skeleton mask. The application of this threshold allowed the exclusion of brain areas with

low FA, including peripheral small tracts where there may be high between-subject variability, from further analysis (Smith et al., 2006).

The local FA-maximum was projected onto this white-matter skeleton. Subsequently, the voxel location of the local FA maximum was employed to project the respective FA, MD, AD and RD values from that voxel onto the skeleton. Differences in microstructure measures between the two groups were assessed using voxel-wise independent t-tests, where two +/- +/- different contrasts were used (WT > $Dlg2^{+/-}$, and $Dlg2^{+/-}$ > WT). The randomize function (part of FSL) was used, together with the TFCE algorithm (S. M. Smith & Nichols, 2009), generating cluster-size statistics based on 1000 random permutations. For multiple comparison correction, FWE correction was first used with a threshold of $p < 0.05$. Subsequently, in order to investigate more subtle changes, the analysis was repeated with the less conservative FDR correction (Benjamini and Hochberg, 1995), which has been previously used in rodent imaging data (Sagi et al., 2012; Sierra et al., 2011).

2.2.5. Statistical analysis: use of Bayesian statistics

Statistical analysis was performed using JASP (JASP Team (2020). JASP (Version 0.14.1) [Computer software]) except where the demand equation was fit to data from the modified progressive ratio task in Chapter 5, which used GraphPad Prism (Version 9.0.0). For traditional null-hypothesis significance testing $p < 0.05$ was considered statistically significant. Parametric ANOVA makes the assumptions of normality, sphericity and equality of variances. Levene's test was used to test equality of variances. Where Maunchly's test indicated sphericity was violated Greenhouse-Geisser corrected values are reported. Q-Q plots of the data were used to assess for normality. Although ANOVA is usually robust to this violation, where normality was violated data were re-analysed with the Kruskal-Wallis nonparametric test. In every instance the conclusion of the test was upheld, and these are not reported here.

Traditional null-hypothesis significance testing only assesses how unlikely the observed data is given the assumption of the null hypothesis, and thus $p > 0.05$ does not distinguish evidence for the null hypothesis from data insensitivity (Dienes, 2014). In contrast, Bayesian tests calculate the relative probabilities of observing the data given the null and alternative hypotheses, and thus allow assessment of whether the evidence is in favour of either hypothesis. In this body of work Bayesian statistics have been applied where traditional null-hypothesis significance testing shows a non-significant result for a key effect or interaction where a null result is theoretically informative (in particular, evidence for a lack of a difference across genotypes).

Bayes factors relate to the ratio of probability for the observed data under a model based on the null hypothesis compared with a model based on some specified alternative. When represented as BF01 Bayes factors vary between 0 and infinity, where 1 indicates that the data do not favour either theory more than the other, values greater than 1 indicate increasing evidence for the null over the alternative hypothesis and values less than 1 increasing evidence for the alternative over the null hypothesis. When using Bayes factors to decide whether there is substantial evidence for the null over the alternative, the following conventions suggested by Jeffreys et al (1939/1961) can be followed: a Bayes factor between 1 and 3 gives weak or anecdotal support to the null, a factor between 3 and 10 represents some supporting evidence, while a factor more than 10 indicates strong evidence for the null.

Bayes factors were calculated for factorial ANOVAs in the way described by Rouder, Morey, Speckman, and Province (2012) and Rouder, Morey, Verhagen, Swagman, and Wagenmakers (2016) and implemented using JASP 0.14 and the default prior scale for fixed and random effects and reported as the analysis of effects – this gives a $BF_{exclusion}$ which is equivalent to BF01 when averaging across models including the factor or interaction of interest. Bayes factors for t-tests were calculated as described by Rouder, Speckman, Sun, Morey, & Iverson, 2009 and implemented using JASP 0.14 with the default settings for the Cauchy prior distribution on effect size under the alternative hypothesis.

2.3. Results

2.3.1. Western blots

Of the three proteins analysed only PSD-93 showed consistent decreases across all four brain regions in the $Dlg2^{+/-}$ rats compared to wild-types (Figure 6). Integrated densities were analysed using repeated measures ANOVA with within-subjects factor of brain region (prefrontal cortex, cortex, hippocampus, cerebellum – apart from NR1 where expression in cerebellum was negligible in all cases and thus this region was omitted from the analysis) and between-subjects factor of genotype. Example blots can be seen in Figure 7. Repeated measures ANOVA analysis for PSD-93 showed a significant main effect of genotype ($F(1, 22)$ = 13.680, $p = 0.001$, $n^2_p = 0.383$) demonstrating the success of the heterozygous gene knockout on reducing PSD93 protein levels. There was also a significant main effect of brain region ($F(1.018, 22.400) = 20.893$, $p < 0.001$, $n^2_p = 0.487$) and a genotype × brain region interaction ($F(1.018, 22.400) = 5.166$, $p = 0.032$, $n^2_p = 0.190$). The significant genotype × brain region interaction was followed up with independent samples t-tests. PSD-93 was more abundant in the PFC of wild-types than $Dlg2^{+/-}$ rats ($t(22) = 6.057$, $p < 0.001$, $d = 2.473$), likewise in the hippocampus ($t(22) = 5.378$, $p < 0.001$, $d = 2.195$), cortex ($t(22) = 4.032$, $p < 0.001$, $d = 1.646$) and cerebellum ($t(22) = 2.702$, $p = 0.013$, $d = 1.103$).

Repeated measures ANOVA analysis of PSD-95 levels showed no main effect of genotype with Bayes factor inconclusive ($F(1, 22) = 3.805$, $p = 0.064$, $n^2_p = 0.147$; $BF_{exclusion} = 2.187$), Despite the inconclusive Bayes factor Figure 6B shows that PSD-95 is often more abundant in the wild-types then the $Dlg2^{+/-}$s, differing from the expected pattern of upregulation in the $Dlg2^{+/-}$s to compensate for decreased PSD-93. There was no main effect of brain region ($F(1.722, 37.884) = 0.175$, $p = 0.808$, $n^2_p = 0.008$; $BF_{exclusion} = 18.288$) and no genotype × brain region interaction with Bayes factors providing evidence for the null ($F(1.722, 37.884) = 1.140$, $p = 0.324$, $n^2_p = 0.049$; $BF_{exclusion} = 23.396$).

Similarly for analysis of NR1 NMDA receptor subunit levels there was no main effect of genotype ($F(1, 17) = 0.262$, $p = 0.616$, $n^2_p = 0.015$; $BF_{exclusion} = 3.752$), brain region ($F(1.148, 19.514) = 0.884$, $p = 0.373$, $n^2_p = 0.049$; $BF_{exclusion} = 4.026$) or genotype × brain region interaction ($F(1.148, 19.514) = 0.902$, $p = 0.368$, $n^2_p = 0.050$; $BF_{exclusion} = 8.238$). Thus it seems

that *Dlg2* haploinsufficiency does not have downstream effects on the expression of related proteins.
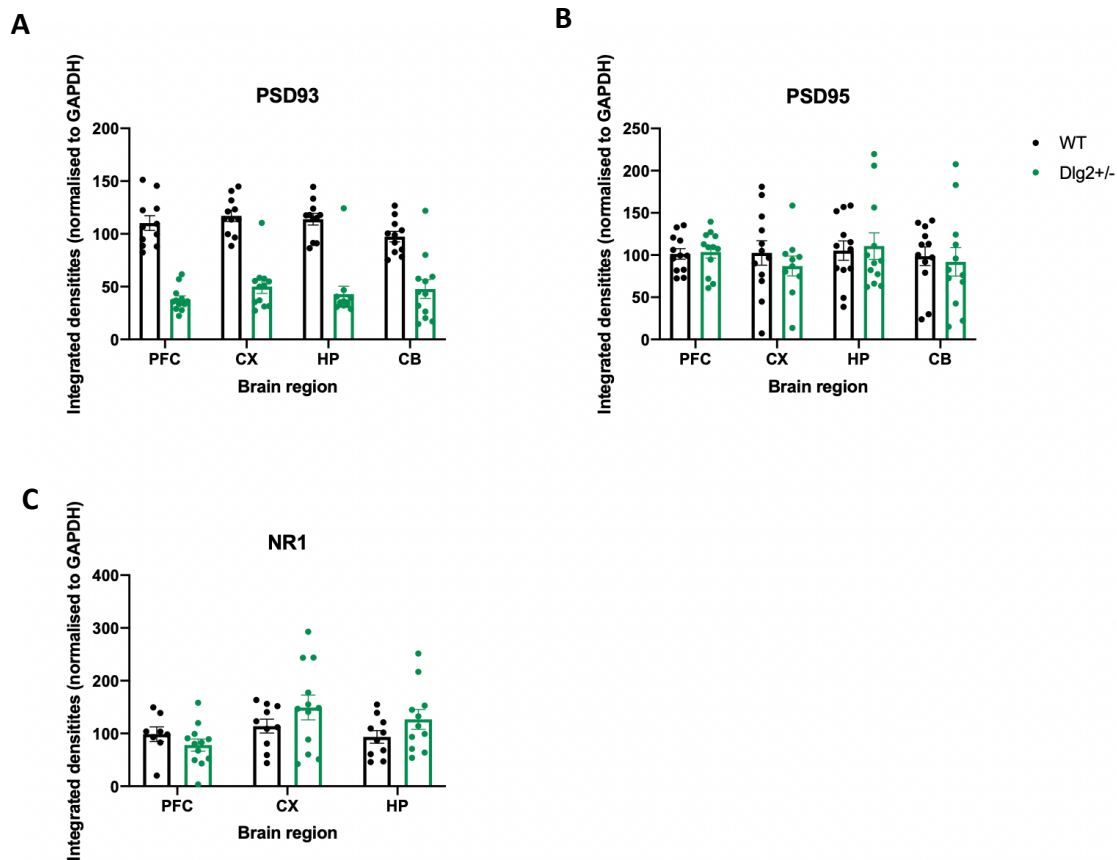
Figure 6: Expression of proteins PSD-93 (A), PSD-95 (B) and NR1 NMDA receptor subunit (C) in *Dlg2*⁺/⁻ and wild-type rats. These were assessed across four brain regions: prefrontal cortex (PFC), cortex (CX), hippocampus (HP) and cerebellum (CB). Cerebellar NR1 expression was too low for analysis thus is not reported. Mean ± SEM integrated density plotted plus individual data points.
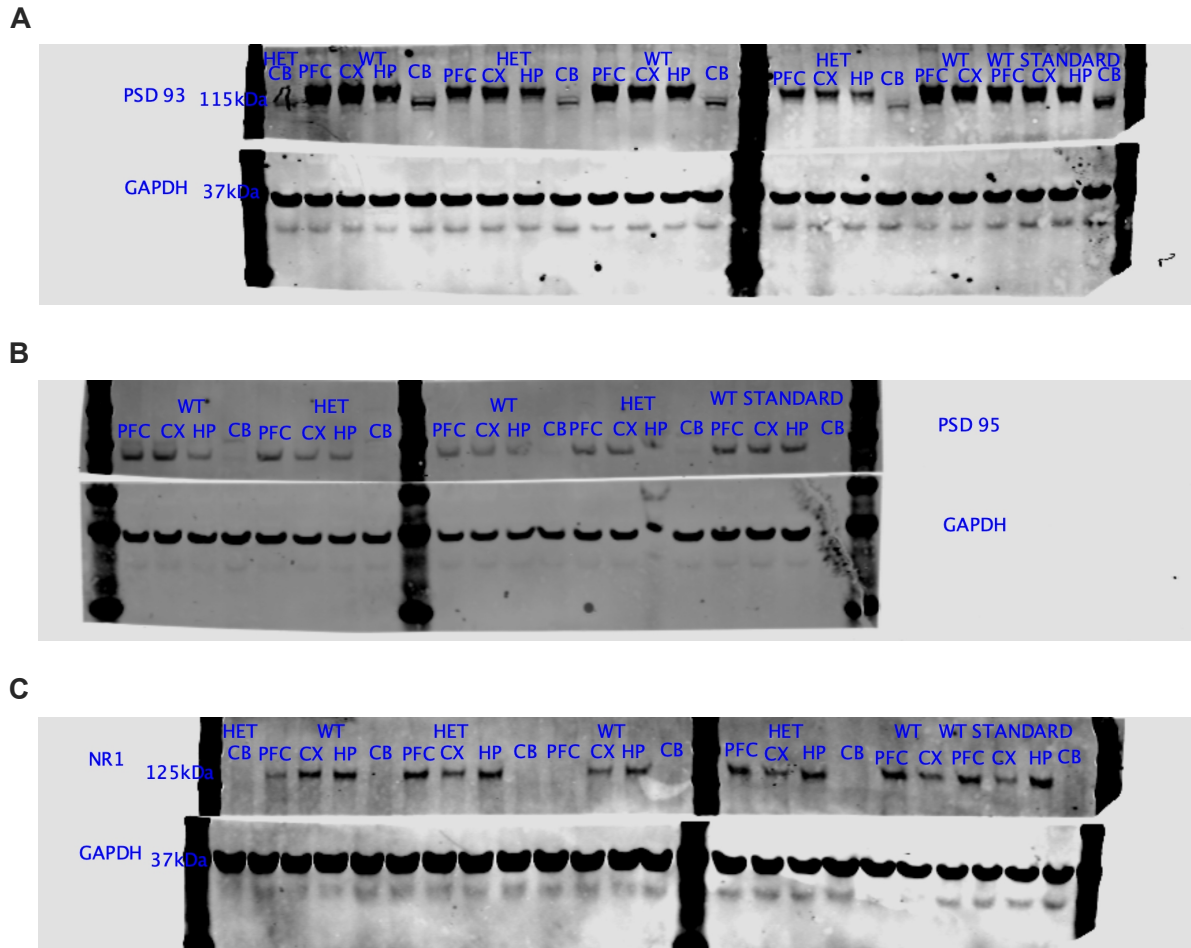
Figure 7: Sample blots showing the same samples and brain regions for PSD93 (A), PSD95 (B) and NR1 (C). Lack of bands for NR1 cerebellum shown.

2.3.2. Volumetric comparison of selected brain structures

Each brain structure was compared across genotypes using independent samples and Bayesian independent samples t-tests. As no differences were found between *Dlg2*$^{+/-}$ and wild-type rats (Figure 8) findings did not require correcting for false-discovery rate. Bayes factors gave inconclusive to anecdotal evidence for null effects. There were no differences in volume for the hippocampus, perirhinal cortex, ventricles, striatum or prefrontal cortex. Reports of t-tests and Bayesian t-tests can be found in Table 3.
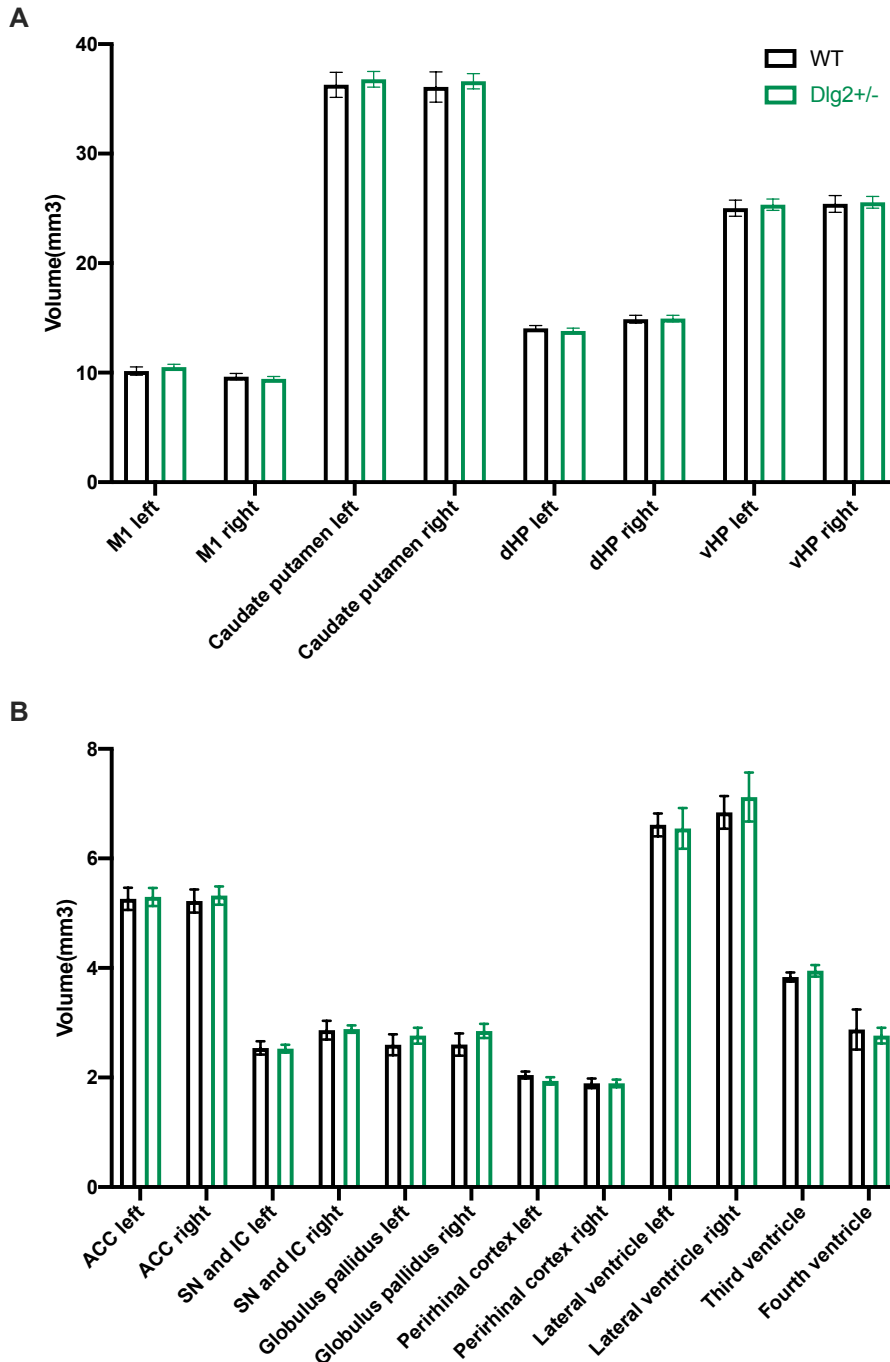
Figure 8: MRI-derived volumes for *Dlg2*+/- rats (green) and wild-types (black) by left and right hemisphere. Mean ± SEM **A)** brain regions with volumes > 10 mm^3: primary motor cortex (M1 left, M1 right), caudate putamen (left, right), dorsal hippocampus (dHP left, dHP right) and ventral hippocampus (vHP left, vHP right) **B)** brain regions with volumes < 10 mm^3: anterior cingulate gyrus (ACC left, ACC right), substantia nigra and internal capsule (SN and IC left, SN and IC right), globus pallidus (left, right), perirhinal cortex (left, right), lateral ventricle (left, right), third ventricle and fourth ventricle.

Table 3: Independent samples t-tests and Bayesian independent-samples t-tests for genotype comparisons of brain volume ROIs.

Brain region	t	df	p	d	BF_{01}
M1 left	0.823	26	0.418	0.312	2.194
M1 right	-0.589	26	0.561	-0.223	2.481
Anterior cingulate left	0.132	26	0.896	0.050	2.808
Anterior cingulate right	0.370	26	0.714	0.140	2.684
Caudate putamen left	0.390	26	0.700	0.148	2.669
Caudate putamen right	0.353	26	0.727	0.134	2.697
Substantia nigra and internal capsule left	-0.097	26	0.923	-0.037	2.816
Substantia nigra and internal capsule right	0.113	26	0.911	0.043	2.812
Globulus pallidus left	0.707	26	0.486	0.268	2.344
Globulus pallidus right	1.063	26	0.297	0.403	1.856
Perirhinal cortex left	-1.112	26	0.276	-0.421	1.785
Perirhinal cortex right	-0.016	26	0.987	-0.006	2.826
Dorsal hippocampus left	-0.650	26	0.521	-0.246	2.412
Dorsal hippocampus right	0.126	26	0.901	0.048	2.809
Ventral hippocampus left	0.350	26	0.729	0.133	2.699
Ventral hippocampus right	0.152	26	0.880	0.058	2.802
Lateral ventricle left	-0.145	26	0.886	-0.055	2.804
Lateral ventricle right	0.503	26	0.620	0.190	2.570
Third ventricle	0.817	26	0.421	0.309	2.202
Fourth ventricle	-0.294	26	0.771	-0.111	2.736

2.3.3. TBSS analysis of whole-brain white matter microstructure

TBSS analysis did not reveal differences in white matter microstructure in either the wild type > $Dlg2^{+/-}$ or wild type < $Dlg2^{+/-}$ directions. This was consistent amongst across methods of familywise error correction. Maps of the t-value (corrected for multiple comparisons) did not reveal regional differences in FA, MD or RD.
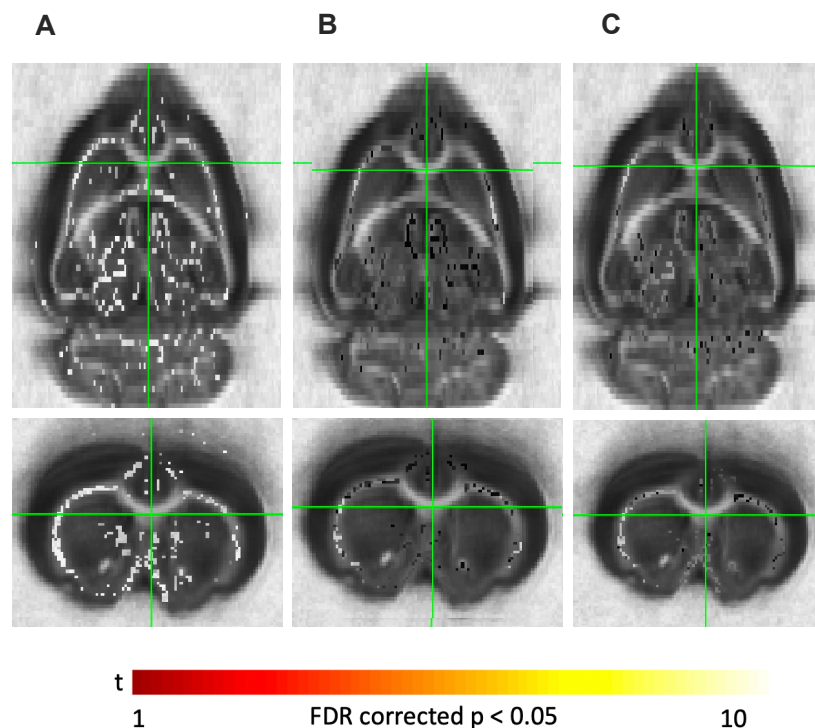
Figure 9: Maps of the t-value (whole brain voxel analysis at p < 0.05 false discovery rate corrected for multiple comparisons) overlayed on a common FA skeleton. These demonstrate no change in **A)** FA **B)** MD or **C)** RA across genotypes in the WT>$Dlg2^{+/-}$ direction. Results are comparable in the $Dlg2^{+/-}$>WT contrast. Crosshair centred on the genu of the corpus callosum, approx. 1.20 mm from Bregma (Paxinos and Wilson, 1997).

2.4. Discussion

This chapter provides the first building blocks of knowledge of the biological consequences of $Dlg2$ haploinsufficiency, both validating it as a mechanistic model of $Dlg2$ deletion on one

chromosome and interrogating further structural consequences of development in such a biological state.

Gene expression is decreased for *Dlg2* transcripts in the hippocampus and prefrontal cortex but not the cerebellum of *Dlg2*$^{+/-}$ rats (Pass, 2019). Western blot data confirms that PSD-93 is reduced in the prefrontal cortex and hippocampus and also finds it reduced in the posterior cortex and cerebellum. Decreased PSD-93 in the cerebellum is interesting given the lack of *Dlg2* mRNA decrease, implying a post-translational mechanism reducing the PSD-93 levels here independently of the genetic lesion. Replication and interrogation of this finding is essential to draw further conclusions. Importantly, gene expression analysis was conducted on 2-month-old rats while quantification of protein was conducted on 4-month-old rats. Within this developmental time frame, it may be that the expression of *Dlg2* also decreases to produce the reduction in PSD-93.

The finding of consistent PSD-93 decreases indicates that the *Dlg2*$^{+/-}$ model is valid at the protein level. Furthermore, no evidence of compensatory changes to related *Dlg2* paralogs *Dlg1*, *Dlg3* or *Dlg4* is seen at the expression level (Pass, 2019) or for PSD-95 at the protein level. Compensation between *Dlg2* and *Dlg4* has been seen in other rodent models where either gene is knocked down or decreased in copy number (Favaro et al., 2018; Winkler, Daher, Wüstefeld, Hammerschmidt, Poggi, Seelbach, Krueger-Burg, et al., 2018). Distinguishing the *Dlg2*$^{+/-}$ rat from these specifies it as a model which can specifically focus on the clinical relevance of *Dlg2* heterozygosity alone. Despite this Dlg2 has many interactors (General Introduction 1.3.4) and this analysis does not preclude biological compensation by other mechanisms either in adulthood or development.

Whether change in PSD-93 levels causes change to NMDAR levels or function remains a key question. Here no change in levels of the NR1 NMDAR subunit were observed across brain regions tested. This provides evidence that there is no gross decrease in NMDARs at the synapse, as NR1 is the only essential subunit for NMDAR formation, thus allowing a good readout of overall NMDAR content (Rudolf et al., 1996). This aligns with previous work demonstrating no change to NMDAR function or synaptic presence when *Dlg2* is decreased

in homozygous knockdown models (Elias et al., 2006; Krüger, Favaro, Liu, Kitlińska, et al., 2013).

However, changes in NMDAR subunit expression and function have also been reported in *Dlg2* knockout models (Zhang et al., 2010) and functional changes indicative of reduced NMDAR currents have been reported in this *Dlg2*$^{+/-}$ rat (Griesius et al., 2022). It could be that even with levels of NMDAR similar to wild-types less NMDAR is functional in *Dlg2*$^{+/-}$ rats. Fewer NMDAR complexes could be anchored at the postsynaptic membrane if *Dlg2* is compromised, and key effectors may be missing from the complexes that are present. Performing co-immunoprecipitation experiments for NMDAR subunits in synaptosomes and then analysing NMDAR binding partners could determine differences in NMDAR complex formation between *Dlg2*$^{+/-}$ and wild-type rats.

Furthermore, PSD-93 only directly binds the NR2B NMDAR subunit (Sato et al., 2008). There is evidence that in vivo the NR2B subunit is expressed at a higher level during early postnatal brain development (Monyer, Burnashev, Laurie, Sakmann, & Seeburg, 1994), while in the adult brain expression of NR2A predominates (Wenzel, Fritschy, Mohler, & Benke, 1997). Thus it would be instructive to investigate whether the ratio of NR2A:NR2B receptors in *Dlg2*$^{+/-}$ rats differs from wild-types both in development and maturity. If the ratio of these subunits were different in the *Dlg2*$^{+/-}$ rats this could have consequences for the electrophysiological properties of neurons, given that NR2A mediates fast current passage while NR2B mediated longer duration depolarisation (Monyer et al., 1992).

It is also notable in Figures 2 and 3 that *DLG2* is expressed in non-brain tissue in humans. Thus, it is possible that psychiatric phenotypes resulting from *DLG2* CNVs could be a consequence of functional disruption in organs outside the brain. *DLG2* mRNA is expressed in the testis and adrenal gland, where changes in *DLG2* expression could have an influence on endocrine function. However, Figure 3 shows that PSD-93 is not enriched in these tissues. Figure 3 also shows that PSD-93 is expressed in the duodenum and rectum. Functional disruption here could bring about psychiatric consequences given recent findings concerning the role of the gut-brain axis and immunity in a variety of psychiatric disorders DLG2 CNVs are associated with (Severance, Prandovszky, Castiglione, & Yolken, 2015;

Srikantha & Hasan Mohajeri, 2019). In light of these ideas, it would be useful to profile the Dlg2 mRNA and protein expression across brain and body tissues in the $Dlg2^{+/-}$ model.

At a whole-brain level no observable changes in the grey matter volume of selected structures or white-matter microstructure were found between genotypes. Thus it is impossible to align a profile of grey and white matter abnormalities in $Dlg2^{+/-}$ rats with either ASD or schizophrenia. The lack of deficits also diverges from the widespread abnormalities seen in rodent models of schizophrenia such as the MAM rat (Chin et al., 2011) and NMDAR-antagonism models using PCP (Barnes et al., 2015), ketamine (Schobel et al., 2013) and MK-801 (Wu et al., 2016). The latter finding adds further evidence to the fact that the $Dlg2^{+/-}$ rat is likely not a NMDAR hypofunction model analogous to these pharmacological models as is also evidenced by the lack of decrease in the NMDAR subunit NR1 in western blots. While abnormalities in brain structures could focus research into psychological or molecular deficits associated with these, this logic cannot be reversed. That is, intact white matter microstructure and grey matter volumes does not indicate that brain function is intact even if the structure of brain anatomy shows no gross defects.

The approaches taken to isolate differences between genotypes in grey and white matter were both limited. Analysing volumetric changes by masking ROIs was undertaken as no suitable template brain exists for the Long Evans rat breed and creating one by averaging the wild-type brains in such a small sample for a VBM approach could have been warped by interindividual variability. Specifying ROIs allows hypothesis-driven determination of key structures, yet this kind of analysis means that there may have been volume changes in structures other than those selected a priori which went unnoticed. The opposite problem is true of the white matter TBSS analysis. The lack of ROI specification when quantifying white-matter microstructure meant that strict measures controlling for family-wise error could have precluded the identification of structural changes in specific tracts. Therefore in the absence of gross abnormalities more subtle changes could have been missed by the hypothesis-free approach taken here.

For grey matter, volume is also only one component of structure. Changes in grey matter density are associated with ASD (e.g. reviewed in Chen et al., 2011) and structural

asymmetry of the hippocampus and amygdala associated with *Dlg2* in Alzheimer's (Wachinger, Nho, Saykin, Reuter, & Rieckmann, 2018). Analysing these components in *Dlg2^+/-* rats would give a more detailed picture of the structural integrity of brain structures. Any findings from MRI analysis should be interrogated further at the molecular level to determine the mechanism, e.g. reductions in dendritic spine density or microglia levels. Rather than just isolating differences in brain structure across genotypes, the consequences of structural variation should be elucidated by correlating grey and white matter changes with relevant psychological or cognitive capabilities as has been done previously (e.g Kühn et al., 2012; Stegmayer et al., 2014).

In conclusion, this work specifies the validity of the *Dlg2^+/-* rat model at the protein level. Only PSD-93, which is targeted with the heterozygous genetic lesion, is decreased in expression. There does not appear to be compensation from the most related paralog (PSD-95) or change to NR1 NMDAR subunit levels. Analysis of MRI data for changes in gross white matter integrity or to grey matter volume in selected structures did not reveal differences between wild-type and *Dlg2^+/-* brain structure. The remaining work in this thesis focuses on behavioural phenotyping of the *Dlg2^+/-* rat model, starting with the assessment of basic psychiatric-relevant phenotypes in Chapter 3.

Summary

- Protein PSD-93, coded by the *Dlg2* gene, is decreased in the *Dlg2^+/-* rat. There is no change to levels of NR1 NMDAR protein or PSD-95 protein in the rat model.
- Volumetric analysis of grey matter in selected structured did not reveal differences between the *Dlg2^+/-* and wild-type rats.
- TBSS analysis of whole-brain white matter microstructure did not reveal differences between the *Dlg2^+/-* and wild-type rats.

Chapter 3

General behavioural phenotyping of *Dlg2+/-* rats

3.1. Introduction

3.1.1. Aims

The *Dlg2+/-* rat is a novel model which has not been subject to any detailed behavioural phenotyping. As a place to start *Dlg2+/-*s and wild-types were compared across a range of tasks which are commonplace and well-validated when phenotyping rodent models of DLG2-associated conditions such as ASD and schizophrenia. These tasks all exploit natural ethological responses (such as a preference for safe locations, investigating conspecifics, startling in response to auditory pulses and so forth) to determine whether there are natural differences within these. These assays do not require extensive training or animal handling removing these as confounds.

The array of tasks chosen here is wide but by no means exhaustive. These behaviours are pertinent to different psychiatric endophenotypes, such as anxiety and social behaviour abnormalities, rather than intending to link the model to any specific disorder. This is useful when considering a genetic lesion that increases risk for a range of conditions. These assays also provide the first fundamental building block when continuing to test *Dlg2+/-*s on tasks which require training or more complex procedures as they ascertain possible confounds such as anxiety, hearing, and responding to stimuli.

3.1.2. Anxiety

Anxiety symptoms are a component in all of the conditions *DLG2*-encompassing genetic variants are associated with, be this schizophrenia (Temmingh & Stein, 2015), autism (White, Oswald, Ollendick, & Scahill, 2009), bipolar (McIntyre et al., 2006) or ADHD (Schatz & Rostain, 2006). In each of these cases the interplay between the specific disorder and anxiety will vary, as indeed it might on a case-by-case basis, with anxiety often preceding diagnosis (Coryell et al., 2009; Grillo, 2018) but also arising from the experience of symptoms and the burden of living with these.

There is also evidence of a direct link between *Dlg2* function and anxiety in an excess retinoid mouse model of depression. In the mouse model *Dlg2* mRNA was significantly decreased in the hippocampus by all-trans retinoic acid (ATRA) injections for 12 days at three weeks of age. *Dlg2* mRNA levels were positively correlated with decreased anxiety-associated behaviours on the open field test, but not the elevated plus maze test of anxiety (Qin, Fang, Shan, Qi, & Zhou, 2020). In the ATRA mouse model other biochemical and physiological effects of ATRA could have precipitated the apparent *Dlg2*-anxiety link. The *Dlg2*$^{+/-}$ model provides a better way to understand the causality between this genetic change and anxiety phenotypes. Furthermore assessing anxiety phenotypes in the *Dlg2*$^{+/-}$ rat model will aid understanding of whether the CNV-associated genetic change contributes to this endophenotype, or whether the comorbidity of anxiety with these conditions in CNV carriers arises due to other factors.

Two established tests of rodent anxiety were used, the open field test (OFT) and elevated plus maze (EPM). These tests exploit the conflict between a rodent's natural tendency to shelter in safe, enclosed spaces and their desire to explore novel environments (Sartori, Landgraf, & Singewald, 2011). Anxious behaviour is quantified by the percentage of time the rodent spends in the open, unprotected maze areas (the central region of the OFT and the open arms of EPM) as opposed to the amount of time spent in the 'safe' regions (the outer region of the OFT and EPM closed arms). There are also a number of ethological measures of anxiety that can be obtained, such as frequency of defecation, and in the EPM stretch-attend postures and head dips (Horii, McTaggart, & Kawaguchi, 2018). Stretch attends occur when the rodent keeps its body close to the floor and its rear legs in the closed arm while investigating the open arm or middle section. When the rat is on an open arm and peers over the platform edge such that its head is fully off the platform, that is a head dip. Anxiety on the EPM is associated with more stretch attend postures, fewer head dips and increased defecation (Humby, Cross, Messer, Guerrero, & Davies, 2016).

Other disease-relevant measures can be obtained in analysing the EPM and OFT. Hyperactivity in responding to a novel environment can be assessed by distance moved and velocity of the rodent. Additionally, scoring the duration and frequency of self-grooming

provides a method of assessing compulsive, repetitive behaviours in rodents which has been reported in rodent models of autism (Silverman, Tolu, Barkan, & Crawley, 2010). Indeed increased self-grooming has been demonstrated in $Dlg2^{-/-}$ mice (Yoo et al., 2020b).

Further direct links between $Dlg2$ and anxiety come from the exploration of $Dlg2^{-/-}$ mice on the EPM, OFT and the light/dark box (Winkler, Daher, Wüstefeld, Hammerschmidt, Poggi, Seelbach, Krueger-burg, et al., 2018; Yoo et al., 2020b). Light/dark box is a similar anxiety assay that involves placing the rodent in an arena where one half is well-lit (e.g. 400 lux) and the other half is dimly-lit (e.g. 4 lux). In a way analogous to staying in closed EPM arms or the OFT periphery rodents demonstrate anxiety by remaining in the 'safe' dimly lit side of the box (Crawley & Goodwin, 1980).

In a procedure where mice were placed in the centre of the open field Winkler et al (2018) found that latency to cross to the peripheral zone, distance travelled, and velocity decreased significantly with decreasing $Dlg2$ gene dosage. That is $Dlg2$ heterozygous mice demonstrated greater hypoactivity in the field than wild-types, with $Dlg2$ homozygotes being more hypoactive than heterozygotes. This occurred despite all rodents spending comparable amounts of time in the different maze zones and there being no difference across genotypes in light/dark box exploration. This indicates that rather than having an anxiety phenotype $Dlg2$ mutants may vary in their locomotor response to novelty.

Hypoactivity in the open field was also observed in $Dlg2^{-/-}$ mice by Yoo et al (2020), who also found that locomotion was comparable across genotypes in a familiar home-cage environment specifying differences as a response to novelty. In contrast to Winkler et al (2018) they observed that mutants spent less time in the centre zone of the open field than controls and showed a significantly decreased duration of exploring in the light chamber of the light/dark box. These behaviours indicate increased anxiety in the $Dlg2^{-/-}$ line, yet also depend on the locomotion of the rodent. There was also no difference between genotypes in exploration on the EPM, leading researchers to match Winkler's (2018) conclusion that $Dlg2^{-/-}$ rats demonstrate an aberrant locomotor response to novel contexts. With these phenotypes present when $Dlg2$ is completely knocked down it will be informative to see if they replicate in the more disorder-relevant heterozygous rat model.

3.1.3. Sensorimotor gating

Sensorimotor gating is a pre-attentive filtering process that is involved in regulating and screening out unwanted or unnecessary sensory inputs. Experimentally, functional sensorimotor gating is measured by pre-pulse inhibition (PPI) which evaluates the reduction of an induced response (startle in rodents and often eyeblink in humans) to an auditory tone after exposure to a weak preceding tone (Swerdlow, 2009).

Deficiencies in sensorimotor gating are a highly heritable behavioural endophenotypes and risk factors for multiple neurodevelopmental conditions. PPI deficits have been best characterised and widely replicated in schizophrenia patients (Mena et al., 2016) but are also present in those with high schizotypy (Wan, Thomas, Pisipati, Jarvis, & Boutros, 2017), unaffected family members, and various rodent models of the disorder (Powell, Zhou, & Geyer, 2009). Decreased PPI has also been documented in obsessive-compulsive disorder, manic bipolar patients, panic disorder, and adults with autism (Ahmari, Risbrough, Geyer, & Simpson, 2012; Ludewig, Ludewig, Geyer, Hell, & Vollenweider, 2002; Mao et al., 2019; Sinclair, Oranje, Razak, Siegel, & Schmid, 2017). Aligning with the role of sensorimotor gating in filtering out irrelevant stimuli, it has been found that PPI correlates with the ability to learn about relevant stimuli in latent inhibition tasks and accuracy on the continuous performance task, cognitive mechanisms related to the identification of goal-irrelevant information in the input stage and the subsequent ability to attend to goal-relevant information in light of this (Jones, Hills, Dick, Jones, & Bright, 2016).

The association of many neurodevelopmental conditions with PPI disruption renders it a good test to apply to a rat model of a cross-disorder CNV with an established link to schizophrenia spectrum disorders. PPI is also a cross-species phenomena and thus has good translational value. Furthermore, the PPI procedure allows assessment of hearing and baseline short-term habituation to auditory stimuli in the *Dlg2*$^{+/-}$ rats, ensuring these processes are intact for later behavioural testing which will rely on normal responses to stimuli.

3.1.4. Change in behaviour with stimulus exposure

Habituation is a simple non-associative form of learning that comprises the decline in the propensity to respond to a stimulus that has become familiar due to prior exposure. It is likely that this is an adaptive response to ensure that attentional resourced are allocated to novel stimuli. Habituation represents another behavioural characteristic that is conserved across species (Rankin et al., 2009) and has repeatedly been observed as abnormal across diverse psychiatric disorders including autism, schizophrenia, ADHD and some dementias, where habituation ability is often predictive of symptom severity (for review see McDiarmid, Bernardos, & Rankin, 2017).

Variations in the method used to study habituation in humans and animal models have limited comparison and confused conclusions concerning the role of habituation in disorder. Habituation is roughly divided into short-term processes (within-day) and longer-term processes (across days), with different neural underpinnings (Stopfer, Chen, Tai, Huang, & Carew, 1996). Short term habituation can be assessed using spontaneous alternation in the Y maze, or more commonly in decrease in locomotor activity in a novel environment (Jones, Watson, & Fone, 2011). This is assessed by rodent's locomotor activity in the OFT and the habituation protocol in the sensorimotor gating battery where rodents startle to an auditory pulse is expected to decrease as the pulse is repeatedly exposed. Short-term habituation tests are also complementary to PPI assessments, which represent short-term an attentional screening process in a different form.

A deficit of short-term habituation has been defined in the *Gria1*$^{-/-}$ model of genetic risk for schizophrenia, a mouse lacking the GRIA1 AMPAR subunit (Austen, Sprengel, & Sanderson, 2017; Sanderson et al., 2009). Given the role of *Dlg2*$^{+/-}$ in developmental AMPAR-related synaptic scaling (Favaro et al., 2018) and mature neuron AMPAR content and transmission (Elias et al., 2006) it may be expected that *Dlg2*$^{+/-}$ rats share this short-term habituation deficit. Sanderson et al. (2007, 2009) observed impaired habituation on Y maze and novel object recognition tests, where knockout mice would explore the recently presented context/ object for the same duration of time as the novel one. The authors theorize that failures of habituation resulting in familiar stimuli appearing salient and driving attention for

longer durations may mechanistically contribute to 'aberrant salience,' the idea that misapplied prediction error learning drives psychosis (Kapur, 2003). They propose that failures of habituation may be particularly deleterious in the prodrome before disorder onset (Barkus et al., 2014).

In the $Dlg2^{+/-}$ model short term processes were investigated by Y-maze spontaneous alternation and novel object recognition (Chapter 4.1.4.) in addition to OFT and sensorimotor measures. Here long-term processes were investigated by studying the habituation to a novel flavour across 12 consecutive days. This complements investigation of short-term processes and has been overlooked in the literature interrogating habituation in schizophrenia. There is evidence that participants with ASD show only short-term deficits and intact long-term habituation processes (Ornitz, Lane, Sugiyama, & de Traversay, 1993). A further advantage of investigating long and short term processes is that they are thought to rely on separable brain systems with short-term habituation reliant on mechanisms intrinsic to sensory processing (Davis, Gendelman, Tischler, & Gendelman, 1982), the striatum and the inferior colliculus (Jordan & Leaton, 1983). Alternatively long-term habituation is thought to rely on the dorsal hippocampus and frontal cortex (Groves, Wilson, & Boyle, 1974). Thus, patterns of deficits in $Dlg2^{+/-}$ rats across these tasks may guide research into which neural systems show deficits and which are intact.

In this assay long-term habituation is assessed by measuring changes in rodents consumption and drinking behaviour to novel flavours across twelve consecutive days. Rats are neophobic and will consume less of a novel, potentially dangerous, solution than one which is familiar and 'safe.' As rodents habituate it would be expected that their consumption of the novel flavour increases, and that they start to 'like' drinking the solution, the latter of which can be investigated by monitoring lick microstructure (Lin, Amodeo, Arthurs, & Reilly, 2012).

While ingesting fluids rats drink in bouts of sustained runs of rapidly occurring rhythmic licks which can be distinguished from other bouts by pauses of a defined length of time. The average number of licks in these bouts (lick cluster size) is lawfully related to the nature of the solution being consumed. Lick cluster size shows a positive, monotropic relationship to

the concentration of palatable fluids such as sucrose (e.g. Davis & Smith, 1992; Spector, Klumpp, & Kaplan, 1998), while lick cluster size decreases monotonically with increasing concentrations of unpalatable quinine solutions (e.g. Hsiao & Fan, 1993; Spector & John, 1998). Because rodent's enjoyment of a solution is reflected in lick cluster size (Chapter 5.1.2., Dwyer, 2012), as rats habituated to the flavours in this assay lick cluster size was expected to increase (Lin et al., 2012).

3.1.5. Social preference and social novelty

Another issue that spans conditions associated with *Dlg2* disruption is abnormal social functioning. This is particularly evident in autism, being one of the key diagnostic features (DSM-5; American Psychiatry Association 2013) and comprising a lack of interest in socialising with others and inability to understand and respond to social cues others read with ease. In schizophrenia and bipolar social withdrawal is well-documented and is associated with poorer disease outcomes (Cannon et al., 1997). As with other domains described here testing a $Dlg2^{+/-}$ rat model on social behaviour provides a way to link a specific biological and genetic mechanism to a complex behavioural domain which occurs across disorder categories.

Social preference, the tendency to actively approach and desire spending time near a conspecific, and social novelty, the preference for known conspecifics rather than strangers, can be tested using the 'three-chamber' test (Rein, Ma, & Yan, 2020a). In the social preference phase of this this test rodents have access to an arena and can choose to spend time engaging with an unknown rodent or an object, with healthy rodents choosing the unknown rodent. In the following social novelty phase the rodent is presented with a choice between the rodent they previously met and a new unfamiliar rodent. They are expected to show understanding of their previous interaction by exploring the unfamiliar conspecific. This test has been widely reported with mice although there has also been success in using it with rats (Templer, Wise, Isabel Dayaw, & Nicole Dayaw, 2018). Deficits on both phases of this task are prevalent in rodent models of autism (Moy et al., 2004) and social preference is reduced in rodent models of schizophrenia negative symptoms (Wilson & Koenig, 2014).

Due to their intimate link predictions concerning the effect of *Dlg2* disruption on social behaviour can be made by looking in to work on direct NMDA receptor disruption. Mice with genetic disruptions to NMDA receptor signalling spend less time with the conspecific in the social preference test (Zoicas & Kornhuber, 2019). This has been shown in knockout mice with disruption to the essential NMDA receptor GluN1 subunit ($NR1^{-/-}$) (Halene et al., 2009), Grin1^{D481N} mice with a point mutation in the GluN1 glycine binding site (Labrie, Lipina, & Roder, 2008) and rodents given NMDA receptor antagonists (McKibben, Reynolds, & Jenkins, 2014). More specifically work by Yoo et al., (2020) implies that the *Dlg2* gene may be particularly involved in social behaviour, with researchers finding that *Dlg2*$^{-/-}$ mice did not show the expected preference for a conspecific on the social preference task. However the opposite finding, with *Dlg2*$^{-/-}$ mice showing hypersociality on the social preference test has been documented (Winkler, Daher, Wüstefeld, Hammerschmidt, Poggi, Seelbach, Krueger-Burg, et al., 2018). Given the link of *Dlg2* and NMDA signalling and the pre-existing conflict of findings with *Dlg2*-targeted mutants, assessment of social behaviour in the more clinically relevant *Dlg2*$^{+/-}$ model was important.

3.1.6. Phencyclidine-induced locomotor response.

Increasing evidence has been found to support the idea that dysfunction to glutamate neurotransmission systems is a primary pathological change in schizophrenia (e.g. Coyle, Tsai, & Goff, 2003; Konradi & Heckers, 2003; Olney & Farber, 1995; Tsai & Coyle, 2002). Key evidence for this is that administration of the NMDAR antagonist PCP is known to produces a transient psychosis-like phenotype in healthy human subjects, resulting in hallucinations and delusions reminiscent of schizophrenia positive symptoms (Cohen, Rosenbaum, Luby, & Gottlieb, 1962; Krystal et al., 1994). Furthermore, in both stabilised and acute schizophrenic patients PCP rekindles or exacerbates these 'positive symptoms' (Javitt & Zukin, 1991). Chronic PCP exposure has become a well-validated pharmacological rodent model of schizophrenia (for review see Castañé, Santana, & Artigas, 2015; Jones, Watson, & Fone, 2011a) which shows a sensitisation reaction in terms of abnormal hyperlocomotion when given an acute PCP dose (Kalinichev et al., 2008). Thus, hyperlocomotion in response to PCP or other NMDAR antagonists has been taken as a biomarker of schizophrenia-like phenotypes, particularly positive symptoms, and glutamatergic abnormalities, in rodent

models relevant to this condition (e.g. Crawford et al., 2020; Didriksen et al., 2017; Enomoto, Noda, & Nabeshima, 2007; Koseki et al., 2012; Nielsen et al., 2017). Given the link between PSD-93 and glutamate transmission (General Introduction 1.3.2 and 1.3.3) and the described NMDAR alteration of function (Griesius et al., 2022) it would be expected that potentiated locomotor response to PCP would be observable in the model. This could reveal an underlying propensity towards psychosis or schizophrenia 'positive symptoms.'

3.2. Methods

Animals

Within this chapter Cohort 2 was used for flavour habituation, Cohort 3 for anxiety tests and sensorimotor gating and Cohort 6 for social preference, social novelty, and PCP hyper-locomotion. A summary of all rat Cohorts used throughout the experimental work in this thesis can be found in Appendix 1.

3.2.1. Elevated plus maze

The elevated plus maze (EPM) consisted of two open arms (45 cm long × 10 cm wide), two closed arms (45 cm long × 10cm wide × 30cm high) and a middle (10 × 10 cm) component forming the shape of a plus elevated 50cm above the ground. Rats were habituated to the testing room for at least 1 hour before testing by being placed there in their home cages. The room was dimly lit, with the light level in the open arms 26 lux, and the light level in the closed arms 15.3 lux. Each rat was placed in the middle compartment with its head facing an open arm and allowed to freely explore the apparatus for 5 minutes. For female rodents vaginal cytology was performed after testing on the maze to determine estruses stage. After each rat the equipment was cleaned with 70% ethanol. Movements of each rat were recorded by a camera mounted 120 cm above the maze and connected to a computer. Videos were recorded as mp4 files and analysed using Ethovision (Noldus Information Technologies 3.0.15, Netherlands). Time spent in the open, closed and middle section of the arms was recorded, with a rat occupying an area defined as when all four paws were within

the space. Head-dips, stretch attend postures and grooming were manually scored. Faecal boli were counted for each animal after each run.

3.2.1. Open field test

The open field apparatus was a 100 × 100 cm square wooden arena with 30cm high walls, painted black. Light levels were 25 lux at the centre of the maze, and 11.8 lux in the corners of the maze. Rats were habituated to the test room for at least 1 hr, then the animal was placed in the middle of the south wall of the arena, with their head pointing towards the wall. Locomotor activity and thigmotaxis were assessed during a 10-minute session. Video of the animal's movements was recorded by a camera mounted 200 cm, above the arena. After testing the arena was cleaned with 70% ethanol and the rat placed in a holding cage until all of its cage mates were tested, at which point they were returned to the home cage. Ethovision software was used to analyse the video recordings for the amount of time rodents spent in the centre (25cm^2 located 25cm from each other wall) and edges of the maze (the rest of the arena), distance moved (cm) and velocity (cm/s). Faecal boli were counted for every rodent.

3.2.3. Sensorimotor gating

Animals underwent a 30-min startle response session in a SR-Lab^{TM} Startle Response System, consisting of 91 trials. Background noise level was set to 70 dB and rats acclimated to this for 5 minutes before trials began. Test pulses lasting 40ms were administered at 120 dB and 105 dB on pulse-alone trials and on pre-pulse + pulse trials test pulses were preceded 70 ms by a 20ms pre-pulse 4, 8 or 16 dB above background. Pulse-alone and pre-pulse + pulse trials were intermixed on a pseudorandom schedule. At the end of the session there were 6 additional pulses of increasing intensity; 70-120 dB to measure increasing startle response. An accelerometer sensor (a clear 10 cm diameter 20 cm long plexiglas cylinder on a plexiglass base) measured the amount of movement of each animal in response to each test pulse. Response to the first three pulses at 120 dB and 105 dB were averaged and analysed as an index of emotional reactivity, then the habituation of startle response was measured by the average of responses to the first six pulse alone trials at each pulse dB. Pre-pulse inhibition

was measured by the inhibition of response to the pulse following pre-pulse presentation was recorded as the average response during a 65 ms window from pre-pulse onset.

3.2.4. Flavour habituation

Rats were trained and tested in 16 custom made drinking chambers (Med Associated Inc., St Albans, USA). Cages were arranged on two shelves, giving 8 top cages and 8 bottom cages. These were 30 × 13 × 13 cm (L × W × H), with steel bar flooring and white plastic walls. Flavours were accessible through drinking spouts attached to 50 ml cylinders, which could be lowered through left or right apertures in the front wall of the chamber by hand. Only the left aperture was used for the current study. A contact sensitive lickometer registered the licks made by rats to the nearest 0.01s once the bottle was available, and MED-PC Software (Med Associates, Inc) recorded the data.

For 12 consecutive days half of the wild-type (n = 14) and $Dlg2^{+/-}$ rats (n = 10) were given 15 minutes to drink 0.4 % saccharin solution, with the other half of the cohort drinking 0.1% saccharin solution, in these boxes. Rats were maintained on a water restriction schedule where they were given 2h access to water in their home cage every day at 4.00 pm in addition to experimental access to fluids beginning at 10.00 am. Normally rats require some initial learning trials to drink reliably with the spouts retracted slightly from the boxes (to prevent accidental contact being recorded as licks) yet these rats were familiar with drinking in these boxes as they had already participated in the lick microstructure assessment described in chapter 5. Thus, the context and drinking was familiar to the rats, and the flavours novel.

Mean consumption of saccharin (g) and mean lick cluster sizes were calculated and used for analysis. A cluster was defined as a set of licks, each separated by an interlick interval of no more than 0.5 s. This criterion was used by Davis and his co-workers who pioneered this technique (e.g., Davis & Perez, 1993; Davis & Smith, 1992) and in many previous studies employing this assay (notably Austen, Sprengel, & Sanderson, 2017; Lydall, Gilmour, & Dwyer, 2010; for a review, see Dwyer, 2012).

3.2.5. Social preference and social novelty

This test utilised the same arena as the open field with two wire mesh chambers (22 cm diameter) weighed down with 2kg weights placed diagonally in opposite corners of the arena as in layout 1 and 2 (Figure 10). The distance between side walls and the chambers was 18 cm, and the distance between them diagonally always 35 cm. Light was 24.8 lux. Rats were placed in the experimental room where the arena was separated from them by a curtain for 1h prior to testing commencing. Each individual rat was given 10 minutes to explore the arena with empty chambers then removed to a separate holding cage for 5 minutes, before being placed back in the arena for the 10-minute social preference and social novelty tests separated by an additional 5-minute break in the holding cage. After each phase equipment was wiped down with 70% ethanol.

In the social preference test one chamber contained an unknown conspecific (Stranger 1) and the other chamber and unknown object, while in the social novelty test one chamber contained a familiar conspecific (Stranger 1) and the other chamber an unknown conspecific (Stranger 2). For each gender two same-gender co-housed wild-type rats were used as Stranger 1 and 2. These rats did not have prior contact with the test rats and were habituated to being in the chambers for 10 minutes before first use as a stranger. Which rat acted and Stranger 1 and 2 was counterbalanced across the rats being tested with a particular stranger pair, as was whether rats were exposed to Layout 1 or 2. In these layouts the position of the chambers varies between tests to prevent exploration being driven by any location-based familiarity.
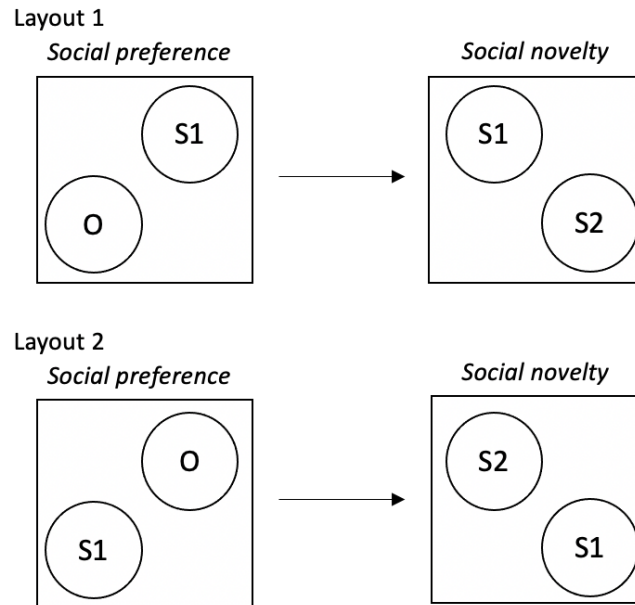
Figure 10: The two possible social preference and social novelty arena layouts used, counterbalanced across genotype and sex. Abbreviations: O for object, S1 for stranger 1 and S2 for stranger 2.

For analysis raw exploration times of the chambers were used in addition to d2 discrimination ratio (Equation 1). Discrimination ratio gives a readout of the difference in exploration time between the two stimuli without the confound of overall tendency to explore for long or short durations. Discrimination ratios are used here and in Chapter 4, and circumvent any issues with analysing raw exploration times with repeated measures ANOVA which assumes independence of observations. Independence is questioned as if a rat is exploring one stimulus it can't be exploring the other, although the rat can be consistently not exploring either stimuli and change exploration across stimuli and no stimuli multiple times in a test session. Here the d2 used for social preference is reported, the d2 for social novelty is calculated in the same way but with Stranger 2 exploration taken from Stranger 1 exploration.

Equation 1:

$$D2 = \frac{Stranger\ 1\ exploration\ time - object\ exploration\ time}{total\ exploration\ time}$$

3.2.6. PCP-induced locomotion

Phencyclidine hydrochloride (PCP) supplied by Sigma-Aldrich, UK, was made into a 5 mg/ml solution using 0.9% (w/w) saline. To examine PCP-induced changes to locomotor activity and stereotyped behaviour rats were placed in 58 x 45 x 60 cm (L × W × H) boxes and recorded with a camera 200 cm above the boxes. Four boxes were used simultaneously but the 60 cm barrier walls prevented rats from interacting with others being tested. Rats were placed in the boxes for a 30-minute habituation period before being injected subcutaneously with a 5 mg/kg dose of PCP and returned to the same box for a further 90 minutes. Ethovision software was used to track distance travelled and velocity.

3.2.7. Statistical analysis

As outlined in Chapter 2.2.5 data were analysed with traditional null-hypothesis significance testing and, where a null finding would be theoretically informative (i.e. evidence for a lack of a difference across genotypes), Bayesian statistics. For traditional null-hypothesis significance testing p< 0.05 was considered statistically significant. For ANOVA analysis where Maunchly's test indicated sphericity was violated Greenhouse-Geisser corrected values are reported. For all analyses that included sex as a factor interactions that included sex were non-significant these can be seen in the Appendix.

3.3. Results

3.3.1. Behaviour on anxiety tests

Figure 11 shows measures from the EPM for time the open arms (11A), head-dips (11B), stretch-attend postures (11C), grooming (11D), distance travelled (11E), velocity (11F) and defecation (11G). There were no genotype differences in the proportion of time spent in the open arms: non-significant main effect of genotype (F (1, 33) = 0.053, p = 0.819, n^2_p = 0.002; $BF_{exclusion}$ = 4.124). There were also no genotype effects in ethological measures including head-dips (F(1,33) = 0.034, p = 0.854, n^2p = 0.001; $BF_{exclusion}$ = 4.266), stretch-attend

postures ($F(1,33) = 0.190$, $p = 0.666$, $n^2p = 0.006$; $BF_{exclusion} = 3.528$), grooming ($F(1,33) = 0.002$, $p = 0.961$, $n^2p = 0.000$; $BF_{exclusion} = 3.816$) or defecation ($F(1,39) = 1.505$, $p = 0.227$, $n^2p = 0.039$; $BF_{exclusion} = 2.471$). There were also no genotype effects in distance travelled ($F(1,33) = 0.082$, $p = 0.776$, $n^2p = 0.002$; $BF_{exclusion} = 4.182$) or velocity ($F(1,33) = 0.082$, $p = 0.776$, $n^2p = 0.002$; $BF_{exclusion} = 4.151$). These findings indicate that $Dlg2^{+/-}$ rats do not appear to have an anxiety phenotype in the EPM.
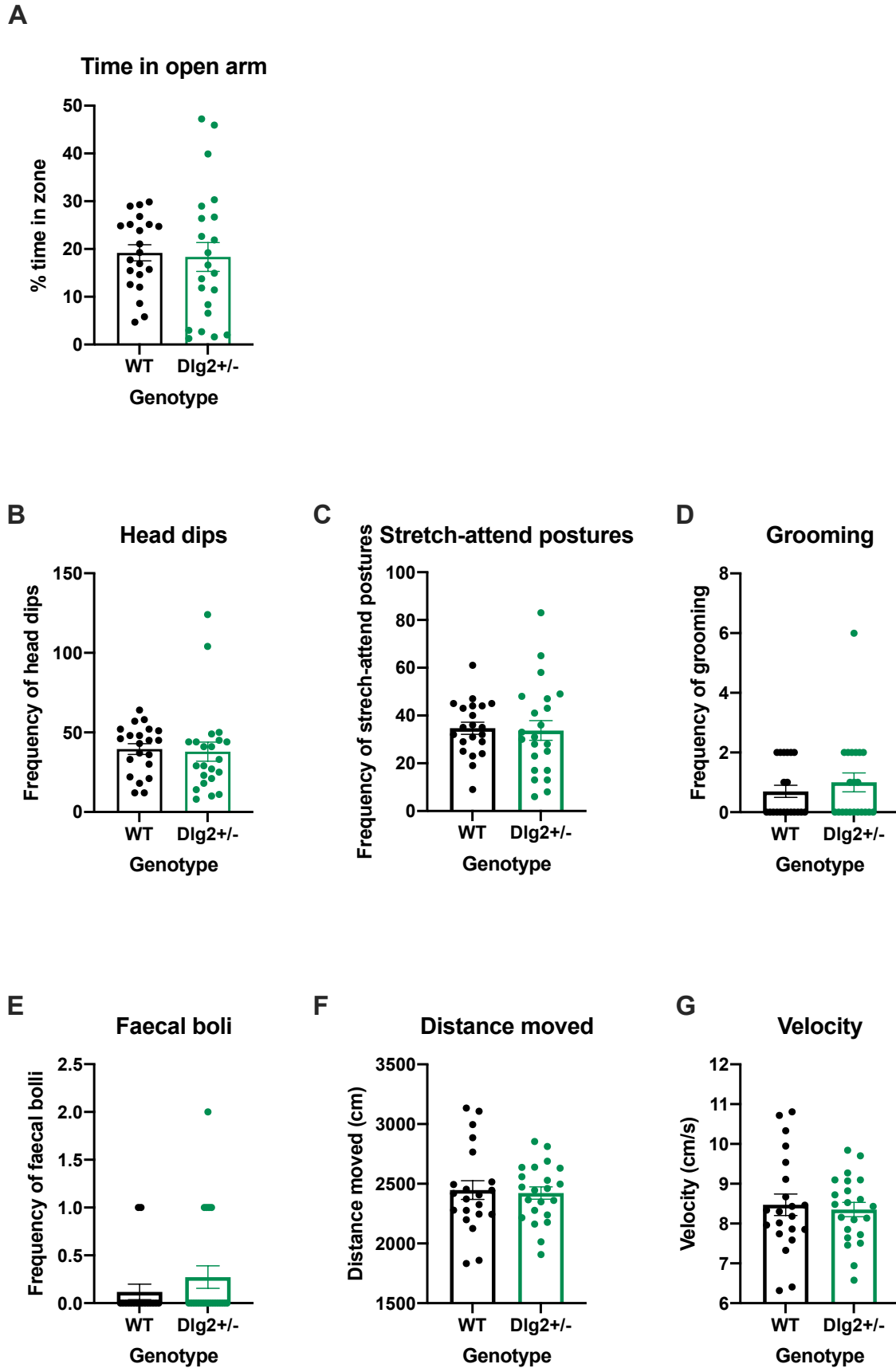
Figure 11: Effect of *Dlg2* heterozygous knockout on anxiety-related behaviour in the EPM.

Mean ± SEM with data points representing individuals **A)** time in zone **B)** head dips **C)** stretch-attend postures **D)** grooming **E)** defecation **F)** distance moved **G)** velocity.

Figure 12 shows the results from the open field for time in the centre and outer zones (12A), velocity (12B), distance moved (12C), and defecation (12D). While there was a general tendency to avoid the central area, there were no genotype differences in the proportion of time spent in centre and outer zones: main effect of zone ($F(1,38) = 9100.383$, $p = < 0.001$, $n^2_p = 0.996$) but no genotype main effect (($F(1, 38) = 0.697$, $p = 0.409$, $n^2_p = 0.018$; $BF_{exclusion} = 7.292$) or zone × genotype interaction ($F(1,38) = 0.232$, $p = 0.633$, $n^2_p = 0.000$; $BF_{exclusion} = 6.064$). Similarly there were no genotype differences in either distance travelled ($F(1,38) = 0.002$, $p = 0.961$, $n^2_p < 0.001$; $BF_{exclusion} = 3.551$) or velocity ($F(1,38) = 0.013$, $p = 0.908$, $n^2_p < 0.001$; $BF_{exclusion} = 3.753$). While there was a suggestion that *Dlg2$^{+/-}$* rats defecated numerically more than WT controls, there was no significant genotype effect ($F(1,38) = 3.769$, $p = 0.060$, $n^2_p = 0.090$; $BF_{exclusion} = 0.880$). Although the BF was inconclusive here, it should be remembered that there was no suggestion of a genotype effect on defecation in the EPM.

Overall while the experiment demonstrated anxiety generally, with the rats avoiding the aversive open central field section, there were no genotype effects on this nor any other measure in the open field.
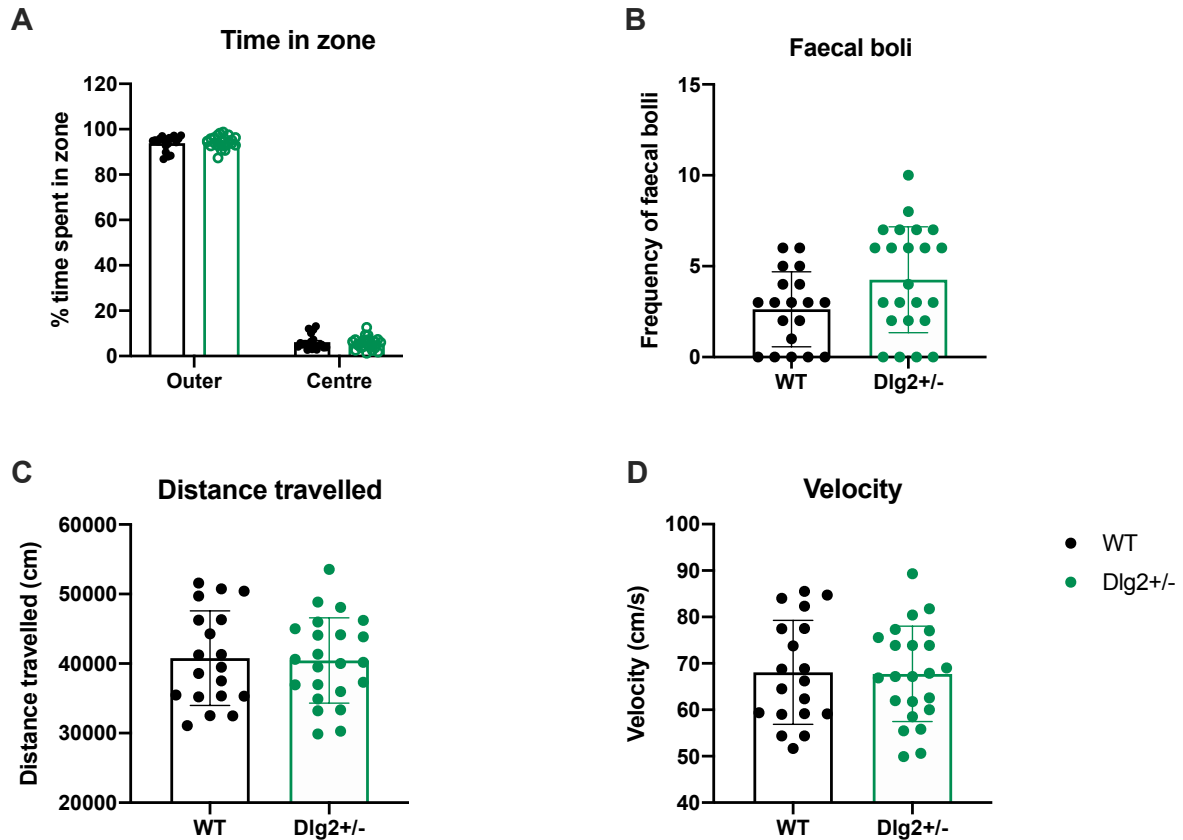
Figure 12: Effect of *Dlg2* heterozygous knockout on open-field measures A) time in zone **B)** defection **C)** distance travelled and **D)** velocity. Mean ± SEM plus individual values.

3.3.2. Sensorimotor gating

Weight-adjusted acoustic startle response to increasing auditory pulses (Figure 13A) was analysed using mixed ANOVA and mixed Bayesian ANOVA with within-subjects factors of pulse (70, 80, 90, 100, 110 and 120 dB) and between-subjects factors of sex and genotype (sex effects and interactions are reported in the supplementary materials). There was a significant main effect of pulse (F(1.089, 43.542) = 29.705, $p < 0.001$, $n^2{}_p$ = 0.426) indicating an increased magnitude of startle response at higher pulse intensities. There was no significant main effect of genotype (F(1, 40) = 0.872, p = 0.356, $n^2{}_p$ = 0.021; $BF_{exclusion}$ = 8.964), nor a genotype × pulse interaction (F(1.089, 43.542) = 0.590, p = 0.460, $n^2{}_p$ = 0.015; $BF_{exclusion}$ = 22.407). Thus startle to increasing auditory stimuli proceeded in the same way for HET and WT animals.

Habituation of startle response was assessed at 120 dB (Figure 13B) and 105 dB (Figure 13C). Mixed ANOVA and Bayesian mixed ANOVA were used to assess habituation to 105 dB and 120 dB pulse alone trials separately with within-subjects factors of trial number and between subjects factors of sex and genotype. At 120 dB rodents acoustic startle response habituated as trials progressed (main effect of trial: $F(2.130, 85.190) = 13.127$, $p < 0.001$, n^2_p = 0.247) but there were no genotype differences in this (non-significant genotype × trial interactions: ($F(2.130, 85.190) = 0.498$, $p = 0.621$, $n^2_p = 0.012$; $BF_{exclusion}$ = 643.328 or main effect of genotype ($F(1, 40) = 0.347$, $p = 0.559$, $n^2_p = 0.009$; $BF_{exclusion}$ = 10.545). This remained the same at 105 dB (main effect of trail: ($F(1.573, 62.934) = 6.058$, $p = 0.007$, n^2_p = 0.132, non-significant genotype × trial interactions: ($F(1.573, 62.934) = 0.519$, $p = 0.555$, n^2_p = 0.013; $BF_{exclusion}$ = 127.064 and genotype main effects ($F((1, 40) = 1.497$, $p = 0.228$, n^2_p = 0.036; $BF_{exclusion}$ = 6.416).

There were also no genotype effects on pre-pulse inhibition at 120 dB (Figure 13D) or 105 dB (Figure 13E). Repeated measures ANOVA and Bayesian repeated measures ANOVA with factors of pre-pulse intensity (4, 8 and 16 dB above background) sex and genotype were used to assess PPI to 105 dB and 120 dB pulse trials separately. At 120 dB rodents demonstrated a greater inhibition of startle with increasing pre-pulse intensity (significant main effect of pre-pulse: $F(2, 80) = 83.401$, $p < 0.001$, $n^2_p = 0.676$) yet no significant genotype × pre-pulse interaction ($F(2, 80) = 1.097$, $p = 0.339$, $n^2_p = 0.027$; $BF_{exclusion}$ = 5.712 or genotype main effect ($F(2, 80) = 0.011$, $p = 0.917$, $n^2_p = 0.000$; $BF_{exclusion}$ = 5.482). As above these findings remained the same at 105 dB (main effect of pre-pulse: $F(1.380, 55.210)$ = 42.203, $p < 0.001$, $n^2_p = 0.513$, non-significant genotype × pre-pulse interactions: $F(1.380, 55.210) = 0.314$, $p = 0.650$, $n^2_p = 0.008$; $BF_{exclusion}$ = 3.713) and genotype main effects ($F(1, 40) = 0.696$, $p = 0.409$, $n^2_p = 0.017$; $BF_{exclusion}$ = 3.437).
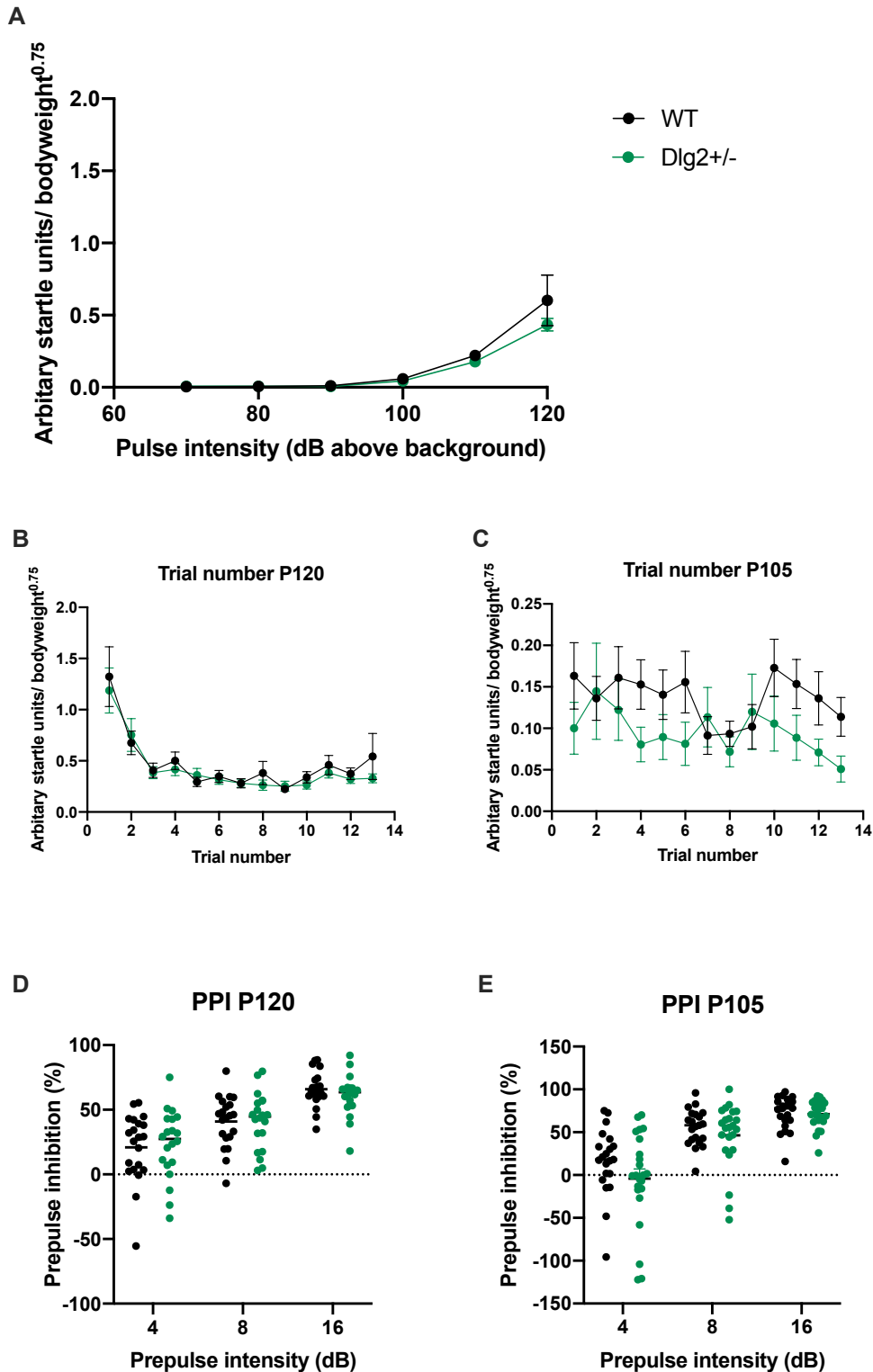
Figure 13: Effect of *Dlg2* heterozygosity on acoustic startle response and pre-pulse inhibition. A) Mean ± SEM weight-adjusted ASR to 70-120 dB pulses above background. **B)** Habituation of startle response through increasing pulse trials. Mean ± SEM ASR shown at 120 dB and **C)** 105 dB. **D)** Mean ± SEM PPI plus individual values by a 4, 8 and 16 dB (above background) pre-pulse on 120 dB pulse and **E)** 105 dB pulse.

3.3.3. Flavour habituation

Consumption of 0.1 and 0.4% saccharin for both genotype groups is shown in Figure 14A. Consumption was analysed by between-subjects ANOVA and Bayesian ANOVA with factors of day (1-12), genotype and flavour. Between-subjects ANOVA revealed a significant main effect of day ($F(6.164, 197.50) = 3.146$, p = 0.005, n2p = 0.067), as consumption varied by day for both concentrations of saccharin. The flavour × day interaction was also significant ($F(6.164, 185.43) = 2.954$, $p = 0.008$, $n^2_p = 0.063$) demonstrating different habituation patterns to the different saccharin concentrations. The day × genotype interaction was non-significant ($F(6.164, 83.24) = 1.326$, $p = 0.244$, $n^2_p = 0.029$; $BF_{exclusion} = 8.962$), while there was a trend towards a day × genotype × flavour interaction ($F(6.164, 124.00) = 1.975$, $p = 0.067$, $n^2_p = 0.043$; $BF_{exclusion} = 2.604$). Main effects of genotype ($F(1, 81.45) = 0.804$, $p = 0.375$, $n^2_p = 0.018$; $BF_{exclusion} = 3.149$) and flavour ($F(1, 122.82) = 1.212$, $p = 0.277$, $n^2_p = 0.027$; $BF_{exclusion} = 0.032$) were non-significant, as was the genotype × flavour interaction ($F(1, 164.33) = 1.622$, $p = 0.210$, $n^2_p = 0.036$ $BF_{exclusion} = 1.792$).
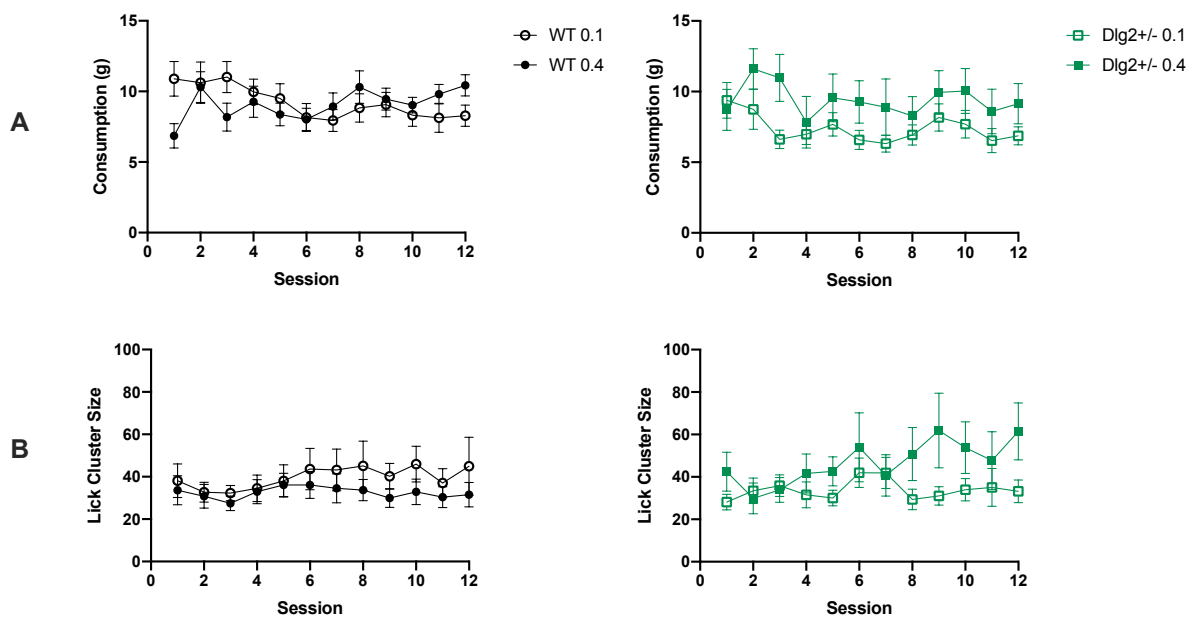
Figure 14: Effect of *Dlg2* haploinsufficiency on flavour habituation. Mean (± SEM) consumption **A)** and lick cluster size **B)** for *Dlg2+/-* and wild-type rats by day across 12 training days.

Changes to lick cluster size to saccharin concentrations are shown in Figure 14B. Between subjects ANOVA and Bayesian ANOVA with factors of day, genotype and flavour revealed a significant main effect of day ($F(5.004, 1600.9) = 2.285$, $p = 0.047$, $n^2_p = 0.049$), as rats habituated to flavours and neophobia diminished lick cluster size changed. All other main effects and interactions were non-significant, including day × flavour ($F(5.004, 2609) = 0.744$, $p = 0.591$, $n^2_p = 0.017$; $BF_{exclusion} > 1000$), and day × genotype × flavour ($F(5.004, 6562) = 1.871$, $p = 0.100$, $n^2_p = 0.041$; $BF_{exclusion} > 1000$) interactions in addition to day × genotype ($F(5.004, 2149) = 0.613$, $p = 0.690$, $n^2_p = 0.014$; $BF_{exclusion} > 1000$), genotype ($F(1, 44) = 0.479$, $p = 0.492$, $n^2_p = 0.011$; $BF_{exclusion} = 5.853$), flavour ($F(1, 44) = 0.231$, $p = 0.633$, $n^2_p = 0.005$; $BF_{exclusion} = 6.664$), and genotype × flavour ($F(1, 44) = 2.759$, $p = 0.104$, $n^2_p = 0.059$; $BF_{exclusion} = 6.524$), effects. Thus, in this experiment lick cluster size did not show the expected increase as rats habituated to either flavour, and there were no genotype effects present on lick cluster size in this experiment. The lack of expected habituation shown in consumption and particularly lick cluster size makes genotype comparisons difficult, although there is no evidence to suggest that $Dlg2^{+/-}$ rats responded differently to flavours across days than wild-types.

3.3.4. Social preference and social novelty

Raw exploration times for conspecific and object in the social preference test are shown in Figure 15A. These data were analysed by mixed model ANOVA and Bayesian ANOVA with the within-subject factor of item (conspecific, object) and between-subjects factors of sex and genotype. The conspecific was explored more than the object (significant main effect of item:($F(1, 50) = 76.012$, $p < 0.001$, $n^2p = 0.603$) however this did not differ with genotype (non-significant item × genotype interaction, $F(1,50) = 0.479$, $p = 0.492$, $n^2p = 0.009$; $BF_{exclusion} = 7.678$ or genotype main effect $F(1, 50) = 0.014$, $p = 0.907$, $n^2p = 0.000279$; $BF_{exclusion} = 7.986$).

The discrimination ratio for social preference is show in in Figure 15B. This was significantly different from 0 for the entire cohort (one-sample t-test $t(53) = 9.988$, $p < 0.001$, $d = 1.359$) reflecting the tendency to explore the conspecific more than the object. There were no

genotype differences in discrimination ratio: non-significant main effect of genotype ($F(1, 50) = 0.850$, $p = 0.361$, $n^2p = 0.017$; $BF_{exclusion} = 4.973$). This reflects the fact that rats explored the conspecific more than the object as expected, yet *Dlg2* haploinsufficiency has no influence on this tendency.

Raw exploration times for the familiar rat (Stranger 1) and the novel rat (Stranger 2) can be seen in Figure 15C. These data were analysed by mixed model ANOVA and Bayesian ANOVA with the within-subject factor of item (Stranger 1, Stranger 2) and between-subjects factors of sex and genotype. The expected difference in exploration based on familiarity was not seen (non-significant main effect of item ($F(1, 50) = 0.960$, $p = 0.332$, $n^2p = 0.019$); $BF_{exclusion}$ = 6.978). The item × genotype interaction was also non-significant ($F(1, 50) = 0.127$, $p = 0.723$, $n^2p = 0.003$; $BF_{exclusion} = 20.094$) as were genotype main effects ($F(1, 50) = 0.206$, $p = 0.652$, $n^2p = 0.004$; $BF_{exclusion} = 6.728$).

The discrimination ratio for the social novelty test is shown in Figure 15D. Discrimination ratio for social novelty was not significantly different from 0 (one-sample t-test $t(53) = 0.644$, $p = 0.522$, $d = 0.088$), meaning that rats did not show the expected preference for exploring the familiar rodent. There were no genotype differences in discrimination ratio: non-significant main effect of genotype ($F(1, 50) = 0.329$, $p = 0.569$, $n^2p = 0.007$; $BF_{exclusion} = 3.882$). It is impossible to draw conclusions concerning the effect of genotype on social novelty in the absence of the expected preference to explore the familiar rat in wild-type controls.
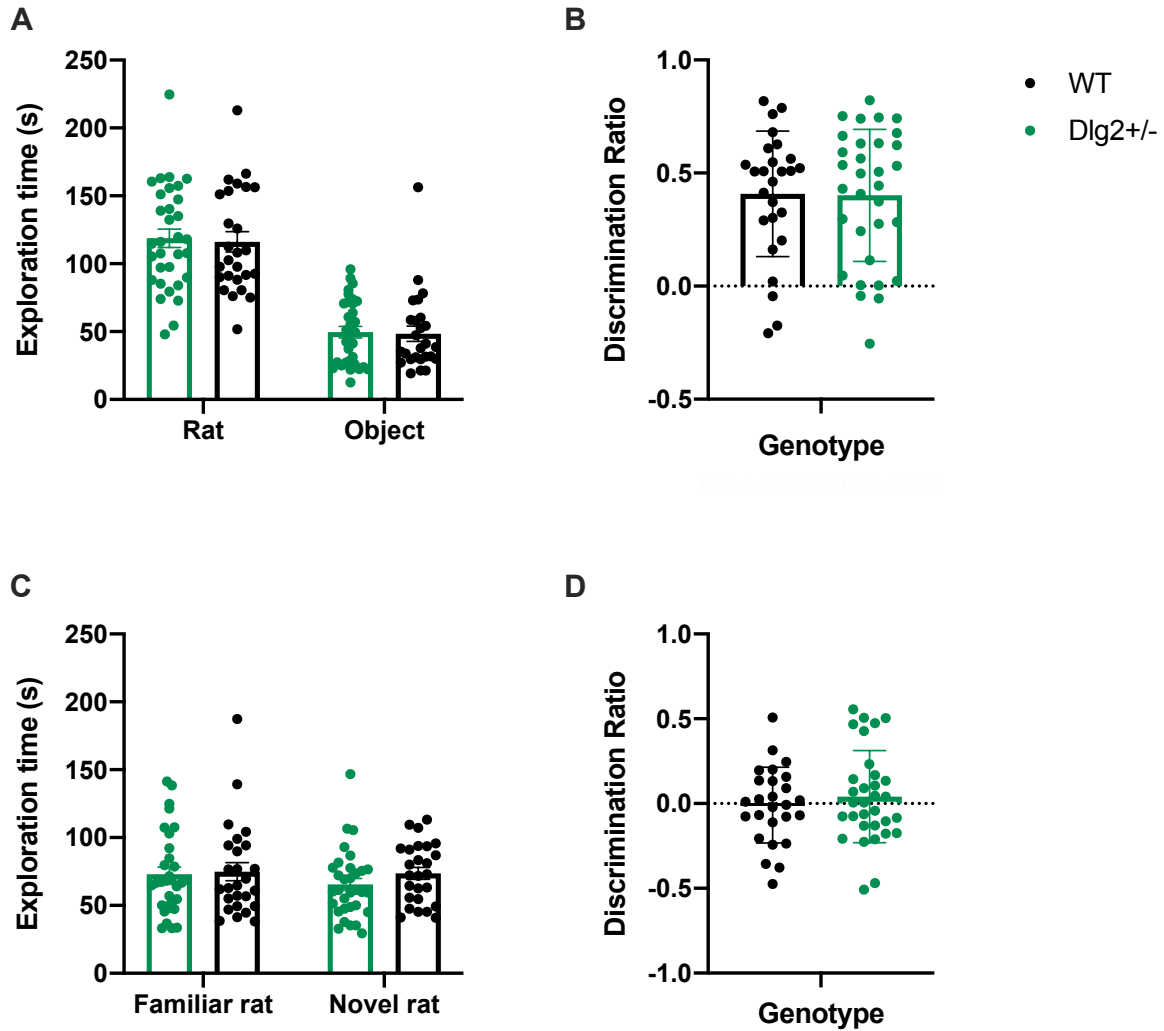
Figure 15: *Dlg2^+/-* and wild-type exploration times on the social preference and social novelty tasks. Mean and SEM ± plus individual values **A)** social preference raw exploration, **B)** social preference d2 discrimination ratio, **C)** social novelty raw exploration and **D)** social novelty d2 discrimination ratio.

3.3.5. PCP-induced locomotion

Figure 16 shows the distance travelled in the arena over the 120-minute test period (30 mins preceding PCP administration and the following 90 mins) for wild-type and *Dlg2^+/-* rats. Distance travelled was analysed using repeated measures ANOVA with the repeated measures factor of time bin (12 × 10-minute bins covering the 120-minute test period) and between-subjects factors of sex and genotype. There was a main effect of time bin (*F*(2.388,

107.477) = 7.446, $p < 0.001$, n^2p = 0.142) as activity varied through rats initial habituation and locomotor activity increased following drug injection. There was a significant time bin × genotype interaction (F(2.388, 107.477) = 5.949, $p = 0.002$, n^2p = 0.117) and main effect of genotype (F(1,45) = 8.074, $p = 0.007$, n^2p = 0.152). Follow up analyses of the time bin by genotype interaction indicated that the $Dlg2^{+/-}$ animals were more active than wild-types on time bin 70 and onwards (smallest t(56) = 2.466, $p = 0.017$, $d = 0.651$) but not on time bin 60 and before (largest t(56) = 1.738, $p = 0.088$, $d = 0.459$).
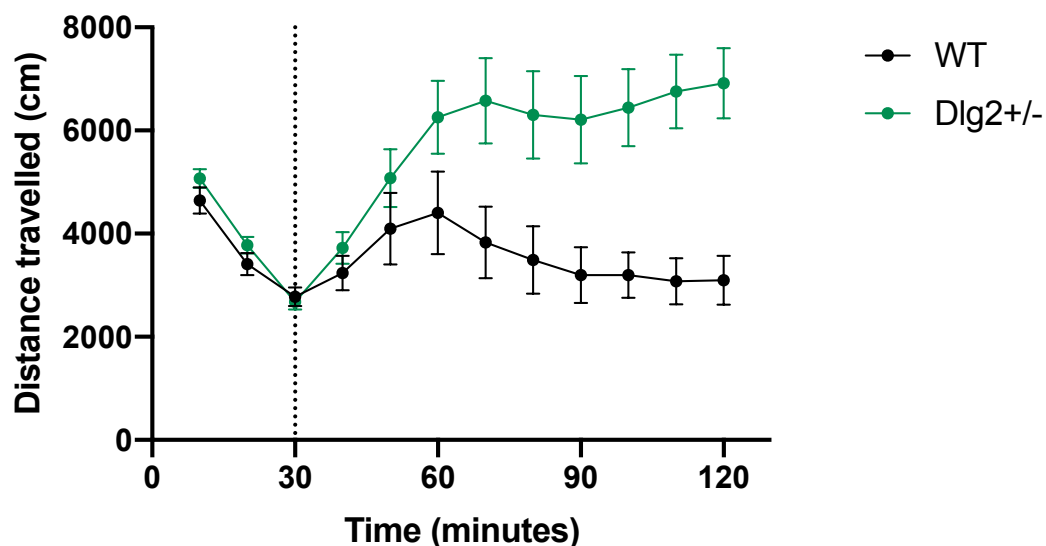
Figure 16: Locomotor activity in response to PCP injection in $Dlg2^{+/-}$ and wild-type rats. Mean ± SEM distance travelled is plotted in 10-minute bins. The dotted line at 30 minutes denotes when the PCP injection occurred.

3.4. Discussion

In this Chapter the consequences of $Dlg2$ haploinsufficiency for anxiety, sensorimotor gating, long-term habituation, social behaviour, and locomotor response to the NMDAR-antagonist PCP were assessed. These tests are widely used with rodents characterise a broad range of disorder-relevant phenotypes, giving an initial screening of basic processes impaired and spared in the new rodent model. It was found that $Dlg2^{+/-}$ rats performed comparably to wild-types on assays of anxiety, sensorimotor gating, long-term habituation,

and social behaviour. This is useful background for further experimentation on the *Dlg2⁺/⁻* model as it means further cognitive and reward processing tasks will not be confounded by differences in anxiety, hearing or responding to stimuli. When locomotor response to PCP challenge was assessed, *Dlg2⁺/⁻* rats demonstrated a potentiated response to the drug. This demonstrates the first behavioural correlate of *Dlg2* heterozygosity and is in line with electrophysiological data demonstrating a change in NMDAR function in *Dlg2* heterozygous rats (Griesius et al., 2022).

These findings have implications for other biologically relevant rodent models, including NMDAR dysfunction models and *Dlg2* knockouts (-/-). Comparisons between complete and heterozygous gene knockout models are important as it allows a distinction to be made between knowledge about the function of a protein and compensative processes and processes which require complete PSD-93 protein levels. As heterozygous models better describe genetic alteration in psychiatric disorders where there are dosage changes rather than the absence of the gene, drawing conclusions from these models can better describe whether a psychiatric endophenotype is dependent on a particular genetic disruption found in patients.

On the open field *Dlg2⁺/⁻* rats did not show evidence of anxiety or changes in locomotor behaviour in response to novelty. There were likely ceiling effects with rats of both genotypes spending > 90% of their time in the peripheral zone. To decrease anxiety in the wild-types and allow a better genotype comparison, light levels in the room could be reduced to decrease fear and encourage exploration. Despite these effects when using time in open field zone to draw conclusions about anxiety all EPM measures demonstrated that *Dlg2⁺/⁻* rats did not show differential anxiety responses to wild-types.

This contrasts with alterations in performance on the open field have been reported in *Dlg2⁻/⁻* mice (Winkler et al 2018; Yoo et al 2020). There is no evidence of hypoactivity to novel environments in the *Dlg2⁺/⁻* rats tested in the open field. The difference in findings in comparison to *Dlg2⁻/⁻* models could show that this is a behaviour resulting from complete ablation of PSD-93 which is rescued by the presence of some protein in heterozygous models. Yet Winkler's (2018) finding of hypoactivity in heterozygotes implies that it may be

a phenotype of relevance across all levels of *Dlg2* disruption. Differences in model generation and species could also contribute to this discrepancy. Task differences are also important, as placing the mouse in the centre of the open field in Winkler et al (2018) could produce an initial fear response that may have influenced subsequent behaviour in the task. Further delineation of the hypoactivity response in *Dlg2* models is required.

Hypoactivity to novel environments may be due to a failure to habituate to stimuli. This was directly tested here in both long- and short-term forms via the flavour habituation experiment and habituation to an auditory pulse in the sensorimotor gating test. *Dlg2^+/-^* rats did not display deficits on either task, although this is more robust for the auditory short-term habituation than the flavour habituation where rats of both genotypes failed to show the expected habituation responses. Piloting flavour habituation to ensure that solution palatability is aversive to begin with, and thus can be habituated to, would be required to adequately assess genotype differences in long-term habituation. It is also possible to measure long-term habituation of the acoustic startle response (e.g. Groves et al., 1974; Jordan & Leaton, 1983; Ornitz et al., 1993) which may give a more straightforward procedure and easy comparison between long- and short-term habituation forms as stimulus modality would be consistent.

Here heterozygous knockout rats showed intact social behaviour, unlike homozygous knockout mice which demonstrated social preference impairment (Yoo et al., 2020). As with anxiety species differences and differences in model generation may also account for discrepancies in findings. A key concern is that Yoo et al (2020) single-housed mice prior to the social test while these rats were kept in home cages with other conspecifics. Therefore, the genotype difference might only manifest in response to social deprivation or when mutant rats are stressed by isolation.

If this discrepancy could not be explained by task differences it may be that having some functional PSD93 might 'rescue' the phenotype and might be sufficient to prevent social deficits. This could be done by PSD93 acting in tandem with PSD95. Previous work has found similarity in social deficits in *PSD95^+/-^* mice and *PSD93^-/-^* mice, with increased expression of PSD93 in the hippocampus of *PSD95^+/-^* mice implying that PSD93 is acting to compensate in

this mouse model (Winkler, Daher, Wüstefeld, Hammerschmidt, Poggi, Seelbach, Krueger-Burg, et al., 2018). In the *Dlg2+/-* rat this could work the other way with PSD95 and some amount of PSD93 being sufficient to give intact social preference performance. Together this may mean that while PSD93 has some role in social behaviours it is not so essential that the genetic haploinsufficiency seen in psychiatric disorders is sufficient to generate social deficits. It's worth noting however that social behaviour is undeniably more varied in humans than rodents, so although heterozygous knockout doesn't produce a marked social preference deficit in rats PSD93 reduction in humans may influence empathy, social cognition, and social motivation in more subtle ways.

Social novelty preference wasn't observed in *Dlg2+/-* or wild-type rats in the present experiments. This could be due to exploration of the conspecific in the social preference phase of the experiment being insufficient for rats to distinguish the two strangers in the subsequent social novelty phase. It's also worth noting that social preference and social novelty as tests to elucidate deficits in models of psychiatric disorder have been best elaborated with mice. Thus the exact constraints on this behaviour, including how long rats should explore the conspecific in the social preference phase to generate a familiarity effect in social novelty are not well described.

A further difference between this work on the heterozygous knockout in comparison to homozygous knockouts is the increase in compulsive grooming in a *Dlg2* knockdown mouse model (Yoo et al., 2020). This was not seen in the heterozygous rats tested here. To draw a firm conclusion from this *Dlg2+/-* rats would need to be tested in the same self-grooming test Yoo et al., (2020) used, where a rat is placed in a fresh home cage without bedding and recorded for 20 minutes of habituation time plus 10 minutes of test. For an easier comparison it would be interesting to know if Yoo et al., (2020) found any grooming differences between constitutive knockouts and wild types on the EPM and OFT which is not reported.

Sensorimotor gating is intact in the *Dlg2+/-* model with rodents showing normal PPI. This is a key departure from a characteristic phenotype seen in schizophrenia patients (Mena et al., 2016) and rodent models (Powell et al., 2009). However *Dlg2+/-* does not model

schizophrenia directly, and functional sensorimotor gating is present in other conditions *Dlg2* disruption associates with such as ADHD (Castellanos et al., 1996; Conzelmann et al., 2010; Feifel, Minassian, & Perry, 2009; Hanlon et al., 2012; Ornitz, Hanna, & de Traversay, 1992), high-functioning ASD (Kohl et al., 2014) and bipolar disorder (Barrett, Kelly, Watson, Bell, & King, 2005; Carroll, Vohs, O'donnell, Shekhar, & Hetrick, 2007). It has been proposed that PPI can be linked to specific neural systems and give evidence as to what is impaired in some disorders relative to others (Swerdlow, Braff, & Geyer, 2016). The ventral striatum/nucleus accumbens (NAC) is a critical structure in regulation of PPI (e.g. Bikovsky et al., 2016; Ma & Leung, 2016; Vadnie et al., 2016) including its interactions with the ventral pallidum and pedunculopontine nucleus via descending efferent projections (reviewed Swerdlow, Geyer, & Braff, 2001; Swerdlow, Weber, Qu, Light, & Braff, 2008). Intact NAC dopaminergic signalling is also critically involved (Swerdlow, Braff, Geyer, & Koob, 1986). Intact PPI in *Dlg2*$^{+/-}$ rats could indicate intact function of these neural systems.

A key phenotype observed in *Dlg2*$^{+/-}$ rats is sensitised locomotor response to the NMDAR-antagonist PCP, with prolonged and exaggerated locomotor responding compared to wild-types. This is a common phenotype in rodent models of psychotic disorders, including other CNV models (Didriksen, Skarsfeldt, & Arnt, 2007; Nielsen et al., 2017). However, in some cases sensitisation to NMDAR-antagonists manifests in different ways with locomotor activity being decreased or comparable to wild-types in the presence of increased stereotyped behaviour, ataxia and catalepsy in schizophrenia-relevant rodent models (e.g. Bygrave et al., 2016). Scoring these behaviours in *Dlg2*$^{+/-}$ and wild-type rodents in response to PCP would have been useful to fully characterise response to this psychostimulant.

Increased locomotion in response to PCP in *Dlg2*$^{+/-}$ rats aligns with the NMDAR deficit observed in heterozygotes (Griesius et al., 2022) to flag a potential glutamate abnormality in these rodents. Sensitisation of locomotor response to NMDAR-antagonists has been associated with similar neural systems to PPI, specifically relying on dopaminergic activity in the midbrain (Jones, Watson, & Fone, 2011; Wilson, Terry, & Jr., 2010). Evidence for this comes from the fact that systematically administered PCP increases the firing rate of dopaminergic neurons (Freeman & Bunney 1984, French et al 1993) and dopamine receptor antagonists including antipsychotics attenuate PCP-induced locomotion (Sturgeon et al

1981, Freed et al 1984). It could be that PCP hyperlocomotion changes in *Dlg2*[+/-]s indicate deficits in dopaminergic processes in this neural system not seen as baseline without NMDAR-antagonist challenge in PPI.

It could also be the case that of enhanced locomotor response to PCP and intact PPI points to potential deficits in components of complementary and overlapping neural systems. NMDAR antagonists increase extracellular glutamate levels in the prefrontal cortex and NAC (B. Adams & Moghaddam, 1998; Moghaddam & Adams, 1998) stimulating non-NMDA receptors to cause cellular activation and locomotion in a way separable to dopaminergic mechanisms (Chartoff, Heusner, & Palmiter, 2005). Supporting this genetically dopamine deficient mice exhibited PCP induced hyperlocomotion (Chartoff et al 2005), and the behavioural effects of PCP correlate more closely with glutamate release in the NAC and PFC than with dopamine release (Adams & Moghaddam, 1998). Dopamine release has been proposed to be an indirect effect of activated glutamate release in these structures (Adams & Moghaddam, 1998; Takahata & Moghaddam, 2003), and locomotor behaviour in dopamine deficit mice has been shown to be further sensitised in the presence of NMDAR antagonists by increasing dopamine with L-DOPA injections (Chartoff et al 2005). Therefore, the enhanced locomotor response to PCP in *Dlg2*^{+/-} rats may be due to corticostriatal glutamatergic signalling abnormalities, with or without concomitant dopamine sensitisation.

Rodent models with disruptions to NMDA signalling have been described to display phenotypes on most measures tested here including anxiety (Barkus et al., 2010), social preference (Zoicas & Kornhuber, 2019) and pre-pulse inhibition (Linderholm et al., 2010). The finding of no impairment on this *Dlg2*^{+/-} rat model, and the finding that *Dlg2*^{+/-} rats have comparable levels of in the NR1 NMDA receptor subunit to wild-types (Chapter 2), implies that the *Dlg2*^{+/-} model cannot be reduced to a model of NMDA receptor misfunction. The effects of PCP on these rats thus likely operate via glutamate signalling on non-NMDA receptors (including metabotropic glutamate receptors and AMPA receptors) as proposed by Chartoff et al (2005), or on some combination of these factors and dopaminergic processes which is not so deleterious to produce deficits on dopamine-influenced tasks such as PPI.

In conclusion *Dlg2*^{+/-} rats were found to perform comparably to wild-types on tests of anxiety (open field and elevated plus maze), sensorimotor gating, pre-pulse inhibition, across-day flavour habituation and social preference. They demonstrate an exaggerated locomotor response to PCP, a NMDAR antagonist, a finding which may highlight specific abnormalities in neurotransmission in specific brain circuits. Intact performance on many of the behavioural domains assessed here, such as anxiety and habituation, will remove these as confounds when continuing investigation into this model using more complex cognitive tasks (Chapter 4).

Summary

- On many well-established tasks assessing ethological responses to stimuli *Dlg2*^{+/-} rats performed comparably to wild-types. These included the elevated plus maze and open field tests of anxiety, sensorimotor gating, pre-pulse inhibition and social preference.

- *Dlg2*^{+/-} rats show an exaggerated locomotor response to acute PCP challenge, a phenotype seen across many psychiatric disorder rodent models including those of CNVs that increase risk for similar conditions to that at 11q14.1 (Didriksen et al., 2017; Nielsen et al., 2017).

Chapter 4

Memory function in *Dlg2*$^{+/-}$ and wild-type rats

4.1. Introduction

4.1.1. Cognitive symptoms of *Dlg2* associated psychiatric disorders

One of the many burdens of the psychiatric disorders *DLG2* CNVs are associated with are described as 'cognitive symptoms.' These refer to difficulties in memory, self-regulatory, linguistic, attentional, and problem-solving capacities. Impairments in this collection of cognitive processes has been proposed to significantly determine functional outcome for patients (Michael Foster Green, 1996; Marder, 2006). This is concerning in light of the finding that medication which can treat other symptoms, such as depression or delusions, has no therapeutic impact on cognitive symptoms and in some cases worsens them (Gore et al., 2011; Lee et al., 2014).

Across the spectrum of cognition different disorders show patterns of impairment that are both diagnostically unique and overlapping (for review see Millan et al., 2016). Caveats in describing exactly what is impaired and spared across disorder categories come from the methods used to test for impairment, ranging from questionnaires to psychometric tests and lab-based behavioural tasks. This chapter will focus on memory impairments in *DLG2*-relevant psychiatric disorders as there are established translational tests for this cognitive domain which were carried out on *Dlg2*$^{+/-}$ and wild-type rats.

Problems with working memory, the 'online' short-term memory ability to manipulate and use information, are characteristic of autism, intellectual disability and schizophrenia all of which have a demonstrated genetic link to *Dlg2* disruption (Habib, Harris, Pollick, & Melville, 2019a; Numminen et al., 2000; Park & Gooding, 2014). This is commonly assessed by psychometric tests such as the Maryland Letter-Number Span Task (digit span) and the Weschler Memory Scale-III Spatial Span Task. Using digit span is the most established working memory test in humans, yet its reliant on linguistics impairs comparison with tests

assessing working memory in animal models. The spatial span task has been seen as a less reliable working memory task in humans due to the recognition component (Wilde, Strauss, & Tulsky, 2004) yet others have argued that it is exactly this which makes it suitable for cross-species comparison (Dudchenko, 2004). There is evidence that humans with intellectual disability, autism and schizophrenia show deficiencies on the spatial span task (Chey, Lee, Kim, Kwon, & Shin, 2002; Habib, Harris, Pollick, & Melville, 2019b; Saeed, 2016).

Where declarative long-term memory is concerned impairments show differing patterns across disorders. Declarative memory is subdivided into episodic and semantic memory. Episodic memory, the conscious recollection of what happened, where and when involving mental time travel to a past event or into an imagined future (Tulving, 2002) shows abnormalities in schizophrenia and autism. In schizophrenia patients this evident on laboratory-based recall and recognition tasks for words and images experienced prior to a set delay (Danion, Huron, Vidailhet, & Berna, 2007). In autism this has been investigated using cued and free recall of 'table top tasks' (tasks which constitute short scenarios happening in front of the participant such as deforming plasticene or viewing drawings) previously completed by participants (Hare, Mellor, & Azmi, 2007).

Semantic memory involves the learning and storing of facts, information and ideas disconnected from a single event or occurrence. Although there is much within-disorder heterogeneity semantic memory is not deficit in autism (Gaigg, Bowler, & Gardiner, 2014), with many parental reports on children with autism noting exaggerated proficiency in this sphere (i.e. a child knowing extensive facts about a narrow topic). Schizophrenia patients show semantic memory impairments as assessed by naming, word-picture matching, verbal fluency and categorisation (Millan et al., 2016). However in both disorders it has been difficult to separate degradation of semantic knowledge from executive function impairments in accessing the correct information and inhibiting that which is not relevant (Doughty & Done, 2009). Indeed across all memory domains tasks which assess one function often require other cognitive processes (Testa & Pantelis, 2009).

To overcome problems caused by the overlap in tests which are prescribed to different 'cognitive symptom' domains there have been certain efforts to delineate the exact nature

of cognitive impairment and reach a consensus view on specific procedures used to evaluate cognitive deficits. This has been carried out for schizophrenia with the MATRICS initiative driven by the National Institute of Mental Health, academia and industry (Marder, 2006). Keeping things simple, testable and translational has marked a departure from linkage of deficits to constructs such as 'episodic' or 'semantic' memory types.

One of the domains identified by MATRICS was visual learning and memory. In the endorsed clinical test battery this domain is assessed by the Brief Visual Memory Test – Revised (BVMT-R, Benedict, Groninger, Schretlen, Dobraski, & Shpritz, 1996; Benedict & Groninger, 1995). On this test schizophrenia patients show deficits in immediate recall, delayed recall and recognition components (Schretlen et al., 2007), however in individuals with autism spectrum disorders visual memory performance was related to general intellectual ability and not disorder specific (Salmanian, Tehrani-Doost, Ghanbari-Motlagh, & Shahrivar, 2012). Young et al (2009) have suggested ways that this domain can be tested in animal models, which has shaped the choice of behavioural tests used in the present research.

Testing of potential pharmacological compounds to ameliorate or prevent cognitive decline in schizophrenia that reduce apparently related phenotypes in animal models of disorder have often failed to show efficacy in patients (Barch, 2010; Buchanan et al., 2011). A mechanism for dealing with the poor predictive validity of drug trials using animal models is to move research away from animal models 'of' a particular disorder and towards the study of animal models for particular symptom domains or biomarkers (Young, Powell, Risbrough, Marston, & Geyer, 2009). The value in testing *Dlg2*^{+/-} rats for any cognitive impairment is that if deficits are found they will be related to a very specific biological mechanism. Given the association of *Dlg2* with disorders that have a specific mnemonic deficit, and evidence of reduced hippocampal LTP in the rat line (Griesius et al., 2022), such impairments in the *Dlg2*^{+/-} rat line were anticipated.

4.1.2. Reference memory in the Morris water maze

One of the most established tasks of spatial memory acquisition and retention in rodents is reference memory in the water maze (Vorhees & Williams, 2006). It has been consistently

applied to test for memory deficits in rodent models of psychiatric disorder (e.g. Hou et al., 2018; Sircar, 2003) and is proposed to align with the MATRICS domain of visual learning and memory (Young et al., 2009). In the water maze reference memory task rodents are placed in an arena filled with opaque water in which there is a platform hidden just below the water's surface. Over successive trials and days the rat is placed at different locations around the maze edge and must learn the platform location to escape the water. Acquisition of spatial memory is assessed as decreases in time taken and distance swum to find the platform, and retention of platform location memory assessed by spending time searching in the relevant location when the platform is not present (Morris, 1984).

The reference memory task requires the rat to use allocentric (surrounding extra-maze) cues to learn the platform location rather than egocentric (body movement) cues. Analogous tasks have been generated for human patients such as navigation through virtual mazes in a multimodal environment (Hanlon et al., 2006; Ku et al., 2003; Kurtz, Baker, Pearlson, & Astur, 2007; Weniger & Irle, 2008), performance on which has been found to differentiate individuals diagnosed with schizophrenia from controls and correlate with some clinical symptoms, giving the task translational utility (Sorkin, Weinshall, Modai, & Peled, 2006).

Another advantage of this task is that specific physiological and neurochemical substrates required for effective performance have been elaborated. Lesion studies in rodents have recurrently highlighted the essential role of the hippocampus, particularly subfields CA1 and CA3 in performing the task (Moser, Moser, & Andersen, 1993; Stubley-Weatherly, Harding, & Wright, 1996). The glutamatergic system is critically involved with NMDA antagonists such as sub chronic administration of PCP and MDMA disrupting acquisition (Podhorna & Didriksen, 2005a; Steckler et al., 2005; Wass et al., 2006). Importantly sub chronic PCP (7 day administration with 24h washout) produced spatial learning and memory deficits when the platform was hidden but not when it was visible, demonstrating that the NMDA antagonist effect in this case was not due to general effects on sensorimotor function (Beraki, Kuzmin, Tai, & Ögren, 2008). NMDA antagonists have not been shown to effect post-training consolidation (Day & Langston, 2006) while AMPA antagonists disrupt

consolidation at this stage (Riedel et al., 1999) pointing to partially separable roles for AMPAR and NMDAR related processes.

Given the outlined impairment of hippocampal NMDAR-related LTP in *Dlg2*^{+/-} rats (Griesius et al., 2022) and the involvement of *Dlg2* in AMPAR and NMDAR signalling it was predicted that heterozygous knockouts would show impaired reference memory acquisition relative to wild-types.

4.1.3. Object recognition tasks

As with reference memory, harnessing rodents preference for novelty to assess object recognition comprises a series of establish tests of learning and memory often applied to rodent models of psychiatric disorder (e.g. Rajagopal, Massey, Huang, Oyamada, & Meltzer, 2014). Certain variations have also been proposed to align with the MATRICS visual learning and memory domain (Young et al., 2009). Four tasks assessing object recognition were carried out with *Dlg2*^{+/-} and wild-type rats, each to assess the capabilities of a different type of learning and memory. These tasks were novel object recognition, object location, object-in-place and the temporal order task shown schematically in Figure 17.

Novel object recognition (NOR) has been used most extensively to characterise animal models or disorder (Grayson et al., 2015). The test consists of two phases. In the sample phase the rodent is presented with two duplicates of a novel object (A, A) in a familiar walled arena. The rodent is then removed from the arena for a delay and when they are re-introduced they are presented with another copy of the now-familiar object (A) and a novel object (B) in this test phase. Rodents which recognise the previously exposed object are expected to spend longer exploring the novel object. As the positions of the object are unchanged between test and sample phases location cues should be redundant. In terms of its clinical validity schizophrenia patients show deficits on 2D object recognition tasks similar to NOR (André Aleman, Hijman, De Haan, & Kahn, 1999; Clare, McKenna, Mortimer, & Baddeley, 1993; Heckers et al., 2000; Tek et al., 2002) as do rodent models of schizophrenia (Powell & Miyakawa, 2006) and autism (Grayson et al., 2015).

Object location (OL) applies the same logic of NOR to test a rodent's learning and memory of spatial information. In the sample phase rats are presented with two objects (A, A) placed apart in the arena as in Figure 17B. For the test duplicates of the sample objects are presented, yet one has changed location by moving to a previously unoccupied corner (A, A). Healthy rodents are expected to show knowledge of this change by exploring the displaced object more.

In the object-in-place (OIP) and temporal order (TOT) tasks recognition of change between sample and test phases requires that subjects combine spatial and non-spatial information into what-where and what-when paired associates. In the OIP sample phase the rat freely explores the arena containing four different objects (A, B, C, D), each one in a different corner. Then, after a delay, in the test phase two of the objects have been swapped as in Figure 17C. Healthy rats explore the swapped pair more than the static pair reflecting their ability to learn object-location pairings (Aggleton & Nelson, 2020). In the TOT pairs of objects are experienced sequentially over two samples (i.e. A, A then B, B), and in the test phase when four objects are presented together rats should spend more time exploring those seen at the more distant time point (i.e. A, A) reflecting understanding of when objects were experienced (Dix & Aggleton, 1999).

Unlike OL or NOR, in the OIP and TOT test phases no individual object or location is novel, and thus contrasting sample and test phases is essential to understanding changes that have occurred between them. This increases task demands and aligns more closely with the 'what-where-when' notion of episodic memory (Dix & Aggleton, 1999). Overall, these tests comprise a battery which builds from object or location memory to more complex use and integration of this information, allowing assessment of gross to subtle behavioural impairments resulting from genetic disruption to a rodent's cognitive capabilities.

This test battery has added appeal as performance on each test has been associated with differing but related brain regions. Lesion evidence suggests that the perirhinal cortex, rather than the hippocampus, is responsible for successful NOR performance (Winters, Forwood, Cowell, Saksida, & Bussey, 2004). Impairment of NOR in perirhinal lesioned rats is thought to be principally a visual recognition deficit as lesioned rats do not show NOR

impairments in the dark (Albasser et al., 2013). Opposingly in OL perirhinal lesions spare performance (Barker, Bird, Alexander, & Warburton, 2007) while hippocampal lesions produce deficits (Barker & Warburton, 2011; Mumby, Gaskin, Glenn, Schramek, & Lehmann, 2002) resulting in a spatial/ non-spatial double dissociation.

As would be expected by the combination of information required lesions to either the hippocampus or perirhinal cortex impair OIP and TOT (Barker & Warburton, 2011). In line with the increased task demands additional brain sites are recruited, with lesions to the medial prefrontal cortex (mPFC) consistently blocking OIP performance yet sparing NOR and OL (Barker et al., 2007; Barker & Warburton, 2015; Warburton & Brown, 2015) Disconnection studies have shown that during OIP and TOT the mPFC functions in cooperation with the hippocampus and perirhinal cortex (Barker & Warburton, 2015; Warburton & Brown, 2015), and that at least for the OIP test glutamatergic transmission involving AMPA and NMDA receptors between these three brain regions is crucial (Warburton, Barker, & Brown, 2013).

In sum not only will a battery of NOR, OL, OIP and TOT screen $Dlg2^{+/-}$ rats for impairments relating to object, spatial or associative recognition memory any pattern of impairment would indicate a possible set of spared and impaired neural substrates. Given the dysfunction in hippocampal LTP and NMDAR related processes (Griesius et al., 2022) it was predicted that $Dlg2^{+/-}$ rats would be impaired on the OL, OIP and TOT tasks.

4.1.4. Spontaneous alternation in the Y-maze

When rodents are placed in a Y shaped arena, they will naturally alternate in their exploration of different maze arms (labelled A, B and C in Figure 27, Kraeuter, Guest, & Sarnyai, 2019). This occurs whether the rodent is allowed to explore the maze freely and can stay in the arms for a variable length of time or whether they remain in their chosen arm for a set duration determined by closing the door to that arm (Cleal et al., 2020). This has traditionally been seen as a measure of working memory in rodents, and thus a way of assessing a key MATRICS domain, as it requires rodents to keep 'online' information about which arm they just visited and use this to choose a different arm sequentially (Vorhees &

Williams, 2014). It is also an established procedure to test short-term habituation in rodents (Sanderson & Bannerman, 2012).

Impairments on the Y-maze have been described in many rodent models of psychiatric disorder (Cleal et al., 2020; Yee & Singer, 2013), yet there are problems when it comes to interpreting what this means. It has been argued that Y-maze alternation is not a suitable read out of 'working memory' because it requires only one piece of information to be kept 'online' and thus is not analogous to span tasks used in humans (Young et al., 2009). The Y-maze does not require 'active maintenance and manipulation of stored information,' leading to arguments that it assesses short term habituation instead of working memory (Sanderson & Bannerman, 2012). Nevertheless, this task provides a read out of rudimentary working memory and certainly short-term habituation, accompanying the long-term habituation described in Chapter 3.1.4. This task is also hippocampus-dependent, with lesions of this brain region dropping alternation to chance levels (Douglas & Isaacson, 2016; Douglas, 1972) and the emergence of spontaneous alternation behaviour coinciding with hippocampal maturation (Blair et al., 2013; Dumas, 2004; Egger, Livesey, & Dawson, 1973). Thus if $Dlg2^{+/-}$s show a deficit on this task it would provide evidence of another short-term cognitive process impaired in these rodents.

The present work comprises two Experiments involving differing memory test batteries for $Dlg2^{+/-}$ and wild-type rats. In Experiment one rats were tested on NOR, OL, OIP and a short reference memory and reversal water-maze Experiment. To follow up on potential findings from Experiment one, Experiment two involved TOT, an OIP procedure with two sample phases to encourage discrimination between sample and test, a longer water maze reference memory test and Y-maze spontaneous alternation.

4.2 Experiment 1

Animals

A summary of all rat Cohorts used can be found in Appendix 1. Within this chapter Cohort 3 was used for Experiment 1 (NOR, OL, OIP, water-maze reference memory and reversal) and

Cohort 4 was used in Experiment 2 (OIP, TOT, water-maze reference memory, Y-maze spontaneous alternation).

4.2.1. Experiment 1 methods

Materials, pretraining and design of object recognition tasks

Investigation of objects occurred in an open-topped arena (62 × 62 cm). Walls (40 cm high) were made of clear Perspex and half occluded by a white trim to focus attention on the contents of the arena while the animal was still able to see visual cues around the room. An overhead camera recorded behaviour. The stimuli consisted of eight objects that varied in colour, shape, and size and were too heavy for rats to displace. Examples of objects can be seen in Figure 18. Four replicas of each object were used so that in between each sample or test phase identical objects could be swapped out to prevent scent cues guiding recognition.

Before the battery of object recognition tasks rats were habituated individually to an empty arena for 10 mins on two consecutive days. On the third habituation day rats were placed in the arena for 10 minutes and allowed to explore an object that was present in the centre of the arena, allowing potential genotype effects in responding to novel objects to be examined. The object used on the third habituation day was not re-used in any of the later recognition tests.

In between individual rats and test phases objects and the arena were cleaned with 70% ethanol. Rats completed the three object recognition tasks (NOR, OL and OIP) in an order counterbalanced for genotype and sex. Due to the length of tasks and the number of rats involved male and female cohorts were run separately, with each battery running for eight consecutive days. After completing one assay (i.e. OIP) each rat had an additional day of habituation before being tested on a different assay (i.e. OL). Amount of time spent exploring objects in sample and test phases was manually scored.

Novel object recognition

The novel object recognition task, shown schematically in Figure 17A, comprised a sample phase and recognition test separated by a 5-minute delay (as in Barker & Warburton, 2011). In the sample two identical objects (e.g. A1 and A2) were placed ~10 cm from the corners on one wall of the arena. The rat was placed in the arena facing the centre of the opposite wall and allowed 40s of exploration of A1 and A2 or if this was not reached 4 minutes in the arena. Exploratory behaviour was defined as the rat directing its nose to the object at <2cm, with climbing on or touching the objects while looking elsewhere not considered to be exploratory. A 3-minute test followed the delay, during which the animal was again placed in the arena and presented with two objects. One of these was a third copy of the sample objects (i.e. A3) and another was a novel object (i.e. B1). The position of the novel / familiar object at test and the objects used as novel and familiar were counterbalanced across sexes and genotypes.

Object location

This test challenged the rats to recognise that a familiar object had changed location and is shown schematically in Figure 17B. It consisted of a sample phase, where two identical objects were placed in the far corners of the arena (i.e. A1 and A2). Rats were allowed either 40s of exploration of both objects or 4 minutes in the arena. Following a 5-minute delay, a test phase began in which rats were allowed to explore copies of the same object (i.e. A3 and A4). One of these copies was in a location experienced during the sample phase, but the other was moved adjacent to its sample phase position, so that the objects were now at a diagonal to each other. The position of the displaced object was counterbalanced across genotypes and sexes.

Object-in-place

This task tested rat's ability to identify when familiar objects appeared in new locations to those seen previously. The sample phase consisted of 5 minutes in which rats could explore four different objects (i.e. A1, B1, C1, D1) set ~10 cm from each corner of the arena. After a

5-minute delay (as in Barker & Warburton, 2011), a 3-minute test phase began when rats could explore copies of the same objects (i.e. A2, B2, C2, D2), but where two objects on one side of the arena had been swapped, changing their location. The pair of objects moved, and the side of the arena set pairs of objects were on in the sample phase was counterbalanced across sexes and genotypes. Time spent exploring the two objects that had changed position was compared with the time spent exploring the two objects that remained in the same positions.
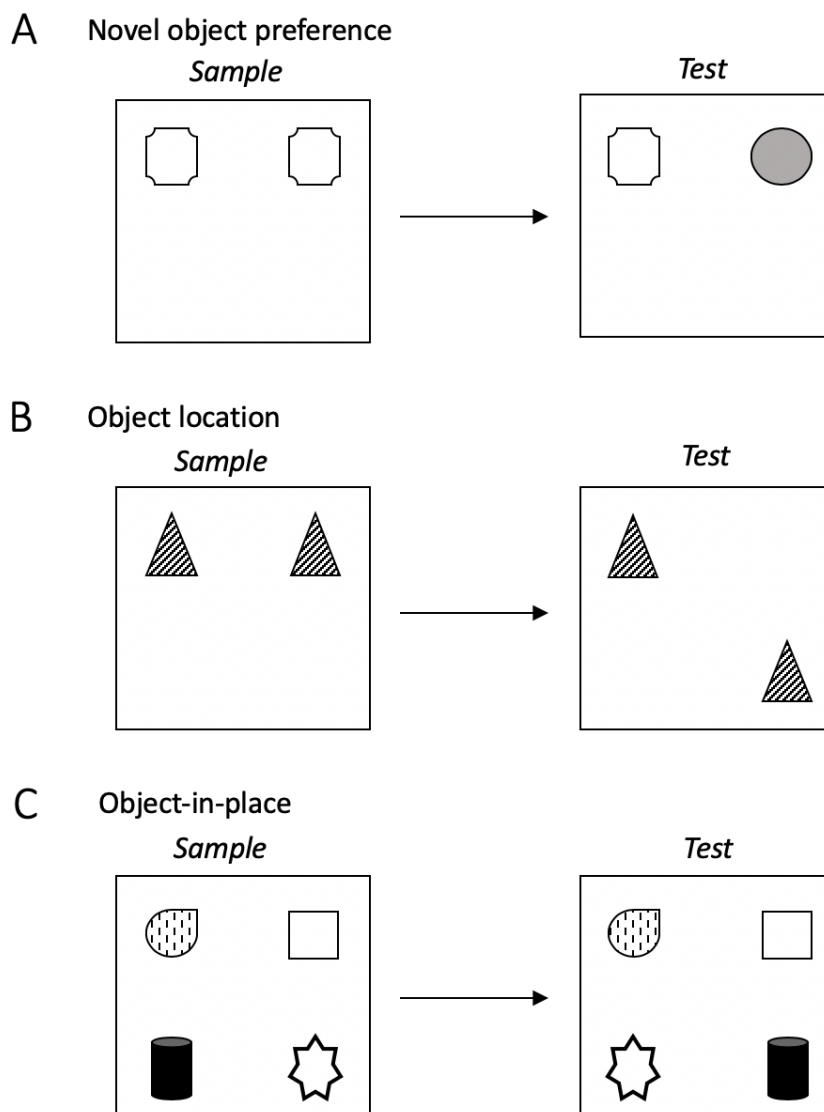
Figure 17: Schematic representations of the object recognition tasks used in Experiment 1.
In the novel object recognition task (A) at test rats would be expected to spend more time exploring the grey circle. In the object location task (B) rats would be expected to explore

the displaced triangle more at test. In the object-in-place task (C) rats would be expected to explore the star and cylinder at test as these have swapped position since the sample phase.

Figure 18: Examples of objects used in the object recognition tasks.

Statistical analysis of object recognition tasks

Both raw object/pair exploration times and discrimination ratios were analysed. Discrimination ratios were calculated as Equation 2:

$$Discrimination\ ratio\ (D2)$$
$$= \frac{Exploration\ of\ novel\ object\ or\ pair - exploration\ of\ familiar\ object\ or\ pair}{Total\ exploration\ time}$$

Analysis of raw exploration time allows for overall genotype or sex effects on exploration to be observed, while discrimination ratio controls for the confound of individual differences in total object investigation on the differential response to familiar and novel stimuli (Ennaceur & Delacour, 1988). Inter-rater reliability established by an observer naive to genotype re-scoring 8 videos of NOR, OL and OIP and calculating the d2 for each rat was $r(6)$

= 0.9251, p = 0.001 for NOR, $r(6)$ = 0.9471, $p < 0.001$ for OL and $r(6)$ = 0.9405, $p < 0.001$ for OIP.

Morris water-maze reference memory and reversal

The white fibreglass maze (2m in diameter, 60 cm deep) was mounted 50cm above the floor. This pool was filled with water (24±1°c) and made opaque by the addition of 50 ml opacifier (ViewPoint, France). The escape platform was 10cm in diameter and rested 2cm below the surface of the water. The maze was in a 4 m^2 room lit by floodlights to 240 lux in the centre of the maze, with the surrounding curtain drawn back and salient pictorial cues on the walls. These cues remained in the same locations throughout the experiment. Rats were tracked with a webcam suspended 1 m above the pool and recorded to DVD. Experiments were controlled and data collected online using Watermaze Software (Edinburgh).

Animals were transported to the experimental room in their home cages, where they remained in a room adjacent to the experimental room before and after their series of daily trials. For the reference memory task, the position of the platform remained constant for each rat throughout all trials while the rat's start position changed from trial to trial. Rats had one of four constant platform positions throughout reference memory acquisition, either North East, North West, South East or South West. Start positions were always North, East, South or West. Platform positions and start positions were counterbalanced across sex and genotype. The acquisition stage consisted of four consecutive days with four trials a day (days 1-4), based on (Gastambide et al., 2015; Young et al., 2009) . Trials were terminated when the rat either located the submerged platform or after 120 s had elapsed. Each rat received all four trials in a row with an inter-trial interval of 30s, in which they were held by the experimenter.

On day five of the experiment only one trial was conducted, a 60s probe trial in which the platform had been removed. On day 6 rats were moved to the reversal stage of the experiment. Here the platform was moved to that directly opposite the reference memory

platform location (e.g. from North West to South East). The reversal phase consisted of four consecutive days with four trials a day (Days 6-9) where the rats start position varied with each trial. On the final testing day (Day 10) the platform was removed for another 60s probe trial.

Morris water-maze reference memory and reversal analysis

How quickly rats found the submerged platform after being released (escape latencies) and distance swum were used as the principal measure of acquisition of platform location memory during the acquisition phases. Differences between sex and genotype groups in swim speed and percentage of time spent within 16.5 cm of the side walls were also examined. Swim speed potentially confounds escape latency and spending a large proportion of the trial near the side walls would indicate a learnt strategy for finding the platform that depends on the platform's proximity to a side wall, and not its spatial location signalled by extra-maze cues.

On probe trials rat's memory of the platform location was assessed by investigating where the rats searched for the platform via the percentage of time spent in each maze quadrant, comparing the target quadrant (where platform was located) with adjacent and opposite quadrants. The accuracy of spatial location memory was measured by number of crossings of the exact location the target platform had been. Swim speed, percentage of time near side walls and latency to first crossing of previous platform location were also analysed on probe trials.

4.2.2. Experiment 1 Results

Habituation to arena

Activity levels during the three ten-minute habituation trials to the empty arena were calculated using Ethovision software. This allows investigation into baseline differences in response to novelty and habituation. In $Dlg2^{-/-}$ mice hypoactivity to novel contexts has been

shown in the open field test (Winkler, Daher, Wüstefeld, Hammerschmidt, Poggi, Seelbach, Krueger-burg, et al., 2018; Yoo et al., 2020a).

Mean velocity and distance travelled for rats of both genotypes in each 10-minute session are plotted in Figure 19A and 19B respectively. These data were analysed with repeated measures ANOVA and Bayesian repeated measures ANOVA within-subjects factor of trial (1, 2 and 4) and between subjects' factors of sex and genotype. Rodents of both genotypes demonstrate an increase in activity on Trial 4 due to a change between the arena presented on Trial 3 and Trial 4. On Trials 1 and 2 the arena was empty, then on Trial 3 the arena contained a single object to assess baseline responses to a novel object on Trial 3 (reported below) before being presented empty again on Trial 4 before object recognition tasks commenced.

For velocity there was a main effect of trial ($F(2, 78) = 10.652$, $p < 0.001$, $n^2_p = 0.215$) reflecting the initial habituation and slight increase in activity following a change of the arena back to empty. There were no genotype effects on habituation with a non-significant main effect of genotype ($F(1, 39) = 0.594$, $p = 0.446$, $n^2_p = 0.015$; $BF_{exclusion} = 8.585$) and genotype × trial interaction ($F(2, 78) = 0.327$, $p = 0.722$, $n^2_p = 0.008$; $BF_{exclusion} = 12.791$). This was much the same for distance travelled with a main effect of trial ($F(2, 78) = 10.495$, $p < 0.001$, $n^2_p = 0.212$), yet no main effect of genotype ($F(1, 39) = 0.274$, $p = 0.604$, $n^2_p = 0.007$; $BF_{exclusion} = 3.244$) nor genotype × trial interaction ($F(2, 78) = 0.520$, $p = 0.597$, $n^2_p = 0.013$; $BF_{exclusion} = 5.052$). For both measures there were main effects of sex, yet no sex × genotype or other sex-based interactions, which are reported in the Appendix A4.1.1. Rats of both genotypes habituated in a similar manner to the novel environment, with no evidence of hypoactivity in response to novelty in the $Dlg2^{+/-}$ line.
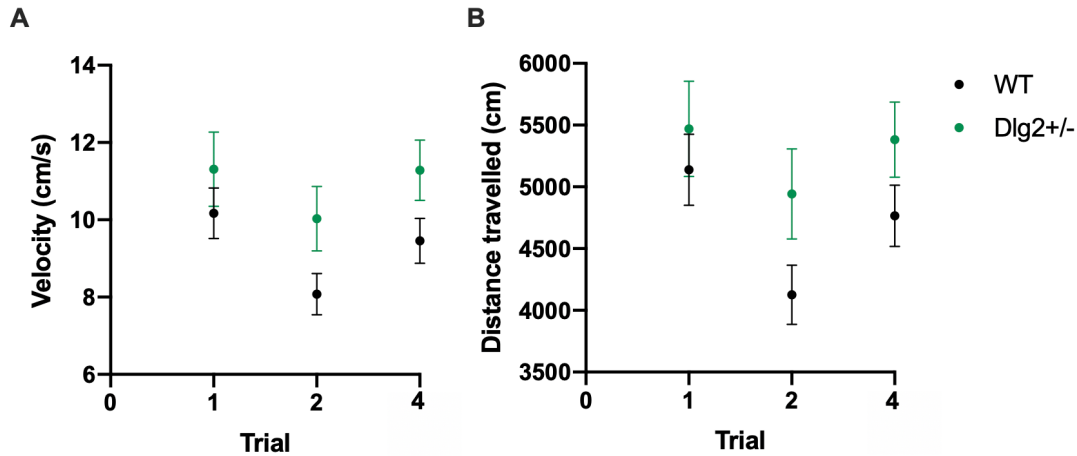
Figure 19: *Dlg2⁺ᐟ⁻* **and wild-type rats' activity in the three 10-minute sessions when habituating to the empty arena to be used for object recognition tasks in Experiment 1.** Mean ± SEM **A)** velocity, **B)** distance travelled.

Responding to a novel object

Baseline genotype differences in responding to a novel object on the third day of habituation were not found, as shown in Figure 20. ANOVA with fixed factors of genotype and sex reported no main effects of genotype ($F(1,40) = 0.063$, $p = 0.804$, $n^2_p = 0.002$; $BF_{exclusion} = 4.058$). Sex effects and interactions were also non-significant and are reported in the Appendix, as for all Experiments in this chapter. Rodents of different genotypes and sexes respond similarly when presented with a novel object, ruling this out as a possible problem with object recognition experiments in this rat line.
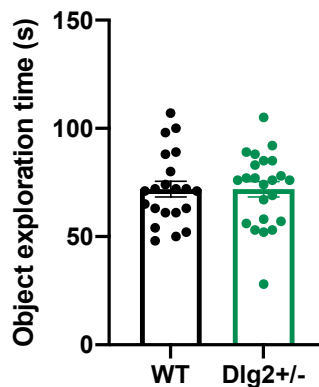
Figure 20: Exploration time for a novel object presented alone during the habituation stage of object recognition experiments. Mean ± SEM with data points representing individuals.

Novel object recognition

Dlg2⁺/⁻ rats performed comparably to wild-types on the novel object recognition task, as shown in by the raw exploration times in Figure 21A, discrimination ratio in Figure 21B. Repeated measures ANOVA, with between subjects factors of genotype and sex and object (novel or familiar object) as a repeated measures factor, found a main effect of object ($F(1,40) = 13.435$, $p < 0.001$, $n^2_p = 0.251$), reflecting rodents propensity to spend longer exploring the novel object. Novel object recognition did not vary with genotype (non-significant object × genotype interaction $F(1,40) = 0.453$, $p = 0.505$, $n^2_p = 0.011$; $BF_{exclusion} = 4.594$, and non-significant genotype main effect $F(1,40) = 0.838$, $p = 0.365$, $n^2_p = 0.021$; $BF_{exclusion} = 5.498$).

Discrimination ratio for NOR was significantly different from 0 (one-sample t-test $t(43) = 5.043$, $p < 0.001$, $d = 0.760$) reflecting the expected increase in exploration of the novel object. One-way ANOVA for discrimination ratio on the novel object exploration task revealed a no effect of genotype ($F(1,40) = 0.063$, $p = 0.804$, $n^2_p = 0.002$; $BF_{exclusion} = 4.240$).

Object location

Rodents of both genotypes explored the displaced object more than the object in the familiar location but there were no genotype effects on this as shown by raw exploration time (Figure 21C) and discrimination ratio (Figure 21D). Repeated measures ANOVA and Bayesian repeated measures ANOVA with between subjects' factors of sex and genotype, and a within subject factor of location (static or displaced) revealed a main effect of location ($F(1,40) = 50.450$, $p < 0.001$, $n^2_p = 0.558$). Object exploration did not differ with genotype (non-significant genotype × location interaction $F(1,40) = 0.889$, $p = 0.351$, $n^2_p = 0.022$; $BF_{exclusion} = 2.857$, and genotype main effect $F(1,40) = 1.370$, $p = 0.249$, $n^2_p = 0.033$; $BF_{exclusion} = 3.250$). Discrimination ratio for OL was significantly different from 0 (one-sample t-test $t(43) = 7.637$, $p < 0.001$, $d = 1.151$) reflecting the expected increase in exploration of the displaced object. The ANOVA on discrimination ratio revealed non-significant main effects

of genotype ($F(1,40)$ = 0.053, p = 0.818, n^2_p = 0.001; $BF_{exclusion}$ = 4.318). *Dlg2^{+/-}* rats are able to remember object location with the same proficiency as wild-types on this task.

Object-in-place task

Raw exploration times and discrimination ratio for the object-in-place task are shown in Figures 21E and 21F respectively. Repeated measures ANOVA with between subjects' factors of genotype and sex and within subjects factors of object (swapped or static) showed a main effect of genotype ($F(1,39)$ = 4.239, p = 0.046, n^2_p = 0.098), and a trend towards an object × genotype interaction ($F(1,39)$ = 3.878, p = 0.056, n^2_p = 0.090; $BF_{exclusion}$ = 0.787).

Discrimination ratio for OIP was not significantly different from 0 for *Dlg2^{+/-}*s (one-sample t-test $t(22)$ = -0.398, p = 0.653 , d = -0.083), yet it was for wild-types ($t(19)$ = 2.080, p = 0.026 , d = 0.465). ANOVA revealed a trend towards a main effect of genotype on the object-in-place discrimination ratios ($F(1,39)$ = 3.619, p = 0.065, n^2_p = 0.085; $BF_{exclusion}$ = 1.021).

OIP performance was relatively poor in all animals, and while there was numerically superior performance in the wild-type animals compared to *Dlg2^{+/-}* (and only wild-type animals were significantly above chance), the Bayes analysis suggests the evidence was entirely inconclusive in terms of group differences. Thus, further work, perhaps using protocols designed to improve OIP performance overall, was required to determine if there is a deficit in *Dlg2^{+/-}* animals.

Interim Summary

Responding to a novel object proceeds the same way across both genotypes, ruling out generalised differences in responding to a novel item as a confound in these object recognition tasks. *Dlg2^{+/-}* rats perform comparably to wild types on the NOR and OL, showing intact memory for object identity and object location. Performance of *Dlg2^{+/-}* rats on the OIP task gives indication of a potential impairment in memory for object-location paired associates although statistical effects are inconclusive.
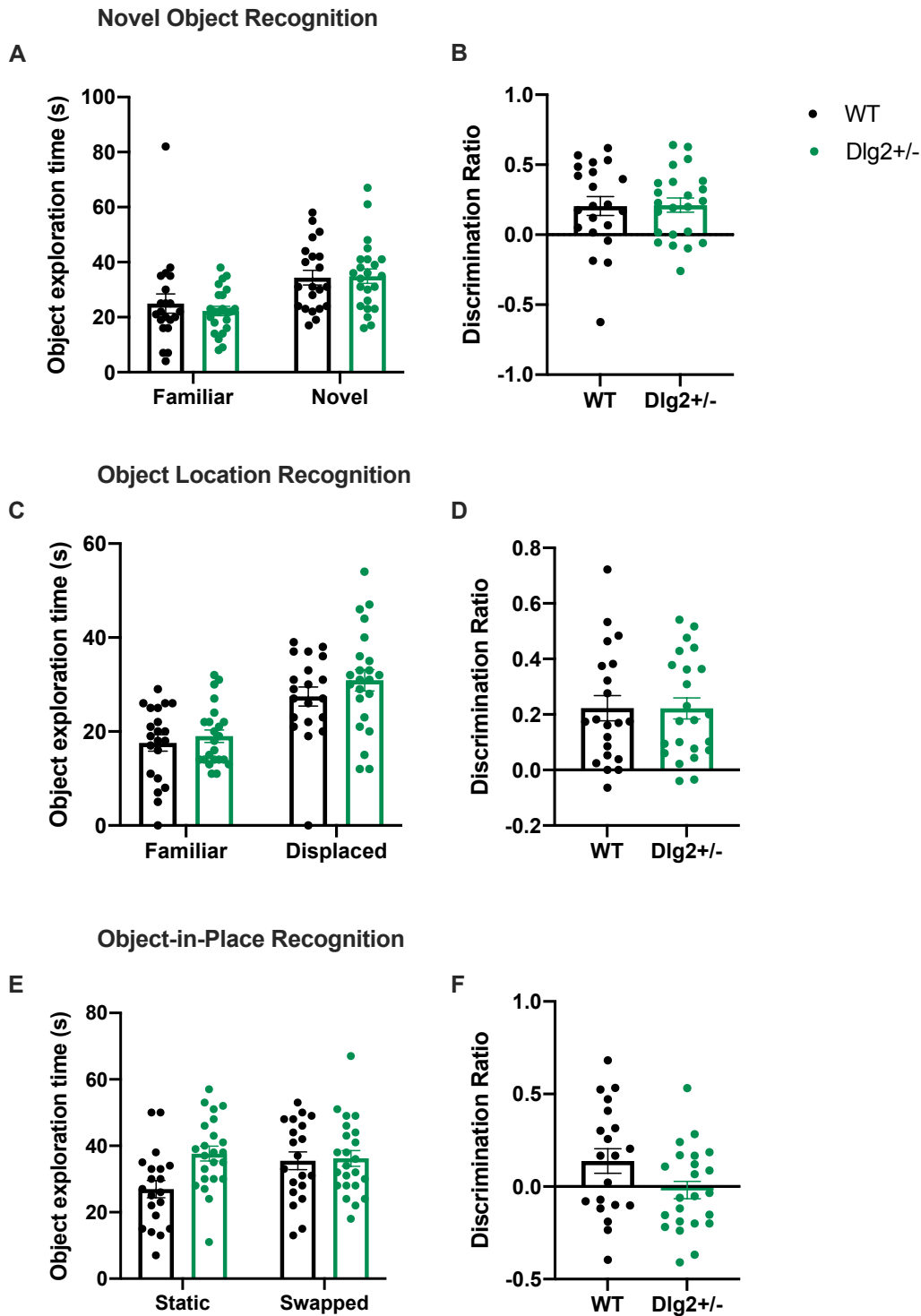
Figure 21: *Dlg2+/-* **and wild-type rats performance on the three object recognition tasks in Experiment 1.** Mean and ± SEM plus individual scores. **A)** Novel object recognition raw exploration **B)** Novel object recognition discrimination ratio **C)** Object location raw exploration **D)** Object location discrimination ratio **E)** Object-in-place raw exploration **F)** Object-in-place discrimination ratio.

Water maze reference memory

Figure 22 shows the mean escape latencies (Figure 22A), distance swum (Figure 22B), swim speed (Figure 22C) and percentage of time near side walls (Figure 22D) of wild-type and $Dlg2^{+/-}$ rats across the first four days of the experiment while rats learned a stable platform location. Means ± SEM are represented for each training day rather than for each trial, as the performance of rats of both genotypes improved over trials within each day yet there were no genotype effects on this. Trial did interact with sex (although not with sex × genotype) which is reported in the Appendix A4.1.6. Escape latencies, distance swum, swim speed and percentage of time near side walls were analysed with repeated measures ANOVA and Bayesian ANOVA with within-subjects factors of day (1-4) and trial (1-16) and between-subjects factors of sex and genotype. Interactions between sex and genotype were all non-significant and are also reported in A4.1.6. of the Appendix.

Escape latency decreased across training days as learning the location of the platform progressed (significant main effects of day: $F(3, 120) = 86.959$, $p < 0.001$, $n^2_p = 0.685$, trial: $F(3, 120) = 36.178$, $p < 0.001$, $n^2_p = 0.475$, and trial × day $F(7.028, 281.140) = 2.445$, $p = 0.019$, $n^2_p = 0.058$). Genotype did not appear to influence this learning with non-significant day × genotype ($F(3, 120) = 0.729$, $p = 0.536$, $n^2_p = 0.018$; $BF_{exclusion} = 65.851$), trial × genotype ($F(3, 120) = 1.696$, $p = 0.172$, $n^2_p = 0.041$; $BF_{exclusion} = 22.356$) or day × trial × genotype ($F(7.028, 281.140) = 0.846$, $p = 0.550$, $n^2_p = 0.021$; $BF_{exclusion} > 1000$) interactions, however there was a trend towards a main effect of genotype ($F(1, 40) = 4.066$, $p = 0.051$, $n^2_p = 0.092$; $BF_{exclusion} = 11.657$), however the Bayes factor suggests that the evidence is actually most consistent with no overall genotype effect.

Distance swum decreased across training days as learning progressed (Figure 22B), shown by significant main effects of day ($F(3, 120) = 62.970$, $p < 0.001$, $n^2_p = 0.612$), trial ($F(3, 120) = 72.399$, $p < 0.001$, $n^2_p = 0.644$, and trial × day ($F(6.670, 266.812) = 5.575$, $p < 0.001$, $n^2_p = 0.122$). Day × genotype ($F(3, 120) = 0.311$, $p = 0.818$, $n^2_p = 0.008$; $BF_{exclusion} = 200.521$), trial × genotype ($F(3, 120) = 0.598$, $p = 0.617$, $n^2_p = 0.015$; $BF_{exclusion} = 134.319$), day × trial × genotype ($F(6.670, 266.812) = 1.262$, $p = 0.271$, $n^2_p = 0.031$; $BF_{exclusion} > 1000$) and genotype

($F(1, 40) = 3.221$, $p = 0.080$, $n^2_p = 0.075$; $BF_{exclusion} = 23.083$) effects were all non-significant with Bayes factors supporting null effects.

While swim speed increased for $Dlg2^{+/-}$ rats and wild-types as learning progressed (significant main effects of day $F(3, 120) = 10.369$, $p < 0.001$, $n^2_p = 0.206$, trial $F(3,120) = 48.253$, $p < 0.001$, $n^2_p = 0.547$ and trial × day ($F(9, 360) = 7.308$, $p < 0.001$, $n^2_p = 0.154$) there were no genotype differences in this (non-significant day × genotype: $F(3,120) = 1.791$, $p = 0.153$, $n^2_p = 0.043$; $BF_{exclusion} = 10.193$, trial × genotype: $F(3,120) = 0.214$, $p = 0.886$, $n^2_p = 0.005$; $BF_{exclusion} = 195.707$, day × trial × genotype ($F(9, 360) = 0.873$, $p = 0.550$, $n^2_p = 0.021$; $BF_{exclusion} > 1000$) and genotype: $F(1,40) = 1.517$, $p = 0.225$, $n^2_p = 0.037$; $BF_{exclusion} = 17.970$ effects). Therefore, any genotype effects on escape latency or distance swum are unlikely to be due to differences in swim speed between genotypes.

Percentage of time spent near the side walls varied with trial (main effect of trial $F(1.059,42.358) = 4.288$, $p = 0.042$, $n^2_p = 0.097$), reflecting the rats increasing understanding that once in the water there was a platform to swim to. There was no main effect of day ($F(1.605, 64.211) = 1.223$, $p = 0.294$, $n^2_p = 0.030$; $BF_{exclusion} = 144.699$) or and trial × day ($F(1.642, 65.678) = 1.386$, $p = 0.256$, $n^2_p = 0.033$; $BF_{exclusion} = 184.866$). There were no effects of genotype on this (non-significant day × genotype: $F(1.605, 64.211) = 0.507$, $p = 0.564$, $n^2_p = 0.013$; $BF_{exclusion} > 1000$, trial × genotype: $F(1.059, 42.358) = 1.585$, $p = 0.216$, $n^2_p = 0.038$; $BF_{exclusion} = 92.989$, day × trial × genotype ($F(1.642, 65.678) = 0.566$, $p = 0.537$, $n^2_p = 0.014$; $BF_{exclusion} > 1000$) and genotype: $F(1, 40) = 1.614$, $p = 0.211$, $n^2_p = 0.039$; $BF_{exclusion} = 43.090$ main effects). Thus rodents of both genotypes did not rely on a strategy of remaining a certain distance from the maze walls to find the platform.
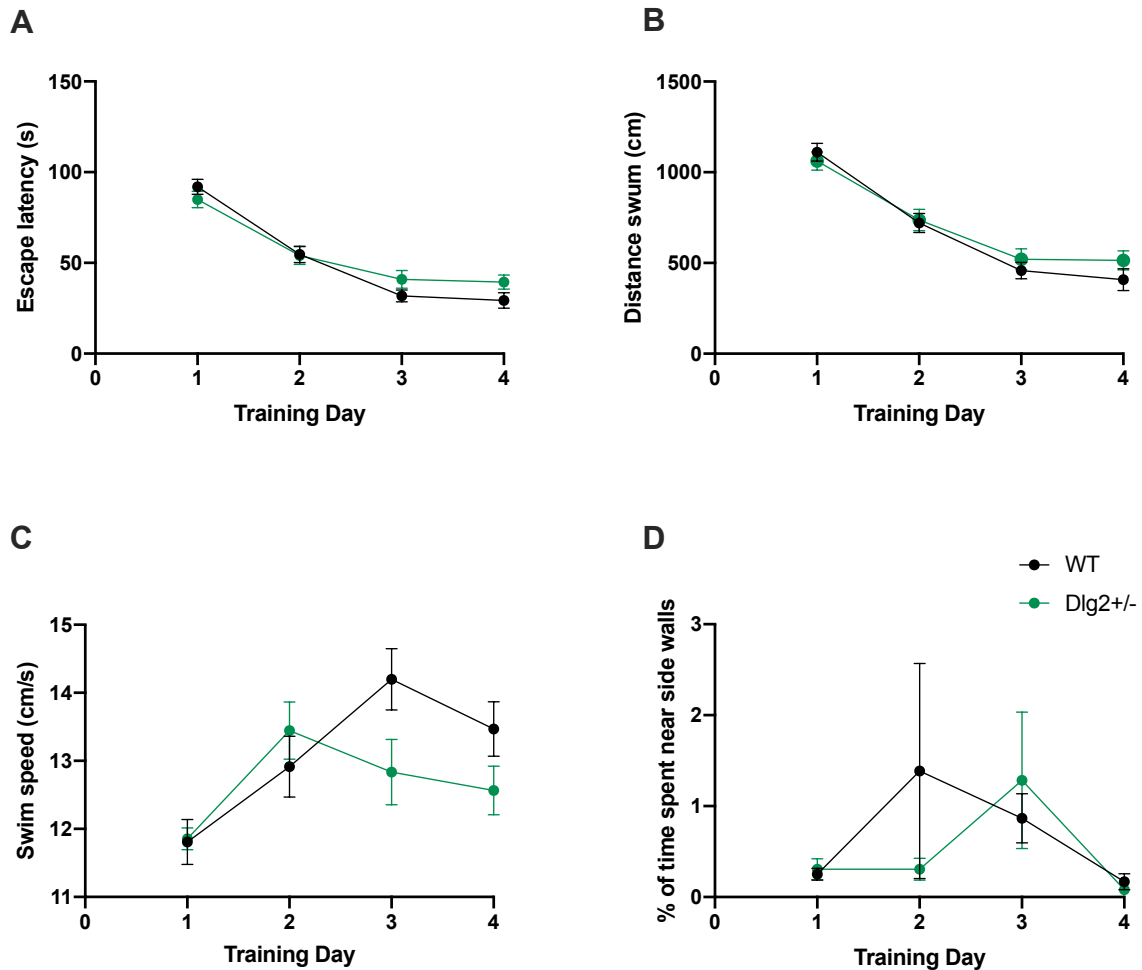
Figure 22: Performance of *Dlg2+/-* and wild-type rats on a four-day reference memory task in the water maze. Escape latencies **(A)**, distance swum **(B)** swim speed **(C)** and percentage of time spent near side walls **(D)** across all four training days in the reference memory task, split by genotype. The latencies, distances and speeds shown for each day are the average of four daily trials and the SEM. Trial is not plotted as interactions with genotype was non-significant (Appendix Table 24).

Reference memory probe test

Wild-type and *Dlg2+/-* spent comparable amounts of time in each quadrant of the maze on the probe test (Figure 23A). Quadrant dwell time was analysed by repeated measures ANOVA and Bayesian repeated measures ANOVA with repeated measures factor of quadrant (that where platform was in training, adjacent right, adjacent left, opposite) and between subjects factors of sex and genotype. In this analysis genotype and sex main effects

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were expected to be non-significant and do not provide valuable information as all rats were in the pool for two minutes in the test trial. However, these are reported as the interactions between these effects and quadrant were useful in determining any differences in where rats of different genotype or sex groups spent their time searching for the platform.

The expected increase in dwell time for the training quadrant was not shown (non-significant main effect of quadrant, $F(2.498, 99.913) = 0.198$, $p = 0.865$, $n^2{}_p = 0.005$; $BF_{exclusion} = 53.179$) and genotype did not influence quadrant dwell time (non-significant quadrant × genotype interaction, $F(2.498, 99.913) = 2.031$, $p = 0.125$, $n^2{}_p = 0.048$; $BF_{exclusion} = 36.867$ and genotype main effect $F(1, 40) = 0.452$, $p = 0.505$, $n^2{}_p = 0.011$; $BF_{exclusion} = 8.714$).

There were no genotype effects on latency to reach platform location (Figure 23B) and number of annulus crossings (Figure 23C). An annulus crossing was defined as the rodent taking a swim path which traversed the diameter of where the platform had been. These were both analysed by ANOVA and Bayesian ANOVA with factors of sex and genotype. Genotype main effects were non-significant for latency ($F(1, 40) = 0.775$, $p = 0.384$, $n^2{}_p = 0.019$; $BF_{exclusion} = 3.443$) and number of annulus crossings ($F(1, 40) = 0.918$, $p = 0.344$, $n^2{}_p = 0.022$; $BF_{exclusion} = 1.623$).

Interim Summary

There was no apparent difference in the ability to learn the platform location between *Dlg2$^{+/-}$* and wild-type rats across the first 4 days of the reference maze task. However rats of both genotypes failed to show memory of the platform location on the day 5 probe test by searching for the platform in the quadrant it had been at during training.
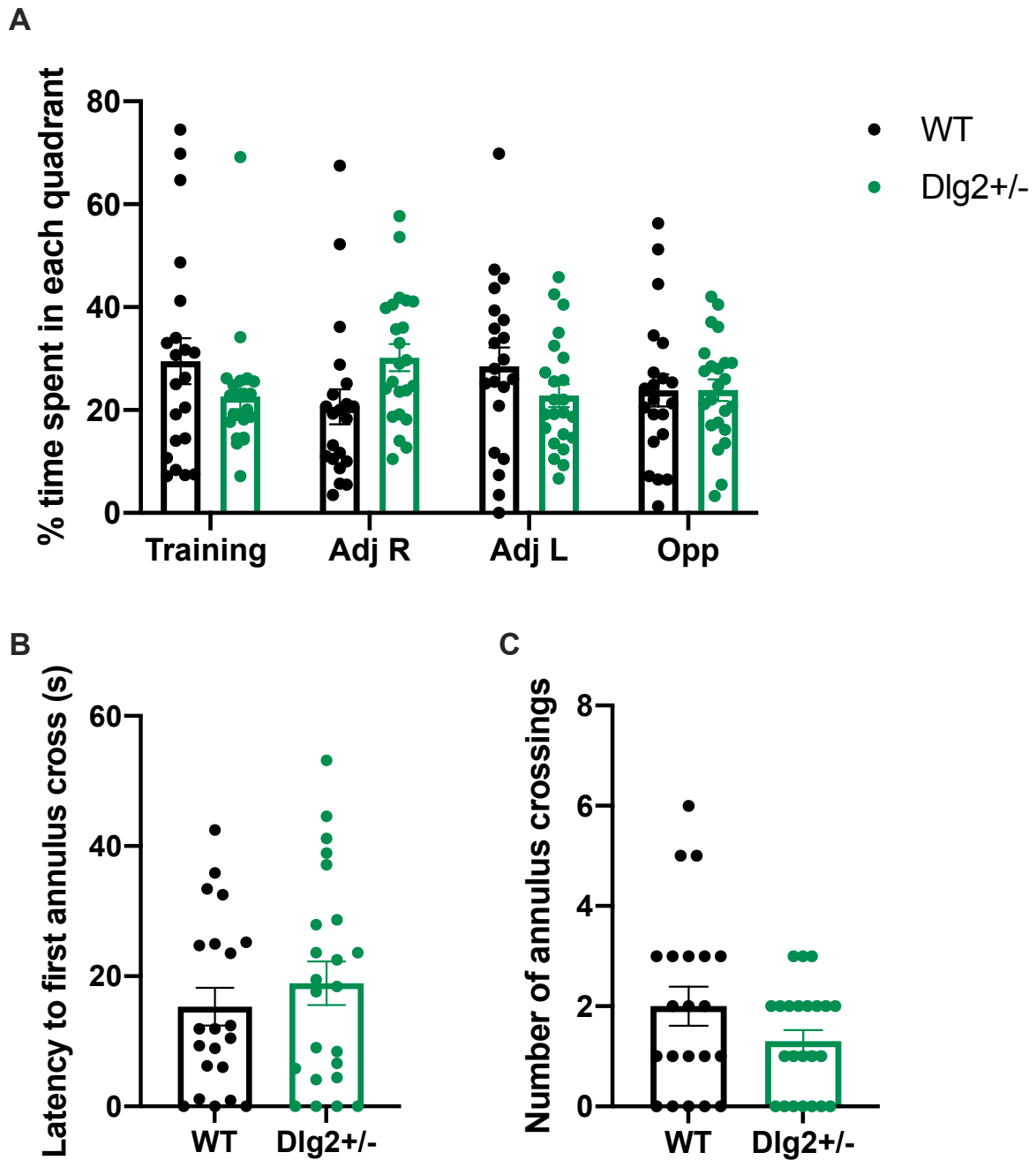
Figure 23: *Dlg2+/-* **and wild-type rats performance on the reference memory probe test.**
Mean ± SEM plus individual values. **A)** Percentage of time spent in each quadrant **B)** Latency to first cross of previous platform annulus **C)** Number of crossings of previous platform annulus location.

Learning a new platform location in the water maze (reversal)

Escape latencies, distance swum, swim speed and percentage of time spent near side walls for different genotype groups in the reversal phase of the water maze task are shown in Figures 24A, 24B, 24C and 24D. As with reference memory trial is not plotted as the performance rats of both genotypes increased similarly across trials within days, and sex × trial interactions are detailed in the Appendix A4.1.8. Escape latencies, distance swum, swim speed and percentage of time spent near side walls were analysed by repeated measures and Bayesian ANOVA with within-subjects factors of day (6-9) and trial (18-34) and between-subjects factors of sex and genotype.

As in the reference stage of the experiment escape latency decreased as rats learned the new platform location with main effects of day (F(2.275, 86.455) = 16.801, p <0.001, n^2_p = 0.307) and trial (F(2.155,81.879) = 52.427, p <0.001, n^2_p = 0.580) but no trial × day interaction (F(6.004, 228.148) = 1.802, p = 0.100, n^2_p = 0.045; BF$_{exclusion}$ = 2.351). Genotype did not influence this (non-significant genotype × day: F(2.275, 86.455) = 1.674, p = 0.190, n^2_p = 0.042; BF$_{exclusion}$ = 74.517, genotype × trial: F(2.155,81.879) = 1.013, p = 0.372, n^2_p = 0.026; BF$_{exclusion}$ = 41.051, genotype × trial × day: F(6.004, 228.148) = 1.600, p = 0.148, n^2_p = 0.040;BF$_{exclusion}$ > 1000 and main effect of genotype (F(1,38) = 0.276, p = 0.602, n^2_p = 0.007; BF$_{exclusion}$ = 35.964).

Distance swum also decreased across days as learning progressed, with main effects of day (F(3, 114) = 13.670, p < 0.001, n^2_p = 0.265) and trial (F(2.076, 78.892) = 70.428, p < 0.001, n^2_p = 0.650) with trial × day approaching significance (F(5.214, 198.150) = 2.226, p = 0.051, n^2_p = 0.055; BF$_{exclusion}$ = 0.246). Genotype did not influence this (non-significant genotype × day: F(3, 114) = 1.023, p = 0.385, n^2_p = 0.026;BF$_{exclusion}$ = 230.157, genotype × trial: F(2.076, 78.892) = 0.828, p = 0.444, n^2_p = 0.021;BF$_{exclusion}$ = 77.145, genotype × trial × day: F(5.214, 198.150) = 1.847, p = 0.102, n^2_p = 0.046;BF$_{exclusion}$ > 1000 and main effect of genotype (F(1, 38) = 0.055, p = 0.815, n^2_p = 0.001; BF$_{exclusion}$ = 40.751).

Swim speed decreased with learning with significant main effects for day (F(3, 114) = 9.257, p < 0.001, n^2_p = 0.196) and trial (F(3, 114) = 15.172, p < 0.001, n^2_p = 0.285) and trial × day

($F(9, 342) = 3.791$, $p < 0.001$, $n^2_p = 0.091$). There was no main effect of genotype on swim speed ($F(1, 38) = 0.391$, $p = 0.535$, $n^2_p = 0.010$; $BF_{exclusion} = 11.223$), genotype × trial: $F(3, 114) = 0.437$, $p = 0.727$, $n^2_p = 0.011$; $BF_{exclusion} = 140.521$ or genotype × trial × day: $F(9, 342) = 0.650$, $p = 0.754$, $n^2_p = 0.017$; $BF_{exclusion} > 1000$. The day × genotype interaction was significant ($F(3, 114) = 3.471$, $p = 0.018$, $n^2_p = 0.084$). With no main effects of genotype on escape latency or distance swum this doesn't provide useful information concerning rodents learning the new platform location.

Percentage of time spent near the side walls did not vary with day (non-significant main effect of day: $F(2.135, 83.282) = 0.513$, $p = 0.613$, $n^2_p = 0.013$; $BF_{exclusion} = 1.114$) but did vary with trial (main effect of trial: $F(1.712, 66.749) = 3.530$, $p = 0.042$, $n^2_p = 0.083$) and trial × day ($F(2.651, 103.371) = 4.042$, $p = 0.012$, $n^2_p = 0.094$). There were no genotype effects on time spent near side walls (non-significant genotype × day: $F(2.135, 83.282) = 0.641$, $p = 0.539$, $n^2_p = 0.016$; $BF_{exclusion} = 209.807$, genotype × trial: $F(1.712, 66.749) = 0.115$, $p = 0.862$, $n^2_p = 0.003$; $BF_{exclusion} = 427.317$ genotype × trial × day: $F(2.651, 103.371) = 0.455$, $p = 0.691$, $n^2_p = 0.012$; $BF_{exclusion} > 1000$ and main effect of genotype: $F(1, 39) = 0.391$, $p = 0.535$, $n^2_p = 0.010$; $BF_{exclusion} = 44.126$). These results show a lack of consistent use of a learnt strategy for finding the platform that depended on its proximity to the side walls. Taken together measures indicated that learning the new platform location during the reversal stage was not influenced by genotype.
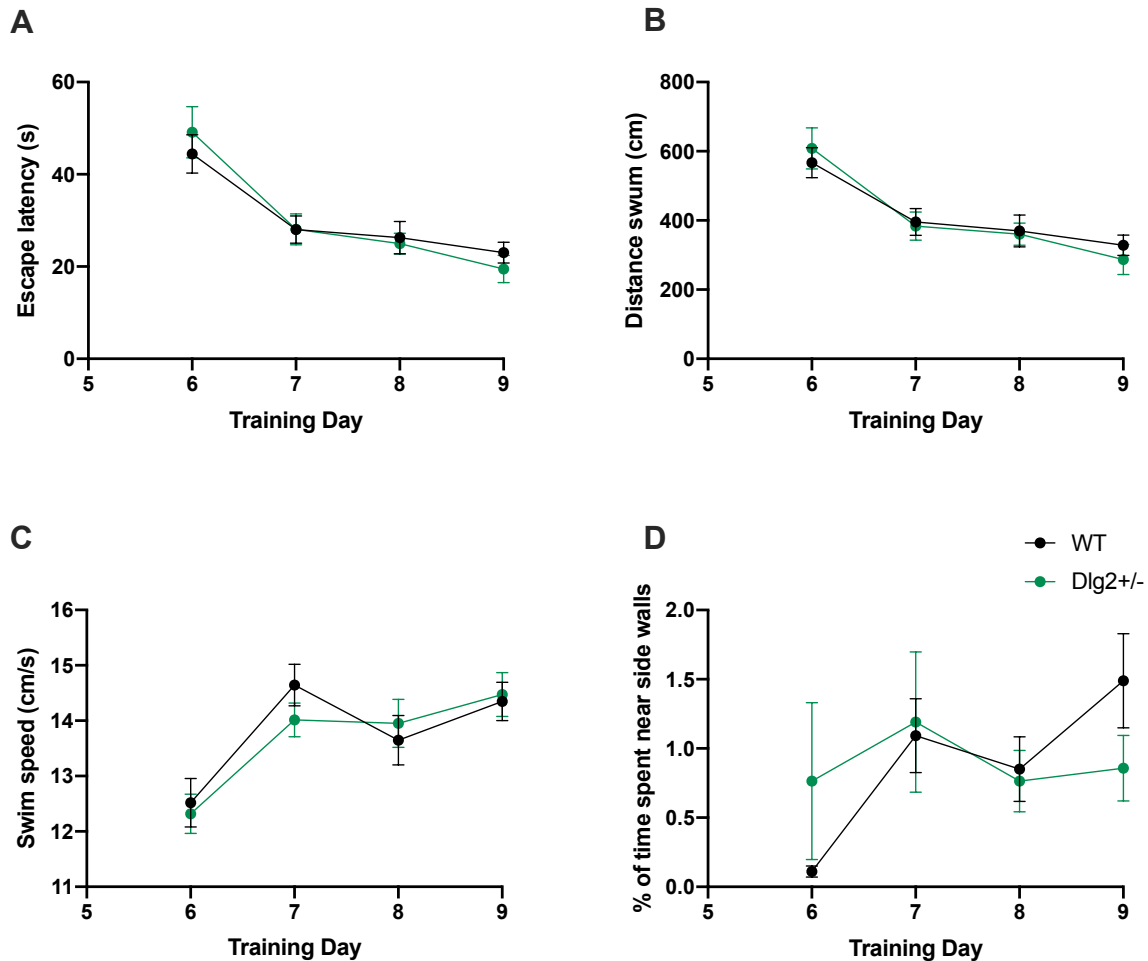
Figure 24: Performance of wild-type and *Dlg2^+/-^* rats after the platform location was changed to the opposite quadrant. Escape latencies **(A)**, distance swum **(B)**, swim speed **(C)** and percentage of time near side walls **(D)**. The latencies, distances and speeds shown for each day are the average of four daily trials and the SEM. Trial is not plotted as interactions with all other variables were non-significant (Appendix Table 24).

Performance on reversal probe test

Quadrant dwell time on the 60s probe test following training with the new platform location is displayed in Figure 25A. Just as in reference memory neither wild-types nor *Dlg2^+/-^* rats demonstrated any difference in the time spent across quadrants (non-significant main effect of quadrant, $F(3, 120) = 0.574$, $p = 0.633$, $n^2_p = 0.014$; $BF_{exclusion} = 41.574$, non-significant genotype × quadrant interaction, $F(3, 120) = 1.602$, $p = 0.196$, $n^2_p = 0.039$; $BF_{exclusion} = 72.235$ and main effect of genotype: $F(1, 40) = 1.071$, $p = 0.307$, $n^2_p = 0.026$; $BF_{exclusion} = 13.748$).
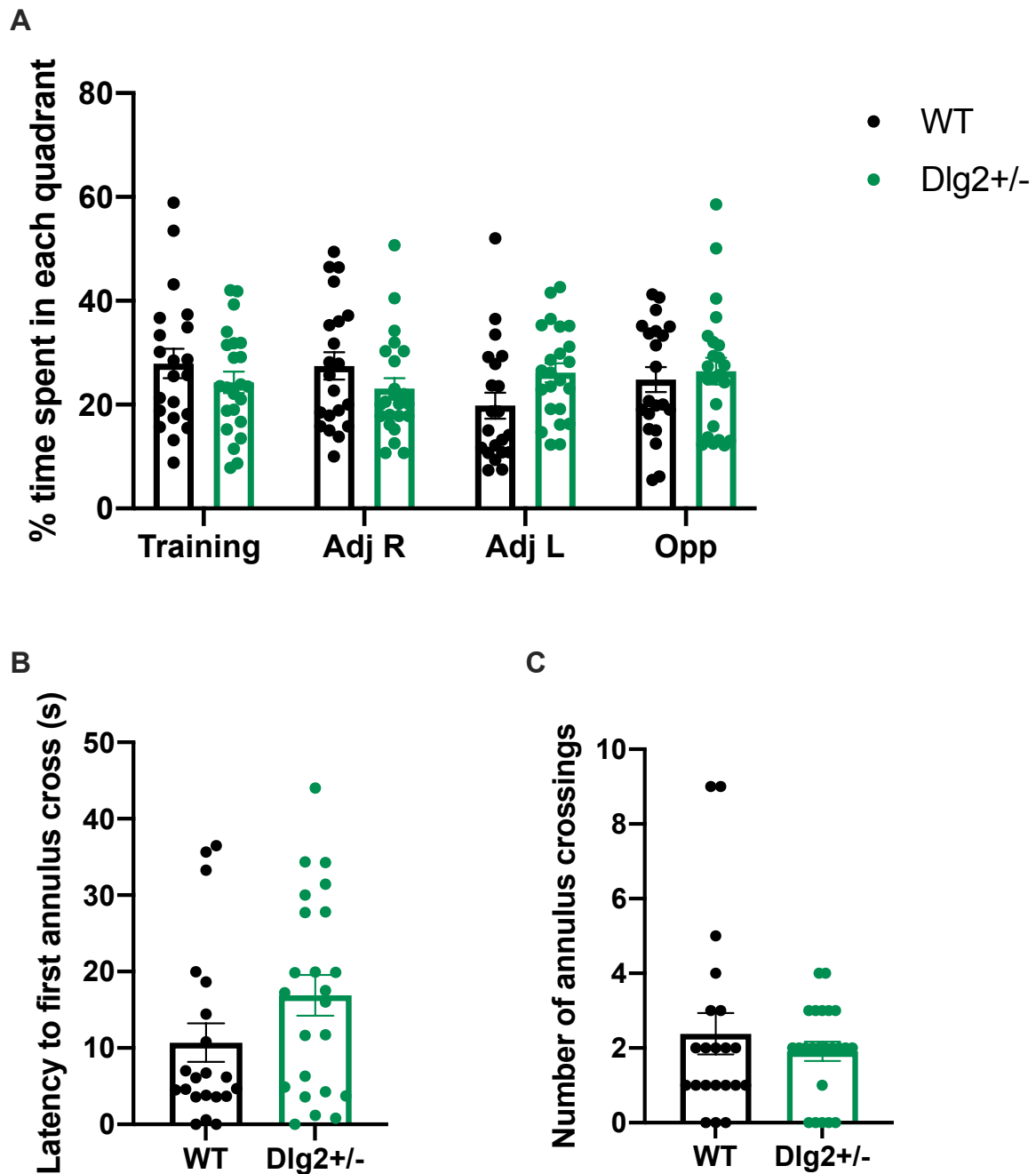
Figure 25: *Dlg2+/-* and wild-type rats performance on the reversal memory probe test.
Mean ± SEM plus individual values. **A)** Percentage of time spent in each quadrant **B)** Latency to first cross of previous platform annulus **C)** Number of crossings of previous platform annulus location.

Other measures of platform location memory, latency to first annulus cross (Figure 25B) and number of annulus crosses (Figure 25C), did not show genotype effects. No main effect of

genotype on latency to first cross ($F(1, 40) = 2.493$, $p = 0.122$, $n^2_p = 0.059$; $BF_{exclusion} = 1.520$) or number of annulus crossings ($F(1, 40) = 0.918$, $p = 0.344$, $n^2_p = 0.022$; $BF_{exclusion} = 3.465$).

Interim Summary

Dlg2$^{+/-}$ rats were just as able to learn the new platform location as wild-types. As with the initial acquisition stage rats of both genotypes failed to show the expected search behaviour in the maze quadrant that had housed the platform, making conclusions about platform location memory difficult.

4.2.3. Experiment 1 Discussion

Potential cognitive deficits arising from heterozygous *Dlg2* deletion were assessed using tests of reference memory and reversal in the Morris water maze, and object recognition depending on object novelty, location and object-in-place memory. There was no indication that *Dlg2* haploinsufficiency impaired performance on NOR or OL recognition. There was a slight indication that *Dlg2$^{+/-}$* rats may be worse at OIP recognition than wild types, although there was no formal support for this from the inferential statistics. To probe this further a version of the object-in-place task with two sample phases was used in Experiment 2, to highlight differences between sample and test phases on this task and clarify if genotype effects are present.

On the reference memory test there was a hint of a possible genotype effect on escape latency. However, neither wild-type nor *Dlg2$^{+/-}$* rats demonstrated the expected increase in time spent searching for the platform in the training quadrant during the probe test, making mnemonic differences across genotypes difficult to interpret and precluding interpretation of 'reversal' learning from day 5 onwards in this task.

It may have been that the short training schedule used (only four four-trial training days rather than ten which have been used in other work e.g. Vann, Wilton, Muir, & Aggleton, 2003; Warburton, Baird, Morgan, Muir, & Aggleton, 2001) resulted in weak memory of the platform location, disrupting search behaviour on the probe test. Perhaps at this early stage

of reference memory acquisition rat's platform location knowledge was based on a string of associated body movements or movements associated with specific extra-maze cues ('knowing how to get there') rather than a stable and complete representation of the maze space ('knowing where.') Thus, a ten-day rerun of the water maze reference memory task with interspersed probe trials was carried out in Experiment 2.

4.3. Experiment 2

4.3.1. Experiment 2 methods

Pretraining and design of object recognition battery

Rats from Cohort 4 were used in all assays described in Experiment 2. The same arena setup and objects used in Experiment 1 were used for Experiment 2 as the new rat cohort were naïve to them. Given the lack of genotype effects in responding to a single novel object (shown in Figure 26) rats in Experiment 2 were individually given three days of ten-minute exposures the empty arena. As in Experiment 1 all objects including replicates and the arena were cleaned with 70% ethanol after being used in any phase of the experiment. The objects used in TOT and OIP were counterbalanced for sex as these experiments were run blind to genotype. All rats were tested on OIP followed by TOT with an additional habituation day in between the two where they were given ten minutes of exposure to the empty arena. Male and female cohorts were run separately across six consecutive days. Object exploration was manually scored online and recorded using Ethovision software allowing for calculation both of exploration time and frequency of visits to objects or pairs.

Object-in-place

The OIP procedure was identical to that used in Experiment 1 except an additional five-minute sample phase followed by five minute delay was added, shown schematically in Figure 26A. This was intended to give the rats more exposure to the first configuration of object pairs to enhance any genotype differences in recognising object-place changes.

Which pair of objects was swapped at test and the side of the arena set object pairs were on was counterbalanced for sex, as it had been for sex and genotype in Experiment 1.

Temporal order task

This task assessed rat's ability to recognise objects based on recency. It entails two sample phases and a test trial (Figure 26B). In each sample phase rats were allowed to explore two copies of one object for five minutes with different objects used for sample phases 1 and 2 (i.e. A1 A2 followed by B1 B2). The delay between the first and second sample phases and the sample and test phase was 1 hour as in Barker & Warburton (2011). During the 3-minute test trial a third copy of the objects from each sample phase was presented (i.e. A3 A4 B3 B4), with one copy of each object being in the same location as in the sample and another in a new location. The objects used in samples 1 and 2 and the layout of objects were counterbalanced across sexes. If temporal order memory is intact rats should spend longer exploring the objects seen in sample 1 than in sample 2 (i.e. the objects presented less recently). Object-place memory can also be assessed on this task if rats explore the objects that are displaced from their sample phase positions at test more than those which are seen in consistent locations. For the expected proportion of exploration to each object and location, see Figure 26B.
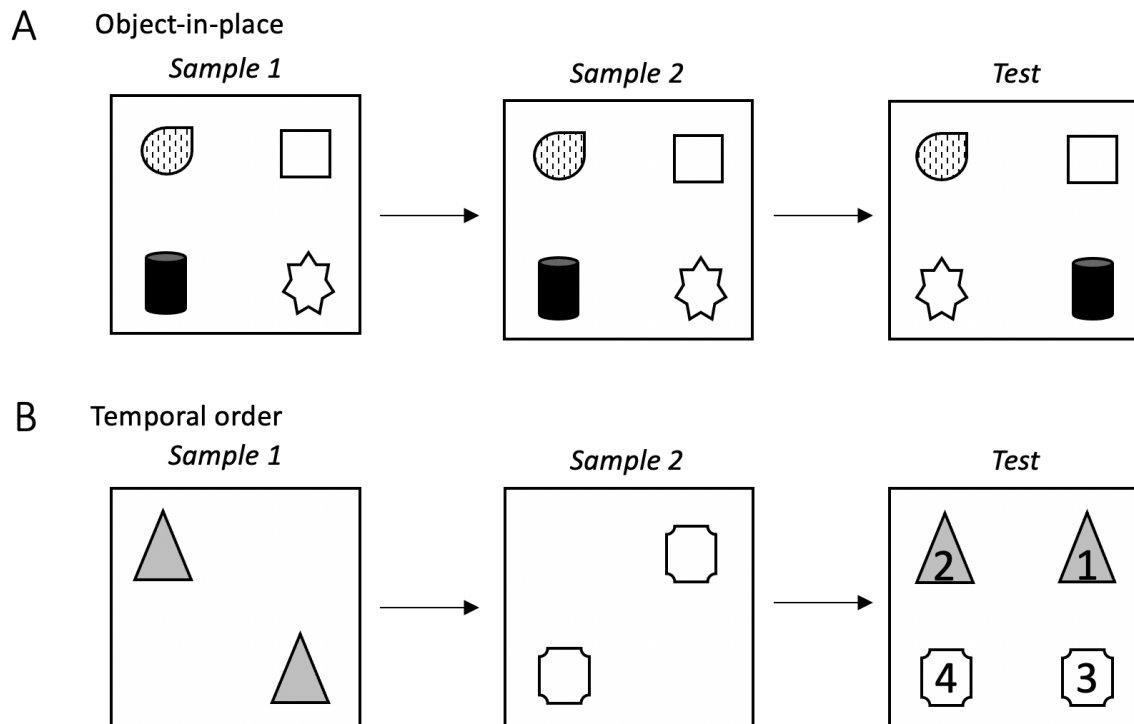
Figure 26: Schematic representations of the object-in-place task (A) and temporal order task (B) used in Experiment 2. The numbers in the test phase of the temporal order task denote the expected hierarchy of object exploration determined by both recency and place information. The object that is in a new location and was experienced at the furthest timepoint is expected to be explored the most (1) and the object that is recently seen and in a familiar location is expected to be explored the least (4).

Morris water-maze reference memory

This was conducted using the same apparatus and set-up as in Experiment 1, yet in Experiment 2 the acquisition was extended to ten days (Vann et al., 2003) with 4 trials per day to get a reliable indication of any genotype differences in reference memory. On days 4, 8 and 10 the first trial was a probe trial where the platform was absent. On days 4 and 8 this probe was followed by 3 platform-present trials. This experiment was run blind to genotype and thus platform location was counterbalanced for sex. As in Experiment 1 escape latency, distance swum, swim speed and percentage of time spent near side walls were analysed on platform-present trials and search behaviour was analysed on platform-absent trials by assessing annulus crossings and percentage time spent in each quadrant.

Spontaneous alternation in the Y-maze

Behavioural testing was carried out in a Y-maze with white wooden floor and clear Perspex walls. Stem (40cm long) and arms (30cm long) were 12 cm wide and 23.5 cm high. Metal doors could be lowered at the entrance of each arm. A low-intensity diffuse illumination of 110 lux was provided above the apparatus and a clear Perspex roof was placed on the maze if once the rat was placed inside to prevent escape. Male rats were run before female rats daily. Before the trial-based procedure began rats were habituated to the empty maze for 10 minutes per day over 3 consecutive days. This afforded an opportunity to assess alternation for the first 5 minutes that rats were placed in the maze in a continuous-trials procedure, where rats could explore the maze and change arms freely without experimenter intervention as in the trial- based procedure that followed. In the trial-based procedure rats were placed in the start arm (i.e. C) and once a rat entered another arm (i.e. A) the door to that arm was closed. After a 30s confinement in the chosen arm the rat was removed and placed in a holding cage in a neutral room for the 60s ITI or back in the holding cage for the 24h ITI before being placed back in the start arm (C). At each ITI rats submitted 5 trials each.
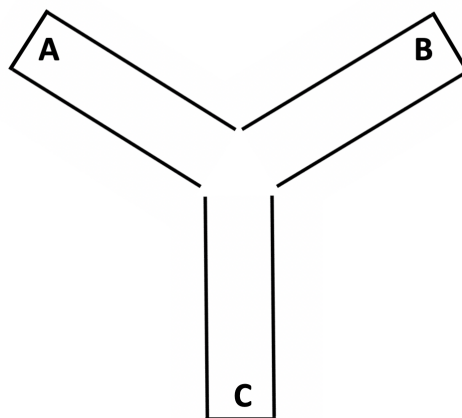
Figure 27: Schematic representation of the Y-maze with each arm labelled as A, B and C.

For analysis maze arms were labelled A, B and C as in Figure 27. Spontaneous alternation % on the continuous trials procedure was calculated using the following formula (Equation 3):

$$\text{Spontaneous alternation \%} = \frac{\text{Number if spontaneous alternations}}{\text{Total number of arm entries} - 2} \times 100$$

For example if the order of arm entry in 5 mins was ABCCBABCABC there would be a total of 6 spontaneous alternations (ABC, CBA, ABC, BCA, CAB, ABC) with 11 arm entries, giving 67% (as in Miedel, Patton, Miedel, Miedel, & Levenson, 2017). On the trial-based procedure spontaneous alternation % was calculated as the percentage of trials on which a rat entered a different arm from that on the previous trial (Deacon & Rawlins, 2006).

4.3.2. Experiment 2 Results

Habituation to arena

The use of Ethovision software to record and score rodents movements in the object recognition tasks allows distance moved and velocity to be conveniently assessed in while rodents were habituated to the arena. This presented a check for any genotype differences in habituation such as hypoactivity to a novel context, as has been shown in *Dlg2*^{-/-} mice (Winkler, Daher, Wüstefeld, Hammerschmidt, Poggi, Seelbach, Krueger-burg, et al., 2018; Yoo et al., 2020a).

Mean velocity and distance travelled for rats of both genotypes in each 10-minute session are plotted in Figure 28A and 28B respectively. These show that although *Dlg2*^{+/-} rats were more active in the arena than wild-types, they habituated in the same manner to the novel context as shown by the same decreases in activity across the three 10-minute sessions. These data were analysed using repeated measures ANOVA and Bayesian repeated measures ANOVA with within-subjects factor of trial (1-3) and between subjects factors of sex and genotype.

For velocity there was a main effect of trial ($F(2, 118) = 63.978$, $p < 0.001$, $n^2_p = 0.520$) reflecting the activity decrease as rats habituated to the arena with increasing experience. The main effect of genotype was also significant ($F(1, 59) = 11.593$, $p = 0.001$, $n^2_p = 0.164$) as *Dlg2+/-* rats traversed at higher velocities than wild-types across all trials. The trial × genotype interaction was non-significant ($F(2, 118) = 1.215$, $p = 0.300$, $n^2_p = 0.020$; $BF_{exclusion}$ =1.326) showing a consistent decrease in activity between sessions for both genotypes, demonstrating comparable habituation processes. The statistical significance of effects and interactions was the same for distance travelled, with main effects of trial ($F(1.805, 106.468)$ = 63.952, $p < 0.001$, $n^2_p = 0.520$) and genotype ($F(1, 59) = 11.859$, $p = 0.001$, $n^2_p = 0.167$), but no trial × genotype interaction ($F(1.805, 106.468) = 1.374$, $p < 0.001$, $n^2_p = 0.520$; $BF_{exclusion}$ = 1.303). Overall, there is no evidence of hypoactivity to a novel environment, with *Dlg2+/-* rats showing more activity than wild-types both in the habituation phase of Experiment 1 (Figure 14) and here where these data show reduced variability in locomotor measures. As in Experiment 1 habituation to the arena occurred comparably for both genotypes.
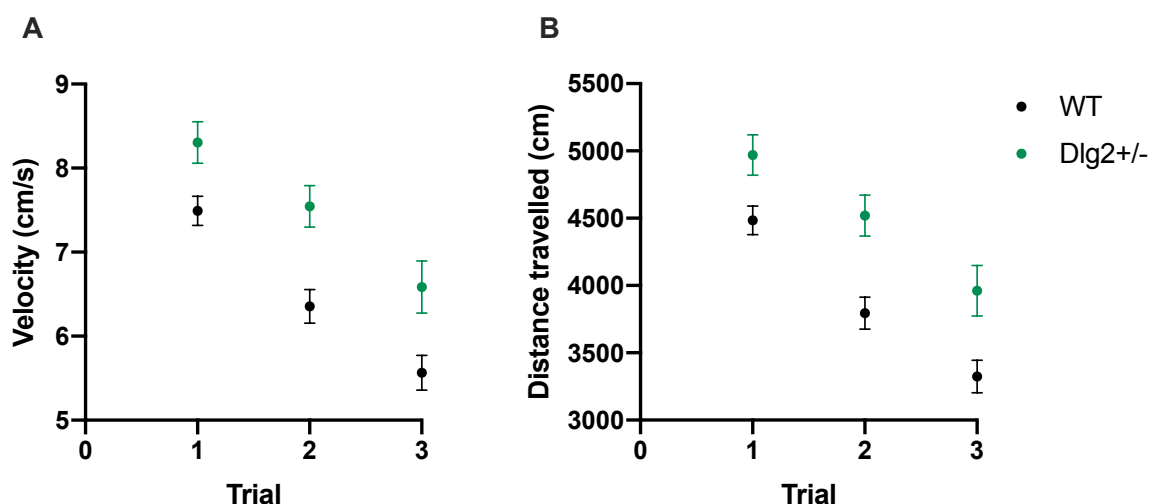
Figure 28: *Dlg2+/-* and wild-type rats' activity in the three 10-minute sessions when habituating to the empty arena to be used for object recognition tasks in Experiment 2. Mean ± SEM **A)** velocity, **B)** distance travelled.

Object exploration in the first object-in-place sample phase

There were no genotypes differences in total exploration time for the four objects combined in the first sample phase of the OIP test as Figure 29 shows. These data were analysed with ANOVA and Bayesian ANOVA with factors of sex and genotype. There was no main effect of genotype ($F(1, 60) = 0.734$, $p = 0.395$, $n^2_p = 0.012$; $BF_{exclusion} = 1.326$). There were sex effects reported in the Appendix A4.2.1. This is similar to the finding of no baseline differences in responding to a novel object assessed in the habituation stage of the object recognition work in Experiment 1. Thus, exploration in the OIP test phase should not have been influenced by baseline differences in object exploration in the sample phases.
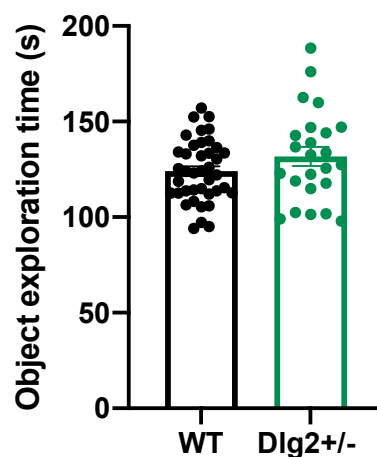
Figure 29: Total object exploration time for wild-types and *Dlg2+/-* rats in the first OIP sample phase. Mean ± SEM plus individual values.

Object-in-place task

Raw exploration time for object pairs (Figure 30A) did not vary with genotype. These data were analysed with repeated measures ANOVA and Bayesian ANOVA with between-subjects factors of sex and genotype and within-subjects factor of object pair (static and swapped). There was a main effect of object pair ($F(1, 60) = 4.642$, $p = 0.035$, $n^2_p = 0.072$) reflecting rats tendency to explore the swapped pair more than the static pair. There were no genotype effects on this (non-significant genotype × pair interaction $F(1, 60) = 0.183$, $p = 0.670$, $n^2_p = $

0.003; BF$_{exclusion}$ = 10.172, non-significant genotype main effect $F(1, 60)$ = 0.014, p = 0.906, n^2_p = 0.000232; BF$_{exclusion}$ = 8.998).

Discrimination ratio for object-in-place is shown in Figure 30B. Discrimination ratio for the test cohort was significantly different from 0 ($t(63)$ = 2.064, p = 0.043, d = 0.258). The main effect of genotype was non-significant ($F(1, 60)$ = 0.071, p = 0.791, n^2_p = 0.001; BF$_{exclusion}$ = 3.327). Although discrimination ratios are small it seems that wild-type and $Dlg2^{+/-}$ rats are able to recognise object-pair associates, but $Dlg2$ heterozygosity does not result in impairments on this task.
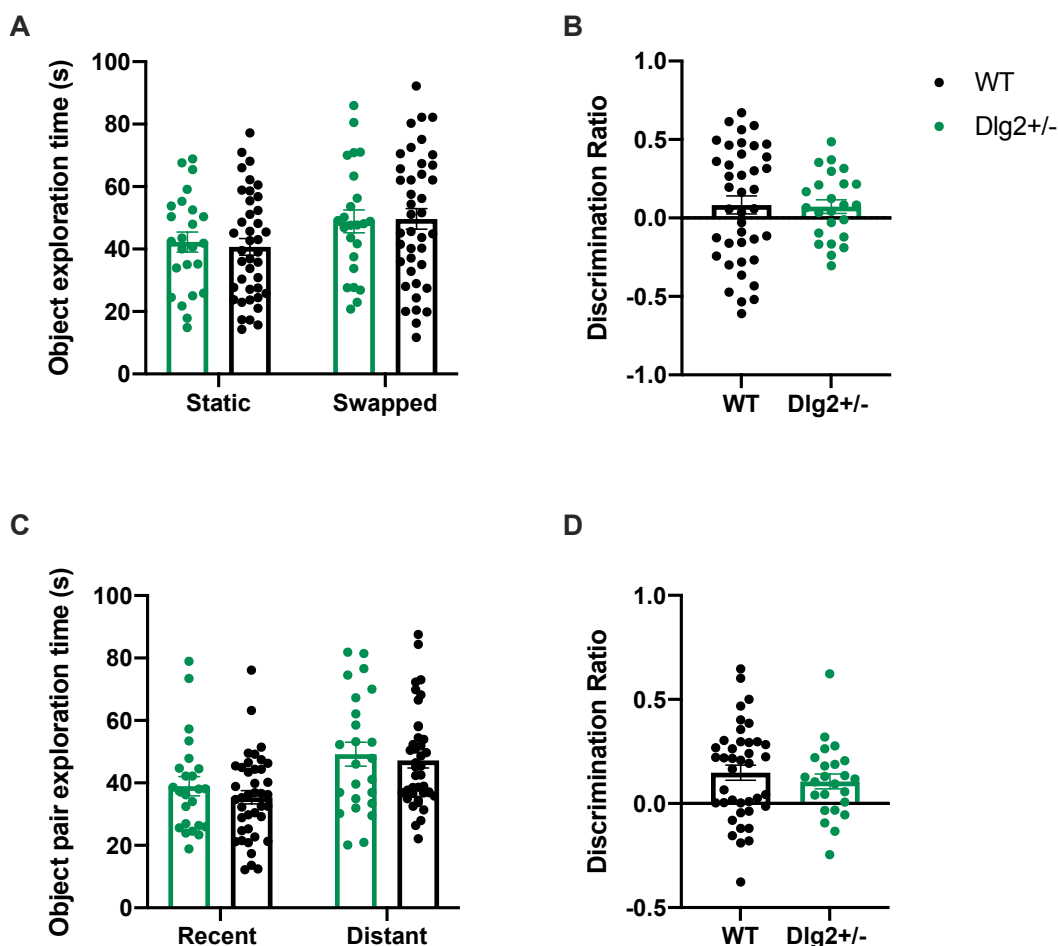
Figure 30: $Dlg2^{+/-}$ and wild-type rats' behaviour in the object-in-place and temporal order tasks. Mean ± SEM plus individual values **A)** Raw exploration time for object-in-place, **B)** object-in-place discrimination ratio **C)** raw exploration for temporal order **D)** temporal order discrimination ratio.

Temporal order task

Raw exploration time to recent and distantly seen pairs on the temporal order task is shown in Figure 30C. These were analysed by repeated measures ANOVA and Bayesian ANOVA with between-subjects factors of sex and genotype and within subjects factors of object pair (recent and distant). There was a main effect of object pair (F(1, 60) = 18.770, $p < 0.001$, $n^2{}_p$ = 0.238) as rodents explored the distantly experienced objects more than those recently experienced. There were no pair × genotype effects (F(1, 60) = 0.297, p = 0.588, $n^2{}_p$ = 0.005; $BF_{exclusion}$ = 4.773), nor overall genotype effects on exploration time (F(1, 60) = 0.109, p = 0.743, $n^2{}_p$ = 0.002; $BF_{exclusion}$ = 4.431).

Discrimination ratio (Figure 30D) for the TOT was significantly different from 0 (t(63) = 5.046, $p < 0.001$, d = -0.631) confirming rodents tendency to explore object pairs dependent on when they were last seen. There were no genotype differences on this (non-significant main effect of genotype (F(1, 60) = 0.992, p = 0.323, $n^2{}_p$ = 0.016; $BF_{exclusion}$ = 3.708). Taken together it seems that wild-type and $Dlg2^{+/-}$ rats have comparable abilities to recognise objects based on recency.

Interim Summary

The OIP assay in Experiment 2 was modified from the version in Experiment 1 to increase performance via the addition of an extra sample phase within which rats could explore the initial layout of objects. Here OIP discrimination is still low but considered across genotypes discrimination ratios were above chance, and Bayes factors for discrimination ratio and the raw analysis give evidence in favour of no genotype effects in OIP recognition. Therefore, resolving the inconclusive nature of the first experiment, $Dlg2$ heterozygosity does not result in impairments on this task. For the TOT there was comparable proficiency of $Dlg2^{+/-}$ and wild-type rats.

Reference memory in the water-maze

Escape latencies, distance swum, swim speed and time spent near side walls on the 10-day reference memory task for wild-type and $Dlg2^{+/-}$ rats are shown in Figure 31A, 31B, 31C and 31D. Trial is not plotted as rodents of both genotypes improved in their performance in a similar manner across trials without genotype interactions, leaving only sex × trial interactions which are detailed in the Appendix A4.1.6. These data were analysed by repeated measures ANOVA and Bayesian ANOVA with repeated measures factors of day (1-10) and trial (1-40) and between-subjects factors of sex and genotype.

Escape latency (Figure 31A) revealed a significant main effect of day ($F(6.431, 385.889) = 95.992$, $p < 0.001$, $n^2_p = 0.615$), trial ($F(2.770, 166.214) = 66.096$, $p < 0.001$, $n^2_p = 0.524$) and a significant day × trial interaction ($F(15.863, 951.794) = 3.118$, $p < 0.001$, $n^2_p = 0.049$) reflecting the decrease in latency as rats learned the platform location over days and trials. This did not vary with genotype, with genotype × day ($F(6.431, 385.889) = 1.097$, $p = 0.364$, $n^2_p = 0.018$; BF$_{exclusion} > 1000$), genotype × trial ($F(2.770, 166.214) = 0.600$, $p = 0.603$, $n^2_p = 0.010$; BF$_{exclusion} > 1000$), genotype × day × trial ($F(15.863, 951.794) = 0.871$, $p = 0.602$, $n^2_p = 0.014$; BF$_{exclusion} > 1000$), and genotype main effects ($F(1, 60) = 0.087$, $p = 0.769$, $n^2_p = 0.001$; BF$_{exclusion} = 76.356$) non-significant with Bayes factors providing strong evidence for the absence of effects.

Distance swum (Figure 31B) also decreased across days and trials as rats learned the platform location (significant main effect of day: $F(6.691, 401.484) = 81.000$, $p < 0.001$, $n^2_p = 0.574$; significant main effect of trial: $F(2.636, 158.137) = 106.719$, $p < 0.001$, $n^2_p = 0.640$) and significant day × trial interaction ($F(15.507, 930.408) = 3.865$, $p < 0.001$, $n^2_p = 0.061$). There was no genotype influence on distance swum as genotype × day ($F(6.691, 401.484) = 0.947$, $p = 0.467$, $n^2_p = 0.016$; BF$_{exclusion} > 1000$), genotype × trial ($F(2.636, 158.137) = 1.340$, $p = 0.265$, $n^2_p = 0.022$; BF$_{exclusion} = 165.086$), genotype × day × trial ($F(15.507, 930.408) = 0.970$, $p = 0.486$, $n^2_p = 0.016$; BF$_{exclusion} > 1000$), and main effects of genotype ($F(1, 60) = 0.064$, $p = 0.809$, $n^2_p = 0.001$; BF$_{exclusion} = 66.186$) were non-significant.

Swim speed increased across days and trials as learning progressed with a significant main effect of day ($F(7.764, 465.810) = 12.089$, $p < 0.001$, $n^2_p = 0.168$) and trial ($F(2.865, 171.892) = 37.904$, $p < 0.001$, $n^2_p = 0.387$), however no day × trial interaction ($F(12.810, 768.589) = 1.336$, $p = 0.187$, $n^2_p = 0.022$; $BF_{exclusion} > 1000$). It also differed with genotype (main effect of genotype ($F(1, 60) = 9.132$, $p = 0.004$, $n^2_p = 0.132$), trail × genotype interaction ($F(2.865, 171.892) = 4.586$, $p = 0.005$, $n^2_p = 0.071$) with a day × genotype interaction that approaches significance ($F(7.764, 465.810) = 1.937$, $p = 0.055$, $n^2_p = 0.031$). The genotype × day × trial interaction is non-significant ($F(12.810, 768.589) = 0.963$, $p = 0.486$, $n^2_p = 0.016$; $BF_{exclusion} > 1000$). In the absence of genotype effects on escape latency and distance swum this isn't noteworthy, although the higher swim speeds of $Dlg2^{+/-}$ rats might indicate a slight motoric difference of ability between genotypes.

Percentage of time near side walls did not vary with day ($F(5.941, 356.470) = 0.890$, $p = 0.502$, $n^2_p = 0.015$; $BF_{exclusion} > 1000$), although there was a main effect of trial ($F(1.457, 87.396) = 4.542$, $p = 0.023$, $n^2_p = 0.070$) likely reflecting rats initial attempts to escape the side walls before they learned they could swim to a platform. The day × trial interaction was non-significant ($F(7.264, 435.810) = 0.831$, $p = 0.565$, $n^2_p = 0.014$; $BF_{exclusion} > 1000$). Main effects of genotype were non-significant ($F(1, 60) = 3.435$, $p = 0.069$, $n^2_p = 0.054$; $BF_{exclusion} = 61.241$), as were day × genotype ($F(5.941, 356.470) = 1.009$, $p = 0.419$, $n^2_p = 0.017$; $BF_{exclusion} > 1000$), trial × genotype ($F(1.457, 87.396) = 1.033$, $p = 0.340$, $n^2_p = 0.017$; $BF_{exclusion} > 1000$) and genotype × day × trial ($F(7.264, 435.810) = 0.961$, $p = 0.461$, $n^2_p = 0.016$; $BF_{exclusion} > 1000$) interactions showing that there was no difference in reliance on this as a strategy to find the platform between genotype groups.
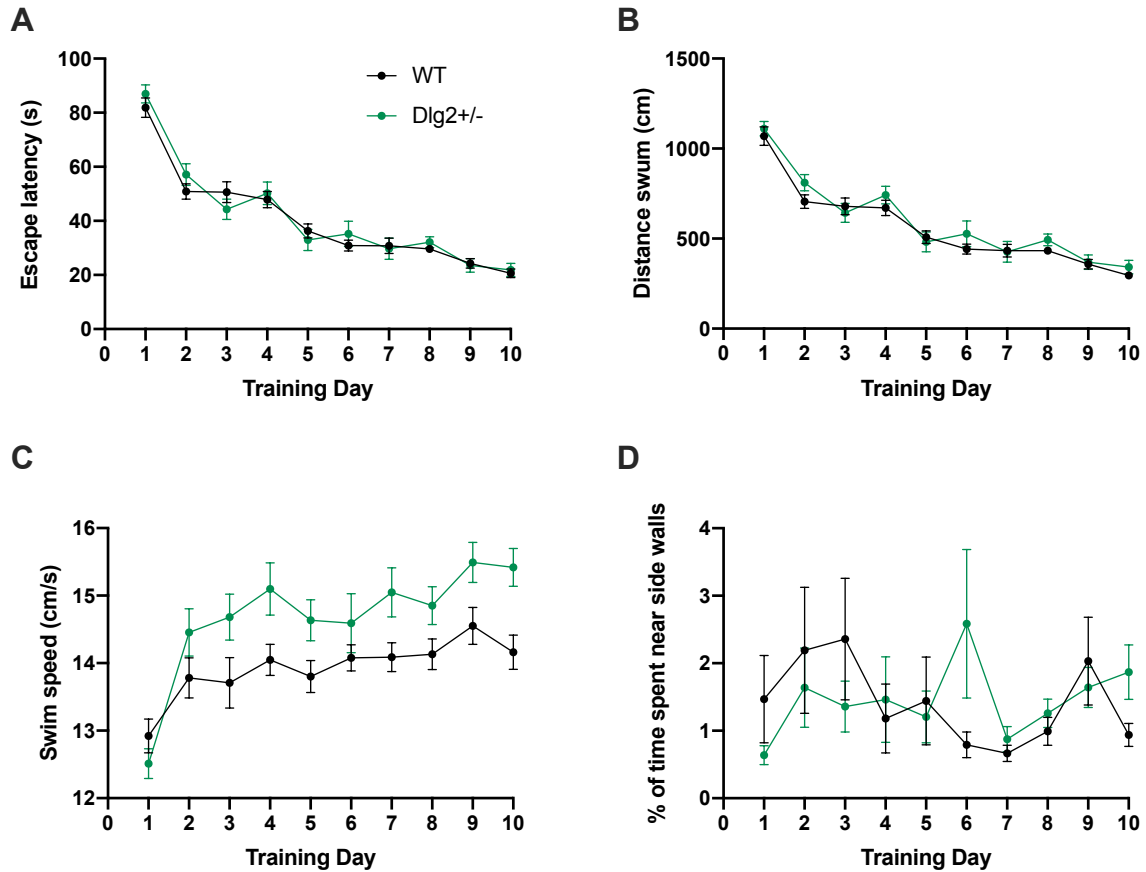
Figure 31: Performance of *Dlg2^+/-* and wild-type rats on a ten-day reference memory task in the water maze. Escape latencies **(A)**, distance swum **(B)** swim speed **(C)** and percentage of time spent near side walls **(D)** across all four training days in the reference memory task, split by sex (male and female) and genotype (*Dlg2^+/-* and wild-type). The latencies, distances and speeds shown for each day are the average of four daily trials and the SEM.

Reference memory probe trials

Three probe trials, the first trial on day 4, 8 and 11 were conducted to assess platform location memory. Each probe was analysed separately using repeated measures and Bayesian repeated measures ANOVA with repeated measures factors of quadrant (training, adjacent right, adjacent left and opposite) and between-subjects factors of sex and genotype.

In the first probe test both wild-type and *Dlg2^+/-* rats spent around equal amounts of time in all four quadrants, as shown in Figure 32A. The main effect of quadrant was non-significant

(F(2.428, 145.708) = 2.651, p = 0.063, $n^2{}_p$ = 0.042; $BF_{exclusion}$ = 4.752), neither was the quadrant × genotype interaction (F(2.428, 145.708) = 0.502, p = 0.642, $n^2{}_p$ = 0.008; $BF_{exclusion}$ = 137.332) nor genotype main effect (F(1, 60) = 0.262, p = 0.610, $n^2{}_p$ = 0.004; $BF_{exclusion}$ = 10.114).

While there was a significant main effect of quadrant in the second probe trial (F(1.720, 103.180) = 6.319, p = 0.004, $n^2{}_p$ = 0.095) this did not reflect the expected increase in exploration of the target quadrant as Figure 32B shows time in this quadrant was in fact not the most explored. There was no genotype effect on quadrant dwell time (non-significant genotype × quadrant interaction (F(1.720, 103.180) = 0.467, p = 0.599, $n^2{}_p$ = 0.008; $BF_{exclusion}$ = 22.072) and genotype main effect ((F(1, 60) = 0.694, p = 0.408, $n^2{}_p$ = 0.011; $BF_{exclusion}$ = 15.326).

In the final probe test after 10 training days rats also failed to spend increased time in the training quadrant searching for the missing platform, as seen in Figure 32C, despite the main effect of quadrant being significant (F(1.675, 100.471) = 9.483, p < 0.001, $n^2{}_p$ = 0.136). As with the other probe trials there were no genotype effects (non-significant genotype × quadrant interaction (F(1.675, 100.471) = 0.586, p = 0.529, $n^2{}_p$ = 0.010; $BF_{exclusion}$ = 28.530) and genotype main effect (F(1, 60) = 0.332, p = 0.567, $n^2{}_p$ = 0.005; $BF_{exclusion}$ = 14.883).
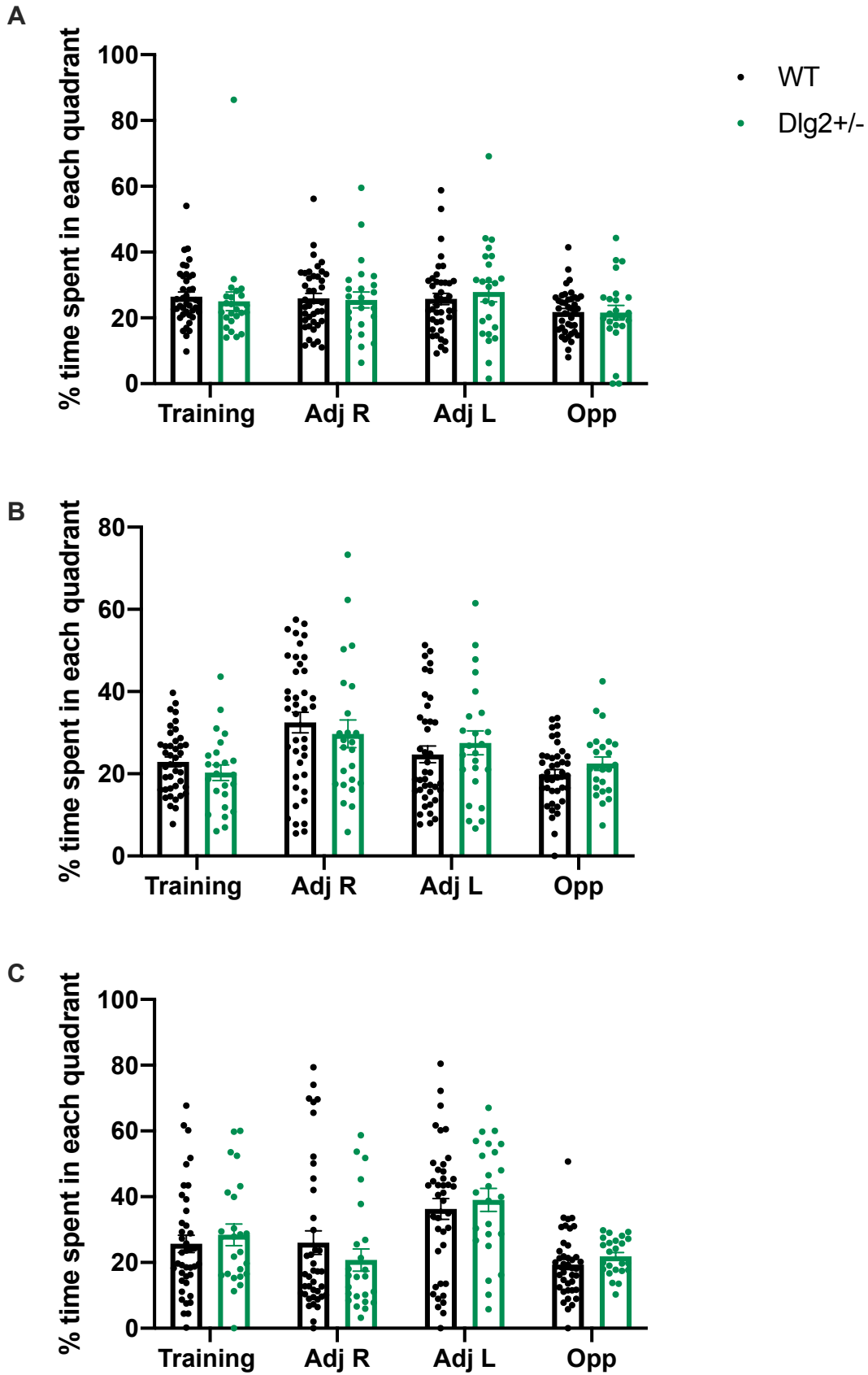
Figure 32: Percentage time spent in each of the four maze quadrants for *Dlg2^+/-^* and wild-type rats on probes 1 (A) 2 (B) and 3 (C). Mean ± SEM plus individual values.

Frequency of annulus crossings (Figure 33A) and latency to first cross (Figure 33B) for each of the three probe tests were analysed by repeated measures ANOVA and Bayesian repeated measures ANOVA with repeated measures factor of probe (1st, 2nd or 3rd probe test) and between subjects factors of sex and genotype. Despite some sex differences all sex × genotype interactions are non-significant, and are shown in Table 34 of the Appendix.

For annulus crossings there was no main effect of probe ($F(2, 120) = 0.611$, $p = 0.545$, $n^2_p = 0.010$; $BF_{exclusion} = 16.592$). There was also no main effect of genotype ($F(1, 60) = 0.078$, $p = 0.781$, $n^2_p = 0.001$; $BF_{exclusion} = 8.403$), not genotype × probe interaction ($F(2, 120) = 0.312$, $p = 0.732$, $n^2_p = 0.005$; $BF_{exclusion} = 79.720$). Thus throughout there were no genotype effects on the accuracy of search for the platform, nor did the number of annulus crossings change across probe tests for rats of either genotype.

ANOVA and Bayesian ANOVA for latency to first annulus cross revealed a main effect of probe ($F(2, 120) = 8.389$, $p < 0.001$, $n^2_p = 0.123$). This likely reflects the fact that rats learned with increasing accuracy where the platform location was as training proceeded, as is also shown by the decreases in escape latency and distance swum over days and trials. There were no genotype effects on this with non-significant main effects of genotype ($F(1, 60) = 3.897$, $p = 0.053$, $n^2_p = 0.061$; $BF_{exclusion} = 2.597$) and genotype × probe interactions ($F(2, 120) = 0.623$, $p = 0.538$, $n^2_p = 0.010$; $BF_{exclusion} = 5.979$). There was a trend towards a main effect of genotype, yet the Bayes factor gives evidence towards a lack of true difference between genotype groups on this measure. Furthermore, as Figure 33B shows, the wild-types are slightly slower than the *Dlg2+/-*s when first crossing the previous platform location indicating that any genotype effect is not due to an impairment in the *Dlg2+/-* rats.
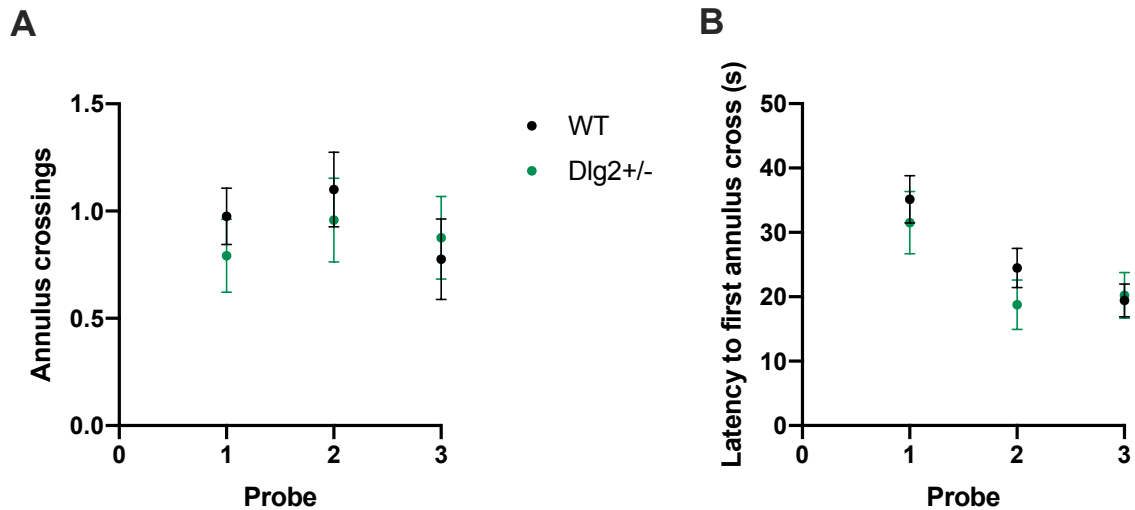
Figure 33: Annulus crossings (A) and latency to first annulus cross (B) across the three probe trials for rats of different genotypes. Mean ± SEM.

Interim Summary

This longer (10-day) version of the reference memory water maze task confirmed that there are no genotype differences in learning the platform location, as measured by latency to reach the platform and the length of swim path to reach it. However even with more training days neither wild-types nor *Dlg2+/-* rats demonstrated evidence of platform location memory by searching in the training quadrant.

Y-maze spontaneous alternation

Continuous trials procedure

Figure 34 shows number of arm entries by maze arm (A, B or C, Figure 34A), total number of arm entries (Figure 34B) and spontaneous alternation (Figure 34C) for *Dlg2+/-* and wild-type rats on the continuous trials procedure.

Repeated measures ANOVA and Bayesian repeated measures ANOVA for number of entries into maze arms A, B and C was carried out to check whether rats had any systematic preference for certain maze arms which would bias the continuous procedure. There was a

significant main effect of maze arm (F(2, 118) = 9.452, $p < 0.001$, n^2_p = 0.138), thus rats did not choose to enter into all arms equally. The main effect of genotype was also significant (F(1, 59) = 6.562, p = 0.013, n^2_p = 0.100), reflecting the fact that $Dlg2^{+/-}$s tended to make more arm entries than wild-types. However the maze arm × genotype interaction was non-significant (F(2, 118) = 1.044, p = 0.355, n^2_p = 0.017; $BF_{exclusion}$ = 1.739).

For all rodents the percentage of spontaneous alternation was higher than chance (50%) as confirmed by a one-sample t-test (t(62) = 4.966, $p < 0.001$, d = 0.626). ANOVA of percentage alternation for the continuous trials procedure revealed a non-significant main effect of genotype (F(1, 59) = 0.443, p = 0.508, n^2_p = 0.007; $BF_{exclusion}$ = 3.474) and sex (F(1, 59) = 0.781, p = 0.381, n^2_p = 0.013; $BF_{exclusion}$ = 3.624) with a significant sex × genotype interaction (F(1, 59) = 5.549, p = 0.022, n^2_p = 0.086; $BF_{exclusion}$ = 2.030). With Bayes factors supporting the lack of sex and genotype main effects and inconclusive for the interaction, this seems to be a random chance crossover interaction with no material difference between wild-types and $Dlg2^{+/-}$s for either sex (Figure 68 Appendix).
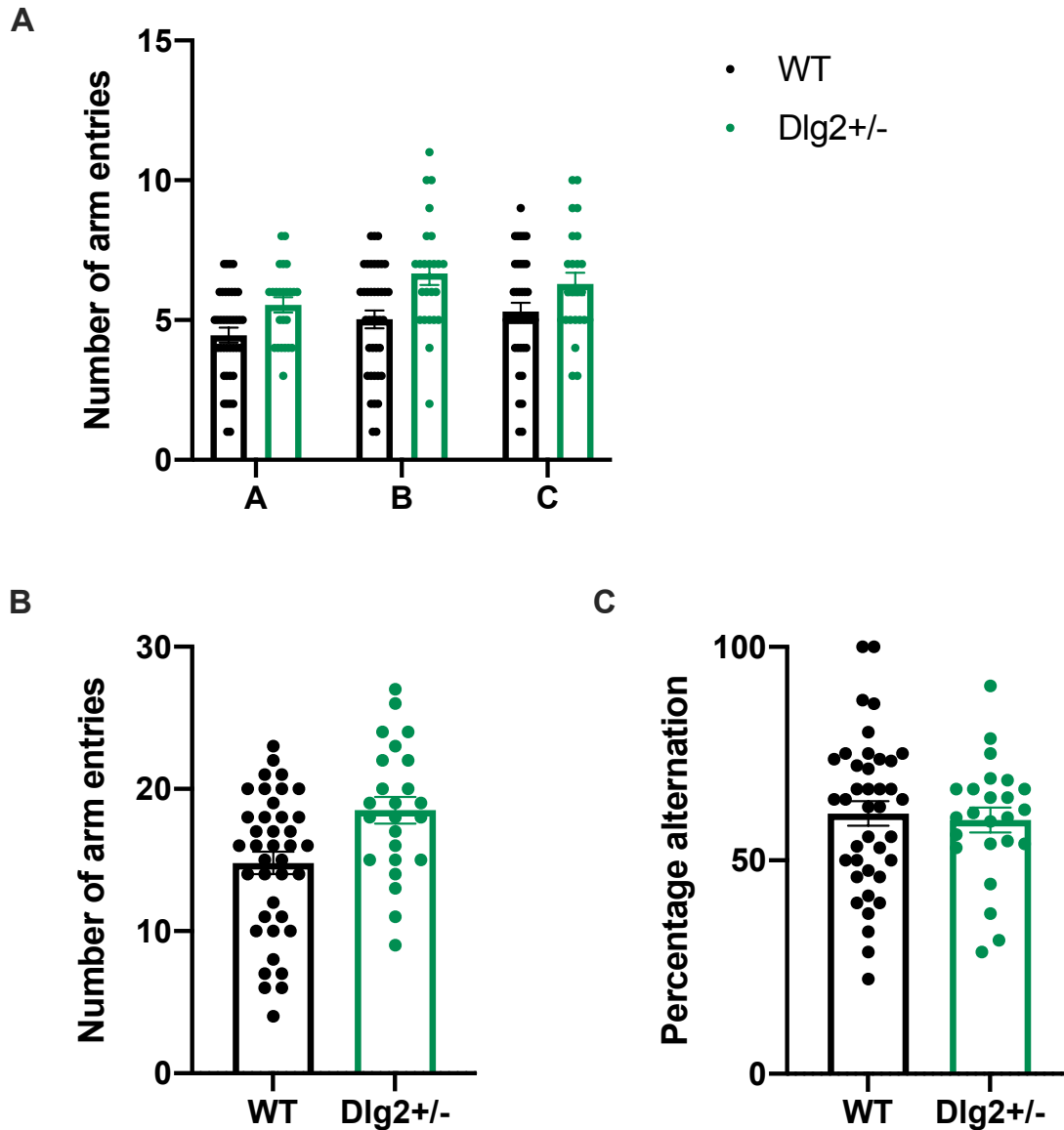
Figure 34: Performance of *Dlg2*^{+/-} and wild-type rats on the continuous-trials Y-maze procedure. Mean ± SEM plus individual values for **A)** number of entries by maze arm **B)** total number of arm entries **C)** spontaneous alternation.

Trial-based procedure

There are no genotype differences in spontaneous alternation on the trial-based procedure at either 1-minute (Figure 35A) or 24-hour (Figure 35B) intertrial intervals. There were no sex effects or interactions found in the trial-based Y-maze procedure as described in Table 38 of the Appendix.

Spontaneous alternation at the 1 minute ITI differed significantly from chance (one-sample t test, $t(63) = 4.848$, $p < 0.001$, $d = 0.606$). ANOVA analysis for the 1 minute ITI showed a non-significant main effect of genotype ($F(1, 60) = 0.283$, $p = 0.597$, $n^2_p = 0.005$; $BF_{exclusion} = 4.805$). At a 24 hour ITI spontaneous alternation was not significantly different from chance for all rats (one-sample t test, $t(63) = 0.475$, $p = 0.318$, $d = 0.059$). ANOVA analysis for the 24 hour ITI found a non-significant main effect of genotype ($F(1, 60) = 0.002$, $p = 0.968$, $n^2_p < 0.001$; $BF_{exclusion} = 34.816$). With Bayes factors supporting the lack of genotype effects, it seems that on the trial-based procedure *Dlg2* heterozygous rats performed comparably to wild-types.
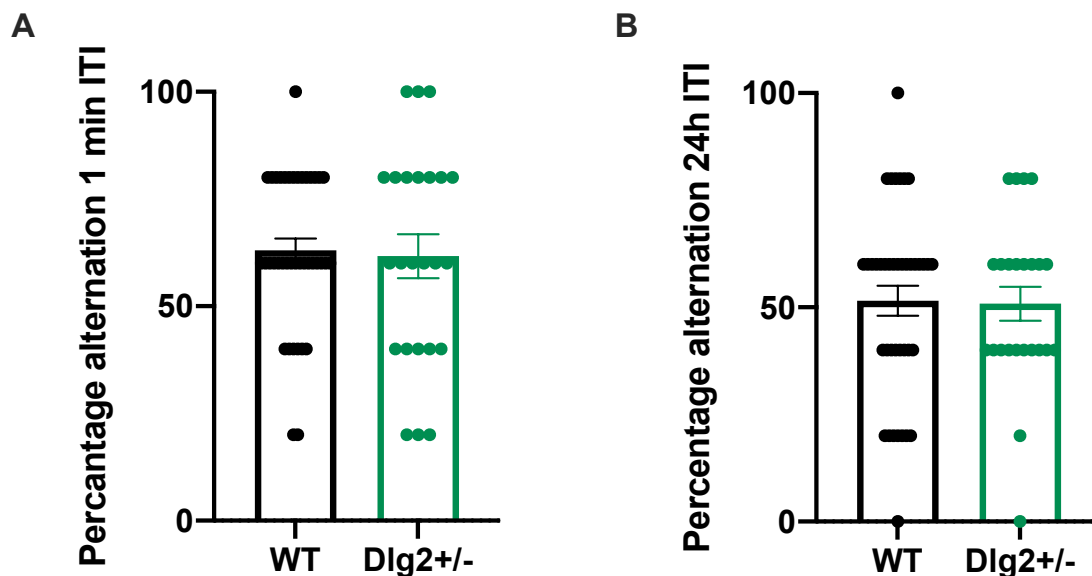
Figure 35: Percentage alternation of *Dlg2$^{+/-}$* and wild-type rats on a trial-based procedure in the Y-maze with an A) 1 minute or B) 24 hour intertrial-interval (ITI). Mean ± SEM plus individual values.

Interim Summary

Despite more arm entries there is no evidence of differences in spontaneous alternation between wild-type and *Dlg2$^{+/-}$* rats on the continuous trials' procedure. The lack of genotype effects holds on a trial-based procedure with a 1-minute intertrial interval, although with a 24h intertrial interval the performance of wild-type and *Dlg2$^{+/-}$* rats is at chance.

4.3.3. Discussion Experiment 2

Adjustments were made to the OIP and reference memory watermaze procedure to highlight potential genotype effects which could not be discounted from Experiment 1. In the OIP, despite having two sample phases in which rats were exposed to object-location associates, discrimination ratios were small at test. Despite this *Dlg2*$^{+/-}$ rats showed the same level of OIP recognition as wild-types. *Dlg2*$^{+/-}$ rats also performed similarly to wild-types on the TOT showing intact recency recognition.

There was no genotype effect on learning the platform location in watermaze reference memory as measured by escape latency or path length when the platform was submerged. However even with 10 days to develop a stable representation of platform location both wild-type and *Dlg2*$^{+/-}$ rats failed to show the expected search behaviour when the platform was removed on probe trials.

Dlg2$^{+/-}$ rats performed as wild-types on Y-maze spontaneous alternation.

4.4 Discussion

The aim of this chapter was to investigate *Dlg2*$^{+/-}$ rats performance on a variety of psychiatric disorder-relevant mnemonic tasks. Of relevance to schizophrenia these were chosen to assess visual learning and memory and working memory MATRICS domains (Young et al 2009). Tasks were also chosen as they are thought to predominantly rely on specific brain regions allowing any impairments in the rat model to be related to neural structures. Many of the tasks are thought to recruit the hippocampus, an area in the *Dlg2*$^{+/-}$ line where electrophysiological experiments have demonstrated disruptions to NMDAR-based neurotransmission and plasticity (Griesius et al., 2022).

The first experiment indicated that *Dlg2*$^{+/-}$ rats performed comparably to wild-types on memory for object identity (NOR) and object location (OL) but that there may be hints towards subtle memory deficits for object-location paired associates in the OIP task. Experiment two clarified that *Dlg2*$^{+/-}$ rats show no deficits in the OIP, with *Dlg2*$^{+/-}$ rats also

showing intact memory for temporal order (TOT). This shows intact memory ability for object identity, location and more episodic-like constructs involving what-when and what-where. In line with work by Barker et al (2007) and Barker and Warburton (2011; 2015) this is a possible indication that medial temporal lobe systems involving the perirhinal cortex and hippocampus, and their interactions with the mPFC are intact in $Dlg2^{+/-}$s. This is in line with the lack of volumetric difference in these structures across genotypes (Chapter 2.3.2.).

However decreased $Dlg2$ gene dosage resulting in PSD-93 reduction in the synapse is likely to have much more subtle effects than lesion or neurotransmitter receptor antagonism studies that have been used to isolate these brain structures as being critical for specific recognition tasks. More subtle impairments in neural plasticity, especially if there are overarching mechanisms of compensation (Griesius et al., 2022), may still allow proficient performance on these tasks.

There is no clear consensus on change to NMDAR associated with PSD-93 alterations. In $Dlg2$ homozygous knockout models no change in NMDAR function has been documented (Elias et al., 2006; Favaro et al., 2018; Krüger, Favaro, Liu, Kitlin´ska, et al., 2013), however Zhang et al., (2010) observed that PSD-93 deficiency in cortical neurons reduces the expression of NR2A and NR2B NMDAR subunits and changes Ca^{2+} influx through NMDARs. No change in NR1 expression in the $Dlg2^{+/-}$ rat was observed (Chapter 2.3.1.) although there is evidence of increased NMDAR currents in the hippocampus of $Dlg2^{+/-}$s (Griesius et al., 2022). Interestingly impairments on OIP, TOT and water maze reference memory were found in NMDA antagonist interventions (Barker & Warburton, 2015b; Van Loon et al., 2016; Warburton et al., 2013), while Griesius et al's (2022) electrophysiological data suggests that $Dlg2^{+/-}$ rats have an increase in NMDA receptor activity. Linearity cannot be assumed and having an increase to a certain process can impact a biological system in a way analogous to loss of components, yet the evidence here indicates that glutamatergic transmission may be sufficient to preserve these memory functions in $Dlg2^{+/-}$s.

Genotype effects on learning the platform location on watermaze reference memory could not be excluded from Experiment 1. Running a longer version of the task with more probe trials clarified that $Dlg2^{+/-}$ rats were just as proficient as wild-types at performing the

reference memory task. Similarly to the intact object recognition performance this is a possible indication that medial temporal lobe glutamatergic function is sufficient in $Dlg2^{+/-}$s to allow task performance, and that more subtle deficits may be expected in a heterozygous model then are present in drug antagonism or lesion models (e.g. Beraki et al., 2008; Moser et al., 1993; Podhorna & Didriksen, 2005b; Stubley-Weatherly et al., 1996). However intact memory ability cannot be confirmed by rodents showing focused search for the platform on probe trials, as neither wild-types nor $Dlg2^{+/-}$s demonstrated this behaviour.

In order to investigate more subtle behavioural deficits there have been recent attempts to move beyond measures such as escape latency and path length to demonstrate spatial acquisition in the Morris water maze (Gehring, Luksys, Sandi, & Vasilaki, 2015; Graziano, Petrosini, & Bartoletti, 2003). These have involved classifying swimming paths into exploration strategies, for example thigmotaxis in the early stages or direct finding (straight paths to the platform) in the later learning stages. Swim paths have been classified in their entirety (Graziano et al., 2003), or to give a more detailed quantification to different learning stages, classified into segments of different behavioural strategies within a single trial (Gehring et al., 2015). The latter method revealed significant and systematic differences in the distribution and composition of exploration strategies across trials used by pubertal stressed and control rats which did not show significant differences in escape latency or path length measures. This approach is open-source and could be applied here to give a more detailed landscape of $Dlg2^{+/-}$ and wild-types task performance.

Long (10 day) and short (4 day) reference memory water maze protocols were used here, neither of which gave the expected increase in time spent in the training quadrant searching for the platform in the probe test. Quadrant dwell time is the most widely reported measure of platform location memory, yet may not be the most sensitive (Maei, Zaslavsky, Teixeira, & Frankland, 2009). Maei et al (2009) compiled data from more than 1600 mouse reference memory probe trials and used Monte Carlo-based methods to simulate experiments with varying sample and effect sizes, discovering that proportion of time in quadrants and number of platform crosses were less effective at detecting group differences than mean proximity to former platform location. These collaborators went on to outline an entropy-based measure to analyse probe trial behaviour which considers how

focused the rodents search is and the degree to which searching is centred on the former platform location (Maei, Zaslavsky, Wang, et al., 2009). This outperformed existing measures in Monte Carlo simulations and was validated in a variety of rodent models of hippocampal dysfunction. Along with semi-automated classification of swimming paths into exploration strategies this provides another measure that could have been used in the present set of experiments to use the richness of water maze positional information to discern subtle behavioural differences between *Dlg2*$^{+/-}$ and wild-type rats.

In their analysis of so many probe trials, Maei et al (2009) observed that search accuracy in control mice peaked at between 10 to 15s and declined thereafter, suggesting that relatively early on in the probe test the mice learned that the platform was absent and shifted search elsewhere. This within-test extinction could have explained the absence of annulus crossing and percentage time in quadrant reflections on platform location memory for the wild types and perhaps also *Dlg2*$^{+/-}$s. The timing of a search accuracy peak likely depends on the rat strain, type of training and experimental set-up, so the control mouse peak outlined isn't best informative of when to look for searching accuracy in the present experiments. Problems of an early shift in search strategy could have been mitigated by training rats to cross the platform multiple times before waiting for removal from the maze, although this presents its own issues with respect to the length of training period used and alteration to other measures such as escape latency and path length.

No genotype effect on spontaneous alternation behaviour in the Y-maze on continuous-trials and discrete-trials procedures was observed. Although continuous-trials procedures have the advantage of decreasing handling stress to the animal these allow the rodent to alternate based on odour trails of its previous arm entries, removing the cognitive component of the task. Thus the agreement between short ITI discrete trials and continuous-trials forms of the task is useful in confirming the absence of alterations in *Dlg2*$^{+/-}$s.

A decrease in delayed alternation after a 1h ITI in the Y-maze, without impairments on the spontaneous alternation continuous trials procedure, has been observed in another schizophrenia and autism relevant CNV mouse model (22q11.2, Tripathi et al 2020). This

was linked to decreased hippocampus-medial PFC LTP in the model (Tripathi et al., 2020), which could (alongside intact OIP performance) bolster the argument that plasticity between these regions is functional in *Dlg2*^{+/-}. Testing an intermediate delay (such as 1h) may be more useful than very short (1 minute) or very long (24h) delays, the latter of which cause a drop off in performance for all rodents. Although what information Y-maze alternation gives about a rodents abilities is hard to discern. Since its conception spontaneous alternation has been linked to stimulus satiation (Glanzer, 1953), curiosity (Dember & Earl, 1957), short-term habituation (Carlton, 1969), foraging strategies (Estes & Schoeffler, 1955) and spatial working memory (Sarter, Bodewitz, & Stephens, 1988).

While this may not be the most relevant task for assessing working memory due to the simplicity of the information required to be kept 'online,' it at least gives a readout of short-term habituation, contextual encoding, and the use of basic spatial information to guide behaviour all of which are shown here to be intact in *Dlg2* heterozygotes. Spontaneous alternation behaviour has been shown to relate directly to hippocampal maturation in longitudinal studies (Dumas, 2004) and experiments with discrete testing ages (Blair et al 2013, Egger et al 1973). Given the important role of *Dlg2* in development (e.g. Chapter 1.3.3., Sanders et al., 2020) different results may be seen if *Dlg2*^{+/-} rats were tested across key timepoints in hippocampal development.

The aim of this chapter was to assess performance of *Dlg2*^{+/-} and wild-type rats on a variety of well-established translational tasks assessing memory, a domain pertinent to the cognitive issues characterising *Dlg2*-CNV related disorders such as schizophrenia, autism and intellectual disability (Millan et al., 2012). On well-established translational tasks broadly related to memory *Dlg2*^{+/-} rats performed comparably to wild-types. Thus, no gross memory impairments manifest in the model, although cognitive symptoms in human disorders have roots in more than memory impairment. For example, MATRICS outlines attention/vigilance, speed of processing, social cognition and problem solving as relevant domains. To rule out the role of PSD-93 in cognitive endophenotypes test batteries that comprise these other cognitive processes would be valuable.

Summary

- *Dlg2$^{+/-}$* rats performed comparably to wild-types when learning to navigate to the platform in training trials of the water maze reference memory task. However intact platform location memory on this task cannot be concluded as rats of neither genotype showed the expected search behaviour on test trials.

- Object recognition tasks were performed comparably between *Dlg2$^{+/-}$* and wild-type rats, showing intact memory of object identity, location, object-in-place and temporal order in the transgenics.

- *Dlg2$^{+/-}$* and wild-types showed comparable spontaneous alternation in the Y-maze.

Chapter 5

Reward processing in *Dlg2*^{+/-} and wild-type rats.

5.1 Introduction

5.1.1. Reward processing and relevance to psychiatric disorder

Anhedonia, a deficit of reward-processing, has been defined as an impaired ability to pursue, experience and/or learn about pleasure (Thomsen, 2015). Symptoms of anhedonia are a core feature of every disorder linked to genetic *DLG2* disruptions. These include depression, ADHD, schizophrenia negative symptoms and the low valuation of rewarding social experiences in autism (DSM-5; American Psychiatry Association 2013). Anhedonia in these conditions is often difficult to treat and contributes heavily to poor quality of life. For example in schizophrenia it has been suggested that negative symptoms contribute more to low functional outcome than positive symptoms (Rabinowitz et al., 2012). The transdiagnostic nature of reward processing deficits makes it a useful candidate to investigate the genetic and biological basis of it through animal models, with the hope of finding common processes that can inspire treatment. Investigating reward processing in a rodent model of a cross-disorder genetic risk is a useful start on this.

As the definition of anhedonia suggests, reward processing can be parsed into several related but dissociable mechanisms. These are hedonic responses ('liking'), motivation ('wanting') and learning comprising value representations, anticipation and decision-making based on reward (Berridge & Robinson, 2003). Hedonic responses have been subdivided further into consummatory responses, relating to pleasure at the point of obtaining a reward, and anticipatory responses which describe pleasure elicited in expectation of reward delivery, the latter of which closely aligns with the idea of 'wanting' (Lewis, Benn, Dwyer, & Robinson, 2019; Martinotti et al., 2012).

Liking, wanting and learning can be distinguished behaviourally, neuroanatomically and neurochemically (Berridge & Robinson, 2003). On the behavioural level it has been

proposed that progressively amplified 'wanting' of substances in the absence of 'liking' characterises drug addiction (Robinson & Berridge, 2013). Physiologically there have been elaborate efforts to functionally map brain hedonic 'hot spots', areas which amplify the 'liking' reaction elicited by pleasurable stimuli in response to certain stimulations (i.e. opioid transfusion) (Peciña, Smith, & Berridge, 2006). Differential treatment of these hotspots, which cluster around limbic structures, can result in dissociations between 'wanting' and 'liking.' For example Castro & Berridge (2014) found that a hedonic hotspot in the nucleus accumbens when stimulated with mu opioids amplified 'liking' reactions to reward, while microinjections to regions outside the hotspot had no effect on 'liking' but amplified 'wanting.'

Despite this separation Berridge and Robinson (2003) describe multiple examples of how liking, wanting, and learning processes interact when responding to reward. These authors and others have described incentive salience, which refers to a motivation for rewards that is driven both by physiological state (e.g. deprivation) and previously learned associations about reward-predicting cues (Berridge, 2016; Berridge, 2012). Once a conditioned stimulus predicts reward it is imbued with incentive salience, and can also elicit approach and consummatory behaviour (Berridge, 2000; Dickinson & Balleine, 2002; Killcross & Blundell, 2002; McClure, Daw, & Read Montague, 2003; Robinson & Berridge, 2003), for example pigeons have been shown to make eating pecks to light cues that predict edible rewards but make drinking pecks for cues that predict liquid reward.

Moreover dopaminergic processes have been described to play little involvement in hedonic responses but be crucial for motivation (Berridge & Robinson, 1998). Despite this, rodents with attenuated dopamine signalling due to dopamine receptor antagonism demonstrate reductions in motivation to work to obtain rewards and a reduction in appetitive hedonic responses to them. The latter has been seen to manifest later, following a series of test sessions (e.g. D'Aquila et al., 2010; Smith & Smith, 2010). This is likely because animals learn the motivational value of the reward has changed which has a knock-on effect on how much the reward is liked. Berridge and Robinson (2003) conclude that the role of specific brain molecules, cells of systems in reward (such as PSD-93 reduction at the synapse) can only be

understood by parsing reward into specific psychological components and probing each of these in turn.

Patterns of impairment across domains of anhedonia will vary both between and within disorder categories. All *DLG2*-associated diseases including schizophrenia (Buckley, Miller, Lehrer, & Castle, 2009), autism (Matson & Nebel-Schwalm, 2007) and intellectual disability (Hurley, 2008) show comorbidity with depression, a disorder defined by deficits in motivation and hedonic processing of reward. Understanding this comorbidity is complex as it is not just that depression may share aetiological processes with these disorders, but a lifetime of struggling with these conditions generates a hardship which could predispose to depression. This is particularly evident in autism where depression becomes increasingly prevalent amongst higher functioning forms of ASD and with increasing age (DeFilippis, 2018). Thus, isolating which components of reward are impaired in these conditions is essential.

In autism it has been proposed that reward dysfunction is critically linked to aberrant social behaviour. The social motivation hypothesis of ASD postulates that an innate decreased reward value of social stimuli leads to less attention to these cues during critical periods of development, resulting in deficient social interaction (Bottini, 2018; Chevallier, Kohls, Troiani, Brodkin, & Schultz, 2012). However, brain imaging studies of ASD patients have found altered activation of brain 'reward hubs,' particularly the striatum, in response to social and non-social rewards such as those associated with patients restricted, repetitive interests (Janouschek et al., 2021). Neural activation does not isolate the psychological components of defunct reward response here. Yet autistic traits in a normal population have been correlated with responses on the Temporal Experience of Pleasure Scale (TEPS) and the Anticipatory and Consummatory Interpersonal Pleasure Scale (ACIPS) questionnaires, which both provide measures of anticipatory and consummatory hedonic responses to social stimuli (ACIPS) and otherwise (TEPS). It was found that ASD traits were negatively associated with reductions in general, and more strongly, social pleasure, pointing to a hedonic deficit in this disorder (Novacek, Gooding, & Pflum, 2016a).

In schizophrenia, a disorder critically linked to the *DLG2* deletion CNV (Rees et al., 2014), research into deficient reward processing has led to what is known as the 'emotion paradox' (Lysaker, Hillis, Leonhardt, Kukla, & Buck, 2014; Strauss & Gold, 2012). This refers to the finding that individuals with schizophrenia report lower levels of trait-like hedonic experience and score more highly on interview-rated measures of anhedonia than controls (Blanchard & Cohen, 2006; Horan, Kring, & Blanchard, 2006), but also report experiencing as much pleasure as controls in response to evocative stimuli in the lab (Berenbaum & Oltmanns, 1992; Kring & Moran, 2008). To account for this it has been proposed that patients have deficits in anticipatory but not consummatory pleasure (Kring, 1999), which has been mostly supported by research using self-report scales that distinguish between present and expected experiences of pleasure (Gard, Kring, Gard, Horan, & Green, 2007). Alternative, but not necessarily mutually exclusive, accounts of the emotion paradox suggests that the core deficit in schizophrenia is the predictive component of anticipatory anhedonia i.e. knowing when a reward will arrive (Knutson, Westdorp, Kaiser, & Hommer, 2000). By either mechanism patients display intact consummatory hedonic responses with impairments in motivation and learning about rewarding stimuli.

The research into schizophrenia provides an example of how distinct reward-related processes can be left defective and intact in psychopathology, and why understanding separable biological and genetic linkages to individual reward-related processes can elucidate disorder aetiology. Given the clinical profiles of *DLG2*-associated disorders a number of hypotheses could be proposed about the reward response profile of *Dlg2*$^{+/-}$ and wild-type rats. Insofar as *Dlg2*$^{+/-}$ models schizophrenia it would be expected that consummatory hedonic responses are intact while anticipatory or 'wanting' based responses show deficits. Alternatively in line with the ASD phenotype *Dlg2*$^{+/-}$ rats may show a generalised deficit of reward comprising reductions in hedonic and motivational components.

5.1.2. Assessment of hedonic response

Attempts to assess hedonic response or 'liking' without the confound of any other reward-related process have focused on measuring sucrose consumption (i.e. the sucrose

preference test), orofacial taste reactivity (TR) reactions and analysing lick microstructure. Each method has potential constraints and caveats. The sucrose preference test involves giving rodents a choice between a rewarding sucrose-containing solution and plain water then measuring consumption of the solutions. Yet using sucrose consumption as a proxy for 'liking' cannot be said to measure consummatory hedonic response in the absence of other aspects of reward processing such as motivation to drink the solution or satiation, and thus is not a reliable hedonic measurement. TR, where evolutionarily conserved facial expressions are analysed in response to pleasant and unpleasant stimuli such as solutions of sucrose and quinine respectively, is a much more sensitive and usefully translational test (Grill & Norgren, 1978). However, TR entails cannulation surgery in order to deliver direct flavour infusions to the oral cavity, which brings about potential confounds in terms of response to the surgical manipulation. Oral cannulation also provides a time constraint of a few weeks on how long responses can be measured and is unsuitable for studies which require hedonic responses to be measured over longer periods or after extended manipulations in a within-subjects design.

This leaves lick microstructure assessment which was carried out here on $Dlg2^{+/-}$ and wild-type rats (described in Chapter 3.1.4.). Lick cluster dissociates from total consumption in that maximum consumption levels occur when palatable solutions are at moderate concentrations yet maximum lick cluster sizes occur when palatable solutions are at high concentrations.

The systematic relationship between the palatability of a solution and lick cluster size suggests that it indicates the hedonic response to the solution consumed. Other evidence for lick cluster size as a hedonic measure comes from the fact that manipulations which influence TR responses cause analogous changes to lick cluster size, for example pairing a palatable taste with LiCl-induced illness results in it generating lick cluster sizes similar to aversive solutions (Baird, St. John, & Nguyen, 2005; Dwyer, Pincham, Thein, & Harris, 2009) and palatable tasted eliciting negative TR patterns (Pelchat, Grill, Rozin, & Jacobs, 1983a). Lick cluster measurements also have translational utility as evidence suggests that foods that are reported to differ in palatability can produce variations in lick microstructure

parameters during human consumption in ways similar variations in consumption by rats (Bellisle, 1989).

Lick microstructure assessment has been useful in probing anhedonia phenotypes in rodent models of psychiatric disorder. Reduced lick cluster size in comparison to control rats to palatable sucrose solutions has been shown in the Wistar Kyoto (WKY) (Wright 2020) and psychosocial cage change stressor (Dwyer 2012) models of depression. This allows for mechanistic interrogation of anhedonia, a key feature of depression, in rodent models. Despite the expectation of intact hedonic responses in rodent models of schizophrenia those targeting specific genetic structures demonstrate reduced lick cluster sizes including the *Cacna1c$^{+/-}$* rat (P. Gasalla, personal communication, September 15, 2021) and *Gria1$^{-/-}$* mice (Austen et al., 2017). Genetic models such as these cannot be expected to model a psychiatric condition in entirety and implicate their specific genetic change with a clinically-relevant phenotype.

In line with the human schizophrenia literature, no change in hedonic response to sucrose was observed in the well-validated sub-chronic PCP rat model of schizophrenia (Lydall et al., 2010). Combining information from the sub-chronic NMDAR antagonism model (Lydall et al 2010) and a genetic AMPAR dysfunction model (e.g. Austen, Sprengel, & Sanderson, 2017) gives mixed predictions about the hedonic profile of *Dlg2$^{+/-}$* rats seeing as PSD-93 interacts with both glutamate receptor types. To understand the specific role of PSD-93 in anhedonia given the cross-disorder and cross-process relevance of this genetic disruption, and the fact that intact hedonic processing is essential for other learning tasks the model could be assessed on, assessing lick microstructure to palatable solutions in the *Dlg2$^{+/-}$* rat is valuable.

5.1.3. Assessment of motivation

There are many established tasks to assess motivation in rodents (for review see Lewis, Benn, Dwyer, & Robinson, 2019). One of the motivational assays first established and most used is the progressive ratio task. This entails training a rodent to lever press for a fixed reward before the number of lever presses (the ratio) required to obtain this reward progressively increases after each reward is received. Motivation to work for reward is

assessed by a rodent's 'breakpoint,' the ratio at which they stop responding (P. A. Randall et al., 2012). Using progressive ratio tasks on rodent models of motivational impairment in schizophrenia and depression have failed to show consistent deficits (Amitai, Powell, & Young, 2019; Leventopoulos, Russig, Feldon, Pryce, & Opacka-Juffry, 2009). This may be because the task does not distinguish between motivational and motor impairments which is necessary when testing genetically or pharmacologically lesioned animals. It is also time-consuming, and task performance may be influenced by habitual responding or impulse control deficits (Salamone, Koychev, Correa, & McGuire, 2015) and the duration of the interval defining the terminal ratio size (Stafford & Branch, 1998), and thus cannot be said to test for 'wanting' alone (Salamone et al., 2015).

An improvement on progressive ratio assays of motivation may be to use a modified version of the task in which a rat must go through a number of epochs of successively increasing fixed ratio reinforcement schedules to obtain reward (Fragale, Beck, & Pang, 2017). This kind of task is quicker to run and lends itself to analyses of motivation and the processing of hedonic value inspired by behavioural economics.

In modified progressive ratio (MPR) tasks rodents go through increasing levels of effort (the price) to obtain a fixed-value reward. This allows a demand curve to be generated, which plots consumption (the number of rewards received) against the fixed ratios that the task advances through. Analysing demand, changes in consumption as a function of price, by curve-fitting data with a demand equation allows parameters relating to motivation and hedonic response of an individual test subject or group to be quantified (Hursh & Silberberg, 2008). A principle of behavioural economics is that consumption is inversely related to price, resulting in demand curves with a negative gradient (Samuelson & Nordhaus, 2010). The rate at which consumption drops off is a measure of the elasticity of demand (Hursh & Silberberg, 2008). The relationship of consumption to price quantifies motivation, in that when motivation to work for the reward is high consumption will be insensitive to price increases and demand inelastic (Fragale et al., 2017; Hursh & Silberberg, 2008).

A well-validated method of analysing changes in demand is by using the exponential demand equation, (Equation 4):

$$log_{10}Q = log_{10}Q_0 + k(e^{-\alpha Q_0 C} - 1)$$

Fitting this equation allows for extraction of the components Q_0 and α (Hursh & Silberberg, 2008). Q_0 is the consumption (or demand) at a theoretical price of 0 and is proposed by Hursch and Silberberg (2008) to be related to the hedonic value of the reinforcer. However, if Q_0 measures consumption when the price is zero it might not be the best metric of hedonic processing. As previously discussed, consumption measures do not give a clear and specific measure of hedonic response as they are susceptible to outside influences such as approach motivation and satiation. Demand elasticity is denoted by α and is inversely related to motivation. Initially this process was used in humans to assess actual and hypothetical reinforcers (Bentzley, Fender, & Aston-Jones, 2013; Reed, Kaplan, Becirevic, Roma, & Hursh, 2016) and compare reinforcers that differ in kind on a single scale (Hursh & Silberberg, 2008).

Fragale et al (2017) used demand curve analysis to understand the motivation and hedonic processing of rodents working for reward in a MPR task. They first validated demand equation fitting to a MPR which used negative reinforcement (escape from foot shock). It was found that α and Q_0 parameters reflected expected changes in motivation and hedonic value across three different shock intensities. Specifically, α decreased as shock increased reflecting increased motivation to escape, and Q_0 increased as shock increased reflecting increased relief at escape from higher shock intensities.

Motivation and hedonic enjoyment of escape from shock and sucrose were then compared in Sprague-Dawley (SD) and WKY rats. WKY rats are depression/anxiety vulnerable and thus were expected to demonstrate an increased motivation for shock cessation and decreased motivation for sucrose reward with no difference in hedonic response compared to SD controls. Supporting this it was found that when negative reinforcement was used α was lower for WKYs than SDs, but with positive reinforcement α was higher for WKYs in comparison to SDs, demonstrating a motivational bias overactive to negative consequences

and underactive to positive consequences in the WKY rodent model. Exponential demand equation fitting was also used by Spiegler, Fortress, & Pang, (2018) in a follow up where SD and WKY rats were tested on a similar MRP procedure that used danger and safety signals to demonstrate that these signals caused differential motivation to avoid shock in WKYs and controls.

Using MPR tasks and demand analysis is preferable to analysing only breakpoints, as the latter gives little information about behaviour at prices other than the breakpoint, while analysing the full demand curve allows a more detailed estimation of motivation across the task (Hursh & Silberberg, 2008). Fragale et al (2017) also quantified breakpoints in the MPR task, defining them as the fixed ratio at which consumption was 0 (where the rat stopped responding) and analysing the attrition of rats completing at least one trial per FR epoch as ratio increased using a survival curve. It was found that this method was less sensitive at demonstrating motivational changes as shock intensity was varied but still displayed the reduced motivational phenotype seen in WKY rats.

Using the analysis of demand curves to quantify differences in motivation and hedonic processing between a rodent model of psychiatric disorder and controls can be applied to *Dlg2*^{+/-} and wild-type rats. To complete this a positive reinforcement MPR was piloted in wild-types to confirm that changes in Q_0 and α reflected changes in the rodents motivational status as elicited by varying reinforcer properties and hunger. Once the optimal motivational manipulation was selected, using 16% sucrose solution as reward under high or low food restriction, the MPR procedure was carried out on *Dlg2*^{+/-} and wild-type rats in both motivational states (2 × 2 design).

Fitting the demand curve to data can be carried out for individual subjects or at the group level for each experimental condition. To embody the 2 × 2 design curves were fit for individual subjects, then parameters Q_0 and α extracted and variation in these across experimental conditions analysed by repeated measures ANOVA. If *Dlg2*^{+/-} rats display reduced motivation relative to controls it would be expected that they show increased α, particularly when less food restricted. The Q_0 parameter from this experiment can be

compared with the results of the lick microstructure assessment of hedonic processing, albeit with consideration of the caveats of using Q_0 as a hedonic measure noted above.

5.1.4. Assessment of reward learning.

Reward learning has been defined as the process by which individuals experience, learn and repeat goal-directed actions that maximise the probability of receiving future rewards (Andre Der-Avakian, Barnes, Markou, & Pizzagalli, 2016). In humans this is typically assessed tasks such as the probabilistic stimulus selection task (PSST) (Frank, Seeberger, & O'Reilly, 2004) and the response bias probabilistic reward task (RBPRT) (Pizzagalli, Jahn, & O'Shea, 2005; Tripp & Alsop, 1999). These tasks both involve decision making based on stimuli with different probabilistic reinforcement schedules. For example, the RBPRT participants must identify which of two ambiguous stimuli (e.g. a short or long mouth on a schematic face) is briefly presented to them to receive monetary feedback. Correct identification of one stimulus is reinforced three times more frequently than the other stimulus, generating in healthy subjects a response bias towards the well-reinforced stimulus. Response bias deficits on this task have been seen in individuals with depression (Pechtel, Dutra, Goetz, & Pizzagalli, 2013; Whitton et al., 2016) and high self-reported anhedonia scores (Pizzagalli et al 2005), but not schizophrenia patients (AhnAllen et al., 2012; Heerey, Bell-Warren, & Gold, 2008). Rodent analogues of these tasks exist, and rodent performance has been shown to vary with dopaminergic manipulations in the same way as human responding has (Der-Avakian, D'Souza, Pizzagalli, & Markou, 2013; Wang, Miura, & Uchida, 2013).

A prerequisite to these tasks entails the ability to learn simple Pavlovian CS-US (i.e. tone-food) and action-outcome associations. The associative learning capability of the *Dlg2+/-* rat model is yet to be sufficiently outlined. There is evidence that *Dlg2+/-* s learn context-shock Pavlovian associations with the same proficiency as wild-types (Pass, 2019) and thus can learn cues signalling negative consequences. As an essential first step in characterising the reward learning of this rat model a Pavlovian discrimination learning task was conducted. This entailed learning which cues predicted food reward and which predicted no outcome. Discrimination learning was chosen as this ability is impaired in human *DLG2* CNV carriers and a *Dlg2-/-* mice (Nithiananantharajah et al., 2013).

Rodents learned two discriminations, one of which involved cues that the rats had been pre-exposed to without consequence. Adding this extension to the discrimination learning task enabled an assessment of latent inhibition by comparing learning of the pre-exposed and non-pre-exposed discrimination cues. Latent inhibition describes a learning effect where pre-exposure to a stimulus without consequence retards subsequent learning about this stimulus. Patients with schizophrenia (e.g. Martins Serra, Jones, Toone, & Gray, 2001), high schizotypy scores (Martins Serra et al., 2001), and depression (Msetfi, Byrom, & Murphy, 2017), and rodent models of these conditions (e.g. Moser, Hitchcock, Lister, & Moran, 2000) have shown a lack of latent inhibition. This has been described as an increased tendency to attend to and learn about irrelevant stimuli in the environment. Such a misallocation of learning ability may result in a world where incorrect associations between stimuli predominate, and stimuli of little consequence take up central cognitive space, resulting in delusional ideology as patients attempt to understand these unusual patterns (aberrant salience theory, Kapur 2003). Reduced latent inhibition may be expected in a schizophrenia-relevant model such as $Dlg2^{+/-}$ and can be assessed alongside appetitive discrimination learning using this preparation.

5.2. Methods

Animals

For piloting the modified progressive ratio task eight female wild-type Sprague Dawley rats were used (PPL 303243, PI Dominic M. Dwyer). These rats had previously been used in a sign and goal tracking task and thus had experience of food pellet rewards and levers but were not trained to act on the lever for reward. Their ad libitum weight before the start of the experiment was 564g (range: 479 – 618 g) and they were maintained at between 85 – 95% of their free feeding weights throughout the food restriction procedure used.

For the lick microstructure assessment and final modified progressive ratio task Cohort 2 was used (for details see Appendix 1). Their mean ad libitum weight before the start of the

experiment was 564g (range: 479 – 618 g) and they were maintained at between 85 – 95% of their free feeding weights throughout the food restriction procedure used.

5.2.1. Lick microstructure assessment

Rats were trained and tested in the sixteen custom-made drinking chambers with contact sensitive lickometers described in Chapter 3.2.4. Rats were trained across five consecutive days for 10 minutes each day to drink 8% sucrose solution from the spouts. During the first session the spout was left to protrude into the cage to encourage drinking, but after this the spout stopped just beyond the opening in the cage to prevent accidental contact. In cases where rats were reticent to drink in the drinking chambers, they were given a five-minute remedial drinking session after all planned drinking sessions had been run that day.

Once all rats were consistently drinking, the test phase began. During test rats drunk both 4% and 16% sucrose solutions for four days each in an order counterbalanced for genotype. Rats were allocated to cages in alternating wild-type/ $Dlg2^{+/-}$ order and the same drinking cage was used for each animal across the experiment. Half of the rats received 4 days of 16% followed by 4 days of 4% and the other half the reverse to implement the counterbalance. The amount of fluid consumed by each rat was measured by weighing the drinking bottle before and after each session. Solutions were made up daily on a weight/ weight basis.

Mean consumption of sucrose (g) and mean lick cluster size for each rat were extracted from the record of licks for analysis. A cluster was defined as a set of licks, each separated by an interlick interval of no more than 0.5 s. This criterion was used by Davis and his co-workers who pioneered this technique (e.g., Davis & Perez, 1993; Davis & Smith, 1992) and in many previous studies employing this assay (notably Austen, Sprengel, & Sanderson, 2017; Lydall, Gilmour, & Dwyer, 2010 for a review, see Dwyer, 2012).

5.2.2. Modified progressive ratio task

Rats were trained and tested in four 30 × 24 × 21 operant chambers (Med Associates, Inc). For this experiment the left chamber wall was fitted with a light (20 cm above the floor) and response lever (3 cm above the floor) between the two apertures used for drinking, and a house light on the opposite side (10 cm above the floor). Sucrose was accessible through drinking spouts attached to 50 ml cylinders, which could be lowered through left or right apertures in one wall of the chamber in an automated fashion. Only the right aperture was used for the current study. Each operant box was housed in a sound-attenuating chamber, and all boxes were controlled by MED-PC software (Med Associated Inc). Only four chambers available and 48 test subjects meant that the experiment had to be run through completely from pre-training to test sequentially for two cohorts of 24 rats.

Although this procedure has been used elsewhere to characterise differences in motivation between rodent strains (Fragale et al., 2017) a pilot ascending FR task was conducted on wild-type rats to specify the conditions that gave the largest change propensity to work for reward. These were different sucrose concentrations (4% and more palatable 16%), varying food restriction and 16% sucrose rewards (restricted to 90% of free feeding weight or given 3× the amount of food given under restriction in g per rat) and varying food restriction as above with sucrose pellet rewards. Findings suggested that the most sensitive contrast was between food restricted and non-restricted rats with 16% sucrose rewards.

$Dlg2^{+/-}$ and wild-type rats were first trained to reliably drink 16% sucrose with one training session across five days. Each session provided 15 minutes of access to sucrose, yet the duration of sucrose access and intertrial intervals were modulated to encourage drinking and acclimatise rats to the five second duration of sucrose access that would serve as a reward in the operant task. All training and test sessions consisted of a number of five-minute epochs. For the first three days rats had access to 3 epochs of 300s sucrose access (day 1), five epochs of 2 × 60 s access separated with a 160s period of no sucrose (day 2), and five epochs of 6 × 20 s access separated with a 55s period of no sucrose (day 3). They then had two days of five epochs with 24 × 5s access separated by a 25s period of no sucrose.

Subsequently, rats were trained to press the lever to receive sucrose rewards over 11 days, with one session a day. All sessions consisted of seven × 5 min epochs (total session length 35 minutes). Firstly, a FR- 1 schedule was used where a single lever press delivered 5s sucrose, followed by an ITI of 20s where the lever was not available. The light above the lever turned on to indicate when it was baited and terminated in the ITI. It took five days of FR-1 to learn to reliably press the lever (> 70 presses, ~ 10 per epoch). Rats were then trained on three days of a VR-5 schedule and three days of a VR-10 schedule to encourage continued pressing for reward. Rats learned to lever press for sucrose reward inconsistently, with a few subjects struggling at each training phase. For struggling rats, the reward ratio on the subsequent epoch was lowered to encourage lever pressing (i.e. from VR-5 to VR-2 or VR-10 to VR-7).

Once rats reliably pressed at each variable ratio rats were tested in an ascending FR schedule. In this procedure the number of lever responses required for sucrose reward was increased every epoch on a quarter logarithmic scale (the number of lever presses required increased in the following manner: 1, 2, 3, 5, 10, 18, 32 as in Fragale et al., 2017). The FR schedule progressed regardless of epoch performance.

During pretraining rats were maintained at 90% free-feeding weight on a food restriction schedule. Rats were then tested with a 16% sucrose reward for four days while food restricted and for four days while not food restricted. Food restriction was counterbalanced for genotype, with half of the wild-types and *Dlg2*^{+/-} rats being restricted first and the other half non-restricted first before this swapped in the repeated measures design.

5.2.3. Appetitive discrimination learning with latent inhibition

Materials

A set of eight chambers were used (23.0 cm × 24.5 cm × 21.0 cm, L × W × H; supplied by Camden Instruments Ltd., UK) arranged in a 4 × 4 array. These consisted of three aluminium walls and an aluminium ceiling. A fourth clear plastic wall served as the door to the

chamber. The floor consisted of a series of stainless-steel rods 0.50 cm in dimeter and 1.5 cm apart (centre-to-centre) below which was a removable tray for cleaning. A food well in the left-hand aluminium wall delivered 45 mg food pellets (TestDiet, Formula 5TUZ). A top-hinged transparent plastic flap guarded access to the food well. Food well entries were automatically recorded when the top-hinged magazine flap was pushed approximately 3 mm. The chamber was sealed in a sound-attenuating outer box for the duration of each trail. A 15 V, 24 W jewel light positioned on the right-hand aluminium wall provided illumination for the trial duration (house light).

Stimuli

Rats learned two discriminations, one auditory and one visual. The auditory stimuli were a 2 kHz tone and a 10 Hz click presented at an intensity of approximately 78 dB by a speaker mounted above the test chamber. The visual stimuli were 3W panel lights mounted on the left wall of the chamber that incorporated the food well, one constant and the other flashing (alternating 0.25 sec on and off). Lights were operated at 24V and mounted 7cm to the left and 7cm to the right of the centre of the food well.

Procedure

A schematic representation of the procedure can be seen in Figure 36. Each rat was pre-exposed to one of the stimulus pairs (tone-click or flashing light-constant light) for 60 non-reinforced pairings in the latent inhibition preparation. Rats received 10 nonreinforced pairings per day for six days over a 40-minute session. Which stimulus pair a rat was pre-exposed to was counterbalanced across genotype, with half of the *Dlg2$^{+/-}$* animals being pre-exposed to flashing-constant and half to tone-click, and likewise for wild-types. To prevent accidental pre-exposure to unintended stimuli from ambient noise between boxes tone-click animals were all run at the same time and flashing-constant rats at the same time. Sessions would start with illumination of the house light, followed by 10 s pseudo-random stimulus presentations with 120 s breaks in between.

Following this, rats were given two days to learn to use the food well. In these sessions rats were given 20 minutes of access to 20 food pellets delivered on a VT 60 s schedule, having to move the plastic flap with their heads to gain access to the well. All rats learned to use the well to receive food.

In the final seven-day test phase of the experiment rats learned both a pre-exposed and a non-pre-exposed discrimination. During these daily 40-minute sessions rats received five presentations of each 10s stimulus (tone, click, flashing and constant light) and 10 rewards contiguous with the reinforced cues. There was a 120 s ITI between stimuli. Which cues for each discrimination (visual and auditory) were reinforced (CS+) and which were non-reinforced (CS-) were counterbalanced for genotype with half of the pre-exposed wild-types having click as a CS+ (tone CS-) and flashing light as a CS+ (constant CS-) and the other half tone as a CS+ (click CS-) and constant light CS+ (flashing CS-) and the same for $Dlg2^{+/-}$s. This also counterbalanced for pre-exposure as half of the wild-types and $Dlg2^{+/-}$s had been pre-exposed to the auditory pair and the other half the visual pair. Stimuli were presented with the same experimental timings as the pre-exposure phase and presented in pseudorandom order.

Pre-exposure

10 X Click
       Tone

➡

Food well training

➡

Training

5 X Click (CS+)
     Tone (CS-)

5 X Constant (CS+)
     Flashing (CS-)
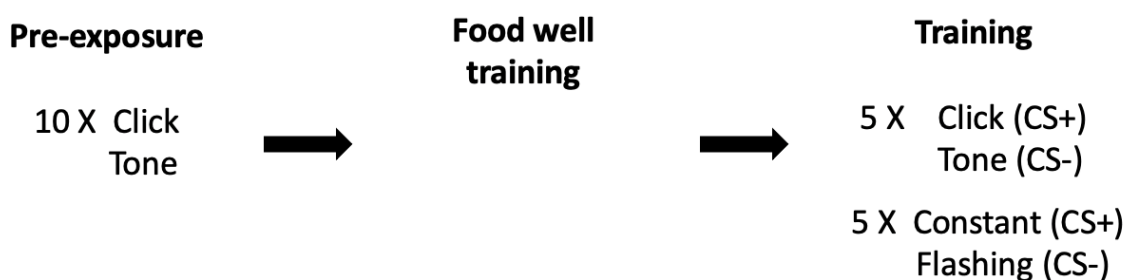
Figure 36: Schematic showing the design of the appetitive discrimination/ latent inhibition Experiment with an example of the stimuli a rat who was pre-exposed to the auditory discrimination would experience. With this preparation the rat would be expected to learn the visual discrimination (seeking food to the constant light CS+ but not to the flashing light CS-) faster than the auditory discrimination.

Data analysis

The number of food well entries (nosepokes) made by the rat for the duration of each stimulus during the seven test days was used to assess acquisition of the two discriminations. A discrimination ratio (below) was used for both pre-exposed and non-pre-exposed discriminations.

$$Discrimination\ ratio = \frac{\Sigma\ (nosepokes\ during\ CS+)}{\Sigma\ (nosepokes\ during\ CS+\ and\ CS-)}$$

A score of 0.50 indicates that the number of nosepokes was the same during the presence of the CS+ and CS-, while scores greater than 0.50 indicate that responding was greater during the presence of the CS+ demonstrating discrimination learning. The raw rates of responding to each stimulus were also collected and analysed for comparison.

5.3. Results

5.3.1. Lick microstructure assessment

Consumption of sucrose was comparable across genotypes (Figure 37A). Repeated measures ANOVA and Bayesian repeated measures ANOVA were used to analyse the test data with genotype as a between subject factor and sucrose concentration (4 v 16%) as a within-subject factor. As Figure 37A shows consumption of sucrose varied with concentration, with rats consuming greater volumes of the 16% solution relative to 4% (main effect of concentration, $F(1,46) = 14.582$, $p = 0.015$, $n^2_p = 0.121$). Genotype had no effect on sucrose consumption as shown by the non-significant main effect of genotype, $F(1,46) = 0.055$, $p = 0.952$, $n^2_p = 0.000$; $BF_{exclusion} = 1.032$. The genotype × concentration interaction was significant $F(1,46) = 7.429$, $p = 0.009$, $n^2_p = 0.139$, reflecting the larger 4-16% based consumption change in the *Dlg2+/-* rats compared to the wild-types as visible in Figure 37. Simple main effects revealed *Dlg2+/-* rats consumed significant more sucrose at 16% than 4% ($p = 0.010$) while wild-types consumption didn't vary with concentration ($p = 0.847$).

Figure 37B shows that lick cluster size also varied with sucrose concentration, as both wild-type and *Dlg2+/-* rats showed larger cluster sizes at 16% than to less palatable 4% (main

effect of concentration, $F(1,46) = 56.074$, $p < 0.001$, $n^2_p = 0.549$). Genotype had no effect on lick cluster size at either concentration (non-significant main effect of genotype, $F(1,46) = 0.642$, $p = 0.427$, $n^2_p = 0.014$; $BF_{exclusion} = 2.531$, non-significant genotype × concentration interaction: $F(1, 46) = 0.002$, $p = 0.965$, $n^2_p = 0.000$; $BF_{exclusion} = 2.774$).

As it was expected that $Dlg2^{+/-}$'s would have lower lick cluster size than wild-types a Bayesian one-tailed independent samples t-test was done on the lick cluster sizes for 4% and 16% conditions, finding evidence for the absence of this expected $Dlg2^{+/-}$ < wild-type effect in both instances (4% $BF_{01} = 7.887$, 16% $BF_{01} = 5.735$).

The variation in both lick cluster and consumption with concentration is expected and informs that the experiment successfully manipulated the hedonic properties of the stimuli. The lack of any reduction in lick cluster size for the $Dlg2^{+/-}$ rats suggests intact hedonic response to stimuli.
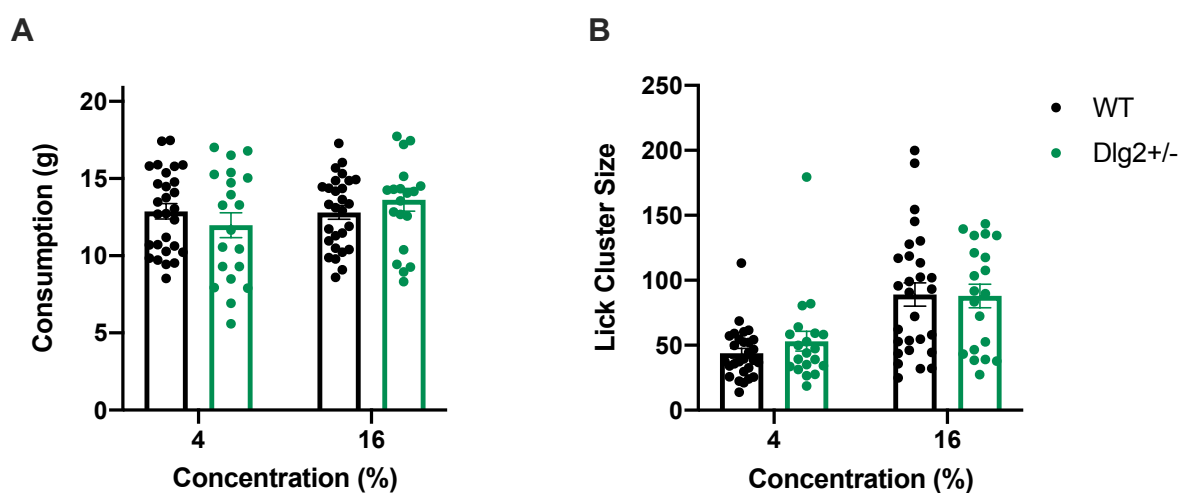
Figure 37: The drinking behaviour of $Dlg2^{+/-}$ and wild-type rats when presented with low and high concentrations of sucrose. Mean ± SEM plus individual values **A)** amount consumed (g), and **B)** lick cluster size.

5.3.2. Modified progressive ratio results

Empirical demand curves

Consumption of rewards as the 'price' (FR) varied was measured as the number of rewards a rat received per epoch. Mean rewards received per epoch indicated how motivated to work for the reward a rodent was and can be used to fit the exponential demand equation to the data and compute the elasticity of demand under different conditions. Mean number of rewards per epoch for each rat was calculated from the last three days of each food restriction condition.

Rewards received per epoch (Figure 38) shows that when more work was required (towards later epochs of the task) the non-restricted rats from both genotype groups obtained fewer rewards, but that genotype doesn't have a large effect on this. Data was analysed using repeated measures ANOVA and Bayesian repeated measures ANOVA with within subjects factors of ratio (1, 2, 3, 5, 10, 18 or 32 lever presses required for reward), restriction (food restricted v non-restricted) and between subjects factor genotype. This found a significant main effect of ratio (F(1.974, 90.792) = 450.445, $p < 0.001$, $n^2_p = 0.907$), and of food restriction (F(1,46) = 29.443, $p < 0.001$, $n^2_p = 0.390$) and ratio × restriction interaction (F(3.324, 152.884) = 5.164, $p = 0.001$, $n^2_p = 0.101$). This shows that rats were less motivated to work for reward at higher ratios and when non-restricted, with lever pressing for reward decreasing more severely at higher ratios when non-restricted compared to when restricted as Figure 38 shows. Thus the task and motivational manipulation were working as intended.

Dlg2 heterozygosity did not influence these motivational effects with no genotype main effect *(F(1,46) = 1.603, $p = 0.212$, $n^2_p = 0.034$; BF$_{exclusion}$ = 4.279)*, ratio × restriction × genotype (F(3.324, 152.884) = 0.583, $p = 0.644$, $n^2_p = 0.013$; BF$_{exclusion}$ > 1000), ratio × genotype (F(1.974, 90.792) = 0.839, $p = 0.541$, $n^2_p = 0.018$; BF$_{exclusion}$ = 22.310) or restriction × genotype *(F(1, 46) = 1.702, $p = 0.198$, $n^2_p = 0.036$; BF$_{exclusion}$ = 4.720)* interactions.
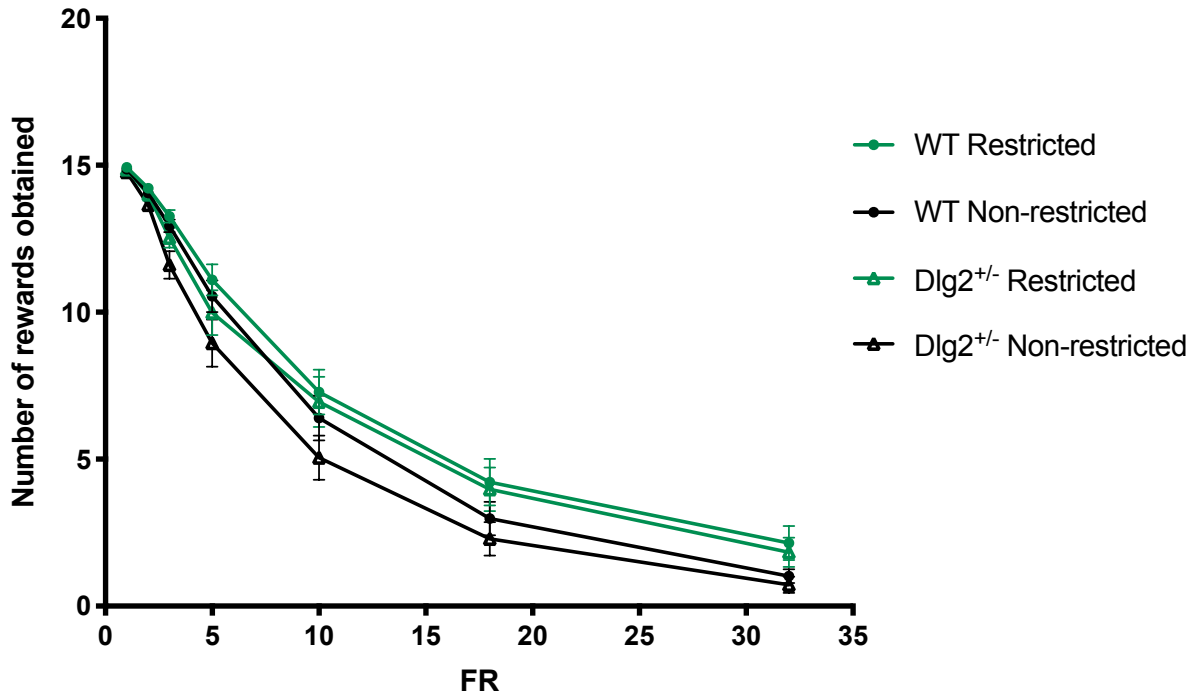
Figure 38: Rewards obtained for *Dlg2^+/-* and wild-type rats at differing food restriction levels as price increases on the MPR task. Mean ± SEM.

Exponential demand curves

In order to derive values of Q_0 and α the exponential demand equation was fitted to lever press data from each individual rat (Figure 39). For the individual fits Q_0 and α were analysed with repeated-measures ANOVA with within-subjects factors of food restriction and between subjects factor of genotype. For α there was a significant main effect of restriction ($F(1, 46) = 20.237$, $p < 0.001$, $n^2_p = 0.306$), validating that this manipulation caused a change in motivation to work for reward. There was no main effect of genotype ($F(1, 46) = 0.729$, $p = 0.398$, $n^2_p = 0.016$; $BF_{exclusion} = 1.791$) nor genotype × restriction interaction ($F(1, 46) = 1.936$, $p = 0.171$, $n^2_p = 0.040$; $BF_{exclusion} = 1.244$).

For Q_0 repeated measures ANOVA gave non-significant main effects of restriction ($F(1, 46) = 0.581$, $p = 0.450$, $n^2_p = 0.012$; $BF_{exclusion} = 5.152$), genotype ($F(1, 46) = 0.849$, $p = 0.362$, $n^2_p = 0.018$; $BF_{exclusion} = 4.024$) and a non-significant genotype × restriction interaction ($F(1, 46) = 0.970$, $p = 0.330$, $n^2_p = 0.021$; $BF_{exclusion} = 10.531$). Bayesian repeated measures ANOVA gave

evidence against all effects as shown. Thus Q_0 of the sucrose reinforcer remained the same for both genotypes across the food restriction schedules.
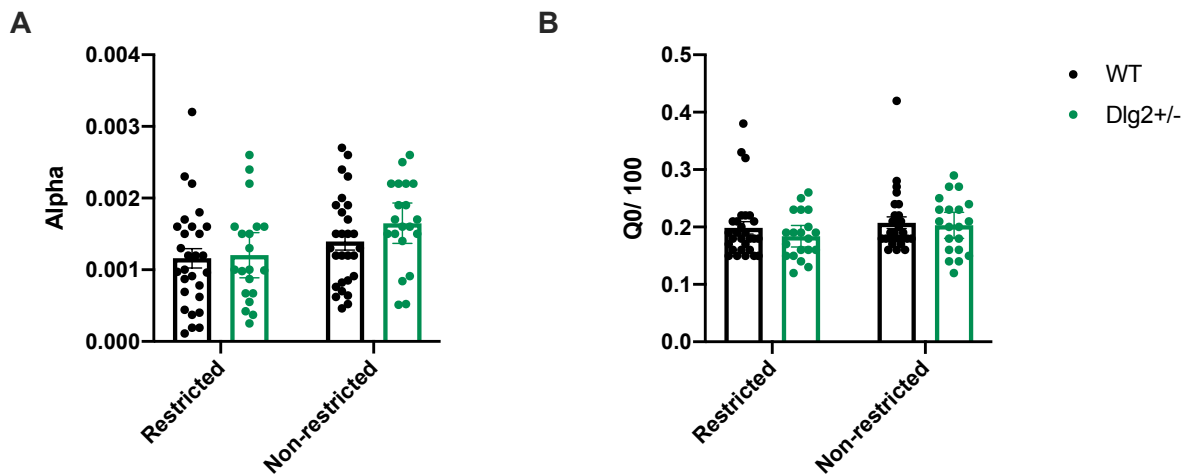
Figure 39: Motivation to consume reward (α) (A) and 'hedonic value' of the reinforcer (Q_0) (B) for *Dlg2+/-* and wild-type rats at both levels of food restriction. Calculated by fitting the exponential demand equation to individual rodent's data and deriving mean value. Graphs show Mean ± SEM plus individual values.

Break point analysis

The effects of genotype and food restriction on break point were investigated as an additional measure of motivation, as has been conducted in previous work using this task (Fragale et al., 2017; Spiegler, Fortress, & Pang, 2017). In line with this, break point was defined as the fixed ratio at which consumption was 0, where the rat stopped responding. These are shown in Figure 40A as survival curves and Figure 40B as summary values plotting the proportion of rats to stop responding at each fixed ratio (their break point) for rodents of different genotypes and food restriction statuses.

In Fragale et al., (2017) break point was analysed by comparison of survival curves with the log-rank (Mantel-Cox) test. This is not appropriate for the repeated measures design used in this experiment as it would treat all conditions as independent. Therefore, a repeated measures ANOVA for break point (FR at which consumption was 0) was carried out with within-subjects factor of restriction and between subject factor of genotype. The main effect of restriction was non-significant ($F(1, 46) = 0.006$, $p = 0.939$, $n^2{}_p = $; $BF_{exclusion} = 6.588$),

as was the genotype main effect ($F(1, 46) = 0.030$, $p = 0.864$, $n^2_p < 0.001$; $BF_{exclusion} = 4.585$) and genotype × restriction interaction ($F(1, 46) = 0.102$, $p = 0.751$, $n^2_p < 0.001$; $BF_{exclusion} = 20.674$). Thus break point defined and analysed in this manner failed to show changes in motivation caused by food restriction unlike the demand equation fitting and raw data analyses reported above – this may well reflect the fact that a large number of animals responded at the highest ratio requirement used here regardless of deprivation condition.
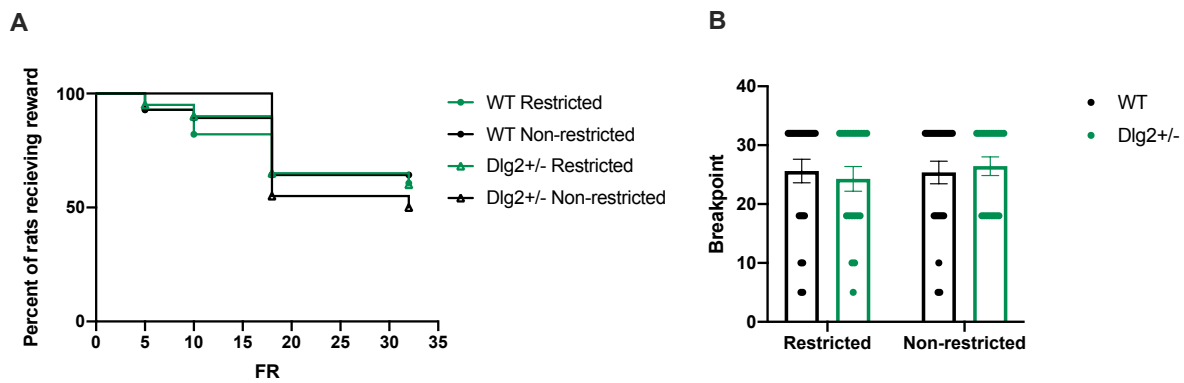
Figure 40: Break point measure for *Dlg2*^+/- and wild-type rats at different levels of food restriction in the modified progressive ratio task. A) Survival curve: for each FR the proportion of rats reaching their breakpoint is represented by a reduction in the survival curve (survival is equivalent to the rats completing at least one trial for the FR). **B)** Mean ± SEM plus individual values breakpoint for rats in different conditions.

5.3.3. Appetitive discrimination learning with latent inhibition

Discrimination ratio (Figure 41A) increases with training across all conditions, with no apparent differences caused by genotype or pre-exposure. Repeated measures ANOVA and Bayesian repeated measures ANOVA with within subjects factors of day (1-7) and exposure (pre-exposed v non-pre-exposed) and between subjects factors of genotype gave a main effect of day ($F(4.913, 226.018) = 4.485$, $p < 0.001$, $n^2_p = 0.089$) reflecting rats learning of the discriminations. There were no main effects of genotype ($F(1, 46) = 0.990$, $p = 0.325$, $n^2_p = 0.021$; $BF_{exclusion} = 13.765$) or day × genotype interactions $F(4.913, 226.018) = 0.986$, $p = 0.426$, $n^2_p = 0.021$; $BF_{exclusion} = 140.337$) showing no genotype effect on speed of learning. Also non-significant were main effects of exposure ($F(1, 46) = 0.158$, $p = 0.693$, $n^2_p =$

0.003;$BF_{exclusion}$ = 21.154), day × exposure (F(4.936, 227.052) = 1.113, p = 0.354, n^2_p = 0.024;$BF_{exclusion}$ = 120.150), exposure × genotype (F(1, 46) = 1.298, p = 0.261, n^2_p = 0.027;$BF_{exclusion}$ = 61.779) and day × exposure × genotype interactions (F(4.936, 227.052) = 0.697, p = 0.624, n^2_p = 0.015;$BF_{exclusion}$ > 1000) showing a lack of slowed learning resulting from pre-exposure, the expected latent inhibition effect, in either genotype group.

Successful discrimination learning by rats of both genotypes is also evident in the raw data for both the non-pre-exposed discrimination (Figure 41B) and the pre-exposed discrimination (Figure 41C). These data were analysed with repeated measures ANOVA and Bayesian repeated measures ANOVA with within-subjects factors of cue (CS+ v CS-), day (1-7) and exposure (pre-exposed v non-pre-exposed) and between subjects factors of genotype. The cue × day interaction (F(4.460, 191.266) = 10.214, p < 0.001, n^2_p = 0.182) and cue main effect (F(1, 46) = 41.444, p < 0.001, n^2_p = 0.474) were significant reflecting rodents learning to respond to the CS+ but not the CS- over training days. The day main effect was non-significant (F(3.136, 144.247) = 1.118, p = 0.345, n^2_p = 0.024;$BF_{exclusion}$ > 1000) as expected, due to the amount of nosepoking remaining constant across days but its allocation starting to vary with learning.

Pre-exposure did not influence learning (non-significant exposure × cue (F(1, 46) = 0.042, p = 0.838, n^2_p < 0.001;$BF_{exclusion}$ = 12.350), exposure × day × cue (F(3.831, 191.266) = 0.241, p = 0.908, n^2_p < 0.001;$BF_{exclusion}$ > 1000) interactions and exposure main effects (F(1, 46) = 0.018, p = 0.895, n^2_p < 0.001;$BF_{exclusion}$ = 1.284) but may have influenced tendency to respond over training days as the exposure × day interaction was significant (F(4.158, 191.266) = 4.305, p = 0.002, n^2_p = 0.086;$BF_{exclusion}$ > 1000). Genotype had no influence on learning as shown by non-significant genotype × cue (F(1, 46) = 1.084, p = 0.303, n^2_p = 0.023;$BF_{exclusion}$ = 4.424), genotype (F(1, 46) = 0.175, p = 0.678, n^2_p = 0.004;$BF_{exclusion}$ = 1.119), genotype × day × cue (F(4.460, 191.266) = 0.660, p = 0.637, n^2_p = 0.014;$BF_{exclusion}$ > 1000) and genotype × day (F(3.136, 144.247) = 0.133, p = 0.946, n^2_p = 0.003;$BF_{exclusion}$ > 1000) effects. Finally genotype and pre-exposure did not interact to alter discrimination learning with non-significant genotype × exposure × cue (F(1, 46) = 0.622, p = 0.434, n^2_p = 0.013;$BF_{exclusion}$ = 32.130), genotype × exposure × day × cue (F(3.831, 191.266) = 0.335, p = 0.847, n^2_p = 0.007;$BF_{exclusion}$ > 1000), genotype × exposure × day (F(4.158, 191.266) = 0.892, p = 0.473, n^2_p =

0.019;$BF_{exclusion} > 1000$) and genotype × exposure ($F(1, 46) = 1.479$, $p = 0.230$, $n^2_p = 0.031$;$BF_{exclusion} = 0.180$) effects.

These results demonstrate that appetitive discrimination learning is not impaired in the *Dlg2*$^{+/-}$ rats. Conclusions about latent inhibition cannot be drawn as the wild-type rats do not show the expected decrease in learning rate with pre-exposure.

A

Discrimination ratio

B

Non-pre-exposed

C
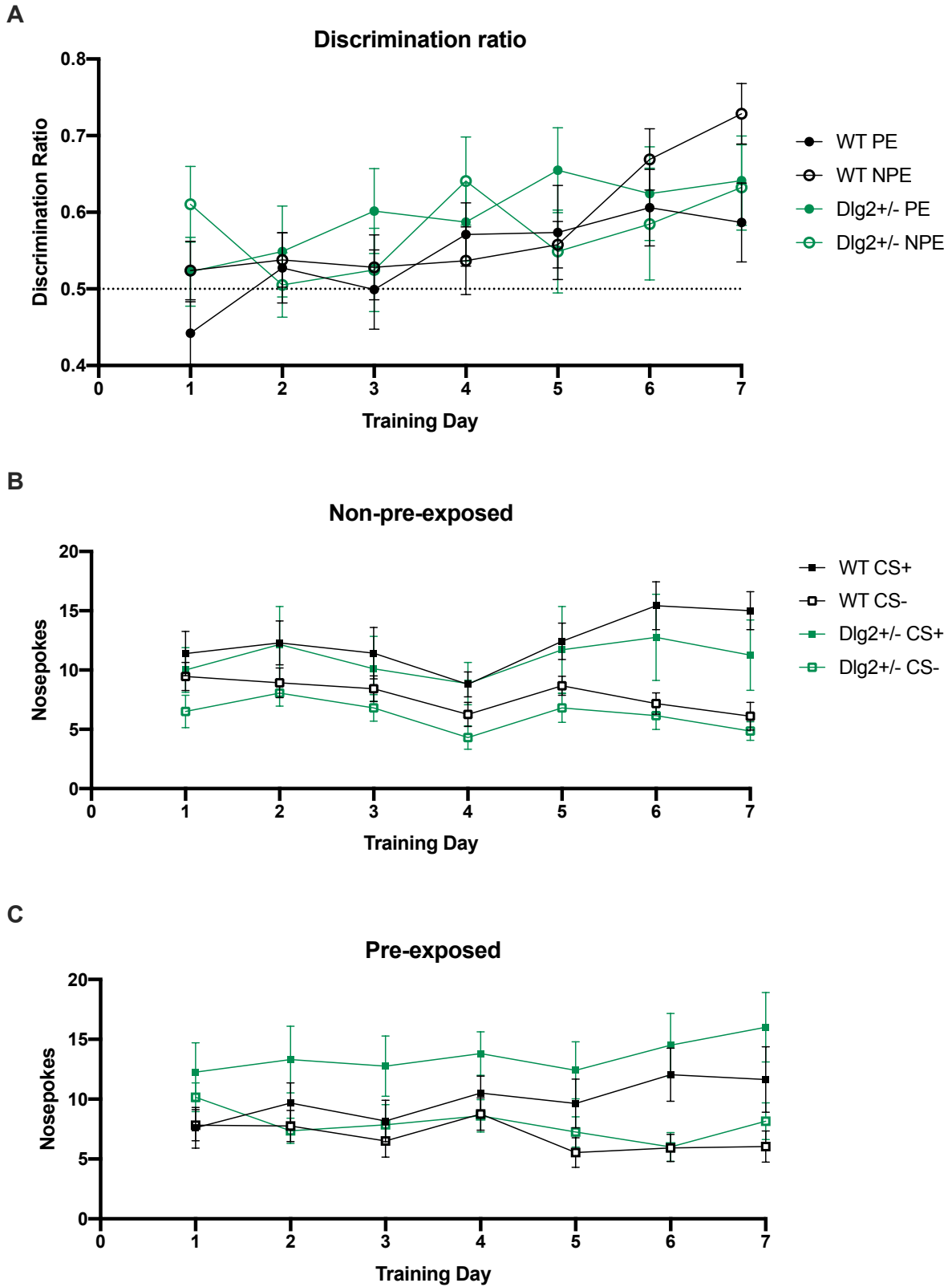
Pre-exposed

Figure 41: Appetitive discrimination learning by *Dlg2⁺/⁻* and wild-type rats over seven training days. Mean ± SEM **A)** discrimination ratio **B)** raw nosepokes to CS+ and CS- for the

non-pre-exposed discrimination **C)** raw nosepokes to CS+ and CS- for the pre-exposed discrimination.

5.4. Discussion

Dlg2^{+/-} and wild-type rats were subjected to a lick microstructure assessment intended to assess consummatory anhedonia, a modified progressive ratio task assessing motivation to work for reward and an appetitive Pavlovian discrimination task to study basic reward learning competency. It was found that *Dlg2*^{+/-} rats showed the same hedonic assessment of sucrose as wild-types on the lick microstructure task, giving evidence against anhedonia phenotypes in this line. On the modified progressive ratio task analysis of the raw data, α (motivation) parameters, and breakpoint-based analysis did not demonstrate a genotype-based difference in the amount of lever pressing to obtain reward at each fixed ratio. Finally, unlike human *DLG2* deletion CNV carriers and *Dlg2*^{-/-} mice (Nithianantharajah et al., 2013) *Dlg2*^{+/-} rats were able to learn to discriminate between CS+ and CS- cues.

There was no evidence of a dissociation like that seen in the 'emotion paradox' of schizophrenia (Gard, Kring, Gard, Horan, & Green, 2007) in these rodents, which would have manifested as intact hedonic response to stimuli with impaired motivation to work to receive them. This is unsurprising given the broad set of conditions *Dlg2* haploinsufficiency is associated with and may indicate that the *Dlg2*^{+/-} rat cannot be considered a model of these reward-related processes schizophrenia. However reward related deficits in ASD and ID are also not mirrored in the *Dlg2*^{+/-} model which shows no clear anhedonia or amotivation, indicating that PSD-93 specifically may be disconnected from reward processing phenotypes of psychiatric disorder.

The MPR task is novel and the assessment of motivation to work for reward in *Dlg2*^{+/-} and wild-type rats could be bolstered with other tasks tapping into reward-related processes in rodents. One candidate is the effort- based choice task, in which rodents must choose between a freely available but smaller or less palatable food reward compared to a larger or more preferred reward that the rodent must expend extra effort to receive. This approach has been validated with rodent models of depression (Nunes et al., 2013) and with

schizophrenia patients (Green, Horan, Barch, & Gold, 2015; Strauss et al., 2011), allowing for a continued translational approach. Comparisons in findings between these motivation tasks and others would be useful to triangulate amotivation in rodents and validate the MPR as a useful measure amongst these.

Furthermore, the breakpoint measure used by Fragale et al (2017) and Spiegler, Fortress, & Pang, (2018) may not be the most sensitive for qualifying differences in motivation, as in the pilot study it did not differentiate between motivational conditions that showed significantly different α values from curve fitting. Fragale et al (2017) note that as the MPR task is not specifically set up for measuring breakpoint it is unlikely to be a very sensitive as a measure. It differs from true breakpoint on progressive ratio in a crucial manner, in that breakpoint on progressive ratio is the point where rodents stop responding while on the MPR it is the last point at which rodents respond, thus these two measures may not relate well to each other. Validating the MRP breakpoint measure by seeing how well this correlates with breakpoint on traditional progressive ratio tasks would be informative and help verify whether this is a useful addition to curve fitting from MPR data.

Interestingly there is evidence of humans with ASD being more willing to expend effort to obtain a monetary reward in an effort for reward task (Damiano, Aloi, Treadway, Bodfish, & Dichter, 2012). Although participants with ASD chose the more difficult task more frequently than controls, their behaviour was less influenced by differences in reward value and probability. It is proposed that this inefficient strategy to obtaining reward may hinder the ability of individuals with ASD to optimally engage or disengage with their environment, contributing to a tendency towards prescribed, repetitive interests (Damiano et al 2012). Assessing *Dlg2+/-* rats on such a task may be more relevant than those outlined here to investigate ASD-relevant phenotypes. In addition, increased effort based decision making was related to repetitive behaviour symptoms across ASDs and controls (Damiano et al 2012). This coherence could be tested for in rodents using markers of repetitive behaviour such as grooming or marble burying, and is of interest in the *Dlg2+/-* line given that increased repetitive behaviour is seen in *Dlg2-/-* mice (Yoo et al 2020).

The ability of *Dlg2*^{+/-} rats to learn operant responses for reward as a component of the MPR task and to learn which cues predict reward on a Pavlovian task demonstrates intact reward learning at a basic level. This provides a framework to assess *Dlg2*^{+/-} rats ability on more complex assays of motivation and reward learning such as the rodent versions of RBPRT and PSST. These tasks not only involve learning about reward, but entail cognitive control, flexibility and decision-making strategies needed to optimise performance for stimuli which are the most likely to result in rewards most often. Accordingly, they recruit brain regions associated with these functions including the anterior cingulate, orbitofrontal cortex, and dopaminergic striatal systems (reviewed in Der-Avakian et al 2016). There are reported abnormalities in these brain regions in schizophrenia (e.g. Adams & David, 2007; McCutcheon, Abi-Dargham, & Howes, 2019; Nakamura et al., 2008), alongside findings of impaired cognitive control in both schizophrenia (Lesh, Niendam, Minzenberg, & Carter, 2011) and ASD (Poljac & Bekkering, 2012) making them good assays to assess these processes in relation to reward in rodent models.

A study comparing reward-related decision making in schizophrenia and ASD found disorder-specific abnormalities in this process. The Iowa Gambling Task (IGT) was used which assesses decision making under uncertainty by measuring participants preference for choosing from four card decks. Participants must learn which decks are overall disadvantageous (coupling large gains with larger losses) or advantageous (coupling small gains with even smaller losses). Two packs are advantageous and two packs disadvantageous, with a further stratification such that within advantageous and disadvantageous packs one deck will include high-frequency and low-magnitude gains/losses and one low-frequency and high-magnitude gains/losses. Both ASD and schizophrenic participants were impaired on the IGT but through different mechanisms. It was found that participants with ASD demonstrated more sensitivity to the magnitude of loss then the frequency of loss, while schizophrenia patients showed more sensitivity to frequency of loss rather than the magnitude (Zhang et al., 2015). Multiple rodent versions of the IGT are available (Pais-Vieira, Lima, & Galhardo, 2007; Rivalan, Ahmed, & Dellu-Hagedorn, 2009; Van Den Bos, Lasthuis, Den Heijer, Van Der Harst, & Spruijt, 2006; Zeeb, Robbins, & Winstanley, 2009) with strong similarities between the human and animal data (De Visser, Baars, Lavrijsen, van der Weer, & van den Bos, 2011; de Visser et al., 2010; van

den Bos, Homberg, & de Visser, 2013; Van den Bos, Jolles, Van der Knaap, Baars, & De Visser, 2012). Testing *Dlg2*^{+/-} rats in such a way could isolate general processes that *Dlg2* deficiency leads to which could contribute to either the schizophrenia or ASD performance profile on the task.

Also using the IGT Saperia et al., (2019) demonstrated that participants with schizophrenia have a reduced tendency towards win-stay strategies while completing the task, although propensity towards lose-shift strategies is intact. This was related to deficits of motivation and cognition in patients, indicating that within disorder deficits may not subscribe to the 'liking, wanting, learning' framework (Robinson & Berridge 2003) and instead transpire in the interactions between domains. This proposes another argument for assessing rodents on more complex reward-learning tasks which require greater integration of information, although using such tasks makes parsing out exactly which psychological processes are altered by *Dlg2* haploinsufficiency increasingly difficult. Using a probabilistic reversal task there is evidence that *Dlg2*^{+/-} rats perform both win-stay and lose-shift strategies with competence while learning initial contingencies yet display an increased tendency towards lose-shift strategy in reversal learning (S. Griesius, personal communication, April 13, 2021). Replication of this effect and interrogation of its relation to other cognitive deficits would be valuable.

In this work the processing and motivation to work for primary food rewards was assessed. Many of the disorders *DLG2* disruptions are associated with have disruption in responding to social reinforcement as a key feature. Using smiles as a reward, Catalano et al., 2018 found that individuals with schizophrenia treated genuine smiles as significantly less rewarding than healthy participants. This could be a precipitating factor in the social withdrawal that characterises schizophrenia (Cullen et al., 2011). Furthermore reduced social hedonic capacity characterises autism and is associated with autistic traits in the general population, indicating that it is a key feature of the broader autistic phenotype (Novacek, Gooding, & Pflum, 2016b). However, *Dlg2*^{+/-} rats demonstrated intact performance on the social preference task (Chapter 3.2.5.) indicating that they also show intact responding to this kind of reward, although this task is unable to specify whether the hedonic quality of interacting with a conspecific was comparable across genotypes.

Conclusions about the latent inhibition learning of *Dlg2*^{+/-} rats cannot be drawn as the expected pre-exposure effect was absent from the wild-types. In the design rodents were not habituated to the experimental chambers before the pre-exposed stimuli were presented in them. This may have resulted in the rodents spending time exploring the new environment during pre-exposure rather than attending to the cues they were being shown. It is also possible that the number of presentations of stimuli during preexposure was not sufficient to induce latent inhibition, although these were designed by consulting literature on latent inhibition designs using the same stimulus modalities (e.g. Channell & Hall, 1983; Grecksch, Bernstein, Becker, Höllt, & Bogerts, 1999; Randall & Kraemer, 1992). The number of presentations required for learning could be rat line specific, as there is evidence that *Dlg2*^{+/-} rats and their wild-type counterparts require larger shock intensities for fear conditioning (Pass, 2019).

The experimental design and cues used may have also contributed to a lack of latent inhibition in learning about the pre-exposed discrimination. As rats were pre-exposed to either a flashing light and constant light or the click and tone, stimuli may have been sufficiently similar to induce perceptual learning of the differences between them based upon latent inhibition to common stimulus elements only (Honey & Bateson, 1996; Symonds & Hall, 1995). This could be expected to facilitate learning about the pre-exposed discrimination, which was not seen in *Dlg2*^{+/-} or wild-type rats, but at very least could account for the lack of pre-exposure effects. A better design would have been to expose rats to stimuli of different modalities (e.g. the click and flashing light) and then train two discriminations (e.g. click-flashing light and tone-constant light) to circumvent this issue.

This series of Experiments gives conclusive evidence for a lack of hedonic deficits and issues with appetitive Pavlovian learning in *Dlg2*^{+/-} rats. There is some evidence of a lack of amotivation, although this would benefit from corroboration using other suitable tasks. The next logical step would also be a more thorough interrogation of reward learning and reward-based decision-making capabilities. These require the ability to learn complex contingencies under various contexts, a process which forms the basis of the reality testing Experiment in Chapter 6.

Summary

- Lick microstructure assessment demonstrated intact hedonic response to palatable stimuli in *Dlg2+/-* rats.

- Assessing lever pressing for a reward at increasing fixed ratios demonstrated no difference in motivation to work for reward across genotypes. This was also seen when the exponential demand equation was fitted to individual task performance to analyse group differences in the parameters α and Q_0 which have been related to motivation and hedonic response to stimuli respectively (Fragale et al., 2018).

- *Dlg2+/-* rats showed intact discrimination learning in an appetitive conditioning paradigm.

Chapter 6

Assessment of reality testing ability in *Dlg2*^{+/-} and wild-type rats

6.1 Introduction

DLG2 genetic disruption has been frequently associated with schizophrenia (Kirov et al., 2012; Nithianantharajah et al., 2013) and bipolar disorder (Noor et al., 2014). Key positive symptoms of these psychotic conditions comprise reality monitoring deficits such as hallucinations and delusions. Hallucinations occur where the patient experiences sensory percepts with no endogenous stimulation in an awake state, with no voluntary control (Aleman, De Haan, & Liester, 1998; Slade & Bentall, 1988). This involves all sensory modalities with prevalence differing depending on the modality involved. The lifetime prevalence for auditory hallucinations in schizophrenia is around 64-80%, for visual hallucinations 23-31%, for tactile 9-19% and 6-10% for the olfactory domain (McCarthy-Jones et al., 2017). Delusions entail false beliefs that are held with a high degree of certainty. These have been estimated to persist chronically following a first psychotic episode in slightly over half of schizophrenia patients (Harrow & Jobe, 2010). There are close connections between these symptom domains, with some suggesting they may be different labels for the same experiences (Fletcher & Frith, 2009). The blurring of the distinction between hallucinations and delusions can be seen in the case of delusions of control, where someone believes their actions are caused by outside influences. Here the patient is 'experiencing' their actions being caused externally, yet this also can be defined as an aberrant belief.

To understand hallucinations and delusions it is important to consider how in the healthy brain sensory evidence might provide for an accurate representation of the world. The predictive coding hypothesis is predicated on the idea that all organisms must build an internal representation of the world based on inferences about noisy, incomplete, and ambiguous sensory evidence (Fletcher, 2017). This is achieved by finding a dynamic balance between incoming sensory data (bottom-up processes) and inferences based on prior knowledge and experience (top-down processes, Rao & Ballard, 1999). By extension an

over-reliance on understanding the world based on what is expected rather than accurately responding to incoming signals tips the balance of conscious experience towards a self-created world.

Over-reliance on a model of the world over current sensory input has been proposed to underly positive symptom pathology in psychosis, explaining both hallucinations (expected percepts) and delusions (a biased interpretation of reality) by this mechanism (Horga, Schatz, Abi-Dargham, & Peterson, 2014; Katharina Schmack et al., 2013; Katharina Schmack, Rothkirch, Priller, & Sterzer, 2017; Teufel et al., 2015). This defines hallucinations and delusions as a maladaptive extreme along a continuum of normal functioning as opposed to a distinct pathological process (Powers, Kelley, & Corlett, 2017). Thus, the existence of hallucinations and delusions at lower rates in the general population (Freeman, 2006; Vellante et al., 2012) is within the purview of such an explanation.

Overweighing expectations when making sense of perceptual input leads to failures in the ability to distinguish internal representations of the world from external representations (reality). Consequently, the ability to differentiate these representations can be assessed in humans (e.g. Powers, Mathys, & Corlett, 2017; Stuke, Kress, Weilnhammer, Sterzer, & Schmack, 2021; Valton et al., 2019). A bias towards remembering imagined events as having actually occurred has also been reported in individuals with schizophrenia using the 'hear-imagine' reality monitoring task where participants are challenged to imagine listening to and listen to a series of words before recalling which were heard and which were imagined (Brunelin et al., 2006; Brunelin et al., 2007). Patients with auditory hallucinations misattributed more words they had imagined as being heard (Brunelin et al., 2006), with patients that experience visual and auditory hallucinations demonstrating this to a greater extent than those who just hallucinate in the auditory domain (Mondino, Dondé, Lavallé, Haesebaert, & Brunelin, 2019). Externalisation bias of this kind has also been reported by Powers, Mathys, & Corlett, (2017). Here researchers repeatedly paired a tone and visual stimulus together, then demonstrated 'conditioned hallucinations' where participants reported hearing the tone in response to the visual stimulus alone. Within the participant group patients with auditory hallucinations reported hearing these conditioned hallucinations more frequently and with higher confidence than controls.

This investigation relies on Pavlovian associations between two stimuli (here a tone and visual stimulus). This generates internal memory representations of the stimuli and their relationship, whereby presentation of one stimulus results in the activation and 'experience' of another (an associatively activated representation). In the case of a conditioned hallucination the presence of the visual stimulus activates the representation of the tone with such detail that it is reported as an external event, with the top-down bias for learned information determining perception being greater in those with a propensity towards hallucinations in this sensory domain. Similar investigations using Pavlovian conditioning can be performed in animal models of disorder (Busquets-Garcia et al., 2017; Koh, Ahrens, & Gallagher, 2018; McDannald et al., 2011), providing the first translational test of positive symptoms which are so definitional of psychosis, but which thus far have been intractable in rodent models (Dwyer, 2018; Koh & Gallagher, 2020; McDannald & Schoenbaum, 2009).

Associatively activated stimulus representations can support learning if paired with a consequence (mediated conditioning, Holland, 1981, 1990) comprising learning about a stimulus that is absent but recalled through Pavlovian associations. The greater the similarity between the associatively activated representation and the veridical stimulus the better mediated conditioning should be supported. Holland (1998) has proposed that in healthy subjects' similarity between associatively activated representations and external stimuli varies as a function of training. With few pairings between two stimuli (e.g. a flavour and a tone) the representations activated by either stimulus alone are perceptual, similar to experiencing the real stimuli, while with more pairings representations become conceptual and abstract, more the idea of the flavour than its taste.

Perceptual representations are expected to support mediated conditioning while conceptual representations are not. In a mediated conditioning design Holland, (1998) gave rats 16 pairings of an auditory stimulus (CS1) and a flavoured sucrose solution unconditioned stimulus (UCS1) and 64 pairings of a different sound (CS2) followed by a distinctly flavoured sucrose solution (UCS2). In a manner counterbalanced across subjects either the lightly trained stimulus (CS1) or the extensively trained stimulus (CS2) would then be paired with LiCl-induced nausea, and consumption of the two flavours (UCS1 and UCS2)

was tested. Mediated conditioning was shown for the lightly trained relationship (CS1-UCS1) with rodents who experienced nausea paired with CS1 consuming less of UCS1. This effect was not seen for the extensively trained relationship (CS2-UCS2) where rodents did show evidence of learning CS2-UCS2, but did not show mediated conditioning, consuming similar amounts of both flavoured solutions under these circumstances.

To evidence that this mediated conditioning effect results from a transition in stimulus representation from perceptual to conceptual Holland, Lasseter, & Agarwal, (2008) evoked a similar procedure with a taste reactivity (TR) measure. Taste reactivity responses are physical reactions rodents make upon fluid consumption which reflect the motivational or evaluative attributes of those fluids (Grill & Norgren, 1978). Preferred substances such as sucrose elicit positive TRs (e.g. tongue protrusion) while less palatable substances such as concentrated salts elicit negative TRs (e.g. gapes). These responses are tied to the evaluative property of the solution rather than their sensory properties, for example if sucrose is paired with LiCl the positive TR is replaced by a negative one (Berridge, Grill, & Norgren, 1981; Pelchat, Grill, Rozin, & Jacobs, 1983b).

Holland et al (2008) gave rats 16 or 112 tone (CS)-sucrose (US) pairings. The value of the sucrose was then either maintained or reduced by pairing with LiCl-induced nausea. Responding to unflavoured water in the presence of the tone CS was then assessed for maintained and reduced groups at both 16 and 112 pairings. Few CS-US pairings resulted in negative TR responses in the presence of the tone for the reduced group and positive TR responses for the maintained group, showing the rats responded to the CS in a manner consistent with drinking sucrose. These taste reactivity responses were absent in the overtrained 112 pairings group. This shows that with overtraining, where representations should be conceptual, there was less perceptual similarity in associatively evoked representations to actual percepts required to support TR responses.

The fact that the representations generated by mediated conditioning paradigms are sensory in nature is also supported Kerfoot, Agarwal, Lee, & Holland, (2007). Using a similar tone-sucrose design as Holland (1998) researchers demonstrated that when mediated conditioning occurred rodents presented with the tone alone would show greater

immediate early gene c-fos activity in the taste area of the insular cortex than CS-US unpaired controls. This aligns with the finding by Powers et al (2017) of greater activation in tone-responsive brain regions during the conditioned hallucinations as assessed by MRI.

Rats which have a 'hallucination-like' phenotype have been proposed to remain in this perceptual representation stage even with overtraining, resulting in their experience of associatively activated stimuli being indistinguishable or close to the physical stimuli and supporting mediated conditioning even with overtraining (Dwyer, 2018; McDannald & Schoenbaum, 2009). This is shown schematically in Figure 42. In addition, if representations remain perceptual in 'psychotic' rats they should show responses to associatively activated stimuli that are akin to responses generated by stimuli that are actually present, such as TRs, at all training extents (Holland, Lasseter, & Agarwal, 2008b; Kerfoot et al., 2007).
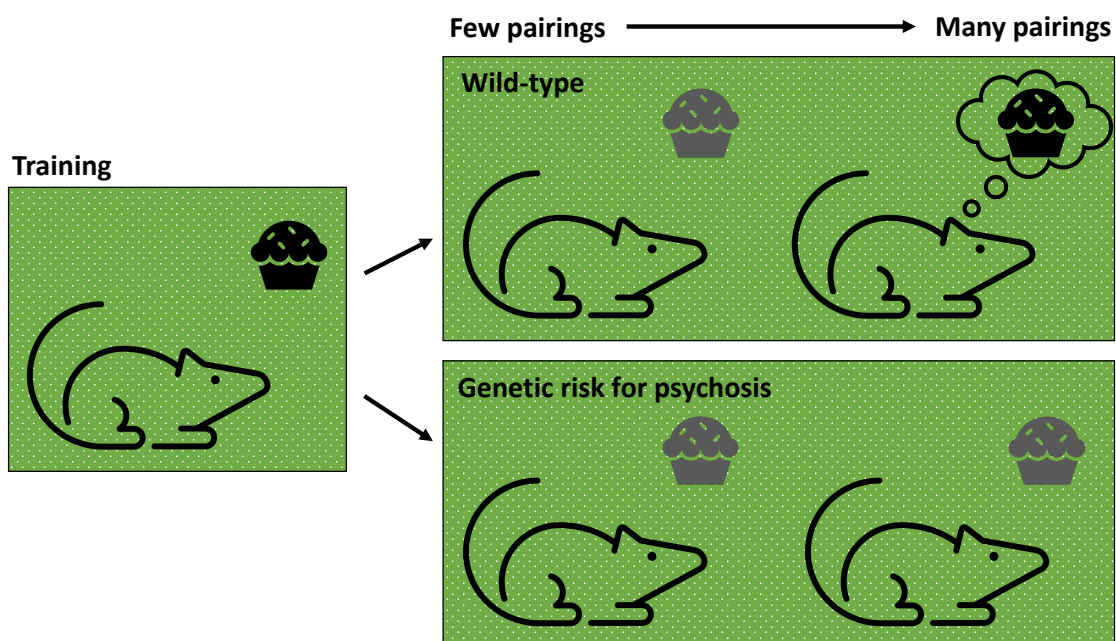
Figure 42: Schematic showing the key difference in learning expected between wild-type rats and those with a propensity towards psychosis. Green boxes denote context, the conditioned stimulus used in the current paradigm. Rats are trained to associate the context with sucrose (denoted by the cupcake). In wild-type rats after a few training trials the context CS evokes a perceptual experience of the sucrose which is expected to support mediated learning. Over many training trials in wild-types the evoked sucrose stimulus becomes a semantic representation, which is not expected to support mediated conditioning. Alternatively in a rat experiencing psychosis this shift does not occur, and even

after many trials the context evokes a perceptual representation of the sucrose, which could support mediated conditioning. Thus mediated learning is expected at all extents of training in rodents with a propensity towards psychosis.

There has already been some success in using mediated conditioning procedures to identify 'hallucination-like' phenotypes in animal models of schizophrenia. A greater propensity for mediated learning with overtraining has been demonstrated in mice exposed to ketamine in adolescence (Koh et al., 2018), rats given neonatal ventral hippocampal lesions (McDannald et al., 2011), mice with the schizophrenia-associated *DISC1* genetic mutation (Fry et al., 2020) and mice given cannabinoids (Busquets-Garcia et al., 2017). Further validation of this general paradigm comes from the fact that mediated learning effects seen in these rodent models are reversed by relevant pharmacological treatments such as progenolone (Busquets-Garcia et al., 2017) and the antipsychotic risperdone in the ketamine model (Koh et al., 2018).

To investigate whether *DLG2* haploinsufficiency may contribute to positive psychotic symptoms a mediated conditioning paradigm was employed. A pilot study was carried out in wild-type rats to determine the number of stimulus pairings required to achieve mediated conditioning at minimal training and lose this effect with overtraining. Subsequently *Dlg2*^{+/-} and wild-type rats were trained to drink water in a neutral context (C) before receiving either minimal or extensive pairings of one flavour (sucrose or saline) with context A and the other in context B. Rats were then re-exposed to context B immediately preceding LiCl induced illness. Mediated learning of flavour-illness associations was assessed through consumption and lick cluster size for flavours in neutral context C. Lick cluster size (Chapter 3.2.4 and 5.2.1) was used as an alternative to TR responses (as used by Holland et al., 2008b; Kerfoot et al., 2007) to capture whether mediated conditioning changed the evaluative phenomenological experience of drinking the flavoured solutions.

Wild-type rats were expected to show mediated conditioning and reduced lick cluster size to the devalued flavour at minimal but not extensive amounts of training. If *Dlg2*^{+/-} rats demonstrated a 'hallucination-like' phenotype it would be expected that they show

mediated conditioning and valence-induced lick cluster size changes at both extents of training.

6.2 Pilot study

Previous investigations have obtained mediated conditioning in a variety of preparations. These have involved learning about odour-flavoured solution (Busquets-Garcia et al., 2017; Koh et al., 2018), tone- flavoured solution (Fry et al., 2020; Holland, 1998) and light-flavoured pellet (McDannald et al., 2011) stimuli. The number of pairings required to obtain mediated conditioning has also been variable, from two (Fry et al., 2020) to sixteen (Holland, 1998). The present study uses a context-flavour design which has previously supported mediated learning (Dwyer 2001). In order to assess whether mediated conditioning would replicate in such a design and, critically, to ascertain how many stimulus pairings were required to demonstrated mediated learning with minimal training and lose this with overtraining a pilot study was carried out. This involved two, four or eight flavour-context pairings before pairing context and LiCl and assessing flavour consumption and lick cluster size for evidence of mediated conditioning.

6.2.1. Method

Animals

Forty-eight male Lister Hooded rats were used (supplied by Charles River, UK) that had been subjects in an entirely separate behavioural Experiment not reported here involving lever press and food pellet rewards, but were naïve to the apparatus and procedures used in the pilot study. Their mean ad libitum weight before the start of the experiment was 295g (range: 284 – 320 g). Animals were housed as described previously (Chapter 2.2.). Research was conducted in accordance with the Home Office regulations under the Animal (Scientific Procedures) Act 1986 (PPL 303243, PI Dominic M. Dwyer).

Materials

All experimental phases took place in the 16 custom-made drinking chambers described in Chapter 3.2.4. The room housing this equipment was used for the three experimental contexts. In the neutral context C lights were on, there was no sound and boxes were cleaned the day before use for minimal scent. In context A the room was dark, illuminated with a red bulb, with no sound. A solution of 5% peppermint flavouring was wiped around the sides of each box with a paper towel to provide scent before a rat was placed in the box. In context B the lights were on, ambient white noise was playing, and standard lab cleaning fluid (Mr Muscle) was wiped around the sides of each box to provide scent. Whether rats were in the top boxes or bottom boxes was confounded with context, for example for a given rat A entailed a top box and B a bottom box, with top/bottom placement varying each day in neutral context C. Sucrose (2%) and saline (1%) were made up daily on a weight/weight basis. Lithium chloride (0.15 M isotonic) was administered intraperitoneally at a volume 10ml/kg of body weight.

Procedure

Table 4: Design of pilot reality testing experiment. The associations detailed would vary depending on counterbalance. CX denotes context (A, B or C). FCX+ refers to the flavour associated with the context in which the rat experienced LiCl induced illness (i.e. here saline). FCX- refers to the flavour associated with the context where no injections were given (i.e. here sucrose). A rat with these pairings would be expected to show a decreased consumption of saline at test.

Condition (number of pairings)	Pre-training		Training		Conditioning		Test	
	CX	Stimulus	CX	Stimulus	CX	Stimulus	CX	Stimulus
2	C	Water	A	Saline	A	LiCl	C	Saline (FCX+)
			B	Sucrose	B	n/a		Sucrose (FCX-)
4	C	Water	A	Saline	A	LiCl	C	Saline (FCX+)
			B	Sucrose	B	n/a		Sucrose (FCX-)

| 8 | C | Water | A | Saline | A | LiCl | C | Saline (FCX+) |
| | | | B | Sucrose | B | n/a | | Sucrose (FCX-) |

Rats were water deprived by restricting their fluid consumption to one hour of home cage water access per day, given approximately one hour after experimental sessions. Fluids were also given daily as part of the experiment. The overall design of the reality testing pilot can be seen in Table 4. Initially rats learned to drink water from bottles with retracted spouts (as described in Chapter 3.2.4.) in context C by giving them five mins drinking per day over four days (Experimental days 1-4). They then were then exposed to context-flavour pairs (e.g. A-sucrose, B-saline) by placing them in the context with access to one bottle of one flavour for five mins. Rats were separated into three experimental groups of sixteen each, one group receiving two context-flavour pairings, one group receiving four pairings and the final group receiving eight pairings. Context-flavour pairs were counterbalanced with half of the rats each of the pairing groups (2, 4 and 8) associating A with sucrose and B with saline and the other half A with saline and B with sucrose.

Each day a different context was run in an order of A, B, B, A (Experimental days 5-8). Across the same four days rats in group two got one of these pairings per day, rats in group four got two of these pairings per day and rats in group eight got four pairings per day in order to ensure each flavour-context pair received two pairings in group two, four pairings in group four and so forth as represented in Table 5.

Table 5: Design of flavour-context pairings for each condition (2, 4 and 8 pairings) across the four training days. In this example A is paired with saline and B with sucrose but across each condition half of the rats received these associations and the other half associated A with sucrose and B with saline to implement the counterbalance.

Condition	Training Day 1		Training Day 2		Training Day 3		Training Day 4	
	CX	**Flavour**	**CX**	**Flavour**	**CX**	**Flavour**	**CX**	**Flavour**
2	A	Saline	B	Sucrose	B	Sucrose	A	Saline
4	(2×) A	Saline	(2×) B	Sucrose	(2×) B	Sucrose	(2×) A	Saline
8	(4×) A	Saline	(4×) B	Sucrose	(4×) B	Sucrose	(4×) A	Saline

Subsequently rats learned a context-illness association, e.g. A-LiCl. This took place over 6 days with contexts run daily in an order of A, B, B, A, A, B (Experimental days 9-14). On each day rats were placed in the context and given water to drink for five mins before those which associated the given context with illness were injected and placed back in their box. All rats then remained in the boxes for 15 minutes. Which context was associated with LiCl induced illness was counterbalanced in a way independent from the context-flavour counterbalance, with half of all rats receiving LiCl injections in A and the other half in B. It is noteworthy that the injection protocol was variable as this method was new to the researcher conducting the experiment, resulting in variable intervals between the cessation of consumption in the context and LiCl administration.

During the six day test phase (Experimental days 15-20) rats were placed in context C and given sucrose or saline to drink for five minutes with consumption (g) and lick cluster recorded. Rats received two days of each flavour alone (i.e. just one bottle of sucrose or saline, giving four days in total to test both flavours) and two days of both sucrose and saline bottles simultaneously for choice. On Experimental days 15, 17, 18 and 20 half of the rats tested were given the two choice test and the other half one bottle of one flavour alone (half FCX+ and half FCX-). On Experimental days 16 and 19 all rats were given one bottle of one flavour (half FCX+ and half FCX-). Testing was carried out in this way to prevent extinction of the aversion conditioning to a flavour (rats drinking more of this flavour as in previous test days it has been paired with no consequence) from uniformly influencing either the choice test or consumption of flavours individually. Consumption in grams and lick cluster size to flavours were averaged across matching tests, averaging consumption of single flavours presented alone across the two test days when these were available (two for sucrose and two for saline to each rat) and across both flavours separately for the choice test.

Despite the counterbalance in whether sucrose or saline was used as the FCX+ and FCX- flavours rats still tended to show a flavour bias, preferring to drink one flavour over the other. To prevent flavour bias from confounding interpretation of consumption determined by flavour valence, where this was present consumption and lick cluster scores were

corrected using the method outlined in Robinson, 2017. This involves generating a correction factor for each flavour by dividing the average consumption of that flavour by the average overall consumption of both flavours and multiplying each consumption score of this flavour by this. For example, for sucrose consumption on the one bottle test a correction factor would be calculated by sucrose average consumption divided by overall sucrose and saline average consumption. Sucrose consumption scores for each rat would then be multiplied by this correction factor. The same analysis was performed on corrected and uncorrected scores for comparison.

To prevent side bias interfering with consumption measures the positioning of the drinking bottle was alternated left-right-left for four days and right-left-right for four days in an alternating manner across all Experimental days.

6.2.2 Pilot study results

Paired samples t-tests were used to assess whether there were baseline differences in consumption of sucrose and saline at test. It was found that significantly more saline was consumed (M = 11.377, SE = 0.316) than sucrose (M = 9.392, SE = 0.387) at test ($t(47)$ = -4.082, p < 0.001, d = -0.589), yet lick cluster sizes to both flavours did not differ significantly (sucrose M = 35.08, SE = 2.989, saline M = 33.43, SE = 3.229, $t(47)$ = 0.615, p = 0.542, d = -0.089). Thus consumption was corrected in the way suggested by Robinson (2017) before analysis while raw lick cluster scores were analysed.

Figure 43A and Figure 43B respectively show corrected consumption and raw lick cluster size to FCX+ and FCX- at two, four or eight flavour-context pairings for rats on the one bottle tests. These were analysed with repeated measures ANOVA with between-subjects factor of number of pairings and within-subjects factor of flavour valence (FCX+ or FCX-). All findings for corrected consumption scores replicated when uncorrected scores were analysed.

Despite the consumption at four pairings for FCX+ being numerically lower than FCX- rather than the equated level across both flavours seen at two and eight pairings, the flavour valence × number of pairings interaction was not significant ($F(2, 45)$ = 1.984, p = 0.149, n^2_p

= 0.081). Neither were flavour valence ($F(1, 45) = 2.881$, $p = 0.097$, $n^2_p = 0.060$) or number of pairings ($F(2, 45) = 0.256$, $p = 0.775$, $n^2_p = 0.011$) main effects.

Likewise for lick cluster size the flavour valence × number of pairings interaction was not significant ($F(2, 45) = 1.884$, $p = 0.164$, $n^2_p = 0.077$), nor the flavour valence ($F(1, 45) = 3.138$, $p = 0.083$, $n^2_p = 0.065$) or number of pairings ($F(2, 45) = 0.165$, $p = 0.848$, $n^2_p = 0.007$) main effects.

However if the difference in consumption to FCX+ and FCX- for the four pairings group is compared with paired samples t-tests there is a significant difference for consumption to flavours of either valence ($t(15) = -2.305$, $p = 0.036$, $d = -0.576$), but not to lick cluster size ($t(15) = -0.357$, $p = 0.726$, $d = -0.089$). This provides evidence for mediated conditioning occurring in the four-pairing group in the pilot as shown by consumption but not lick cluster size. There were no significant differences in consumption to FCX+ and FCX- at two pairings ($t(15) = -0.438$, $p = 0.668$, $d = -0.109$) and eight pairings ($t(15) = 0.061$, $p = 0.952$, $d = 0.015$), nor in lick cluster size to FCX+ and FCX- at two pairings ($t(15) = 1.963$, $p = 0.068$, $d = 0.491$) and eight pairings ($t(15) = 1.061$, $p = 0.306$, $d = 0.265$).
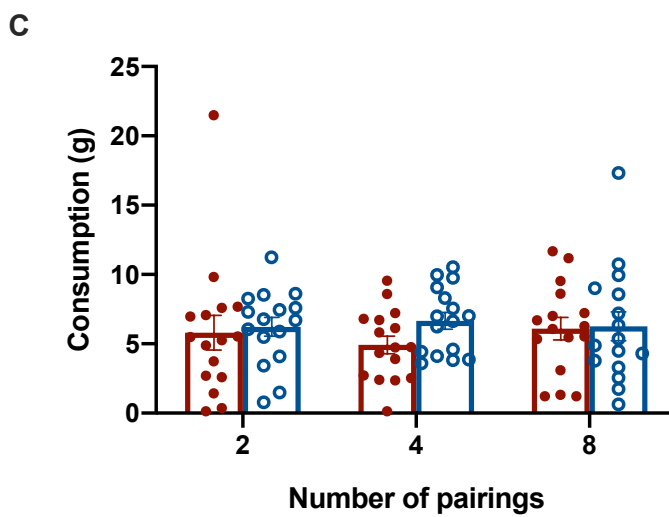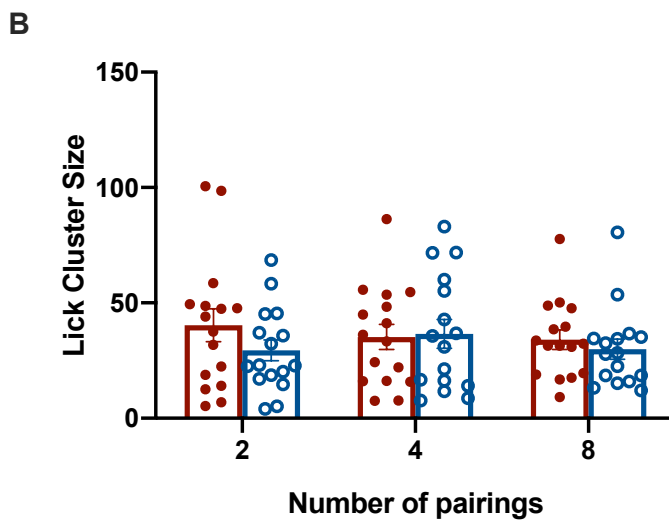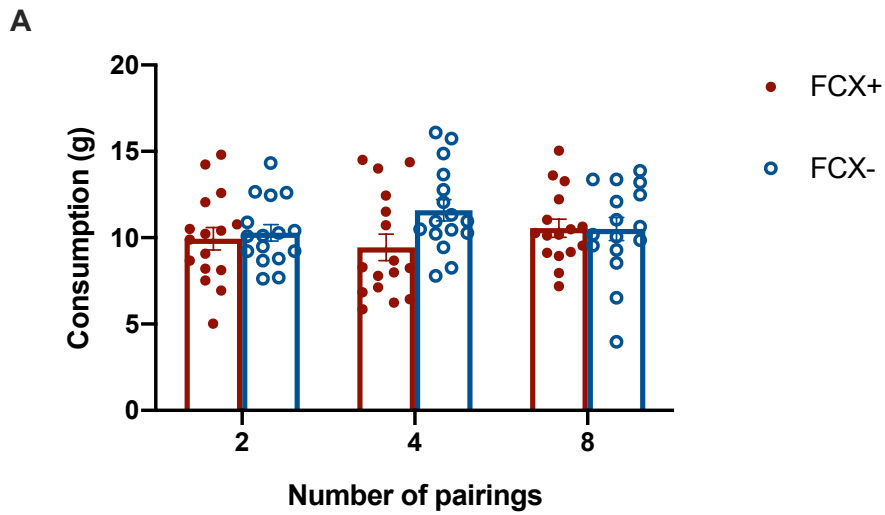
Figure 43: Representation-mediated taste aversion in wild-type rats given 2, 4 or 8 context-flavour pairings. Graphs show mean ± SEM plus individual values for consumption

and lick cluster size of the flavour associated with a context where LiCl was experienced (FCX+) and a flavour associated with a 'safe' injection-free context (FCX-) across pairings in the one bottle test **(A and B)** and consumption of FCX+ and FCX- across pairings on the two-bottle choice test **(C).**

Rats fluid consumption demonstrated flavour bias towards sucrose on the two-bottle tests (sucrose M = 7.581 SE = 0.367, saline M = 4.404 SE = 0.468, $t(47)$ = 4.225, $p < 0.001$, d = 0.610) and thus as in the one bottle test the Robinson (2017) correction was applied. Lick cluster sizes for the two-bottle test were not analysed as swapping between bottles when making a choice disrupts the accuracy of measurement. Corrected consumption scores for FCX+ and FCX- across the three extents of training are shown in Figure 43C.

Repeated measures ANOVA for corrected consumption on the two bottle choice test did not reveal a flavour valence × number of pairings interaction ($F(2, 45)$ = 0.319, p = 0.729, $n^2{}_p$ = 0.014). Main effects of flavour valence ($F(1, 45)$ = 0.824, p = 0.369, $n^2{}_p$ = 0.018) and number of pairings ($F(2, 45)$ = 0.181, p = 0.835, $n^2{}_p$ = 0.008) were also non-significant. These findings replicated on analysis of the uncorrected consumption scores. Thus the occurrence of mediated learning is not supported at any of the levels of training on the two bottle test.

6.2.3. Pilot study discussion

There is evidence of representation-mediated conditioning in the pilot study occurring at four flavour-context pairings, in that consumption on the one bottle test was lower to the FCX+ than the FCX-. This was not present at two or eight pairings. This is not clearly demonstrated in the repeated measures ANOVA analysis flavour valence × number of pairings interaction but can be seen when the four pairing group is analysed alone. There were issues with the pilot study that may have contributed to insensitive data such as the variability in LiCl injection protocol mentioned in the methods, which should not be a problem in conducting this experiment a second time with $Dlg2^{+/-}$ and wild-type rats. Thus as four pairings looks sufficient to give mediated conditioning in this design (as the 'few' pairings condition) with a mediated conditioning effect not seen at eight pairings (the

'many' pairings condition) flavour-context associations were experienced as four or eight pairings in the Experiment testing the mediated conditioning of *Dlg2*^{+/-} and wild-type rats.

6.3 Reality testing Experiment

6.3.1. Animals

Cohort 5 was used for the reality testing Experiment (as described in Appendix 1). Their mean ad libitum weigh before the start of the experiment was 424g (range: 266 – 727g).

6.3.2. Procedure

Table 6: Design of reality testing experiment. The associations detailed would vary depending on counterbalance. FCX+ refers to the flavour associated with the context in which the rat experienced LiCl induced illness (i.e. here saline). FCX- refers to the flavour associated with the context where no injections were given (i.e. here sucrose). A rat with these pairings would be expected to show a decreased consumption of saline at test.

Condition	Pre-training		Training		Conditioning		Test	
	CX	Stimulus	CX	Stimulus	CX	Stimulus	CX	Stimulus
4 pairings wild-type	C	Water	4*A 4*B	Saline Sucrose	A B	LiCl n/a	C	Saline (FCX+) Sucrose (FCX-)
4 pairings *Dlg2*^{+/-}	C	Water	4*A 4*B	Saline Sucrose	A B	LiCl n/a	C	Saline (FCX+) Sucrose (FCX-)
8 pairings wild-type	C	Water	8*A 8*B	Saline Sucrose	A B	LiCl n/a	C	Saline (FCX+) Sucrose (FCX-)
8 pairings *Dlg2*^{+/-}	C	Water	8*A 8*B	Saline Sucrose	A B	LiCl n/a	C	Saline (FCX+) Sucrose (FCX-)

The Experiment was conducted as in the pilot except using only four and eight context-flavour pairing groups as shown in Table 6. Rats were water restricted by restricting their fluid consumption to one hour of drinking per day, with 12h of water access every seven days. Fluids were also given daily as part of the experiment. Throughout the experiment male rats were run before female rats. The experiment was run blind to genotype and thus rats were sorted into four and eight pairing conditions based on progenitor breeding pair and sex with the assumption that *Dlg2* heterozygosity should show a roughly Mendalian distribution.

To recap, the experiment entailed four days of learning to drink water in the drinking chambers in neutral context C (Experimental days 1-4). This was followed by four days of context-flavour pairings (e.g. context A- sucrose and context B – saline) in contexts A and B, presented over the four days in order A, B, B, A (Experimental days 5-8). *Dlg2*$^{+/-}$ and wild-type rats in the four pairings group received two context-flavour pairings per day to give four pairings of each flavour and context in total, while *Dlg2*$^{+/-}$ and wild-type rats in the eight pairings group received four context- flavour pairings per day to give a total of eight pairings of each flavour with each context. As the Experiment was run blind to genotype counterbalancing of which flavour was associated with which context was carried out with half the rats of each sex receiving A- sucrose B- saline and the other half vice versa, with an equal distribution of rats from each progenitor pair in each flavour- context group.

Subsequently rats received six days of illness-context pairings (Experimental days 9-14 with contexts in order A, B, B, A, A, B) where rats drunk water for 5 minutes, received an injection if they were in the FCX+ and then remained in the drinking chambers for 15 minutes. Half of the training A- sucrose B- saline group and half of the A-saline B-sucrose training group received LiCl in A and half LiCl in B to implement the counterbalance for sex and progenitor pair. Finally one bottle flavour consumption and lick cluster size and two bottle saline/sucrose choice tests were given in neutral context C for six days (Experimental days 15-20). On Experimental days 15, 17, 18 and 20 half of the rats tested were given the two choice test and the other half one bottle of one flavour alone (half FCX+ and half FCX-). On Experimental days 16 and 19 all rats were given one bottle of one flavour (half FCX+ and half FCX-).

To prevent side bias interfering with consumption measures the positioning of the drinking bottle was alternated left-right-left for four days and right-left-right for four days in an alternating manner across the training phases of the experiment.

6.3.3. Results

Drinking behaviour after conditioned taste aversion
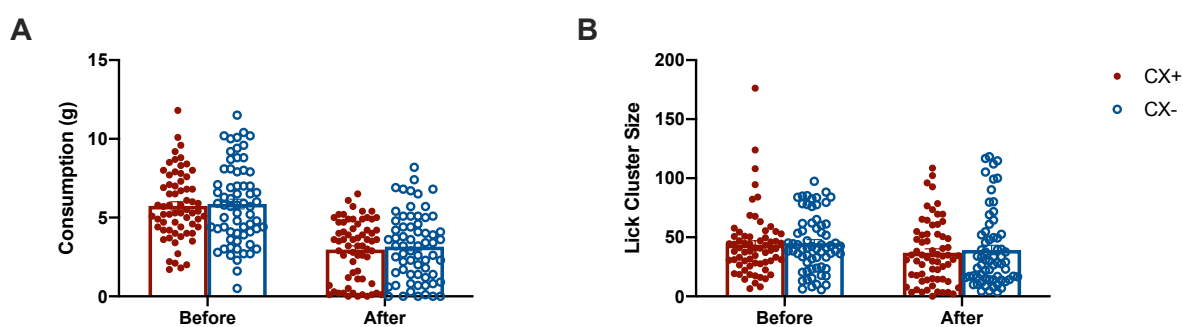
Figure 44: Consumption (A) and lick cluster size (B) in the five minutes of drinking water before LiCl injections and after two LiCl injections in CX+ across the contextual taste aversion period of the Experiment. Mean ± SEM plus individual values.

Figure 44 shows consumption and lick cluster size in the five minutes of drinking on the first session of context-LiCl pairing and following two LiCl injections in the CS+ and CS- contexts. This shows a decrease in overall consumption and lick cluster size in both contexts but doesn't show evidence of a differential response to CS+ and CS- (repeated measures ANOVA context (CS+/CS-) × time (before/after) interaction: $F(1, 64) = 0.048$, $p = 0.827$, $n^2_p = 0.00075$). This may be because consumption and lick cluster size are perhaps not the most sensitive measure of aversion to the context (Best, Brown, & Sowell, 1984; Boakes, Westbrook, & Barnes, 1992), and due to the experimental design they are calculated prior to the final context-LiCl injection, after which the strongest aversion would be expected. Furthermore, as shown in the following section mediated learning was observed, suggesting that the context aversion measure was successful.

Mediated learning

As with the pilot rats showed flavour bias. On the one bottle test more saline was consumed (M = 9.054g, SE = 0.419) than sucrose (M = 6.513g, SE = 0.324), while lick clusters for sucrose (M = 53.925g, SE = 5.758) were significantly higher than those for saline (M = 41.885g, SE = 4.042). On the two bottle test more saline (M = 6.164g, SE = 0.448) was consumed than sucrose (M = 4.689g, SE = 0.287). Thus consumption and lick cluster values were corrected using the method outlined in the pilot (Robinson, 2017).

To determine how best to apply the correction repeated measures ANOVA for both one and two bottle consumption and lick cluster size at test was carried out with within-subjects factors of flavour (sucrose vs saline) and between subjects factors of sex, genotype and number of pairings. For one bottle consumption this analysis revealed only a main effect of flavour (F(1, 57) = 21.698, p < 0.001, n^2_p = 0.276) and no interactions, suggesting it was appropriate to apply the correction to all consumption data rather than applying it individually based on genotype or sex. This was also true of one bottle lick cluster size (main effect of flavour: F(1, 57) = 4.215, p = 0.045, n^2_p = 0.069). For two bottle consumption there was a main effect of flavour (F(1, 57) = 6.246, p = 0.015, n^2_p = 0.099), and a significant flavour × sex × genotype × number of pairings interaction (F(1, 57) = 4.517, p = 0.038, n^2_p = 0.073) caused by female rats at four pairings preferring sucrose to saline while all other animals preferred saline to sucrose. Here the correction was also applied to all consumption data. Comparisons between findings on the analysis of corrected scores and uncorrected scores revealed agreement in findings between the two. Learning in this experiment proceeded the same way for wild-type and *Dlg2*$^{+/-}$ both sexes (non-significant sex × genotype × flavour and sex × genotype × flavour × pairing interactions), thus mostly non-significant sex effects and are presented in Table 39 of the Appendix.

Figure 45A shows that one bottle corrected consumption of FCX+ is decreased relative to FCX- at both four and eight pairings for *Dlg2*$^{+/-}$ and wild-type rats. These data, in addition to lick cluster size and two bottle consumption, were analysed using repeated measures ANOVA with within-subjects factors of flavour (FCX+ vs FCX-) and between subjects factors of number of pairings, sex and genotype. There was a main effect of flavour (F(1, 57) =

12.396, $p < 0.001$, $n^2_p = 0.179$), showing differing consumption for flavours devalued and non-devalued through their association to the contexts (mediated conditioning) in this procedure. The flavour × pairings × genotype interaction non-significant ($F(1, 57) = 0.023$, $p = 0.879$, $n^2_p < 0.001$; $BF_{exclusion} = 220.498$) neither was the flavour × pairings interaction ($F(1, 57) = 0.292$, $p = 0.591$, $n^2_p = 0.005$; $BF_{exclusion} = 14.277$). Bayes factors giving evidence for null effects show that mediated conditioning proceeded in the same way at both four and eight pairings across genotypes.

The non-significant flavour × genotype interaction ($F(1, 57) = 0.882$, $p = 0.352$, $n^2_p = 0.015$; $BF_{exclusion} = 9.153$) shows that there were no genotype differences in ability to learn the mediated devaluation. Also non-significant with Bayes factors supporting the null were main effects of genotype ($F(1, 57) = 0.120$, $p = 0.730$, $n^2_p = 0.002$; $BF_{exclusion} = 18.279$), main effects of pairings ($F(1, 57) = 0.170$, $p = 0.682$, $n^2_p = 0.003$; $BF_{exclusion} = 20.509$) and genotype × number of pairings ($F(1, 57) < 0.001$, $p = 0.988$, $n^2_p < 0.001$; $BF_{exclusion} = 44.380$).

As Figure 45B shows lick cluster size for FCX+ and FCX- did not vary across genotype and pairing groups. Repeated measures ANOVA for lick cluster size did not reveal main effects of flavour ($F(1, 57) = 1.235$, $p = 0.271$, $n^2_p = 0.021$; $BF_{exclusion} = 9.393$), failing to reflect the mediated learning effect consumption does. Interactions of flavour × pairings was non-significant with a Bayes factor supporting the null ($F(1, 57) = 3.277$, $p = 0.076$, $n^2_p = 0.054$; $BF_{exclusion} = 5.533$) showing, as consumption does, that number of pairings does not influence mediated conditioning in this preparation. The flavour × genotype ($F(1, 57) = 1.246$, $p = 0.269$, $n^2_p = 0.021$; $BF_{exclusion} = 6.818$), flavour × genotype × pairings ($F(1, 57) = 0.418$, $p = 0.520$, $n^2_p = 0.007$; $BF_{exclusion} = 21.230$), main effect of pairings ($F(1, 57) = 1.057$, $p = 0.308$, $n^2_p = 0.018$; $BF_{exclusion} = 8.472$) and genotype × number of pairings ($F(1, 57) = 1.823$, $p = 0.182$, $n^2_p = 0.031$; $BF_{exclusion} = 4.868$) were all non-significant with Bayes factors supporting null effects as genotype and pairings did not interact to influence lick cluster response to conditioned stimuli.

There was a main effect of genotype on lick cluster size in this Experiment ($F(1, 57) = 4.885$, $p = 0.031$, $n^2_p = 0.079$), with *Dlg2$^{+/-}$* rats showing larger lick clusters than wild-types (Figure

45B). This provides further support to the lick cluster size finding outlined in Chapter 5 which concludes that *Dlg2*$^{+/-}$ rats do not show anhedonia phenotypes.

Consumption on the two-bottle choice test to FCX+ and FCX- from rats of different genotypes is shown in Figure 45C. Rats did not show evidence of mediated conditioning in the choice test (non-significant main effect of flavour: $F(1, 57) = 3.132$, $p = 0.082$, $n^2_p = 0.052$; $BF_{exclusion} = 3.541$, and flavour × pairings interaction: $F(1, 57) = 0.811$, $p = 0.372$, $n^2_p = 0.014$; $BF_{exclusion} = 14.308$). There were no genotype differences in learning or consumption on the two bottle test (non-significant flavour × genotype: $F(1, 57) = 0.532$, $p = 0.469$, $n^2_p = 0.009$; $BF_{exclusion} = 16.342$, flavour × pairings × genotype: $F(1, 57) = 0.006$, $p = 0.937$, $n^2_p < 0.001$; $BF_{exclusion} = 113.466$ and genotype × pairings: $F(1, 57) = 0.245$, $p = 0.622$, $n^2_p = 0.004$; $BF_{exclusion} = 47.294$ interactions and main effects of genotype: $F(1, 57) = 0.771$, $p = 0.384$, $n^2_p = 0.013$; $BF_{exclusion} = 20.684$ and pairings: $F(1, 57) = 0.899$, $p = 0.347$, $n^2_p = 0.016$; $BF_{exclusion} = 19.535$).
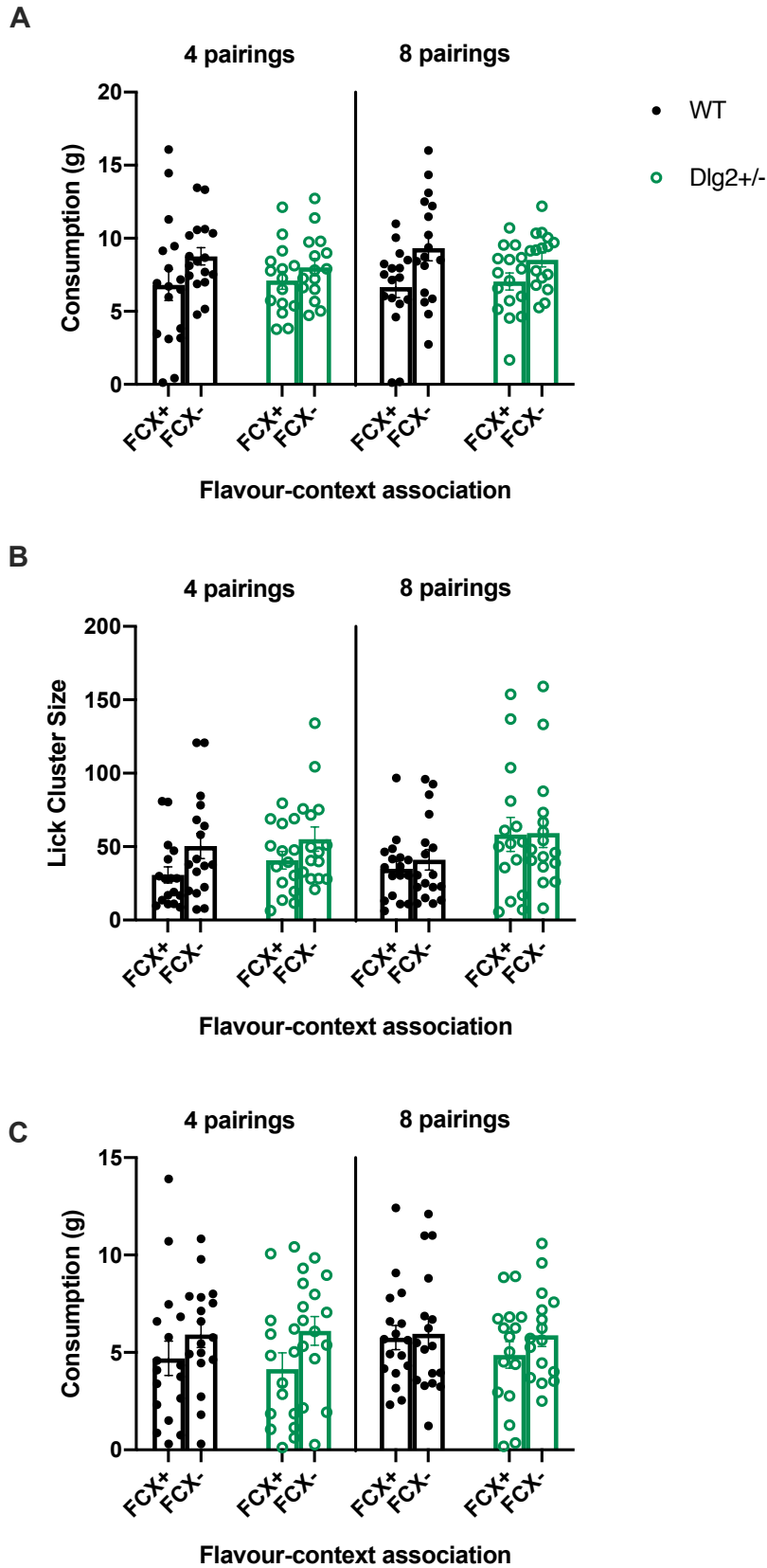
Figure 45: *Dlg2+/-* **and wild-type performance in representation-mediated taste aversion at 4 and 8 context-flavour pairings**. Consumption and lick cluster size of the flavour associated

with a context where LiCl was experienced (FCX+) and a flavour associated with a 'safe' LiCl-free context (FCX-) in the one bottle test **(A and B)** and consumption of FCX+ and FCX- on the two-bottle choice test **(C).**

6.4. Discussion

The normal performance of a conditioned response (CR) is thought to be mediated by the activation of a representation of the unconditioned stimulus (US) associated with it. Holland (1990) has proposed that the nature of this representation determines the nature of the unconditioned response (UR) and the ability of the US representation to support further learning. Specifically, with few pairings the CS evokes a sensory memory of the US that facilitates mediated conditioning. With further pairings the CS accesses a US representation which codes only its motivational or semantic significance and not its sensory properties, and which is less likely to enter new associations (Holland 1998). Mediated conditioning and the stimulus representations which govern it have been used to interrogate the degree to which a rodent is able to distinguish between mnemonically evoked representations of stimuli and those which are physically present in the environment (Busquets-Garcia et al 2017; McDannald et al 2011; Koh et al 2018). Thus it provides the first translational assay of positive symptoms in rodent models of psychotic disorders.

The findings in this Chapter demonstrate that mediated conditioning was similar after four and eight pairings in both *Dlg2*$^{+/-}$ and wild-type rats. An absence of enhancement of mediated conditioning in the *Dlg2*$^{+/-}$ rats at either degree of training suggests that this rat model does not demonstrate issues in reality testing. Consequently, the *Dlg2* gene may not be involved in the generation of schizophrenia positive symptom endophenotypes.

This takes a step towards ruling-out the role of *Dlg2* from NMDA-receptor processes in generating excessively strong or overactive predictions from hierarchically higher brain regions to lower brain regions. According to the predictive coding framework this results in hallucinations or delusions from expectation-based override of sensory processing. Ranson et al., (2019) demonstrated that mice treated with MK-180 NMDAR antagonist showed evidence of a large elevation in activity of projections from the ACC to V1, resulting in net

suppression of V1 activity, validating the idea that in NMDAR specific models of psychosis aberrant predictive processing may be implemented biologically. Ranson et al (2019) didn't show concomitant behavioural phenotypes associated with elevated top-down neural activity, but Koh et al (2018) demonstrated reality testing impairments using a ketamine NMDAR antagonism model. As the *Dlg2*$^{+/-}$ rat model is more subtle and specific than pharmacological NMDAR antagonism models it allows a specific conclusion that the PSD-93 scaffold itself is not critical for any NMDAR-dependent processes in the aetiology of positive symptoms in psychosis.

The present findings also have implications for Holland's (1998) ideas concerning stimulus representation throughout learning, as for wild-types mediated conditioning proceeded at both four and eight flavour-context pairings. It is reasonable to explain this by stating that in this design both four and eight pairings evoked perceptual representations, leading to the observation of mediated conditioning at both extents of training. This highlights a problem with Holland's (1998) ideas in that there is no clear prediction about when a shift from 'perceptual' to 'conceptual' representations can be expected, and whether such shifts may vary with stimulus modality.

For 'minimal' amounts of training Holland (2008) used 16 flavour-sound pairings, which would encompass both intervals used here. However other designs involving flavour- light pairings failed to find evidence of mediated learning in wild-types at both six and 12 pairings, proposing that for wild-types representations must already be conceptual at this stage (McDannald 2011). Even within modalities there are inconsistencies, with Busquets-Garcia (2017) showing odour-flavour mediated learning at three but not six pairings, and Koh et al (2018) failing to show odour-flavour mediated conditioning at three pairings. Furthermore, the amount of training required to change stimulus representations may also vary with species. In the pilot study there was an indication of mediated learning at four but not eight pairings in Lister Hooded rats but this was absent from rats of a Long Evans background. This body of literature gives support for the idea that there is a difference in the representations generated from minimal and extensive extents of training and how these are learned about, but there is no reliable, replicated consensus on what constitutes either training extent. Before being applied to rodent models of psychiatric disorder it

would be best to delineate when mediated conditioning can be expected and which representations guide this.

The use of a lick microstructure assessment of hedonic response to the associatively activated stimulus was intended to distinguish the type of stimulus representations being evoked. Holland (2008) used TR responses to this end, reporting them at minimal but not extensive training. Lick cluster size as a measurement of hedonic stimulus processing is claimed to be analogous to TR, in that both analyses seem broadly sensitive to the same manipulations (Dwyer, 2008). Therefore, it was predicted that at perceptual levels of processing with minimal training lick cluster size should reflect aversion to the associatively activated flavour. Lick cluster size did show larger differences between the flavour associated with the aversive context and the neutral context at four pairings than eight pairings, which is in line with Holland's (2008) findings. However, this difference was not statistically significant and the Bayes factor ($BF_{exclusion}$ = 5.533) gave support for no effect.

This could provide evidence against the idea that perceptual representations were evoked at four and eight pairings, showing mediated conditioning in the absence of this. However, a safer conclusion may be that lick cluster size is an inadequate read-out of the sensory 'experience' of an evoked stimulus representation in the present experiment. It may have been that lick microstructure could initially represent the evoked valence early in the test session or on the earlier test days (which were closer to conditioning). However, as consumption proceeded the intrinsically hedonic properties of the flavours which water-restricted rats enjoyed drinking may have become more prominent. In line with this there is evidence that lick cluster responses in conditioned taste aversion studies extinguish faster than consumption measures (Dwyer, 2009; Dwyer, Gasalla, & López, 2013). Thus, by the test phase lick microstructure may have failed to capture the mediated learning effect which was still evident in consumption measures.

Similar studies have used immediate early gene expression in sensory cortices as a measure of whether the associatively activated stimulus was perceptual in nature (e.g. Kerfoot et al 2007). This approach would have been untenable in this design which relies on presenting rats with flavoured solutions at test. Immediate early gene expression to mark activity in

sensory cortex after minimal and extensive training would be valuable in a tone-flavour design like Holland (2008) and thus far has not been conducted. It would also give a mechanism by which the degree of expression could be tracked as training proceeds, giving a biological readout of how much sensory activation and evoked stimulus generates to determine when representations may become less sensory and more conceptual in nature.

In conclusion mediated conditioning occurred similarly after four and eight pairings in both *Dlg2+/-* and wild-type rats. This contrasts with Holland's (1998) proposals of a change from perceptual to conceptual processing across training, and implies that under-expression of *Dlg2* may not contribute to positive symptoms such as hallucinations. It is possible that both intervals used here comprise 'minimal' training where representations should have still been perceptual. What represents short and long training intervals to obtain these differing stimulus representations remains to be outlined.

Summary

- *Dlg2+/-* rats and wild-types both show evidence of mediated conditioning in the reality testing task at both extents of training.
- As the attenuation of mediated conditioning with overtraining was not seen in the wild-types, it is impossible to determine conclusively whether continued mediated conditioning in the presence of overtraining is seen in the *Dlg2+/-* rats. This makes it difficult to draw conclusions about the role of *Dlg2* heterozygosity in reality testing.

Chapter 7

General Discussion

7.1. Summary of findings

Identifying risk variants for psychiatric disease in large-scale genetic studies provides a useful springboard for isolating biological and behavioural endophenotypes that contribute to multiple conditions. This can be conducted by using enrichment analysis to find behavioural domains or biological pathways risk variants converge in, e.g. the clustering of genes which are associated with increased schizophrenia and neurodevelopmental disorder risk within those that code for postsynaptic density effectors (Fromer et al., 2014). Taking this further by modelling individual risk variants in rodents, such as *DLG2* haploinsufficiency in CNVs, provides an additional mechanism to isolate cross-disorder biological and psychological processes that precipitate disorder.

To this end the primary question addressed by this thesis is the following: how might *Dlg2* heterozygosity contribute to psychiatric disorder? This was addressed by a biological and behavioural characterisation of a novel *Dlg2$^{+/-}$* rat model. The *Dlg2$^{+/-}$* model was shown to be valid at the protein level, with reductions seen in PSD-93 without obvious compensation by change in PSD-95 or NMDAR subunit levels (Chapter 2.3.1.). This matches the specificity of decrease shown in Dlg2 mRNA levels (Pass, 2019). It also confirms that the model was appropriate to investigate the question without other primary biological confounds or disorder risk factors.

Dlg2$^{+/-}$ rats were not more anxious than wild-types (Chapter 3.3.1). Nor did they demonstrate deficits in short (Chapter 4.3.2.) or long-term habituation (Chapter 3.3.3.), abnormal pre-pulse inhibition (Chapter 3.3.2.) or social behaviour (Chapter 3.3.4.). This is all in contrast to previous findings in homozygous knockout models (Winkler, Daher, Wüstefeld, Hammerschmidt, Poggi, Seelbach, Krueger-Burg, et al., 2018; Yoo et al., 2020a). On basic tasks assessing ethological responses which are usually awry in rodent models of *Dlg2* relevant disorders *Dlg2$^{+/-}$* rats showed intact performance, except for an increased

locomotor response to the NMDAR-antagonist PCP which is discussed in detail in section 7.2.5.

$Dlg2^{+/-}$ rats performed comparably to wild-types on tasks assessing many memorial processes despite changes in hippocampal plasticity (Griesius et al., 2022). This involved working memory as assessed by Y-maze spontaneous alternation (Chapter 4.3.2.), long-term forms of recognition memory based on object identity (Chapter 4.2.2.), object location (Chapter 4.2.2.), recency (Chapter 4.3.2.) and object-location paired associates (Chapter 4.2.2. and 4.3.2.), and reference memory in the Morris water maze (Chapter 4.3.2.).

$Dlg2^{+/-}$ rats did not show alteration in their ability to process and respond to rewards. Using Berridge and Robinsons's (2009) topography no deficits were found in the hedonic response to reward ('liking', Chapter 5.2.1.), motivation to work for reward ('wanting' Chapter 5.2.2.) or the ability to learn about reward (Chapter 5.2.3.). Thus, it is likely that $Dlg2$ does not contribute to symptoms of psychiatric disorders resulting from changes to reward processing. Finally, a method of assessing the propensity towards hallucinations and delusions in rodents was piloted and run with $Dlg2^{+/-}$ rats (Chapter 6). On this task they responded as wild-types, although this is not sufficient to rule out schizophrenia positive symptom pathology in these rodents (see section 7.2.6).

Null findings supported by Bayesian statistics in many of the behavioural domains investigated removes them as a confound on other assays. The lack of deficits on anxiety tasks removes this as a constraint on behaviour in tasks requiring exploration (e.g. social preference). The ability of $Dlg2^{+/-}$ rats to habituate to an auditory tone and perform novel object recognition demonstrates intact hearing and basic visual ability in these rats which is fundamental to ensuring accurate measures on many behavioural tasks (e.g. operant conditioning). These reassurances allow research on the $Dlg2^{+/-}$ rat to move forward without concern for these constraints.

Changes to grey matter volume and white matter integrity were interrogated with MRI. DTI data analysed with TBSS did not find alteration in white matter microstructure measured across genotypes. To assess grey matter volume neuroanatomical regions of interest (ROI)

were chosen based on regions that typically show volume changes in relevant rodent models (i.e. NMDA antagonism models) and human patients with ASD or schizophrenia. Volumetric changes were not observed in any of the ROIs sampled (Chapter 2.3.2.). This is unsurprising given the phenotypic subtlety of the *Dlg2+/-* model, as catastrophic alterations to grey and white matter would be expected to result in similarly severe behavioural deficits. In line with this there were no changes in regions relevant to the object recognition battery such as the perirhinal cortex, hippocampus and medial prefrontal cortex.

7.2. Key areas of focus in analysing the *Dlg2+/-* rat model

7.2.1. Comparison to NMDAR dysfunction models

It would be reasonable to postulate that as PSD-93 is a key synaptic scaffold for the NMDAR, having less functional PSD-93 at the synapse would have a knock-on effect and disrupt NMDAR-associated processes. This could occur by anchoring less NMDA in the PSD, although there is evidence from Chapter 2.3.1. that there is the same amount of NMDA in synaptosomes between *Dlg2+/-* rats and wild-types, although this does not give information concerning the anchorage, location or complex constituents of NMDARs in the synapse which could still be abnormal. Alterations in NMDAR binding partners at the synapse of *Dlg2+/-*s could be interrogated by the co-immunoprecipitation experiment outlined in Chapter 2, although this is still only the first step needed to specify molecular changes surrounding the interaction of these two proteins.

The combined evidence suggests that the *Dlg2+/-* model cannot be reduced to a model of impaired NMDA function. NMDA-antagonism rodent models show impairments of learning, memory (e.g. Lee & Zhou, 2019) and social behaviour (e.g. Becker & Grecksch, 2004; Bruins Slot, Kleven, & Newman-Tancredi, 2005; Ellenbroek & Cools, 2000; Neill et al., 2010; Sams-Dodd, 1999; Snigdha & Neill, 2008) that *Dlg2+/-* rats do not show. Work by Griesius et al (2022) recording from *Dlg2* heterozygous knockout hippocampal neurons demonstrated that increased NMDAR currents in neurons are entirely offset by concomitant reduction in electrical input resistance caused by an increase in potassium channel expression. Thus, biological compensation for a primary NMDAR deficit in the rat model may explain the lack

of NMDAR-associated phenotypes. This means that when considering compounds to ameliorate deficits caused by decreased *Dlg2* dosage in human CNV carriers it may be wise to focus on drugs other than those which directly target NMDAR and instead target biological substrates involved in this process of compensation. In line with this Griesius et al (2022) also discovered that potassium channel block of activation of muscarinic M1 receptors rescued dendritic integration and synaptic plasticity changes, isolating these as substrates to focus treatment on.

7.2.2. Social behaviour

With social behaviour abnormalities seen in NMDAR dysfunction (Becker & Grecksch, 2004; Bruins Slot, Kleven, & Newman-Tancredi, 2005; Ellenbroek & Cools, 2000; Neill et al., 2010; Sams-Dodd, 1999; Snigdha & Neill, 2008) and *Dlg2*$^{-/-}$ mice (Winkler, Daher, Wüstefeld, Hammerschmidt, Poggi, Seelbach, Krueger-Burg, et al., 2018; Yoo et al., 2020b), the intact performance of *Dlg2*$^{+/-}$ rats on the social preference task represents a key departure in the abilities of *Dlg2*$^{+/-}$ rats from those in related models. However, a more through interrogation of social function in the *Dlg2*$^{+/-}$ rat is warranted, given the status of changes to social behaviour as key symptoms of the disorders *Dlg2* increases risk for. In autism impaired social behaviour and social communication is a core and disabling feature (American Psychiatric Association, 2013) which is often evident from early infancy (Young, Brewer, & Pattison, 2003). In schizophrenia social withdrawal comprises a key negative symptom that often precedes psychosis (Larson, Walker, & Compton, 2010). Schizophrenia is also characterised by more subtle deficits to social cognition, the perceptual processes that lead to accurate recognition of dispositions and intentions of oneself and others (Brothers, 1990). This involves impairments to the capacity to understand others mental states (theory of mind, Montag et al., 2011), empathy (Lehmann et al., 2014) and emotion recognition (Novic, Luchins, & Perline, 1984).

Social preference is a small corner of the gamut of social behaviours that is relevant to *Dlg2*-associated conditions. Assessing social preference and social novelty by the time spent exploring conspecifics in different chambers is a task typically applied to mice (Rein, Ma, & Yan, 2020b). An assessment that is more detailed and relevant to human social interaction is

observing play behaviour in rodent dyads. Using a rat model offers a key advantage here as the social play behaviour of mice involves only a small subset of the complex playful activities rats display (Pellis & Pasztor, 1999; Whishaw, Metz, Kolb, & Pellis, 2001). It is best to quantify rat social play in adolescence by measuring the frequency and duration of specific behaviours involving flipping a playmate onto their back (pinning), pouncing, and chasing (Trezza, Baarendse, & Vanderschuren, 2010). The initiation of these behaviours by a specific individual can be assessed, as can the responsiveness of an individual to the play of another wild-type or mutant rat. There is evidence for disorder sensitivity in measuring play as it is impaired in rat models of autism (Chomiak, Karnik, Block, & Hu, 2010; Schneider & Przewłocki, 2005). Assessing play would also allow for the study of communication, an area overlooked by this thesis, as ultrasonic vocalisations can be measured during play (Knutson, Burgdorf, & Panksepp, 1998) and have shown reductions in rat models of autism (Gzielo et al., 2021).

$Dlg2^{+/-}$ rats did not show anhedonia to sucrose or a clear lack of motivation to work for food reward, yet sensitivity and motivation towards social rewards was not assessed. It has been proposed that the dissociation of rewards into hedonic ('liking'), motivational ('wanting') and cognitive ('learning') components applies to social rewards as it does to food and drug rewards (Trezza, Campolongo, & Vanderschuren, 2011). This may be the case, yet no analogous lick microstructure or TR response measurement exists to assess the hedonic response to social interaction. This is a shame given the fact that reduced hedonic value of social stimuli is characteristic of autism, schizophrenia, and depression (American Psychiatric Association, 2013). With the advent of learning-based approaches for the parcellation of rodent ultrasonic vocalisation (USV) components using artificial neural networks (e.g. DeepSqueak; Coffey, Marx, & Neumaier, 2019) it may be possible to determine which USV components elicited during social play relate to hedonic responses, and quantify differences in these between transgenic and wild-type animals.

The 'learning' component of responding to social reward could be assessed by conditioned place preference (CPP). This task requires rodents to learn Pavlovian associations between distinctive areas of an apparatus and the presence of a conspecific. Learning is demonstrated by a preference to spend time in this location on subsequent exposure to the

apparatus when the conspecific is removed. As *Dlg2^+/-* rats are able to learn Pavlovian associations, reduction in CPP may indicate that social interaction is not a rewarding enough US to facilitate learning. An appropriate US in such a task could be social play rather than social interaction per se, as investigations have shown that if drug treatments render a conspecific non-playful CPP does not develop (Trezza, Damsteegt, & Vanderschuren, 2009) and avoidance of the play partner is conditioned (Calcagnetti & Schechter, 1992). However, it may be that drug treatment produced a partner whose social interaction was disrupted as a whole rather than just removing the play component.

Motivation to socialise can be assessed using operant tasks where social rewards are used as reinforcers. This procedure is not well-elucidated in rodents, and the necessity of using social play rather than mere social interaction as a reinforcer would provide problems with ensuring the same duration and value of reinforcer across trials and participants. Evans et al., (1994) demonstrated that mature female rats would press a lever for access to a castrated male, and that responding for this was comparable with responding for food reward under conditions of extinction, fixed ratio and deprivation. Thus as play becomes less important with rodents age social interaction alone could be used to measuring motivation to access reward ('wanting') and the ability to learn about reward.

Working for social reward could be incorporated in a task analogous to the MPR, where single-housed rats could work at varying ratios for interaction with a conspecific. A lengthy piloting process would be required to determine whether rodents would lever press in such a task and what duration/ nature of social interaction would serve as a reinforcer. A further advantage of using a MPR is that fitting the demand equation to data allows the comparison of the motivation to receive different kind of reinforcer on a common scale, giving a meaningful comparison between motivation for food and social rewards.

As human social behaviour is complex, social defects within disorder often comprise specific social-cognitive impairments, e.g. misreading of facial expressions in schizophrenia (Goghari & Sponheim, 2013). Atypical empathy characterises autism (Harmsen, 2019) and schizophrenia (Bonfils, Lysaker, Minor, & Salyers, 2016) and could be measured in rodents. Mice and rats have been shown to alter their behaviour by observing conspecifics. This

demonstrates the social transmission of pain (Langford et al., 2006), fear (Chen, Panksepp, & Lahvis, 2009) and food preference (Choleris, Guo, Liu, Mainardi, & Valsecchi, 1997). Applying these tests to rodent models of autism and schizophrenia, including *Dlg2*^{+/-}, may provide an opportunity to assess more human-relevant changes to social behaviour.

7.2.3. Assessment of positive symptoms in the *Dlg2*^{+/-} rat model

Pharmacologically induced hyperactivity and disrupted PPI have frequently been reported as indicators of schizophrenia positive symptoms in rodents (Jones, Watson, & Fone, 2011; Wilson & Terry., 2010). This argument seems predicated on the logic that these are generated by mesolimbic dopamine hyperactivity. Increased dopaminergic activity in this region is believed to underlie hallucinations and delusions in human psychosis, given the ability of antipsychotic medication to reverse positive symptoms (Howes & Kapur, 2009). Indeed, antipsychotics also reverse hyperactivity and PPI deficits in rodent models (Anastasio & Johnson, 2008; Phillips, Wang, & Johnson, 2001; Wang et al., 2001), leading to the argument that these assays have predictive validity (respond in an expected way to intervention) with respect to capturing positive symptoms. Furthermore, these two behavioural assays represent a fundamental inability to habituate. This has been proposed to be the cognitive result of elevated midbrain dopamine that mediates the link between altered neurotransmission and hallucinations and delusions in humans (e.g. Kapur, 2003).

There are imperfections to this argument. In humans' clinical studies have shown that patients with schizophrenia show increased presynaptic dopamine function in the associative striatum, defined by its dense connectivity from frontal and parietal associative cortices (Howes et al., 2009, 2013; Kegeles et al., 2010; Winton-Brown, Fusar-Poli, Ungless, & Howes, 2014). This is contra to the previous assumption that this was driven by the limbic striatum, which receives inputs from the hippocampus, amygdala and medial orbitofrontal cortex (Kapur, 2003). Subjects at high risk of developing schizophrenia show similar presynaptic dopamine abnormalities in the associative striatum (Roiser et al., 2013). However psychostimulant-induced locomotion in rodents is heavily associated with dopaminergic activation in the limbic striatum (Kesby, Eyles, McGrath, & Scott, 2018) resulting in an anatomical misalignment with the human phenotype.

The nature of PPI and hyperactivity seem distant from behaviours that would indicate a hallucination or a delusion. Using these as a readout of positive symptoms is intended as a proxy measure, to isolate rodents that may be experiencing hallucinations and delusions, rather than demonstrating the presence of these experiences. This approach is indirect and lacks specificity. Impaired PPI or abnormal locomotor response to psychostimulants indicates dopaminergic hyperactivity which is also associated with negative symptoms of schizophrenia. It has been proposed that reduced phasic dopamine responses in the midbrain for relevant stimuli may lead to these being neglected, as phasic responses are allocated to irrelevant stimuli, leading to delusion and negative symptoms such as anhedonia and amotivation (Maia & Frank, 2017). This is seen clinically as acute administration of PCP in healthy humans results in a psychotic-like state accompanied by progressive withdrawal and poverty of speech which capture some negative symptom domains (Luby, Cohen, Rosenbaum, Gottlieb, & Kelley, 1959). In rodents acute PCP dosing causes social withdrawal (Sams-Dodd, 1995) and impairments to cognition (Egerton, Reid, McKerchar, Morris, & Pratt, 2005). This is similar for PPI which in human patients correlates with both positive and negative symptoms (Braff, Swerdlow, & Geyer, 1999; Meincke et al., 2004).

A measure that better captures the true nature of what comprises a hallucination or delusion is the reality testing protocol outlined in Chapter 6. It may also involve the same neurotransmission systems as these experience as there is evidence that exaggerated mediated conditioning may arise from a dysregulation of the dopamine system. Treatment with the antipsychotic haloperidol effectively reduced the tendency to evoke internal representations in *DISC1* mice (Fry et al., 2020), and treatment with a different antipsychotic attenuated mediated learning in a sub-chronic ketamine exposure mouse model (Koh et al., 2018). Which exact striatal components (i.e. associative or limbic) mediate conditioned hallucinations should be determined to interrogate alignment with conditioned hallucinations and positive symptoms in patients.

Dlg2$^{+/-}$ rats show increased locomotor response to PCP (Chapter 3.3.5.) in the absence of PPI abnormalities (Chapter 3.3.2.) or evidence of exaggerated mediated conditioning (Chapter

6.3.3.). This is not sufficient to conclude a lack of positive symptoms in these rodents, especially given that the reality testing procedure requires delineation of the training extent at which mediated conditioning is no longer apparent in wild-types. This is required to accurately determine whether *Dlg2$^{+/-}$* rodents would still show mediated learning at this point, as it hypothesized in rodents experiencing schizophrenia positive symptoms (Dwyer, 2018; McDannald et al., 2011; McDannald & Schoenbaum, 2009). A starting point for this could be the approach taken by Fry et al (2020). These researchers demonstrated that after extensive training of a tone and sucrose association *DISC1* mice demonstrated a lick microstructure profile to water in the presence of the tone as though they were drinking sucrose, while wild-types showed lower lick cluster sizes appropriate to the palatability of water. Requiring no aversive conditioning, this could determine whether *Dlg2$^{+/-}$* rats have associatively evoked representations that are perceptual even after overtraining. It could also be assessed repeatedly as the number of pairings increases to observe the point at which perceptual representations become conceptual, although one would have to be careful of the confound of extinction through presenting the tone without sucrose repeatedly.

Kesby et al (2018) propose that to capture positive symptoms in rodents it is essential to find deficits on behavioural tasks which are relevant to the specific dopaminergic profile of positive symptomology. Specifically, tasks which rely on the associative striatum. They give outcome devaluation and serial reversal learning as suggestions. Outcome devaluation involves updating action-outcome responding based on newly learned information. Serial reversal learning entails updating behavioural response based on a series of changes to response-outcome contingencies. Those diagnosed with schizophrenia show difficulties with both tasks (Chudasama & Robbins, 2006; Morris, Quail, Griffiths, Green, & Balleine, 2015). Furthermore, outcome devaluation issues are related to deficits in the PFC and associative striatum, yet serial reversal learning only relies on the associative striatum and not the PFC. This allows for a readout of exactly which brain regions are compromised, and a true indication that the associative striatum is involved. They are cognitive tasks that do not capture the essence of hallucinations or delusions, but if proxy measures based on dopaminergic profile are to be used, these may be the most accurate ones.

An alternative protocol to assess hallucinations is outlined by Schmack, Bosc, Ott, Sturgill, & Kepecs, (2021). These researchers defined hallucinations as false percepts held with a high degree of certainty. Thus they developed analogous tasks for mice and humans that involved probing hallucination-like percepts by means of high confidence false detections on an auditory signal detection task. This involved playing an auditory stimulus which, on around half of trials, embedded a tone signal amongst a noisy background. Humans made button-press responses to report the tone while mice nose-poked into choice ports. Humans reported confidence via questionnaire, mice via investing variable time durations to earn a reward with longer durations indicating higher confidence. This task seems valid as high-confidence false detections increased after ketamine manipulations in mice and correlated with increased self-reported hallucinations in humans. This task is also true to the neurobiology of positive symptoms, as hallucination-like percepts were preceded by elevated striatal dopamine levels and could be induced by optogenetic stimulation of mesolimbic dopamine neurons.

Working with animal models will always entail analogical arguments, thus choosing behavioural tasks best matched to symptom domains is essential. Triangulation through multiple methods such as these, with the addition of direct measurements of region-specific dopamine in $Dlg2^{+/-}$ rats, would be the best method for determining whether this genetic variant increases risk for positive symptoms.

7.2.4. Intact memory and associative learning in the $Dlg2^{+/-}$ rat model

The summation of work in this thesis builds a clear picture that the $Dlg2^{+/-}$ rat demonstrates intact memory and associative learning ability. This is demonstrated for a variety of memory tasks in Chapter 4 as well as the ability to learn Pavlovian associations (Chapter 5.3.3), operant associations (Chapter 5.3.2) and learn about associatively accessed representations (Chapter 6.3.3.). Maintaining a high ability to learn about different contingencies removes a potential confound when planning other behavioural probes for the $Dlg2^{+/-}$ model and paves the way for assessing more complex disease-relevant learning preparations. It is worth mentioning a caveat of this conclusion, which is that only appetitive preparations were

assessed. However normal acquisition, extinction and recall of Pavlovian associations between context and footshock in *Dlg2+/-* rats have been demonstrated (Pass, 2019).

Dlg2+/- rats learning proficiency furthers the evidence that the *Dlg2+/-* model cannot be reduced to a model of NMDAR dysfunction, as NMDAR-antagonism models typically demonstrate deficits on tasks such as discrimination learning (Lee & Zhou, 2019). This also provides an informative contrast to a *Dlg2-/-* model which has been described as showing impairments on a variety of learning and memory tasks. *Dlg2-/-* mice are deficit on extinction, discrimination learning, reversal learning and the ability to learn and remember object-location pairs (visuo-spatial discrimination learning) (Nithianantharajah et al., 2013). Nithianantharajah et al (2013) propose that *Dlg2* plays a specific role in flexibly using learned information, and it may be that even a reduced amount of PSD-93 protein in tandem with its paralogs is sufficient to support cognition of this kind.

In every mental disorder *Dlg2* has been related to deficits exist on a continuum. Capabilities and states which are symptomatic of disorder manifest in the general population yet become problematic by occurring at extremes. Autism is defined as a spectrum disorder for this reason, with difficulties in the key symptoms (social/ communication deficits and repetitive behaviours) manifesting with varying severity. In a model that is true to the psychiatric phenotype as *Dlg2+/-* is, and in which deficits are likely subtle, pushing the abilities of these rodents further with more complex behavioural tasks may isolate where deficits lie.

As *Dlg2+/-* rats can learn simple CS-US and action-outcome associations, as well as discriminations, their capabilities could be investigated further using a task in which correct responses are dictated by the particular combination of cues present. A biconditional discrimination (Table 7) requires subjects to learn the correct responses where task-setting cues dictate different responses to the same stimuli.

Table 7: Design of a biconditional experiment. Letters denote stimuli, + and – indicates combinations of stimuli which result in positive (+) or negative (-) outcomes.

Discrimination	
Biconditional	Simple
AV+ BV-	CX+ DX+
AW- BW+	CY- DY-

Here each cue is paired with both reinforcement and non-reinforcement thus to learn when reinforcement will occur the combination of cues must be learned. This task has good translational utility and can be performed similarly on humans, with those with high schizotypy (Haddon et al., 2011) demonstrating impairments.

This task has the added benefit of being 'hippocampal' (Albasser et al., 2013). The battery of tasks in Chapter 4 were chosen to interrogate a possible behavioural component to the alteration in hippocampal plasticity outlined by Griesius et al., (2022). However, the tasks in Chapter 4 are commonly impaired by gross hippocampal disruption such as lesions and pharmacological inactivation which would be expected to produce more severe deficits than a subtle change to plasticity. It may be that the tasks in Chapter 4 were too simple to isolate a subtle behavioural deficit, thus increasing task difficulty and requiring a greater integration of information could challenge this further. This is also a benefit of testing $Dlg2^{+/-}$ rats on cognitive tasks which recruit brain regions key to the aitiology of schizophrenia positive symptoms such as outcome devaluation and serial reversal learning as mentioned in section 7.2.3.

7.2.5. Locomotor response to phencyclidine

Altered psychostimulant sensitivity has been shown in other psychosis-relevant CNV rodent models, the 22q11.2 (Didriksen et al., 2017) and 1q21.1 (Nielsen et al., 2017) microdeletion mouse models. In the 22q11.2 mouse this manifested as an exaggerated locomotor response to PCP and ketamine. In the 1q21.1 mouse exaggerated locomotor behaviour was seen in response to amphetamine but was not significant with administration of PCP, yet PCP resulted in sensorimotor gating impairments. As administration of non-competitive

NMDAR antagonists in healthy rodents and humans can induce a behavioural syndrome isomorphic to positive and negative symptoms of schizophrenia (Javitt & Zukin, 1991; Snyder, 1980; Tamminga, 1998) and exacerbates positive and negative symptoms in schizophrenic patients (Itil, Keskiner, Kiremitci, & Holden, 1967; Lahti, Koffel, Laporte, & Tamminga, 1995; Malhotra et al., 1997) the finding of PCP sensitivity in these rodent CNV models may highlight a general psychosis susceptibility.

Findings such as these contribute to the hypoglutamaterigic hypothesis of psychosis (Moghaddam & Javitt, 2012). However, it is difficult to use these findings to determine which biological processes are altered in CNV rodent models such as $Dlg2^{+/-}$. Acute PCP administration has been reported to activate serotonergic, glutamatergic, noraderenergic, cholinergic and neurotensinergic transmission in rodents and monkeys (Deutch, Tam, Freeman, Bowers, & Roth, 1987; Hertel et al., 1995; Jentsch, Elsworth, Redmond, & Roth, 1997).

Of particular relevance for further investigation, given the idea that PCP hyperlocomotion can be used as a readout of neurotransmission changes associated with schizophrenia positive symptoms (section 7.2.3), is the relationship between PCP and dopamine. By probing electrophysiology of substantia nigra dopamine neurons in mouse brain slices Piccart, Tschumi, and Beckstead (2019) demonstrated that acute PCP affected dopamine D2 autoreceptor-mediated currents. Autoreceptor signalling was attenuated by PCP through dopamine transporter inhibition and G-coupled potassium ion channel inhibition. Seeing as repeated activation of D2 autoreceptors induces long-term depression of inhibitory input and increased dopamine neuron activity (Beckstead & Williams, 2007; Piccart, Courtney, Branch, Ford, & Beckstead, 2015), this could mean that PCP produces the increased midbrain dopamine firing thought to be key in schizophrenia aitiology (Kapur et al 2003). Electrophysiological assessment of dopaminergic activity in $Dlg2^{+/-}$ rat midbrain slices would be relevant especially given evidence towards a reduced frequency of excitatory, but not inhibitory, spontaneous postsynaptic currents in mouse $Dlg2^{-/-}$ dorsolateral striatum (Yoo et al., 2020a). However this thesis has given scant evidence for the replication of homozygous phenotypes in heterozygous mutants.

Increased locomotor activity in psychiatric disorder models, including the sub-chronic PCP model, does not occur spontaneously but is observed after an acute PCP dose (Abekawa, Honda, Ito, Inoue, & Koyama, 2002; Clark, Johnson, Wright, Monn, & Schoepp, 2002; Fletcher, Tenn, Rizos, Lovic, & Kapur, 2005; Hanania, Hillman, & Johnson, 1999; Johnson, Phillips, Wang, & Kevetter, 1998; Martinotti et al., 2012; Scalzo & Holson, 1992; Tenn, Kapur, & Fletcher, 2005; Xu & Domino, 1994). In this sense the acute drug trigger seems to comprise an additional challenge to an already compromised system that reveals symptomology. This is in line with 'multiple hits' hypotheses of disorder which state that it is a critical cumulation of certain combinations of genetic and environmental risk factors which tip an individual over the threshold to determine disorder (Davis et al., 2016; Reich, Cloninger, & Guze, 1975; Tsuang, Stone, & Faraone, 2001). Acute PCP administration could comprise another 'hit' that reveals a system with a fundamental propensity towards disorder. This in mind, and given the evidence that PCP triggers an increase in midbrain dopamine burst firing (Piccart et al., 2019), some behavioural domains outlined in this thesis could be re-tested under the influence of an acute PCP dose. Reality testing, social behaviour, PPI, and appropriate cognitive tasks would be good candidates for this. It is hypothesised that PCP would trigger changes to behaviour of all rats that would be more exaggerated in $Dlg2^{+/-}$s.

7.3. Sex effects

Sex effects are apparent in the disease progression of all conditions associated with $Dlg2$ disruption. In schizophrenia male: female incidence approximates 1.4:1 with older onset predominating in women (André Aleman, Kahn, & Selten, 2003; Drake et al., 2016; Hafner, Maurer, Loffler, & Riecher-Rossler, 1993; Hambrecht, Maurer, Häfner, & Sartorius, 1992; Leung & Chue, 2000). ASD is known to have a male preponderance with the male:female ratio being reported as 4.5:1 (Christensen et al., 2016), as is moderate to severe intellectual disability with the male:female ratio being 1-2:1 (Werling & Geschwind, 2013). Most disorders with a neurodevelopmental component have higher incidences in males (Werling & Geschwind, 2013).

In addition to the social factors that contribute to sex-related changes in incidence or reporting of these psychiatric disorders, which are beyond the scope of this thesis, biological sexual dimorphisms may contribute to these ratios. Furthermore sex differences must be understood to ascertain the most relevant biomarkers and treatment regimens for disease. Mistakenly assuming that female behaviour is akin to male behaviour, yet more variable due to hormonal cycles (Becker et al., 2005), results in all-male cohorts predominating in this research leaving this key area of understanding overlooked. Thus, when modelling disease risk in rodents it is important to include a characterisation of how the risk manipulation influences both sexes.

In humans with ASD females tend to show increased functional social behaviour (Head, McGillivray, & Stokes, 2014; Lai et al., 2011) and less repetitive behaviours compared to males (Mandy et al., 2012; Zwaigenbaum et al., 2012). In humans these apparent differences may be due to subtle differences in the expression of these phenotypes e.g. females with autism may have better social skills yet conflict in social relationships may be harder for autistic females to cope with than autistic males or non-autistic females (Sedgewick, Crane, Hill, & Pellicano, 2019). Likewise sex differences in key phenotypes have been described in many rodent models of ASD (Jeon et al., 2018). $Dlg2^{+/-}$ females performed as males in the social preference test (Appendix A3.3) and no sex-related changes were seen in duration or frequency of grooming (Appendix A3.1). However using a more detailed and comprehensive battery of social tests as outlined in section 7.2.2 may reveal more subtle sex differences in $Dlg2^{+/-}$ social behaviour.

In schizophrenia men have poorer premorbid adjustment and present with worse negative symptoms then women (Leung & Chue, 2000; Morgan, Castle, & Jablensky, 2008; Vázquez-Barquero et al., 1996), with specific positive symptoms such as paranoia (Bardenstein & McGlashan, 1990) and auditory hallucinations (Suessenbacher-Kessler et al., 2021) being shown as more prevalent in women by some studies. In the $Dlg2^{+/-}$ model no sex-related changes were seen in the assessment of propensity towards positive symptoms (Appendix A6.1.). Overall, no sex-related differences in behaviour were observed in the $Dlg2^{+/-}$ rat.

Sex differences are also seen in the neuroanatomy of those with *DLG2*-related disorders. In men with schizophrenia MRI and post-mortem studies reported larger lateral and third ventricles and smaller medial temporal volumes including the hippocampus and amygdala (Flaum et al., 1995; Lauriello et al., 1997). In ASD MRI volumetrics has revealed a variety of sex × diagnosis effects in regions associated with limbic, visual, auditory/ language and default mode network regions (Cauvet et al., 2019; Ecker et al., 2017; Irimia et al., 2018; Postema et al., 2019; Schaer, Kochalka, Padmanabhan, Supekar, & Menon, 2015). Although typically these results suggest that males have more extreme grey matter changes with disorder, only scanning and analysing volumes of brain structures from a male *Dlg2$^{+/-}$* cohort is a limitation of the work presented here.

Multiple-threshold multifactorial liability models propose that the male bias in prevalence for neurodevelopmental disorders occurs due to liability thresholds differing with sex (Reich et al., 1975). Specifically, females require a greater risk factor load to manifest a clinical diagnosis (Jacquemont et al., 2014; Martin, Hamshere, Stergiakouli, O'Donovan, & Thapar, 2014; Robinson, Lichtenstein, Anckarsäter, Happé, & Ronald, 2013). An excess of deleterious autosomal CNVs in females compared with males has been shown in a neurodevelopmental disorders cohort and an ASD cohort (Jacquemont et al., 2014). Females with ASD (Gilman et al., 2011; Levy et al., 2011; Sanders et al., 2015) and schizophrenia (Han et al., 2016) have been found to possess a higher burden of de novo CNVs than males with these conditions. A trend towards increased de novo CNV rate has been shown in unaffected female siblings of ASD patients (Sanders et al., 2015). Isolating sex-specific compensative processes and sex effects in the *Dlg2$^{+/-}$* model, as with other CNV models, is essential in determining the protective processes female carriers have which affords them the ability to withstand more mutations than males before being affected by disorder.

Finally, it is useful to remember that the human social world has nuances animal modelling cannot approximate. The 1998 World Health report states that 'women's mental health is inextricably linked to their status in society. It benefits from equality and suffers from discrimination' (World Health Organisation, 1998). The underdiagnosis of female mental health issues and their alienation from treatment settings linked to their social status is an issue to be resolved at many levels within research and clinical settings (World Health

Organisation, 2002). Prioritising the inclusion of male and female rodent models when phenotyping is an equality that sets the tone for continued understanding and prioritisation of sex differences in disease when translating research from lab to clinic.

7.4. *Dlg2* and development

Conditions associated with *DLG2*-encompassing CNVs are neurodevelopmental, with symptoms thought to result from abnormal brain development processes. This includes ASD, ADHD, and intellectual disability (Morris-Rosendahl & Crocq, 2020). Schizophrenia has also been described as a neurodevelopmental condition (Rund, 2018) with evidence for a developmental aitiology coming from brain cytoarchitectural changes, premorbid cognitive and motor symptoms, and pre-natal environmental risk factors (Murray, Lewis, & Lecturer, 1987; Weinberger, 1987). The genetic overlap in risk factors between schizophrenia and these conditions provides further support that it may be in part a neurodevelopmental condition (Girirajan et al., 2011; International Schizophrenia Consortium et al., 2009; Rees et al., 2014; Sebat, Levy, & McCarthy, 2009; Williams et al., 2010). Further supporting *DLG2* as having a developmental role is the description of rare missense variants in the gene leading to delayed puberty in humans (Jee et al., 2020).

In addition to increasing risk for neurodevelopmental conditions there is direct evidence that *DLG2* plays a role in cortical development processes. In humans *DLG2* mRNA is present from eight weeks post-conception (Kang et al., 2011), as well as in vitro throughout all stages of cellular differentiation from embryonic stem cells to cortical projection neurons (van de Leemput et al., 2014). $DLG2^{-/-}$ CRISPR-Cas9 engineered human embryonic stem cells were found to show multiple abnormalities in developmental processes as they were differentiated into cortical excitatory neurons (Sanders et al., 2020). The mRNA expression of key markers for typical stages as cells become more specialised in early corticogenesis was downregulated in $DLG2^{-/-}$ lines. For the expression of some developmental markers this recovered to wild-type levels at a later point in differentiation. Genes downregulated included those contributing to the development of cell morphology, connectivity and active properties leading to the development of neurons with long, sparsely branched neurites and immature action potentials (Sanders et al., 2021). There was also an enrichment for rare

and common variants conferring a wide range of disorders including schizophrenia and ASD amongst the genes under expressed implying that changes to developmental time course associated with *Dlg2* disruption have relevance in the genesis of these conditions (Sanders et al., 2021).

Developmental changes at a young age that may contribute to disorder have been widely evidences in humans with ASD and schizophrenia. In autism certain prognostic features, such as reduced eye contact (Jones & Klin, 2013) and atypical preverbal vocalizations (Esposito, Hiroi, & Scattoni, 2017), could signal the future onset of ASD at least in a subpopulation (Estes et al., 2015; Ozonoff et al., 2018; Zwaigenbaum, Bryson, & Garon, 2013). Furthermore, children and adolescents who later develop schizophrenia lag in working memory, attention, and processing speed (Bora & Murray, 2014; Bora & Pantelis, 2015; David, Malmberg, Brandt, Allebeck, & Lewis, 1997; Davidson et al., 1999; Fuller et al., 2002; Meier et al., 2014; Reichenberg et al., 2010; Seidman et al., 2016; Woodberry, Giuliano, & Seidman, 2008).

All *Dlg2*^{+/-} and wild-type rodents in this thesis were tested in adulthood. It may be that by adulthood sufficient compensatory processes have developed to allow proficient performance on tasks relevant to the endophenotypes tested. With the finding that in humans behavioural abnormalities beginning in early childhood precede a diagnosis of disorder, there is a good argument for assessing the abilities of much younger *Dlg2*^{+/-} rodents. This could begin with measuring ultrasonic vocalisations in rodent pups in response to separation from the mother and nest, with reduced USVs taken as an indicator of early deficits in social communication. Lower rates of USVs have been shown in multiple rodent models of autism (e.g. Malkova, Yu, Hsiao, Moore, & Patterson, 2012; Wyatt et al., 2013) including pups lacking the gene for neuroligin-4 which binds *Dlg2* (Jamain et al., 2008).

Assessing social play and associated USVs in adolescence is also appropriate for developmental phenotyping. As is comparison of the performance of *Dlg2*^{+/-} rats on cognitive tasks such as object recognition and reward sensitivity tasks such as lick microstructure at old and younger timepoints. Biologically a large degree of work could be done to observe developmental changes in cellular and brain morphology, gene and protein

expression and synaptic plasticity in the $Dlg2^{+/-}$ model. It is also a priority to discover whether the alterations seen in $DLG2^{-/-}$ human cell lines would persist in a more clinically relevant $DLG2^{+/-}$ line.

7.5. The function of *DLG2* in psychiatric disorder

The phenotypes described in *Dlg2* homozygous knockout rodent and cellular models have not replicated in this *Dlg2* heterozygous model. These include hypolocomotion in the open field (Winkler, Daher, Wüstefeld, Hammerschmidt, Poggi, Seelbach, Krueger-Burg, et al., 2018; Yoo et al., 2020a), changes to social behaviour (Winkler, Daher, Wüstefeld, Hammerschmidt, Poggi, Seelbach, Krueger-Burg, et al., 2018; Yoo et al., 2020a), increased self-grooming (Yoo et al., 2020a) and cognitive deficits including object-location memory, extinction, and discrimination learning (Nithianantharajah et al 2012). It may be the case that *Dlg2* is essential to the normal performance of these behaviours, but having *Dlg2* present (albeit reduced in quantity) is sufficient for them to proceed. There is precedent for this as reduced dosage of other *Dlg* genes is sufficient for functioning, with the presence of a single copy of *Dlg1* resulting in a cognitive phenotype comparable to wild-types in mice while full deletion is embryonic lethal (Nithianantharajah et al 2012).

If the methodology of behavioural tasks is matched across studies comparison of homozygous and heterozygous mutants is instructive. Homozygous models provide useful information on which psychological and biological processes rely on *Dlg2*, while heterozygous models discern which processes may be critically disrupted in disorder, or isolate mechanisms of compensation which risk disruption by outside risk factors. However, there were differences in the way in which tasks were run which could partially account for this disparity. The touchscreen presentation of learning tasks by Nithianantharajah et al (2012) could have increased task difficulty in comparison to analogous tasks used here, e.g. recognising object-location paired associates must be easier when a rodent can touch, smell and see them in a 3D arena in the OIP task rather than viewing 2D shapes on screen. The increase in task difficulty may have made subtle learning and cognitive issues more apparent.

Winkler et al (2018) concluded the PSD-93^{-/-}s have increased sociability based on direct dyadic social interaction while Yoo et al (2020) concluded deficit social interaction from reduced conspecific approach on the social preference test. Furthermore, in the social preference protocol used by Yoo et al (2020) mice were single housed for three days prior to the experiment. This would have assessed social preference under conditions of deprivation and stress rather than assessing ecologically valid interest in a conspecific. These additional challenges may have provided risk factors in addition to *Dlg2* gene disruption which may have combined to generate a change in behaviour. Even with these concerns the comparison between homozygous and the psychiatric relevant heterozygous model can isolate domains on which further investigation is warranted, with the idea that deficits may be more subtle in the heterozygotes.

Subtle phenotypes in heterozygous models are not surprising given that in humans *Dlg2*-encompassing CNVs are risk factors and not determinant of psychiatric disorder. Reggiani et al (2017) describe affected probands from neurotypical maternal carriers of 11q14.1 CNVs, and Nithianantharajah et al (2012) a similar CNV carrier without diagnosed psychiatric disorder. Disruption to *Dlg2*, as with all genetic risk factors, may predispose a biological system towards disorder while a cumulation of other risk factors is essential to precipitate it. The increased locomotor activity to PCP provides an example of this where a drug risk factor that triggers psychosis symptoms in humans already at risk for schizophrenia (Itil et al., 1967; Lahti et al., 1995; Malhotra et al., 1997) results in a markedly different and symptomatic response from *Dlg2*[+/-]s in comparison to wild-types. This in mind gene × environment interactions could be assessed in the *Dlg2*^{+/-} rat, such as prepubertal stress or maternal separation in infancy. If phenotypes were evident in *Dlg2*[+/-]rats given additional environmental hardship it would evidence that *Dlg2* CNVs do not cause catastrophic deficits alone but predispose a system towards this. The interplay between risk factors in causing certain phenotypes in *Dlg2*[+/-]s could isolate specific pathways which cause the association of *Dlg2* with a heterogenous set of conditions (ASD, schizophrenia, bipolar, intellectual disability and ADHD) caused by a particular combination of factors.

Finally, the *Dlg2*^{+/-} rat only models CNVs which reduce *Dlg2* copy number, and not *Dlg2* duplication CNVs. Duplications of *DLG2* also manifest in the human populations and

increase risk for the same range of conditions (Kirov et al., 2012). If remaining faithful to an investigation of the psychiatric relevance of *DLG2*, any phenotypes seen with the deletion model should also be investigated in duplication models. In humans with 16p11.2 CNVs 'mirror phenotypes' have been shown where deletion and duplication mutations have opposing effects on aspects of brain structure and cognition (Leblanc & Nelson, 2016; Qureshi et al., 2014). If similar findings occurred for *Dlg2* deletion and duplication models this could help isolate biological processes which must occur at certain developmental timepoints or within specific ranges to protect against disorder.

7.6 Conclusions

To assess how disruption to the *Dlg2* gene in human CNVs might trigger psychiatric disorder, a *Dlg2$^{+/-}$* rat model was created and assessed for alterations in protein expression and brain structure and on a range of disorder-relevant behavioural tasks. The model has construct validity in that the PSD-93 protein is significantly decreased in heterozygotes without changes to related proteins PSD-95 or NR1 NMDAR subunit. No white matter microstructural differences were observed across genotypes, and out of selected ROIs no changes in grey matter volume were observed between brain structures in *Dlg2$^{+/-}$* and wild-type rats. No deficits were found in anxiety, long and short-term habituation, social behaviour, memory function, reward processing or reality testing abilities. A differential psychostimulant sensitivity was observed in *Dlg2$^{+/-}$* rats, showing a more sustained and exaggerated locomotor response to PCP administration than wild-types. This finding alongside the lack of replication of homozygous knockout phenotypes in *Dlg2* heterozygotes implies that heterozygous knockdown of *Dlg2* may predispose a biological system towards disorder without being catastrophic enough in itself to result in severe deficits. How *Dlg2* haploinsufficiency interacts with other disorder risk factors, be these environmental or biological, is likely crucial in understanding how this manipulation causes disease and which of the associated psychiatric disorders manifest.

Appendix 1.

Details of rat cohorts used throughout the Experimental work in this thesis

Rats were bred at and supplied by Charles River, UK. Animals were housed in groups from two to four in standard cages with environmental enrichment (wooden chews and cardboard tubes) and maintained on a 12h light-dark cycle. Research was conducted in accordance with the Home Office regulations under the Animal (Scientific Procedures) Act 1986 (PPL 303243, PI Dominic M. Dwyer or PPL 303135, PI Jeremy Hall).

Cohort 1

Used for preliminary work not reported in this thesis.

Cohort 2

Table 8: Genotype and sex information for rat cohort 2

	$Dlg2^{+/-}$	WT	Total
Male	20	28	48

Used under PPL 303243, PI Dominic M. Dwyer

Experiments participated in in temporal order:

- Modified progressive ratio task (Chapter 5.1.3.).
- Lick microstructure assessment (Chapter 5.1.2.).
- Flavour habituation (Chapter 3.2.4).
- Ex vivo MRI scans (subset of rodents, Chapter 2.2.4).
- Tissue used for Western blot (subset of rodents, Chapter 2.3.1).

Cohort 3

Table 9: Genotype and sex information for rat cohort 3

	$Dlg2^{+/-}$	WT	Total
Male	12	8	20

Female	9	15	24
Total	21	24	44

Used under PPL 303135, PI Jeremy Hall

Experiments participated in in temporal order:

- Elevated plus maze (Chapter 3.2.1.).
- Open field test (Chapter 3.2.2.).
- Pre-pulse inhibition and sensorimotor gating (Chapter 3.2.3.).
- Object recognition battery – NOR, OL, OIP (Chapter 4.2.1.).
- 4-day reference memory and reversal in the water maze (Chapter 4.2.1.).

Cohort 4

Table 10: Genotype and sex information for rat cohort 4

	Dlg2+/-	WT	Total
Male	9	23	32
Female	15	17	32
Total	24	40	64

Used under PPL 303135, PI Jeremy Hall

Experiments participated in in temporal order:

- OIP with two samples (Chapter 4.3.1.).
- TOT (Chapter 4.3.1.).
- 10-day water maze reference memory task (Chapter 4.3.1.).
- Y-maze spontaneous alternation (Chapter 4.3.1.).

Cohort 5

Table 11: Genotype and sex information for rat cohort 5

	Dlg2+/-	WT	Total
Male	13	18	31

Female	18	16	34
Total	31	34	65

Used under PPL 303135, PI Jeremy Hall

Experiments participated in in temporal order:

- Reality testing (Chapter 6).

Cohort 6

Table 12: Genotype and sex information for rat cohort 6

	Dlg2$^{+/-}$	WT	Total
Male	19	14	33
Female	13	12	25
Total	32	26	58

Used under PPL 303135, PI Jeremy Hall

Experiments participated in in temporal order:

- Social preference and social novelty tasks (Chapter 3.2.5.).
- PCP hyperlocomotion (Chapter 3.2.6.).

Appendix 2.

Sex effects in the characterisation of the *Dlg2⁺/⁻* rat

The use of male-only cohorts when investigating psychiatric risk with rodent models is prevalent in the literature but risks conclusions that overlook sex differences in how these risk factors operate. For such reasons, the current experimental work used both male and female animals to allow sex differences to be investigated. However, as noted in the main text there were generally no sex by genotype interactions for the measures considered. Thus the main text report focused on genotype effects regardless of sex, while here we report the full results of the analyses including sex as a variable (oestrus stage was included as a variable initially – but had no effects so was not reported here). These support the conclusion that the current results are consistent with the *Dlg2⁺/⁻* manipulation being similar (or similarly absent) in males and females.

Chapter 3

A3.1 Behaviour on anxiety tests

A3.1.1 Elevated Plus Maze

As shown in Figure 50 sex had no effect on any measures recorded in the EPM including time in closed and open arms (Figure 50A), head dips (Figure 39B), stretch-attend postures (Figure 50C), grooming (Figure 50D), distance travelled (Figure 50E), velocity (Figure 50G) or defecation (Figure 50F). All main effects and interactions (along with Bayes factors providing evidence for the null effects) in the EPM assays can be seen in Table 13.

Figure 50: Effect of *Dlg2* heterozygous knockout and sex on anxiety-related behaviour in the EPM. Mean ± SEM with data points representing individuals **A)** time in zone **B)** head dips **C)** stretch-attend postures **D)** grooming **E)** distance moved **F)** velocity **G)** defecation.

Table 13: Repeated measures ANOVA and Bayesian ANOVA inferential statistics for analyses including sex as a factor on EPM measures

Analysis	Effect	F	p	n^2_p	BF$_{exclusion}$

Time in open arm	Genotype main effect	(1,33) = 0.053	0.819	0.002	4.124
	Sex main effect	(1,33) = 0.419	0.522	0.013	3.676
	Genotype × sex	(1,33) = 0.378	0.543	0.011	8.590
Head dips	Genotype main effect	(1,33) = 0.034	0.854	0.001	4.266
	Sex main effect	(1,33) = 0.054	0.818	0.002	4.222
	Genotype × sex	(1,33) = 0.107	0.746	0.003	9.677
Stretch attend postures	Genotype main effect	(1,33) = 0.190	0.666	0.006	3.528
	Sex main effect	(1,33) = 0.088	0.769	0.003	3.578
	Genotype × sex	(1,33) = 1.493	0.230	0.043	5.343
Grooming	Genotype main effect	(1,33) = 0.002	0.961	0.000	3.816
	Sex main effect	(1,33) = 0.001	0.970	0.000	3.825
	Genotype × sex	(1,33) = 0.748	0.393	0.022	7.897
Distance travelled	Genotype main effect	(1,33) = 0.082	0.776	0.002	4.182
	Sex main effect	(1,33) = 0.092	0.764	0.003	4.170
	Genotype × sex	(1,33) = 0.003	0.995	0.000	10.242

Velocity	Genotype main effect	(1,33) = 0.082	0.776	0.002	4.151
	Sex main effect	(1,33) = 0.092	0.763	0.003	4.139
	Genotype × sex	(1,33) = 0.003	0.995	0.000	9.743
Defecation	Genotype main effect	(1,39) = 1.505	0.227	0.039	2.471
	Sex main effect	(1,39) = 0.00000460	0.995	0.002	3.970
	Genotype × sex	(1,39) = 0.120	0.731	0.008	5.940

A3.1.2 Open Field

Sex also had no effect on open-field hyperactivity and anxiety measures as shown in Figure 51, including time in centre and peripheral zones (Figure 51A), velocity (Figure 51B), distance travelled (Figure 51C) and defecation (Figure 51D). Inferential statistics including sex for these measures can be found in Table 14, which shows significant sex effects for distance travelled and velocity in the open field yet no sex × genotype interactions.
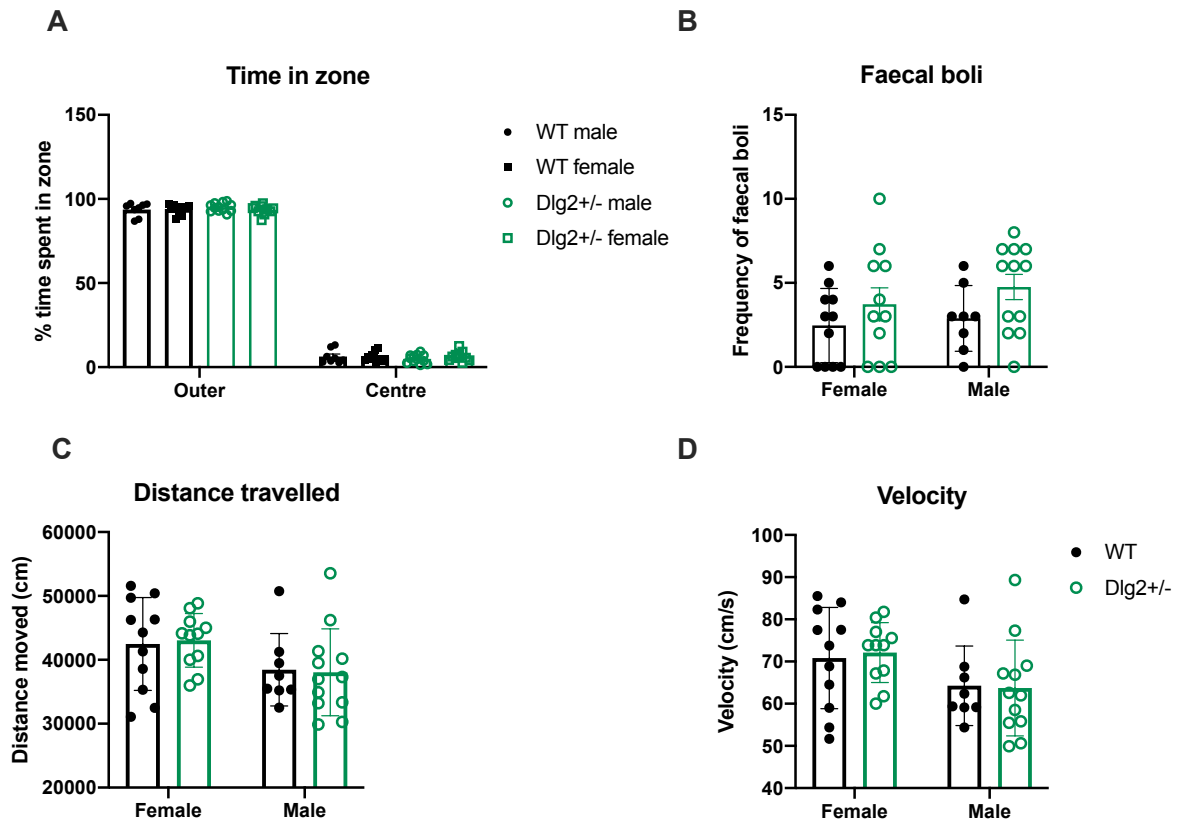
Figure 51: Effect of *Dlg2* heterozygous knockout and sex on open-field measures. A) time in zone **B)** velocity **C)** distance travelled and **D)** defection. Mean ± SEM with data points representing individuals.

Table 14: Repeated measures ANOVA and Bayesian ANOVA inferential statistics for sex effects and interactions on open field measures

Analysis	Effect	F	p	n^2_p	$BF_{exclusion}$
Time in zone	Zone main effect	(1,38) = 9100.383	< 0.001	0.996	0.000
	Genotype main effect	(1,38) = 0.697	0.409	0.018	7.292
	Sex main effect	(1,38) = 0.222	0.641	0.006	7.169
	Genotype × sex	(1,38) = 0.095	0.759	0.003	19.332

	Zone × genotype	(1,38) = 0.232	0.633	0.000	6.064
	Zone × sex	(1,38) = 0.362	0.551	0.000	4.372
	Zone × genotype × sex	(1,38) = 0.973	0.330	0.000	42.124
Velocity	Genotype main effect	(1,38) = 0.013	0.908	0.000	3.753
	Sex main effect	(1,38) = 5.481	0.025	0.126	0.453
	Genotype × sex	(1,38) = 0.083	0.775	0.002	3.462
Distance travelled	Genotype main effect	(1,38) = 0.002	0.961	0.000	3.551
	Sex main effect	(1,38) = 5.526	0.024	0.127	0.434
	Genotype × sex	(1,38) = 0.061	0.806	0.002	3.853
Defecation	Genotype main effect	(1,38) = 3.769	0.060	0.090	0.880
	Sex main effect	(1,38) = 0.792	0.379	0.020	2.601
	Genotype × sex	(1,38) = 0.138	0.712	0.004	3.300

A3.2 Sensorimotor gating

Rats of different sexes performed comparably on tests of startle to increasing auditory stimuli (Figure 52A), habituation of startle response at 105 dB (Figure 52B) and 120 dB (Figure 52C) and pre-pulse inhibition at 105 dB (Figure 52D) and 120 dB (Figure 52E). All

main effects and interactions (along with Bayes factors providing evidence for the null effects) in the EPM assays can be seen in Table 15.

A

B

Trial number P105

C

Trial number P120

D

PPI P105

E

PPI P120
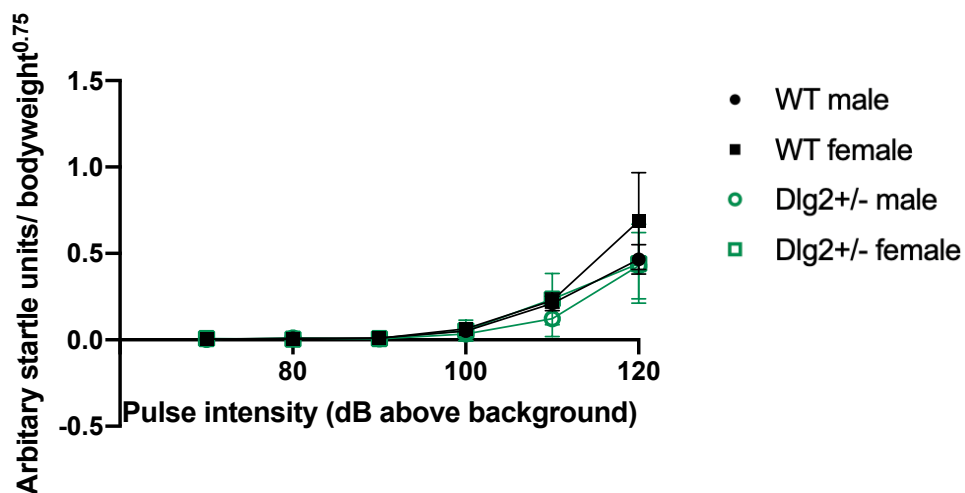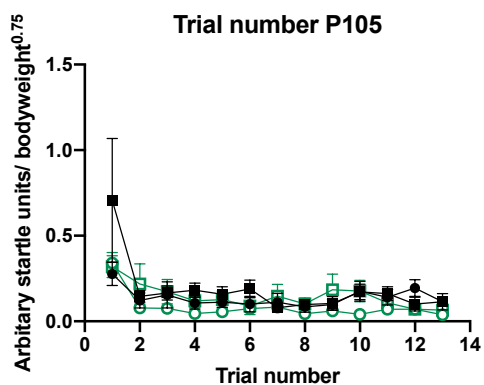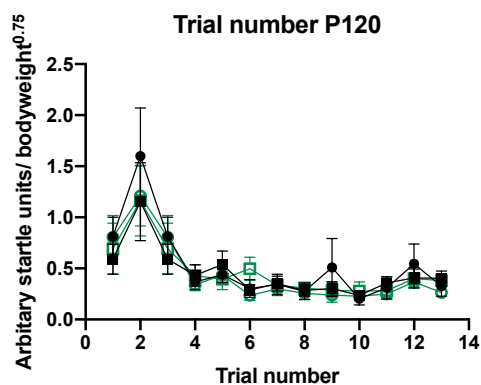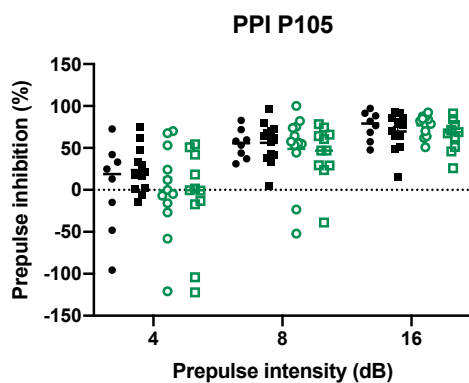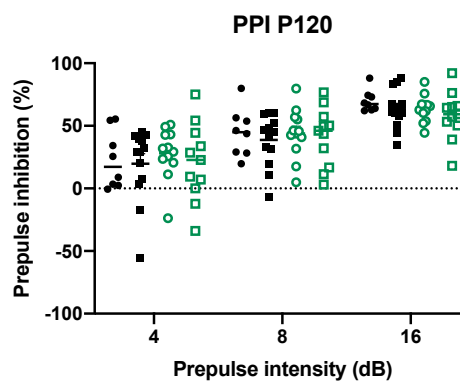
Figure 52: Effect of *Dlg2* heterozygosity and sex on acoustic startle response and pre-pulse inhibition. A) Mean ± SEM weight-adjusted ASR to 70-120 dB pulses above background.

B) Habituation of startle response through increasing pulse trials. Mean ± SEM ASR shown at 120 dB and **C)** 105 dB. **D)** Mean ± SEM with data points representing individuals PPI by a 4, 8 and 16 dB (above background) pre-pulse on 120 dB pulse and **E)** 105 dB pulse.

Table 15: Repeated measures ANOVA and Bayesian ANOVA inferential statistics for sex effects and interactions on sensorimotor gating measures

Analysis	Effect	F	p	$n^2{}_p$	$BF_{exclusion}$
Weight-adjusted ASR to 70-120 dB pulses above background	Pulse main effect	(1.089, 43.542) = 29.705	< 0.001	0.426	< 0.0001
	Genotype main effect	(1, 40) = 0.872	0.356	0.021	8.964
	Sex main effect	(1, 40) = 0.858	0.360	0.021	9.573
	Genotype × sex	(1, 40) = 0.056	0.815	0.001	38.490
	Genotype × pulse	(1.089, 43.542) = 0.590	0.460	0.015	22.407
	Sex × pulse	(1.089, 43.542) = 0.450	0.522	0.011	37.906
	Genotype × sex × pulse	(1.089, 43.542) = 0.485	0.505	0.012	9759.370
Habituation of startle response (105 dB)	Trial main effect	(1.573, 62.934) = 6.058	0.007	0.132	< 0.0001

	Sex main effect	(1, 40) = 2.127	0.153	0.050	4.607
	Genotype main effect	(1, 40) = 1.497	0.228	0.036	6.416
	Genotype × sex	(1, 40) = 0.056	0.813	0.001	18.698
	Trial × genotype	(1.573, 62.934) = 0.519	0.555	0.013	127.064
	Trial × sex	(1.573, 62.934) = 0.681	0.476	0.017	102.095
	Trial × sex × genotype	(1.573, 62.934) = 1.247	0.288	0.030	63776.307
Habituation of startle response (120 dB)	Trial main effect	(2.130, 85.190) = 13.127	< 0.001	0.247	< 0.0001
	Sex main effect	(1, 40) = 0.012	0.913	0.000	10.694
	Genotype main effect	(1, 40) = 0.347	0.559	0.009	10.545
	Genotype × sex	(1, 40) = 0.072	0.790	0.002	32.699
	Trial × genotype	(2.130, 85.190) = 0.498	0.621	0.012	643.328
	Trial × sex	(2.130, 85.190) = 1.160	0.320	0.028	174.990

	Trial × sex × genotype	(2.130, 85.190) = 0.543	0.594	0.013	5964000
PPI 105 dB	Pre-pulse main effect	(1.380, 55.210) = 42.203	< 0.001	0.513	< 0.0001
	Sex main effect	(1, 40) = 1.495	0.229	0.036	6.523
	Genotype main effect	(1, 40) = 0.696	0.409	0.017	3.437
	Genotype × sex	(1, 40) = 1.106	0.299	0.027	8.717
	Pre-pulse × genotype	(1.380, 55.210) = 0.314	0.650	0.008	3.713
	Pre-pulse × sex	(1.380, 55.210) = 0.104	0.827	0.003	7.207
	Pre-pulse × genotype × sex	(1.380, 55.210) = 1.952	0.163	0.047	87.336
PPI 120 dB	Pre-pulse main effect	(2, 80) = 83.401	< 0.001	0.676	< 0.0001
	Sex main effect	(1, 40) = 0.902	0.348	0.022	4.447
	Genotype main effect	(1, 40) = 0.011	0.917	0.000	5.482
	Genotype × sex	(1, 40) = 0.0001929	0.989	0.000	9.240
	Pre-pulse × sex	(2, 80) = 0.030	0.970	0.001	10.064

	Pre-pulse × genotype	(2, 80) = 1.097	0.339	0.027	5.712
	Pre-pulse × genotype × sex	(2, 80) = 0.177	0.838	0.004	206.171

A3.3 Social preference and social novelty

There were no sex effects on exploration in the social preference or social novelty tasks as seen for social preference raw exploration time (Figure 53A) and discrimination ratio (Figure 53B) and social novelty raw exploration time (Figure 53C) and discrimination ratio (Figure 53D). All main effects and interactions (along with Bayes factors providing evidence for the null effects) in the social preference and novelty assays can be seen in Table 16.
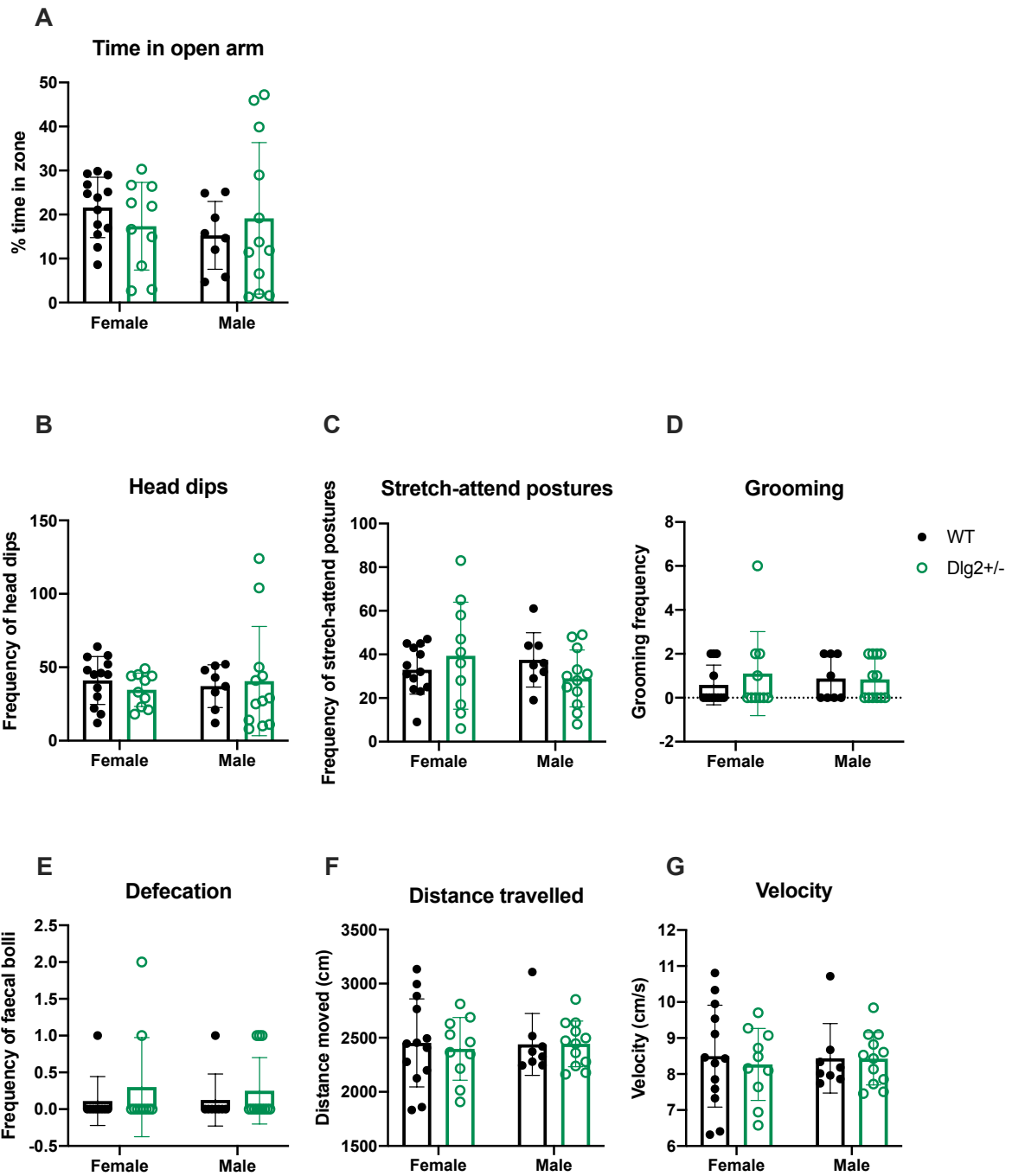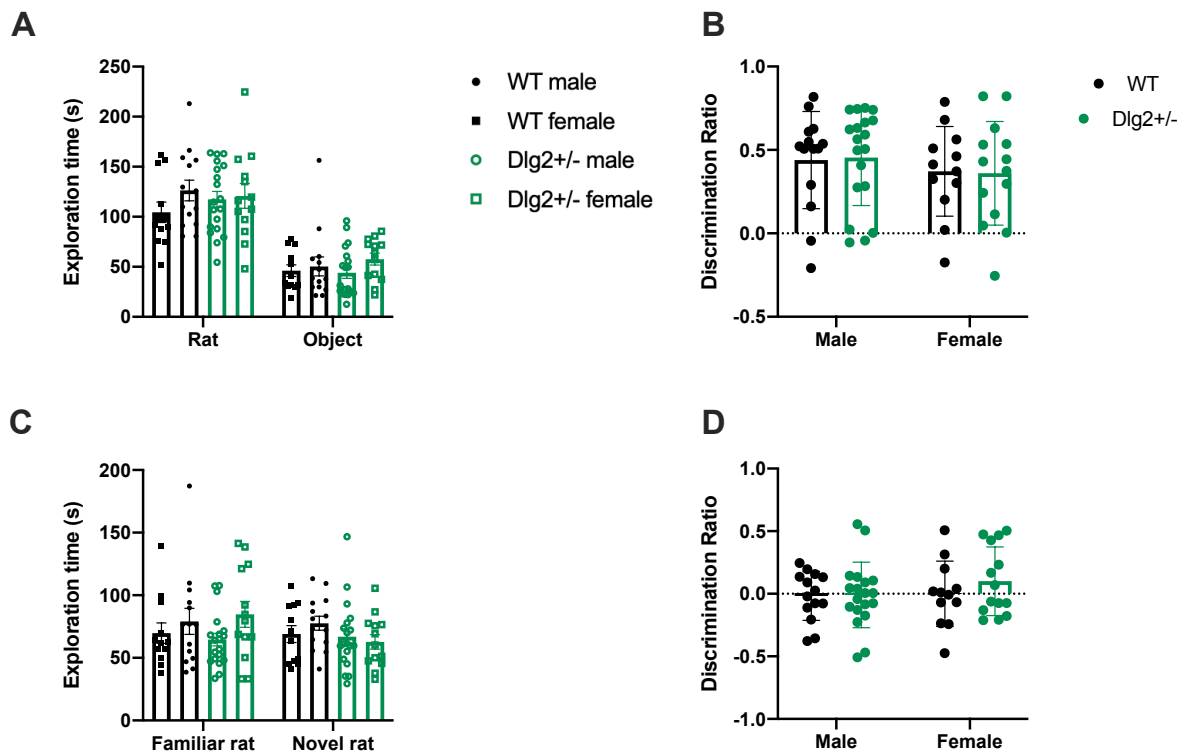

Figure 53: Effect of *Dlg2* heterozygosity and sex on the social preference and social novelty tasks. Mean and SEM ± with data points representing individuals **A)** raw exploration on the social preference task, **B)** d2 discrimination ratios on the social preference task, **C)**

raw exploration on the social novelty task and **D)** d2 discrimination ratios on the social

novelty task.

Table 16: Repeated measures ANOVA and Bayesian ANOVA inferential statistics for sex effects and interactions on social preference raw exploration and d2 scores

Analysis	Effect	F	p	$n^2{}_p$	BF$_{exclusion}$
Social preference raw exploration time (s)	Item main effect	(1, 50) = 76.012	< 0.001	0.603	0.000
	Genotype main effect	(1, 50) = 0.014	0.907	0.000279	7.986
	Sex main effect	(1, 50) = 0.108	0.744	0.002	6.845
	Genotype × item	(1,50) = 0.479	0.492	0.009	7.678
	Genotype × sex	(1, 50) = 1.737	0.193	0.034	9.851
	Item × sex	(1, 50) = 0.391	0.535	0.008	3.923
	Item × genotype × sex	(1, 50) = 0.003	0.957	0.0000577	46.918
Social preference discrimination ratio	Genotype main effect	(1, 50) = 0.850	0.361	0.017	4.973
	Sex main effect	(1, 50) = 0.894	0.349	0.018	2.451
	Genotype × sex	(1, 50) = 0.337	0.564	0.007	8.268

Social novelty raw exploration time (s)	Item main effect	(1, 54) = 1.066	0.306	0.019	6.978
	Genotype main effect	(1, 54) = 0.618	0.435	0.011	6.728
	Sex main effect	(1, 54) = 0.009	0.924	0.0001689	8.398
	Genotype × item	(1, 54) = 0.664	0.419	0.012	20.094
	Genotype × sex	(1, 54) = 2.611	0.112	0.046	9.598
	Item × sex	(1, 54) = 1.259	0.267	0.023	13.001
	Item × genotype × sex	(1, 54) = 1.408	0.241	0.025	66.256
Social novelty discrimination ratio	Genotype main effect	(1, 54) = 0.825	0.368	0.015	3.882
	Sex main effect	(1, 54) = 1.084	0.302	0.020	3.083
	Genotype × sex	(1, 54) = 0.632	0.430	0.012	6.903

A3.4 PCP-induced locomotion

All sex effects and interactions in the analysis of locomotor activity pre and post PCP administration were non-significant as shown in Table 17. Despite this it is worth noting that the Bayes factor for the sex × genotype interaction is inconclusive, and Figure 54 suggests that the exaggerated PCP hyperlocomotion finding in *Dlg2+/-* rats is larger in females.
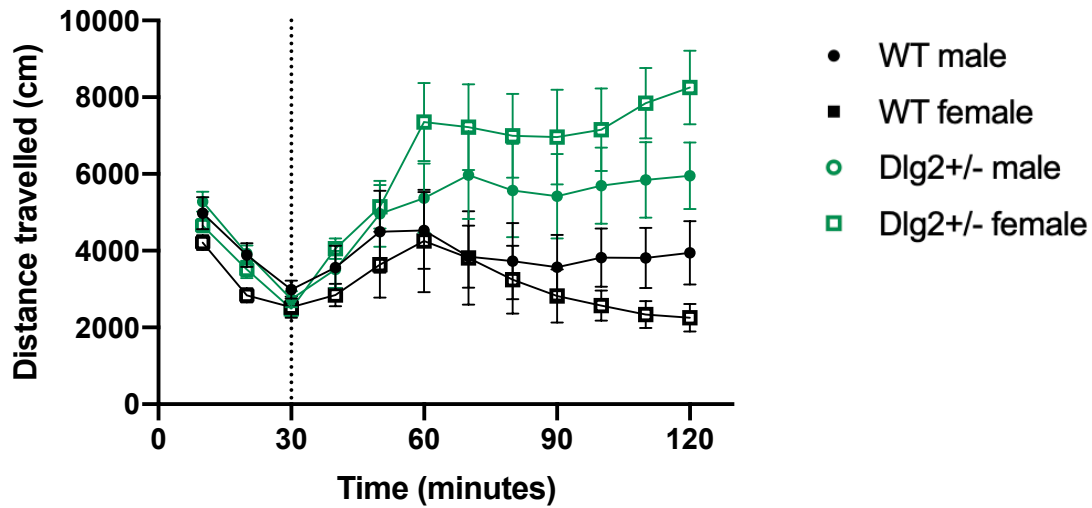
Figure 54: Locomotor activity in response to PCP injection in male and female *Dlg2*⁺/⁻ and wild-type rats. Mean ± SEM distance travelled is plotted in 10-minute bins. The dotted line at 30 minutes denotes when the PCP injection occurred.

Table 17: Sex effects and interactions from repeated measures ANOVA and Bayesian repeated measures ANOVA of distance moved over the 2h rats spent in the arena.

Effect	F	p	n^2_p	$BF_{exclusion}$
Time bin main effect	(2.388, 107.477) = 7.446	< 0.001	0142	< 0.0001
Sex main effect	(1,45) = 0.027	0.870	0.000599	4.546
Genotype main effect	(1,45) = 8.074	0.007	0.152	< 0.0001
Genotype × sex	(1,45) = 2.196	0.145	0.047	2.210
Time bin × sex	(2.388, 107.477) = 1.039	0.367	0.023	35.628
Time bin × genotype	(2.388, 107.477) = 5.949	0.002	0.117	< 0.0001

Time bin × sex × genotype	(2.388, 107.477) = 0.881	0.433	0.019	253.282

Chapter 4

A4.1. Experiment 1

A4.1.1. Habituation to arena

Male rats showed higher activity levels than females across all trials when habituating to an empty arena as shown by velocity Figure 55A and distance travelled Figure 55B. This is reflected by the main effect of sex for velocity ($F(1, 39) = 43.552$, $p < 0.001$, $n^2_p = 0.528$) and distance travelled ($F(1, 39) = 31.396$, $p < 0.001$, $n^2_p = 0.446$). There were no sex differences in habituation with sex × trial interactions non-significant for both measures, and no genotype effects on this with sex × genotype and sex × genotype × trial interactions non-significant as shown in Table 18.
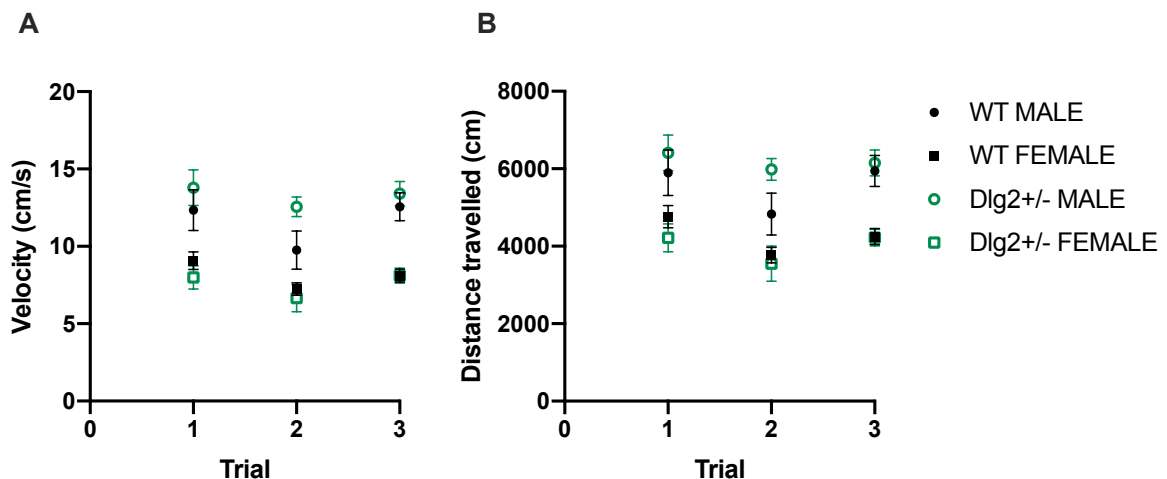
Figure 55: Male and female *Dlg2$^{+/-}$* and wild-type rats' activity in the three 10-minute sessions when habituating to the empty arena to be used for object recognition tasks in Experiment 1. Mean ± SEM **A)** velocity, **B)** distance travelled.

Table 18: Main and interaction effects from repeated measures ANOVA and Bayesian ANOVA analysis of velocity and distance travelled across three 10-minute habituation sessions to the empty object recognition arena in Experiment 1.

Measure	Effect	F	p	$n^2{}_p$	$BF_{exclusion}$
Velocity	Main effect trial	(2, 78) = 10.652	< 0.001	0.215	0.003
	Main effect genotype	(1, 39) = 0.594	0.446	0.015	8.585
	Main effect sex	(1, 39) = 43.552	< 0.001	0.528	< 0.001
	Sex × genotype	(1, 39) = 1.883	0.178	0.046	3.705
	Trial × sex	(2, 78) = 0.204	0.816	0.005	6.345
	Trial × genotype	(2, 78) = 0.327	0.722	0.008	12.791
	Trial × sex × genotype	(2, 78) = 0.803	0.452	0.020	71.080
Distance	Main effect trial	(2, 78) = 10.495	< 0.001	0.212	0.003
	Main effect genotype	(1, 39) = 0.274	0.604	0.007	3.244
	Main effect sex	(1, 39) = 31.396	< 0.001	0.446	< 0.001
	Sex × genotype	(1, 39) = 1.321	0.257	0.033	1.933
	Trial × sex	(2, 78) = 0.250	0.780	0.006	2.377
	Trial × genotype	(2, 78) = 0.520	0.597	0.013	5.052

	Trial × sex × genotype	(2, 78) = 0.828	0.441	0.021	31.264

A4.1.2. Responding to a novel object

There were no sex differences in baseline responding to a novel object (Figure 56), precluding this as a concern for object recognition tests. Non-significant main effects and interactions from ANOVA and Bayesian ANOVA are presented in Table 19.
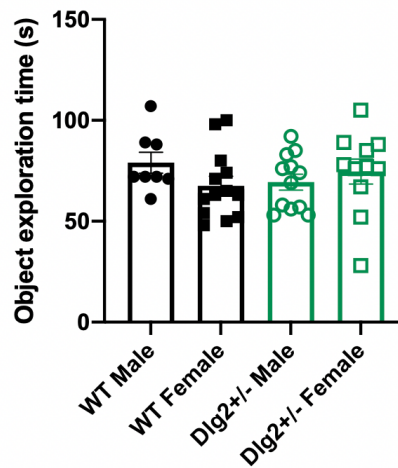
Figure 56: Exploration time for a novel object presented alone during the habituation stage of object recognition experiments. Mean ± SEM with data points representing individuals.

Table 19: Sex effects and interactions from ANOVA and Bayesian ANOVA of exploration time to a novel object.

Effect	F	p	n^2_p	BF$_{exclusion}$
Genotype	(1,40) = 0.063	0.804	0.002	4.058
Sex	(1,40) = 0.363	0.550	0.009	3.747
Genotype × sex	1,40) = 2.632	0.113	0.062	4.383

A4.1.3. Novel object recognition

There were no interactions with sex on the novel object recognition task as shown by raw exploration time (Figure 57A) and discrimination ratio (Figure 57B). Non-significant effects and interactions are shown in Table 20.

Table 20: Main and interaction effects for a repeated measures ANOVA and Bayesian repeated measures ANOVA of object exploration time in the novel object recognition test.

Analysis	Effects	F	p	n^2p	BF$_{exclusion}$
Raw exploration times	Main effect of object	(1, 40) = 13.435	< 0.001	0.251	0.002
	Main effect of sex	(1, 40) = 6.677	0.014	0.143	1.390
	Main effect of genotype	(1, 40) = 0.838	0.365	0.021	5.498
	Sex × genotype	(1, 40) = 1.146	0.291	0.028	5.344
	Object × sex	(1, 40) = < 0.001	0.993	0.000	2.438
	Object × genotype	(1, 40) = 0.453	0.505	0.011	4.594
	Object × sex × genotype	(1, 40) = 1.351	0.252	0.033	15.272
Discrimination ratio	Genotype main effect	(1,40) = 0.063	0.804	0.002	4.240
	Sex main effect	(1,40) = 0.363	0.550	0.009	4.198
	Sex × genotype	(1,40) = 2.632	0.113	0.062	5.906

A4.1.4. Object location recognition

Sex did not influence object location recognition as shown by raw exploration time (Figure 57C) and discrimination ratio (Figure 57D). Non-significant main effects and interactions for sex are shown in Table 21.

Table 21: Main and interaction effects found in repeated measures ANOVA and Bayesian repeated measures ANOVA analysis of exploration time on the object location task.

Analysis	Effects	F	p	n^2p	$BF_{exclusion}$
Raw exploration times	Main effect of location	(1, 40) = 50.450	< 0.001	0.558	< 0.001
	Main effect of sex	(1, 40) = 0.00004314	0.995	0.000	5.249
	Main effect of genotype	(1, 40) = 1.370	0.249	0.033	3.250
	Sex × genotype	(1, 40) = 0.205	0.653	0.005	7.766
	Location × sex	(1, 40) = 0.429	0.516	0.011	4.673
	Location × genotype	(1, 40) = 0.889	0.351	0.022	2.857
	Location × sex × genotype	(1, 40) = 0.601	0.443	0.015	21.807
Discrimination ratio	Genotype main effect	(1,40) = 0.053	0.818	0.001	4.318
	Sex main effect	(1,40) = 1.636	0.208	0.039	2.443
	Sex × genotype	(1,40) = 0.470	0.497	0.012	6.600

A4.1.5. Object-in-place recognition

Sex did not influence object location recognition as shown by raw exploration time (Figure 57E) and discrimination ratio (Figure 57F). Non-significant main effects and interactions for sex are shown in Table 22.

Table 22: Main and interaction effects found in a repeated measures ANOVA of object pair exploration time in the object-in-place test phase.

Analysis	Effects	F	p	np2	BF$_{exclusion}$
Raw exploration times	Main effect of object	(1, 40) = 2.053	0.160	0.050	1.640
	Main effect of sex	(1, 40) = 8.722	0.005	0.000	0.598
	Main effect of genotype	(1, 40) = 4.239	0.046	0.098	0.857
	Sex × genotype	(1, 40) = 2.971	0.093	0.071	1.178
	Object × sex	(1, 40) = 0.0003357	0.985	0.000	2.998
	Object × genotype	(1, 40) = 3.878	0.056	0.090	0.787
	Object × sex × genotype	(1, 40) = 0.202	0.656	0.005	3.997
Discrimination ratio	Genotype main effect	(1,39) = 3.619	0.065	0.085	1.021
	Sex main effect	(1,39) = 0.130	0.720	0.003	3.623
	Sex × genotype	(1,39) = 0.305	0.584	0.008	4.069
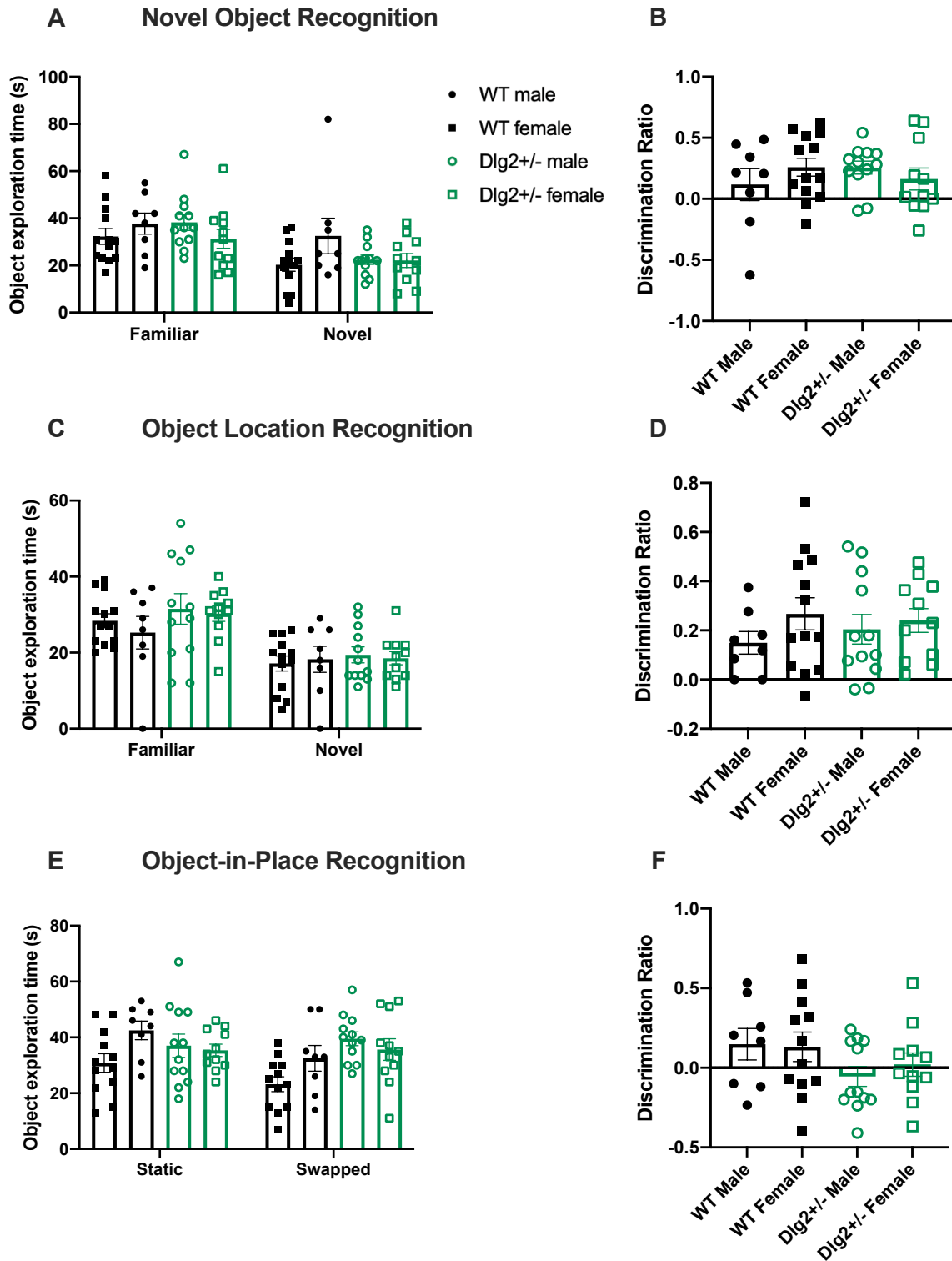
Figure 57: Male and female *Dlg2^{+/-}* and wild-type rats performance on the three object recognition tasks in Experiment 1. Mean and ± SEM plus individual scores. **A)** Novel object recognition raw exploration **B)** Novel object recognition discrimination ratio **C)** Object location raw exploration **D)** Object location discrimination ratio **E)** Object-in-place raw exploration **F)** Object-in-place discrimination ratio.

A4.1.6. Water maze reference memory

Figure 58 shows the mean escape latencies (Figure 58A), distance swum (Figure 58B), swim speed (Figure 58C) and percentage of time near side walls (Figure 58D) for rats of both sexes and genotypes on the reference memory task. There were no sex × genotype interactions for any measure (Table 23). It is of note that there were significant main effects of sex on distance swum ($F(1,40) = 5.545$, $p = 0.024$, $n^2p = 0.122$) and significant sex × day ($F(3, 120) = 9.447$, $p < 0.001$, $n^2p = 0.191$) and sex × trial × day ($F(7.028, 281.140) = 2.086$, $p = 0.045$, $n^2p = 0.050$) interactions for escape latency. These sex effects reflected differences in swim speed between sexes, with females swimming consistently faster than males at all stages in the experiment as shown in Figure 58C (main effect of sex on swim speed: $F(9,40) = 48.371$, $p < 0.001$, $n^2p = 0.547$). Sex differences in swim speed should not have influenced genotype effects on escape latency and path length, as sex × genotype interactions on swim speed were non-significant ($F(1,40) = 0.219$, $p = 0.642$, $n^2p = 0.005$; $BF_{exclusion} = 17.280$).
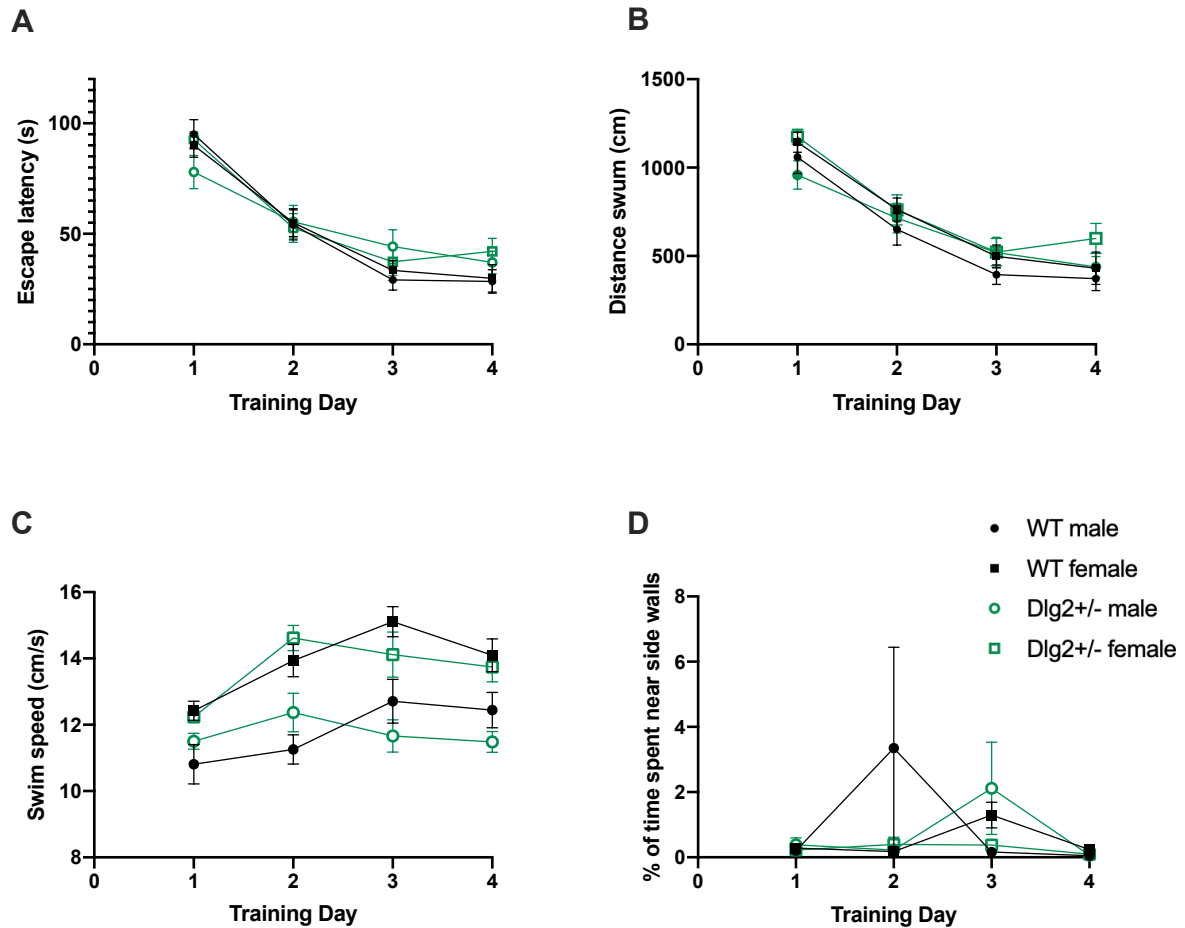
Figure 58: Performance of *Dlg2⁺/⁻* and wild-type rats of both sexes on a four-day reference memory task in the water maze. Escape latencies **(A)**, distance swum **(B)** swim speed **(C)** and percentage of time spent near side walls **(D)** across all four training days in the reference memory task, split by sex (male and female) and genotype (*Dlg2⁺/⁻* and WT). The latencies, distances and speeds shown for each day are the average of four daily trials and the SEM.

Table 23: Table showing main and interaction effects for repeated measures ANOVA and Bayesian repeated measures ANOVA on escape latency, distance swum, swim speed and percentage of time spent near side walls on the reference memory task.

Analysis	Effect	F	P	n^2p	BF$_{exclusion}$
Escape latency	Main effect of day	$(3, 120) = 86.959$	< 0.001	0.685	< 0.001

	Main effect of trial	(3, 120) = 36.178	< 0.001	0.475	< 0.001
	Main effect of sex	(1, 40) = 2.937	0.094	0.068	< 0.001
	Main effect of genotype	(1, 40) = 4.066	0.051	0.092	11.657
	Genotype × sex	(1, 40) = 0.676	0.416	0.017	12.793
	Day × genotype	(3, 120) = 0.729	0.536	0.018	65.851
	Day × sex	(3, 120) = 9.447	< 0.001	0.191	< 0.001
	Day × genotype × sex	(3, 120) = 1.190	0.317	0.029	283.721
	Trial × genotype	(3, 120) = 1.696	0.172	0.041	22.356
	Trial × sex	(3, 120) = 1.366	0.257	0.033	11.629
	Trial × genotype × sex	(3, 120) = 0.664	0.576	0.016	> 1000
	Day × trial	(7.028, 281.140) = 2.445	0.019	0.058	1.086
	Day × trial × genotype	(7.028, 281.140) = 0.846	0.550	0.021	> 1000
	Day × trial × sex	(7.028, 281.140) = 2.086	0.045	0.050	7.731

	Day × trial × genotype × sex	(7.028, 281.140) = 0.522	0.818	0.013	> 1000
Distance swum (cm)	Main effect of day	(3, 120) = 62.970	<0.001	0.612	<0.001
	Main effect of trial	(3, 120) = 72.399	<0.001	0.644	<0.001
	Main effect of sex	(1, 40) = 5.545	0.024	0.122	12.423
	Main effect of genotype	(1, 40) = 3.221	0.080	0.075	23.083
	Day × genotype	(3, 120) = 0.311	0.818	0.008	200.521
	Genotype × sex	(1, 40) = 0.316	0.577	0.008	49.123
	Day × sex	(3, 120) = 0.398	0.755	0.010	103.446
	Day × genotype × sex	(3, 120) = 0.894	0.447	0.022	> 1000
	Trial × genotype	(3, 120) = 0.598	0.617	0.015	134.319
	Trial × sex	(3, 120) = 0.349	0.790	0.009	119.806
	Trial × genotype × sex	(3, 120) = 0.310	0.818	0.008	> 1000
	Day × trial	(6.670, 266.812) = 5.575	<0.001	0.122	<0.001
	Day × trial × genotype	(6.670, 266.812) = 1.262	0.271	0.031	> 1000

	Day × trial × sex	(6.670, 266.812) = 0.399	0.896	0.010	> 1000
	Day × trial × genotype × sex	(6.670, 266.812) = 0.580	0.764	0.014	> 1000
Swim speed (cm/s)	Main effect of day	(3, 120) = 10.369	< 0.001	0.206	< 0.001
	Main effect of trial	(3,120) = 48.253	<0.001	0.547	0.000
	Main effect of sex	(1, 40) = 48.371	<0.001	0.547	< 0.001
	Main effect of genotype	(1, 40) = 1.517	0.225	0.037	17.970
	Day × genotype	(3,120) = 1.791	0.153	0.043	10.193
	Genotype × sex	(1, 40) = 0.219	0.642	0.005	17.280
	Day × sex	(3,120) = 1.801	0.151	0.043	1.714
	Day × genotype × sex	(3,120) = 0.488	0.691	0.012	454.410
	Trial × genotype	(3,120) = 0.214	0.886	0.005	195.707
	Trial × sex	(3,120) = 0.936	0.426	0.023	20.588
	Trial × genotype × sex	(3,120) = 0.792	0.501	0.019	> 1000
	Day × trial	(9, 360) = 7.308	<0.001	0.154	<0.001

	Day × trial × genotype	(9, 360) = 0.873	0.550	0.021	> 1000
	Day × trial × sex	(9, 360) = 1.322	0.224	0.032	61.098
	Day × trial × genotype × sex	(9, 360) = 0.737	0.675	0.018	> 1000
Percentage of time near walls	Main effect of day	(1.605, 64.211) = 1.223	0.294	0.030	144.699
	Main effect of trial	(3,120) = 4.288	0.007	0.097	2.514
	Main effect of sex	(1, 40) = 0.877	0.355	0.021	39.953
	Main effect of genotype	1, 40) = 1.614	0.211	0.039	43.090
	Sex × genotype	(1, 40) = 3.829	0.057	0.087	140.479
	Day × genotype	(1.605, 64.211) = 0.507	0.564	0.013	> 1000
	Day × sex	(1.605, 64.211) = 0.407	0.623	0.010	> 1000
	Day × genotype × sex	(1.605, 64.211) = 0.767	0.442	0.019	> 1000
	Trial × genotype	(1.059, 42.358) = 1.585	0.216	0.038	92.989

	Trial × sex	(1.059, 42.358) = 1.575	0.217	0.038	46.072
	Trial × genotype × sex	(1.059, 42.358) = 2.037	0.160	0.048	> 1000
	Day × trial	(1.642, 65.678) = 1.386	0.256	0.033	184.866
	Day × trial × genotype	(1.642, 65.678) = 0.566	0.537	0.014	> 1000
	Day × trial × sex	(1.642, 65.678) = 0.417	0.621	0.010	> 1000
	Day × trial × genotype × sex	(1.642, 65.678) = 0.810	0.428	0.020	> 1000

A4.1.7. Reference memory probe

Sex differences and sex × genotype interactions did not manifest on the probe test, as shown by quadrant dwell time (Figure 59A), latency to first annulus cross (Figure 59B) and number of annulus crosses (Figure 59C). Inferential statistics from repeated measures ANOVA and Bayesian ANOVA for quadrant dwell time can be found in Table 24 and ANOVA and Bayesian ANOVA analyses for latency to first cross and number of crosses in Table 25.
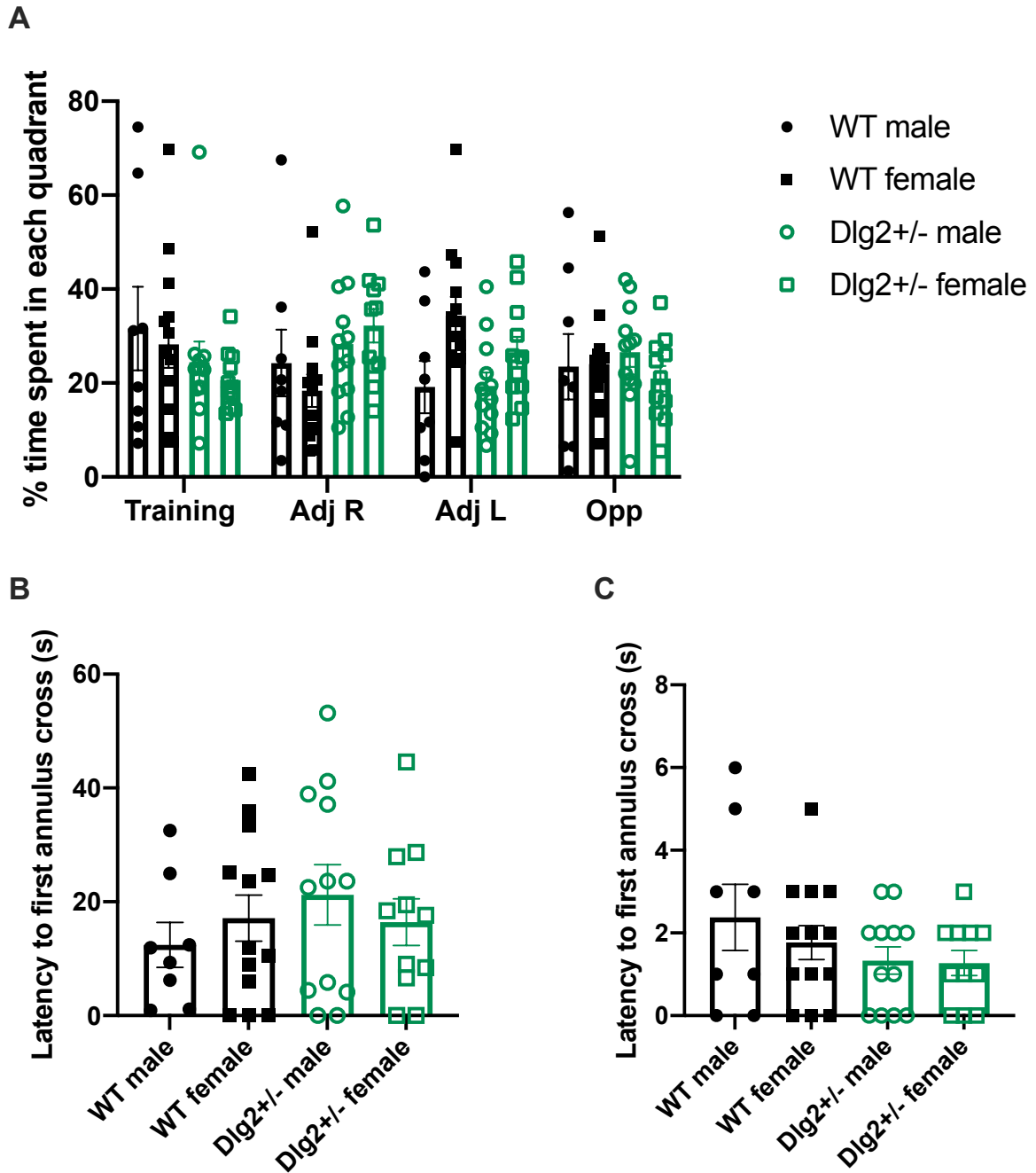
Figure 59: Male and female *Dlg2⁺ᐟ⁻* and wild-type rats performance on the reference memory probe test. Mean ± SEM plus individual values. **A)** Percentage of time spent in each quadrant **B)** Latency to first cross of previous platform annulus **C)** Number of crossings of previous platform annulus location.

Table 24: Main and interaction effects from repeated measures ANOVA and Bayesian repeated measures ANOVA on percentage time spent in each quadrant on the reference memory probe test.

Effects	F	p	n^2p	$BF_{exclusion}$
Main effect of quadrant	(2.498, 99.913) = 0.198	0.865	0.005	53.179
Main effect of sex	(1, 40) = 1.518	0.225	0.037	13.425
Main effect of genotype	(1, 40) = 0.452	0.505	0.011	8.714
Sex × genotype	(1, 40) = 0.525	0.473	0.013	63.507
Quadrant × sex	(2.498, 99.913) = 1.944	0.138	0.046	55.298
Quadrant × genotype	(2.498, 99.913) = 2.031	0.125	0.048	36.867
Quadrant × sex × genotype	(2.498, 99.913) = 0.659	0.552	0.016	471.822

Table 25: Main and interaction effects found in ANOVA and Bayesian ANOVA for number of annulus crossings and latency to first annulus cross.

Analysis	Effect	F	p	n^2p	$BF_{exclusion}$
Number of crossings	Main effect of sex	(1, 40) = 0.468	0.498	0.012	3.658
	Main effect of genotype	(1, 40) = 0.918	0.344	0.022	1.623
	Sex × genotype	(1, 40) = 0.918	0.344	0.022	4.818
Latency to first cross	Main effect of sex	(1, 40) = 0.00009845	0.992	0.000	4.286
	Main effect of genotype	(1, 40) = 0.775	0.384	0.019	3.443

	Sex × genotype	(1, 40) = 1.063	0.309	0.026	6.934

A4.1.8. Learning a new platform location in the water maze (reversal)

There were no sex × genotype, day × genotype × sex, trial × genotype × sex or day × trial × genotype × sex interactions on escape latency (Figure 60A), distance swum (Figure 60B), swim speed (Figure 60C) or percentage of time near side walls (Figure 60D) in the reversal stage of watermaze reference memory. These are shown in the inferential statistics Table 26. For escape latency there was a significant day × trial × sex interaction (F(6.004, 228.148) = 2.889, p = 0.010, n^2_p = 0.071) caused by females showing a more pronounced escape latency decrease across trials for the first few days of the reversal test.   For distance swum there was a main effect of sex (F(1, 38) = 4.756, p = 0.035, n^2_p = 0.111), reflecting females tendency overall to swim longer distances as shown in Figure 49B. The day × trial × sex interaction (F(5.214, 198.150) = 2.324, p = 0.042, n^2_p = 0.058) for distance swum was also significant, reflecting the larger distance decrease with trials for the first few days in females compared to males. Females consistently swum faster than males (main effect of sex for swim speed (F(1, 38) = 7.002, p = 0.012, n^2_p = 0.156). Females also showed less of a tendency to stay close to the side walls across days (significant day × sex interaction for percentage of time near side walls F(2.135, 83.282) = 4.348, p = 0.014, n^2_p = 0.100) and across trials (significant trial × sex interaction for percentage of time near side walls F(1.712, 66.749) = 4.131, p = 0.026, n^2_p = 0.096).
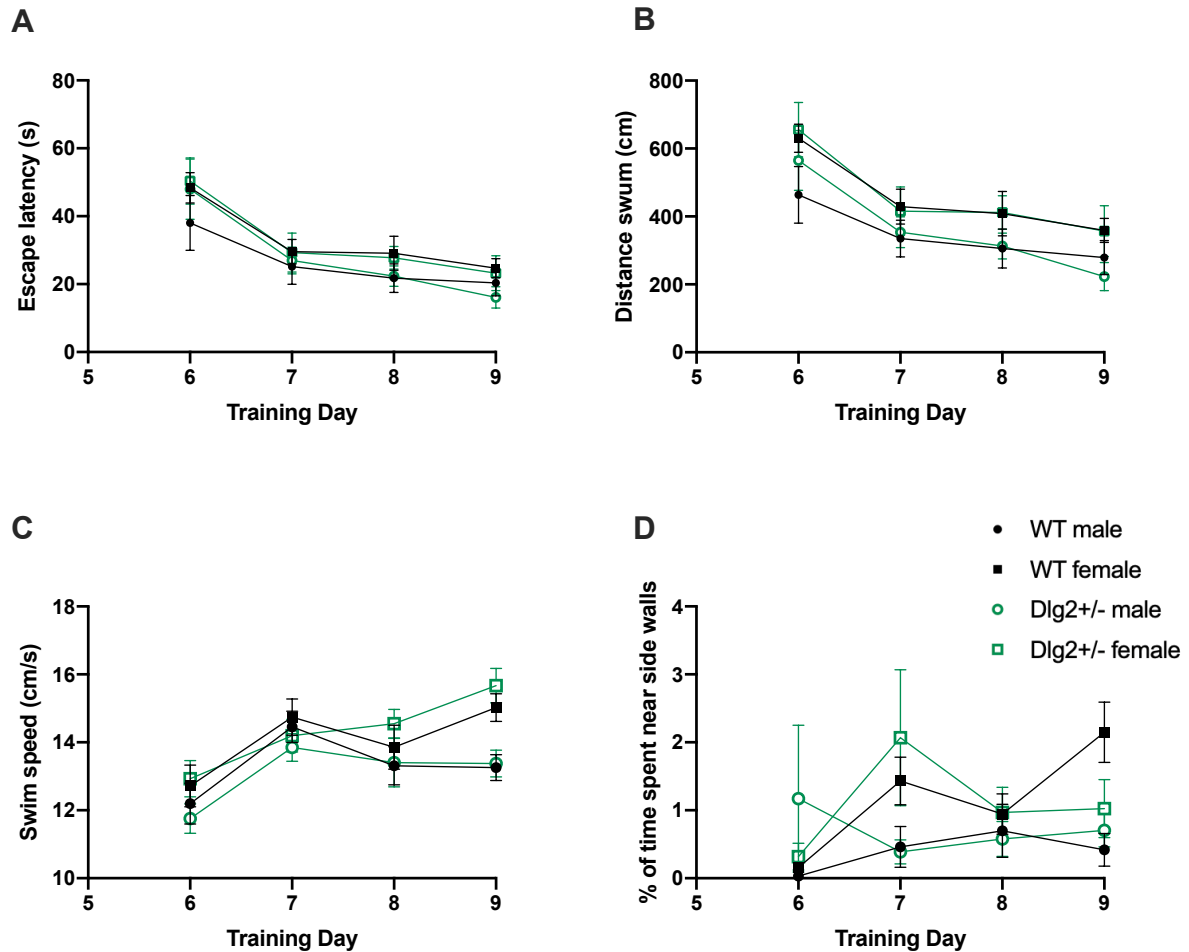
Figure 60: Performance of wild-type and *Dlg2$^{+/-}$* rats of both sexes after the platform location was changed to the opposite quadrant. Escape latencies **(A)**, distance swum **(B)**, swim speed **(C)** and percentage of time near side walls **(D)**. The latencies, distances and speeds shown for each day are the average of four daily trials and the SEM.

Table 26: Repeated measures ANOVA and Bayesian repeated measures ANOVA effects and interactions for escape latency, distance swum, swim speed and percentage of time near walls during water-maze reversal.

Analysis	Effect	*F*	*p*	*n²p*	BF$_{exclusion}$
Escape latency	Main effect of day	(2.275, 86.455) = 16.801	<0.001	0.307	> 1000
	Main effect of trial	(2.155,81.879) = 52.427	<0.001	0.580	> 1000
	Main effect of sex	(1,38) = 1.547	0.221	0.039	20.299

	Main effect of genotype	(1,38) = 0.276	0.602	0.007	35.964
	Sex × genotype	(1,38) = 0.351	0.557	0.009	80.719
	Day × genotype	(2.275, 86.455) = 1.674	0.190	0.042	74.517
	Day × sex	(2.275, 86.455) = 0.642	0.548	0.017	165.797
	Day × genotype × sex	(2.275, 86.455) = 0.961	0.396	0.025	> 1000
	Trial × genotype	(2.155,81.879) = 1.013	0.372	0.026	41.051
	Trial × sex	(2.155,81.879) = 1.763	0.175	0.044	12.565
	Trial × genotype × sex	(2.155,81.879) = 0.391	0.693	0.010	> 1000
	Day × trial	(6.004, 228.148) = 1.802	0.100	0.045	2.351
	Day × trial × genotype	(6.004, 228.148) = 1.600	0.148	0.040	> 1000
	Day × trial × sex	(6.004, 228.148) = 2.889	0.010	0.071	> 1000
	Day × trial × genotype × sex	(6.004, 228.148) = 1.123	0.350	0.029	> 1000
Distance swum (cm)	Main effect of day	(3, 114) = 13.670	< 0.001	0.265	< 0.001
	Main effect of trial	(2.076, 78.892) = 70.428	< 0.001	0.650	< 0.001
	Main effect of sex	(1, 38) = 4.756	0.035	0.111	7.606
	Main effect of genotype	(1, 38) = 0.055	0.815	0.001	40.751

	Day × genotype	(3, 114) = 1.023	0.385	0.026	230.157
	Day × sex	(3, 114) = 0. 767	0.515	0.020	68.513
	Day × genotype × sex	(3, 114) = 0. 758	0.520	0.020	> 1000
	Trial × genotype	(2.076, 78.892) = 0.828	0.444	0.021	77.145
	Trial × sex	(2.076, 78.892) = 1.627	0.202	0.041	8.225
	Trial × genotype × sex	(2.076, 78.892) = 0.218	0.812	0.006	> 1000
	Day × trial	(5.214, 198.150) = 2.226	0.051	0.055	0.246
	Day × trial × genotype	(5.214, 198.150) = 1.847	0.102	0.046	> 1000
	Day × trial × sex	(5.214, 198.150) = 2.324	0.042	0.058	54.642
	Day × trial × genotype × sex	(5.214, 198.150) = 0.828	0.535	0.021	> 1000
Swim speed (cm/s)	Main effect of day	(3, 114) = 9.257	< 0.001	0.196	< 0.001
	Main effect of trial	(3, 114) = 15.172	< 0.001	0.285	< 0.001
	Main effect of sex	(1, 38) = 7.002	0.012	0.156	0.795
	Main effect of genotype	(1, 38) = 0.391	0.535	0.010	11.223
	Genotype × sex	(1, 38) = 1.548	0.221	0.039	7.640
	Day × genotype	(3, 114) = 3.471	0.018	0.084	2.959
	Day × sex	(3, 114) = 2.480	0.065	0.061	1.595
	Day × genotype × sex	(3, 114) = 1.262	0.291	0.032	18.021

	Trial × genotype	(3, 114) = 0.437	0.727	0.011	140.521
	Trial × sex	(3, 114) = 0.673	0.570	0.017	49.751
	Trial × genotype × sex	(3, 114) = 0.661	0.578	0.017	> 1000
	Day × trial	(9, 342) = 3.791	< 0.001	0.091	0.007
	Day × trial × genotype	(9, 342) = 0.650	0.754	0.017	> 1000
	Day × trial × sex	(9, 342) = 0.880	0.543	0.023	408.184
	Day × trial × genotype × sex	(9, 342) = 0.469	0.895	0.012	> 1000
Percentage of time near walls	Main effect of day	(2.135, 83.282) = 0.513	0.613	0.013	1.114
	Main effect of trial	(1.712, 66.749) = 3.530	0.042	0.083	0.838
	Main effect of sex	(1, 39) = 1.814	0.186	0.044	1.807
	Main effect of genotype	(1, 39) = 0.391	0.535	0.010	44.126
	Sex × genotype	(1, 39) = 0.137	0.713	0.004	53.279
	Day × genotype	(2.135, 83.282) = 0.641	0.539	0.016	209.807
	Day × sex	(2.135, 83.282) = 4.348	0.014	0.100	0.361
	Day × genotype × sex	(2.135, 83.282) = 0.633	0.544	0.016	> 1000
	Trial × genotype	(1.712, 66.749) = 0.115	0.862	0.003	427.317
	Trial × sex	(1.712, 66.749) = 4.131	0.026	0.096	0.513

	Trial × genotype × sex	(1.712, 66.749) = 1.169	0.311	0.029	> 1000
	Day × trial	(2.651, 103.371) = 4.042	0.012	0.094	0.149
	Day × trial × genotype	(2.651, 103.371) = 0.455	0.691	0.012	> 1000
	Day × trial × sex	(2.651, 103.371) = 2.419	0.078	0.058	0.157
	Day × trial × genotype × sex	(2.651, 103.371) = 0.156	0.907	0.004	> 1000

A4.1.9. Performance on reversal probe test

There were no sex × genotype interactions or sex effects on quadrant dwell time (Figure 61A), latency to first annulus cross (Figure 61B) or number of annulus crossings (Figure 61C) on the reversal probe test. Inferential statistics for dwell time can be seen in Table 27 and for first annulus cross and number of crossings in Table 28.
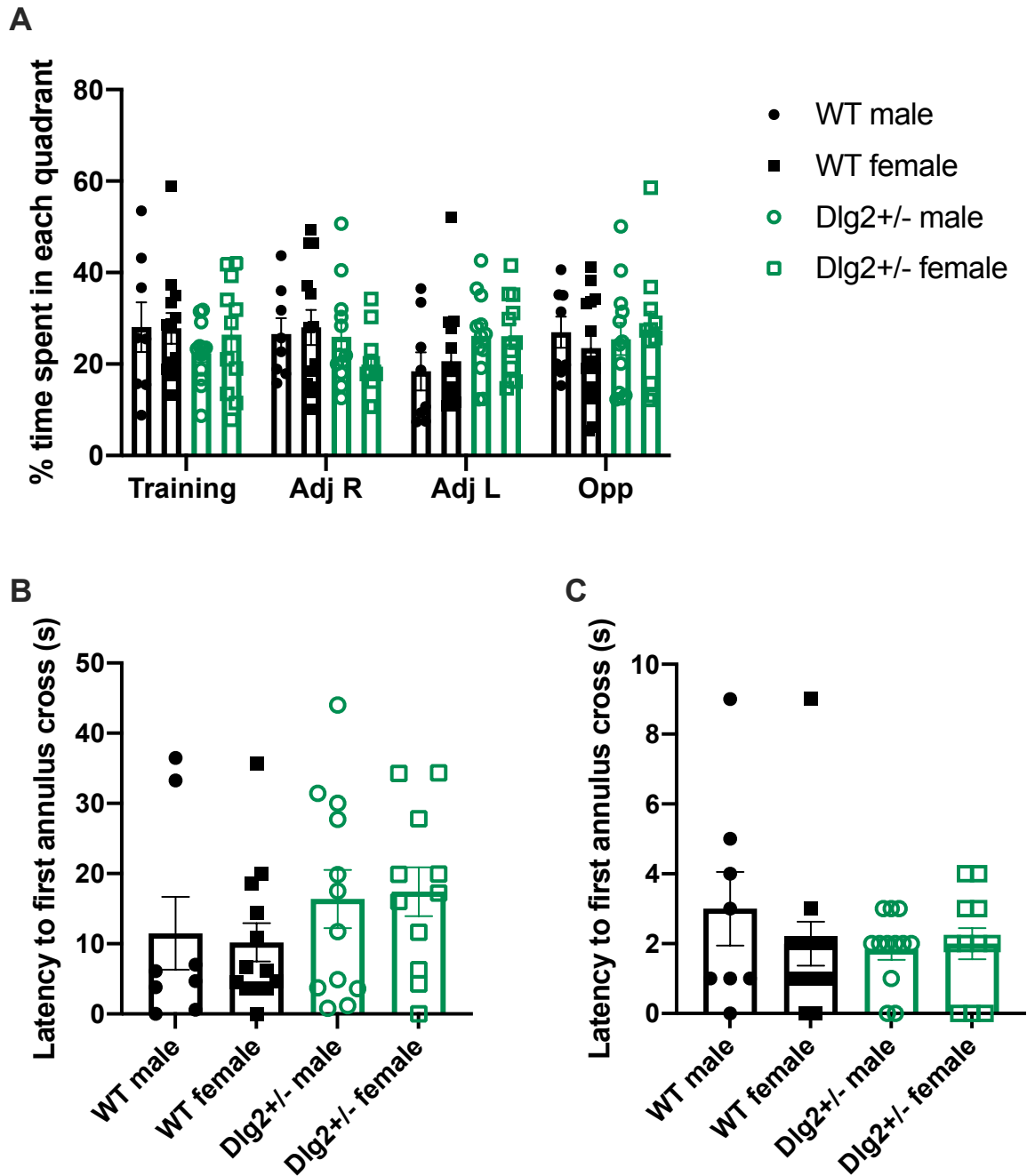
Figure 61: Male and female *Dlg2+/-* and wild-type rats performance on the reversal memory probe test. Mean ± SEM plus individual values. **A)** Percentage of time spent in each quadrant **B)** Latency to first cross of previous platform annulus **C)** Number of crossings of previous platform annulus location.

Table 27: Main and interaction effects from a repeated measures ANOVA on percentage time spent in maze quadrants in the reversal probe test.

Effects	F	p	n^2p	$BF_{exclusion}$
Main effect of quadrant	(3, 120) = 0.574	0.633	0.014	41.574
Main effect of sex	(1, 40) = 2.341	0.134	0.055	14.534
Main effect of genotype	(1, 40) = 1.071	0.307	0.026	13.748
Sex × genotype	(1, 40) = 0.002	0.964	0.000	70.270
Quadrant × sex	(3, 120) = 0.223	0.880	0.006	321.452
Quadrant × genotype	(3, 120) = 1.602	0.196	0.039	72.235
Quadrant × sex × genotype	(3, 120) = 0.587	0.625	0.014	> 1000

Table 28: Main and interaction effects from ANOVA analysis of number of annulus crossings and latency to first annulus cross on the reversal probe test.

Analysis	Effect	F	p	n^2p	$BF_{exclusion}$
Number of crossings	Main effect of sex	(1, 40) = 0.468	0.498	0.012	3.957
	Main effect of genotype	(1, 40) = 0.918	0.344	0.022	3.465
	Sex × genotype	(1, 40) = 0.918	0.344	0.022	6.891
Latency to first cross	Main effect of sex	(1, 40) = 0.001	0.974	0.000	4.193
	Main effect of genotype	(1, 40) = 2.493	0.122	0.059	1.520
	Sex × genotype	(1, 40) = 0.094	0.760	0.002	5.758

A4.2. Experiment 2

A4.2.1. Habituation to arena

There were sex differences when habituating to the arena used for object recognition assays as shown by velocity (Figure 62A) and distance travelled (Figure 62B). Inferential statistics for arena habituation are shown in Table 29. Females were more active than males in terms of velocity and distance travelled (main effect of sex for velocity: $F(1, 59) = 10.815$, $p = 0.002$, $n^2_p = 0.155$; main effect of sex for distance travelled: $F(1, 59) = 12.464$, $p < 0.001$, $n^2_p = 0.174$). Females also demonstrated greater decreases in activity between each habituation session, particularly between sessions 2 and 3 as Figures 51A and 51B show. This is reflected in the trial × sex interactions for velocity ($F(2, 118) = 9.486$, $p < 0.001$, $n^2_p = 0.139$) and distance travelled ($F(1.805, 106.468) = 9.097$, $p < 0.001$, $n^2_p = 0.134$). There was however no genotype effect on this as shown by the non-significant trial × sex × genotype interactions (velocity: $F(2, 118) = 1.112$, $p = 0.332$, $n^2_p = 0.018$; $BF_{exclusion} = 1.731$; distance travelled: $F(1.805, 106.468) = 1.178$, $p = 0.308$, $n^2_p = 0.020$; $BF_{exclusion} = 1.468$).
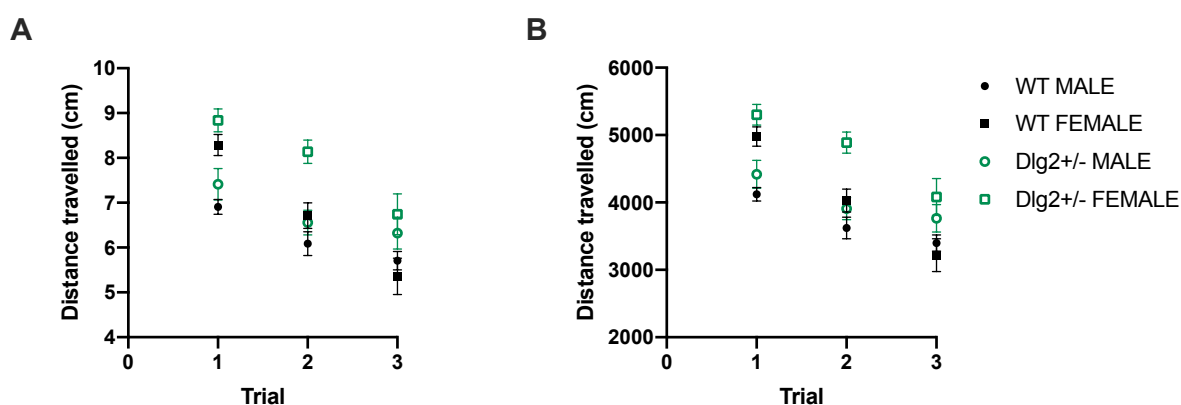
Figure 62: Male and female *Dlg2$^{+/-}$* and wild-type rats' activity in the three 10-minute sessions when habituating to the empty arena to be used for object recognition tasks in Experiment 2. Mean ± SEM **A)** velocity, **B)** distance travelled.

Table 29: Main and interaction effects from repeated measures ANOVA and Bayesian ANOVA analysis of velocity and distance travelled across three 10-minute habituation sessions to the empty object recognition arena.

Measure	Effect	F	p	n^2_p	$BF_{exclusion}$
Velocity	Main effect trial	$(2, 118) = 63.978$	< 0.001	0.520	0.000
	Main effect genotype	$(1, 59) = 11.593$	0.001	0.164	0.027
	Main effect sex	$(1, 59) = 10.815$	0.002	0.155	< 0.001
	Sex × genotype	$(1, 59) = 1.633$	0.206	0.027	0.666
	Trial × sex	$(2, 118) = 9.486$	< 0.001	0.139	0.002
	Trial × genotype	$(2, 118) = 1.215$	0.300	0.020	1.326
	Trial × sex × genotype	$(2, 118) = 1.112$	0.332	0.018	1.731
Distance	Main effect trial	$(1.805, 106.468) = 63.952$	< 0.001	0.520	< 0.001
	Main effect genotype	$(1, 59) = 11.859$	0.001	0.167	0.021
	Main effect sex	$(1, 59) = 12.464$	< 0.001	0.174	< 0.001
	Sex × genotype	$(1, 59) = 1.757$	0.190	0.029	0.714
	Trial × sex	$(1.805, 106.468) = 9.097$	< 0.001	0.134	0.002
	Trial × genotype	$(1.805, 106.468) = 1.374$	< 0.001	0.520	1.303

	Trial × sex × genotype	(1.805, 106.468) = 1.178	0.308	0.020	1.468

A4.2.2. Object exploration in the first object-in-place sample

Total exploration times for objects in the first OIP sample phase for $Dlg2^{+/-}$ and wild-type rats of both sexes are shown in Figure 63. When analysed with ANOVA and Bayesian ANOVA these data revealed a main effect of sex ($F(1, 60) = 9.973$, $p = 0.002$, $n^2_p = 0.143$; $BF_{exclusion} = 0.154$) reflecting the fact that females had an overall higher exploration time than males. There was also a sex × genotype interaction ($F(1, 60) = 4.415$, $p = 0.040$, $n^2_p = 0.069$; $BF_{exclusion} = 0.563$) as from the females the $Dlg2^{+/-}$ rats explored more than wild-types however within the males the wild-types explored more than the $Dlg2^{+/-}$s. Given that Bayes factors are inconclusive and that there are no other sex × genotype interactions, particularly on baseline exploration of a single object as assessed in Experiment 1, or on the following object-in-place test phase for Experiment 2, this significant interaction is not sufficient to suggest that the lack of genotype effects on object-in-place performance is related to sex. All main and interaction effects from this analysis are shown in Table 30.
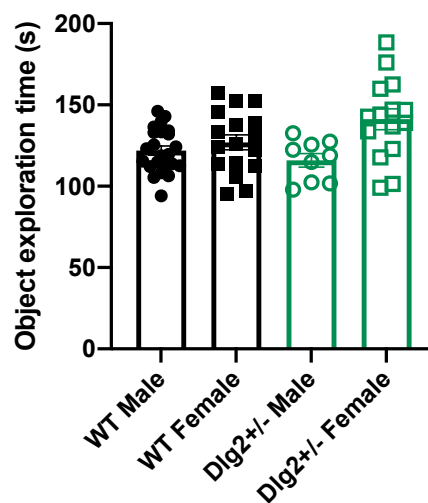
Figure 63: Total object exploration time for wild-types and $Dlg2^{+/-}$ rats of both sexes in the first OIP sample phase. Mean ± SEM plus individual values.

Table 30: Main and interaction effects from ANOVA and Bayesian ANOVA analysis of total object exploration time in the object-in-place sample phase.

Effects	F	p	$n^2{}_p$	BF$_{exclusion}$
Sex	(1, 60) = 9.973	0.002	0.143	0.154
Genotype	(1, 60) = 0.734	0.395	0.012	1.326
Sex × genotype	(1, 60) = 4.415	0.040	0.069	0.563

A4.2.3. Object-in-place task

Raw exploration times for static and swapped objects (Figure 64A) and discrimination ratio (Figure 64B) for the object-in-place task did not vary with sex. Non-significant main effects and interactions for ANOVA and Bayesian ANOVA analyses of these data are found in Table 31.

Table 31: Main and interaction effects from repeated measures ANOVA and Bayesian ANOVA analysis of object pair exploration time in the object-in-place test phase.

Analysis	Effects	F	p	$n^2{}_p$	BF$_{exclusion}$
Raw exploration times	Main effect of pair	(1, 60) = 4.642	0.035	0.045	0.538
	Main effect of sex	(1, 60) = 1.434	0.236	0.009	6.267
	Main effect of genotype	(1, 60) = 0.014	0.906	< 0.001	8.998
	Sex × genotype	(1, 60) = 0.014	0.907	< 0.001	22.971
	Pair × sex	(1, 60) = 0.605	0.440	0.006	5.215
	Pair × genotype	(1, 60) = 0.183	0.670	0.002	10.172

	Pair × sex × genotype	(1, 60) = 0.506	0.480	0.005	104.067
Discrimination ratio	Genotype main effect	(1, 60) = 0.071	0.791	0.001	3.327
	Sex main effect	(1, 60) = 0.780	0.381	0.013	4.950
	Sex × genotype	(1, 60) = 0.405	0.527	0.007	10.716
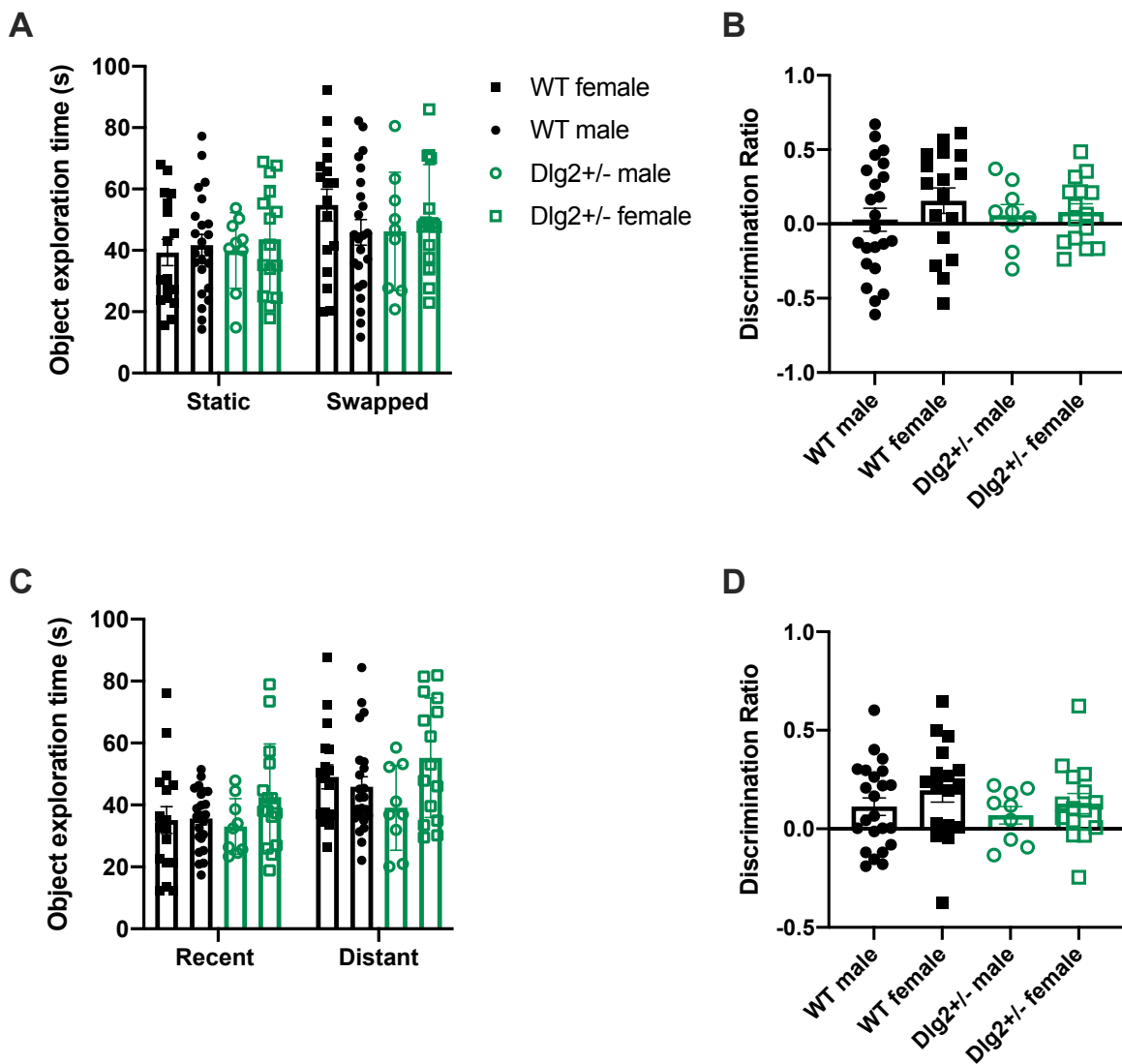
Figure 64: Male and female *Dlg2+/-* and wild-type rats' behaviour in the object-in-place and temporal order tasks. Mean ± SEM plus individual values **A)** Raw exploration time for

object-in-place, **B)** object-in-place discrimination ratio **C)** raw exploration for temporal order, **D)** temporal order discrimination ratio.

A4.2.4. Temporal order task

As Figure 64A shows sex did influence raw exploration time with females exploring more than males (significant main effect of sex ($F(1, 60) = 4.897$, $p = 0.031$, $np^2 = 0.075$)) but there were no sex × genotype ($F(1, 60) = 3.211$, $p = 0.078$, $np^2 = 0.051$; $BF_{exclusion} = 2.712$), sex × pair ($F(1, 60) = 1.087$, $p = 0.301$, $np^2 = 0.018$; $BF_{exclusion} = 2.317$) or sex × genotype × pair ($F(1, 60) = 0.086$, $p = 0.770$, $np^2 = 0.001$; $BF_{exclusion} = 19.007$) interactions. Sex had no effect on discrimination ratio as shown by Figure 64B and non-significant effects and interactions in Table 32.

Table 32: Main and interaction effects from repeated measures ANOVA and Bayesian ANOVA analysis of object pair exploration time in the temporal order test phase.

Analysis	Effects	F	p	n^2_p	$BF_{exclusion}$
Raw exploration times	Main effect of pair	(1, 60) = 18.770	< 0.001	0.097	< 0.001
	Main effect of sex	(1, 60) = 4.897	0.031	0.042	1.592
	Main effect of genotype	(1, 60) = 0.109	0.743	< 0.001	4.431
	Sex × genotype	(1, 60) = 3.211	0.078	0.028	2.712
	Pair × sex	(1, 60) = 1.087	0.301	0.006	2.317
	Pair × genotype	(1, 60) = 0.297	0.588	0.002	4.773
	Pair × sex × genotype	(1, 60) = 0.086	0.770	< 0.001	19.007

Discrimination ratio	Genotype main effect	(1, 60) = 0.992	0.323	0.016	3.708
	Sex main effect	(1, 60) = 1.628	0.207	0.026	2.718
	Sex × genotype	(1, 60) = 0.042	0.838	< 0.001	7.164

A4.2.5. Watermaze reference memory

The performance of wild-type and $Dlg2^{+/-}$ rats of both sexes on escape latency, distance swum, swim speed and percentage of time spent near side walls are shown in Figures 65A, 65B, 65C and 65D. These were analysed with repeated-measures ANOVA and Bayesian ANOVA as outlined in Table 33.

There was a main effect of sex for escape latency ($F(1, 60) = 16.560$, $p < 0.001$, $n^2_p = 0.216$), but no sex × genotype interaction ($F(1, 60) = 0.218$, $p = 0.642$, $n^2_p = 0.004$; $BF_{exclusion} = 78.089$). In addition sex main effects were significant for distance swum ($F(1, 60) = 27.978$, $p < 0.001$, $n^2p = 0.318$) as was the trial × sex ($F(2.636, 158.137) = 2.825$, $p = 0.047$, $n^2p = 0.045$) interaction. The sex × genotype interaction was non-significant with a Bayes factor evidencing the null ($F(1, 60) = 0.690$, $p = 0.409$, $n^2_p = 0.011$; $BF_{exclusion} = 68.953$), showing that while females may have escaped faster and swum further than males this did not differ with genotype.

Sex effects in escape latency and distance swum are likely due to swim speed varying with sex, as females swam faster than males (Figure 65C). There was a significant main effect of sex on swim speed ($F(1, 60) = 15.198$, $p < 0.001$, $n^2p = 0.202$) and significant day × sex ($F(7.764, 465.810) = 3.688$, $p < 0.001$, $n^2p = 0.058$), trial × sex ($F(2.865, 171.892) = 4.595$, $p = 0.005$, $n^2p = 0.071$) and day × trial × sex interactions ($F(12.810, 768.589) = 9.248$, $p = 0.532$, $n^2p = 0.009$). The sex × genotype interaction was non-significant ($F(1, 60) = 0.006$, $p = 0.941$, $n^2_p < 0.001$; $BF_{exclusion} = 11.249$).

Females showed a consistently greater likelihood to spend time near the side walls (significant main effect of sex (F(1, 60) = 121.834, p < 0.001 , n^2p = 0.670). This varied with trial (sex × trial interaction approaching significance: F(1.457, 87.396) = 3.395, p = 0.053, n^2_p = 0.054) but there was no sex × genotype interaction (F(1, 60) = 1.651, p = 0.204, n^2_p = 0.027; $BF_{exclusion}$ = 83.101). Overall on the watermaze reference memory task female rats demonstrated a faster escape latency and distance swum which may have been due to their higher swim speeds. They also had a greater tendency to rely on swimming close to the side walls to find the platform. For all measures there were no genotype differences on these sex effects.

Table 33: Repeated measures ANOVA and Bayesian repeated measures ANOVA effects and interactions for escape latency, distance swum, swim speed and percentage of time near walls during the 10-day water-maze reference memory.

Analysis	Effect	F	p	n^2_p	$BF_{exclusion}$
Escape latency	Main effect of day	(6.431, 385.889) = 95.992	< 0.001	0.615	0.000
	Main effect of trial	(2.770, 166.214) = 66.096	< 0.001	0.524	0.000
	Main effect of sex	(1, 60) = 16.560	< 0.001	0.216	0.177
	Main effect of genotype	(1, 60) = 0.087	0.769	0.001	76.356
	Sex × genotype	(1, 60) = 0.218	0.642	0.004	78.089
	Day × genotype	(6.431, 385.889) = 1.097	0.364	0.018	> 1000
	Day × sex	(6.431, 385.889) = 1.728	0.108	0.028	377.802
	Day × genotype × sex	(6.431, 385.889) = 1.389	0.214	0.023	> 1000
	Trial × genotype	(2.770, 166.214) = 0.600	0.603	0.010	> 1000

	Trial × sex	(2.770, 166.214) = 2.048	0.114	0.033	34.903
	Trial × genotype × sex	(2.770, 166.214) = 1.256	0.291	0.021	> 1000
	Day × trial	(15.863, 951.794) = 3.118	< 0.001	0.049	< 0.001
	Day × trial × genotype	(15.863, 951.794) = 0.871	0.602	0.014	> 1000
	Day × trial × sex	(15.863, 951.794) = 0.664	0.829	0.011	> 1000
	Day × trial × genotype × sex	(15.863, 951.794) = 0.890	0.580	0.015	> 1000
Distance swum (cm)	Main effect of day	(6.691, 401.484) = 81.000	< 0.001	0.574	< 0.001
	Main effect of trial	(2.636, 158.137) = 106.719	< 0.001	0.640	< 0.001
	Main effect of sex	(1, 60) = 27.978	< 0.001	0.318	0.006
	Main effect of genotype	(1, 60) = 0.064	0.809	0.001	66.186
	Sex × genotype	(1, 60) = 0.690	0.409	0.011	68.953
	Day × genotype	(6.691, 401.484) = 0.947	0.467	0.016	> 1000
	Day × sex	(6.691, 401.484) = 1.070	0.381	0.018	> 1000
	Day × genotype × sex	(6.691, 401.484) = 1.110	0.356	0.018	> 1000

	Trial × genotype	(2.636, 158.137) = 1.340	0.265	0.022	165.086
	Trial × sex	(2.636, 158.137) = 2.825	0.047	0.045	10.155
	Trial × genotype × sex	(2.636, 158.137) = 2.317	0.086	0.037	> 1000
	Day × trial	(15.507, 930.408) = 3.865	< 0.001	0.061	< 0.001
	Day × trial × genotype	(15.507, 930.408) = 0.970	0.486	0.016	> 1000
	Day × trial × sex	(15.507, 930.408) = 0.667	0.824	0.011	> 1000
	Day × trial × genotype × sex	(15.507, 930.408) = 0.898	0.569	0.015	> 1000
Swim speed (cm/s)	Main effect of day	(7.764, 465.810) = 12.089	< 0.001	0.168	0.000
	Main effect of trial	(2.865, 171.892) = 37.904	< 0.001	0.387	0.000
	Main effect of sex	(1, 60) = 15.198	< 0.001	0.202	< 0.001
	Main effect of genotype	(1, 60) = 9.132	0.004	0.132	1.261
	Genotype × sex	(1, 60) = 0.006	0.941	< 0.001	11.249
	Day × genotype	(7.764, 465.810) = 1.937	0.055	0.031	34.035
	Day × sex	(7.764, 465.810) = 3.688	< 0.001	0.058	0.006

	Day × genotype × sex	(7.764, 465.810) = 1.304	0.241	0.021	> 1000
	Trial × genotype	(2.865, 171.892) = 4.586	0.005	0.071	11.738
	Trial × sex	(2.865, 171.892) = 4.595	0.005	0.071	5.205
	Trial × genotype × sex	(2.865, 171.892) = 0.380	0.758	0.006	> 1000
	Day × trial	(12.810, 768.589) = 1.336	0.187	0.022	> 1000
	Day × trial × genotype	(12.810, 768.589) = 0.963	0.486	0.016	> 1000
	Day × trial × sex	(12.810, 768.589) = 9.248	0.532	0.009	> 1000
	Day × trial × genotype × sex	(12.810, 768.589) = 1.294	0.211	0.021	> 1000
Percentage of time near walls	Main effect of day	(5.941, 356.470) = 0.890	0.502	0.015	> 1000
	Main effect of trial	(1.457, 87.396) = 4.542	0.023	0.070	22.411
	Main effect of sex	(1, 60) = 121.834	< 0.001	0.670	< 0.001
	Main effect of genotype	(1, 60) = 3.435	0.069	0.054	61.241
	Sex × genotype	(1, 60) = 1.651	0.204	0.027	83.101

	Day × genotype	(5.941, 356.470) = 1.009	0.419	0.017	> 1000
	Day × sex	(5.941, 356.470) = 1.044	0.396	0.017	> 1000
	Day × genotype × sex	(5.941, 356.470) = 0.966	0.447	0.016	> 1000
	Trial × genotype	(1.457, 87.396) = 1.033	0.340	0.017	> 1000
	Trial × sex	(1.457, 87.396) = 3.395	0.053	0.054	25.454
	Trial × genotype × sex	(1.457, 87.396) = 1.386	0.252	0.023	> 1000
	Day × trial	(7.264, 435.810) = 0.831	0.565	0.014	> 1000
	Day × trial × genotype	(7.264, 435.810) = 0.961	0.461	0.016	> 1000
	Day × trial × sex	(7.264, 435.810) = 0.698	0.679	0.012	> 1000
	Day × trial × genotype × sex	(7.264, 435.810) = 0.798	0.593	0.013	> 1000
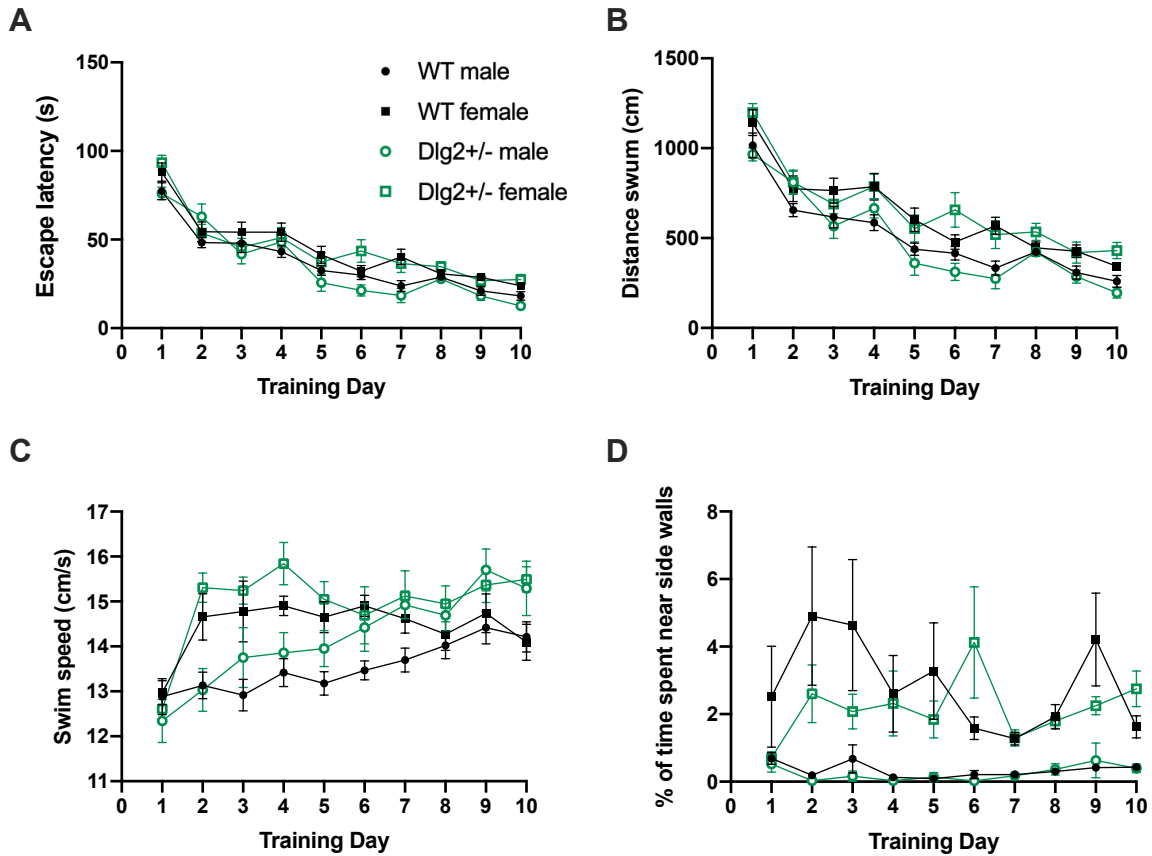
Figure 65: Performance of *Dlg2^{+/-}* and wild-type rats of both sexes on a ten-day reference memory task in the water maze. Escape latencies **(A)**, distance swum **(B)** swim speed **(C)** and percentage of time spent near side walls **(D)** across all four training days in the reference memory task, split by sex (male and female) and genotype (HET and WT). The latencies, distances and speeds shown for each day are the average of four daily trials and the SEM.

A4.2.6. Watermaze reference memory probe test

Dwell time in maze quadrants during the three probe tests (Figures 66A, 66B and 66C) did not vary with sex, as shown by the non-significant sex main effects and interactions for the repeated measures ANOVA and repeated measures Bayesian ANOVA analysis of each probe test (Table 34).

Table 34: Main and interaction effects from a repeated measures ANOVA on percentage time spent in maze quadrants in the 10-day reference memory probe test.

Probe trial	Effects	F	p	n^2_p	BF$_{exclusion}$
1 (Day 4, Trial 13).	Main effect of quadrant	(2.428, 145.708) = 2.651	0.063	0.042	4.752
	Main effect of sex	(1, 60) = 0.016	0.899	< 0.001	17.778
	Main effect of genotype	(1, 60) = 0.262	0.610	0.004	10.114
	Sex × genotype	(1, 60) = 0.016	0.899	< 0.001	106.998
	Quadrant × sex	(2.428, 145.708) = 1.359	0.260	0.022	33.675
	Quadrant × genotype	(2.428, 145.708) = 0.502	0.642	0.008	137.332
	Quadrant × sex × genotype	(2.428, 145.708) = 1.227	0.300	0.020	> 1000
2 (Day 8, Trial 29).	Main effect of quadrant	(1.720, 103.180) = 6.319	0.004	0.095	< 0.001
	Main effect of sex	(1, 60) = 0.018	0.894	< 0.001	15.834
	Main effect of genotype	(1, 60) = 0.694	0.408	0.011	15.326
	Sex × genotype	(1, 60) = 0.171	0.681	0.003	93.210
	Quadrant × sex	(1.720, 103.180) = 0.637	0.508	0.011	27.002

	Quadrant × genotype	(1.720, 103.180) = 0.467	0.599	0.008	22.072
	Quadrant × sex × genotype	(1.720, 103.180) = 0.409	0.634	0.007	> 1000
3 (Day 11, Trial 45).	Main effect of quadrant	(1.675, 100.471) = 9.483	< 0.001	0.136	< 0.001
	Main effect of sex	(1, 60) < 0.001	0.990	< 0.001	15.493
	Main effect of genotype	(1, 60) = 0.332	0.567	0.005	14.883
	Sex × genotype	(1, 60) = 0.814	0.371	0.013	79.945
	Quadrant × sex	(1.675, 100.471) = 0.260	0.732	0.004	24.034
	Quadrant × genotype	(1.675, 100.471) = 0.586	0.529	0.010	28.530
	Quadrant × sex × genotype	(1.675, 100.471) = 0.928	0.384	0.015	> 1000
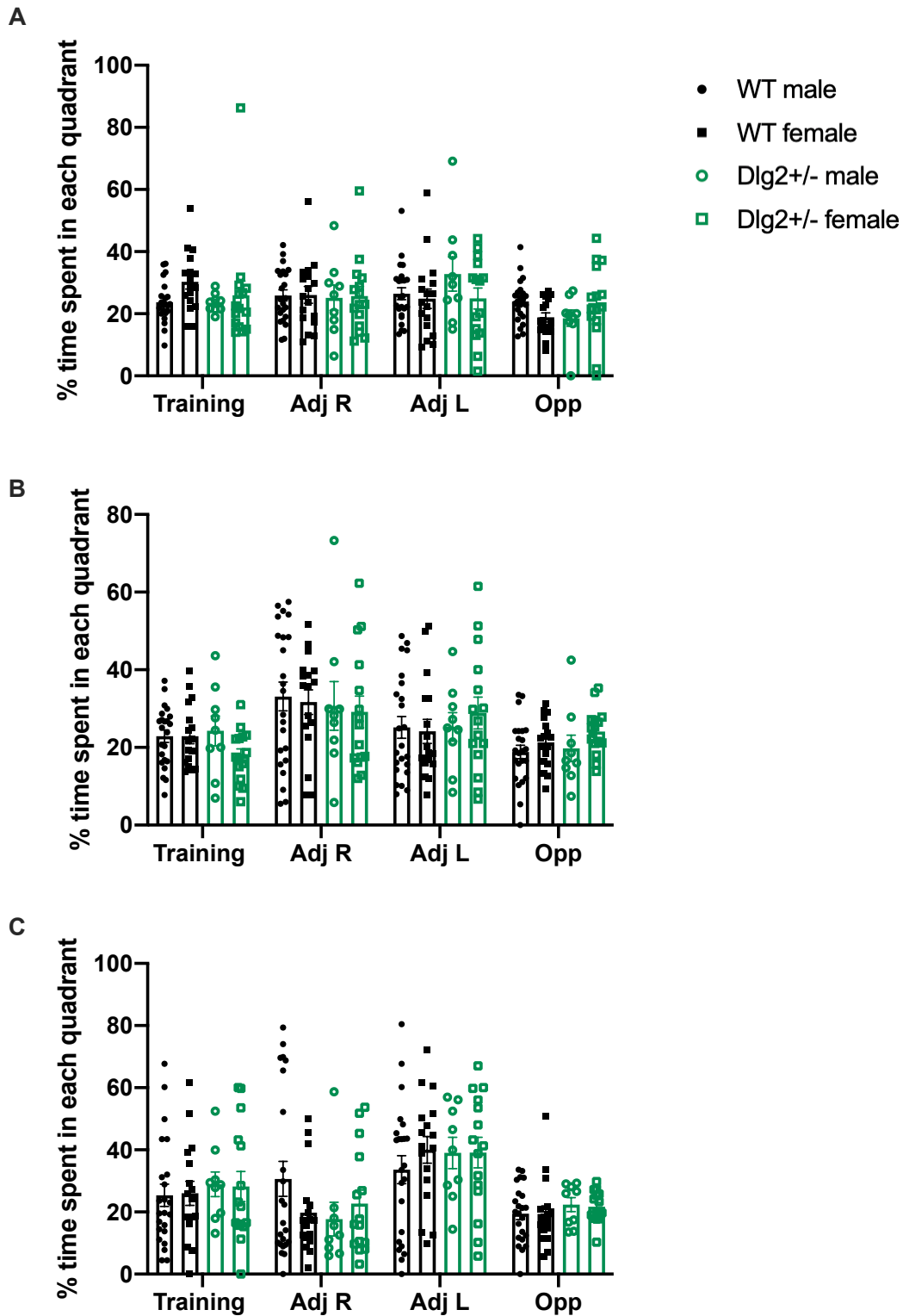
Figure 66: Percentage time spent in each of the four maze quadrants for *Dlg2$^{+/-}$* and wild-type rats of both sexes on probes 1 (A) 2 (B) and 3 (C). Mean ± SEM plus individual values.

As Figure 67A shows males of both genotypes tended to perform more annulus crossings than females, reflected by the significant main effect of sex (F(1, 60) = 10.952, p = 0.002, $n^2{}_p$

= 0.154). This did not vary significantly across probe trials as shown by the non-significant probe × sex interactions ($F(2, 120) = 0.273$, $p = 0.761$, $n^2_p = 0.005$; $BF_{exclusion} = 26.630$). There were no genotype effects on this with sex × genotype ($F(1, 60) = 2.386$, $p = 0.128$, $n^2_p = 0.038$; $BF_{exclusion} = 5.189$) and probe × sex × genotype interactions non-significant ($F(2, 120) = 0.305$, $p = 0.738$, $n^2_p = 0.005$; $BF_{exclusion} > 1000$).

Males latency to annulus cross was significantly smaller than females as shown in Figure 67B and reflected by the main effect of sex on latency to cross ($F(1, 60) = 24.595$, $p < 0.001$, $n^2_p = 0.291$). This did not change across probe trials (non-significant probe × sex interaction: $F(2, 120) = 2.138$, $p = 0.122$, $n^2_p = 0.034$; $BF_{exclusion} = 0.785$), nor were there genotype effects on this (non-significant sex × genotype interaction: $F(1, 60) = 0.557$, $p = 0.458$, $n^2_p = 0.009$; $BF_{exclusion} = 3.130$; and probe × sex × genotype interaction $F(2, 120) = 0.533$, $p = 0.588$, $n^2_p = 0.009$; $BF_{exclusion} = 24.198$).

Table 35: Main and interaction effects from repeated measures ANOVA and Bayesian repeated measures ANOVA analysis of number of annulus crossings and latency to first annulus cross on the three reference memory probe tests.

Measure	Effect	F	p	n^2_p	$BF_{exclusion}$
Frequency of annulus crossings	Main effect probe	(2, 120) = 0.611	0.545	0.010	16.592
	Main effect sex	(1, 60) = 10.952	0.002	0.154	0.406
	Main effect genotype	(1, 60) = 0.078	0.781	0.001	8.403
	Sex × genotype	(1, 60) = 2.386	0.128	0.038	5.189
	Probe × sex	(2, 120) = 0.273	0.761	0.005	26.630
	Probe × genotype	(2, 120) = 0.312	0.732	0.005	79.720

	Probe × sex × genotype	(2, 120) = 0.305	0.738	0.005	> 1000
Latency to first annulus cross	Main effect probe	(2, 120) = 8.389	< 0.001	0.123	0.002
	Main effect sex	(1, 60) = 24.595	< 0.001	0.291	0.004
	Main effect genotype	(1, 60) = 3.897	0.053	0.061	2.597
	Sex × genotype	(1, 60) = 0.557	0.458	0.009	3.130
	Probe × sex	(2, 120) = 2.138	0.122	0.034	0.785
	Probe × genotype	(2, 120) = 0.623	0.538	0.010	5.979
	Probe × sex × genotype	(2, 120) = 0.533	0.588	0.009	24.198
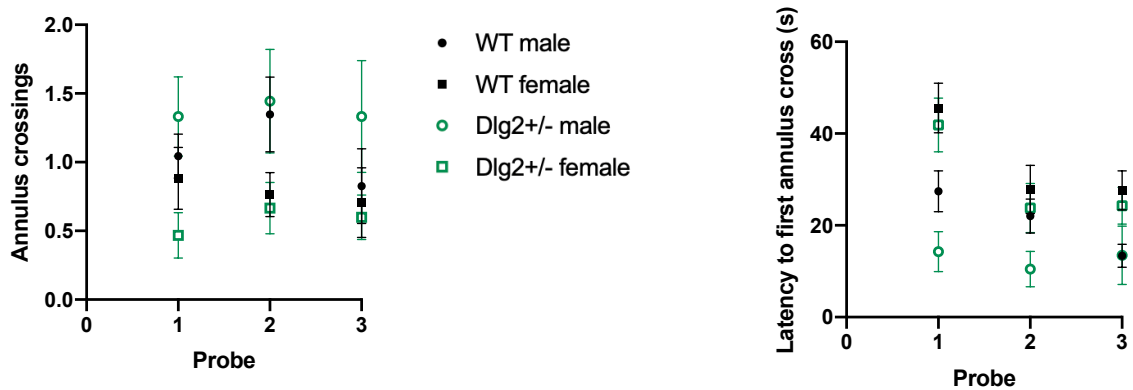
Figure 67: Annulus crossings (A) and latency to first annulus cross (B) across the three probe trials for rats of different genotypes and sexes. Mean ± SEM.

A4.2.7. Y-maze spontaneous alternation

Continuous trials procedure

There were no sex effects on number of arm entries as shown in Figure 68A and 68B and confirmed by repeated measures ANOVA and Bayesian ANOVA analysis showing non-significant sex effects and interactions in Table 36 and 37.

Table 36: Main and interaction effects from repeated measures ANOVA and Bayesian ANOVA of number of entries made into each maze arm (A, B and C) in the Y-maze using a continuous trials procedure.

Effect	F	p	$n^2{}_p$	BF$_{exclusion}$
Main effect maze arm	(2, 118) = 9.452	< 0.001	0.138	0.003
Main effect genotype	(1, 59) = 6.562	0.013	0.100	0.200
Main effect sex	(1, 59) = 2.281	0.136	0.037	1.012
Sex × genotype	(1, 59) = 1.497	0.226	0.025	1.233
Maze arm × genotype	(2, 118) = 1.044	0.355	0.017	1.739
Maze arm × sex	(2, 118) = 2.854	0.062	0.046	0.631
Maze arm × sex × genotype	(2, 118) = 0.159	0.853	0.003	10.402

Figure 68: Performance of *Dlg2+/-* and wild-type rats of both sexes on the continuous-trials Y-maze procedure. Mean ± SEM plus individual values for **A)** number of entries by maze arm **B)** total number of arm entries **C)** spontaneous alternation.

Table 37: Main and interaction effects from repeated measures ANOVA and Bayesian ANOVA of number of entries made into each maze arm (A, B and C) in the Y-maze using a continuous trials procedure.

Effect	F	p	n^2_p	BF$_{exclusion}$
Main effect maze arm	(2, 118) = 9.452	< 0.001	0.138	0.003

Main effect genotype	(1, 59) = 6.562	0.013	0.100	0.200
Main effect sex	(1, 59) = 2.281	0.136	0.037	1.012
Sex × genotype	(1, 59) = 1.497	0.226	0.025	1.233
Maze arm × genotype	(2, 118) = 1.044	0.355	0.017	1.739
Maze arm × sex	(2, 118) = 2.854	0.062	0.046	0.631
Maze arm × sex × genotype	(2, 118) = 0.159	0.853	0.003	10.402

Trial based procedure

There were no sex effects or sex × genotype interactions on spontaneous alternation with an intertrial interval of 1 minute (Figure 69A) or 24 hours (Figure 69B). Non-significant effects and interactions can be found in Table 38.
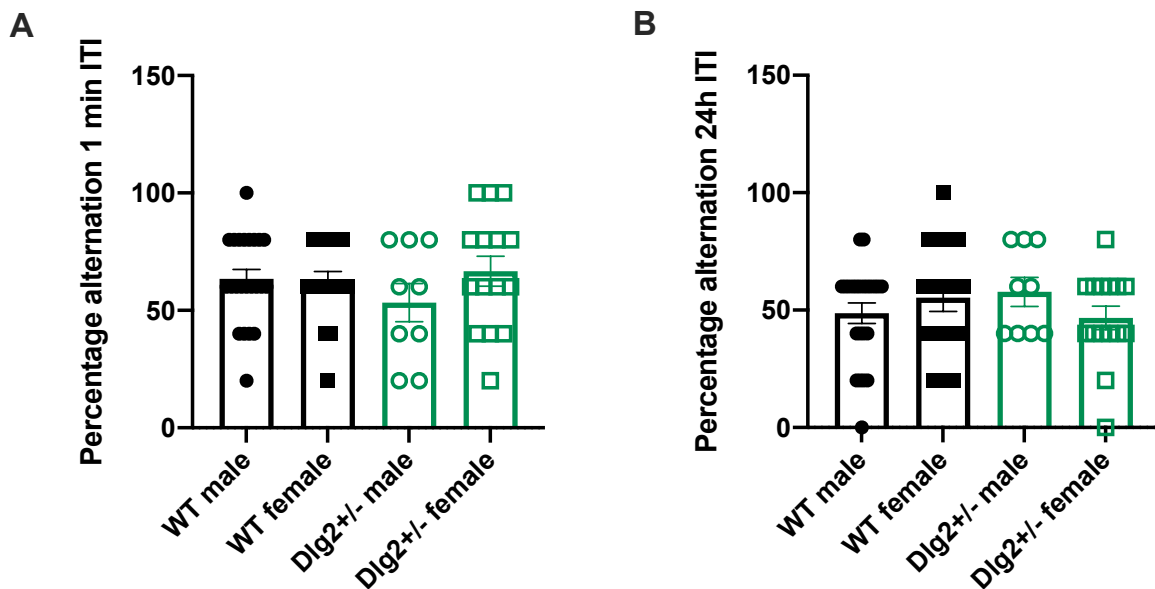
Figure 69: Percentage alternation of *Dlg2$^{+/-}$* and wild-type rats of both sexes on a trial-based procedure in the Y-maze with an A) 1 minute or B) 24-hour intertrial-interval (ITI). Mean ± SEM plus individual values.

Table 38: ANOVA and Bayesian ANOVA main effects and interactions for percentage alternation at 1 minute and 24 hour intertrial intervals in the trial-based Y-maze procedure.

Intertrial interval	Effect	F	p	$n^2{}_p$	BF$_{exclusion}$
1 minute	Main effect genotype	(1, 60) = 0.283	0.597	0.005	4.805
	Main effect sex	(1, 60) = 1.239	0.270	0.020	4.125
	Sex × genotype	(1, 60) = 1.738	0.192	0.028	7.546
24 hour	Main effect genotype	(1, 60) = 0.002	0.968	< 0.001	4.816
	Main effect sex	(1, 60) = 0.163	0.688	0.003	4.958
	Sex × genotype	(1, 60) = 2.509	0.118	0.040	6.390

Chapter 6

A6.1. Reality testing

There are no sex differences in learning about FCX+ and FCX- on the reality testing task as shown by one bottle consumption (Figure 70A), one bottle lick cluster size (Figure 70B) and two bottle 'choice' consumption (Figure 70C). For all measures sex × flavour, sex × flavour × pairings, sex × genotype × flavour and sex × genotype × flavour × pairing interactions are non-significant, as shown in Table 39. On the two bottle choice test there was a significant sex × genotype interaction (F(1, 57) = 4.973, p = 0.030, $n^2{}_p$ = 0.080) on overall consumption caused by wild-type males consuming more fluid overall than wild-type females, and *Dlg2*$^{+/-}$ females consuming slightly more than *Dlg2*$^{+/-}$ males as Figure 70C shows. This interaction is for overall consumption and there are no interactions between sex, genotype, pairings and

flavour on consumption. Thus, this sex × genotype effect does not demonstrate differences in reality monitoring ability for either sex or genotype.
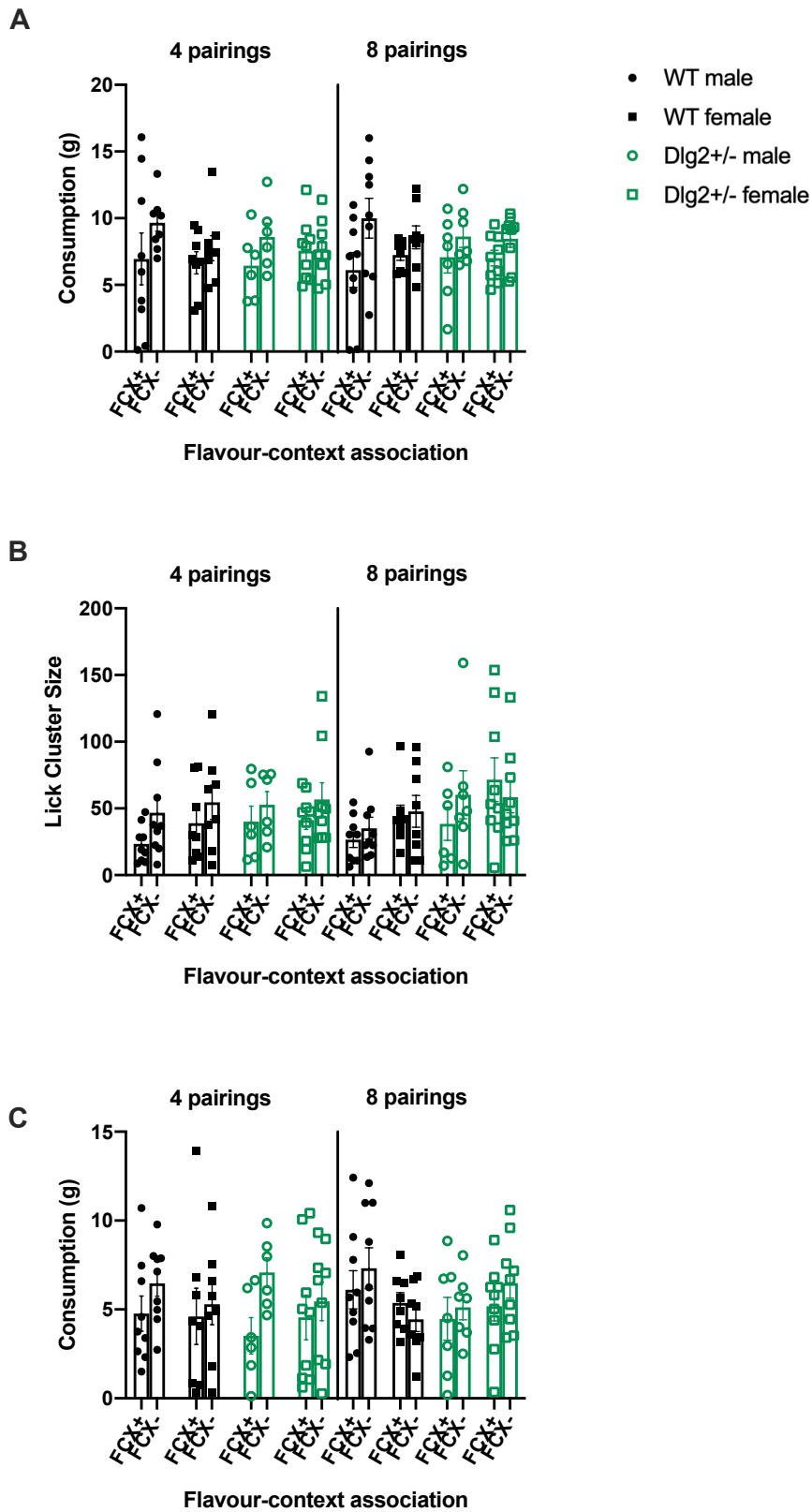
Figure 70: Performance of *Dlg2⁺/⁻* and wild-type rats of both sexes in representation-mediated taste aversion at 4 and 8 context-flavour pairings. Graphs show corrected consumption and lick cluster size of the flavour associated with a context where LiCl was

experienced (FCX+) and a flavour associated with a 'safe' LiCl-free context (FCX-) in the one bottle test **(A and B)** and corrected consumption of FCX+ and FCX- on the two-bottle choice test **(C).**

Table 39: Repeated measures ANOVA and Bayesian repeated measures ANOVA main effects and interactions for corrected one bottle consumption and lick cluster size and two bottle consumption in the reality testing Experiment test.

Analysis	Effect	F	p	n^2_p	BF$_{exclusion}$
One bottle consumption (g)	Flavour main effect	(1, 57) = 12.396	< 0.001	0.179	0.038
	Genotype main effect	(1, 57) = 0.120	0.730	0.002	18.279
	Sex main effect	(1, 57) = 0.340	0.562	0.006	11.435
	Pairings main effect	(1, 57) = 0.170	0.682	0.003	20.509
	Sex × genotype	(1, 57) = 0.295	0.589	0.005	27.040
	Sex × pairings	(1, 57) = 0.127	0.723	0.002	29.899
	Genotype × pairings	(1, 57) < 0.001	0.988	< 0.001	44.380
	Sex × genotype × pairings	(1, 57) = 0.283	0.597	0.005	349.353
	Flavour × sex	(1, 57) = 2.481	0.121	0.042	3.590
	Flavour × genotype	(1, 57) = 0.882	0.352	0.015	9.153
	Flavour × pairings	(1, 57) = 0.292	0.591	0.005	14.277

	Flavour × sex × genotype	(1, 57) = 0.236	0.629	0.004	73.682
	Flavour × sex × pairings	(1, 57) = 0.065	0.800	0.001	97.709
	Flavours × genotype × pairings	(1, 57) = 0.023	0.879	< 0.001	220.498
	Flavour × sex × genotype × pairings	(1, 57) = 0.521	0.473	0.009	> 1000
One bottle lick cluster size	Flavour main effect	(1, 57) = 1.235	0.271	0.021	9.393
	Genotype main effect	(1, 57) = 4.885	0.031	0.079	2.012
	Sex main effect	(1, 57) = 0.713	0.402	0.012	11.942
	Pairings main effect	(1, 57) = 1.057	0.308	0.018	8.472
	Sex × genotype	(1, 57) = 0.744	0.392	0.013	8.349
	Sex × pairings	(1, 57) = 0.003	0.955	< 0.001	16.362
	Genotype × pairings	(1, 57) = 1.823	0.182	0.031	4.868
	Sex × genotype × pairings	(1, 57) = 0.081	0.777	0.001	52.486
	Flavour × sex	(1, 57) = 0.029	0.865	< 0.001	21.393
	Flavour × genotype	(1, 57) = 1.246	0.269	0.021	6.818

	Flavour × pairings	(1, 57) = 3.277	0.076	0.054	5.533
	Flavour × sex × genotype	(1, 57) = 0.148	0.702	0.003	97.259
	Flavour × sex × pairings	(1, 57) = 0.002	0.967	< 0.001	32.107
	Flavour × genotype × pairings	(1, 57) = 0.418	0.520	0.007	21.230
	Flavour × sex × genotype × pairings	(1, 57) = 0.007	0.935	< 0.001	> 1000
Two bottle consumption(g)	Flavour main effect	(1, 57) = 3.132	0.082	0.052	3.541
	Genotype main effect	(1, 57) = 0.771	0.384	0.013	20.684
	Sex main effect	(1, 57) = 1.469	0.231	0.025	16.624
	Pairings main effect	(1, 57) = 0.899	0.347	0.016	19.535
	Sex × genotype	(1, 57) = 4.973	0.030	0.080	21.119
	Sex × pairings	(1, 57) = 0.017	0.897	< 0.001	43.067
	Genotype × pairings	(1, 57) = 0.245	0.622	0.004	47.294
	Sex × genotype × pairings	(1, 57) = 2.931	0.092	0.049	291.779
	Flavour × sex	(1, 57) = 1.014	0.318	0.017	12.685

	Flavour × genotype	(1, 57) = 0.532	0.469	0.009	16.342
	Flavour × pairings	(1, 57) = 0.811	0.372	0.014	14.308
	Flavour × sex × genotype	(1, 57) = 0.050	0.824	< 0.001	107.488
	Flavour × sex × pairings	(1, 57) = 0.181	0.672	0.003	194.427
	Flavours × genotype × pairings	(1, 57) = 0.006	0.937	< 0.001	113.466
	Flavour × sex × genotype × pairings	(1, 57) = 0.749	0.391	0.013	> 1000

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